



BRIEF REPORT

Decreased Muscle Strength of Knee Flexors is Associated with Impaired Muscle Insulin Sensitivity in Non-Diabetic Middle-Aged Japanese Male Subjects

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ABSTRACT

Introduction: Reduced muscle strength is a high risk factor for type 2 diabetes mellitus, and this association is especially strong in non-obese male individuals. However, it remains unclear how reduced muscle strength affects susceptibility to diabetes. We have examined whether lower limb muscle strength is associated with insulin resistance in non-obese Japanese male subjects.

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Methods: Measurements from 64 non-diabetic, non-obese, middle-aged Japanese men were analyzed. Insulin sensitivity in muscle was measured using the hyperinsulinemic-euglycemic clamp. Isometric muscle strength of the knee extensor and flexor muscles was evaluated using a dynamometer.

Results: Lower muscle strength of knee flexors, but not knee extensors, was associated with impaired muscle insulin sensitivity (knee flexor muscles: low, medium, and high strength was 6.6 ± 2.2 , 7.3 ± 2.0 , and 8.8 ± 2.2 mg/kg per minute, respectively, p for trend < 0.05 ; knee extensor muscles: low, medium, and high strength was 7.3 ± 2.5 , 7.5 ± 2.2 , and 7.8 ± 2.3 mg/kg per minute, respectively, p for trend = 0.73). Knee flexor muscle strength was also identified as an independent determinant of insulin sensitivity in the multiple regression analysis ($\beta = 0.274$, $p = 0.036$).

Conclusions: Diminished strength of knee flexor muscles, but not knee extensor muscles, was associated with muscle insulin sensitivity in non-diabetic, non-obese Japanese male subjects.

Keywords: Insulin sensitivity; Muscle strength; Non-obese

Key Summary Points

The exact mechanisms of impaired muscle strength in persons with type 2 diabetes are still unclear.

The aim of this study was to examine whether lower limb muscle strength is associated with insulin resistance in non-diabetic men.

The results showed that lower limb flexor muscle strength, but not extensor muscle strength, was associated with insulin sensitivity in muscle.

Muscle strength weakness was also associated with insulin resistance before the onset of type 2 diabetes.

INTRODUCTION

Reduced muscle strength is a risk factor for type 2 diabetes mellitus (T2DM) [1], and muscle strength is lower in patients with T2DM than in their non-diabetic counterparts [2]. In addition, reduced muscle strength predicts cardiovascular disease and all-cause mortality in community-dwelling populations [3] and patients with T2DM [4]. Thus, enhancing muscle strength may help prevent the onset of T2DM and improve prognosis in pre-diabetic subjects as well as patients with T2DM. While reduced muscle strength in T2DM can be caused both by decreased muscle mass [5] and impaired muscle contractile function [6], the exact mechanism and time course of the latter in patients with T2DM are still unclear. Previous studies have demonstrated that decreased knee extensor muscle strength, adjusted by muscle mass [7] or lean body mass [8], is associated with insulin resistance as determined by the homeostasis model assessment of insulin resistance (HOMA-IR) in non-diabetic elderly [7] and healthy younger individuals [8]. These data suggest that insulin resistance that is closely associated with

decreased muscle contractile function may be present prior to T2DM onset. However, these previous studies used HOMA-IR as a surrogate marker for whole-body insulin resistance [7] and did not precisely evaluate insulin sensitivity using the hyperinsulinemic euglycemic clamp, the gold standard for assessing tissue-specific insulin sensitivity. In another study, HOMA-IR was found to be only weakly correlated with clamp-determined muscle insulin sensitivity ($r = -0.63$) and was not correlated with hepatic insulin sensitivity [9]. Thus, it remains unclear whether insulin sensitivity determined by hyperinsulinemic euglycemic clamp is associated with muscle strength in non-diabetic subjects.

In this context, we designed the present study to investigate the association between muscle strength and muscle insulin sensitivity as determined by hyperinsulinemic euglycemic clamp in non-diabetic, middle-aged Japanese men.

