Apelin-transgenic mice exhibit a resistance against diet-induced obesity by increasing vascular mass and mitochondrial biogenesis in skeletal muscle

Toshihiro Yamamoto a,⁎, Yugo Habata a, Yoshio Matsumoto a, Yoshitaka Yasuhara a, Tadatoshi Hashimoto a, Hitomi Hamajyo b, Hisashi Anayama b, Ryō Fujii c, Hiromitsu Fuse a, Yasushi Shintani a, Masaaki Mori a

a Pharmacology Research Laboratories, Pharmaceutical Research Division, Takeda Pharmaceutical Company Limited, Osaka, Japan
b Development Research Center, Pharmaceutical Research Division, Takeda Pharmaceutical Company Limited, Osaka, Japan
c Discovery Research Center, Pharmaceutical Research Division, Takeda Pharmaceutical Company Limited, Osaka, Japan

⁎ Corresponding author. Tel.: +81 6 6300 6142; fax: +81 6 6300 6306.
E-mail address: Yamamoto_Toshihiro@takeda.co.jp (T. Yamamoto).

1. Introduction

Apelin is an endogenous ligand of the G-protein-coupled 7-transmembrane receptor, APJ [1]. The human apelin (Apln) gene is located on the long arm of the X chromosome and encodes a 77-amino acid pre-proapelin protein; it is cleaved by proteases and is secreted as apelin-36—a 36-amino acid full-length apelin—as well as in shorter forms such as apelin-17 and apelin-13. These peptides are biologically active and are able to be cross-activated between human and mouse APJ [1,2].

Apelin/APJ signalling mediates a wide range of physiological actions [3] such as vascular physiology [4–6], blood pressure [7], immune responses [8], and glyco-lipid metabolism [9,10]. Recently, apelin was identified as an adipokine that is upregulated by insulin and in conjunction with obesity [11]. Higuchi et al. report that the repeated administration of apelin-13 in diet-induced obese (DIO) mice results in a decrease in adiposity via the increase of uncoupling protein (UCP) expression in brown adipose tissue and skeletal muscle [9]. Dray et al. also demonstrated that apelin-13 lowers plasma glucose levels by increasing glucose utilisation in skeletal muscle in both normal and DIO insulin-resistant mice [10]. These results strongly suggest that apelin stimulates energy metabolism.

Although there are several reports describing the phenotypes of apelin-knockout (apelin-KO) and conditional apelin-transgenic (apelin-Tg) mice, most described vascular physiology and pathology. Apelin-KO mice exhibit impaired retinal vascularisation and decreased angiogenic responses in their corneas due to vascular endothelial growth factor (VEGF)-α and fibroblast growth factor (FGF)-2 stimulation [5], decreased vascular diameter and density [4], upregulated API expression in vascular smooth muscle, and decreased neointima formation in the carotid ligation [12]; keratinocyte-specific apelin-Tg mice exhibit increased vascular diameter in the dermis [13]. On the other hand, only a single report provides information...
regarding the effect of apelin on energy metabolism and describes the decrease of insulin sensitivity in skeletal muscle of apelin-KO mice [14]. Since adipose accumulation is closely related to blood vessel formation for oxygen and energy supply [15], it is thought that apelin/APJ signalling may influence the energy metabolism of obesity and diabetes via dysfunctions in angiogenesis and/or vascular maturation in metabolic tissues. However, there are no reports concerning the anti-obesity and/or anti-diabetic actions of apelin from a vascular pathophysiological viewpoint.

Therefore, we examined the effect of apelin/APJ signalling on obesity by using human apelin-Tg mice. Our study aims to determine whether the stimulation of apelin/APJ signalling decreases adiposity and if so, whether this action is mediated by the modulation of vascular pharmacological action. Unexpectedly, the expression of genes related to lipid metabolism in skeletal muscle of apelin-Tg mice was not different compared to the non-Tg control, although apelin-Tg mice exhibited DIO resistance without affecting food intake. On the other hand, the mRNA expression of angiopeptin1 (Ang1), a key molecule for vascular maturation, and its receptor, endothelium-specific receptor tyrosine kinase 2 (Tie2), were significantly upregulated in the skeletal muscle of apelin-Tg mice. It is interesting that the vascular mass, aerobic type-I muscle fibre ratio, and mitochondrial content increased in the skeletal muscle of apelin-Tg mice at HFD-fed condition. These results suggest that the DIO resistance of apelin-Tg mice is caused by high energy expenditure stimulated by increases in mitochondrial biogenesis and oxygen consumption in conjunction with increased vascular mass in skeletal muscle.

