INTRODUCTION

In both preclinical and clinical studies during drug development, alanine transaminase (ALT) and aspartate transaminase (AST) have long been used as potent blood markers to detect hepatocellular injury. In general, ALT is superior to AST in ability to detect hepatocellular injury because of its higher abundance in hepatocytes and the stronger degree of correspondence between its circulating level and the morphological severity of injury (Travlos et al., 1996; Fujii, 1997). In contrast, in some cases, especially rats, ALT is inferior to AST because of its relatively lower activity in hepatocytes and shorter half-life in circulation. It has therefore been proposed that in rats glutamate dehydrogenase (GLDH) and sorbitol dehydrogenase (SDH) are more useful than ALT in detecting injury, based on their large increases and persistence following injury and their high degree of sensitivity in detecting injury and specificity for the liver (Travlos et al., 1996; O’Brien et al., 2002).

In preclinical studies, circulating levels of hepatocellular enzymes sometimes slightly but clearly increase in the absence of any morphological hepatocellular injury. This is probably due to leakage of these enzymes from the hepa-
tocytes into the circulation, either through membrane with increased permeability or membrane blebs without subsequent hepatocellular death (Gores et al., 1990; Hoffman et al., 1999; Ramaiah, 2007). However, the causes of this phenomenon are likely to include not only alteration of the hepatocyte membrane but also the induction of hepatocellular enzymes themselves by drug treatment. Gluconeogenesis is considered a possible inducer of hepatocellular enzymes, given their primary roles as enzymes and several related types of in vivo evidence. Transaminases are originally ubiquitous enzymes involved in several central metabolic pathways including hepatic gluconeogenesis. Under certain gluconeogenic conditions, ALT catalyzes the reaction from alanine to pyruvate (Sakagishi, 1995) while AST participates in the malate-aspartate shuttle and supplies oxaloacetate (Horio et al., 1988). Other hepatocellular enzymes, GLDH and lactate dehydrogenase (LDH), are also known to play roles in gluconeogenesis, by catalyzing the conversions of glutamate and lactate, respectively (Brooks, 2002; Brosnan, 2000). Hepatic transaminase activities are increased in vivo under certain gluconeogenic conditions in animals in response to glucocorticoid, glucagon, dietary protein, and fasting (Rosen et al., 1959; Horio et al., 1988).

Here, we present findings of increases in circulating transaminase levels without evidence of hepatocellular injury in mice treated with the selective β3-adrenoceptor agonist BRL 37344, (±)-(R*,R*)-[4-[(2-(3-Chlorophenyl)-2-hydroxyethyl)amino]propyl]phenoxy]acetic acid. To clarify whether this resulted from a β3-adrenoceptor-stimulating effect of BRL 37344, we examined the inhibitory effect of the selective β3-adrenoceptor antagonist SR 59230A, 3-(2-Ethylphenyl)-1-[[1S]-1,2,3,4-tetrahydro-naphth-1-yl][amino]-(2S)-2-propanol, on the increase in circulating transaminase levels induced by BRL 37344. We also considered the relationship between this BRL 37344-induced increase in circulating transaminase levels and glucose metabolism by measuring blood components relevant to glucose metabolism.

**MATERIALS AND METHODS**

**Animals**

Male specific-pathogen-free Crlj:CD1(ICR) mice were obtained from Charles River Japan (Kanagawa, Japan). The animals were housed individually in aluminum breeding cages in a temperature (21-25°C), humidity (40-70%)-, and lighting (L/D:12/12)-controlled barrier-system room. They were fed CE-2 commercial diet (CLEA Japan, Tokyo, Japan) and provided water ad libitum. After one week of quarantine, they were subjected to experiments. Age at the start of experiments was 7 weeks. This study was performed in accordance with the Guidelines for Animal Care and Experimentation of Kissei Pharmaceutical Co., Ltd.

**Blood and liver sampling**

Blood samples were collected from the caudal vena cava of mice at the designated time points, under nonfasted condition with use of isoflurane anaesthesia, with disposable syringes containing heparin sodium as an anticoagulant. The collected blood was separated into plasma and stored deeply frozen until use. The animals were then euthanized by exsanguination and the liver was collected and immediately fixed in 10% phosphate-buffered formalin for histopathological examination.