METHODS

Study Subjects

We enrolled 70 subjects with a body mass index (BMI) of ≥ 23.0 to < 25.0 kg/m² from the baseline dataset of the Sportology Center Core Study, a single-center, prospective, observational study on the mechanisms underlying metabolic abnormalities in non-diabetic, non-obese, middle-aged (aged 30–50 years) Japanese men [9]. We included those subjects with BMI between 23 and 25 kg/m² based on the World Health Organization's proposal of BMI ≥ 23 kg/m² and BMI ≥ 25 kg/m² being the thresholds of overweight and obesity, respectively, in Asians [10], with Asian males with BMI ranging from 23 to 25 kg/m² supposed to be classified as "at risk" [10]. Subjects who were being treated for hypertension, lipid disorders, diabetes, cardiovascular disease, chronic lung disease, cancer, renal failure, serious hepatic dysfunction, hepatitis B, and hepatitis C were excluded from the study. No muscle strength data were available for six of the 70 subjects who participated in the study; consequently,

data on 64 subjects were included in this analysis. All individuals gave written informed consent to participate in this study, which was approved by the ethics committee of Juntendo University and conducted according to the principles of the Declaration of Helsinki (2008).

Study Design

The design of the Sportology Center Core Study has been described previously in detail [11]. Briefly, all subjects underwent a baseline evaluation which included screening conducted in examinations carried out in three visits. In the examinations, the subject underwent oral glucose tolerance test, peak oxygen uptake ($VO_{2\text{peak}}$) test, lower limb muscle strength test, brief-type self-administered diet history questionnaire, and measurement for daily physical activity level by an accelerometer (Lifecorder; Suzuken, Nagoya, Japan). We also evaluated intramyocellular lipid (IMCL) and intrahepatic lipid (IHL) by ^1H -magnetic resonance spectroscopy (MRS), total body fat content and fat-free mass (FFM) using the bioimpedance method (InBody; BIOSPACE, Tokyo, Japan), and visceral fat area (VFA) and subcutaneous fat area (SFA) by magnetic resonance imaging (MRI). Each subject then underwent two-step euglycemic hyperinsulinemic clamp tests; each step lasted 180 min, with constant insulin infusions of 10 and 20 $\text{mU}/\text{m}^2/\text{min}$. Glucose disappearance during the second step was used as an index of muscle insulin sensitivity.

Two-Step Hyperinsulinemic Euglycemic Clamp Test

The subjects were instructed to consume a weight-maintenance standard diet and limit their daily physical activity to their mean daily physical activity level $\pm 10\%$ during the 3 days immediately before the clamp test. After an overnight fast, a two-step hyperinsulinemic euglycemic glucose clamp was performed with an artificial endocrine pancreas (STG 22; Nikkiso, Shizuoka, Japan) with glucose tracer. Briefly, after securing an intravenous cannula in the forearm, a bolus dose [$200 \text{ mg}/\text{m}^2$ body

surface area (BSA)] of $[6,6\text{-}^2\text{H}_2]\text{glucose}$ (Cambridge Isotope Laboratories, Tewksbury, MA, USA) was injected intravenously. Constant infusion was continued at a rate of 2 mg/m^2 BSA per minute for 3 h (-180 to 0 min) to measure endogenous glucose production (EGP) at fasting state [12]. This was followed by a primed insulin infusion (40 mU/m^2 per minute followed by 20 mU/m^2 per minute, each lasting 5 min) and continuous insulin infusion at 10 mU/m^2 per minute for 3 h (first step) (0–180 min). In the second step of the clamp, after a priming insulin infusion (80 mU/m^2 per minute followed by 40 mU/m^2 per minute, each lasting 5 min), insulin was infused continuously at 20 mU/m^2 per minute for 3 h (180–360 min). We used a warming blanket for arterialization of the hand vein, and plasma glucose level in arterialized blood was maintained at approximately 95 mg/dl by a variable 20% glucose infusion containing approximately 2.5% $[6,6\text{-}^2\text{H}_2]\text{glucose}$. Blood samples were obtained for biochemical analysis at 10-min intervals during the last 30 min of the steady-state period of the first and second steps of the clamp. Enrichment of $[6,6\text{-}^2\text{H}_2]\text{glucose}$ in plasma was measured by high-performance liquid chromatography (LTQ-XL-Orbitrap mass spectrometer; Thermo Fisher Scientific, Waltham, MA, USA) as described previously [9].

Calculations

A steady-state equation was used to calculate the rates of EGP and glucose disappearance (Rd) at each step [9]. EGP and Rd were normalized by BSA and FFM, respectively [9]. We calculated percentage reduction of EGP at the first step and used the result as an index of hepatic insulin sensitivity [13]. Rd at the second step was used as an index of muscle insulin sensitivity [14].