2. Materials and methods

2.1. Animals

To generate the apelin-Tg mice, we used a human ubiquitin C (hUbc) promoter to drive the ubiquitous expression of the human Aphn gene [16]. Furthermore, a human cytomegalovirus immediate-early enhancer was ligated upstream of the hUbc promoter for increased expression. The homology arms of the gtRosa26 site with the Ascl site were added because we had initially planned to generate apelin-Tg mice by inserting an expression vector into the gtRosa26 site by homologous recombination. A human Aphn CDNA and an SV40 polyadenylation signal sequence were inserted into the plasmid containing the sequences mentioned above. The expression vector was digested and purified by agarose gel electrophoresis. In Tris–EDTA buffer (1 mmol/L Tris–HCl and 0.1 mmol/L EDTA; pH 8.0), 3 μg/mL expression vector was injected into the pronuclei of fertilised oocytes obtained from C57BL/6J mice (Clea Japan, Inc., Tokyo, Japan). The integration of transgenes into genomic DNAs was detected by PCR with primers recognising the expression vector (forward: GAGTCGCGCAGCTTGTTGACCAGC, reverse: AGATCTTCAGAAAGG-CATGGGTCCTT ATGGGAG) using tail DNA. Transgenic offspring were obtained by breeding with C57BL/6J mice (Clea Japan, Inc.). Non-transgenic offspring were used as controls.

We used male mice in all examination. At 6 weeks of age, all animals were divided into 2 diet groups at random: one group received a standard diet (SD) with CE-2 (4.6% calorie fat; Clea Japan, Inc.) and the other received a high-fat diet (HFD) with D12451 (45% calorie fat; Research Diets, New Brunswick, NJ, USA). The mice were comprised 6 channels, the measurements were continuously taken several times in the same week of age, summed, and then analysed as final data.

2.2. Plasma parameter measurement

For periodical measurements, 10 μL blood was collected from the tail vein and immediately mixed with 40 μL saline supplemented with 10 units heparin on ice. Plasma (5× diluted) was then separated by centrifugation at 14,000 × g for 5 min at 4 °C. At the endpoint, plasma was prepared by decapsulation simultaneously when tissue samples were removed. Plasma separation was carried out as described above with 2.5% (v/v) heparin (1000 units/mL). Blood was collected in the morning under non-fasting conditions or 24 h after fasting conditions. Samples were stored at −80 °C until analysis.

Blood glucose (Roche Diagnostics, Tokyo, Japan), plasma leptin (R&D systems, Minneapolis, MN, USA), plasma insulin (Morinaga Institute of Biological Science Inc., Kanazawa, Japan), and plasma adiponectin (Otsuka, Tokyo, Japan) levels were measured using each assay reagent according to the manufacturer’s protocol. Plasma glucose (PG), triacylglycerol (TG), total cholesterol (TC), non-esterified fatty acid (NEFA), total ketone bodies (TKB), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured by an Automatic Analyser system 7180 (Hitachi, Tokyo, Japan). Plasma apelin was measured by an enzyme immunoassay (EIA) method as previously reported [2].

2.3. Body fat measurement

Body fat and lean weight were measured by an EchoMRL-900 (Aloka, Tokyo, Japan). Each weight was normalised against body weight.

2.4. Body temperature measurement

The rectal temperature of 26-week-old mice was measured at room temperature (25 °C) at 9:30 am using a rodent telemeter thermometer BAT-12 (Physitemp Instrument Inc., Clifton, NJ, USA) equipped with a rectal probe RET-3 (Muromachi Kikai Co., Ltd., Tokyo, Japan) inserted 2 cm into the rectum. To avoid temperature rises induced by stress, cages were taken out one at a time for each measurement.

2.5. Indirect calorimetry measurement

In vivo indirect calorimetry was performed using a Small Animal Colorimetry Measurement system MK-5000RQ/06 (Muromachi Kikai Co., Ltd., Tokyo, Japan). Constant airflow (0.5 mL/min) was drawn through the chamber and monitored by a flow meter. To calculate oxygen consumption (VO2), carbon dioxide excretion (VCO2), and respiratory quotient (RQ; ratio of VCO2/VO2), gas concentrations were continuously monitored every 5 min at the inlet and outlet of the scaled chambers over a 24 h period. At 16 and 50 weeks of age, apelin-Tg and non-Tg control mice with median body weights corresponding to their strain were chosen and their indirect calorimetry was measured in an experimental chamber at 25 °C±1 °C, 50±5% humidity under a 12-h light–dark cycle (lights on from 7:30 am to 7:30 pm) with free access to food and tap water. Because the system comprised 6 channels, the measurements were continuously taken several times in the same week of age, summed, and then analysed as final data.

2.6. Tissue removal

After decapitation, the whole brain, lungs, heart, kidneys, liver, quadriceps muscle, epididymal adipose, mesenteric adipose tissue, and brown adipose tissue were removed. Samples were stored at −80 °C until analysis.
2.7. Tissue apelin concentration measurement

Tissue apelin concentration was measured by the inhibitory activity against forskolin-induced cAMP production as we described previously [17]. Tissue samples (approximately 100–200 mg) were boiled in 5 mL distilled water for 5 min. After the samples were chilled in an ice bath, acetic acid (1%, 1 mol/L) was added, and the samples were homogenised. The homogenate was filtered with a cell strainer (#352340; Becton Dickinson), and centrifuged (12,000 × g for 20 min) at room temperature. An apelin-containing fraction was then obtained from the supernatant after separation using a Sep-Pak C18 plus Cartridge (Waters Corporation, Milford, MA, USA). In brief, after the Sep-Pak C18 plus Cartridge was washed with 3 mL methanol and equilibrated with 3 mL 0.1% trifluoroacetic acid (TFA). The homogenised supernatant was applied to the column and subsequently washed twice with 3 mL TFA. A basic peptide fraction including apelin was then eluted from the column with 3 mL 40% acetonitrile (CH3CN) containing 0.1% TFA. Collected fractions were lyophilised by a centrifugal concentrator (Thermo Savant, Holbrook, NY, USA) and were stored as measurement samples at −80 °C until analysis. Measurements were carried out after the fractions were re-dissolved with a measurement buffer.

