Plasma 2-hydroxyglutarate, a promising prognostic biomarker candidate for skeletal muscle injury in Fischer 344 rats

Hisakuni Obayashi1,2, Naoko Kobayashi3, Yoshikazu Nezu4, Takashi Yamoto3, Mitsuyuki Shirai1 and Fumitoshi Asai1

1Laboratory of Veterinary Pharmacology, School of Veterinary Medicine, Azabu University, 1-17-71 Fuchinobe, Chuo-ku, Sagamihara, Kanagawa 252-5201, Japan
2Research Function, Daiichi-Sankyo Co., Ltd., 1-2-58 Hiromachi, Shinagawa-ku, Tokyo 140-8710, Japan
3Medicinal Safety Research Laboratories, Daiichi-Sankyo Co., Ltd., 1-16-13 Kitakasai, Edogawa-ku, Tokyo 134-8630, Japan
4Rare Disease Laboratories, Daiichi-Sankyo Co., Ltd., 1-2-58 Hiromachi, Shinagawa-ku, Tokyo 140-8710, Japan

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ABSTRACT — Previously, we have demonstrated the potential of plasma 2-hydroxyglutarate (2HG) as an easily detectable biomarker for skeletal muscle injury in rats. Here, we examined whether plasma 2HG was superior to conventional skeletal muscle damage biomarkers, including aspartate aminotransferase (AST), creatine kinase (CK), and skeletal muscle-type CK isoenzyme (CK-MM) levels, in rats. Skeletal muscle injury was induced in 4- or 9-week-old male Fischer 344 rats by cerivastatin (CER) or tetramethyl-p-phenylenediamine (TMPD) administration. Plasma 2HG levels were measured on days 4, 8, and 11 (CER group) and at 6 and 24 hr post-administration (TMPD group). Plasma AST, CK, and CK-MM activities and histopathological changes in the rectus femoris muscle were evaluated at the study endpoints. In the CER group, AST, CK, and CK-MM increased in 4- and 9-week-old rats, whereas increases in CK (4- and 9-week-old rats) and CK-MM (4-week-old rats) were not obvious in the TMPD group. In both 4- and 9-week-old rats, plasma 2HG increased on day 8 and at 24 hr post-administration in the CER and TMPD groups, respectively. Histopathological analysis revealed myofiber vacuolation and necrosis in both groups. The histopathological damage to the rectus femoris muscle was more severe in the CER than in the TMPD group. Increased plasma 2HG was associated with CER- and TMPD-induced skeletal muscle injuries in rats and was not affected by age differences or repeated blood collection. The results suggest that plasma 2HG is superior to CK and CK-MM as a biomarker for mild skeletal muscle injury.

Key words: Biomarker, Muscle injury, 2-Hydroxyglutarate, Rats

INTRODUCTION

Chemical- or drug-related muscle toxicity, ranging from muscle pain to rhabdomyolysis, is induced by treatment with antilipidemic and hypocholesterolemic drugs, anesthetics, immunosuppressants, proton pump inhibitors, and other compounds (Nozaki et al., 2004; Oshima, 2011; Tanaka et al., 2014). Early detection of skeletal muscle toxicity is important because it critically influences medical care and drug discovery, considering that skeletal muscle toxicity can develop into life-threatening rhabdomyolysis. Aspartate aminotransferase (AST) and creatine kinase (CK) are widely used as traditional biomarkers for skeletal muscle disorders. CK is a dimeric protein consisting of two distinct polypeptide subunits, M (muscle type) and B (brain type), and exists as three isoenzymes, CK-MM, CK-BM, and CK-BB. Since the types of CK isozymes vary in different tissues, lesion sites can be identified using CK-MM as a skeletal muscle marker, CK-MB as a cardiac muscle marker, and CK-BB as a brain tissue marker.