^1H -MRS and MRI

The IMCL values of the right tibialis anterior and soleus muscles and the IHL of segment 6 in the liver were measured by ^1H -MRS (VISART EX V4.40; Toshiba, Tokyo, Japan) [15, 16]. After the measurements, IMCL was quantified by methylene signal intensity (S-fat) using the

creatine signal (Cre) as the reference, and calculated as the ratio S-fat/Cre. IHL was quantified by S-fat using H₂O as the internal reference, and calculated as the percentage of H₂O + S-fat [S-fat × 100/(H₂O + S-fat)] [15, 16]. VFA and SFA were measured with MRI as described previously [16]. Briefly, T1-weighted trans-axial scans were obtained, and VFA and SFA at the fourth and fifth lumbar interspaces were measured as described previously using specific software (AZE Virtual Place, Tokyo, Japan) [16].

Knee Extensor/Flexor Muscle Strength Test

Knee extensor/flexor muscle strength was measured using the Cybex770 system (Cybex Division of Lumex, Ronkonkoma, NY, USA). The isometric peak torques of knee extensors/flexors were measured at an angular velocity of 60° for 30 s. During the test, participants were encouraged to perform maximal muscle contractions. The isometric peak torques (Nm) of knee extensors/flexors were adjusted by body weight (kg).

Statistical Analysis

Study subjects were categorized into tertiles of low, medium, and high, respectively, according to knee extensor/flexor muscle strength. Data are presented as mean ± standard deviation. Data of the three groups were compared by one-way analysis of variance. The dose–response relationship in the three groups was analyzed using the Jonckheere Terpstra test. Multiple linear regression was used to analyze the relationship between muscle insulin sensitivity and muscle strength and other risk factors. This study included adiponectin, VFA, and VO_{2peak} as covariates, all of which were correlated with muscle insulin sensitivity in our previous study [9]. All statistical tests were two-sided with a 5% level of significance.

RESULTS

The physical characteristics of the subjects categorized by muscle strength tertile are

summarized in Tables 1 and 2. The muscle strength of knee extensors was approximately threefold greater than that of knee flexors, and both strengths were positively associated with each other. Both strengths were negatively associated with percentage body fat, VFA, and SFA, and were positively associated with FFM (*p* for trend < 0.05). Although both muscle strengths were associated with several indices representing adiposity, only knee flexor muscle strength was negatively associated with elevated free fatty acids, fasting plasma insulin and HOMA-IR, and reduced adiponectin. Further, knee flexor muscle strength was positively associated with insulin sensitivity in muscle. In contrast, knee extensor muscle strength was not associated with insulin sensitivity in muscle. These same relations between muscle strength and insulin sensitivity were observed when muscle strength was adjusted by FFM instead of body weight. In addition, we investigated the relationship between muscle strength and insulin sensitivity using multiple linear regression analysis. Due to the small number of study subjects, we included a limited number of covariates in the multiple regression analysis: age, muscle strength, visceral fat area, adiponectin, and VO_{2peak}. Knee flexor muscle strength ($\beta = 0.274$, $p = 0.036$) and adiponectin ($\beta = 0.239$, $p = 0.042$) were identified as independent determinants of insulin sensitivity (Table 3, Model 1). When we included free fatty acids instead of adiponectin in the model, only knee flexor muscle strength was significant ($\beta = 0.277$, $p = 0.041$) (Table 3, Model 2).

DISCUSSION

The results of the present study show that in this population of non-obese, non-diabetic Japanese male subjects, reduced knee flexor muscle strength was associated with impaired insulin sensitivity in muscle. Interestingly, knee extensor muscle strength was not associated with insulin sensitivity in muscle in this population.

Reduced muscle strength of the knee flexors, but not the knee extensors, was associated with muscle insulin resistance as determined by