Apelin concentration was calculated as the IC50 against the intracellular cAMP increase stimulated by 2 μmol/L forskolin using APJ-overexpressing Chinese hamster ovary (CHO) cells transfected with an APJ-expressing plasmid. The cells were cultured in a 96-well plate at the cell density of 3 × 104 cells/100 μL with αMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% dialysed foetal bovine serum (Invitrogen) and penicillin (100 units/mL)–streptomycin (100 μg/mL) mixture (Invitrogen) at 37 °C in 5% CO2. One day after incubation, each well was washed with 150 μL measurement buffer (Hanks’ balanced salt solution, 20 mmol/L HEPES (pH 7.3), 0.1% bovine serum albumin, and 0.2 mmol/L 3-isobutyl-1-methylxanthine) and subsequently pre-incubated with 150 μL/well measurement buffer at 37 °C for 30 min. After pre-incubation, measurement samples (100 μL/well) dissolved with measurement buffer were added with or without forskolin and incubated at 37 °C for 30 min. Intracellular cAMP concentration was then measured using cAMP enzyme immunoassay Biotrik TM (GE Healthcare, Buckinghamshire, UK). Apelin concentration was calculated using a standard curve with apelin-13 (Peptide Institute Inc., Osaka, Japan).

2.8. Real-time quantitative polymerase chain reaction

Messenger RNAs were amplified by polymerase chain reaction (PCR) and quantified by real-time quantitative PCR (qRT-PCR) as described below. Total tissue RNA was prepared from tissues using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Hiden, Germany) according to the manufacturer’s protocol. RNA was quantified using a Spectrophotometer ND-1000 (NanoDrop products, Wilmington, DE, USA) to measure the absorbance at 260 nm relative to that at 280 nm. Complementary DNA (cDNA) was synthesised using a GeneAmp PCR System 7700 (Applied Biosystems, Carlsbad, CA, USA) from total RNA (100 ng) in a volume of 50 μL using TaqMan Reverse Transcription Reagent (Applied Biosystems) under the following conditions as cycling parameters: 25 °C for 10 min, 48 °C for 30 min, and 95 °C for 5 min. TaqMan premix (20×) probe/primers were purchased from Applied Biosystems (Suppl. Table S1). Using an ABI PRISM HT7700 sequence detector (Applied Biosystems), PCR amplification was performed in 20 μL solution containing 25 ng relative cDNA template in TaqMan Universal Gene Expression Master Mix (Applied Biosystems) according to the following protocol: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles at 95 °C for 15 s and 60 °C for 1 min. For each transcript, real-time PCR was conducted in duplicate, and the amplified transcripts were quantified using the 36B4 mRNA expression level as an internal control.

2.9. Mitochondrial DNA measurement

Total DNA of the quadriceps muscle was prepared using QIAamp DNA Micro Kit (Qiagen) according to the manufacturer’s protocol. The mitochondrial content in the tissues was substituted with the copy number of mitochondrial NADH dehydrogenase subunit 1 (ND1) using genomic DNA β-actin (Actb) using the qRT-PCR method as described above. We constructed the primer sequences for normal ND1 (forward: CCTGACCCATAGCCATATAATGATTAT; reverse: CCTGATACCTTTGGACTTCTTG) and Actb (forward: AAATCTGTTGAGCATCAAAAAGA; reverse: GCCCATCCTGCCGCTGCAA), and used them to conduct the measurements.

2.10. Immunohistochemical analysis

Paraffin-embedded sections (4-μm thick) of quadriceps muscle tissues from 57-week-old apelin-Tg and non-Tg control mice, which were fixed overnight in 4% paraformaldehyde containing 0.1% phosphate buffer, were cut using a microtome and subjected to an immunohistochemical technique employing rat anti-mouse CD31 antibody (#6430-0758; Serotec, Oxford, UK) for capillary characterisation as a general method. In brief, sections were collected on glass slides, fixed, de-waxed, washed with distilled water (DW), incubated in 0.1% 0.05 mol/L Tris-buffered saline at 37 °C for 30 min, and washed again with DW. The sections were then incubated with methanol containing 0.3% hydroxy peroxide (H2O2) at room temperature for 30 min, washed again with DW, rinsed with phosphate-buffered saline (PBS), and incubated with the rabbit serum from the ABC rat IgG kit (PK-6104; Vector Laboratories, Burlingame, CA, USA) at room temperature for 30 min. Sections were then incubated with a primary anti-mouse CD31 antibody overnight at 4 °C. On the following day, the slides were washed with PBS, incubated with biotinylated rat IgG from the same kit for 30 min at room temperature, washed again with PBS, incubated with ABC complex from the same kit for 30 min at room temperature, washed again with PBS, and then incubated with the 3,3′-diaminobenzidine (DAB). The sections were subsequently washed with DW, counterstained with haematoxylin, dehydrated, cleaned, and finally mounted. In the analysis, we confirmed that the primary antibody was specifically stained.