However, novel biomarkers that are superior to the conventional skeletal muscle biomarkers, AST and CK, or that can be combined with conventional biomarkers to
compensate for disadvantages of AST and CK are needed since AST and CK display low sensitivity and specificity, e.g., due to interference caused by hemolysis (Matsuzawa et al., 1993; Bohlmeyer et al., 1994; Sorichter et al., 1999; Koseoglu et al., 2011). Besides, AST and CK values vary with age (Cherian and Hill, 1978; Morandi et al., 2006). Because of individual and age-related differences in biological reactions with chemical substances, it is necessary to consider these factors in biomarker research (Ishikawa et al., 2014). Several new biomarkers, including skeletal muscle troponin I (sTnl), myosin light chain 3 (Myl3), and fatty acid-binding protein 3 (FABP3), have been recommended for use with CK to monitor drug-induced skeletal muscle injury (Muntean et al., 2017). Muscle-specific microRNAs (e.g., miR-133a) have also been suggested as useful and reliable biomarkers for skeletal muscle injury in experimental animals (Laterza et al., 2009; Mizuno et al., 2011). However, several drawbacks are associated with the use of these biomarkers, including their rapid clearance and alteration due to renal dysfunction (Tononura et al., 2012).

Previously, we have performed an unbiased metabolomic analysis to identify new biomarker candidates for skeletal muscle toxicity that have better sensitivities than that of CK and discovered plasma 2-hydroxyglutarate (2HG) as a specific and easily detectable biomarker for skeletal muscle injury in rats (Obayashi et al., 2017). Since there is no other report showing the possibility of using plasma 2HG as a biomarker for skeletal muscle disorders, further research is needed to verify its practicality as a myopathy marker. 2HG is in a redox equilibrium with the tricarboxylic acid (TCA) cycle intermediate α-ketoglutarate (αKG) and is produced from αKG by malate dehydrogenase and lactate dehydrogenase A (Intlekofer et al., 2015). Although the pathophysiology and mechanisms underlying statin-associated muscle disease are not yet fully understood, it has been reported that mitochondrial disorders can cause statin-induced myopathy (Sirvent et al., 2005b, 2008, 2012; Hou et al., 2017; Ramachandran and Wierzbicki, 2017; Allard et al., 2018). Plasma 2HG is produced in mitochondria and the cytoplasm (Prensner and Chinnaiyan, 2011) and is thought to increase as a result of mitochondrial dysfunction (Oldham et al., 2015; Intlekofer et al., 2015). Because of the difference in the 2HG production processes, 2HG has two enantiomers, namely, L (L-2HG) and D (D-2HG) (Chalmers et al., 1980; Duran et al., 1980). Moreover, recent data have revealed that 2HG is generated in mitochondria and the cytoplasm by mutant variants of isocitrate dehydrogenase (IDH) 1 and IDH2, respectively (Prensner and Chinnaiyan, 2011).

The purpose of this study was to examine the usefulness of plasma 2HG as a biomarker for skeletal muscle toxicity. We employed two distinct animal models of skeletal muscle toxicity to evaluate the sensitivity of 2HG for skeletal myopathy in comparison with that of AST, CK, and muscle-type CK (CK-MM) and to examine the influence of age differences and repeated blood sampling on plasma 2HG levels.

MATERIALS AND METHODS

Chemicals

Cerivastatin (CER) was synthesized by Chemtec Labo., Inc. (Tokyo, Japan). Tetramethyl- p-phenylenediamine (TMPD) was purchased from Sigma (St. Louis, MO, USA). CER was mixed with powdered ingredients of a purified diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) at a concentration of 20 or 40 ppm. TMPD was resuspended in a 0.5% methylcellulose (MC) solution (#400; Nacalai Tesque, Inc., Kyoto, Japan).

Animals

Male Fischer 344 rats at 3 and 8 weeks of age were purchased from Charles River Japan, Inc. (Kanagawa, Japan). Animals were housed in stainless steel wire bracket cages in a room maintained at a temperature of 21-26°C, relative humidity of 30-70%, and a 12-hr light (7:00 to 19:00) cycle. All animals were allowed free access to water and a commercial rodent diet. The rats were acclimated for 1 week before experiments. All experimental procedures were performed in accordance with the guidelines of the Animal Care and Use Committee of Daiichi Sankyo Co., Ltd. (Tokyo, Japan). The experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Daiichi Sankyo Co., Ltd.