Table 1 Physical characteristics of the study subjects

| Physical parameters | Knee flexor muscle strength according to tertile | | | p value | Knee extensor muscle strength according to tertile | | | p value |
|--------------------------------|--|--------------------------|----------------------------|---------|--|--------------------------|----------------------------|---------|
| | Low (N = 21) | Medium (N = 22) | High (N = 21) | | Low (N = 22) | Medium (N = 20) | High (N = 22) | |
| | | | | | | | | |
| Flexor strength (Nm/kg) | 0.89 ± 0.08 | 1.11 ± 0.06 | 1.41 ± 0.17 | – | 0.99 ± 0.17 | 1.13 ± 0.19 [†] | 1.29 ± 0.25 ^{†,‡} | < 0.01* |
| Extensor strength (Nm/kg) | 2.70 ± 0.26 | 2.88 ± 0.35 [†] | 3.12 ± 0.37 ^{†,‡} | < 0.01* | 2.54 ± 0.23 | 2.88 ± 0.13 | 3.27 ± 0.23 | – |
| Age (year) | 42.0 ± 5.6 | 42.9 ± 4.9 | 42.1 ± 4.9 | 0.82 | 42.1 ± 6.5 | 41.9 ± 4.7 | 43.1 ± 3.7 | 0.66 |
| BMI (kg/m ²) | 24.0 ± 0.5 | 24.0 ± 0.6 | 23.9 ± 0.6 | 0.69 | 24.2 ± 0.4 | 23.8 ± 0.5 [†] | 23.9 ± 0.6 | 0.02* |
| Fasting plasma glucose (mg/dl) | 98.0 ± 5.1 | 96.5 ± 8.0 | 95.4 ± 7.8 | 0.50 | 94.6 ± 4.8 | 97.6 ± 7.3 | 97.8 ± 8.4 | 0.25 |
| Fasting plasma insulin (μU/ml) | 7.1 ± 2.0 | 6.1 ± 3.2 | 5.0 ± 2.2 [†] | < 0.01* | 6.1 ± 2.0 | 6.1 ± 2.9 | 5.6 ± 3.1 | 0.76 |
| HbA1c (%) | 5.0 ± 0.2 | 4.8 ± 0.2 | 4.8 ± 0.3 | 0.15 | 5.0 ± 0.2 | 4.8 ± 0.2 | 4.8 ± 0.3 | 0.58 |
| Triglyceride (mg/dl) | 167.1 ± 93.8 | 161.1 ± 79.6 | 121.1 ± 71.8 | 0.15 | 167.1 ± 93.8 | 161.1 ± 79.6 | 121.1 ± 71.8 | 0.25 |
| HDL-C (mg/dl) | 49.6 ± 11.2 | 54.6 ± 16.5 | 60.7 ± 12.7 [†] | 0.04* | 54.1 ± 15.8 | 56.4 ± 13.5 | 54.6 ± 13.6 | 0.86 |
| LDL-C (mg/dl) | 123.2 ± 27.8 | 127.3 ± 25.2 | 124.3 ± 34.3 | 0.89 | 134.8 ± 33.6 | 118.6 ± 25.8 | 121.0 ± 24.8 | 0.14 |
| Free fatty acid (μEq/L) | 426.5 ± 113.7 | 380.6 ± 93.4 | 362.5 ± 112.2 | 0.15* | 386.1 ± 119.9 | 378.8 ± 101.7 | 402.1 ± 105.1 | 0.78 |
| Adiponectin (μg/ml) | 3.6 ± 1.7 | 3.9 ± 1.9 | 4.6 ± 1.8 | 0.10* | 4.0 ± 2.1 | 3.9 ± 1.7 | 3.8 ± 1.8 | 0.95 |
| HOMA-IR | 1.7 ± 0.5 | 1.5 ± 0.8 | 1.1 ± 0.6 [†] | 0.02* | 1.4 ± 0.5 | 1.5 ± 0.7 | 1.4 ± 0.9 | 0.95 |

Values in table are presented as the mean ± standard deviation (SD)
 BMI body mass index, HbA1c hemoglobin A1c, HDL-C high-density lipoprotein cholesterol, LDL-C low-density lipoprotein cholesterol, HOMA-IR homeostasis model assessment of insulin resistance
 *^{†,‡}p value, one-way analysis of variance for continuous variables. [†]p < 0.05 vs. Low tertile, [‡]p < 0.05 vs. Medium tertile for Tukey–Kramer or Games-Howel post hoc test; *p < 0.05 for Jonckheere–Terpstra test