Fibre typing was performed by using an anti-human slow troponin I monoclonal antibody (MAB) (#01869-96; Kanto Chemical Co. Ltd., Tokyo, Japan) and an anti-human fast troponin I MAB (#01868-96; Kanto Chemical Co. Ltd.) as described by Matsumoto et al. [18]. The de-waxed sections were incubated with REALTM Target Retrieval Solution (S2031; Daco, Glostrup, Denmark) at 121 °C for 15 min, washed with DW, incubated with methanol containing 0.3% H2O2 at room temperature for 30 min, washed again with DW, incubated with blocking reagent A from the Mouse Stain Kit (#414321; Nichirei, Tokyo, Japan) at room temperature for 60 min, washed with PBS, and then incubated with primary anti-human slow troponin I MAB #01869-96 overnight at 4 °C. On the following day, the slides were washed with PBS, incubated with blocking reagent B from the same kit for 10 min at room temperature, washed again with PBS, incubated with the Simple Stain Mouse MAX-PO (Nichirei) for 10 min at room temperature, washed again with PBS, and then incubated with the 3,3′-diaminobenzidine (DAB). The sections were subsequently washed with DW, counterstained with haematoxylin, dehydrated, cleaned, and finally mounted. In the analysis, we confirmed that the primary antibody was specifically stained.

Sections were observed using a ZEISS Axiosplan microscope (Carl Zeiss, Oberkochen, Germany), and each image was acquired (400 × 300 pixels) with an OLYMPUS DP20 digital camera (Olympus, Tokyo, Japan). Each image was processed and further analysed using Adobe Photoshop Elements 6 (Adobe Systems Inc., San Jose, CA, USA). In brief, for the analysis of double-stained sections, each DAB- or haematoxylin-positive area was extracted and their pixels were
measured automatically. The quantification of the ratio of CD31-positive endothelial cells per field was normalised by the number of muscle fibres in the same field. The ratio of type-I muscle fibres per field was also quantified as the ratio against the total number of muscle fibres. We also performed staining using anti-human fast troponin I MAb #01868-96 and confirmed that the positively stained areas were turned over by using anti-human slow troponin I MAb #01869-96 (data not shown). We used 4 separate sections in each sample. Section preparation, photography, and tissue analysis were carried out by an experimenter blinded to treatment conditions.

2.11. Statistical analysis
Statistical analysis was performed using the SAS Version 8.2 (SAS Institute Inc., CA, USA). Data are expressed as a mean ± S.D. unless otherwise specified. Statistical analysis was performed using parametric Dunnett test, nonparametric Steel test or parametric Student’s t-test. P-values less than 0.05 were considered statistically significant.

3. Results

3.1. Apelin-transgenic mice
We obtained 6 founders by injection of an apelin-expression vector into fertilised oocytes, and apelin-Tg offspring were obtained from 3 founders (data not shown). These apelin-Tg offspring were fertile and did not exhibit any apparent developmental or behavioural abnormalities. In these 3 founders of apelin-Tg mice, we measured apelin contents in several tissues by a biological assay using APJ-overexpressing CHO cells as described in Section 2.7, and then we selected the founder which showed the highest concentrations of apelin in these tissues and used as apelin-Tg strain for the following experiments.

The apelin contents in brain, epididymal adipose tissue, liver, and skeletal muscle were higher in apelin-Tg mice than in non-Tg control mice (Fig. 1).

3.2. Decrease in body weight gain in HFD-fed apelin-Tg mice
To determine the efficacy of apelin on reducing adiposity, we first compared the body weights of apelin-Tg mice fed the SD or HFD with those of non-Tg control mice. The body weights of apelin-Tg mice at 6 weeks old were already lower than those of the non-Tg controls (23.0 ± 1.6 g vs. 24.9 ± 1.3 g, respectively; Mean ± S.D.; P < 0.01) (Fig. 2A). After 20 weeks of continuous SD feeding from 6 weeks of age, the body weight gain of apelin-Tg mice was similar to that of the non-Tg control mice (8.3 ± 1.4 g vs. 9.5 ± 1.7 g, respectively), although the overall body weights of apelin-Tg mice were lower than those of the non-Tg control mice (31.4 ± 2.2 g vs. 34.1 ± 2.0 g, respectively; P < 0.01) (Fig. 2B). The food intake of both groups of mice was not altered during the 20 weeks (Fig. 3).