Dosing

In the CER-induced model of skeletal muscle toxicity, rats (n = 5 per dose group) were fed a diet supplemented with 20 ppm CER (4-week-old rats), 40 ppm CER (9-week-old rats), or a commercial diet only as a control for 10 days. In the TMPD-induced model, 4- and 9-week-old rats were orally administered TMPD at a dose level of 9 mg/kg or 0.5% MC as a control. Because age-related differences in statin-induced muscular injury were previously reported (Reijneveld et al., 1996), we used 4- and 9-week-old rats in the current study. The dose of each compound was selected based on previously reported pathological studies of skeletal muscle injury (Obayashi et al., 2011; Pritt et al., 2008) and our preliminary data.
Sample collection
Blood samples (approximately 100 μL) were obtained for plasma 2HG analysis by jugular venipuncture on days 4, 8, and 11 of treatment (CER group) and at 6 and 24 hr after dosing (TMPD group). Blood samples were collected from rats in a conscious state because the effects of anesthesia on plasma 2HG levels are unknown. To measure plasma AST, CK, and CK-MM, blood samples were obtained from the abdominal aorta under isoflurane anesthesia on day 11 for the CER group and at 24 hr after dosing for the TMPD group. Blood was heparinized and centrifuged (15,000 rpm, 10 sec for 2HG analysis; 3,000 rpm, 15 min for AST, CK, and CK-MM analysis) to obtain plasma samples. The plasma samples were stored at –80°C until use. After the last blood sampling, the animals were euthanized humanely by exsanguination, and the rectus femoris muscle was excised for histopathological examination.

Plasma 2HG analysis
Analysis of plasma 2HG was conducted at Daiichi Sankyo RD Novare Co., Ltd. (Tokyo, Japan). Plasma samples (40 μL) were deproteinized by adding 120 μL of methanol, followed by centrifugation (3,000 rpm, 5 min). Then, 100 μL of each supernatant fraction was mixed with 100 μL of water for liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis. Subsequently, the supernatants were pretreated on an Oasis MAX μElution plate (Waters Corporation, Milford, MA, USA). To 80 μL of each lysate sample, 100 μL of d<sub>5</sub>-3HG was added as an internal standard, in addition to 20 μL of water. The mixtures were transferred to the extraction plate, which was preconditioned with 200 μL of methanol and 200 μL of water. Then, the extraction plate was washed with 200 μL of 50% methanol in water, and the analyte was eluted with 100 μL of 2% formic acid in methanol. The eluent was mixed with 100 μL of water, and the resulting mixture was injected into an LC-MS/MS system consisting of an ACQUITY UPLC instrument (Waters Corporation) and a Xevo TQ MS instrument (Waters Corporation). The analyte and internal standard were separated from foreign substances using a Hypersil column (2.1 mm × 150 mm, 5 μm; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Mobile phase A consisted of methanol/water/formic acid (100/900/5, v/v/v); mobile phase B consisted of methanol/water/formic acid (900/100/5, v/v/v), and the flow rate was 300 μL/min. The analyte and internal standard were ionized using electrospray ionization in a negative ion mode and detected with multiple reaction monitoring transitions of m/z 147 to 129 and 152 to 89, respectively. The MS parameters were as follows: capillary voltage, 2.0 kV; source temperature, 150°C; and desolvation temperature, 600°C.

Plasma AST, CK, and CK-MM analysis
Plasma AST and CK levels were measured using a standard commercial assay kit (L-Type Wako; Wako Pure Chemical Industries, Ltd., Osaka, Japan) on an automated biochemical analyzer (7180; Hitachi High-Technologies Corporation, Tokyo, Japan), in accordance with the manufacturer’s instructions. CK isoenzymes (CK-BB, CK-MB, and CK-MM) were electrophoretically separated using a rapid-type electrophoresis device (REP8JF71000; Helena Laboratories, Saitama, Japan), and the relative percentage of each isoenzyme was calculated. The absolute activity of CK-MM was determined from the total CK activity and the relative percentage of CK-MM.

