# Studies on mechanisms of antiepilepsy and antiobesity in experimental animal models

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# Contents

**List of Abbreviations**

| Abbreviation | Term |
|---|---|
| ACC | Acetyl-CoA carboxylase |
| AD | Afterdischarge |
| BMI | Body mass index |
| BSA | Bovine serum albumin |
| CaM kinase II | Calcium calmodulin-dependent protein kinase II |
| CSF | Cerebrospinal fluid |
| DG | Diacylglycerol |
| DGAT | Diacylglycerol acyltransferase |
| DMSO | Dimethylsulfoxide |
| DPP-IV | Dipeptidyl peptidase IV |
| EEG | Electroencephalogram |
| EDTA | Ethylenediaminetetraacetic acid |
| EGTA | Ethylene glycol tetraacetic acid |
| EGTA/AM | EGTA tetra acetoxymethyl ester |
| EIA | Enzyme immunoassay |
| FAS | Fatty acid synthase |
| G-3-P | Glycerol-3-phosphate |
| GABA | γ-aminobutyric acid |
| GLP-1 | Glucagon-like peptide-1 |
| HSVLF | High sucrose very low fat |

| Abbreviation | Term |
|---|---|
| i.c.v. | Intracerebroventricular |
| KO | Knockout |
| LCFA | Long chain fatty acid |
| LXR | Liver X receptor |
| 2-MG | sn-2-monoacylglycerol |
| MGAT | Monoacylglycerol acyltransferase |
| MGL | Monoacylglycerol lipase |
| mtGPAT | Mitochondrial glycerol 3-phosphate acyltransferase |
| MTP | Microsomal triglyceride transfer protein |
| NASH | Nonalcoholic steatohepatitis |
| $O_2$ | Oxygen |
| PKC | Protein kinase C |
| PYY | Peptide YY |
| QOL | Quality of life |
| RQ | Respiratory quotient |
| SCD1 | Stearoyl-CoA desaturase 1 |
| SD | Sprague Dawley |
| SREBP-1c | Sterol regulatory element-binding protein 1-c |
| TG | Triglyceride |
| TLC | Thin layer chromatography |

# Chapter 1

# General Introduction

Epilepsy is one of the most serious and common brain disorders affecting individuals worldwide (Johnston and Smith, 2010). Epilepsy is characterized by an enduring predisposition to generate epileptic seizures (Fisher et al., 2005). In general, epilepsy starts in children or in individuals older than 60 years of age. The prevalence of epilepsy in subjects over 70 years of age is twice that in children, and the incidence increases significantly with age for every decade after 60 years of age (Martin et al., 2014; Stephen and Brodie, 2000). Since the number of elderly people is rapidly increasing, the treatment of seizures and epilepsy will become a greater concern in the near future (Leppik, 2001). Therefore, the medical care for epilepsy will be extremely important in preparation for the coming of an ultra-aged society in Japan.

Epilepsy causes patients to experience recurring and uncontrollable seizures (Duncan et al., 2006; Litt and Echauz, 2002). An epileptic seizure is due to abnormal excessive neuronal excitability in the brain detected using electroencephalogram (EEG). Patients may experience strange emotions, convulsions or lose consciousness. There are numerous possible causes for epileptic seizures, including illness, brain injury, and abnormal brain development. However, the cause is unknown in many cases. Epileptic seizures are divided into two main types, focal seizures and generalized seizures (Engel, 2001). In general, generalized seizures affect both sides of the brain at once. In contrast, focal seizures start in a single area of the brain called the “focus” of the seizures. Sometimes focal seizures spread from one side to both sides of the brain and are called secondarily generalized seizures (Berg et al., 2010). If an individual experiences a

secondarily generalized seizure, the person becomes unconscious and will usually have a tonic-clonic seizure. Understandably, the quality of life (QOL) in epilepsy patients is poor and the frequency of seizures is considered one of the most relevant determinants of poor QOL scores (Guekht et al., 2007). Therefore, controlling seizures is particularly important for epilepsy treatment.

The treatment for epileptic seizures is mainly through medications, such as carbamazepine, valproate and the benzodiazepines (Loscher and Schmidt, 2011). Even if there are numerous antiepilepsy drugs, the QOL in epilepsy patents is still not favorable. The majority of patients diagnosed with new-onset epilepsy achieve seizure freedom with the available drugs. However, about one-third of these patients experience uncontrollable seizures and the newer antiepilepsy drugs have only minimally improved outcomes in patients whose seizures are refractory to older agents (Brodie et al., 2012). Therefore, clarifying a novel mechanism for antiepileptic effects is urgently needed.

Epileptic seizures are difficult to reproduce in *in vitro* assay systems. Consequently, numerous animal models for epileptic seizures have been used to elucidate the mechanism of action of antiepilepsy effects. In particular, amygdaloid kindling is one of the most common animal models of epilepsy and is a phenomenon in which repeated administration of an initially subconvulsive electrical stimulus eventually results in intense motor seizures (French et al., 2013; Loscher, 2011). This model has been widely accepted as a model of secondary generalized seizures and has provided key insights into epilepsy (Goddard, 1967; Maru and Goddard, 1987).

The central histaminergic system is known to have numerous physiological functions, such as the management of circadian rhythm, body temperature, learning, memory, appetite and epilepsy (Haas et al., 2008). In fact, it has been suggested that the

histaminergic neuron system is involved in seizures in rats (Kamei et al., 1998). Therefore, stimulating histaminergic neuron system by antagonizing of histamine $H_3$ receptors, which act as autoreceptors in presynaptic histaminergic neurons and control histamine turnover, have been considered a potential drug target for the treatment of central nervous system disorders, such as epilepsy (Bialer et al., 2015; Sadek et al., 2014). However, the mechanism of the antiepilepsy effects of histamine is still unclear. In Chapter 2, the mechanism of the antiepilepsy effect of histamine was investigated using the amygdaloid kindling model to elucidate a new strategy for epileptic seizure treatments.

Obesity has been increasing at epidemic proportions and has now become a critical problem (Lavie et al., 2009). In adults, overweight is defined as a body mass index (BMI) of 25 to 29.9 kg/$m^2$ and obesity is defined as BMI greater than or equal to 30 kg/$m^2$. Recently, nearly 70% of adults in the United States have been classified as overweight or obese compared with fewer than 25% 40 years ago (Flegal et al., 2010). Obesity increases not only body weight but also the risk of insulin resistance, type 2 diabetes, dyslipidemia, hypertension, fatty liver disease and atherosclerosis (Lavie et al., 2009; Van Gaal et al., 2006). Recently, obesity has been pointed out as being the primary cause of metabolic syndrome as a multiplex risk factor for cardiovascular disease and premature death (Grundy et al., 2004; Isomaa et al., 2001). According to the National Institutes of Health, obesity and overweight together are the second leading cause of preventable deaths in the United States. Therefore, the medical care for obesity is also important considering diversification of diet, in particular a shift to fat-based diets in Japan.

There are many factors that contribute to obesity. Particularly, a combination of excessive energy intake and lack of energy expenditure is considered to explain most cases of obesity (Hill et al., 2012). Therefore, the main treatment for obesity is lifestyle changes (Fock and Khoo, 2013; Jensen et al., 2014). Although lifestyle changes, such as diet and physical exercise, can be successful in reducing body weight, dietary modifications and exercise are difficult to continue (Eshghinia and Mohammadzadeh, 2013; Lang and Froelicher, 2006). As another treatment option for obesity, some medications are available (Jindal et al., 2013; Okuma et al., 2015). Since the cause of obesity is an imbalance between food intake and energy expenditure (Bouchard, 2008), a reduction in food intake has been one of the main strategies for obesity treatment (Kang and Park, 2012; Valsamakis et al., 2014). However, drugs and compounds that reduce food intake by affecting the central nervous system have psychological adverse effects, such as emotional changes. In particular, rimonabant, which is an anorectic antiobesity drug that suppresses the cannabinoid system, has been withdrawn from the market because of potentially serious central nervous system adverse effects including depression and suicide attempts.

Similar to the central nervous system, the gastrointestinal tract reportedly plays an important role in satiety (Chaudhri et al., 2008; Konturek et al., 2004). Indeed, long chain fatty acid (LCFA), one of the digestive products of triglyceride (TG), decreases food intake through intestinal feedback signaling such as satiety hormone secretion (Feltrin et al., 2008; French et al., 2000), suggesting that the control of food intake by peripheral signaling may be a potential strategy for treating obesity that avoids serious psychological adverse effects. Sn-2-monoacylglycerol (2-MG) is one of the digestive

products of TG as well as LCFA. Some investigations suggest that 2-MG is involved in obesity. For example, knockout (KO) mice of monoacylglycerol acyltransferase (MGAT) 2, which catalyzes the synthesis of diacylglycerols (DG) from 2-MG, are protected against high fat diet induced obesity (Tsuchida et al., 2012; Yen et al., 2009). In addition, transgenic mice that over-express monoacylglycerol lipase (MGL) specifically in the small intestine displayed obesity and adiposity (Chon et al., 2012). However, in contrast to LCFA, the physiological role of 2-MG on food intake has not been well investigated. In Chapter 3, the effects of intestinal 2-MG on food intake were investigated to elucidate the physiological function of 2-MG. In addition to food intake, since LCFA reportedly not only decreases food intake but also induces diarrhea (Ammon and Phillips, 1974), the effect of 2-MG on diarrhea induction was also investigated and compared with that of LCFA.

Since 2-MG is a substrate of MGAT2, the inhibition of MGAT2 is considered to increase intestinal 2-MG and may alter food intake and body weights. In MGAT2 KO mice (Banh et al., 2015; Tsuchida et al., 2012; Yen et al., 2009), MGAT2 deficiency led to decreased body weight gain when the mice were fed a high-fat diet but not when the mice were fed a normal diet. In addition, MGAT2 KO mice were protected from metabolic disorders, such as glucose intolerance and fatty liver. Therefore, MGAT2 inhibition is a potential novel strategy for the treatment of obesity and related diseases. However, the effect of MGAT2 inhibition on food intake is controversial. Moreover, the mechanism of the antiobesity effects of MGAT2 inhibition, such as changes in intestinal lipid components by inhibiting MGAT2, has not been sufficiently investigated.

JTP-103237 is a novel MGAT inhibitor that selectively inhibits MGAT2 and is generated at Japan Tobacco Central Pharmacological Research Institute. In Chapter 4,

the effects of MGAT inhibitor, JTP-103237, on body weight, food intake and glucose tolerance were investigated in a high fat diet induced obesity model to elucidate the antiobesity effects of MGAT inhibition. In addition, to clarify the mechanism of the antiobesity effects, intestinal lipid composition was investigated after MGAT inhibitor treatment.

Fatty liver disease, which is characterized by an increase in hepatic TG content, is associated with obesity (Fabbrini et al., 2010) and is observed in up to 94% of obese individuals. Fatty liver disease is rapidly becoming the most common liver disease worldwide, including Japan (Bellentani et al., 2010; Jimba et al., 2005). The incidence of fatty liver disease is expected to increase in the future, since onset is strictly linked to lifestyle habits, in particular the excessive intake of sucrose which is known to increase hepatic TG content (Yasutake et al., 2014). Although fatty liver disease is regarded as a precursor of numerous diseases, such as nonalcoholic steatohepatitis (NASH), diabetes and atherosclerosis (Hamaguchi et al., 2005; Lonardo et al., 2015; Targher et al., 2007), medications for this disease are not yet available. Therefore, an improvement in both obesity and fatty liver disease is considered possible meaningful treatment. MGAT2 is mainly expressed in intestinal tissues but is also expressed in extraintestinal tissues, such as liver (Hall et al., 2012; Mostafa et al., 1993). Some reports have pointed out that hepatic MGAT2 may be involved in fatty liver disease (Hall et al., 2012; Nelson et al., 2014). However, the roles of MGAT in the liver are still unclear. In Chapter 5, to clarify the role of MGAT2 in the liver, the effects of MGAT inhibitor, JTP-103237, on hepatic TG content was investigated in a high sucrose diet induced fatty liver model. Furthermore, the effects of MGAT inhibition on TG synthesis and de novo lipogenesis

in the liver were also investigated to clarify the mechanisms of MGAT inhibition and its effects on hepatic lipid metabolism.

# Chapter 2

# Mechanism of the inhibitory effect of histamine on amygdaloid-kindled seizures in rats

## INTRODUCTION

We have demonstrated that the central histaminergic system is closely related to epilepsy. For instance, histidine and metoprine showed inhibitory effects against amygdaloid-kindled seizures at doses that showed increases in histamine contents in the brain (Kamei et al., 1998). In addition, $H_3$ receptor antagonists, thioperamide and clobenpropit, inhibited amygdaloid-kindled seizures, and this effect was antagonized by $H_1$ receptor antagonists (Kakinoki et al., 1998). The inhibitory mechanism of histamine on kindled seizures is, however, still unclear.

Conversely, it is well known that histamine $H_1$ receptors are coupled with phospholipase C–dependent breakdown of phosphoinositides, which regulates the intracellular calcium concentration (Leurs et al., 1995). As a result, various tissue responses are observed, and the activation of other enzymes such as protein kinase C (PKC) and calcium calmodulin-dependent protein kinase II (CaM kinase II) was recognized (Hill et al., 1997; Leurs et al., 1995). In contrast to these findings, intracellular and extracellular calcium concentrations are known to be related to seizure activity. Reportedly, incubation of hippocampal slices in very low calcium-containing medium can induce synchronous bursting activity (Jefferys and Haas, 1982). It has been also found that exposure of the stimulation site to artificial cerebrospinal fluid (CSF) containing normal levels of calcium can completely block the kindled seizure event (Davies and Peterson, 1989). Furthermore, exposure of the stimulation site to artificial

CSF without calcium resulted in a return of the complete seizure. On the basis of these reports, inhibition of amygdaloid-kindled seizures induced by histamine appears to be closely associated with increased intracellular calcium levels in rats.

The present study was performed to clarify the mechanism of the inhibitory effect of histamine, especially the participation of calcium ions, on amygdaloid-kindled seizures in rats.

## MATERIALS AND METHODS

### Animals and Chemicals

Male Wistar rats, 7–8 weeks old and weighing 200–250 g (N = 80), were used (Nippon SLC, Shizuoka, Japan). All animals were maintained in an air-conditioned room with controlled temperature (24 ± 2°C) and humidity (55 ± 15%). The animals were given food and water ad libitum. All procedures involving animals were conducted in accordance with the guidelines of the Animal Care and Use Committee, Faculty of Pharmaceutical Sciences, Okayama University.

The chemicals used were histamine dihydrochloride (Wako, Osaka, Japan), diphenhydramine hydrochloride (Sigma, St. Louis, MO, U.S.A.), zolantidine dimaleate (kindly provided by Smith Kline and Beecham), calcium chloride dihydrate (Wako), A23187 (Sigma), ethylene glycol tetraacetic acid (EGTA) (Sigma), EGTA tetra acetoxymethyl ester (EGTA/AM) (Nacalai Tesque, Kyoto, Japan), calphostin C (Wako) and KN62 (Wako). Histamine and calcium chloride were dissolved in artificial CSF containing 119 mM NaCl, 3.3 mM KCl, 1.3 mM CaCl2, 1.2 mM MgCl2, 0.5 mM Na2HPO4, 21 mM NaHCO3, and 3.4 mM d-glucose. EGTA was dissolved in calcium-free artificial CSF. EGTA/AM dissolved in calcium-free artificial CSF

containing 10% dimethylsulfoxide (DMSO). A23187, calphostin C, and KN62 were dissolved in artificial CSF containing 10% DMSO. Diphenhydramine was dissolved in saline. Histamine, calcium chloride, A23187, EGTA, EGTA/AM, calphostin C, and KN62 were injected intracerebroventricular (i.c.v.). I.c.v. injection was performed through an injection cannula fitted inside the guide cannula, and for all drugs, 5 μl per head was given within 30s (Kamei et al., 1983). Diphenhydramine was administered intraperitoneally. When the drug test was done, the amygdala of the rat was stimulated electrically, and the threshold of kindled seizures (seizure stage, 5; AD duration ~50 s) was determined. Then the drugs were injected i.c.v. or i.p. Drugs were administered at intervals of ≥7 days when the same animals were used for repeated experiments.

**Surgery**

Under pentobarbital anesthesia (35 mg/kg, i.p., Nembutal, Abbot Laboratories, North Chicago, IL, U.S.A.), the rats were fixed to a stereotaxic apparatus (Narishige, SR-5, Tokyo, Japan), and electrodes were implanted into the right amygdala (A: 5.0, L: 5.0, H: −2.5) according to the atlas of de Groot (De Groot, 1959). The electrodes were bipolar twisted stainless steel wires 200 μm in diameter. A guide cannula made of stainless steel tubing with an outside diameter of 700 μm was implanted into the right lateral ventricle (A: 5.4, L: 1.5, H: 3.0). Electrodes were connected to a miniature receptacle, which was embedded in the skull with dental cement. At least 2 weeks was allowed for recovery from surgery.

**Experimental Procedures in Kindled Seizures**

The animals were placed in a Plexiglas observation chamber (20×35×25 cm).

Bipolar EEGs were recorded with an electroencephalograph (EEG-7209; Nihon Kohden, Tokyo, Japan). Bipolar stimulation of the amygdala was applied every day with a constant electric stimulator (SEN-3301, SS-102J; Nihon Kohden) and continued until a generalized convulsion was obtained. Stimulation parameters were pulse duration of 1.0 ms, frequency of 60 Hz, and train duration of 1.0 s at intensity just sufficient to induce an afterdischarge (AD, 100–300 μA). Convulsive behavior was divided into five stages: (a) jaw movement, (b) head nodding, (c) forelimb clonus, (d) kangaroo posture, and (e) kangaroo posture and falling back (Fig. 1). AD duration was defined as high-voltage spike-and-wave complex observed in the right amygdala. After the animals developed the final stage of generalized seizures (kangaroo posture and falling back), stimulation was repeated for 5 more days to establish completely kindled rats. Then the animals were rested for 7 days. Seizure stage (convulsive behavior) and AD duration were used as indices of kindled seizures.

**Histologic Experiments**

After the experiments, the animals were killed, and localization of the electrodes in the brain was verified histologically. In this experiment, localization of the electrodes in the brain was checked grossly during dissection of the brain region.

**Statistical Analysis**

All data are expressed as the mean ± or + standard deviation. The statistical significance of differences in AD duration was determined by analysis of variance (ANOVA) with Dunnett's test. For statistical analysis of seizure stage, the Kruskal–Wallis test with post hoc Steel’s test was used.

## RESULTS

### Effects of Histamine on Amygdaloid-kindled Seizures

The effects of histamine on amygdaloid-kindled seizures are shown in Fig. 2 and 3. I.c.v. injection of histamine caused dose-dependent inhibition of both seizure stage and AD duration of amygdaloid-kindled seizures. At a dose of 2 μg, it elicited no significant inhibition, but at a dose of 5 μg, histamine caused significant inhibition of both seizure stage (20 and 30 min after injection) and AD duration (10, 20, 30, and 45 min). At a dose of 10 μg, significant inhibition was noted from 10 to 90 min after injection in both seizure stage and AD duration. Pretreatment with $H_1$ receptor antagonists, diphenhydramine (5 and 10 mg/kg, intraperitoneally), resulted in significant antagonism of histamine-induced inhibition (Table 1). However, the $H_2$ receptor antagonist zolantidine showed no antagonism of the inhibition of kindled seizures induced by histamine, even at a dose of 50 mg/kg, intraperitoneally (Table 1).

### Effects of Calcium Chloride and A23187 on Amygdaloid-kindled Seizures

I.c.v. injection of calcium chloride at doses of 10–50 μg caused dose-dependent inhibition of amygdaloid-kindled seizures (Fig. 4). For seizure stage, significant inhibition was observed at a dose of 50 μg (20 and 30 min after injection). AD duration was shortened by calcium chloride at 20 μg (30 min) and 50 μg (30 min; Fig. 3 and 4). A23187, a calcium ionophore, also significantly inhibited seizure stage at a dose of 10 μg (30 min). AD duration was also shortened by 5 μg (30 min) and 10 μg (30 min; Fig. 4).

**Effects of Calcium Chloride and A23187 on the Inhibition of Amygdaloid-kindled Seizures Induced by Histamine**

I.c.v. injection of calcium chloride at a dose of 10 μg, which showed no significant effect on amygdaloid-kindled seizures when used alone, significantly potentiated the effects of histamine (2 μg). Similar results were observed with A23187 (Table 2).

