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E. 知的財産権の出願・登録状況

なし

研究成果の刊行に関する一覧表

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REVIEW

Translational research of novel hormones: lessons from animal models and rare human diseases for common human diseases

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Abstract Since the 1980s, a number of bioactive molecules, now known as cardiovascular hormones, have been isolated from the heart and blood vessels, particularly from the subset of vascular endothelial cells. The natriuretic peptide family is the prototype of the cardiovascular hormones. Over the following decade, a variety of hormones and cytokines, now known as adipokines or adipocytokines, have also been isolated from adipose tissue. Leptin is the only adipokine demonstrated to cause an obese phenotype in both animals and humans upon deletion. Thus, the past two decades have seen the identification of two important classes of bioactive molecules secreted by newly recognized endocrine cells, both of which differentiate from mesenchymal stem cells. To assess the physiological and clinical implications of these novel hormones, we have investigated their functions using animal models. We have also developed and analyzed mice overexpressing transgenic forms of these proteins and knockout mice deficient in these and related genes. Here, we demonstrate the current state of the translational research of these novel hormones, the natriuretic peptide family and leptin, and discuss how lessons learned from excellent animal models and rare human diseases can provide a better understanding of common human diseases.

Keywords Natriuretic peptide family (ANP, BNP, CNP) · Leptin · Translational research · Animal models · Genetically engineered mice

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Although a multitude of animal models have been developed to emulate various diseases, there are a few excellent animal models that mimic human disease remarkably well, such as spontaneously hypertensive rats (SHR) [1] and hereditary obese mice, ob/ob mice [2]. These models are very useful for translational research into the common human diseases, hypertension and obesity. Lessons from research on SHR, an excellent animal model for hypertension research, developed at Kyoto University led us to investigate the clinical importance of cardiovascular hormones and adipokines using appropriate animal models that mimic human diseases beyond species differences. In this review, we discuss the current state of translational research of the natriuretic peptide family and leptin and discuss the ways in which animal models and rare human diseases can educate about common human diseases.

Translational research of natriuretic peptide family

The natriuretic peptide family consists of three structurally related peptides, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) [3]. The biological actions of natriuretic peptides are mediated by activation of two subtypes of membranous guanylyl cyclase (GC), GC-A and GC-B, leading to

intracellular accumulation of cyclic guanine monophosphate (cGMP) [4]. The rank order of potency to induce cGMP production via GC-A is ANP ≥ BNP >> CNP, while that via GC-B is CNP > ANP ≥ BNP [5]. Thus, ANP and BNP serve as endogenous ligands for GC-A, while CNP is specific for GC-B. A third natriuretic peptide receptor with no intracellular GC domain, dubbed the clearance receptor (C-receptor), is thought to be engaged in the receptor-mediated degradation of natriuretic peptides [4]. The ANP, BNP/GC-A system plays a pivotal role in the regulation of cardiovascular homeostasis, as demonstrated by their augmentation in various pathophysiological states such as heart failure [6–10], myocardial infarction [11, 12], cardiac hypertrophy [13, 14], and hypertension [15–17]. ANP and BNP are cardiac hormones secreted primarily by the atrium and ventricle of the heart, respectively [10, 17], with strong diuretic, natriuretic, and vasodilatory activities [6, 7, 10]. ANP and BNP are used in the treatment of heart failure [18, 19] and serve as sensitive biochemical markers for heart failure and cardiac hypertrophy [8–10]. ANP infusion therapy has currently reached a greater than 30% share among drugs given for acute congestive heart failure in Japan.

CNP, the third member of natriuretic peptide family, was first purified from porcine brain [20]. While CNP is the primary natriuretic peptide in the human brain [21], it is also produced by vascular endothelial cells [22–24] and macrophages [25]. This hormone functions in the regulation of vascular endothelial function and arteriosclerosis via local effects, not by acting as a circulating hormone [26–28]. These observations indicate that CNP acts as an autocrine/paracrine regulator and as a neuropeptide [21].

The distribution of the natriuretic peptide system overlaps with the distribution of the renin–angiotensin system [21, 29–33], prompting us to examine the functional relationship of the natriuretic peptide system and the renin–angiotensin system. We demonstrated an antagonistic relationship between these two systems, both in their peripheral functions as well as their central actions [34–39]. Furthermore, the natriuretic peptide system has therapeutic implication in vascular regeneration in patients with arteriosclerosis obliterans [40].