**Blood biochemistry**

The following plasma components were determined with a 7180 automatic analyzer (Hitachi High-Technologies, Tokyo, Japan) according to the indicated methods: ALT and AST activities by the standard methods of the Japan Society of Clinical Chemistry; GLDH activity by the standard method of the German Society of Clinical Chemistry; guanine deaminase (GUA) activity, non-esterified fatty acid (NEFA), pyruvate (PA), and lactate (LA) levels by enzyme methods; and glucose (GLU) level by the hexokinase method. Insulin and glucagon levels were measured by enzyme-linked immunosorbent assays (ELISA) with an insulin assay kit (Morinaga Institute of Biological Science, Kanagawa, Japan) and a glucagon assay kit (Yamaihara Institute, Shizuoka, Japan).

**Histopathology**

The liver fixed with 10% phosphate-buffered formalin was embedded in paraffin, sectioned at 3 µm-thickness, stained with hematoxylin-eosin, and microscopically examined.

**Experimental design**

Experiment 1: The time-course changes in levels of plasma components and hepatic histopathology following administration of the selective β3-adrenoceptor agonist BRL 37344 were determined. BRL 37344 sodium salt hydrate was obtained from Sigma Chemical Co. (St. Louis, MO, USA). It was dissolved in physiological saline and administered intraperitoneally to mice as a single dose of 1 or 10 mg/kg body weight. The dose levels were determined based on a preliminary experiment performed with doses ranging from 1 to 100 mg/kg of BRL 37344, in which ele-
viation of circulating NEFA levels indicated that the pharmacological lipolytic effect of BRL 37344 plateaued at 10 mg/kg. Control mice received physiological saline alone in the same fashion. Blood and liver samples were collected at 0.5, 4, 8, and 24 hr post-dose of BRL 37344 for blood biochemical and histopathologic examination.

Experiment 2: The effects of the selective β3-adrenoceptor antagonist SR 59230A on circulating levels of blood components following administration of the β3-adrenoceptor agonist BRL 37344 were evaluated. SR 59230A oxalate salt was obtained from Sigma Chemical Co. It was suspended uniformly in physiological saline and administered intraperitoneally to mice once at a dose of 1 or 3 mg/kg body weight. Thirty minutes after the administration of SR 59230A, BRL 37344 dissolved in physiological saline was administered intraperitoneally to mice as a single dose at 10 mg/kg body weight. The dose of SR 59230A was determined based on a preliminary experiment performed with doses ranging from 1 to 20 mg/kg, in which doses above 5 mg/kg dose-dependently increased ALT levels by 1.4 to 3.1-fold and AST levels by 2.1 to 13-fold from baseline due to toxicity, while those up to 3 mg/kg were not toxic. The dose of BRL 37344 was set based on the results of experiment 1. Blood samples were collected 4 hr post-dose of BRL 37344 for blood biochemical examination.

Statistics

All values are expressed as the mean ± S.E. Comparisons between the control group and treated groups were first performed with Bartlett's test for homogeneity of variation, followed by Duncan’s parametric multiple or non-parametric multiple range test for comparisons. P values < 0.05 were considered significant.

RESULTS

Effect of β3-adrenoceptor agonist on blood biochemistry and liver (experiment 1)

The time-course changes in levels of blood components following administration of the β3-adrenoceptor agonist BRL 37344 are shown in Fig. 1. The β3-adrenergic stimulating effect of BRL 37344 was confirmed by its lipolytic effect of increasing in NEFA levels from 0.5 to 8 hr post-dose and by its insulinotropic effect of increasing insulin levels in dose-proportional fashion from 0.5 to 4 hr post-dose. Corresponding to the increases in insulin levels, GLU levels were decreased dose-dependently at 4 and 8 hr post-dose in the BRL 37344-treated mice. The levels of both gluconeogenic precursors PA and LA were rapidly increased in proportion to dose at 0.5 hr post-dose. PA level was again increased at 8 hr post-dose. There were no changes in level of the gluconeogenic hormone glucagon in BRL 37344-treated mice. In association with these changes in glucose metabolism, transaminase (ALT and AST) levels were increased maximally and in dose-proportional fashion at 4 hr post-dose in the BRL 37344-treated mice. The significant increase in AST levels at 10 mg/kg persisted up to 24 hr post-dose. While transaminase levels were increasing, there were no significant increases in the levels of other hepatocellular enzymes, GLDH, LDH, and GUA, in the BRL 37344-treated mice, except for a slight increase in GLDH level only at 24 hr post-dose. Neither necrosis nor degeneration was observed in the hepatocytes of the BRL 37344-treated mice up to 24 hr post-dose.