**Effects of EGTA and EGTA/AM on the Inhibition of Amygdaloid-kindled Seizures Induced by Histamine**

EGTA at a dose of 20 μg, i.c.v., showed a significant antagonizing effect on the inhibition of kindled seizures induced by histamine. The same results were obtained with EGTA/AM at doses of 5 and 10 μg, i.c.v. (Table 3). EGTA and EGTA/AM showed no significant effects on kindled seizures when used separately at doses of 20 and 10 μg, respectively (Table 3).

**Effects of Calphostin C and KN62 on the Inhibition of Amygdaloid-kindled Seizures Induced by Histamine**

Pretreatment with calphostin C, a PKC inhibitor, resulted in no significant antagonism of the histamine-induced inhibition of kindled seizures even at a dose of 20 μg, i.c.v. Conversely, pretreatment with KN62, a CaM kinase II inhibitor, showed a significant antagonizing effect on histamine-induced inhibition of amygdaloid-kindled seizures at a doses of 5 and 10 μg, i.c.v., in both seizure stage and AD duration (Fig. 5). Calphostin C and KN62 showed no significant effect on kindled seizures when used separately at doses of 20 and 10 μg, respectively (data not shown).

## DISCUSSION

In the present study, it was confirmed that the $H_1$-histaminergic system is closely related to amygdaloid-kindled seizures by i.c.v. injection of histamine and i.p. injection of $H_1$ and $H_2$ receptor antagonists. It also was found that inhibition of amygdaloid-kindled seizures induced by i.c.v. injection of histamine was potentiated by i.c.v. injection of calcium chloride and A23187 at doses showing no apparent effects on amygdaloid-kindled seizures when used alone. Conversely, high doses of calcium chloride and A23187 dose-dependently inhibited amygdaloid-kindled seizures. It has been reported that decreases in extracellular free calcium level resulted in increases in seizure activity in CA1 hippocampus pyramidal cells (Jefferys and Haas, 1982). In addition, it has been reported that CSF containing normal levels of calcium is able to block kindled seizures and that CSF without calcium resulted in the return of seizures (Davies and Peterson, 1989). This may be why changes in intracellular calcium levels are important in causing kindled seizures, and that inhibition of amygdaloid-kindled seizures induced by histamine is closely associated with the calcium ion influx pathway. This suggestion also was supported by the present findings that i.c.v. injection of calcium chelators (EGTA and EGTA/AM) antagonized the inhibition of amygdaloid-kindled seizures induced by histamine.

The i.c.v. injection of CaM kinase II inhibitor KN62 antagonized the inhibition of kindled seizures induced by histamine. However, the PKC inhibitor calphostin C showed no antagonism of the effect of histamine. Butler et al. (Butler et al., 1995) reported that CaM kinase mutant mice exhibit a marked increase in brain excitability evident in epileptic seizures; however, PKC mutant mice are not associated with increased excitability. CaM kinase II is the major calcium-regulated transduction system

in the brain (Browning et al., 1985; Levitan, 1994; Nairn et al., 1985). CaM kinase II also has been reported to play a crucial role in modulation of neurotransmitter release (Llinas et al., 1985), neurotransmitter biosynthesis (Yamauchi and Fujisawa, 1983), and neuronal excitability (Churn et al., 1991). Models of increased neuronal excitability such as kindling (Bronstein et al., 1990; Bronstein et al., 1992; Goldenring et al., 1986) and seizure activity (Kochan et al., 2000; Perlin et al., 1992) show significantly reduced CaM kinase II activity. Reportedly, CaM kinase II was activated by histamine via $H_1$ receptors (Hill et al., 1997; Leurs et al., 1995). This is why histamine caused inhibition of kindled seizures, and KN62 antagonized the effect of histamine. However, because of the limited number of compounds and concentrations used and the complexities associated with i.c.v. dosage, these conclusions must be regarded as tentative.

It has been reported that inhibition of amygdaloid-kindled seizures induced by histaminergic drugs was antagonized by bicuculline, a specific γ-aminobutyric acid $(GABA)_A$ receptor antagonist (Ishizawa et al., 2000). In addition, CaM kinase II activation reportedly modulates $GABA_A$ receptor function (Churn and DeLorenzo, 1998; Machu et al., 1993; Wang et al., 1995) and GABA release (Ishikawa et al., 1990). Activation of CaM kinase II–dependent phosphorylation caused an increase in $GABA_A$-receptor binding and resulted in a decrease of neuronal excitability by increasing GABA function.

Therefore, we propose the hypothesis that the mechanism of inhibition of amygdaloid-kindled seizures induced by histamine may be associated with the CaM kinase II activation pathway.

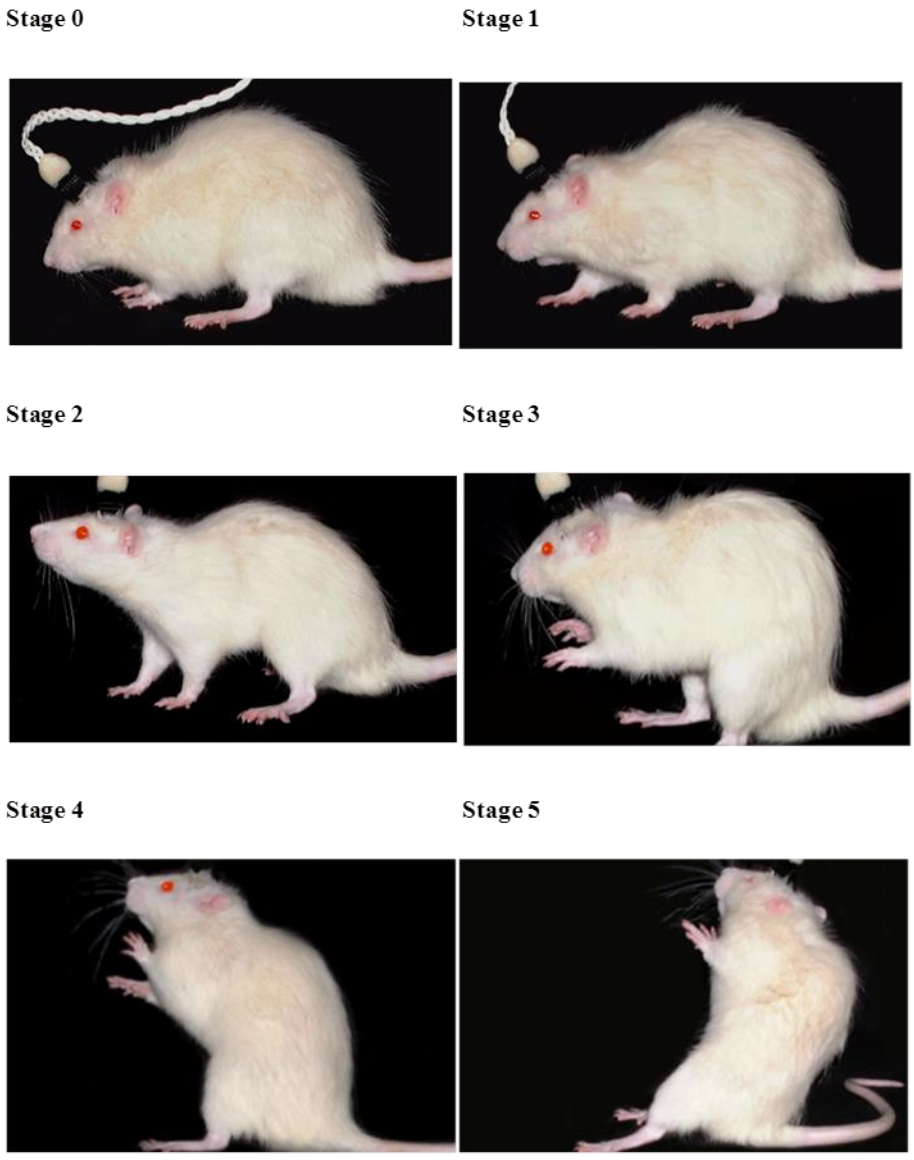


**Figure 1.** Developmental stages of amygdaloid kindled seizures.

Stage 0; normal, Stage 1; jaw movement, Stage 2; head nodding, Stage 3; forelimb clonus, Stage 4; kangaroo posture, Stage 5; kangaroo posture and falling back.

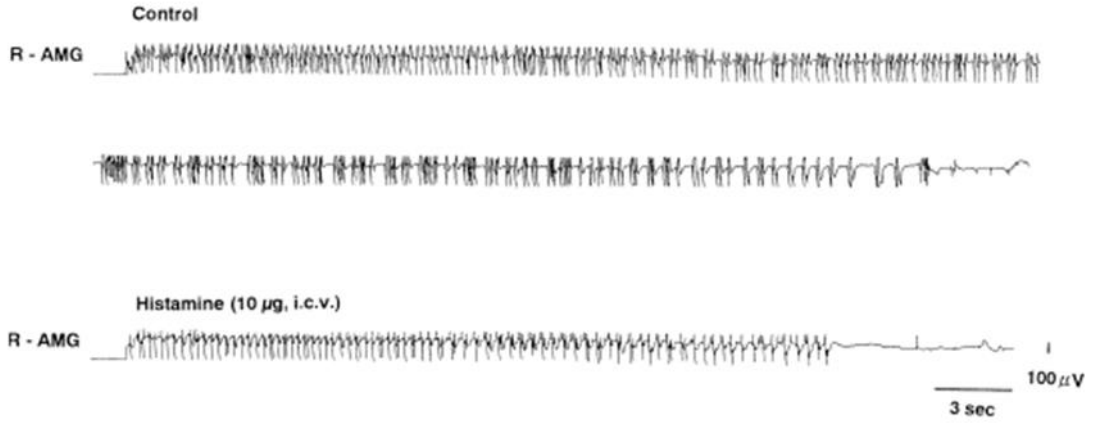


**Figure 2.** Representative example for the effects of histamine on amygdaloid-kindled seizures in rats. Control: Twenty minutes after saline (5 μl, i.c.v.) injection. Histamine : Twenty minutes after Histamine (10 μg/5 μl, i.c.v.) injection.

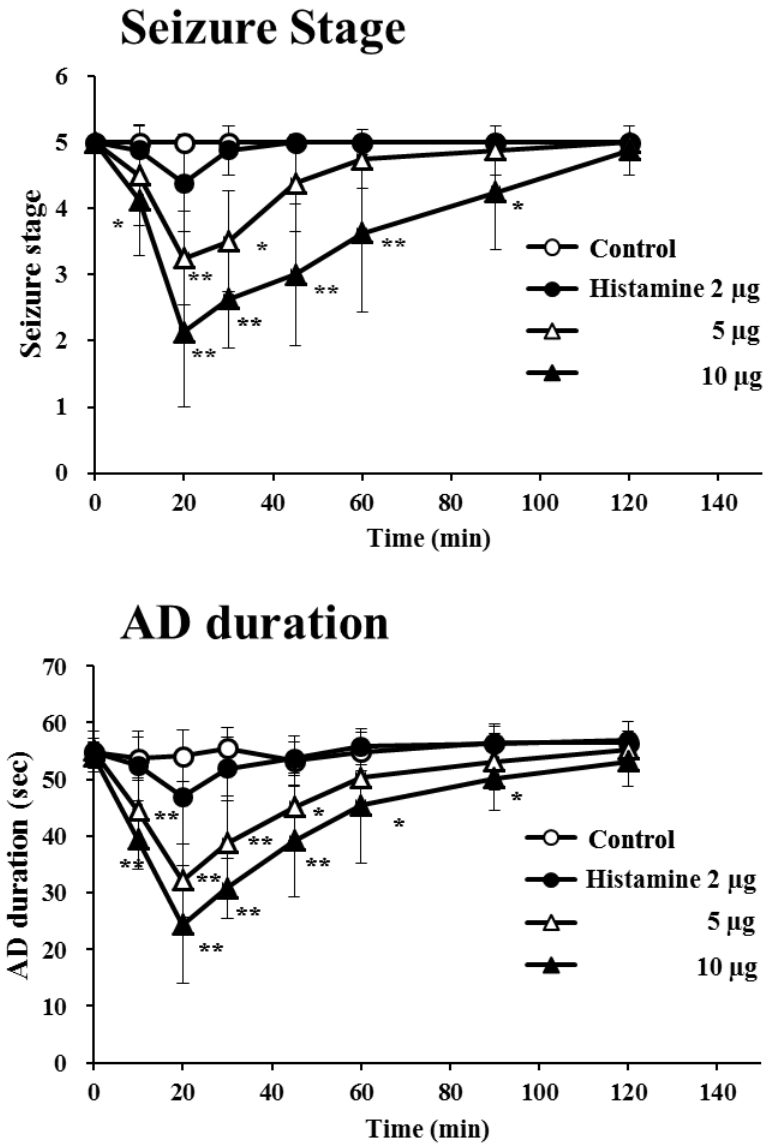


**Figure 3.** Effects of histamine on amygdaloid-kindled seizures in rats. Data shown as means ± standard deviation (n = 8). *p<0.05, **p<0.01; significantly different from the Control group.

**Table 1.** Effects of $H_1$ and $H_2$ receptor antagonists on the inhibition of amygdaloid-kindled seizures by histamine (10 μg, i.c.v.).

| Drugs | Dose (mg/kg) | Seizure stage | AD duration (sec) |
|---|---|---|---|
| Histamine | - | 2.1 ± 1.4 | 24.4 ± 11.8 |
| Histamine | 1 | 2.7 ± 2.2 | 32.8 ± 15.5 |
| + | 5 | 4.3 ± 1.2* | 42.8 ± 12.5* |
| Diphenhydramine | 10 | 4.7 ± 0.7** | 47.5 ± 9.8** |
| Histamine | 10 | 2.5 ± 1.6 | 29.5 ± 13.1 |
| + | 20 | 2.7 ± 2.5 | 33.2 ± 21.1 |
| Zolantidine | 50 | 2.7 ± 2.3 | 26.1 ± 19.5 |

Data shown as means ± standard deviation (n = 8).

*p<0.05, **p<0.01; significantly different from the Histamine treated group.

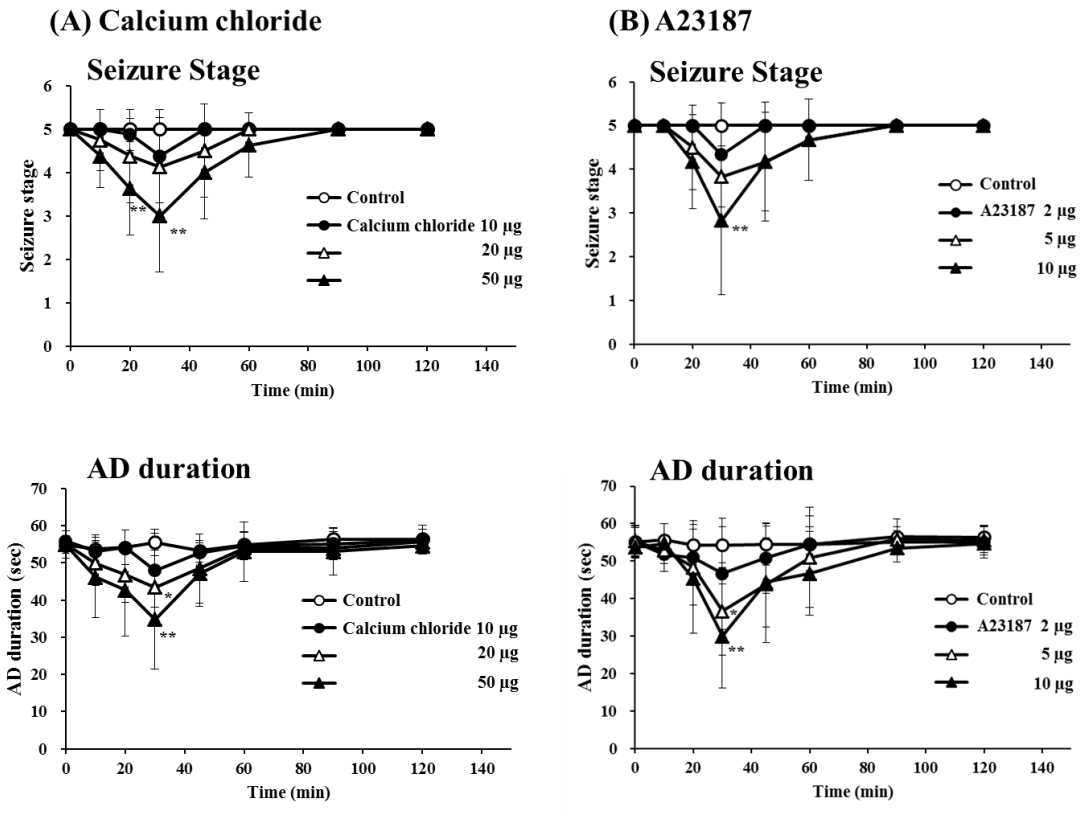


**Figure 4.** Effects of calcium chloride (A) and A23187 (B) on amygdaloid-kindled seizures in rats. Data shown as means ± standard deviation (n = 8). *p<0.05, **p<0.01; significantly different from the Control group.

**Table 2.** Effects of calcium chloride and A23187 on the inhibition of amygdaloid-kindled seizures induced by histamine (2 μg, i.c.v.).

| Drugs | Dose (μg, i.c.v) | Seizure stage | AD duration (sec) |
|---|---|---|---|
| Histamine | - | 4.5 ± 0.9 | 44.6 ± 5.6 |
| Histamine + Calcium chloride | 5 | 4.1 ± 1.1 | 39.6 ± 8.9 |
| | 10 | 2.6 ± 1.2* | 28.4 ± 5.8** |
| Histamine | - | 4.6 ± 0.8 | 46.7 ± 7.5 |
| Histamine + A23187 | 1 | 4.0 ± 1.4 | 37.4 ± 15.3 |
| | 2 | 2.6 ± 1.8* | 27.9 ± 16.1* |

Data shown as means ± standard deviation (n = 8).

*p<0.05, **p<0.01; significantly different from the Histamine treated group.

**Table 3.** Effects of EGTA (20 μg, i.c.v.) and EGTA/AM (10 μg, i.c.v.) on the inhibition of amygdaloid-kindled seizures induced by histamine (10 μg, i.c.v.).

| Drugs | Dose (mg/kg) | Seizure stage | AD duration (sec) |
|---|---|---|---|
| Histamine | - | 2.1 ± 1.4 | 28.0 ± 10.9 |
| EGTA | - | 5.0 ± 0.0 | 56.0 ± 5.0 |
| Histamine | 5 | 2.8 ± 1.9 | 33.2 ± 16.9 |
| + | 10 | 3.8 ± 1.9 | 42.2 ± 8.7 |
| EGTA | 20 | 4.6 ± 0.7* | 48.0 ± 7.8* |
| Histamine | - | 2.3 ± 1.7 | 24.4 ± 9.7 |
| EGTA/AM | - | 5.0 ± 0.0 | 53.8 ± 4.9 |
| Histamine | 2 | 2.5 ± 2.1 | 25.0 ± 18.5 |
| + | 5 | 4.4 ± 0.7* | 40.8 ± 7.1* |
| EGTA/AM | 10 | 4.8 ± 0.5** | 49.4 ± 5.9** |

Data shown as means ± standard deviation (n = 8).

*p<0.05, **p<0.01; significantly different from the Histamine treated group.