Mice with genetic alterations in the ANP, BNP/GC-A system

Genetically engineered mice are useful tools to study the complex phenotypic effects of an altered gene in living animals. Overexpression or deficiency of each member of the natriuretic peptide family or its receptors has been generated through transgenic (Tg) or knockout (KO) technologies [41–45]. We generated Tg mice expressing BNP under the control of the serum amyloid P (SAP) component promoter, which targets hormone expression to the liver [43]. BNP-Tg mice exhibited a 100-fold increase in plasma BNP concentrations with concomitant elevations in plasma cGMP concentrations. These mice displayed significantly lower blood pressures and smaller hearts than non-Tg littermates. These results indicate that BNP functions in the long-term cardiovascular regulation and may be useful as a long-term therapeutic agent. In addition, the proteinuria and renal dysfunction observed in anti-GBM nephritis [46], the nephrosclerosis induced by subtotal nephrectomy [47], and the manifestations of diabetic nephropathy [48] were ameliorated in BNP-Tg mice compared to those in wild-type mice, indicating a possible application for the natriuretic peptide family in the treatment of renal disorders.

We also generated mice bearing a targeted disruption of the BNP gene [44]. At baseline, BNP-KO mice did not show any signs of systemic hypertension or ventricular hypertrophy; however, these animals developed multifocal fibrotic lesions within the cardiac ventricle even in the absence of additional stresses; these lesions increased in size and number in response to ventricular pressure overload, demonstrating that BNP is an antifibrotic factor acting within the ventricle of the heart as an autocrine/paracrine regulator for ventricular remodeling [44]. In addition to these cardiovascular manifestations, BNP-Tg mice exhibited marked skeletal overgrowth via endochondral bone formation [49]. Nevertheless, BNP-KO mice did not possess any skeletal abnormalities [44]. The skeletal overgrowth seen in BNP-Tg mice that express elevated plasma concentrations of BNP was similar to that seen in cartilage-specific CNP-Tg mice [49]. As the BNP/GC-A system does not have an abnormal skeletal phenotype [41, 42, 45], we postulated that the markedly increased circulating levels of BNP (100-fold greater than wild-type mice) may cross-react with GC-B to stimulate endochondral bone growth, even though the affinity of BNP for GC-B is lower than that for GC-A. This interpretation is supported by the finding that the skeletal overgrowth observed in BNP-Tg mice was not abrogated by a genetic deficiency of GC-A in BNP-Tg mice [50].

ANP transgenic mice expressing elevated levels of circulating ANP under the control of mouse transthyretin promoter [41] exhibited decreased arterial blood pressure without the induction of diuresis or natriuresis. ANP-KO mice and GC-A-KO mice displayed salt-sensitive and salt-resistant hypertension, respectively [42, 45]. Studies using GC-A-KO mice implicated the involvement of GC-A in antihypertrophic actions in the heart [51–53]. A more detailed analysis of GC-A was performed using mice bearing a conditional knockout of GC-A and indicated the importance of GC-A in vascular endothelial-cell-mediated blood pressure control [54–56].

As for the regulation of ANP and BNP gene expression, neuron-restrictive silencer elements (NRSEs) are located in the 5′-flanking region of the BNP gene and the 3′-untranslated region of the ANP gene [57]. The neuron-restrictive silencer factor (NRSF) can thus repress ANP promoter activity through binding to NRSE [58]. Studies examining dominant-negative NRSF Tg mice expressed under the control of the α-myosin heavy-chain promoter have demonstrated that NRSF plays an important role in the gene expression of both ANP and BNP and in the progression of cardiac dysfunction and lethal arrhythmia associated with heart failure [59].

Genetically engineered mice of the CNP/GC-B system

We generated mice with a targeted disruption of the CNP gene; the resultant CNP-KO mice exhibited markedly short stature due to impaired bone growth [60]. Mammalian bones are formed through two different mechanisms, endochondral ossification and membranous ossification. Most mammalian bones are formed through endochondral ossification, a process during which chondrocytes in the growth plate undergo proliferation, hypertrophy, cell death, and osteoblastic replacement [61]. The short-stature phenotype of CNP-KO mice resulted from impaired bone growth through endochondral ossification [60]. CNP-Tg mice with targeted overexpression of CNP at the growth plate cartilage exhibited prominent overgrowth of those bones formed through endochondral ossification [62]. GC-B-KO mice exhibit the same short-stature phenotype as observed in CNP-KO mice [63], demonstrating that the CNP/GC-B system is a physiologically important stimulator of endochondral bone growth. Dominant-negative GC-B transgenic rats displayed blood-pressure-independent cardiac hypertrophy, suggesting evidence linking GC-B signaling to the control of cardiac growth [64].

cGMP-dependent protein kinase (cGK) has been identified as a molecule activated downstream of the natriuretic peptide family and GC system [65]. Mice depleted with the gene of one subtype of cGK, cGKII (cGKII-KO mice), exhibit a short-stature phenotype secondary to impaired endochondral bone growth [66], similar to that observed in CNP-KO mice [60]. We demonstrated that cGKII affected endochondral bone growth by functioning downstream of the CNP/GC-B system by showing that the impaired endochondral bone growth observed in cGKII-KO mice could not be rescued by targeted overexpression of CNP in the growth plate cartilage [67].