3.3. DIO resistance of apelin-Tg mice is based on the decrease of body fat weight gain
To elucidate the cause of the DIO resistance of apelin-Tg mice, we measured body fat mass in both HFD-fed apelin-Tg and control mice using MRI. The ratio of body fat mass in apelin-Tg mice was significantly lower than that in the non-Tg control mice (35.4 ± 1.9 % vs. 39.6 ± 2.5 %, respectively; P < 0.01) (Fig. 4A). On the other hand, the ratio of body lean mass was not significantly different between apelin-Tg and non-Tg control mice (55.1 ± 2.9 % vs. 51.2 ± 3.9 %, respectively) (Fig. 4B). This
analysis suggested that the decrease in the body weight gain of HFD-fed apelin-Tg mice is mainly based on the decrease in adipose tissue weight gain.

3.4. Plasma profiles in apelin-Tg mice

The concentrations of BG, PG, TG, TC, NEFA, TKB, insulin, leptin, adiponectin, and apelin in blood or plasma were not significantly different between apelin-Tg and non-Tg control mice under non-fasting and fasting conditions (Table 1).

3.5. Upregulation of body temperature in HFD-fed apelin-Tg mice

Under the SD feeding condition, the rectal temperatures between apelin-Tg and non-Tg control mice were similar (35.7±0.2 °C vs. 35.7±0.4 °C, respectively). On the other hand, the rectal temperatures of apelin-Tg mice were higher than those of the non-Tg control mice under the HFD feeding condition (36.4±0.3 °C vs. 37.4±0.5 °C, respectively; P<0.01) (Fig. 5).

3.6. Increase in oxygen consumption in HFD-fed apelin-Tg mice

We measured the oxygen consumption at 2 time points. At first, we measured it at 16 weeks of age when body weight gain in HFD-fed apelin-Tg mice was already lower significantly than that of HFD-fed non-Tg control mice. Second, we measured the oxygen consumption again at 50 weeks of age before we performed immunohistochemical

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**Table 1**

| Blood or plasma profile in Apelin-Tg and non-Tg control mice at 26-week-old. |
|-----------------|-----------------|-----------------|-----------------|
|                  | SD              | HFD             |                  |
|                  | Cont            | Tg              | Cont            | Tg              |
|                  | P               |                 | P               |                 |
| BG (mg/dL)       | fed 162±13 (5)  | 163±16 (7)      | 0.89            | 200±35 (5)      | 199±27 (7)      | 0.74            |
|                  | fast 191±54 (5) | 203±23 (7)      | 0.61            | 213±40 (5)      | 232±51 (7)      | 0.50            |
| PG (mg/dL)       | fed 179±19 (10) | 171±16 (12)     | 0.26            | 214±26 (10)     | 196±31 (12)     | 0.15            |
|                  | fast 152±32 (10)| 161±12 (7)      | 0.99            | 205±33 (5)      | 197±28 (7)      | 0.72            |
| TG (mg/dL)       | fed 102±59 (10) | 98±31 (12)      | 0.84            | 49±20 (10)      | 44±15 (12)      | 0.52            |
|                  | fast 48±31 (5)  | 47±15 (7)       | 0.97            | 58±27 (5)       | 47±12 (7)       | 0.91            |
| TC (mg/dL)       | fed 78.7±21 (10)| 78±13 (12)      | 0.90            | 227±61 (10)     | 186±64 (12)     | 0.15            |
|                  | fast 114±17 (5) | 110±12 (7)      | 0.84            | 200±73 (5)      | 185±62 (7)      | 0.60            |
| NEFA (mEq/L)     | fed 989±305 (10)| 1006±381 (12)   | 0.91            | 1184±358 (10)   | 1050±471 (12)   | 0.47            |
|                  | fast 1134±169 (5)| 1077±227 (7)   | 0.64            | 1396±221 (5)    | 1130±239 (7)    | 0.12            |
| TKB (μM)         | fed 111±38 (10) | 122±41 (12)     | 0.53            | 113±71 (10)     | 140±74 (12)     | 0.41            |
|                  | fast 1425±391 (5) | 1306±259 (7) | 0.91            | 1876±733 (5)    | 1354±790 (7)    | 0.25            |
| Insulin (ng/mL)  | fed 2.2±1.1 (5) | 1.5±0.93 (7)    | 0.24            | 9.4±7.8 (5)     | 10±8.0 (7)      | 0.90            |
|                  | fast 0.68±0.39 (5)| 0.95±0.84 (7) | 0.52            | 2.8±1.8 (5)     | 2.1±0.8 (7)      | 0.39            |
| Leptin (ng/mL)   | fed 16±9.0 (5)  | 13±4.8 (7)      | 0.46            | 96±30 (5)       | 97±18 (7)       | 0.93            |
|                  | fast 5±3.7 (5)  | 4.0±1.8 (7)      | 0.44            | 69±24 (5)       | 67±20 (7)       | 0.85            |
| Adiponectin (μg/mL) | fed 39±12 (5)  | 41±8.5 (7)      | 0.79            | 52±11 (5)       | 50±9.8 (7)      | 0.74            |
|                  | fast 41±11 (5)  | 37±10 (7)       | 0.48            | N.T.            | N.T.            | -               |
| Apelin (ng/mL)   | fed 2.6±1.1 (5) | 3.7±1.7 (7)     | 0.23            | 2.3±0.6 (5)     | 2.2±0.8 (7)     | 0.86            |
|                  | fast 1.8±0.5 (5) | 2.0±0.4 (7)   | 0.43            | 1.9±0.7 (5)     | 2.4±0.7 (7)     | 0.18            |