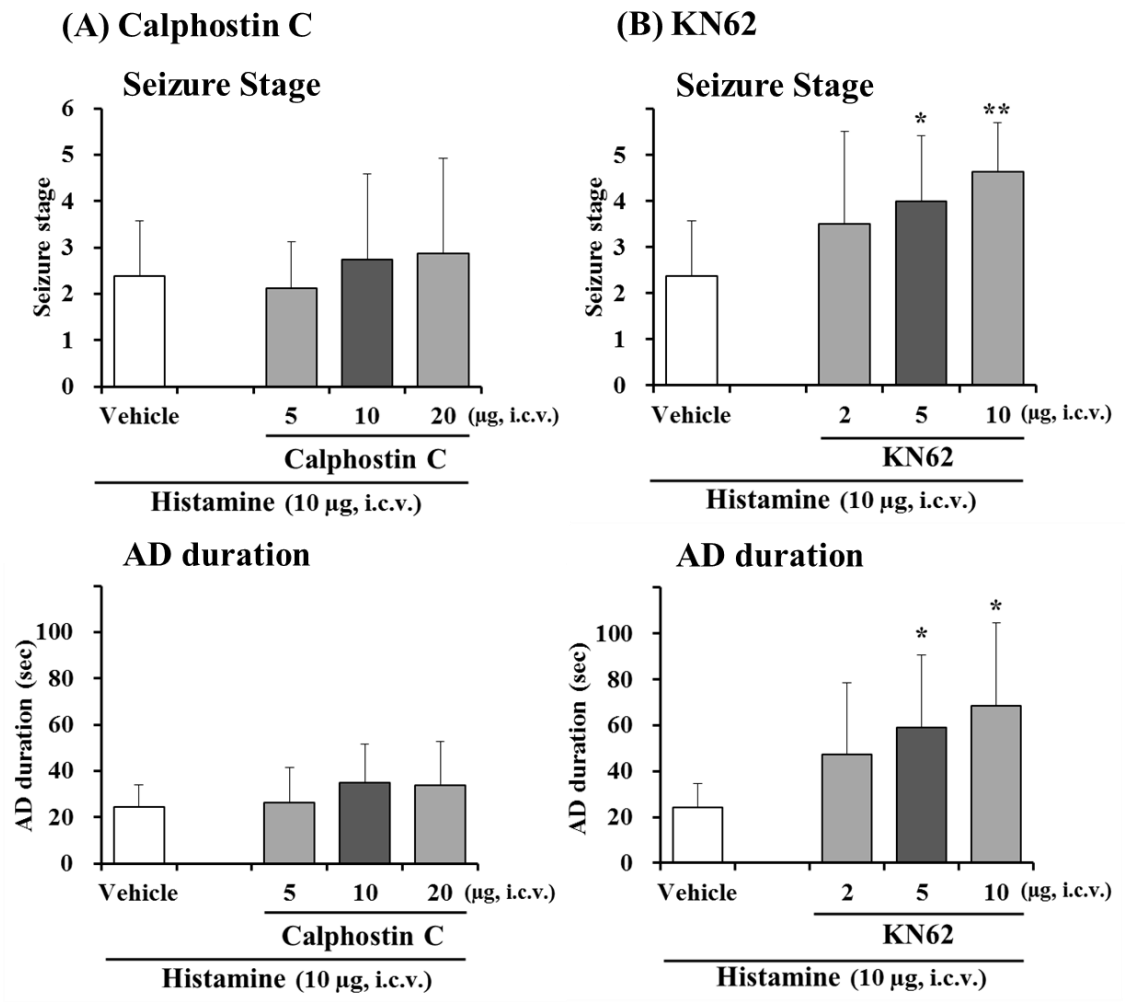


**Figure 5.** Effects of calphostin C (A) and KN62 (B) on the inhibition of amygdaloid-kindled seizures induced by histamine. Data shown as means + standard deviation (n = 8).*p<0.05, **p<0.01; significantly different from the Vehicle and Histamine treatment group.

# Chapter 3

# Intrajejunal infusion of 2-monoacylglycerol reduced food intake without inducing diarrhea in rats

## INTRODUCTION

The physiological regulation of food intake involves signals from the gastrointestinal tract as well as from the central nervous system. Indeed, dietary fat, carbohydrate and protein stimulate satiety signals (Leidy et al., 2015; Chapman et al., 1999; Blundell et al., 1994). Understandably, since overconsumption of nutrients consequently causes an over intake of calories and obesity, this satiety signal may be against overeating. On the other hand, overconsumption of nutrients also causes malabsorption, which leads to gastrointestinal symptoms.

LCFA and 2-MG are both digestive products of dietary TG hydrolysis. Typically, these digestive products are absorbed in the upper intestine, however, if these products reach to the distal intestine, they may stimulate a feedback signal for satiety (Samra, 2010; Spiller et al., 1984; Welch et al., 1985). The function of LFCA, one of the digestive products of TG, in the intestine has been well investigated. LCFA reportedly suppresses appetite in humans (Feltrin et al., 2004) and rats (Ogawa et al., 2012; Woltman and Reidelberger, 1995). The mechanism of induction of satiety by intestinal LCFA is mainly understood via gastrointestinal peptide secretion such as glucagon-like peptide-1 (GLP-1), peptide YY (PYY) and cholecystokinin. On the other hand, LCFA stimulates not only satiety, but gastrointestinal symptoms. The malabsorption of LCFA is known to induce diarrhea (Yoshioka et al., 1986). In contrast, the function of 2-MG in

the intestine is still unclear. In the present study, the effect of 2-MG on food intake and diarrhea was evaluated and compared to the effect of LCFA in rats. To correctly investigate the effect in the intestine, 2-MG and LCFA were directly infused into the jejunum.

## MATERIALS AND METHODS

### Animals

Male Sprague Dawley (SD) rats were purchased from Charles River Laboratories (Yokohama, Japan). Rats were maintained with free access to water and a normal chow diet (CRF-1, Charles River Japan) and housed in a room controlled for temperature at 23 ± 3°C and humidity of 55 ± 15% in 12-h light/dark cycles (lights on from 8:00 AM to 8:00 PM). All procedures were conducted according to guidelines from Japan Tobacco's Animal Care Committee.

### Itrajejunal Cannulation

Male SD rats were purchased from Charles River Laboratories (Yokohama, Japan). Rats were maintained with free access to water and a normal chow diet (CRF-1, Charles River Japan) and housed in a room controlled for temperature at 23 ± 3°C and humidity of 55 ± 15% in 12-h light/dark cycles (lights on from 8:00 AM to 8:00 PM). All procedures were conducted according to guidelines from Japan Tobacco's Animal Care Committee.

Prior to surgery, the rats were fasted overnight and then anesthetized with 50 mg/kg of intraperitoneal pentobarbital sodium (Abbott Laboratories, Chicago, IL). After abdominal celiotomy, a polyethylene tube (SP28; I.D. 0.4 mm, O.D. 0.8;mm, Natsume,

Tokyo, Japan) was inserted into the duodenum (5 cm from the pylorus). The end of the tube reached the proximal jejunum (10 cm from the pylorus). The tube was fixed to the entrance of the intestine with silk sutures. The opposite end of the tube was threaded through the opening in the abdominal wall and tunneled subcutaneously to the dorsal surface of the neck, where a Dacron mesh anchor button (DC95; Instech Solomon, Plymouth Meeting, PA) was implanted. The tube was also fixed to the ventral abdominal wall and the anchor button mesh using silk sutures. The rats were allowed to recover for 1 week and become sufficiently adapted to the continuous infusion apparatus (Instech Solomon) before experiments were performed.

**Feeding Test**

In the feeding test, the chow was removed and immediately after that, 2-MG (2-monolein, synthesized by Japan Tobacco Central Pharmacological Research Institute), LCFA (linoleic acid, commercial grade (~60% linoleic acid, ~30% oleic acid), Sigma, St. Louis, MO), or saline were infused intrajejunally for 4 hour in rats. Since rats mainly feed during the dark period, the feeding was restarted at beginning of dark period (1 hour after starting the infusion). Cumulative food intake was measured for 3 hours, 6 hours and 23 hours.

**Cecal Water Contents**

Whole cecal contents were collected from scarified rats at 4 hours after infusion of respective lipids or saline. Total water contents were calculated from the differences in cecal contents weight before and after drying.

**GLP-1 levels**

At 2 hours after starting the infusion (1 hours after starting feeding), the rats were anesthetized with diethyl ether and blood was collected from the portal vein into siliconized tubes on ice containing approximately 5% of ethylenediaminetetraacetic acid (EDTA), aprotinin, and a dipeptidyl peptidase IV (DPP-IV) inhibitor (final concentrations of EDTA, aprotinin and DPP-IV inhibitor (Millipore Corporation) were 6 mmol/l, $1\times10^3$ KIU/ml and 50 μmol/l, respectively). Plasma was isolated by centrifugation at 10,000 x g for 30 min at 4°C. Plasma active GLP-1 levels were measured via a sandwich enzyme immunoassay (EIA) using assay kits (Millipore)..

**Statistical Analysis**

The differences of food intake, incidence of diarrhea and cecal water contents between saline and respective lipid infusions were compared using Paired t-tests, Fisher's exact test and Turkey's multiple comparison tests, respectively.

**RESULTS**

**The Effects of Intrajejunal 2-MG and LCFA Infusion on Food Intake in Rats.**

In the experiment 1, we evaluated the effect of 2-MG and LCFA on food intake in three separate crossover studies. 2-MG at a rate of 200 μL/hr, 300 μL/hr and LCFA at a rate of 200 μL/hr were infused to separate animals. The jejunal infusion of respective lipids or saline was performed on separate days. In experiment 1, the intrajejunal infusion of 2-MG resulted in reduced food intake at both infusion rate of 200 μL/hr and 300 μL/hr in rats (Fig. 6). This effect in the 300 μL/hr 2-MG treated group appeared to be clearer than in the 200 μL/hr 2-MG treated group. The effect in the 300 μL/hr of

2-MG treated group was continuously observed after the completion of infusion. Similar to another report (8), the intrajejunal infusion of LCFA reduced food intake in our experiment. The efficacy in the 300 μL/hr 2-MG and the 200 μL/hr LCFA treated groups seemed comparable. In the preliminarily study, both 2-MG and LCFA tended to increase portal plasma GLP-1 levels (26 and 29 pM in the saline treated group (n=2), 107, 76 and 190 pM in the 2-MG treated group (n=3) and 218, 134 and 115 pM in the LCFA treated group (n=3)).

**Comparison of the effects of intrajejunal 2-MG, LCFA and saline infusion on diarrhea in rats**

As shown in Table 4, in LCFA treated rats, diarrheal stools were observed in 4 out of 6 rats 23 hours after starting feeding and this change was statistically significant. In contrast, in 2-MG treated rats, diarrheal stools were not observed in all rats.

**Comparison of the effects of intrajejunal 2-MG, LCFA and saline infusion on cecal water contents in rats**

As shown in Fig. 7, the intrajejunal infusion of LCFA significantly increased cecal water contents. The valuse in LCFA treated group was 2 times higher as compared to that in saline treated group. In contrast, the intrajejunal infusion of 2-MG did not change cecal water contents.

**DISCUSSION**

In experiment 1, the intrajejunal infusion of 2-MG significantly reduced food intake. Similar to another report (Ogawa et al., 2012), the intrajejunal infusion of LCFA

reduced food intake and the efficacy in the 300 μL/hr 2-MG and the 200 μL/hr LCFA treated groups seemed comparable.

As mentioned above, nutrients are known to simulate satiety to help prevent overeating. Considering this, 2-MG, one of the digestive products of TGs, may stimulate satiety signals as a feedback mechanism for overeating. In the preliminarily study, both 2-MG and LCFA tended to increase portal plasma GLP-1 levels. Therefore, increases in GLP-1 levels may partly contribute to the effect of 2-MG on food intake. However, the exact mechanisms for the satiety effect of 2-MG and decreases in food intake are still unclear. Further investigations are needed to clarify the contribution of gastrointestinal peptide secretion (including PYY and cholecystokinin) and/or the vagal afferent pathway for this effect of 2-MG.

In experiment1, when food weight was measured, we noticed that some diarrheal stools were observed in LCFA treated rat but not in 2-MG treated rats. We therefore postulated that the sensitivity for gastrointestinal symptoms, such as diarrhea, is different between LCFA and 2-MG. We then compared the effect of intrajejunal infusion of 2-MG and LCFA on diarrhea (experiment 2). LCFA induced diarrhea in 4 out of 6 rats 23 hours after starting feeding. In contrast, 2-MG did not induce diarrhea in all rats. LCFA reportedly inhibited water and electrolyte absorption in the ileum, which may lead to steatorrhea and diarrhea in humans (Ammon and Phillips, 1974). Therefore, diarrhea induced by LCFA is interpreted that secretory diarrhea. Indeed, intrajejunal infusion of LCFA induced watery diarrhea in experiment 2. However, even though the efficacy of 2-MG on food intake reduction was almost the same as compared to LCFA, 2-MG did not induce diarrhea. To investigate into the mechanism of secretory diarrhea induction, we evaluated the water content in cecal contents in 2-MG (300 μL/hr) and

LCFA (300 μL/hr) treated rats at 3 hours after starting feeding in preliminary study. 2-MG was less likely to increase water content in the cecum as compared with LCFA. From these, there is a probability that the difference of reactivity on fluid secretion between 2-MG and LCFA contributes their diarrhea inducibility. Although in-depth investigations are needed to confirm these mechanisms, the difference of diarrhea inducibility by both TG digestive products is quite an interesting phenomenon.

We demonstrated that jejunal infusion of 2-MG reduced food intake in rats. The deletion of MGAT2, which converts 2-MG to DG, is known to reduce food intake in high fat diet fed mice (Nelson et al., 2014). In addition, transgenic mice that overexpress MGL, which converts monoacylglycerols to LCFA and glycerol specifically in small intestine, were hyperphagic (Chon et al., 2012).

In conclusion, these results may contribute to understanding the phenomenon in MGAT2 deficient mice, MGL transgenic mice and MGAT inhibitors. In addition, 2-MG did not induce diarrhea at the condition that it comparably reduced food intake as compared to LCFA, suggesting that 2-MG may become a unique and useful fat substitute in clinical, which stimulate satiety without inducing diarrhea.

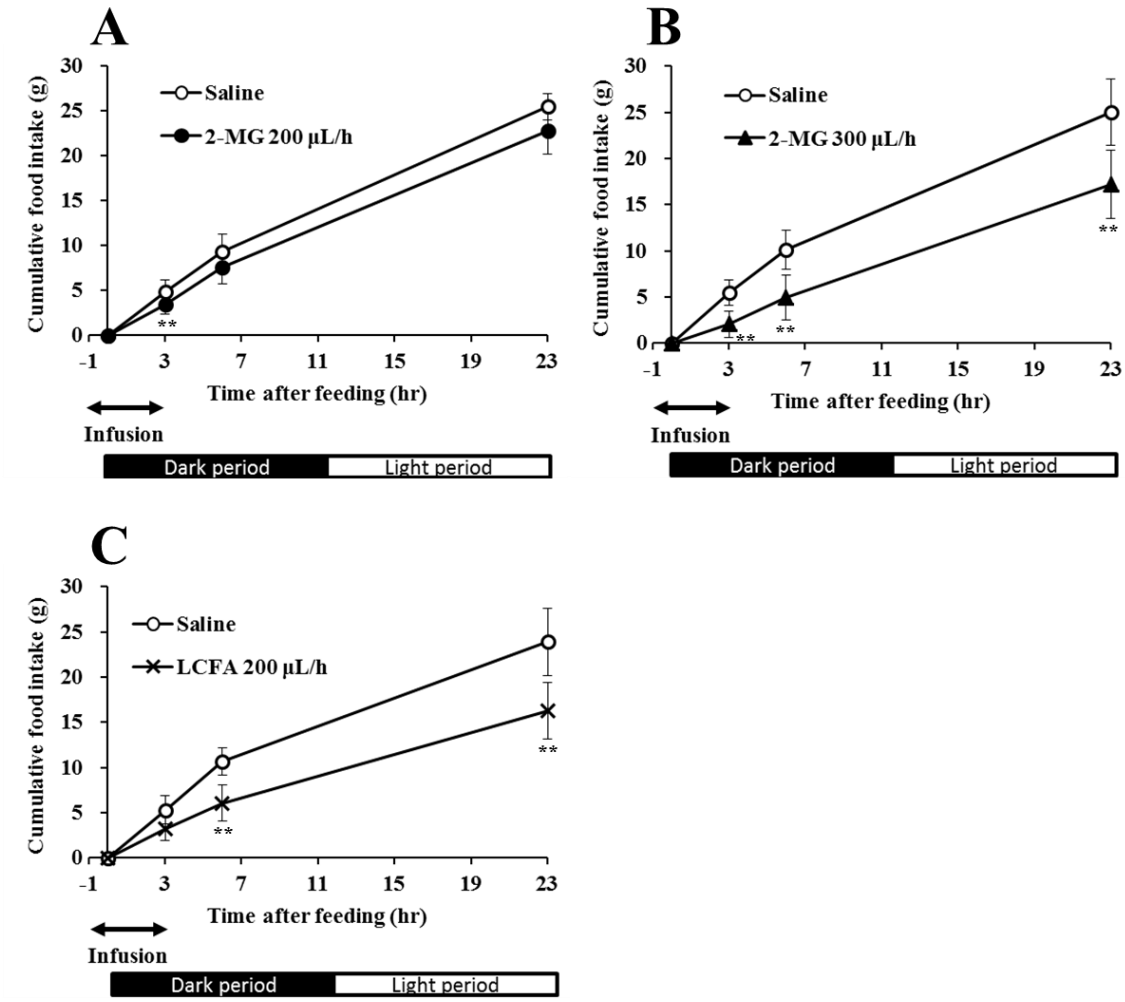


**Figure 6.** The effects of intrajejunal saline (A), 2-MG (B) and LCFA (C) infusion on cumulative food intake in rats. Data shown as means ± standard deviation ((A) n = 8, (B) and (C) n = 6). ***p* < 0.01; significantly different from the Saline treatment.

**Table 4.** Incidence of diarrhea after intrajejunal infusion of 2-MG (300 μL/hr, 4 hours) and LCFA (200 μL/hr, 4 hours) in rats.

| Treatment | Saline | 2-MG | LCFA |
|---|---|---|---|
| Number of animals observed diarrhea | 0 | 0 | 4 |
| Total number of animals | 6 | 6 | 6 |
| Significance | - | - | * |

*P<0.05; significantly different from the Saline and 2-MG treatment.

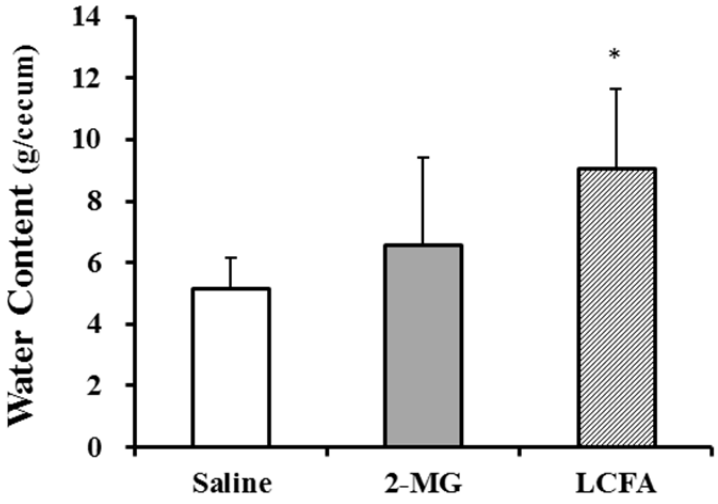


**Figure 7.** The effects of intrajejunal 2-MG, LCFA and saline infusion on cecal water contents in rats. Data shown as means + standard deviation (n = 5-6). *P<0.05; significantly different from the Saline treatment.

# Chapter 4

# JTP-103237, a novel monoacylglycerol acyltransferase inhibitor, modulates fat absorption and prevents diet-induced obesity

## INTRODUCTION

The prevalence of obesity is increasing around the world and effective treatment strategies are urgently needed to address the obesity epidemic (Halford et al., 2010; Rodgers et al., 2012). Obesity results from an imbalance between energy intake and energy expenditure (Hill et al., 2012). Understandably, an energy imbalance is caused by excessive dietary fat intake (Prentice and Poppitt, 1996) which has been implicated as promoting not only obesity, but also metabolic diseases such as type 2 diabetes (Bray and Popkin, 1998; van Dam et al., 2002).

MGAT catalyze in the first step of TG synthesis and are involved in dietary fat absorption. The formation of DG from 2-MG and fatty acyl CoA by MGAT is considered the rate-limiting step of triacylglycerol synthesis in intestine (Senior and Isselbacher, 1962; Yen and Farese, 2003). The glycerol 3-phosphate pathway, which is another pathway for TG synthesis, is mainly involved in the *de novo* TG synthesis in most tissues (Lehner and Kuksis, 1996). Three subtypes of MGAT have been identified. Among these, MGAT2 is highly expressed in the intestine and is expressed in both humans and rodents (Yen and Farese, 2003). Thus, it is likely that MGAT2 plays an important role in dietary fat absorption. In fact, the absorption of fat into circulation was significantly reduced in MGAT2 KO mice (Tsuchida et al., 2012). In addition, diet induced obesity, glucose intolerance and fatty livers were prevented in MGAT2 KO mice (Yen et al., 2009). Moreover, MGAT2 KO mice demonstrated increased energy

expenditure as compared to wild-type mice and the differences were most pronounced during the feeding period, suggesting that MGAT2 modulates diet-induced thermogenesis (Nelson et al., 2011; Yen et al., 2009). Thus, there is a strong possibility that inhibiting MGAT2 is a promising strategy for metabolic disorders caused by excessive fat intake, such as obesity and type 2 diabetes.