Histopathology
To confirm the occurrence of skeletal muscle disorders, the rectus femoris muscle was used for histopathological analysis because it shows the most severe changes among three skeletal muscle types (soleus muscle, rectus femoris muscle, and tibialis anterior muscle), as has been observed in our previous study (Obayashi et al., 2017). The muscles from all control and treated rats were fixed in 10% buffered neutral formalin and embedded in paraffin. Muscle sections (approximately 2-μm thick) were prepared by routine methods, stained with hematoxylin and eosin, and examined by light microscopy. Histopathological grading was employed to analyze the presence and pathological severity of lesions as follows: being within normal limits (−) or showing slight (+) or moderate (++) damage (Mann et al., 2012).

Statistical analysis
Quantitative data are expressed as the mean ± S.D., unless otherwise stated. All quantitative data were statistically analyzed using an F-test, followed by the Student’s t-test or the Aspin-Welch t-test to determine significant differences between treated and control groups. All statistical analyses were performed using the SAS® software, release 8.2 (SAS Institute, Inc., Cary, NC, USA). A P-value < 0.05 was considered statistically significant.

RESULTS
Plasma 2HG levels
Plasma 2HG levels were determined in rats treated with CER and TMPD starting at 4 weeks of age (Fig. 1A, 1B) and 9 weeks of age (Fig. 1C, 1D) and were compared with those in the respective vehicle control rats. One of
the five CER-treated rats in the 9-week-old group died on day 11. The plasma 2HG levels showed an increased trend in the CER-treated group after day 8 of treatment and in the TMPD-treated group at 6 hr after dosing. Meanwhile, repeated blood collection did not affect plasma 2HG levels.

No significant differences in the plasma 2HG levels were observed between the 4-week-old control and CER-treated rats (Fig. 1A) on day 4. In contrast, the plasma 2HG levels in the CER-treated rats were significantly higher than those in the control rats on day 8 (2.20 ± 0.37 and 1.60 ± 0.20 μM, respectively; *P < 0.05) and day 11 (2.53 ± 0.53 and 1.29 ± 0.16 μM, respectively; **P < 0.01). At 4 weeks of age, the 2HG plasma levels in the TMPD-treated rats at 9 weeks of age were significantly higher than those in the control rats at 6 hr (1.87 ± 0.37 and 1.30 ± 0.27 μM, respectively; **P < 0.01) and 24 hr (2.28 ± 0.35 and 1.85 ± 0.21, respectively; *P < 0.05) after dosing.

In the 9-week-old group, plasma 2HG levels were significantly (P < 0.05) higher in the CER-treated rats than in the control rats on day 8 (1.74 ± 0.26 and 1.31 ± 0.12 μM, respectively; Fig. 1C). In addition, plasma 2HG levels in the TMPD-treated rats at 9 weeks of age were significantly higher (P < 0.05) than those in the control rats 24 hr after dosing (1.87 ± 0.37 and 1.30 ± 0.27 μM, respectively; Fig. 1D).

**Conventional biomarkers of skeletal muscle injury**

Plasma AST, CK, and CK-MM levels were determined in the rats treated with CER and TMPD starting at 4 weeks of age (Fig. 2A, 2B) and 9 weeks of age...
Plasma 2HG as a biomarker for skeletal muscle toxicity

(Fig. 2C, 2D) and were compared with those in the respective vehicle-treated control rats. In both 4- and 9-week-old rats, AST, CK, and CK-MM showed high values in the CER-treated group, but changes in CK and CK-MM were not obvious in the TMPD-treated group. In particular, significantly higher plasma levels ($P < 0.01$) of AST, CK, and CK-MM were observed in the CER-treated 4-week-old rats than in the control rats on day 11 of treatment (Fig. 2A). In contrast, only plasma AST significantly increased ($P < 0.001$) in the TMPD group (Fig. 2B).