Abbreviations: SD, standard diet; HFD, high-fat diet; Cont, non-Tg control; Tg, apelin-Tg; P, P value between Cont and Tg; BG, blood glucose; PG, plasma glucose; TC, plasma triglyceride; TG, plasma total cholesterol; NEFA, plasma non-esterified fatty acid; TKB, plasma total keton-bodies; N.T., not tested. Data are expressed as mean±S.D. of 5–12 mice (animal number).
analysis in order to confirm whether it is continuing higher level in HFD-fed apelin-Tg mice. As shown in Fig. 6, we found a difference between apelin-Tg and non-Tg control mice with respect to the oxygen consumption rate (VO₂); the VO₂ values of HFD-fed apelin-Tg mice were greater than those of HFD-fed non-Tg control mice. At 16 weeks old, the VO₂ values of apelin-Tg mice in dark and light cycles were 61.9±7.1 and 59.0±6.1 mL/min/kg (Mean ± S.D.), respectively, which were 15–17% higher (P<0.01) than the corresponding values in non-Tg control mice (52.5±3.7 and 51.2±3.0 mL/min/kg, respectively) (Fig. 6A). The carbon dioxide excretion rate (VCO₂) of apelin-Tg mice was also greater than that of non-Tg control mice (Fig. 6B; P<0.01), although the respiratory quotient (RQ) was not significantly different among groups (Fig. 6C). At 50 weeks old, the VO₂ values of apelin-Tg mice also increased, and their RQ values were unchanged (data not shown). The body weight change and food intake during the period of indirect calorimetry measurement was not different between apelin-Tg and non-Tg control mice.

3.7. Upregulation of vascular maturation-related genes in HFD-fed apelin-Tg mice

To investigate the molecular mechanism underlying body temperature and oxygen consumption increases in HFD-fed apelin-Tg mice, comprehensive gene expression analyses were performed in the skeletal muscle, brown and white adipose tissue of SD- and HFD-fed apelin-Tg and non-Tg mice, and compared them with each other. Interestingly, in the skeletal muscle of HFD-fed apelin-Tg mice, the genes of vascular maturation relating factor Ang1/2 and its receptor Tie1/2, angiogenic factor Vegfa and its receptor Vegfr2, endothelial cell marker protein Pcan1, transcriptional factors relating to lipid metabolism Ppara, Foxo1 and Erra, mitochondrial fatty acid oxidation enzymes Pdk4 and Cpt1b, and uncoupling protein Ucp3 were upregulated compared to SD-fed apelin-Tg mice (Fig. 7A, P<0.05–0.001). Strikingly, we observed significant upregulation of Ang1 and Tie2 mRNAs, which are both key molecules for vascular maturation signalling, in skeletal muscle in HFD-fed apelin-Tg mice compared with non-Tg control mice (Fig. 7B; P<0.01). Pcam1 and Vegfr2 mRNA levels were also increased (P<0.05–0.001), although that of Vegfr2 mRNA was not altered. Transcriptional factors related to lipid metabolism, Ppara and Foxo1, were slightly increased (P<0.05). Mitochondrial biogenesis-related genes Pgc1a and Erra, tended to be upregulated compared to the non-Tg control mice; however, the difference was not statistically significant. The expression of UCP3 mRNA was also significantly increased (P<0.01); those of Cpt1b, Acadl, Acadm, and Cox4a, lipid metabolism-related genes in mitochondria, were not altered. On the other hand, in the skeletal muscle of SD-fed apelin-Tg mice, the expressions of Tie1 and Tie2 mRNAs but not Ang1 mRNA were slightly increased (P<0.05); the mRNA expressions of Vegfa and Vegfr2 were unchanged compared to those of the non-Tg control mice (Fig. 7C).

3.8. Increased vascular mass, mitochondrial content, and type-I muscle fibre ratio in HFD-fed apelin-Tg mice

It is interesting to note that we found that the number of endothelial cells per muscle fibre increased 2 fold in the skeletal...
muscle in HFD-fed apelin-Tg mice compared to that in the non-Tg control mice \( (P<0.001) \); however, the number of nuclei was not different between groups (Fig. 8A). This difference was not observed in SD-fed mice. In addition, the type-I muscle fibre ratio in HFD-fed apelin-Tg mice was not different from that of SD-fed apelin-Tg mice, although that of the ratio in non-Tg control mice decreased 37\% \( (P<0.05) \) (Fig. 8B). Therefore, the ratio of type-I muscle fibres in the HFD-fed apelin-Tg mice increased 2-fold compared to that in the HFD-fed non-Tg control mice \( (P<0.05) \). Fig. 8C shows slow troponin I muscle fibres stained brown with DAB in the skeletal muscle of non-Tg control and apelin-Tg mice. The mRNA expressions of \( Tnni1 \), a protein marker of type-I muscle fibres, and \( Mb \), an oxygen storage protein present in muscles, increased \( (P<0.01-0.001) \) in the skeletal muscle of HFD-fed apelin-Tg mice; the mRNA expression of \( Tnni2 \), a protein marker of type-II muscle fibres, decreased \( (P<0.01) \) (Fig. 8D). The gene expressions of \( Mef2 \) (a muscular transcription factor gene), \( mtTFA \) (mitochondrial biogenesis-regulating factor gene), and \( MnSOD \) (an oxygen radical scavenger gene) were not altered in this tissue. Under the SD feeding condition, the expressions of all of these genes were not different between apelin-Tg and non-Tg control mice (Fig. 8E). Furthermore, the mitochondrial \( ND1 \) DNA copy number was up to 40\% greater in the skeletal muscle of HFD-fed apelin-Tg mice \( (P<0.01) \), although it did not change in the SD-fed mice (Fig. 8F).