Although the physiological role of MGAT2 has been actively researched using MGAT2 KO mice, there have been no reports about the detailed pharmacological profile of MGAT2 inhibitors. Recently, we discovered the novel MGAT2 inhibitor, JTP-103237, which is a 7-(4,6-Di-tert-butyl-pyrimidin-2-yl)-3-(4-trifluoromethoxy-phenyl)-5,6,7,8-tetrahydro-[1,2,4]triazolo[4,3-a]pyrazine derivative. In this study, we evaluated the effects of JTP-103237 on lipid metabolism in the intestine, energy expenditure and diet-induced obesity.

## MATERIALS AND METHODS

### Animals and Chemicals

All animals were purchased from Charles River Laboratories (Yokohama, Japan). The animals were maintained on CRF-1 (Charles River Japan), as standard laboratory chow diets, and water ad libitum. For the evaluation of food consumption and for repeated administration studies, animals were given free access to water and experimental diets. The diets contained 3.1% and 35% (w/w) fat were purchased from Oriental Yeast Co. (Osaka, Japan). The animals were housed under specific pathogen-free conditions in a room controlled for temperature at 23 ± 3°C and humidity of 55 ± 15% in 12-h light/dark cycles (lights on from 8:00 AM to 8:00 PM). All

procedures were conducted according to guidelines from Japan Tobacco's Animal Care Committee.

JTP-103237 was synthesized in the Central Pharmaceutical Research Institute of Japan Tobacco Inc. (Osaka, Japan). [1-$^{14}$C] oleoyl-coenzyme A (oleoyl-CoA) was purchased from Amersham Biosciences. [carboxyl-$^{14}$C] triolein was purchased from PerkinElmer. All other chemicals were standard reagent grade.

**Enzyme Assays**

Recombinant human MGAT2 or human MGAT3 were cloned into pcDNA3.1 (+) vectors and transiently transfected into COS-7 cells using lipofectamine 2000. Membrane fractions of COS-7 cells were isolated as enzyme sources.

Recombinant human diacylglycerol acyltransferase (DGAT) 2 was cloned into pFASTBAC1 vectors and expressed in Sf9 insect cells using a baculovirus expression system. Sf9 cells were infected and membrane fractions isolated as enzyme sources as described by Cases et al (Cases et al., 1998). The reaction mixtures for human MGAT2 and human MGAT3 enzyme assays contained 100 mM Tris-HCl (pH 7.5), 250 mM Sucrose, 5 mM MgCl2, 0.05% bovine serum albumin (BSA), 0.05 mM 2-oleoyl glycerol and 1 μg of protein/ml of recombinant human MGAT2 or 15 μg of protein/ml of recombinant human MGAT3. The reaction mixtures for the human DGAT2 enzyme assay contained 100 mM Tris-HCl (pH 7.5), 250 mM Sucrose, 1 mM EDTA, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate,, 0.1% BSA, 0.2 mM 1,2-oleoyl-sn-glycerol and 30 μg of protein/ml of recombinant human DGAT2. Serial dilutions of JTP-103237 with DMSO were added to the reaction mixture at a final concentration of 5% DMSO. The reaction was initiated by adding 25 μM of [1-$^{14}$C]

oleoyl-CoA for a final volume of 100 µl and the mixture was incubated for 15 min at 25°C. The reaction was terminated by adding 500 µl chloroform: methanol (2:1) solvent. After mixing, the reaction mixture was centrifuged (2000 x g, 10 min) and lipids extracted in the organic phase were separated by thin layer chromatography (TLC) using a hexane:diethylether:acetic acid (80:30:2) solvent system. The radioactivity of synthesized [1-$^{14}$C] DG was measured using the FLA-7000 imaging system (Fuji film, Tokyo, Japan). Human, rat and mouse intestinal microsomal MGAT enzyme assays were performed in the same manner as the MGAT2 enzyme assay, except for the use of 15 µg/ml of human intestinal microsomes (KAC, Kyoto, Japan), 6 µg/ml rat intestinal microsomes or 4 µg/ml mouse intestinal microsomes instead of recombinant human MGAT2. IC50 values were calculated in a semilogarithmic proportional manner from the two points flanking 50% inhibition.

**Evaluation of Intestinal MGAT Activity**

These experiments were performed in order to select the dose in *in vivo* experiments. JTP-103237 (10, 30 and 60 mg/kg) or vehicle (0.5% methylcellulose) was administered orally to SD rats (7 weeks old). Small intestines were collected over time at 1, and 8 h after administration of JTP-103237 from individual rats. Collected portions of the intestine (almost 100 mg) were carefully washed and weighed. All of these samples were collected in the same portion of the small intestine. These samples were homogenized with 1 ml/100 mg tissue of homogenate buffer consisting of 0.25 M sucrose, 100 mM of Tris-HCl and a zirconia ball (YTZ® ball, Nikkato Corporation) using a mixer mill (Retsch Co., Ltd.). The homogenate was subjected to centrifugation at. 10,000 x g for 30 min at 4°C and the resulting supernatant was designated the

enzyme fraction (S9 fraction). MGAT activity in the enzyme fraction was measured using the same method described above.

In mice (8-week old male C57BL/6J mice), JTP-103237 (10, 30 and 100 mg/kg) or vehicle (0.5% methylcellulose) was administered orally and small intestines were collected 2 h after administration. The method for S9 perpetration and measurement of MGAT activity were also the same as the method mentioned above and the percent of the MGAT activity in the control group was calculated. Since the number of animals in these studies was 3 or less, statistical analysis was not performed.

**Evaluation of Fat Absorption**

12 to 14-week old male C57BL/6J mice were fasted before turning off the lights on the day prior to administration. JTP-103237 (100 mg/kg) or vehicle (0.5% methylcellulose) was administered orally to fasted mice. 30 min after administration, 54 μCi/kg of [1-$^{14}$C] triolein in a 2% lipid emulsion was orally gavaged. Immediately prior to this procedure, 10% of Triton WR-1339 was intravenously administered as 5 ml/kg body weight. Blood samples were collected 30 and 60 min after lipid loading from ophthalmic veins and radioactivity was measured with a scintillation counter.

**Evaluation of Distribution of Lipid Components in the Intestine**

The method of compound administration and radiolabeled lipid loading were the same as in the evaluation of fat absorption. Three segments of small intestine were individually and periodically collected 15, 30 and 60 min after lipid loading. Lipids were extracted from tissues, and were developed and separated via TLC. The signal intensity of TG, DG, LCFA and 2-MG fractions on a TLC plate were measured using a

FLA-7000 imaging system as described above. The equivalent radioactivity in each sample was calculated using a correction factor obtained from the standard sample.

**GLP-1 and PYY Levels after Lipid Loading**

7-week old male SD rats were fasted before turning off the lights on the day prior to administration. JTP-103237 (30 mg/kg) or vehicle (0.5% methylcellulose) was subsequently orally administered 30 min prior to the loading of the 10 ml/kg of lipid emulsion (Intralipos® Injection 20%, Otsuka Pharmaceutical Factory, Inc., Japan). Blood was collected from the portal vein 60 min after loading the lipid emulsion into siliconized tubes containing approximately 5% of EDTA, aprotinin, and a DPP-IV inhibitor (final concentrations of EDTA, aprotinin and DPP-IV inhibitor (Millipore Corporation) were 6 mmol/l, $1\times10^3$ KIU/ml and 50 μmol/l, respectively) in blood. Plasma active GLP-1 and total PYY concentrations were measured via a sandwich EIA using assay kits (Millipore).

**Feeding Test in Rats**

7-week old male Wistar rats were acclimatized to a 3.1% or 35% (w/w) fat diet for 14 days. Rats were fasted for 24 h before turning off the lights on the day prior to administration. Immediately after the lights were turned off, the diets were changed to diets containing JTP-103237 (the final dosages were 32.3 mg/kg/day [3.1% fat diet] and 28.4 mg/kg/day [35% fat diet], respectively). Feeding was resumed immediately after administration and food was weighed at 0.5, 1, 2, 4, 6, 8, and 24 h. Cumulative food consumption was calculated from the differences in food weight before and after feeding.

**Anti-obesity Study in a Diet-induced Obesity model**

Experiment 1 (subchronic (1 week) treatment): 6-week old male C57BL/6J mice were provided a 3.1% or 35% (w/w) fat diet ad libitum for 5 weeks in order to establish a condition of obesity, after which animals were used. JTP-103237 was administered orally as a food admixture for 7 days (the final dosages were 29.0 mg/kg/day [3.1% fat diet] and 8.7 mg/kg/day [35% fat diet], respectively). Body weight and food weight were measured on Day 0 and Day 7 to assess the effects on body weight gain and food consumption.

Experiment 2 (chronic treatment): 6-week old male C57BL/6J mice were provided a 35% (w/w) fat diet ad libitum for 5 weeks in order to establish a condition of obesity. JTP-103237 was administered orally as a food admixture for 4 weeks (the final dosage was 11.4 mg/kg/day). Body weight and food weight were measured on Day 0 and every 7 days to assess the effects on body weight gain and food consumption. After 4 weeks of treatment with JTP-103237, mice were acclimated individually in metabolic cages, and oxygen ($O_2$) consumption and respiratory quotient (RQ) were measured using a monitoring system MK-5000RQ (Muromachi Kikai, Tokyo, Japan) after mice were acclimated to the system for 1 day on a 35% fat diet. Measurements were taken from each cage about every 6 min. Data were normalized by lean body mass and were averages of every hour.

Experiment 3 (comprehensive evaluation): 4-week old male BDF1 mice ([C57BL/6N × DBA/2N]: F1) were provided a 35% (w/w) fat diet ad libitum for 3 weeks. JTP-103237 and orlistat were administered orally as a food admixture for 41 days (the final dosages were 13.5 mg/kg/day [JTP-103237] and 17.6 mg/kg/day

[orlistat], respectively). Body weight and food weight were measured on Day 0 and every 2 weeks to assess the effects on body weight and food consumption. On Day 13, mice were fasted overnight. The next day, a glucose solution (1 g/kg) was administered intraperitoneally. Prior to and 30, 60 and 120 min after glucose loading, blood was collected from the orbital vein and plasma glucose levels were measured. On Day 26, blood was collected from the orbital vein and plasma glucose, insulin and TG levels were measured. On Day 41, epididymal fat and liver were collected and the wet weight of fat tissue was measured. Total lipid was extracted from liver tissues in chloroform-methanol (2: 1, v/v) and hepatic TG content was measured.

**Statistical Analysis**

Data are expressed as mean ± or + standard deviation. All statistical analysis was performed with statistical software Statlight 2000 (Yukms Corp., Tokyo, Japan). In all studies except “Experiment 3”, a comparative study with orlistat in diet induced obesity mice, a Student’s t-test was performed provided that homogeneity was confirmed by an F test. In the case that homogeneity was not confirmed by an F test, a Welch’s test was performed. In the study of “Experiment 3”, to compare against not only the control group but also the orlistat treated group, a Bartlett’s homogeneity of variance test was performed followed by a Turkey's multiple comparison test for data with equal variances The level of significance was two-sided 5%.

## RESULTS

### Specificity and Species Differences of JTP-103237

The chemical structure of JTP-103237 is shown in Fig. 8. JTP-103237 inhibited recombinant MGAT2 activity, but did not inhibit recombinant DGAT2, which belongs to the same family as MGAT2 (Shi and Cheng, 2009). JTP-103237 also inhibited MGAT3 but the IC50 value for MGAT3 was approximately 300 times higher than that of MGAT2 (Table 5).

JTP-103237 potently inhibited MGAT activity in the intestinal S9 fraction without remarkable species differences among humans, rats and mice (Table 6).

### Effect on Intestinal MGAT Activity

The intestinal MGAT activity after administration of JTP-103237 was evaluated. In rats, the values of the percent of the MGAT activity in the control group in 10, 30 and 60 mg/kg at 1 h after dosing were 10.5, 9.5 and 9.5%, respectively. Those at 8 h after dosing are 22.8, 12.2 and 11.6%, respectively. In mice, those in 10, 30 and 100 mg/kg at 2 h after dosing are 54.6, 42.5 and 35.1%, respectively.

### Effect on Fat Absorption

Next, we evaluated the rate of fat absorption after a single dose of JTP-103237 by challenging the animals using a lipid emulsion containing $^{14}$C-labeled triolein. The radioactivity in circulating blood increased in a time-related manner in the vehicle group. In the JTP-103237 treated group, radioactivity in circulating blood was significantly reduced compared with the control group (Fig. 9).

**Effect on Distribution of Lipid Components**

Subsequently, we evaluated the contents of lipid components after lipid loading in three segments of the small intestine, from proximal to distal. The lipid components are shown in Fig. 10. In the proximal small intestine, radioactive TGs in the JTP-103237-treated group were significantly decreased as compared with those in the vehicle group. Meanwhile, in the middle small intestine, those were comparable between the two groups. Interestingly, in the distal small intestine, those in the JTP-103237 group increased as compared in the vehicle group (radioactivity in the vehicle group had undetectable levels 30 and 60 min after lipid loading), in contrast to the proximal intestine. The results for radioactive DGs were similar to those of TG. Specifically, JTP-103237 tended to decrease radioactive DGs in the proximal intestine and increased in the distal intestine. JTP-103237 significantly increased radioactive LCFA in the proximal intestine 15 min after lipid loading. In the middle and distal small intestine, JTP-103237 tended to increase radioactive LCFA 30 and 60 min after lipid loading. Radioactive 2-MGs in the JTP-103237-treated group significantly increased, in particular 15 and 30 min after lipid loading. These changes on 2-MG with JTP-103237 administration were more apparent than those of other lipid components.

**Effect on GLP-1 and PYY Levels after Lipid Loading**

We next evaluated the effects of JTP-103237 on plasma GLP-1 and PYY levels after lipid loading. JTP-103237 increased plasma PYY levels 60 min after lipid loading (Fig. 11). Plasma GLP-1 levels in the JTP-103237 treated group seemed to be higher as compared with those in the control group, but this change was not statistically significant (20.1 ± 13.7 pmol/l in the control group and 35.0 ± 20.4 pmol/l in the

JTP-103237 treated group, respectively. P value was 0.169 by t-test).

**Effect on Food Intake**

Since JTP-103237 increased substrates of MGAT in the intestine, we next evaluated the effect of JTP-103237 on food intake when fed a low fat diet (3.1% fat diet) or a high fat diet (35% fat diet). JTP-103237 significantly decreased cumulative food intake in rats fed a 35% fat diet. In contrast, JTP-103237 did not alter food intake in rats fed a 3.1% fat diet (Fig. 12). In addition, JTP-103237 decreased body weight gain from 0 to 24 h after treatment in rats fed a 35% fat diet, but not a 3.1% fat diet (data not shown).

**Effect on Diet-induced Obesity**

Next, we evaluated whether JTP-103237 treatment prevented diet-induced obesity. First, we investigated body weight and food intake in mice fed a 35% fat diet or low fat diet 3.1% fat diet with sub-chronic treatment (7 days) of JTP-103237. As shown in Fig. 13, JTP-103237 significantly decreased body weight and body weight gain. In addition, these effects were not observed in the mice fed a 3.1% fat diet.

We also evaluated the effects of chronic treatment (28 days) of JTP-103237 on body weight and food intake in the mice fed a 35% fat diet. As shown in Fig. 14, JTP-103237 significantly and consistently decreased body weight gain and food intake.

**Effect on Energy Expenditure**

We performed indirect calorimetry in mice fed a 35% fat diet after 4 weeks of treatment with JTP-103237 to evaluate the effects on 24 h energy expenditure and RQ.

The $O_2$ consumption in the light phase did not change in the JTP-103237 treated mice. Meanwhile, JTP-103237 significantly increased $O_2$ consumption in the early dark phase, during which mice would mainly feed (Fig. 15). JTP-103237 did not affect RQ during the evaluation period.

**Effect on glucose tolerance, fat weight and hepatic TG in Diet Induced Obesity mice**

To assess the effects on obesity-related parameters, we evaluated glucose tolerance, fat weight and hepatic TGs in comparison with orlistat, a pancreatic lipase inhibitor. In this experiment, since glucose tolerance in BDF1 mice was reportedly more impaired than that in C57BL/6 (Karasawa et al., 2009), we used high fat fed BDF1 mice.

As shown in Fig. 16, JTP-103237 significantly decreased body weight and the efficacy of JTP-103237 was comparable with orlistat during the entire evaluation period. JTP-103237 decreased food intake. In contrast, orlistat significantly increased food intake. JTP-103237 significantly decreased plasma glucose levels after glucose loading, but this effect of orlistat was not significant. As shown in Table 7, JTP-103237 tended to decrease plasma insulin and TG levels. In addition, JTP-103237 significantly decreased fat weight and hepatic TG content.

**DISCUSSION**

A number of studies indicate that inhibition of MGAT2 is a novel and potential strategy for metabolic diseases or disorders caused by excessive fat intake (Gao et al., 2013; Nelson et al., 2011; Tsuchida et al., 2012; Yen et al., 2009). In this study, we

evaluated the effects of JTP-103237, which is a novel MGAT2 inhibitor discovered in Japan Tobacco Central Pharmacological Research Institute, on fat absorption and energy metabolism.

JTP-103237 selectively inhibited recombinant human MGAT2 and potently inhibited MGAT activity in intestinal S9 fractions without notable species differences among humans, rats and mice. JTP-103237 inhibited intestinal MGAT activity in rats, indicating that JTP-103237 can inhibit intestinal MGAT in *in vivo* experiments. This effect reached a maximum at a dose of 30 mg/kg and above at 1 and 8 h in rats. Although the potency of JTP-103237 intestinal MGAT inhibition seemed to be lower than that in rats, JTP-103237 also inhibited MGAT in in mice. In addition, no significant toxicological effect was observed in the preliminary 2 weeks toxicological study (repeated administration) of JTP-103237 in rats at doses of 100 and 1000 mg/kg. From these, we set the dose in the single dose study at 30 mg/kg in rats and 100 mg/kg in mice.

MGAT2 has been suggested as playing an important role in dietary fat absorption in the intestine (Grigor and Bell, 1982; Yen and Farese, 2003; Yen et al., 2014). We therefore first evaluated the rate of fat absorption after a single dose of JTP-103237. JTP-103237 significantly decreased radioactivity in circulating blood as compared with the control group. The rate of fat absorption in MGAT2 KO mice is reportedly partly reduced when the entry of dietary fat into circulation is delayed (Nelson et al., 2014; Yen et al., 2009). Taken together, consistent results were obtained between inhibitor and KO mice.

We next investigated the effects of JTP-103237 on the contents of lipid components in the small intestine after lipid loading. JTP-103237 significantly but

slightly decreased radioactive TGs. Notable increases in LCFAs and 2-MGs, which are substrates of MGAT, were observed after lipid loading, indicating these changes were as a result of MGAT inhibition. The decrease in radioactive DGs was not clear in this study. There are some possible reasons shown below. First, the determination of DG levels may be difficult since it is an intermediate product of triacylglycerol synthesis. In addition, it has been reported that MGAT2 demonstrates weak DGAT activity (Cao et al., 2003a; Cao et al., 2003b). Although the effect of JTP-103237 on DGAT activity by MGAT2 has not been evaluated, it may contribute to the modulation of DG levels.

Interestingly, JTP-103237 increased the amount of lipids in the distal small intestine as compared with vehicle, suggesting the reduction of fat absorption in the proximal small intestine with JTP-103237 administration may result in more lipids reaching the distal intestine. Dietary lipids in the distal intestine are known to increase plasma GLP-1 and PYY, which is secreted from L cells in the distal intestine (Chaudhri et al., 2006). In addition, MGAT2 deficiency leads to an increase in plasma GLP-1 levels after lipid loading (Okawa et al., 2009; Yen et al., 2009). Thus, we investigated whether JTP-103237 increases plasma GLP-1 and PYY levels after lipid loading. JTP-103237 increased plasma PYY levels 60 min after lipid loading, in which the lipids in the distal intestine increased in the above study. Plasma GLP-1 levels in the JTP-103237 treated group was slightly higher as compared with those in the control group but this difference was not statistically significant.

GLP-1 and PYY are known as satiety hormones produced in response to meals. In addition, other lipid absorption inhibitors, such as microsomal triglyceride transfer protein (MTP) inhibitors and DGAT1 inhibitors, reportedly decreased food intake (Hata et al., 2011; Tomimoto et al., 2015). From these, we hypothesized that JTP-103237

alters food intake by modulating lipid components in the small intestine by inhibiting intestinal MGAT. In the present study, JTP-103237 decreased food intake in rats fed a high fat diet (35% fat diet), but these effects were not observed in the rats fed a low fat diet (3.1% fat diet), indicating that the satiety effect of JTTP-103237 would be dietary fat-dependent.

