Effects of fenofibrate and its combination with lovastatin on the expression of genes involved in skeletal muscle atrophy, including FoxO1 and its targets

Haruka Ajima1, Yuko Kai2, Junya Fujimaki1, Shiiori Akashi1, Akihito Morita1, Osamu Ezaki2, Yasutomi Kamei2,3 and Shinji Miura1,2

1Laboratory of Nutritional Biochemistry, Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan
2Department of Nutritional Science, National Institute of Health and Nutrition, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8636, Japan
3Laboratory of Molecular Nutrition, Graduate School of Environmental and Life Science, Kyoto Prefectural University, 1-5 Hangi-cho, Shimogamo, Sakyo-ku, Kyoto, 606-8522, Japan

(Received July 30, 2020; Accepted October 21, 2020)

ABSTRACT — Fibrates and statins have been widely used to reduce triglyceride and cholesterol levels, respectively. Besides its lipid-lowering effect, the side effect of muscle atrophy after fibrate administration to humans has been demonstrated in some studies. Combination therapy with fibrates and statins also increases the risk of rhabdomyolysis. FoxO1, a member of the FoxO forkhead type transcription factor family, is markedly upregulated in skeletal muscle in energy-deprived states and induces muscle atrophy via the expression of E3-ubiquitine ligases. In this study, we investigated the changes in FoxO1 and its targets in murine skeletal muscle with fenofibrate treatment. High doses of fenofibrate (greater than 0.5% (wt/wt)) over one week increased the expression of FoxO1 and its targets in the skeletal muscles of mice and decreased skeletal muscle weight. These fenofibrate-induced changes were diminished in the PPARα knockout mice. When the effect of combination treatment with fenofibrate and lovastatin was investigated, a significant increase in FoxO1 protein levels was observed despite the lack of deterioration of muscle atrophy. Collectively, our findings suggest that a high dose of fenofibrate over one week causes skeletal muscle atrophy via enhancement of FoxO1, and combination treatment with fenofibrate and lovastatin may further increase FoxO1 protein level.

Key words: Muscle atrophy, Forkhead box-containing protein O1, Fenofibrate, Lovastatin, Muscle atrophy F-box

INTRODUCTION

Dyslipidemia is a well-established risk factor of cardiovascular disease and is estimated to account for more than half of the global cases of coronary artery disease (Shanmugasundaram et al., 2010). Dyslipidemia is qualitatively defined as an impairment of lipid metabolism characterized by high levels of triglyceride (TG), low levels of high-density lipoprotein cholesterol (HDL-C), and higher small and dense low-density lipoprotein (sd-LDL) than normal levels (Miccoli et al., 2008). Although combination lifestyle therapies, such as exercise and diet interventions, are an efficacious, preliminary means of improving cholesterol levels in patients diagnosed with dyslipidemia (Varady and Jones, 2005), this pathology is difficult to manage because: 1) it could have a genetic/familiar component in its genesis, and 2) many other extra-cardiac diseases at relatively high incidence and prevalence can cause impairment in lipid control, besides alimentary intake (diabetes mellitus, chronic kidney disease, drugs and alcohol abuse) (Qi et al., 2012; Vodnala et al., 2012). Thus, fibrates and statins have been widely used in clinical practice to reduce triglyceride and cholesterol levels, respectively.

Fibrates, the agonists of peroxisome proliferator-activated receptor α (PPARα), are used either as monother-
Fenofibrate has multiple blood lipid-modifying actions, such as decreasing blood triglyceride level and increasing HDL-C level (Miura et al., 2005; van der Hoogt et al., 2007). These effects are thought to be due to the enhancement of β-oxidation (Pruijboom-Brees et al., 2005) and activation of lipoprotein lipase (Ziouzenkova et al., 2003). Besides the lipid-lowering effect of fibrates, some studies have revealed the occurrence of muscle atrophy as a side effect after the administration of fibrates in humans (Hodel, 2002; Muscari et al., 2003). Besides the lipid-lowering effect of fibrates, which are inhibitors of 3-hydroxy-3-methyl-glutaryl Coenzyme A (HMG-CoA) reductase, are generally well tolerated but can have severe myopathic effects, albeit relatively infrequently (Thompson et al., 2003). Although the synergistic clinical benefits of combination therapy with fibrates and statins have been reported (Papadakis et al., 1999; Kiortsis et al., 2000; Gavish et al., 2000; Farnier and Picard, 2001), the use of these drugs, especially in combination therapy, is associated with the risk of rhabdomyolysis (Hodel, 2002; Shek and Ferrill, 2001).