In the 9-week-old rats, the plasma AST levels were significantly higher ($P < 0.05$) in the CER-treated rats than in the control rats, but no statistically significant differences were observed in the plasma levels of CK and CK-MM (Fig. 2C). In addition, no differences in the plasma levels of AST, CK, and CK-MM were found between the TMPD-treated rats and control rats at 9 weeks of age (Fig. 2D).

**Histopathology**

Histopathological examination resulted in no abnormal findings in any muscle samples in the control groups (Tables 1 and 2, Fig. 3A-D). In the CER-treated group, a marked variation in the fiber size, with moderately vacuolated (arrows in Fig. 3E, 3G) or necrotic fibers and cellular infiltration, was obvious in the rectus femoris muscle in both 4-week-old (Table 1, Fig. 3E) and 9-week-old (Table 2, Fig. 3G) rats. In the TMPD-treated rats, moderately vacuolated fibers (arrows in Fig. 3F, 3H) and a few necrotic fibers were noted in both 4-week-old (Table 1, Fig. 3F) and 9-week-old (Table 2, Fig. 3H) rats.
DISCUSSION

To evaluate the potential of plasma 2HG as a biomarker for skeletal muscle toxicity, we measured the plasma concentrations of 2HG in rats with skeletal muscle injury and compared those with the levels of conventional biomarkers such as AST, CK, and CK-MM. The novel findings of this investigation are: 1) plasma 2HG is a more sensitive indicator than CK-MM for TMPD-induced mild skeletal myopathy; 2) plasma 2HG levels were not significantly affected by repeated blood collection; and 3) no significant difference in plasma 2HG levels was found between immature and young adult rats with skeletal myopathy. The finding that the sensitivity of plasma 2HG was superior to that of the conventional skeletal muscle biomarkers at an early stage of skeletal muscle injury was reproducible. These results suggest that plasma 2HG may serve as a promising novel biomarker for skeletal muscle toxicity.

Skeletal muscles are susceptible to drug-related injury because of their mass, high degree of blood flow, and mitochondrial energy metabolism (Valiyil and Christopher-Stine, 2010). An early evaluation of their myotoxic potential is important for the development of new drugs and has long mainly relied on the measurement of CK and AST. Although these biomarkers are useful diagnostic tools for assessing myopathies in humans, their preclinical utility has been limited by their marked variability and the lack of muscle specificity, particularly in rats (Bohlmeyer et al., 1994; Sorichter et al., 1999). Previously, we have demonstrated the potential of plasma 2HG as a specific and easily detectable biomarker for skeletal muscle injury in rats (Obayashi et al., 2017).

In this study, we used CER and TMPD, which induce skeletal muscle injury through distinct mechanisms (Munday, 1988, 1990; De Pinieux et al., 1996; Nishimoto et al., 2003). The histopathological analysis confirmed the presence of skeletal myopathy in the rectus femoris muscle of rats treated with CER or TMPD. However, the histopathological changes were more profound in the CER-treated rats than in the TMPD-treated rats. The onset of skeletal myopathy was relatively gradual in the CER-treated rats but became acute in the TMPD-treated rats, consistent with our previous report (Obayashi et al., 2017). In addition, the plasma AST level significantly increased in the CER-treated rats at 4 and 9 weeks of age in the TMPD-treated rats at 4 but not 9 weeks of age. Although no statistical differences were found in the plasma levels of CK or CK-MM between the control and CER-treated rats at 9 weeks of age or between the TMPD-treated rats of different sexual maturities, the CK and CK-MM values in the CER-treated group clearly increased, reflecting the histopathological severity. These data are consistent with a report showing that a skeletal

Table 1. Histopathology of the rectus femoris muscle in 4-week-old rats treated with cerivastatin or tetramethyl-p-phenylenediamine.