Table 2 Clinical characteristics of the study subjects

| Clinical parameters | Knee flexor muscle strength according to tertile | | | <i>p</i> value | Knee extensor muscle strength according to tertile | | | <i>p</i> value |
|---|--|-------------------------|---------------------------|----------------|--|-------------------------|-------------------------|----------------|
| | Low (<i>N</i> = 21) | Medium (<i>N</i> = 22) | High (<i>N</i> = 21) | | Low (<i>N</i> = 22) | Medium (<i>N</i> = 20) | High (<i>N</i> = 22) | |
| Percentage body fat | 23.9 ± 3.9 | 22.5 ± 4.0 | 19.5 ± 5.1 [†] | < 0.01* | 24.6 ± 3.0 | 21.4 ± 5.6 [†] | 20.0 ± 4.0 [†] | < 0.01* |
| FFM (kg) | 53.1 ± 4.9 | 55.4 ± 5.6 | 57.1 ± 5.1 [†] | 0.05* | 52.1 ± 4.3 | 56.5 ± 6.0 [†] | 57.1 ± 5.0 [†] | < 0.01* |
| VFA (cm ²) | 105.1 ± 21.6 | 93.6 ± 32.5 | 79.2 ± 35.4 [†] | 0.03* | 98.4 ± 30.3 | 101.0 ± 34.5 | 79.8 ± 29.3 | 0.06* |
| Subcutaneous fat area (cm ²) | 141.3 ± 29.3 | 124.3 ± 35.2 | 106.0 ± 45.2 [†] | 0.01* | 136.8 ± 31.3 | 125.6 ± 44.2 | 109.4 ± 38.5 | 0.06* |
| <i>VO</i> _{2peak} (ml/kg per minute) | 31.5 ± 4.7 | 30.3 ± 7.8 | 34.1 ± 8.0 | 0.21 | 31.2 ± 7.5 | 32.7 ± 6.5 | 32.2 ± 7.5 | 0.79 |
| Daily physical activity (METs·h) | 4.2 ± 1.3 | 5.0 ± 1.3 | 4.5 ± 1.1 | 0.15 | 4.7 ± 1.2 | 4.9 ± 1.5 | 4.2 ± 1.1 | 0.20 |
| IMCL in TA (S-fat/Cre) | 2.7 ± 1.2 | 2.8 ± 1.6 | 3.4 ± 1.8 | 0.37 | 2.8 ± 1.3 | 3.5 ± 1.9 | 2.6 ± 1.4 | 0.17 |
| IMCL in SOL (S-fat/Cre) | 13.2 ± 6.9 | 11.1 ± 4.3 | 16.1 ± 8.4 | 0.06 | 12.0 ± 4.7 | 14.5 ± 9.3 | 13.9 ± 6.3 | 0.47 |
| Muscle insulin sensitivity (mg/FFM kg per minute) | 6.6 ± 2.2 | 7.3 ± 2.0 | 8.8 ± 2.2 [†] | < 0.01* | 7.3 ± 2.5 | 7.5 ± 2.2 | 7.8 ± 2.3 | 0.73 |

Values in table are presented as the mean ± SD

Cre Creatine, FFM fat-free mass, IMCL Intramyocellular lipid, METs metabolic equivalents, S-fat methylene signal intensity, SOL soleus muscle, TA tibialis anterior muscle, VFA visceral fat area, *VO*_{2peak} peak oxygen consumption
p value, one-way analysis of variance for continuous variables. [†]*p* < 0.05:vs. Low tertile for Tukey–Kramer or Games-Howel post hoc test; **p* < 0.05 for Jonckheere–Terpstra test

Table 3 Multiple linear regression for relationship between muscle strength and insulin sensitivity

| Muscle strength and model covariates | Knee flexor muscle strength | | Knee extensor muscle strength | |
|---|-----------------------------|----------------|-------------------------------|----------------|
| | β | <i>p</i> value | β | <i>p</i> value |
| Model 1 | | | | |
| Flexor strength (Nm/kg) | 0.274 | 0.036 | – | – |
| Extensor strength (Nm/kg) | – | – | 0.060 | 0.477 |
| Age | – 0.002 | 0.990 | 0.071 | 0.586 |
| Adiponectin ($\mu\text{g/ml}$) | 0.239 | 0.042 | 0.286 | 0.021 |
| VFA (cm^2) | – 0.164 | 0.223 | – 0.244 | 0.081 |
| $\dot{V}\text{O}_{2\text{peak}}$ (ml/kg per minute) | 0.176 | 0.182 | 0.223 | 0.104 |
| Model 2 | | | | |
| Flexor strength (Nm/kg) | 0.277 | 0.041 | – | – |
| Extensor strength (Nm/kg) | – | – | 0.029 | 0.827 |
| Age | – 0.008 | 0.994 | 0.076 | 0.588 |
| Free fatty acids ($\mu\text{Eq/l}$) | – 0.072 | 0.562 | – 0.097 | 0.458 |
| VFA (cm^2) | – 0.230 | 0.109 | – 0.333 | 0.024 |
| $\dot{V}\text{O}_{2\text{peak}}$ (ml/kg per minute) | 0.175 | 0.214 | 0.225 | 0.128 |