FoxO1, a member of the FoxO forkhead type transcription factors, is markedly upregulated in skeletal muscle in energy-deprived states, such as fasting and severe diabetes (Kamei et al., 2003). Transgenic mice specifically overexpressing FoxO1 in skeletal muscle weigh less than wild-type control mice, suggesting that FoxO1 leads to impaired skeletal muscle function (Kamei et al., 2004). The mRNA encoding two E3-ubiquitin ligases, namely atrogin-1 (muscle atrophy F-box, MAFbx) and MuRF1 (muscle RING finger-1), are significantly upregulated during muscle atrophy. In fact, the overexpression of atrogin-1 in myotubes causes atrophy, whereas mice deficient in either atrogin-1 or MuRF1 are resistant to atrophy (Bodine et al., 2001; Gomes et al., 2001). Previous results suggest that the FoxO1 transcription factors play a role in the regulation of E3-ubiquitin ligases, such as atrogin-1 and MuRF1 (McLoughlin et al., 2009; Majumder et al., 2018); therefore, FoxO1 may induce skeletal muscle atrophy by upregulating atrogin-1 and MuRF1. Fibrates increase the expression of E3-ubiquitin ligases mediated by PPARα and stimulate the ubiquitination of skeletal muscle proteins, and this consequently stimulates FoxO1 activation-mediated protein degradation (Ringseis et al., 2013). Statin administration was demonstrated to result in the dephosphorylation of FoxO (1 and 3) transcription factors and the induction of ubiquitin and lysosomal proteolysis via the upregulation of FoxO downstream target genes, such as atrogin-1 and MuRF1 (Mallinson et al., 2009).

Previous reports suggest that fibrates and statins upregulate FoxO1 levels. In order to evaluate the dose and duration of fibrate administration and the mechanisms of atrophy induction, we sought to determine dose- and time- and PPARα-dependent changes in FoxO1 and its targets upon treatment with fenofibrate. Furthermore, whether the combination of fibrates and statins further activates FoxO1, when compared with their respective individual administration has yet to be clarified. Therefore, we investigated the effects of combination treatment with fibrates and statins on FoxO1 and its target gene expression.

**MATERIALS AND METHODS**

**Animals**

C57BL/6J mice were purchased from Japan SLC Inc. (Shizuoka, Japan). PPARα knockout mice (B6;129S4-Ppara<sup>tm1Gonz</sup>/J) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were maintained under a 12-hr light/dark cycle at a constant temperature of 22°C and had free access to water and a normal chow diet (MF; Clea Japan, Tokyo, Japan) until treatment initiation. Mice were handled in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and our institutional guidelines. The animal experiments were reviewed and approved by the National Institute of Health and Nutrition Ethics Committee on Animal Research (No. 0610) and the Institutional Animal Care and Use Committee of the University of Shizuoka (No. 195259).

**Experiment 1**

Eight-week-old male mice were treated with several doses of fenofibrate as a dietary supplement in a high-carbohydrate (HC) diet ad libitum for one week. Before the experiment, mice were randomly divided into seven groups (n = 5) and administered the HC diets supplemented without (0%; control group) or with 0.01, 0.03, 0.1, 0.3, 0.5, and 1% (wt/wt) of fenofibrate. According to calories, the HC diet contained 68% carbohydrate, 10% fat, and 22% protein. High oleic safflower oil was used as the source of fat. After one week of treatment, mice were sacrificed by cervical dislocation under light anesthesia with isoflurane.