An increase in LCFA in the intestine is known to lead to the suppression of appetite (Feinle et al., 2003; Feinle et al., 2001; O'Donovan et al., 2003). As shown in Chapter 3, since jejunal infusion of 2-MG reduced food intake, an increase in 2-MG in the intestine also leads to the suppression of appetite. In addition, mice with intestine specific MGL overexpression, which show a decrease in 2-MG species in the intestine, were found to be hyperphagic and displayed reduced energy expenditure (Chon et al., 2012) and intestine-specific deletion of MGAT2 reportedly induced satiety in a dietary fat-dependent manner (Nelson et al., 2014). From these, it is suggested the substrate of MGAT might modulate satiety and JTP-103237 suppressed food intake by modulating intestinal lipid component via MGAT inhibition.

JTP-103237 prevented diet induced obesity in accordance with reports using MGAT2 KO mice (Nelson et al., 2014; Tsuchida et al., 2012; Yen et al., 2009). In addition, JTP-103237 increased $O_2$ consumption only in the early dark phase in diet-induced obesity mice. Since rats mainly feed during the dark phase, this effect might be associated with the modulation of dietary fat absorption by inhibiting MGAT. The detailed mechanism by which MGAT inhibition triggers $O_2$ consumption is unclear; however, an increase in $O_2$ consumption in MGAT2 KO mice was consistently reported (Nelson et al., 2014; Tsuchida et al., 2012; Yen et al., 2009). As previously described, mice with intestine-specific MGL overexpression, which show a decrease in 2-MG

species in the intestine, reportedly displayed reduced energy expenditure (Chon et al., 2012), suggesting that intestinal 2-MG may be involved in energy expenditure.

In order to comprehensively investigate the potential of JTP-103237 as an anti-obesity agent, we evaluated obesity-related parameters in comparison with orlistat, a pancreatic lipase inhibitor, in high fat fed BDF1 mice. The efficacy of JTP-103237 on body weight reduction was comparable with orlistat and the magnitude of the effect was above 10%. Interestingly, orlistat increased food intake in contrast to JTP-103237, which decreased food intake. JTP-103237 significantly decreased plasma glucose levels after glucose loading and the glucose curve during glucose tolerance tests in the JTP-103237 treated group was lower than that in the orlistat treated group. In addition, JTP-103237 significantly decreased fat weight and hepatic TG content.

The intestine has important sensing and signaling roles in the regulation of energy homeostasis, in addition to a role in the digestion and absorption of nutrients (Badman and Flier, 2005). In this study, JTP-103237 modulated intestinal lipid components and distribution by inhibiting MGAT2. We considered this effect of JTP-103237 in the intestine as leading to the amelioration of energy homeostasis in the entire body by reducing fat intake and increasing energy expenditure. Understandably, this effect should contribute to body weight reduction and improvement of glucose metabolism.

On the other hand, although MGAT2 in the intestine plays an indispensable role in energy metabolism, it has been reported the possibility that MGAT2 in other tissues may also contribute to it (Gao et al., 2013). In addition, MGAT1 antisense oligonucleotides treatment improved glucose tolerance and hepatic insulin sensitivity (Soufi et al., 2014). We tried to create recombinant human MGAT1 but we could not

obtain the membrane fraction which exhibited sufficient MGAT activity. Thus, unfortunately, we have not evaluated the effect of JTP-103237 on MGAT1 activity. Considering the bioavailability of JTP-103237 (the values in rats were 29.0 and 26.4% at 10 and 100 mg/kg, respectively. Those in mice were 20.8 and 12.0% at 10 and 100 mg/kg, respectively), it is probable that the inhibitory effects of JTP-103237 on MGAT in other tissues affected energy metabolism. These points will likely be the subject of future investigation.

Although there is room for further investigation including clinical trials to prove showing these beneficial effects in human, JTP-103237 is expected to be a drug for the treatment of obesity and metabolic diseases that has a novel mechanism of action and unique properties, such as peripherally modulating food intake and enhancing energy expenditure.

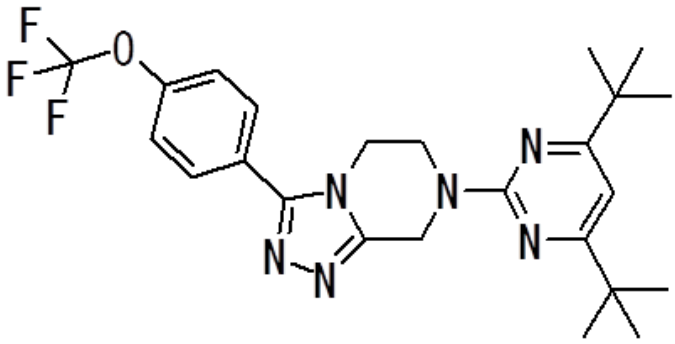

**Figure 8.** Chemical structure of JTP-103237

**Table 5.** Inhibitory effects of JTP-103237 on human MGAT2, human MGAT3 and human DGAT2.

| | MGAT2 | MGAT3 | DGAT2 |
|---|---|---|---|
| $IC_{50}$ (μM) | 0.019 | 6.423 | > 30 |

Enzyme activity was determined using membrane fractions containing recombinant enzymes from COS-7 (MGATs) or Sf9 (DGAT2) cells.

**Table 6.** **I**nhibitory effects of JTP-103237 on human, rat and mouse small intestinal MGAT activity

| | Human | Rat | Mouse |
|---|---|---|---|
| $IC_{50}$ (μM) | 0.004 | 0.002 | 0.002 |

Enzyme activity was determined using rat and mouse small intestinal microsomes.

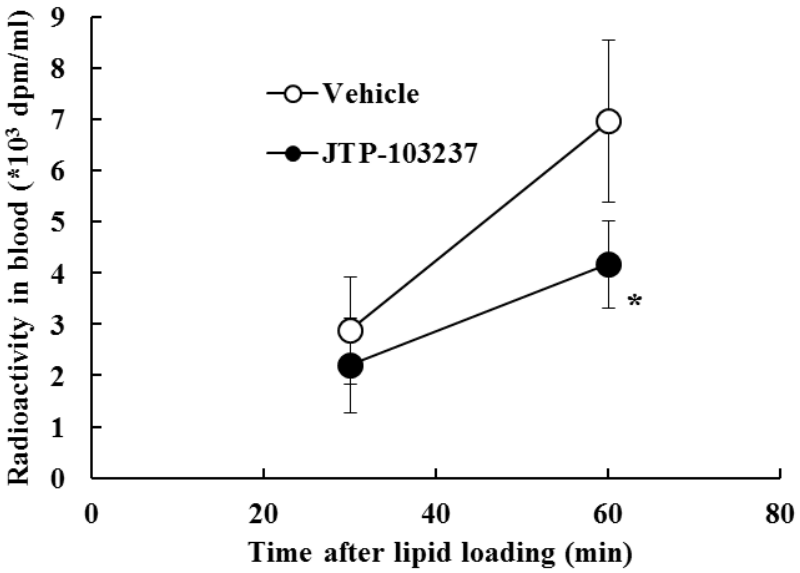


**Figure 9.** Plasma radioactivity after administration of lipid emulsions containing $^{14}C$ triolein in JTP-103237 treated mice. Data shown as means ± standard deviation (n = 4). *P<0.05; significantly different from the Vehicle group.

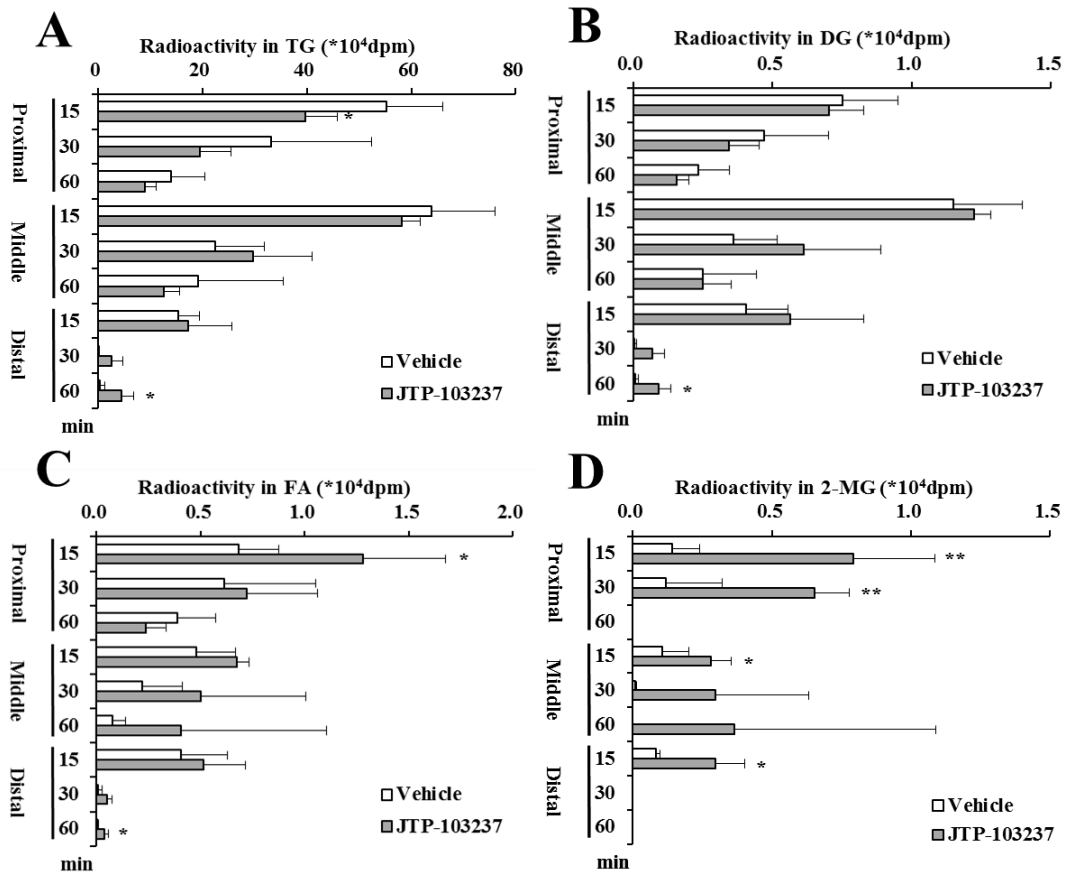


**Figure 10.** Distribution of TG (A), DG (B), LCFA (C) and 2-MG levels (D) in each segment of the small intestine after administration of lipid emulsions containing $^{14}C$ triolein in JTP-103237 treated mice. Data shown as means + standard deviation (n = 4). *P<0.05, **P<0.01; significantly different from the Vehicle group.

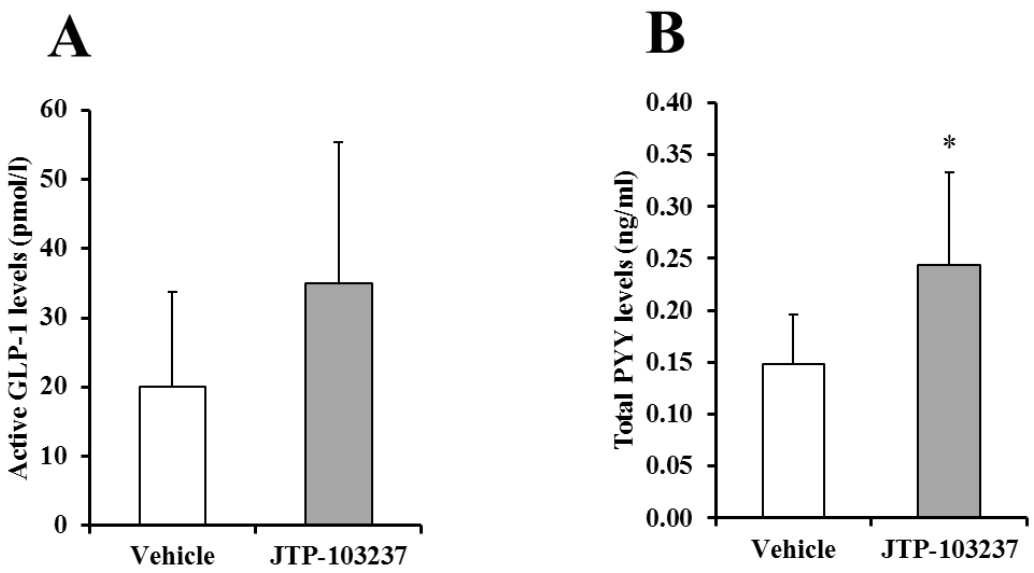


**Figure 11.** Effect of JTP-103237 on GLP-1 (A) and PYY (B) levels after administration of lipid emulsions in rats. Data shown as means + standard deviation (n = 6). *P<0.05; significantly different from the Vehicle group.

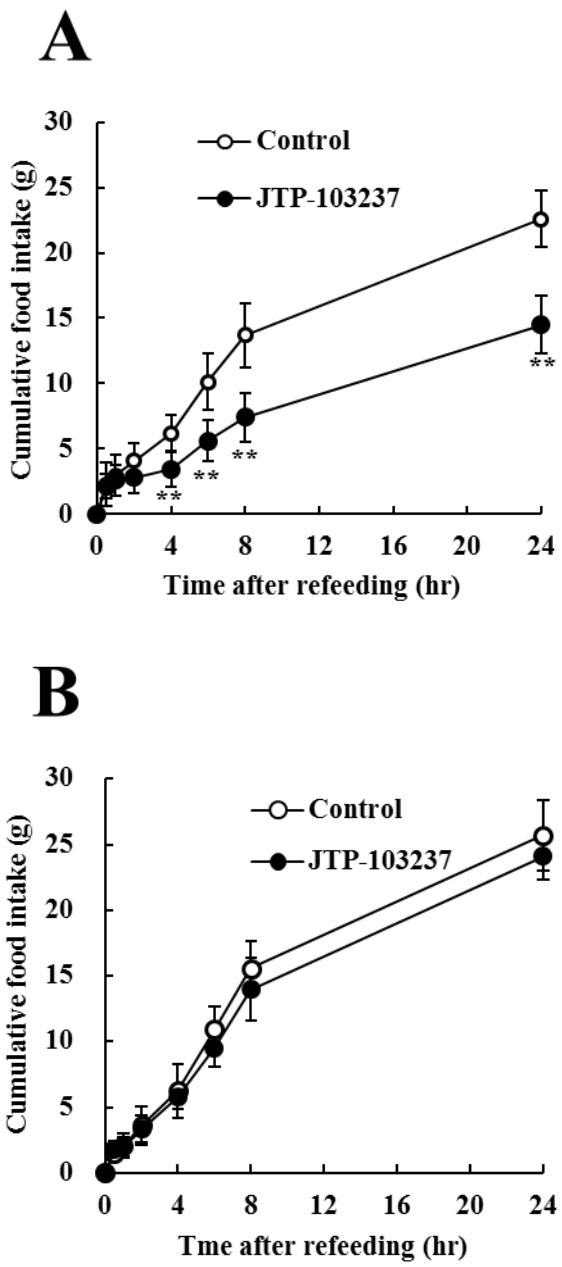


**Figure 12.** Effect of JTP-103237 on food intake in rats fed a high fat diet (35% fat diet, A) or a low fat diet (3.1% fat diet, B). Data shown as means ± standard deviation (n = 6). **P<0.01; significantly different from the Control group.

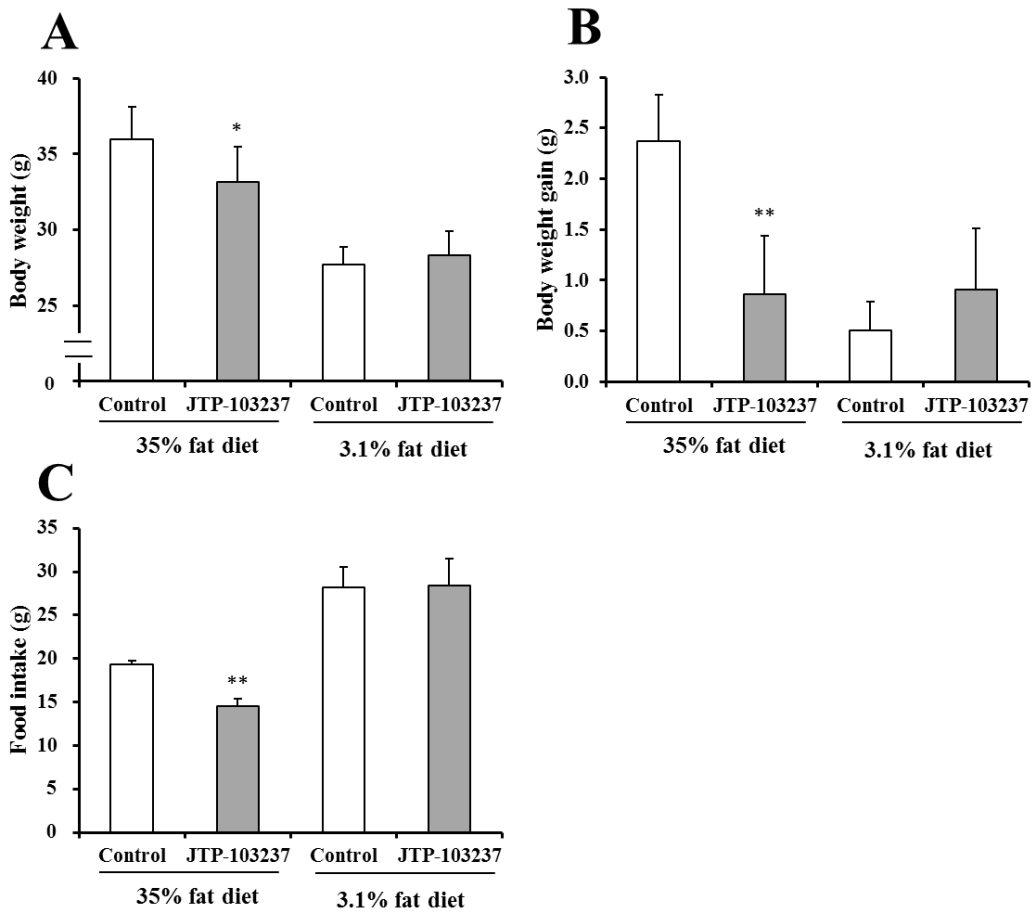


**Figure 13.** Effect of JTP-103237 on body weight (A), body weight gain (B) and food intake (C) after subchronic treatment in C57BL/6J mice fed a high fat diet (35% fat diet) or a low fat diet (3.1% fat diet). Data shown as means + standard deviation (n = 6). *P<0.05, **P<0.01; significantly different from the Control group.

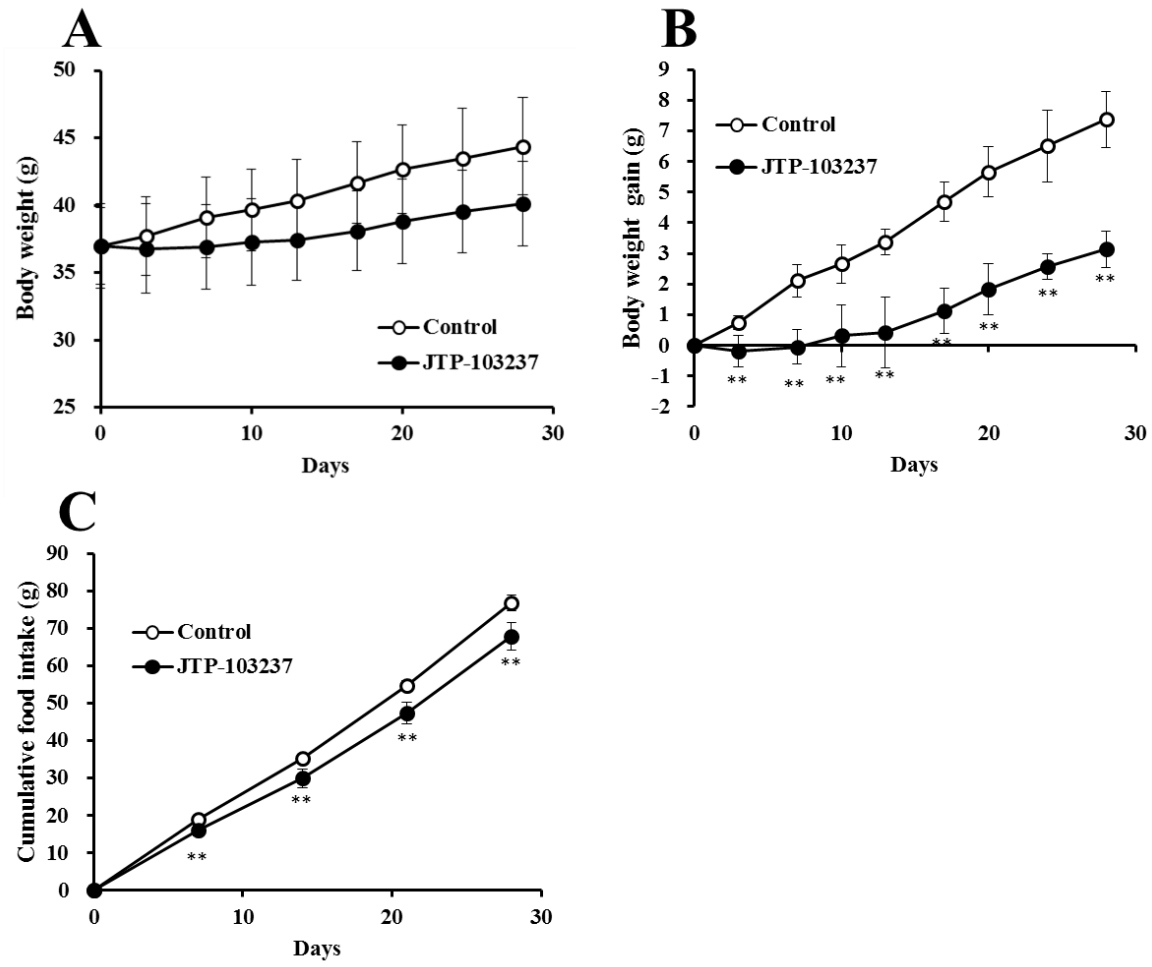


**Figure 14.** Effect of JTP-103237 on body weight (A), body weight gain (B) and food intake (C) in C57BL/6J mice fed a high fat diet (35% fat diet). Data shown as means ± standard deviation (n = 6). *P<0.05, **P<0.01; significantly different from the Control group.