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<th>Observation</th>
<th>CER</th>
<th>TMPD</th>
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<tr>
<td></td>
<td>0 ppm</td>
<td>20 ppm</td>
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<tr>
<td></td>
<td>n = 5</td>
<td>n = 5</td>
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<tr>
<td>Vacuolar degeneration, muscle fibers</td>
<td>–</td>
<td>++ (5)</td>
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<tr>
<td>Necrosis, muscle fibers</td>
<td>–</td>
<td>++ (5)</td>
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<tr>
<td>Cellular infiltration</td>
<td>–</td>
<td>+/++ (5)</td>
</tr>
</tbody>
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Grade: –, within normal limits; +, slight injury; +/++, slight to moderate injury; ++, moderate injury. Numbers in parentheses represent the number of animals with pathological changes. CER, cerivastatin Na; TMPD, tetramethyl-p-phenylenediamine.

Table 2. Histopathology of the rectus femoris muscle in 9-week-old rats treated with cerivastatin or tetramethyl-p-phenylenediamine.

<table>
<thead>
<tr>
<th>Observation</th>
<th>CER</th>
<th>TMPD</th>
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<tbody>
<tr>
<td></td>
<td>0 ppm</td>
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<td>n = 5</td>
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<tr>
<td>Vacuolar degeneration, muscle fibers</td>
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<td>Necrosis, muscle fibers</td>
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<td>++ (4)</td>
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<tr>
<td>Cellular infiltration</td>
<td>–</td>
<td>+ (4)</td>
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Grade: –, within normal limits; +, slight injury; +/++, slight to moderate injury; ++, moderate injury. Numbers in parentheses represent the number of animals with pathological changes. CER, cerivastatin Na; TMPD, tetramethyl-p-phenylenediamine.
muscle disorder, accompanied by elevated CK and CK-MM levels, was induced following CER administration (Seachrist et al., 2005).

Plasma 2HG levels increased significantly in the 4- and 9-week-old rats treated with CER or TMPD. In contrast, no significant increase in AST was observed in the 9-week-old TMPD-treated rats. In addition, no significant increases in the plasma levels of CK and CK-MM were observed in the CER-treated rats at 9 weeks of age or in the TMPD-treated rats at 4 or 9 weeks of age. Furthermore, the AST, CK, and CK-MM plasma levels showed a greater variability than did those of 2HG in the CER-treated rats with severe skeletal muscle injury but not in the TMPD-treated rats. Thus, the plasma 2HG level appears to be a more sensitive biomarker than are CK and CK-MM levels for detecting skeletal muscle injury in rats. The reason for higher sensitivity of plasma 2HG in detecting skeletal muscle injury is unclear. One possible explanation is that CK is typically abundant in muscle tissues and leaks from muscle cells following injury, whereas 2HG levels specifically increase in skeletal muscle tissues under these conditions (Obayashi et al., 2017). Another possible reason is differences in the half-lives of these markers. The half-life of AST in the blood is approximately 2.3 hr in rats, 3.3 to 4.4 hr in dogs, and 4 to 46 hr in humans (Evans, 1996a), and that of CK is approximately 0.6 hr in rats, 0.6 to 16.2 hr in dogs (Evans, 1996b), up to 9 hr in rabbits, and 12 hr in humans (Walker, 2006). As far as we are aware, the 2HG blood half-life is unknown, and it is unclear whether it is diffi-

Fig. 3. Histopathological analysis of muscle fibers. Rectus femoris muscle tissues from 4-week-old rats treated with 0 ppm CER (A) or 20 ppm CER (E) were collected on day 11, and those from 4-week-old rats treated with 0 mg/kg TMPD (B) or 9 mg/kg TMPD (F) were collected at 24 hr after dosing. Tissues from 9-week-old rats treated with 0 ppm CER (C) or 40 ppm CER (G) were collected on day 11, and those from 9-week-old rats treated with 0 mg/kg TMPD (D) or 9 mg/kg TMPD (H) were collected at 24 hr after dosing. The following findings were noted: (A-D) no abnormalities; (E, G) marked variation in fiber sizes, with moderately vacuolated (arrow) and moderately necrotic fibers; (F, H) moderately vacuolated (arrow) and slightly necrotic fibers. Hematoxylin and eosin staining. Scale bar = 100 μm. CER, cerivastatin Na; TMPD, tetramethyl-p-phenylene diamine.
cult to eliminate 2HG from the blood. However, since the data of our previous report (Obayashi et al., 2017) have been reproduced, 2HG seems to be useful as a biomarker.