**Experiment 2**

Eight-week-old male mice were treated with 0.5% (wt/wt) of fenofibrate as a dietary supplement in the HC-diet for one day, two days, three days, one week, or two weeks ad libitum (n = 4). Control mice were administered the HC-diet ad libitum. After the indicated times, mice were sacrificed by cervical dislocation under light...
anesthesia with isoflurane.

**Experiment 3**
Seven to eight-week-old male mice were treated with 0.5% (wt/wt) of fenofibrate as a dietary supplement in the HC-diet for one week (n = 5). Because mice administered fenofibrate had a decrease in food intake, two fenofibrate treatment control groups were prepared: the first control included mice administered the HC diet *ad libitum* while the second control included mice administered the HC diet under calorie-restricted conditions (HC restrict) for one week. Mice in the HC-restricted group were given 3 g/day of food and the same amount of calories was administered to mice treated with fenofibrate. Twelve-week-old PPARα knockout mice, and the control, wild-type 129S1/SvImJ mice, were untreated or treated with 0.5% (wt/wt) of fenofibrate as a dietary supplement in the HC diet (n = 4). Each mouse was fed 3 g/day for one week. After one week of treatment, mice were sacrificed by cervical dislocation under light anesthesia with isoflurane.

**Experiment 4**
Seven-week-old male mice were treated with 0.5% (wt/wt) of fenofibrate, 0.13% (wt/wt) of lovastatin, or both drugs as a dietary supplement in the HC-diet *ad libitum* for one week (n = 4-5). After one week of treatment, mice were sacrificed by cervical dislocation under light anesthesia with isoflurane.

**Quantitative real-time RT-PCR**
The skeletal muscles were dissected and maintained in liquid nitrogen. Total RNA was extracted from the gastrocnemius using RNA iso plus (Takara Bio Inc., Shiga, Japan). Reverse transcription (RT) was performed with PrimeScript RT reagent Kit with a gDNA Eraser (Takara Bio Inc.). Quantitative real-time RT-PCR (qPCR) was conducted with SYBR Premix Taq II (Takara Bio Inc.). Mouse-specific primer pairs were used as shown in Table 1. Results were normalized to the expression of 36B4 or 18S rRNA.

**Western blot analysis**
Homogenates were prepared from frozen gastrocnemius aliquots using RIPA buffer (Merck Millipore, Temecula, CA, USA). Thereafter, the protein concentrations in the homogenates were determined with a bicinchoninic acid protein assay kit (Takara Bio Inc.) with bovine serum albumin as the standard. From each homogenate, 20 μg of protein was separated on 10-12% SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad laboratories, Inc., Hercules, CA, USA). Primary antibodies against FoxO1 (#2880S; Cell Signaling Technology, Boston, MA, USA) and atrogin-1/Fbx32 (#EPR9148(2), ab168372; Abcam, Cambridge, UK), MuRF1 (#EPR6431(2), ab172479; Abcam) and HRP-linked secondary antibodies against rabbit-IgG (#2729S; Cell Signaling Technology) were used. Blots were detected using the chemiluminescence reagent, ECL Prime Western Blotting Reagent (GE Healthcare, Buckinghamshire, UK), and signal intensities were quantified using a C-DiGit blot scanner (LI-COR bioscience, Lincoln, NE, USA, USA).

**Statistical analysis**
All data are expressed as mean ± SEM. Data were analyzed by one-way ANOVA. When differences were significant, each group was compared using the Tukey–Kramer test with JMP version 11 (SAS Institute, Cary, NC, USA). Statistical significance was defined as P < 0.05.