Effects of β3-adrenoceptor antagonist on β3-adrenoceptor agonist-induced changes in transaminase levels (experiment 2)

The effects of the selective β3-adrenoceptor antagonist SR 59230A on the alteration of ALT, AST and insulin levels by the β3-adrenoceptor agonist BRL 37344 were evaluated 4 hr post-dose of BRL 37344 and are shown in Fig. 2. The time point of evaluation was determined based on experiment 1, in which blood transaminase levels were maximally increased 4 hr post-dose. The magnitude of elevation of insulin level by BRL 37344 alone was significantly decreased by pre-treatment with SR 59230A in proportion to dose, confirming its β3-adrenergic inhibitory effect. The magnitude of elevation of ALT level by BRL 37344 alone tended to decrease in proportion to SR 59230A doses, even though there were no statistical differences. The magnitude of elevation of AST was not decreased with 1 mg/kg of SR 59230A.

DISCUSSION

Treatment with the selective β3-adrenoceptor agonist BRL 37344 increased the circulating transaminase levels in mice without causing increases in the circulating levels of other hepatocellular enzymes or any type of morphological hepatocellular injury. Pre-treatment with the β3-adrenoceptor antagonist SR 59230A partly inhibited these increases in transaminase levels by BRL 37344 even though it was not significant due to hepatotoxicity at higher doses of SR 59230A. However, propranolol, a β1- and β2-adrenoceptor antagonist, at sufficient doses was not effective in inhibiting the increase in circulating transaminase levels induced by BRL 37344 in our own experiments. In particular, propranolol ranging from 10 to 50 mg/kg, which are sufficient to inhibit β1- and β2-
adrenoceptor-mediated effects (Kohout et al., 2001), did not inhibit increases in transaminase levels by 100 mg/kg of BRL 37344 in mice. These findings indicated that the increases in levels of circulating transaminases by BRL 37344 were due at least in part to β3-adrenoceptor-mediated effect. Blood components relevant to glucose and lipid metabolisms exhibited dynamic changes, starting with insulin and NEFA secretions, in the BRL 37344-treated mice. Specifically, BRL 37344 initially increased insulin and NEFA 0.5 hr post-dose, findings considered attributable to enhanced insulin secretion from the pancreas (Atef et al., 1996) and enhanced lipolysis in the adipose tissues (Lönnqvist et al., 1995), which thus involved β3-adrenoceptor-stimulating effects. Thereafter, GLU was decreased 4 and 8 hr post-dose nevertheless the animals used in this study were normoglycemic but not hyperglycemic, suggesting the disruption of glucose homeostasis. Along with the dynamic changes in levels of insulin, NEFA and GLU, the levels of both gluconeogenic precursors PA and LA were increased 0.5 hr post-dose and then returned to baseline by 4 hr post-dose. In this study, although there were no changes directly suggestive of enhanced gluconeogenesis such as an increase in the gluconeogenic hormone glucagons, the excessive changes in glucose metabolism could have been sufficient to induce gluconeogenesis within 24 hr post-dose of BRL 37344. It may thus be that transaminases were induced as early as 4 hr post-dose by enhanced gluconeogenesis, as sug-

Fig. 1. Effects of BRL 37344 on blood components in mice. Mice received a single intraperitoneal dose of BRL 37344 (1 or 10 mg/kg). Values are the mean ± S.E. of results for three to four male mice at 0.5, 8 and 24 hr post-dose, and for seven to eight male mice at 4 hr post-dose. Experiments were repeated at 4 hr post-dose to assess reproducibility. *P < 0.05, **P < 0.01 versus control mice, P < 0.05, #P < 0.01 versus mice treated with 1 mg/kg of BRL 37344.
gested by the finding that alanine, the substrate of ALT, is the most effective gluconeogenic precursor (Yamamoto et al., 1974). Additionally, the increased NEFA is considered to be probably involved in this mechanism to increase transaminases in enhanced gluconeogenesis, given that NEFA β-oxidation produces acetyl-CoA that activates pyruvate carboxylase activity, NADH and ATP, which are used for gluconeogenesis (Chen et al., 1999; Roden et al., 2000).