4. Discussion

The HFD-fed apelin-Tg mice exhibited DIO resistance with increasing oxygen consumption and \( Ucp3 \) mRNA expression in
skeletal muscle as well as the results in which the repeated administration of apelin-13 to DIO mice reported by Higuchi et al. [9]. Individual energy expenditure greatly depends on ATP production via oxidative phosphorylation and heat production by UCP activation in mitochondria. Thus, we first speculated that the DIO resistance of apelin-Tg mice is caused by the increase in mitochondrial functioning. However, the expressions of mitochondrial genes relating to oxidative phosphorylation, such as Cpt1a/b, Acadl, Acadm, Ccps, and Cox4a, were not altered in skeletal muscle of apelin-Tg mice, although apelin concentration in this tissue was about 10-fold higher than that of non-Tg control mice with an increase of Apln mRNA expression. In contrast, the expression levels of the key genes for vascular maturation, Ang1 and Tie2, were significantly upregulated in the tissues of HFD-fed apelin-Tg mice. It is well known that Ang1/Tie2 signalling reinforce
tight junctions in blood vessel endothelium by producing adhesion molecules, and migrating mural cells to the endothelium, followed by the promotion of blood vessel maturation [19,20]. Thus, we subsequently hypothesised that the DIO resistance of apelin-Tg mice is caused by the increase in vascular mass in skeletal muscle. Apelin is reported to enlarge capillary tube formation in conjunction with VEGFα [4,21]. We also observed an increased number of vascular endothelial cells per muscle fibre in the skeletal muscle of HFD-fed apelin-Tg mice, suggesting that the DIO resistance of apelin-Tg mice is correlated with vascular formation in skeletal muscle.

Ang1/Tie2 signalling regulates vascular quiescence and angiogenesis balance by altering the levels of VEGFα [22]. In an angiostatic state in which only a limited amount of VEGFα exists, Ang1 released from mural cells activates Tie2 at points of endothelial cell–cell contact, followed by an angiostatic phase for maintaining vascular quiescence. In contrast, when VEGFα levels exceed the limits by certain triggers, Tie2 is anchored to the extracellular matrix; as a result, Ang1/Tie2 signalling is diminished and switches to VEGFα/VEGFR2 signalling, which induces an angiogenic state. It is not clear whether the increase in endothelial cell numbers in skeletal muscle of HFD-fed apelin-Tg mice is accompanied by an increase in blood vessel number or calibre size, or a combination of both. In any case, the increase in vascular mass in apelin-Tg mice may be the cause behind expanded vascular diameter, because Vegfa gene expression was unchanged in these tissues. Since individual VO2 is positively related to vascular density (i.e., the bloodstream) [23], the VO2 increase in observed apelin-Tg mice may be dependent on the increase of vascular mass in skeletal muscle. Therefore, we speculate that the VO2 increase in HFD-fed apelin-Tg mice relates to the increase of mitochondria content in skeletal muscle that increases energy expenditure via quantitative increase in oxidative phosphorylation rather than qualitative increase in each mitochondrial function.

In the brown adipose tissue (BAT), Ang1 and Tie2 mRNA expressions were also significantly increased in company with Ucp1 mRNA expression in HFD-fed apelin-Tg mice (Suppl. Fig. S1, P<0.01). Since the maturation of vascular network shifts to a metabolically active state in BAT [15], the increase in Ang1 and Tie2 mRNA expressions may be one of the reasons for increasing energy expenditure in HFD-fed apelin-Tg mice. On the other hand, angiogenesis in white adipose tissue (WAT) is reported to increase WAT mass [15]; however, Vegfa gene expression levels in the WAT of apelin-Tg mice did not change (Suppl. Fig. S2). Because Ang1/Tie2 gene expression in WAT also shows a tendency to increase, this may be another reason why apelin-Tg mice exhibited DIO resistance.