**RESULTS**
Fenofibrate treatment decreases skeletal muscle mass and increases FoxO1 mRNA levels in a dose-dependent manner
C57BL/6J mice were treated with several doses of fenofibrate as a dietary supplement in the HC-diet for

<table>
<thead>
<tr>
<th>Target</th>
<th>Sense sequence (5‘→3’)</th>
<th>Antisense sequence (5‘→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>36B4</td>
<td>GCCCTGCACTCTCGTTTC</td>
<td>TGGCCAGGACGCCTTGT</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>GGGACGCTGAGAAACGCC</td>
<td>GGGTCGGGAGTGGGTAATT</td>
</tr>
<tr>
<td>FoxO1</td>
<td>TTTCTCCACATGACAGAGCA</td>
<td>AATTCAGCTCCCCATCTCC</td>
</tr>
<tr>
<td>atrogin-1</td>
<td>AGTGAGGACCGCTAC</td>
<td>GATCAAACGCTTCC</td>
</tr>
<tr>
<td>MuRF1</td>
<td>ACGAGAAGAAGACCCGAGCTG</td>
<td>CTTGGCACCCTTGAGAAGAAGAAGG</td>
</tr>
<tr>
<td>MCAD</td>
<td>TGGATCTGTCAGGCGGAGATT</td>
<td>GGTCACATAGACCTGAGAAGAAGA</td>
</tr>
</tbody>
</table>

FoxO1, forkhead box protein O1; atrogin-1, muscle atrophy F-box (MAFbx); MuRF1, muscle RING finger-1; MCAD, medium-chain acyl-CoA dehydrogenase
one week. As shown in Fig. 1A, food intake was dose-dependently decreased in mice supplemented with 0.3, 0.5, and 1% of fenofibrate. As food intake decreased, a subsequent decrease in body weight, skeletal muscle (gastrocnemius and quadriceps) weight, and epididymal white adipose tissue weight was observed following treatment with fenofibrate. Additionally, fenofibrate treatment increased liver weight; however, the largest increase in liver weight was obtained with a dose of 0.1%. To determine whether treatment with fenofibrate dose-dependently influences FoxO1 and its target gene expression, the levels of these mRNAs in the gastrocnemius were examined (Fig. 1B). FoxO1 mRNA levels increased at doses of 0.5% and 1%. The expression of atrogin-1 and MuRF1, which are positively regulated by FoxO1, also increased at these doses. The expression level of medium-chain acyl-CoA dehydrogenase (MCAD) was measured in the gastrocnemius. Fenofibrate treatment at doses greater than 0.3% increased MCAD mRNA expression in the skeletal muscles.

FoxO1 mRNA level increases one or two weeks after fenofibrate treatment

A time course study of fenofibrate treatment was performed with a fixed dose of 0.5%. As shown in Fig. 2A, a decrease in food intake was observed one day after the initiation of fenofibrate treatment and this continued for 2 weeks. Further, decreases in body weight, skeletal muscle weight, and epididymal white adipose tissue weight were observed before one week of fenofibrate treatment. These decreases were gradually augmented with feeding periods. The increase in liver weight reached a plateau at 3 days after fenofibrate treatment. To determine whether the changes in FoxO1 and its target gene expression were synchronized with muscle weight change, we determined the levels of their mRNA in the gastrocnemius (Fig. 2B). Compared with mice administered the HC diet alone, a significant increase in FoxO1 and its target genes, such as atrogin-1 and MuRF1 expressions were observed 1 or 2 weeks after fenofibrate treatment. The MCAD mRNA expression level in skeletal muscles was increased after 2 days of treatment.

Decrease in skeletal muscle mass and increase in FoxO1 and its target gene expression are not caused by a reduction in food intake

To determine the contribution of the decrease in food intake to the phenotypic alterations in mice administered fenofibrate, the same food amount consumed by mice administered 0.5% fenofibrate was administered to control mice. As shown in Fig. 3A, the mice consumed the entire food portion assigned. Therefore, no difference was found in food intake between mice administered the calorie-restricted diet and those administered fenofibrate. Although calorie restriction reduced body weights, significant decreases in body weight and gastrocnemius weight were observed between calorie-restricted mice and fenofibrate-supplemented mice. Further, an increase in liver weight was observed in mice administered fenofibrate. As shown in Fig. 3B, calorie-restriction did not affect FoxO1, MCAD, atrogin-1, and MuRF1 mRNA expression in the skeletal muscle, suggesting that the increase in these mRNAs in the skeletal muscles owing to fenofibrate administration did not occur because of a decrease in food intake.