p value: multiple linear regression analysis adjusted for all factors in the table

hyperinsulinemic euglycemic clamp. In contrast, a study using HOMA-IR showed that reduced knee extensor muscle strength was associated with insulin resistance in the elderly [7] and that muscle strength of both the knee flexors and extensors was associated with insulin resistance in healthy young subjects [8]. The Rd used as an index of muscle insulin sensitivity in the present study was only weakly correlated with HOMA-IR ($r = -0.63$) [11]. In addition, in the present study we analyzed only non-obese Japanese male subjects, which differs from the study populations in previous studies in terms of age and BMI [7, 8]. These variations in subjects and methods used to evaluate insulin sensitivity may explain the differences in study results.

Similar to previous studies in non-diabetic elderly and young subjects [7, 8], we observed a link between decreased muscle strength and muscle insulin resistance. Results from an animal model also showed that muscle contractile function was reduced in parallel with impaired

insulin sensitivity during administration of a high-fat diet before diabetes onset [17]. Regarding the causal relation between these factors, a meta-analysis showed that resistance training, which increases muscle strength, improved markers of insulin resistance in subjects with metabolic syndrome [18]. On the other hand, Marsh et al. investigated the effect of an insulin sensitizer, pioglitazone, on knee extensor muscle strength and leg muscle power during voluntary weight loss in non-diabetic older adults [19] and showed that in women, pioglitazone potentiated the effect of resistance training on muscle power but not muscle strength, and did not alter lean body mass. These data suggest that improving insulin resistance might enhance muscle function independent of muscle mass change, an approach that may be applicable to diabetic patients with reduced muscle strength. In fact, muscle strength has been reported to be reduced in diabetic patients [20].

It is still unclear why the strength of knee flexors, but not extensors, was associated with insulin sensitivity in this study. It has been shown that type I muscle fibers have a higher glucose-handling capacity than type II muscle fibers [21]. Interestingly, results from an animal model recently showed that muscle strength was reduced in parallel with insulin resistance during a high-fat diet, but that this occurred only in the extensor digitorum longus muscle, which contains predominantly type II fibers, and not in the soleus muscle, which comprises mainly type I fibers [17]. Knee flexor muscles have been reported to have a relatively high proportion of type II fibers [22], and thus the strength of knee flexors is more likely to be associated with insulin sensitivity than that of extensors. In addition, knee extensors are generally used more during physical activity than knee flexors, and thus the association between insulin sensitivity and knee extensor muscle strength might be diminished by major covariates, such as physical activity. Taken together, decreased knee flexion muscles strength might be a better marker for impaired muscle insulin sensitivity than knee extensor muscle strength.

The present study has several limitations. First, the analysis was conducted on only Japanese male subjects. Since glucose and lipid metabolism and fat distribution vary by sex and ethnicity, our results may not be applicable to females and other ethnic groups. Another possible limitation is the relatively small number of subjects. However, two-step hyperinsulinemic euglycemic clamp is very time consuming (approx. 10 h), and therefore the inclusion of more than 60 non-diabetic subjects may represent a strength rather than a limitation. Finally, intervention studies are clearly required to confirm the causality of the parameters identified in the present study.

CONCLUSION

Knee flexor muscle strength was associated with muscle insulin sensitivity in non-diabetic, middle-aged Japanese male subjects. Further studies are needed to generalize these findings.

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Authorship Contributions. YS, YT, and KT performed the research and contributed to the study design, data collection, interpretation of results, and writing and editing of the manuscript. YS, KT, SK, TF, YF, HE, KW, TK, TY, HK, DS, SK, and RS participated in data collection and analysis, and contributed to the discussion. RK contributed to the discussion. HW contributed to the study design and reviewed and edited the manuscript.

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Compliance with Ethics Guidelines. All individuals gave written informed consent to participate in this study, which was approved by

the ethics committee of Juntendo University and conducted according to the principles of the Declaration of Helsinki (2008).

Data Availability. All data generated or analyzed during this study are included in this published article.

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