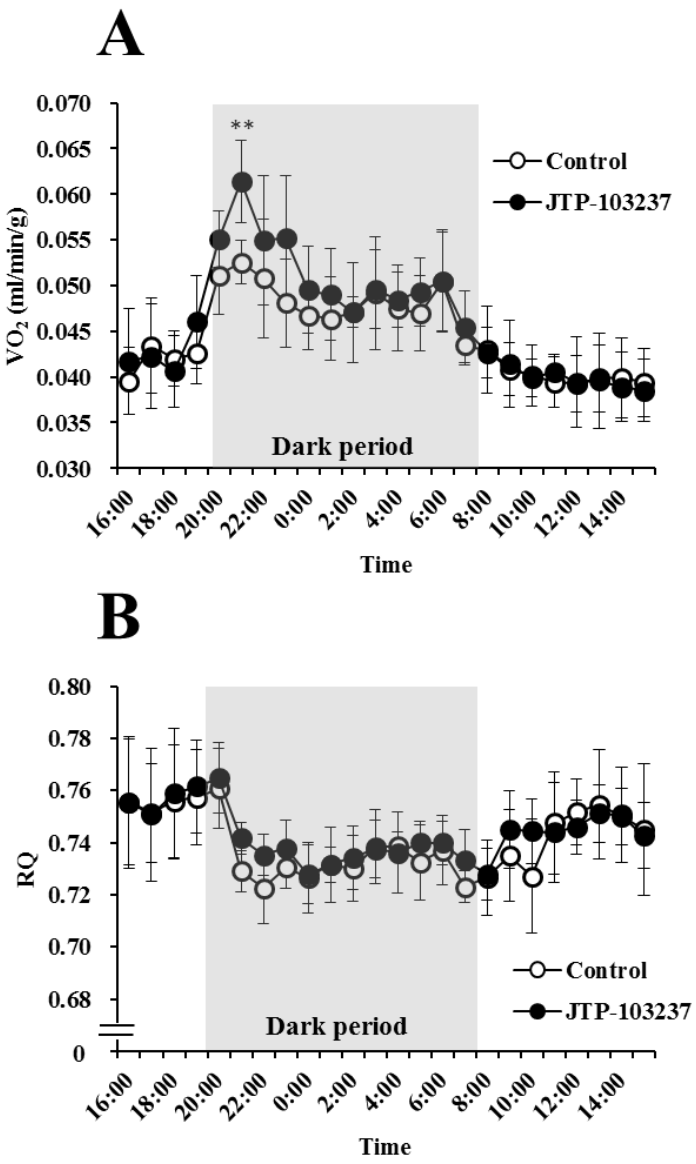


**Figure 15.** Effect of JTP-103237 on $O_2$ consumption ($VO_2$, A) and RQ (B) in C57BL/6J mice fed a high fat diet (35% fat diet). Data shown as means ± standard deviation (n = 6). **P<0.01; significantly different from the Control group.

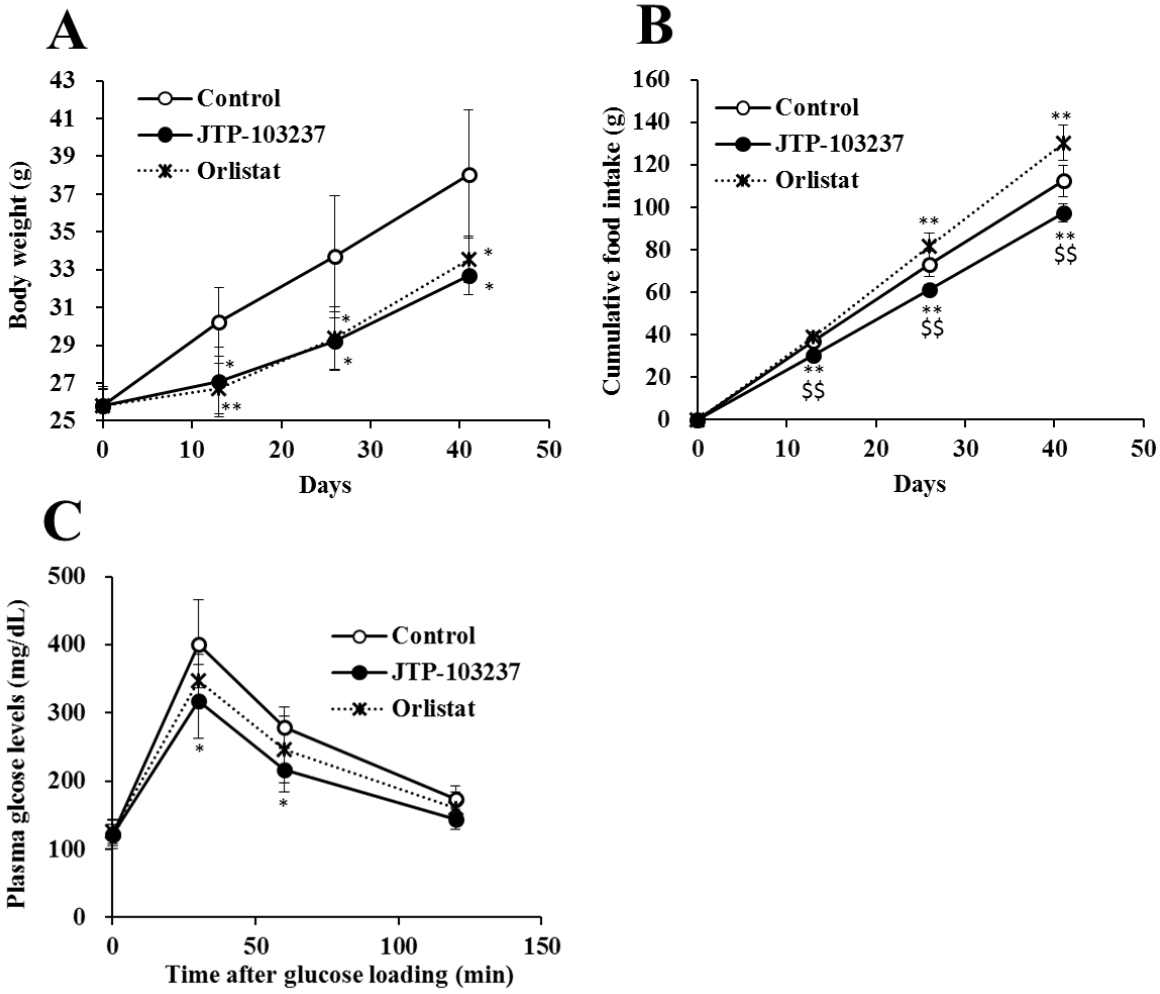


**Figure 16.** Effect of JTP-103237 on body weight (A), food intake (B) and blood glucose concentration following an intraperitoneal glucose loading (C) in BDF1 mice fed a high fat diet (35% fat diet). Data shown as means ± standard deviation (n = 6). *P<0.05, **P<0.01; significantly different from the Control group. $$P<0.01; significantly different from the Orlistat treated group.

**Table 7.** Effects of JTP-103237 on biochemical parameters, fat weight and hepatic TG in diet induced obesity mice (BDF1 mice).

| Treatment | Control | JTP-103237 | Orlistat |
|---|---|---|---|
| Glucose (mg/dl) | 203 ± 21 | 210 ± 17 | 211 ± 11 |
| Insulin (mg/dl) | 10.3 ± 8.4 | 6.0 ± 2.9 | 5.1 ± 4.0 |
| TG (mg/dl) | 182 ± 86 | 150 ± 76 | 175 ± 90 |
| Fat weight (g) | 2.1 ± 0.5 | 1.4 ± 0.2** | 1.4 ± 0.3* |
| Hepatic TG (mg/g liver) | 103 ± 43 | 50 ± 12* | 48 ± 8* |

Data shown as means ± standard deviation (n = 6).

*P<0.05, **P<0.01; significantly different from the Control group.

# Chapter 5

# JTP-103237, a monoacylglycerol acyltransferase inhibitor, prevents fatty liver and suppresses both triglyceride synthesis and de novo lipogenesis

## INTRODUCTION

Fatty liver disease is the most common cause of liver dysfunction worldwide (Attar and Van Thiel, 2013; LaBrecque et al., 2014). Fatty liver disease is a condition defined by excessive fat accumulation in the form of TG in the liver, namely fatty liver. The exact cause of fatty liver disease is still unknown. However, both obesity and insulin resistance likely have a strong influence on the disease process. Understandably, with the growing epidemic of obesity and diabetes, the prevalence and impact of fatty liver disease continues to increase (Vernon et al., 2011). In addition, fatty liver disease is considered to be associated not only with obese and diabetes onset, but also with cardiovascular disease. Inevitably, the economic burden associated with fatty liver disease will increase; however, effective treatments are not yet available, and hence the need for pharmacological treatments is fully justified. Fatty liver is considered to result from an imbalance of lipid metabolism (Musso et al., 2009). Thus, ameliorating hepatic lipid metabolism appears to be a potential strategy for the treatment of fatty liver disease and other related diseases.

There are two major pathways, the glycerol-3-phosphate (G-3-P) pathway and the 2-MG pathway, for TG synthesis (Shi and Cheng, 2009). The G-3-P pathway is a de novo pathway present in most tissues. The MGAT pathway plays a predominant role on dietary fat absorption. MGAT catalyze the first step of TG synthesis. The formation of

DG from 2-MG and fatty acyl CoA facilitated by MGAT is considered the rate-limiting step of triacylglycerol synthesis in this pathway (Senior and Isselbacher, 1962; Yen and Farese, 2003).

The relevance of MGAT activity to hepatic TG metabolism is still unclear. However, liver MGAT activity reportedly increases in diabetic rats (Mostafa et al., 1993) and this pathway is active in neonatal rats (Bhat et al., 1993). In addition, there is evidence that MGAT2 and MGAT3 were overexpressed in patients with fatty liver disease (Hall et al., 2012). As shown in Chapter 4, JTP-103237 selectively inhibited MGAT2 but not DGAT2 and prevented high fat diet induced obesity through the inhibition of intestinal MGAT2. In addition, JTP-103237 reduced hepatic TG content in high fat diet induced obese mice. However, since JTP-103237 reduced food intake and body weight through the inhibition of intestinal MGAT, the contribution of liver MGAT activity on hepatic TG reduction requires further investigation.

In this study, we investigated whether liver MGAT activity is relevant to hepatic lipid metabolism using JTP-103237.

## MATERIALS AND METHODS

### Animals and Chemicals

Male C57BL/6J mice were purchased from Charles River Laboratories (Yokohama, Japan). Mice were maintained with free access to water and either a normal chow diet (CRF-1, Charles River Japan) or a high sucrose very low fat (HSVLF) diet (D08030601, Research Diets Inc.) The caloric contributions (% fat: % carbohydrate: % protein; kcal/g) in HSVLF diets as indicated by the manufacturer are 2.6: 76.7: 20.7; 3.53 kcal/g, respectively. Animals were housed under specific pathogen-free conditions

in a room controlled for temperature at 23 ± 3°C and humidity of 55 ± 15% in 12-h light/dark cycles (lights on from 8:00 AM to 8:00 PM). All procedures were conducted according to guidelines from Japan Tobacco's Animal Care Committee

JTP-103237 was synthesized in the Central Pharmaceutical Research Institute within Japan Tobacco Inc. (Osaka, Japan). [1-$^{14}$C] oleoyl-coenzyme A was purchased from Amersham biosciences. [1-$^{14}$C] oleic acid, [1-$^{14}$C] palmitic acid and [1-$^{14}$C] acetate were purchased from PerkinElmer Japan. D-[U-$^{14}$C]-glucose was purchased from GE Healthcare. All other chemicals were standard reagent grade.

**Measurement of Blood Chemistry and Hepatic TG Content**

Blood samples were collected from the orbital vein in fed state and plasma glucose, TG and total cholesterol levels were measured by an enzymatic method (glucose: LiquiTech glucose HK test Roche Diagnostics, Switzerland), TG: Determiner L TGII (Kyowa Medex Co, Tokyo, Japan), total cholesterol: Determiner L TCII (Kyowa Medex Co, Tokyo, Japan). Plasma insulin levels were measured by an enzyme-linked immunosorbent assay (Rat insulin assay kit (Morinaga Institute of Biological Science, Tokyo, Japan). Liver was collected from mice in fed state and a portion of the liver that was approximately 100 mg, 0.5 ml of methanol, and zirconia beads were added to tubes. The portion of liver was homogenized using a mixer mill (MM300 Retch) (25 Hz, 10 min). To the homogenized solution, 1 ml of chloroform was added and mixed thoroughly. The mixture was then centrifuged (10,000 g, 5 min, 4C$^{o}$) and the resulting supernatant collected. Solvents contained in 0.5 ml of the supernatant were dried under a stream of nitrogen gas. To the residue, 0.5 ml of 2-propanol was added to reconstitute the residue. The TG concentration of the 2-propanol solution was determined by an

enzymatic method.

**Evaluation on Liver MGAT Activity**

Liver S9 fractions were isolated through centrifugation as described in Chapter 4. The MGAT assay was performed at room temperature in the presence of 100 mM Tris-HCl (pH 7.5), 250 mM Sucrose, 5 mM $MgCl_2$, 0.05% BSA (Sigma-Aldrich, St. Louis, MO), 0.05 mM 2-oleoyl glycerol and 25 μM [1-$^{14}$C] oleoyl-CoA. The radioactivity of synthesized [1-$^{14}$C] DG was separated using TLC and analyzed as MGAT activity.

**Evaluation of Hepatic Lipid Synthesis**

Livers were collected in fed state from mice and a portion of the liver that was approximately 100 mg was added to reaction medium (DMEM low glucose containing 500 μM [1-$^{14}$C] acetate (29.0 μCi/ml)). After 90 min, the reaction was stopped by fast cooling. Lipids were extracted from portions of the liver, and separated via TLC using a hexane:diethylether:acetic acid (80:30:2) solvent system. The signal intensity of the TG and DG fraction on a TLC plate was measured using an FLA-7000 imaging system. The radioactivity equivalent in each sample was calculated using a correction factor obtained from the standard sample and the ratio of lipid synthesis per 90 min per gram of liver was also calculated.

The lipid extract was saponified with 1.5N NaOH (90 min, 4C$^{o}$). After saponification, lipids were re-extracted and separated via TLC using the same process described above. The signal intensity of the LCFA fraction on a TLC plate was measured and the radioactivity equivalent in each sample was calculated in the same

manner as previously mentioned. The rate of LCFA synthesis (de novo lipogenesis) was calculated as nmol of radioactivity incorporated into LCFA fractions per 90 min per gram of liver.

**Evaluation of Hepatic Lipid Components after Administration of LCFAs**

10-week old male C57BL/6J mice were acclimatized to an HSVLF diet for 5 weeks. JTP-103237 was administered orally to mice in fed state at a dose of 100 mg/kg. After 60 min, 30 μM/kg of oleic acid containing 135 μCi/kg of [1-$^{14}$C] oleic acid was intraperitoneally administered. After 15 min, mice were sacrificed and a portion of the liver that was approximately 350 mg was collected. Lipids were extracted and separated via TLC. The signal intensity of TG, DG and LCFA fractions on a TLC plate were measured using an FLA-7000 imaging system as described above. The ratio of each lipid component to total lipids was calculated.

**mRNA quantification with real-time quantitative PCR**

Total RNA was extracted from the liver. RNA was transcribed into complementary DNA using M-MLV reverse transcriptase and random primers (Invitrogen, Carlsbad, CA). The reaction mixture was incubated for 10 min at 25°C, 1 h at 37°C, and 5 min at 95°C. Real-time PCR quantification was performed in a 50-μL reaction mixture with an automated sequence detector combined with the ABI Prism 7700 Sequence Detection System software (Applied Biosystems, Foster City, CA). The reaction mixture contained 50 ng of synthesized cDNA, 3.5 mM MgCl2, 0.3 μM primers, 0.1 μM probes, and 1.25 units of Ampli Taq Gold®. Cycle parameters were 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. The expression of

mRNA levels were normalized using 18s rRNA levels. The following primers and FAM-conjugated probes were designed using Primer Express software (Applied Biosystems): sterol regulatory element-binding protein 1-c (SREBP-1c) (forward, ATCGGCGCGGAAGCTGTCGGGGTAGCGTC; reverse, TGAGCTGGAG CATGTCTTCAA; probe, ACCACGGAGCCATGGATTGCACATT), stearoyl-CoA desaturase1 (SCD1) (forward, TCTCCAGTTCTTACACGACCACC; reverse, GGA CGGATGTCTTCTTCCAGGT; probe, CCTCCGGAAATGAACGAGAGAAGGTGA AG), fatty acid synthase (FAS) (forward, GGTCTATGCCACGATTCTGAAT; reverse, GGAATGTTACACCTTGCTCCTT; probe, CACCAATACAGATGGCAG), acetyl-CoA carboxylase (ACC) (forward, TGGATGATGGTCTGAAGGCAG; reverse, CCTCTGAGGCCTTGATCATCAC; probe, TGAGGAAGTTGGCTATCC), mitochondrial glycerol 3-phosphate acyltransferase (mtGPAT) (forward, GCAATGG CGTACTTCATGTGTT; reverse, CATGGAAGCCATCATAGCTTGCAGCATC; probe, GCACCTCTTATTCAGGACTGCA), DGAT 2 (forward, ATCCTCATGTAC ACCTTCTGCACAG; reverse, ACCTGGCTGGCATTTGACTGGAACA; probe, AT CTCCTGCCACCTTTCTTGG), and 18s rRNA (purchased from Applied Biosystems).

**Glucose Utilization in Fat Tissue**

Small portions (approximately 200 mg) of epididymal and mesenteric adipose tissues were incubated in Hank's balanced salt solution (pH7.4) containing D-[U-$^{14}$C]-glucose in the absence or presence of insulin (1 or 10 nmol/l) at 37°C for 2 h. After stopping the reaction through the addition of 0.05 mol/l $H_2SO_4$, the $^{14}CO_2$ produced was trapped in filter paper. Radioactivity in the filter paper was measured using a liquid scintillation counter (TRI-CARB 2500TR, Packard BioScience, Waltham,

MA, USA).

**Statistical Analysis**

Data are expressed as the mean ± or + standard deviation. All statistical analysis was performed with statistical software Statlight 2000 (Yukms Corp., Tokyo, Japan). A Student's t-test was performed provided that homogeneity was confirmed by an F test. In the case that homogeneity was not confirmed by an F test, a Welch's test was performed. A Dunnett's multiple comparison test was performed in the multiple-group study provided that homogeneity was confirmed by a Bartlett's homoscedasticity test. In the case that homogeneity was not confirmed by a Bartlett's homoscedasticity test, a Steel's multiple comparison test was performed. A Pearson correlation test was used to analyze the correlation between hepatic TG content and MGAT activity. Differences were considered significant if p was <0.05 (2-sided).