In this study, we examined the influence of repeated blood sampling on plasma 2HG levels. The plasma 2HG concentrations in the control groups of the 4- and 9-week-old rats ranged from 1.12 to 2.18 μM and from 0.89 to 1.94 μM, respectively. The range of the plasma 2HG concentrations found in this study was consistent with that found in a previous report (Dang et al., 2016), and the data indicated that the plasma 2HG levels were low (less than 300 μM) in rats without skeletal muscle injury. In addition, low variability in the plasma 2HG levels was noted in this study. The levels of conventional biomarkers of skeletal muscle injury, such as AST and CK, which leak through disrupted cell membranes, increase with tissue damage at the blood sampling site following puncture and hemolysis. Indeed, CK activity has been reported to vary from 100 to 1,300 IU/L even in naïve rats, due to differences in blood handling (Yerroum et al., 1999; Goicoechea et al., 2008) and in blood sampling sites (Matsuzawa et al., 1993). Thus, plasma 2HG is unlikely to be affected by repeated blood collection.

In this study, the plasma 2HG levels increased following skeletal muscle injury in both 4- and 9-week-old rats. It is known that 2HG is produced as a metabolite in the TCA cycle (Prensner and Chinnaiyan, 2011), independently of hormones, which vary during individual growth stages. Thus, the 2HG plasma level appears to be independent of the age in rats.

An increase in plasma 2HG suggests the presence of a mitochondrial disorder in the skeletal muscle. Mitochondrial abnormalities in statin-induced myopathies have been consistently observed for other statins (such as simvastatin, rosuvastatin, and pravastatin) in multiple species, including humans (Bergman et al., 2003; Gambelli et al., 2004; Seachrist et al., 2005; Sirvent et al., 2005a; Westwood et al., 2005, 2008). Mitochondrial oxidation of TMPD is important for initiating its myotoxicity because muscles are particularly rich in mitochondria and largely depend on aerobic metabolism to meet their energy requirements (Munday et al., 1990). We propose that the accumulation of 2HG diverts electrons from the TCA cycle and affects the electron transport mechanism, thus contributing to the CER- and TMPD-induced muscular dysfunction.

Our study has several limitations. We did not evaluate the organ or species specificity of plasma 2HG as a biomarker for muscle injury. Therefore, further studies will be needed to assess the plasma 2HG levels in other models of skeletal muscle toxicity and in other general models, including those of hepatic, renal, or cardiac toxicity in rats and other species. In addition, to establish 2HG as a biomarker for skeletal muscle disorders, a comparison between plasma 2HG and other skeletal muscle injury biomarkers, such as sTnI, Myl3, and FABP3, is necessary. Further, to confirm the usefulness of plasma 2HG as a biomarker for skeletal muscle injury, background data for several parameters, such as the animal species, genetic strain, sex, and age, are needed.

This study demonstrated that plasma 2HG concentrations were more closely associated with skeletal muscle injury than were plasma CK-MM levels, suggesting that the monitoring of plasma 2HG will contribute to the detection of skeletal muscle toxicity in experimental and clinical settings. Furthermore, by employing plasma 2HG as a biomarker for skeletal muscle injury, the number of animals needed for drug discovery experiments, especially for toxicology studies, can be reduced because blood samples may be collected repeatedly from the same animal, without sacrificing animals prior to examination.

In conclusion, this study demonstrated that plasma 2HG represents a promising candidate biomarker for detecting mild skeletal muscle toxicity. Plasma 2HG is more sensitive than CK and CK-MM, and no differences were noted between younger and older rats (4- vs. 9-week-old) or after repeated blood collection. Further investigation into the use and limitations of 2HG is warranted.

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

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