For the first time, we demonstrated that the activation of apelin/APJ signalling increases the muscle fibre type-II/I ratio in skeletal muscle. The weights of skeletal muscle in C57BL/6J mice (background strain of apelin-Tg mice) increased under the HFD feeding condition (unpublished observation). Since lean body weight did not differ between apelin-Tg and non-Tg control mice, the degree of muscle weight increase in apelin-Tg mice should be almost the same as that of the non-Tg control mice. Therefore, the increased type-I muscle fibre ratio in HFD-fed apelin-Tg mice may be due to the greater increase in type-I muscle fibre than type-II muscle fibre. It is interesting to clarify in the future that the increase in the type-I muscle fibre ratio in HFD-fed apelin-Tg mice is a result of whether a muscle fibre type shift from type-II to type-I or an increase of type-I muscle fibre occurred. The increased gene expressions of Tnni2 and Mb and the decreased Tnni2 gene expression in the skeletal muscle of HFD-fed apelin-Tg mice do not contradict the increase in the type-I muscle fibre ratio. In addition, the mitochondrial ND1 DNA copy number as a mitochondrial biogenesis parameter was upregulated in the skeletal muscle of HFD-fed apelin-Tg mice. These results corroborate the report in which the repeated administration of apelin-13 to rats increased mitochondrial content in skeletal muscle [24]. On the other hand, mtTFA gene expression was not altered in skeletal muscle in either apelin-Tg or non-Tg control mice, although this gene may be upregulated at any time point in the process of DIO formation.

PGC-1α was determined to be a factor that increases the mitochondrial biogenesis of BAT under cold exposure [25] and is a key molecule that stimulates NRF1 transcription in conjunction with other transcriptional cofactors [26]. The expressions of Pgc1a and Erra tended to increase in the skeletal muscle of HFD-fed apelin-Tg mice, although the difference was not significant. Frier et al. reported that Pgc1b, but not Pgc1a, mRNA expression is increased in skeletal muscle as a result of apelin-13 administration in rats and is accompanied by an increase in mitochondrial content [24]. They also observed that apelin-13 does not change the phosphorylation levels of 5′-AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC). Therefore, the mitochondria biogenesis activation in the skeletal muscle of HFD-fed apelin-Tg mice may be caused by a PGC-1α-independent pathway. Since plasma adiponectin levels were also unchanged in apelin-Tg mice, the adiponectin-induced AMPK activation may not be involved in the DIO resistance of apelin-Tg mice. Therefore, it would be interesting to clarify which molecule is responsible for changing the muscle fibre type and what kind of signalling is involved in this DIO resistance. In addition, the increased expression of genes related to vascular maturation in the BAT of HFD-fed apelin-Tg mice might upregulate energy metabolism in conjunction with increased heat production via UCP1. These results are in agreement with a report in which the repeated administration of apelin-13 to DIO mice increased both Ucp1 expression and body temperature [9].
It was expected that the insulin sensitivity is increased in HFD-fed apelin-Tg mice since their body adiposity was decreased compared to that of non-Tg control mice. Therefore, we performed oral glucose tolerance test (OGTT), and its result showed that glucose utilisation tended to increase in HFD-fed apelin-Tg mice compared to that in non-Tg control mice although the difference was not statistically significant (Suppl. Fig. S3).

The correlation between blood apelin concentration and metabolic diseases, such as obesity and diabetes, is not clear; at present there are several reports indicating that both increases and decreases of blood apelin levels influence patients' conditions [27–29]. Although tissue apelin levels of apelin-Tg mice were higher than those of the non-Tg control mice, their plasma apelin content was unchanged compared to that of the non-Tg control mice when measured by an EIA. We have experienced that plasma apelin is not detected stably by the assay for cAMP production-inhibitory activity as described in Section 2, the reason for this is not clear, we speculate that the circulating active apelin may be reduced as a result of its rapid degradation or its interaction with plasma proteins. Mesmin et al. reported that circulating apelin isoforms were unstable and not detected by liquid chromatography/tandem mass spectrometry (LC/MS/MS) assay although they were detected by the EIA [30]. Thus, future studies are required for measuring precisely circulating functional apelin amounts and for determining isoforms.

In conclusion, we observed increases in vascular mass, type-I muscle fibre ratio, and mitochondrial biogenesis in the skeletal muscle of apelin-Tg mice; these are the main plausible reasons behind the DIO resistance acquisition of these mice. Interestingly, we found a remarkable increase in the gene expressions of Ang1 and Tie2 in the skeletal muscle and BAT of apelin-Tg mice; the signalling of these genes is important for blood vessel maturation. On the other hand, the expression of genes related to mitochondrial lipid metabolism and oxidative phosphorylation in apelin-Tg mice was not different compared to the non-Tg controls. We hypothesise that the regulation of energy metabolism via blood vessel physiology in skeletal muscle may be important for acquiring DIO resistance in conjunction with an increase in heat production via UCP expression in apelin-Tg mice (Fig. 9). From this perspective, apelin/APJ signalling may have an important role in the regulation of metabolism-related diseases.

Acknowledgments

The authors express their deep gratitude to Drs. Hideaki Nagaya and Hiroyuki Odaka of the Pharmaceutical Research Division, Takeda Pharmaceutical Company Limited for their helpful advice and discussion. The present study was conducted with financial support from Takeda Pharmaceutical Company Limited.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.jbbagen.2011.05.004.

References