**RESULTS**

**Effects on Hepatic TG and Liver MGAT activity in HSVLF diet fed mice**

To investigate whether JTP-103237 decreases liver lipid synthesis by inhibiting liver MGAT activity, hepatic TG content in HSVLF diet fed mice was evaluated.

In HSVF fed mice, hepatic TG content increased as compared to those in normal diet fed mice (Fig. 17 B). JTP-103237 significantly decreased hepatic TG content at 100 mg/kg/day for 21 days (Fig. 17 A). Although, the values were not statistically significant, JTP-103237 tended to decrease hepatic TG content at 100 mg/kg/day for 35 days (Fig. 17 B). In HSVF fed mice, MGAT activity increased as compared to those in normal diet fed mice and JTP-103237 significantly inhibited

MGAT activity at 100 mg/kg/day for 35 days (Fig. 18 A). In addition, there was a statistically significant correlation between hepatic TG and MGAT activity (Fig. 18 B).

**Effects on body weight, food intake, fat weight and biochemical parameters in HSVLF diet fed mice**

As shown in Table 8, JTP-103237 did not alter body weight and cumulative food intake in HSVLF diet fed mice. On the other hand, JTP-103237 decreased fat weight, plasma glucose and total cholesterol levels at a dose of 100mg/kg/day. In addition, plasma insulin levels were tended to be decreased in JTP-103237 treated groups and it was statistically significant at 30 mg/kg/day. Although, these values were not statistically significant, JTP-103237 tended to decrease plasma TG levels..

**Effect on Hepatic Lipid Synthesis in HSVLF Diet Fed Mice**

Subsequently, the effect of chronic treatment of JTP-103237 on hepatic lipid synthesis in isolated liver tissues in HSVLF diet fed mice was evaluated. JTP-103237 significantly suppressed TG and DG synthesis from acetates (Fig. 19, A). In addition, lipid extracts were saponified through treatment with NaOH to investigate de novo lipogenesis (LCFA synthesis). As a result, JTP-103237 also significantly decreased de novo lipogenesis (Fig. 19, B).

In order to clarify the time sequence of the effects, lipogenesis after a single or subchronic treatment of JTP-103237 was evaluated. JTP-103237 significantly suppressed lipogenesis after 7 days of treatment but did not suppress lipogenesis after a single treatment (Fig. 20).

**Effect on Lipogenic Gene Expression**

The mRNA levels of lipogenic genes (SREBP-1c, SCD1, FAS, ACC, mtGPAT and DGAT2) in the liver in chronic JTP-103237 treated HSVLF diet fed mice were measured using real-time PCR. SREBP-1c, SCD1 and DGAT2 genes expression was significantly decreased in JTP-103237 treated mice as compared to control (Fig. 21). FAS, ACC and mtGPAT genes expression did not yield statistically significant differences due to the large variability observed, but tended to decrease as compared to control.

**Effects on Hepatic Lipid Components after Administration of LCFAs**

The amount of lipid components after a single treatment of JTP-103237 was subsequently analyzed using $^{14}C$ labeled oleic acid. The differences were not statistically significant; however, JTP-103237 tended to decrease the ratio of TG (product of MGAT) to total labeled lipids ($p=0.06$). In addition, JTP-103237 significantly increased the ratio of LCFAs (substrate of MGAT) to total labeled lipids (Table. 9). However, since separating the bands for 2-MG was difficult, the amount of 2-MG could not be evaluated in this experiment. The ratio of DG did not change in this study, which was likely due to the difficulty in determining DG levels, since DG is an intermediate product of triacylglycerol synthesis.

**Effects on insulin sensitivity in isolated fat tissues**

To investigate the effects of JTP-103237 in other tissues, adipose glucose oxidation in HSVLF diet fed mice was evaluated. Glucose oxidation capability increased with insulin in both epididymal and mesenteric fat tissue. As shown in Fig. 22,

it was not statistically significant, but glucose oxidation in JTP-103237 treated adipose tissues tended to increase and these effects with insulin were clearer than those without insulin. The P value in mesenteric fat tissue with 10 nM insulin was 0.0977.

## DISCUSSION

MGATs are known to play important roles in intestinal TG absorption. In addition to intestinal MGAT activity, there are a few reports describing liver MGAT activity (Bhat et al., 1993; Hall et al., 2012; Mostafa et al., 1993). Regarding subtype, very low expression levels of both MGAT1 and MGAT2 were reportedly detected in mouse liver (Cao et al., 2003b; Yen et al., 2002) Although it was shown that systemic MGAT2 KO mice are resistant to high fat diet induced obesity and fatty livers (Yen et al., 2009), the role of hepatic MGAT has been still unclear. On the other hand, in a recent report, it was indicated MGAT2 in extraintestinal tissues may contribute to the regulation of energy metabolism (Gao et al., 2013; Nelson et al., 2014). For example, intestine-specific expression of MGAT2 in systemic MGAT2 KO mice partly restored the metabolic changes. In addition, MGAT1 antisense oligonucleotides treatment, reportedly, improved fatty liver and hepatic insulin sensitivity (Soufi et al., 2014). Therefore, we hypothesized that extraintestinal MGAT, in particular hepatic MGAT, might be involved in hepatic lipid metabolism.

In the present study, we investigated the effects of MGAT inhibition on hepatic lipid metabolism using JTP-103237, a novel MGAT inhibitor. As shown in previous report, JTP-103237 inhibited MGAT2 activity, but did not inhibit DGAT2 activity, which belongs to the same family as MGAT. In addition, the IC50 value for MGAT3 was approximately 300 times higher than that of MGAT2. However, we have not

evaluated the effect of JTP-103237 on MGAT1 activity since we could not obtain the membrane fraction which exhibited sufficient MGAT1 activity. Therefore, there is a probability that JTP-103237 inhibits not only MGAT2 but MGAT1 in the present study.

In order to focus on liver MGAT activity, we used HSVLF diets in the present study, which induced fatty livers due to high sucrose content (Miyazaki et al., 2007). Since the caloric contribution of fat in HSVLF diet was only 2.6%, the effects by intestinal MGAT inhibition were considered small. Indeed, JTP-103237 did not reduce body weight and food intake in HSVLF diet fed mice in the present study in contrast to the study using high fat diet fed mice in Chapter 4.

JTP-103237 decreased hepatic TG content and inhibited MGAT activity in HSVLF diet fed mice. Moreover, the relationship between hepatic TG content and MGAT activity was significantly correlated, suggesting that decreases in carbohydrate-induced fatty liver might be due to hepatic MGAT inhibition.

We next investigated lipid synthesis in the liver in HSVLF diet fed mice after chronic treatment with JTP-103237. JTP-103237 decreased TG and DG synthesis after chronic treatment. These results were within expectation; however, surprisingly, JTP-103237 decreased LCFA synthesis from acetate (de novo lipogenesis). MGAT catalyzes the synthesis of DG from 2-MG and is the key enzyme for TG synthesis. Understandably, this is not relevant to LCFA synthesis. We suspected non-specific inhibitory effects on enzymes related to LCFA synthesis and we, thus, investigated the effects of JTP-103237 on lipogenesis after a single treatment. Results demonstrated that JTP-103237 did not decrease de novo lipogenesis after a single treatment but decreased lipogenesis after 7 days of treatment. From these findings, the decrease in de novo lipogenesis by JTP-103237 is considered to be not due to an acute effect of hepatic

MGAT inhibition.

Next, to understand how JTP-103237 decreased carbohydrate-induced fatty liver, we analyzed genes involved in lipogenesis. Interestingly, the expression of SREBP-1c, SCD1 and DGAT2 genes was significantly decreased with chronic treatment of JTP-103237. Preliminarily, the expression of MGAT1 and MGAT2 gene were also evaluated. The expression of MGAT1 was not changed and that of MGAT2 tended to be decreased ($p=0.078$, Student's t-test). However, since the copy number of these mRNA was low in comparison with other mRNAs, the data have not been shown. In addition, the expression of LXR and other SREBPs were not evaluated but these are subjects for future investigation. SREBP-1c is a key regulator of fatty livers (Ahmed and Byrne, 2007) and the overexpression of SREBP-1c in the liver of transgenic mice is known to manifest as severe fatty livers (Matsuda et al., 2001). SREBP-1c is predominantly involved in the regulation of insulin-responsive genes, which control lipogenesis (Foretz et al., 1999). In particular, SCD1, which is regulated via SREBP-1c, plays an important role in fatty liver and insulin resistance (Flowers et al., 2006). Indeed, liver-specific SCD1 deficiency completely blocked carbohydrate-induced lipogenesis (Flowers et al., 2008).

LCFAs, which are substrates of MGAT, are known to inhibit SREBP-1c expression by antagonizing activation of the liver X receptor (LXR) signaling (Jump et al., 1994; Ou et al., 2001). We hypothesized that LCFAs accumulated by inhibiting hepatic MGAT might inhibit LXR activation and consequent SREBP-1c expression. Next, to investigate this hypothesis, we evaluated the effects of a single treatment of JTP-103237 on hepatic lipid components after administration of LCFAs. Results demonstrated that JTP-103237 significantly increased the ratio of LCFAs, and tended to

decrease the ratio of TG in liver. These results suggested the possibility that hepatic MGAT inhibition may decrease not only TG synthesis, but also de novo lipogenesis via suppression of LXR/SREBP-1c signaling pathway by increasing LCFA. As described above, JTP-103237 decreased DGAT2 gene expression. It is known that DGAT2, which is strongly involved in fatty liver (Wang et al., 2010), was not regulated by SREBP-1c but LXR (Han et al., 2011; Horton et al., 2003). This result also indicated that JTP-103237 modulates the LXR/SREBP-1c signaling pathway. Our postulated mechanisms on the decrease in hepatic lipid synthesis by JTP-103237 are shown in Fig. 23.

The World Gastroenterology Organization stated that fatty liver disease and NASH represent a major global public health problem that is pandemic (LaBrecque et al., 2014). However, there are no specific drugs for the treatment of these diseases. Recently, the inhibition of hepatic de novo lipogenesis has been considered to be a potential therapeutic strategy for fatty liver disease (Lambert et al., 2014). The contribution of de novo lipogenesis to total hepatic TG production in normal subjects is small. However, the contribution of de novo lipogenesis to total hepatic TG production in patients with fatty liver disease is much higher (Fabbrini et al., 2010). In the present study, JTP-103237 prevented carbohydrate-induced fatty liver and suppressed both TG synthesis and de novo lipogenesis, suggesting that MGAT inhibition might be potential target for treatment of fatty liver disease and NASH.

JTP-103237 decreased plasma glucose levels and total cholesterol, reduced the accumulation of epididymal fats and tended to decrease plasma insulin levels. Since it had been observed the reduction of fat tissue and plasma insulin levels, the insulin sensitivity in adipose tissue was investigated. Although the changes were not

statistically significant due to large variation, JTP-103237 tended to increase adipose tissue glucose oxidation with insulin in HSVLF diet fed mice. In mesenteric fat tissue, the percentages for control group with insulin were 193% and 148% (with 1 and 10 nM insulin, respectively) whereas that without insulin was 125%. There is room for further investigation regarding the role of MGAT in extraintestinal tissue including muscle, but it may be probable that MGAT involves in systemic energy metabolism and insulin sensitivity.

From these findings, JTP-103237 is expected to display beneficial effects in the treatment of carbohydrate induced metabolic disorders, including fatty liver disease, adiposity and diabetes. As shown in Chapter 4, JTP-103237 also prevented fat induced obesity, fatty liver formation and glucose intolerance Taken together, MGAT inhibitor would be effective in metabolic diseases, regardless of whether these diseases are caused by excessive fat intake or carbohydrate intake.

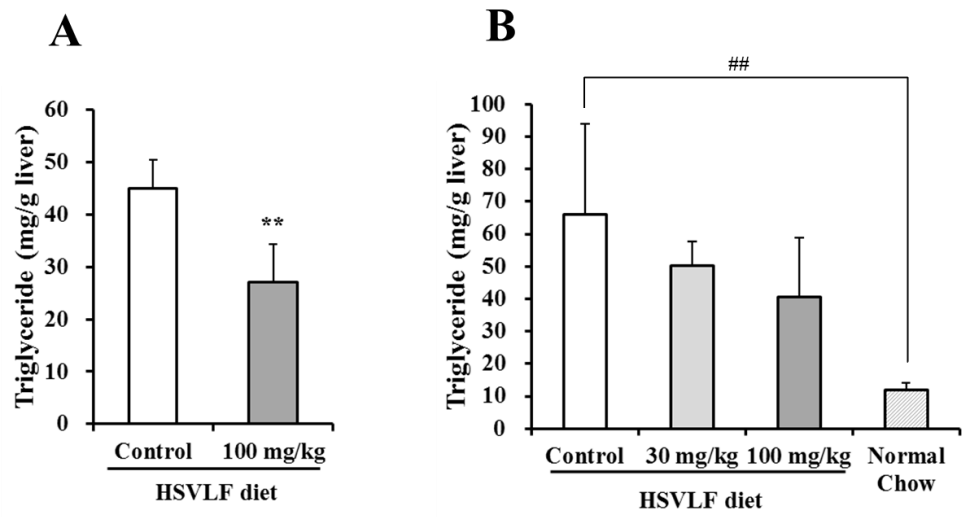


**Figure 17.** Hepatic TG content after chronic treatment with JTP-103237 for 21 days (A) and 35 days (B). Data shown as means + standard deviation ((A) n = 6 and (B) n = 6-8). **P<0.01; significantly different from the Control group. ## P<0.05; significantly different from the Normal Chow fed group.

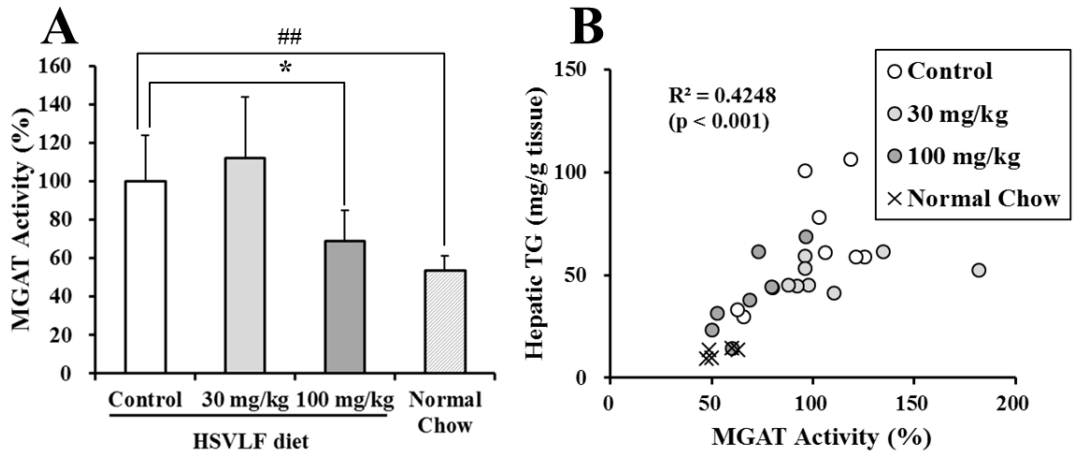


**Figure 18.** Hepatic MGAT activity after chronic treatment with JTP-103237 for 35 days (A) and the relationship between hepatic MGAT activity and TG content (B). Data shown as means + standard deviation (n = 6-8). *P<0.05; significantly different from the Control group. ## P<0.01; significantly different from the Normal Chow fed group.

**Table 8.** The effects of JTP-103237 on biochemical parameters (Day 24) and fat weight (Day 37) in HSVLF diet fed mice.

| Chow | Normal | HSVLF | | |
|---|---|---|---|---|
| Treatment | None | None | JTP-103237 | |
| | | | 30 mg/kg/day | 100 mg/kg/day |
| Body weight (g) | 27.5 ± 0.2 | 27.2 ± 1.8 | 26.9 ± 0.7 | 26.7 ± 1.7 |
| Food intake (g) | 122 ± 4 | 113 ± 7# | 116 ± 4 | 116 ± 8 |
| Fat weight (g) | 0.54 ± 0.06 | 0.65 ± 0.15 | 0.60 ± 0.10 | 0.49 ± 0.11* |
| Glucose (mg/dl) | 186 ± 14 | 215 ± 41 | 197 ± 26 | 179 ± 18* |
| Insulin (mg/dl) | 2.0 ± 2.0 | 3.5 ± 2.1 | 1.5 ± 1.1* | 1.7 ± 0.8 |
| TG (mg/dl) | 144 ± 23 | 74 ± 26# | 59 ± 13 | 58 ± 21 |
| Cholesterol (mg/dl) | 103 ± 2 | 125 ± 16## | 123 ± 10 | 96 ± 9** |

Data shown as means ± standard deviation (n = 6-8).

*P<0.05, **P<0.01; significantly different from the Control group.

#P<0.05, ##P<0.01; significantly different from the Normal Chow fed group.

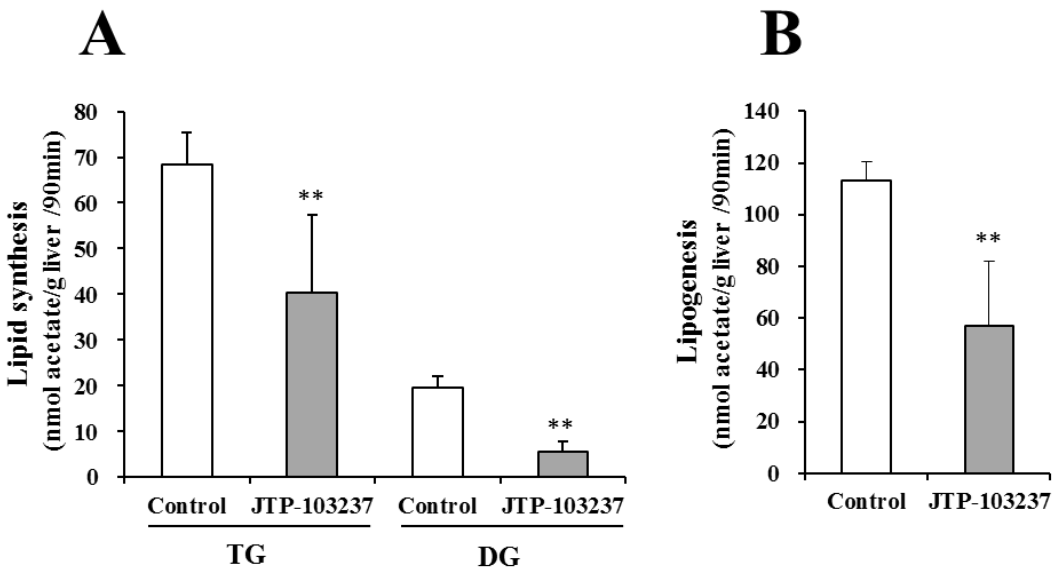


**Figure 19.** Effects of JTP-103237 (100 mg/kg) after 21 days of treatment on TG and DG synthesis (A) and lipogenesis (B) in HSVLF diet fed mice. Data shown as means + standard deviation (n = 5). **P<0.01; significantly different from the Control group.

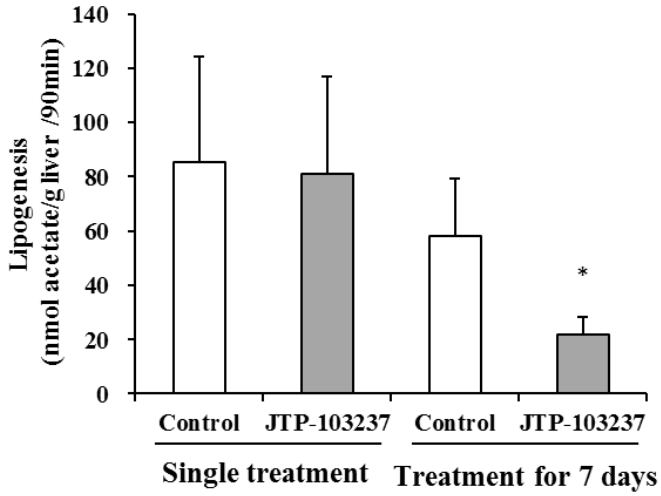


**Figure 20.** Effects of JTP-103237 (100 mg/kg) after a single or 7 days of treatment on lipogenesis in HSVLF diet fed mice. Data shown as means + standard deviation (n = 5). *P<0.05; significantly different from the Control group.