PPARα is involved in the fenofibrate-induced induction of FoxO1 and its target genes and the reduction of muscle mass

To elucidate the contributions of PPARα to the reduction of muscle mass and the expression of FoxO1 in response to fenofibrate, the PPARα knockout mice and control wild-type mice were treated with fenofibrate (Fig. 4). In wild-type 129S1/SvImJ mice, fenofibrate treatment decreased gastrocnemius weight and increased liver weight. The expression of FoxO1, atrogin-1, and MuRF1 also increased upon fenofibrate administration. However, these fenofibrate-induced changes were diminished in PPARα knockout mice, suggesting that PPARα was involved in the induction of FoxO1 and its target genes and the reduction of muscle mass.

Combination treatment with fenofibrate and lovastatin significantly increases FoxO1 and atrogin-1 protein levels but does not cause a further decrease in skeletal muscle mass

To determine whether combination treatment with fibrates and statins further enhances FoxO1 and its target expression, mice were treated with 0.5% (wt/wt) of fenofibrate, 0.13% (wt/wt) of lovastatin, or a combination of both drugs (fenofibrate + lovastatin) as a dietary supplement in the HC diet for one week. Although the administration of lovastatin alone did not alter body weight and skeletal muscle weight, the administration of fenofibrate alone and its combination with fenofibrate and lovastatin could significantly decrease these weights (Fig. 5A). Any significant difference in body weight and skeletal muscle weight was not found between mice administered the fenofibrate diet and those administered the combination. We proceeded to examine the expression of FoxO1 and atrogin-1 at the mRNA (Fig. 5B) and protein levels (Fig. 5C). The administration of lovastatin alone did not
Fig. 1. Dose-dependent changes in body weight, tissue weight, and gene expression owing to treatment with different doses of fenofibrate (FF) (%, wt/wt) for one week. Eight-week-old male C57BL/6J mice were treated with several doses of fenofibrate as a dietary supplement in the high-carbohydrate diet ad libitum for one week. A, total food amount consumed in one week, body weight, gastrocnemius (Gastro) weight, quadriceps (Quadri) weight, liver weight, and epididymal white adipose tissue (Epi. WAT) weight. B, relative mRNA levels of FoxO1, MCAD, atrogin-1, and MuRF1 in the gastrocnemius. Total food amounts consumed in one week have been shown as g/mouse, which was the total value of each cage divided by the number of mice in the same cage. The other values have been expressed as mean ± SEM (n = 5). *, p < 0.05; **, p < 0.01; ***, p < 0.001 vs 0% fenofibrate.
Fig. 2. Time-course effects of fenofibrate on body weight, tissue weight, and gene expression. Eight-week-old male C57BL/6J mice were treated with 0.5% (wt/wt) of fenofibrate (0.5% FF) as a dietary supplement in the diet for the indicated times ad libitum. Control mice were fed with the high carbohydrate-diet ad libitum (HC). A, total food amount consumed, body weight, gastrocnemius (Gastro) weight, quadriceps (Quadri) weight, liver weight, and epididymal white adipose tissue (Epi. WAT) weight at each time-point. B, relative mRNA levels of FoxO1, MCAD, atrogin-1, and MuRF1 in the gastrocnemius. Total food amounts consumed at each time-point are shown as g/mouse which were total values of each cage divided by the number of mice in the same cage. The other values have been expressed as mean ± SEM (n = 4). *, p < 0.05; **, p < 0.01; ***, p < 0.001 vs HC-diet at the same time-point.
Fig. 3. Effects of food intake on the fenofibrate-mediated changes in body weight, tissue weight, and gene expression. Eight-week-old male C57BL/6J mice were treated with 0.5% (wt/wt) of fenofibrate (FF) as a dietary supplement in the HC diet for one week. Two FF treatment control groups were prepared: the first control included mice administered the high carbohydrate-diet ad libitum (HC) while the second control included mice administered the HC-diet under calorie-restricted conditions (HC restrict) for one week. Mice in the HC-restricted group and FF group were given 3 g/day of food. A, total food amount consumed in one week, body weight, gastrocnemius (Gastro) weight, quadriceps (Quadri) weight, liver weight, and epididymal white adipose tissue (Epi. WAT) weight. B, relative mRNA levels of FoxO1, MCAD, atrogin-1, and MuRF1 in the gastrocnemius. Total food amounts consumed in one week have been shown as g/mouse which were total values of each cage divided by the number of mice in the same cage. The other values have been expressed as mean ± SEM (n = 5). Bars without a common superscript letter are statically different (p < 0.05).