Two examples are available of pharmacologically increased circulating transaminase levels not resulting from direct hepatic toxicity. The first is related to altered glucose metabolism just as the case of BRL 37344. Voglibose, an α-glucosidase inhibitor that lowers GLU levels by delaying the intestinal absorption of carbohydrates, increases ALT levels without causing any morphological hepatic injury in rats. This effect is completely eliminated in rats fed a glucose-supplemented diet, demonstrating that pharmacological disruption of glucose metabolism increases circulating ALT levels (Suzuki et al., 1991). The other is due to the stimulation of peroxisome proliferator-activated receptor (PPAR) α. PPAR α agonists, such as fenofibrate and AZD4619, increase circulating transaminase levels without increase in other hepatotoxic markers in humans and rats (Thulin et al., 2008; Kobayashi et al., 2009). This has been demonstrated both in vitro and in vivo due to the increased expression of transaminase protein induced by PPAR α activation (Edgar et al., 1998; Thulin et al., 2008; Kobayashi et al., 2009). PPAR α stimulation is known to enhance fatty acids β-oxidation (Reddy, 2001), from which products to enhance gluconeogenesis might be produced. Thus, circulating transaminase levels are increased due to the PPAR α-mediated changes in lipid metabolism possibly affecting glucose metabolism.

Of the several hepatocellular enzymes, GUA could be useful in detecting hepatocellular injury, especially in cases in which transaminases are secondarily induced by alterations in glucose metabolism by drug such as BRL 37344. This is because GUA, which is not involved in glucose metabolism, was not increased while transaminase levels were increasing in this study. In addition, GUA has been known to be abundant in the liver and recognised as highly useful in detecting hepatocellular injury in both humans and animals. In humans, the clinical significance of determination of circulating GUA levels has been demonstrated for various hepatic diseases such as acute and chronic hepatitis, and is equal or superior to that of transaminases (Ito et al., 1982; Nishikawa et al., 1984; Shiota et al., 1989). In animals as well, circulating GUA levels have been shown to rapidly increase in CCl₄-intoxicated rats and dogs (Bowkiewicz-Surma and

Fig. 2. Effects of SR 59230A on BRL 37344-induced increases in the blood components of mice. Mice received a single intraperitoneal dose of SR 59230A (1 or 3 mg/kg) at 0.5 hr before a single intraperitoneal dose of BRL 37344 (10 mg/kg). Plasma was collected 4 hr post-dose of BRL 37344. Values are fold-changes relative to control values (from experiment 1). Values are the mean ± S.E. of results for four male mice given BRL 37344 alone and seven male mice given SR 59230A. *P < 0.05 versus mice given BRL 37344 alone.
Krawczyński, 1967; Ito et al., 1982). In our own experiments as well, the usefulness of measurement of GUA was confirmed in mice and rats treated with concanavalin A, a hepatotoxic lectin, which was found to be equal or superior to ALT and GLDH in ability to detect hepatocellular injury (data not shown).

In conclusion, we found that the β₃-adrenoceptor agonist BRL 37344 increased circulating transaminase levels in mice in the absence of increases in other hepatocellular enzymes or any hepatocellular injury. The β₃-adrenoceptor antagonist SR 59230A partly inhibited the increases in transaminase levels induced by BRL 37344. Blood components relevant to glucose metabolism exhibited dynamic changes in BRL 37344-treated mice. Our findings suggest the possibility that circulating transaminase levels are increased as pharmacological effects of drugs disrupting glucose metabolism, and suggest that hepatotoxic markers should be selected considering these effects to distinguish between acceptable pharmacology and toxicity.

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REFERENCES