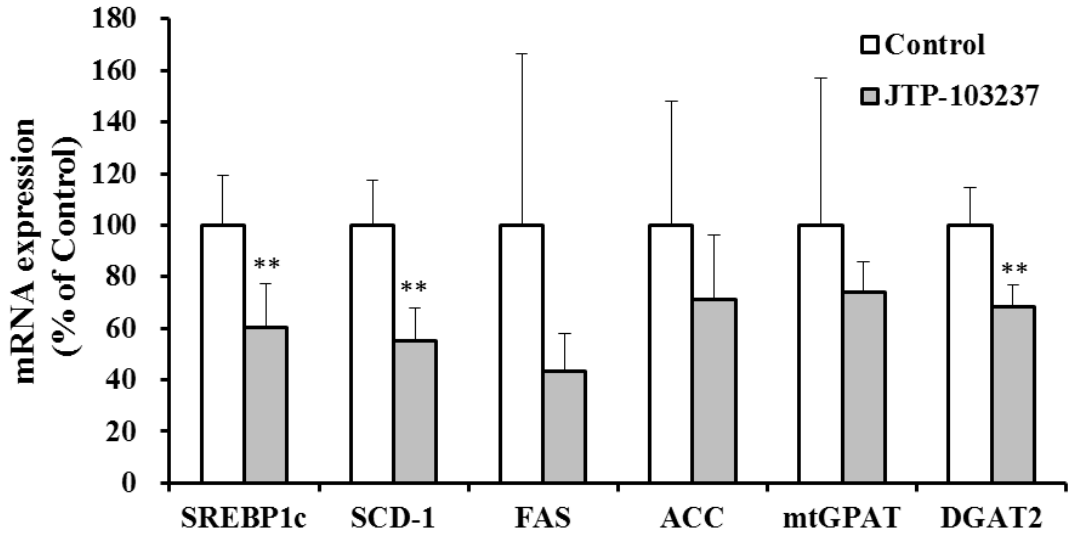


**Figure 21.** Effects of JTP-103237 (100 mg/kg) after 29 days of treatment on lipogenic gene expressions. Data shown as means + standard deviation (n = 6). **P<0.01; significantly different from the Control group.

**Table 9.** Effects of single treatment of JTP-103237 (100 mg/kg) on lipid composition after administration of $^{14}$C oleic acid in HSVLF diet fed mice.

| | TG (%) | DG (%) | LCFA (%) |
|---|---|---|---|
| Control | 72.1±2.2 | 17.7±2.9 | 5.0±0.5 |
| JTP-103237 | 69.8±1.3 | 20.0±1.6 | 5.5±0.3* |

Data shown as means ± standard deviation (n = 6).

*P<0.05; significantly different from the Control group.

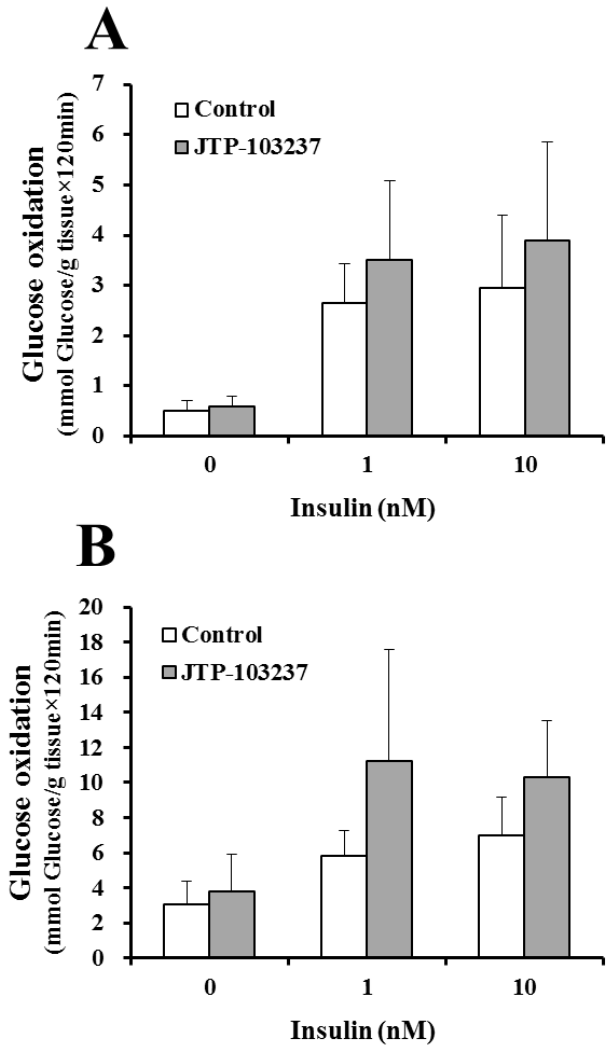


**Figure 22.** Effects of JTP-103237 (100 mg/kg) after 44 days of treatment on glucose utilization in epididymal fat tissue (A) and mesenteric fat tissue (B) Data shown as means + standard deviation (n = 5).

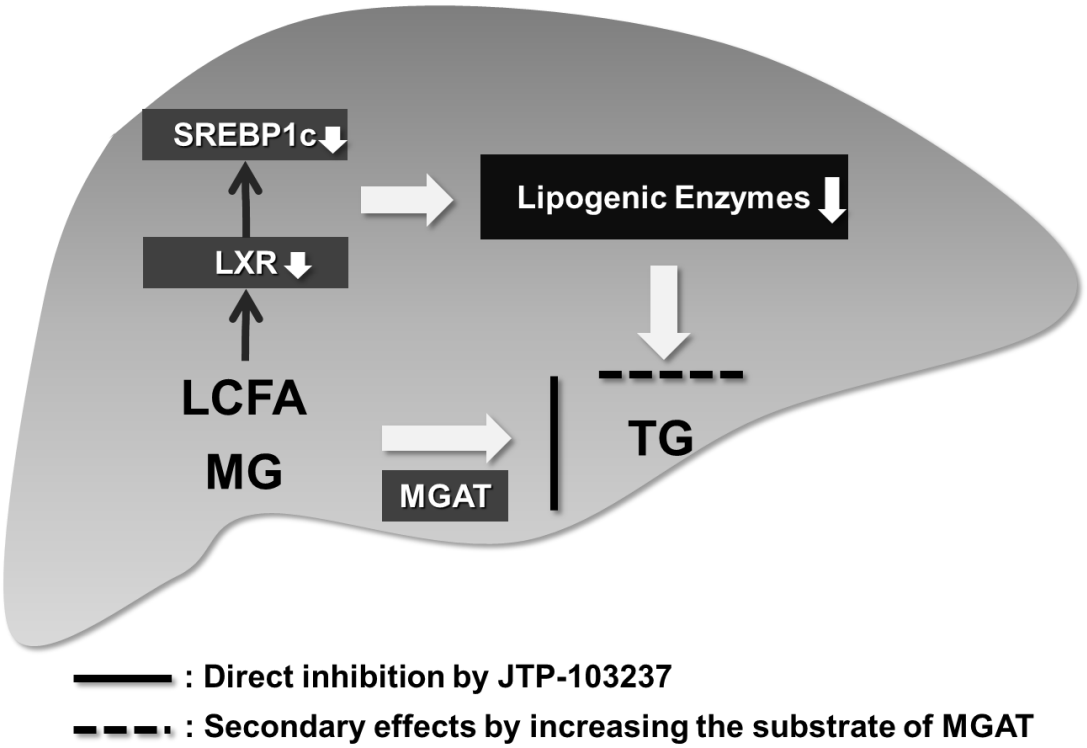


**Figure 23.** Postulated mechanisms on decrease in hepatic lipid synthesis by an MGAT inhibitor. TG synthesis via the MGAT pathway may be suppressed through the direct inhibition of MGAT. As a result of MGAT inhibition, LCFAs, which are substrates of MGAT, may increase and it suppresses SREBP-1c and related gene expression via antagonizing LXR signaling (secondary effects through the inhibition of MGAT). Hepatic TG lipid synthesis would be suppressed by the MGAT inhibitor via direct and indirect (secondary) effects.

# Chapter 6

# General Discussion

Epilepsy is defined as a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures. Epilepsy can occur at all ages, and there are numerous possible presentations and causes (Duncan et al., 2006). Although incidence in childhood has fallen over the past three decades, this reduction is matched by an increase in elderly people. As mentioned in Chapter 1, the QOL in epilepsy patients is worse than the general population (Guekht et al., 2007). In addition, overall, individuals with epilepsy have a 1.6 to 11.4-fold higher mortality rate (Laxer et al., 2014; Ridsdale et al., 2011). Despite the existence of some antiepilepsy drugs, adverse effects remain a leading cause of treatment failure and 30% to 40% of patients with epilepsy fail to respond to antiepilepsy drugs or other treatments (Perucca and Tomson, 2011; Perucca and Gilliam, 2012). With this environment, providing new therapeutic strategies for epilepsy treatment is particularly important.

The histaminergic system reportedly plays a significant role in several brain disorders (Baronio et al., 2014). Histamine is considered to be an anticonvulsive neurotransmitter as low levels of histamine are associated with epileptic seizures (Hirai et al., 2004; Kiviranta et al., 1995). However, the association between the histaminergic system and epilepsy is still in its infancy and the mechanism of the antiepilepsy effects of histamine is also unclear. In Chapter 2, the inhibitory effect and mechanism of histamine on epileptic seizures were investigated using the amygdaloid kindling model, which is an animal model commonly used for epilepsy. I.c.v injection of histamine caused dose-dependent inhibitions of amygdaloid kindling seizure and this effect of

histamine was attenuated with histamine $H_1$ receptor antagonist, but not $H_2$ receptor antagonist (Chapter 2: Fig. 3, Table 1). In addition, the inhibitory effect of histamine was potentiated by increasing calcium levels and was attenuated by calcium chelators (Chapter 2: Tables 2 and 3). Histamine is known to induce cytosolic calcium increases via the histamine $H_1$ receptor pathway (Leurs et al., 1995). CSF containing normal levels of calcium is reportedly able to block kindled seizures and that CSF without calcium resulted in the return of seizures (Davies and Peterson, 1989), suggesting that intracellular calcium levels are important in causing epileptic seizures and that the inhibitory effects of histamine on epileptic seizures are closely associated with calcium signaling. PKC and CaM kinase II are major protein kinases activated via $H_1$ receptor and calcium signaling (Kawakami et al., 2003). In the present study, the inhibitory effects of histamine on kindling seizure were attenuated by inhibiting CaM kinase II, but not by inhibiting PKC (Chapter 2: Fig. 5).

CaM kinase II activation reportedly modulates $GABA_A$ receptor functions, which play fundamental role on epileptic seizures (Churn and DeLorenzo, 1998; Machu et al., 1993; Wang et al., 1995). In addition, the inhibitory effects on amygdaloid kindling seizure by stimulating the histaminergic neuron system were attenuated by $GABA_A$ receptor antagonist, bicuculline (Ishizawa et al., 2000). Taking all of this information together, it is suggested that histamine inhibits epileptic seizures via stimulating $GABA_A$ receptor signaling through the activation of CaM kinase II.

GABA-related drugs, such as sodium valproate and diazepam, are used for the treatment of epilepsy (Sirven and Waterhouse, 2003). However, these drugs have some unwanted adverse effects, such as lethargy, dizziness, and behavioral and cognitive impairment (Laxer et al., 2014). On the other hand, the central histaminergic neuron

system reportedly induces wakefulness and improves cognitive functions (Dere et al., 2010; Panula and Nuutinen, 2013; Schneider et al., 2014). Therefore, although histamine related compounds stimulate $GABA_A$ receptor signaling, these compounds may not cause drowsiness, lethargy and cognitive impairment in contrast to sodium valproate and diazepam. Currently, histamine $H_3$ receptor antagonists, which stimulate histaminergic neurons, are being developed for the treatment of central nervous system disorders, including epilepsy (Bialer et al., 2015; Sadek et al., 2014). Although further investigations regarding the properties of the effect of histamine-related compounds on epilepsy are required, the present study results may contribute to revealing novel mechanisms of antiepilepsy effects through the histaminergic neuron system.

In addition to epilepsy, the obesity epidemic is a global issue, including within Japan, and there are no signs of the epidemic abating. Obesity is the most prevalent cause of metabolic syndrome, which increases the risk of diabetes and cardiovascular disease (Despres and Lemieux, 2006). Recent reports pointed out that one in five deaths in the United States are associated with obesity, which is more than three times higher than previous estimates (Masters et al., 2013). Therefore, preventive and treatment strategies for obesity are urgently needed to stop the epidemic.

As shown in Chapter 1, controlling appetite is one of the promising strategies for obesity treatment. However, despite numerous antiappetite drugs having been used to manage obesity over the years, most have now been withdrawn from the market due to serious adverse effects, including depression and suicide attempts (Kang and Park, 2012). On the other hand, controlling appetite via peripheral signals is considered to be a promising strategy to treat obesity. For example, inhibitors of DGAT1 stimulated

satiety from peripheral signaling (Tomimoto et al., 2015). Similar results have been reported in a study using an MTP inhibitor (Hata et al., 2011). These satiety effects might be via peripheral signals since these effects were observed with an increase in intestinal LCFA, which reportedly promotes satiety by acting on the intestine (Feltrin et al., 2004).

LCFA and 2-MG are both digestive products of dietary TG hydrolysis. LCFA is known to reduce food intake and induce diarrhea. However, the functions of 2-MG on food intake and diarrhea have not been investigated. In Chapter 3, the intrajejunal infusion of 2-MG decreased food intake and did not induce diarrhea in conditions where comparable reduction in food intake was observed with LCFA (Chapter 3: Fig. 6, Table 4). The results suggest that an increase in intestinal 2-MG may be a novel strategy for obesity treatment through a reduction in food intake.

MGAT is involved in intestinal lipid absorption and catalyzes the synthesis of DG from 2-MG and long-chain fatty acyl-CoA. In Chapter 4, a novel inhibitor of MGAT, JTP-103237, reduced TG absorption and led to the accumulation of its substrates, 2-MG and LCFA, after lipid loading (Chapter 4: Figs. 9 and 10). The accumulation of 2-MG was more clearly associated with the administration of the MGAT inhibitor rather than that of LCFA. In addition, MGAT inhibitor increased PYY, which is the satiety hormone secreted from the intestine (De Silva and Bloom, 2012), after loading the TG emulsion (Chapter 4: Fig. 11). Taken together with results from Chapter 3, the increase in intestinal 2-MG by inhibiting MGAT was considered to lead to a decrease in food intake and body weight. Indeed, MGAT inhibitor decreased food intake in a dietary fat dependent manner (Chapter 4: Fig. 12). After chronic treatment with the MGAT inhibitor, body weight gain and cumulative food intake in high fat diet

fed mice were decreased (Chapter 4: Figs. 13 and 14). These results suggested that MGAT inhibition in the intestine contributes to satiety and antiobesity effects by increasing 2-MG. In addition, since the MGAT inhibitor did not change body weight gain and food intake in low fat diet fed mice (Chapter 4: Fig. 13), these effects of the MGAT inhibitor are considered not to be related to the central nervous system but to be related to fat absorption in the intestine.

The efficacy of the MGAT inhibitor on body weight reduction was comparable with orlistat, which is an antiobesity drug (Chapter 4: Fig. 16A). In this conditions, orlistat increased food intake in contrast to the MGAT inhibitor (Chapter 4: Fig. 16B). Since orlistat is a pancreatic lipase inhibitor, this drug may increase TG and decrease 2-MG and FA in the intestine after fat intake. Considering this point, the differences in satiety effects between the MGAT inhibitor and orlistat may be dependent on accumulated fat. Orlistat is known to cause oily diarrhea (Li and Cheung, 2009). LCFA also causes diarrhea by inhibiting water and electrolyte absorption in the ileum in humans (Ammon and Phillips, 1974; Yoshioka et al., 1986). Inhibitors of DGAT1 and MTP, which inhibit TG absorption and increase intestinal FA, caused diarrhea and prevented dose escalation (Denison et al., 2014; Hussain and Bakillah, 2008). In the present study, both the 2-MG and MGAT inhibitor reduced food intake but did not induce diarrhea in rats. From these data, increases in 2-MG in the intestine are expected to be a meaningful and useful approach for obesity treatment to avoid gastrointestinal symptoms, such as diarrhea.

In addition to the reduction in food intake, the MGAT inhibitor enhanced $O_2$ consumption in mice fed a high fat diet (Chapter 4: Fig.15). MGAT2 KO mice reportedly exhibited increased energy expenditure (Nelson et al., 2011; Nelson et al.,

2014; Tsuchida et al., 2012; Yen et al., 2009). Although the detailed mechanism on increasing $O_2$ consumption by MGAT inhibition is unclear, MGAT inhibition may exhibit beneficial effects on food intake and energy expenditure, both of which are the main causes of obesity.

Obesity is known to be associated with fatty liver disease (Fabbrini et al., 2010). Fatty liver disease is considered to be a precursor of metabolic diseases and a risk factor for cardiovascular disease, and a growing epidemic worldwide (Hamaguchi et al., 2005; Lonardo et al., 2015; Targher et al., 2007) In Chapter 3, the MGAT inhibitor decreased hepatic TG content in a high fat diet fed mice (Chapter 4: Table 7). However, this reduction in hepatic TG contents in a high fat diet induced obesity model did not exclude the influence of body weight and food intake reduction by inhibiting intestinal MGAT. MGAT is known to be mainly expressed in intestinal tissue but also is expressed in extraintestinal tissues, such as the liver (Hall et al., 2012; Mostafa et al., 1993). In addition, hepatic MGAT expression is reportedly increased in patients with fatty liver disease (Hall et al., 2012). In Chapter 5, the role of MGAT on hepatic lipid metabolism was investigated in HSVLF diet induced fatty liver model. The inhibitory effects of intestinal MGAT may contribute very slightly in this model since the content of fat in the HSVLF diet is very low. In this condition, the MGAT inhibitor reduced hepatic TG content and hepatic MGAT activity (Chapter 5: Figs. 17 and 18A). Moreover, hepatic TG content was significantly correlated with hepatic MGAT activity (Chapter 5: Fig. 18B), suggesting that this reduction in hepatic TG content may be due to hepatic MGAT inhibition. In addition, the MGAT inhibitor suppressed both TG synthesis and de novo lipogenesis (Chapter 5: Fig. 19).

Although the detailed mechanism of the suppressive effect of the MGAT inhibitor on de novo lipogenesis is unclear, chronic treatment with the MGAT inhibitor decreased the mRNA expression of SREBP-1c (Chapter 5: Fig. 21), which is a key regulator of hepatic de novo lipogenesis (Foretz et al., 1999). In addition, a single treatment with the MGAT inhibitor did not suppress de novo lipogenesis, but significantly increased the ratio of LCFA to total labeled lipids after an injection of LCFA (Chapter 5: Fig. 20, Table 9). The postulated mechanism of the suppressive effects on de novo lipogenesis is shown in Chapter 5, Fig. 23. LCFA, which is a substrate of MGAT, reportedly suppresses SREBP-1c and related gene expression (Jump et al., 1994; Ou et al., 2001; Pawar et al., 2003). Thus, the suppression of de novo lipogenesis with MGAT inhibitor treatment may be a secondary effect of MGAT inhibition via a reduction in SREBP1c expression. There are many factors involved in causing fatty liver disease. Among them, de novo lipogenesis is reportedly more likely to directly contribute to fatty liver disease than dietary fat supply (Basaranoglu et al., 2015). Taken together, it is suggested that MGAT inhibition may be a potential novel approach for the treatment of obesity patients with fatty liver disease.

Obesity is also known to be associated with an increased risk of developing insulin resistance and diabetes (Kahn et al., 2006). The effects of MGAT inhibition on glucose metabolism were also evaluated in Chapters 4 and 5. The MGAT inhibitor significantly improved glucose tolerance in high fat diet fed mice and this effect was more clearly observed that of orlistat (Chapter 4: Fig. 16C). In HSVLF diet fed mice, the MGAT inhibitor reduced plasma glucose levels and tended to increase glucose oxidation after insulin treatment in adipose tissues (Chapter 5: Fig. 22, Table 8). Although further investigations are required in future studies, the antiobesity effect of

MGAT inhibition may ameliorate not only obesity but also related diseases, such as fatty liver disease and diabetes, and beneficially contribute to preventing metabolic syndrome.

In conclusion, the present study clarified novel antiepilepsy and antiobesity effects in experimental animal models. The study in Chapter 2 revealed that the antiepileptic effect of histamine is closely associated with the CaM kinase II activation pathway. Studies in Chapter 3 to 5 revealed that intestinal 2-MG reduced food intake without inducing diarrhea, and increase in intestinal 2-MG by inhibiting MGAT reduced food intake and body weight. Moreover, it is suggested that MGAT inhibition ameliorates obesity-related metabolic disorders, such as fatty liver disease and diabetes. These results may contribute to the clarification of the physiological roles of histaminergic neuron system, intestinal 2-MG and MGAT, and to the discovery of novel treatment approach for epilepsy and obesity.

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