Fig. 4. Involvement of PPARα activation in fenofibrate-induced expression of FoxO1 and its target mRNA and in decreased muscle weight. Twelve-week-old PPARα knockout mice and control wild-type (WT) 129S1/SvImJ mice were treated with 0.5% (wt/wt) fenofibrate (FF), as a high carbohydrate-diet dietary supplement for one week. Control mice were administered the high carbohydrate-diet under calorie-restricted conditions (HC restrict) for one week. Each mouse was fed 3 g/day of food. A, total food amount consumed in one week, body weight, gastrocnemius (Gastro) weight, quadriceps (Quadri) weight, liver weight, and epididymal white adipose tissue (Epi. WAT) weight. B, relative mRNA levels of FoxO1, MCAD, atrogin-1, and MuRF1 in the gastrocnemius. Values are expressed as mean ± SEM (n = 4). Bars without a common superscript letter are statistically different (p < 0.05).
Fig. 5. Effects of fenofibrate, lovastatin, or the combination of both drugs on body weight, tissue weight, gene expression, and protein expression levels. Seven-week-old male C57BL/6J mice were treated with 0.5% (wt/wt) of fenofibrate (FF), 0.13% (wt/wt) of lovastatin (Statin), or both drugs (FF+Statin) as a dietary supplement in the HC-diet ad libitum for one week. A, total food amount consumed in one week, body weight, gastrocnemius (Gastro) weight, quadriceps (Quadri) weight, liver weight, and epididymal white adipose tissue (Epi. WAT) weight. B, relative mRNA levels of FoxO1, atrogin-1, and MuRF1 in the gastrocnemius. C, relative protein levels of FoxO1 and atrogin-1. Total FoxO1, atrogin-1, and MuRF1 were normalized by the values obtained from Ponceau staining as total protein content on the membrane. Total food amounts consumed in one week have been shown as g/mouse which were total values of each cage divided by the number of mice in the same cage. The other values have been expressed as mean ± SEM (n = 3-5). Bars without a common superscript letter are statically different (p < 0.05).
alter the mRNA and protein expression levels of FoxO1 and its target genes. Furthermore, although there was no significant difference in the expression of FoxO1 mRNA and its target genes between mice administered fenofibrate alone or in combination with lovastatin, the protein levels of FoxO1 and atrogin-1 were significantly increased in mice administered the combination compared to those administered fenofibrate alone.

**DISCUSSION**

In the present study, we sought to determine the effects of administering fenofibrate alone and in combination with lovastatin on the expression of genes involved in skeletal muscle atrophy, such as FoxO1 and its targets. Based on our findings, fenofibrate treatment decreased skeletal muscle mass and increased the expression of FoxO1 and its targets in a dose-dependent man-
ner. Moreover, the decrease in skeletal muscle mass was found to be gradually augmented with feeding periods, and the expression of FoxO1 and its targets were significantly increased at 1 or 2 weeks after fenofibrate treatment. According to our findings, these changes occurred dependently on PPARα and did not occur because of a reduction in diet intake. Furthermore, although an enhancement in skeletal muscle loss was not observed, combination treatment with fenofibrate and lovastatin could significantly increase FoxO1 protein level. Our findings suggest that the administration of a high dose of fenofibrate over a week causes skeletal muscle atrophy concomitantly with increasing FoxO1 and combining fenofibrate with lovastatin may further increase the level of the FoxO1 protein.

To determine whether the induced activation of muscular PPARα is due to fenofibrate administration, we measured the mRNA level of MCAD, the key enzyme for fatty acid oxidation and a PPARα target gene in skeletal muscle. As the MCAD mRNA level increased with fenofibrate treatment at doses greater than 0.3%, PPARα activation was confirmed in the skeletal muscle of mice administered the fenofibrate diet. As shown in Fig. 1, as MCAD expression increased at doses greater than 0.3%, a subsequent decrease in skeletal muscle weight was observed. Furthermore, at doses greater than 0.5%, an increase in the expression of FoxO1 and its target genes, such as atrogin-1 and MuRF1, was observed, suggesting that the stimulation of muscular PPARα with fenofibrate and decrease in muscle mass via FoxO1-induced E3-ubiquitin ligase expression occurred simultaneously, at doses of 0.3-1%. On the other hand, MCAD upregulation began after two days of fenofibrate treatment, and the enhanced expression of FoxO1 and its target genes was not observed until one week of fenofibrate treatment. The sensitivity of PPARα dependent gene expression against fenofibrate differs between the target genes (Jo et al., 2017). Such a phenomenon is also observed in target genes of other transcription factors. For example, different foods affect the expression levels of SCD1 and FAS, which are SREBP-1c target genes, although the timing differs for each gene (Yamazaki et al., 2011). In addition, the effects of histone methylation inhibitors on the transcriptional activation of p53 differ depending on the target gene (Fan et al., 2015). Therefore, chromatin status has been suggested to affect target gene expression levels.

Although discrepancies were observed for certain gene expression levels, fenofibrate mediated PPARα activation was required for muscular expression of FoxO1 and its target genes, because fenofibrate-induced expression of these genes decreased in PPARα knockout mice. This finding aligns with those of previous reports, where treatment with pharmacological PPARα agonist-induced expressions of downstream target genes of FoxO1, atrogin-1 and MuRF1, in skeletal muscle has been reported to diminish due to the lack of PPARα (Ringseis et al., 2013). The promoter regions of atrogin-1 and MuRF1 do not contain functional PPAR response element (PPRE) (Ringseis et al., 2013). Since several PPRE sites were identified in the FoxO1 promoter (Nahlé et al., 2008), fenofibrates might upregulate atrogin-1 and MuRF1 via PPRE-dependent FoxO1 expression. The increased expression of muscular FoxO1 could also result from indirect mechanism(s) of PPARα mediated effects on other organs, such as the liver. For example, at night, activation of PPARδ promotes lipogenesis in the liver and increases phosphatidylcholine (PC) (18:0/18:1) secretion into the blood stream. This process is followed by PC (18:0/18:1)-stimulated fatty-acid oxidation via PPARα in skeletal muscle (Liu et al., 2013). We have previously shown that plasma PC profiles change depending on dietary carbohydrate–fat ratios (Inoue et al., 2017). Notably, the levels of PC (16:0/16:1) and PC (16:0/18:1) increased as the dietary carbohydrate–fat ratio increased in humans and mice, and these PCs act as ligands for PPARα. These results suggest that fibrate-induced PPARα activation caused directly or indirectly leads to FoxO1 mediated induction of atrogin-1 and MuRF1 and subsequent skeletal muscle mass reduction.

In the present study, statin-induced atrophy was not observed in mice administered lovastatin alone. This finding could be attributed to the difficulty in establishing the myopathy mouse model via statin administration (Meador and Huey, 2011; Muraki et al., 2012). As shown in Fig. 5, the administration of lovastatin alone did not alter the expression of FoxO1 and its targets at the mRNA and protein levels. However, lovastatin administration significantly increased FoxO1 and atrogin-1 protein expression in the presence of fenofibrate. The difference between mRNA and protein levels of FoxO1 may be explained by the prior finding that FoxO transcription factors are generally regulated at the post-transcriptional level by phosphorylation, dephosphorylation, acetylation, and deacetylation (Wang et al., 2014). Acetylation may regulate the function of FoxO1 by increasing the sensitivity of phosphorylation, thereby causing the degradation of the FoxO1 protein (Matsuzaki et al., 2005). This finding suggests that decreasing FoxO1 acetylation inhibits its degradation, thereby causing its enhanced accumulation. Previously, statins were reported to enhance PPARα activity in a synergistic manner with fibrates (Martin et al., 2001). In addition, statins could activate AMP-acti-
vated protein kinase (AMPK) (Mendieta et al., 2020). Moreover, AMPK is thought to be a downstream effector of PPARα (Chen et al., 2012). Because AMPK regulates FoxO1 by decreasing its acetylation (Wang et al., 2011), our findings suggest that combination treatment may further increase FoxO1 protein levels through the PPARα/AMPK/FoxO1 pathway. However, further studies are required to demonstrate the involvement of this pathway. Although we hypothesized that combination treatment with fenofibrate and lovastatin aggravates skeletal muscle atrophy when compared with effects of the administration of each drug alone, a decrease and increase in muscle weight was not observed. Such findings suggest that the fenofibrate-induced expression of FoxO1 was sufficient to stimulate the expression of E3-ubiquitine ligases for atrophy.

Based on a daily 0.5% fenofibrate and 0.13% lovastatin in the diet is equivalent to doses of approximately 800 mg and 190 mg/kg body weight, respectively. However, because of differences in absorption and pharmacokinetics between mice and humans, the respective dosage of fenofibrate and lovastatin was estimated to be 3,900 mg and 920 mg/body, when a human equivalent dose (HED) coefficient of 0.081 was employed (Nair and Jacob, 2016). Evidently, these dosages are higher than the respective clinical dosages of fenofibrate (maximum daily dose 160 mg/body) and lovastatin (maximum daily dose 80 mg/body). In some diseases or metabolic dysfunctions, these dosages may be associated with impairment in drug metabolism and the elimination and increase in the level of compounds and their metabolites in blood. According to the results of a genome-wide study, a single-nucleotide polymorphism (SNP) in the gene, SLC01B1, located on chromosome 12 and encoding OATP1B1 (which was previously demonstrated to regulate the hepatic uptake of statins), was strongly associated with the risk of myopathy with high-dose statin treatment (Link et al., 2008). As the above factors may partly contribute to the interindividual variability in susceptibility to fibrate- and statin-induced myotoxicity, we opted to administer high doses in the present study.

In conclusion, treatment with a high dose of fenofibrate could enhance the expression of FoxO1 and its targets, thereby causing skeletal muscle atrophy. Moreover, combination treatment with fenofibrate and lovastatin could further increase the protein levels of FoxO1 compared to fenofibrate administration alone. Recent studies have indicated that diabetes-related muscle atrophy is prevented in FoxO-KO mice (O’Neill et al., 2019), and muscle atrophy during cachexia is prevented via the inhibition of FoxO transcriptional activity (Reed et al., 2012). Collectively, these findings indicate that inhibitors of FoxO1 activity may become one of the treatment strategies not only for secondary sarcopenia, but also muscle atrophy caused by fibrates or combination with statins as the side effects.

ACKNOWLEDGMENTS

This work was supported in part by grants-in-aid for scientific research (KAKENHI 21300240, 26282184) from the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT, Tokyo), research grants from the Japanese Ministry of Health, Labor and Welfare (Tokyo), and a grant from the Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO, Japan). We are indebted to members of the Laboratory of Nutritional Biochemistry (Graduate School of Nutritional and Environmental Sciences, University of Shizuoka) and Department of Nutritional Science (National Institute of Health and Nutrition) for their technical assistance.

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

REFERENCES


Inoue, M., Senoo, N., Sato, T., Nishimura, Y., Nakagawa, T., Miyoshi, N., Goda, T., Morita, A. and Miura, S. (2017): Effects of the dietary carbohydrate-fat ratio on plasma phosphatidylcho-
Fenofibrate and lovastatin-induced expression of FoxO1 and its targets