Multiple spontaneous animal models with impairments in the CNP/GC-B system have been identified [68–71]. Two strains of dwarf mice, with an autosomal recessive mutant gene, named cn/cn [68] and short-limbed dwarfism (SLW) mice [69], possess spontaneous loss-of-function mutations in the *GC-B* gene. Spontaneous mutant mice with a loss-of-function mutation in the CNP gene, named long bone abnormality (Lbab) mice, exhibit short-stature owing to their impaired endochondral bone growth [70], and this phenotype could be abrogated by targeted overexpression of CNP in the growth plate cartilage [71].

Clinical application of CNP and its analogs for skeletal dysplasia

To explore the potential applications of CNP and its analogs for clinical use, we attempted to apply the strong effect of CNP and GC-B on endochondral bone growth to skeletal dysplasia, a group of genetic disorders characterized by severely impaired bone growth [72]. Achondroplasia (Ach), the most common form of skeletal dysplasia characterized by short-limbed dwarfism, is caused by constitutive activation of fibroblast growth factor (FGF) receptor 3 [73]. The current therapy for Ach is limited to distraction osteogenesis [74], an orthopedic procedure; no efficient medical therapies have been developed as yet. We demonstrated that targeted overexpression of a CNP transgene in the growth plate cartilage of a mouse model of achondroplasia (Ach mice) rescues their impaired bone growth and short-stature phenotypes [62] (Fig. 1). To elucidate the molecular

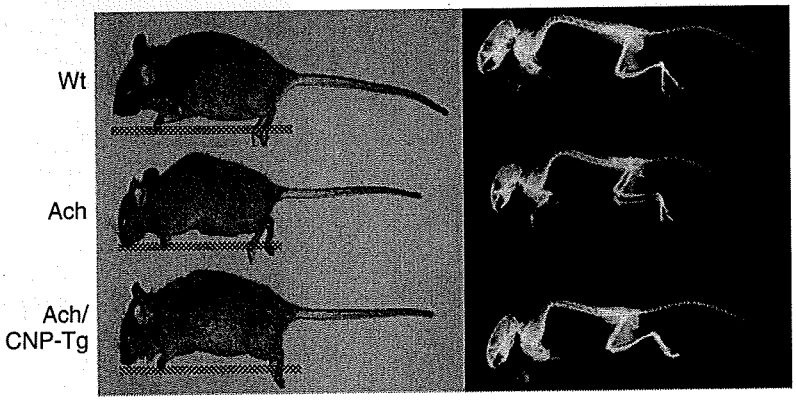

Fig. 1 Rescue of achondroplastic mice (Ach mouse) by targeted overexpression of CNP in growth plate cartilage. From *top* to *bottom* are shown the gross appearance (*left panel*) and skeletal phenotype (*right panel*, soft X-ray picture) of female wild-type mice (*Wt*), Ach mice (*Ach*), and Ach mice overexpressing CNP in the growth plate cartilage (*Ach/CNP-Tg*) at an age of 3 months

mechanism by which CNP ameliorates achondroplasia, we examined the effect of CNP on extracellular signal-regulated kinase (ERK) signaling. CNP inhibited FGF2-stimulated phosphorylation of ERK in a dose-dependent manner through cGMP activation via GC-B ligation, ultimately increasing matrix synthesis by chondrocytes [62].

We also demonstrated that systemic and continuous administration of synthetic CNP is safe and effective to reverse the impaired bone growth seen in Ach mice [75] (Fig. 2). The safety and efficacy of systemic CNP administration in preclinical studies with the observation that CNP has only a minimal effect of blood pressure in humans [76] suggest that systemic administration of CNP or CNP analogs provides a novel therapeutic strategy for the treatment of human skeletal dysplasia, including Ach.

One form of human skeletal dysplasia, acromesomelic dysplasia type Maroteaux, is caused by loss-of-function mutations in the GC-B gene [77]. This implicates the CNP/GC-B system as a physiologically important enhancer of endochondral bone growth in humans, suggesting a clinical application for CNP and CNP analogs to multiple types of human skeletal dysplasia [75].

In the near future, idiopathic short stature, a common disease of short-stature phenotype with an unknown etiology, and bone fracture, the healing of which is made through endochondral ossification, would be the next avenues to explore for a therapeutic effect of CNP treatment.

Translational research of leptin

Leptin, an adipocyte-derived hormone originally identified from hereditary obese mice (ob/ob mice) [78], plays crucial physiologic roles in the regulation of energy expenditure and food intake [79–83]. Mice [84] and rats [85, 86] bearing mutations in leptin receptors demonstrate identical phenotypes as ob/ob mice. The Koletsky rat, an obese substrain of SHR serving as a model of metabolic syndrome exhibiting both hypertension and morbid obesity, was discovered to carry an additional nonsense mutation of the leptin receptor [86].

In obese animals and subjects, plasma leptin concentrations are increased in proportion to the degree of adiposity [87–89], indicating that leptin is a satiety signal communicating the size of adipose stores to the brain [90–92] and that leptin resistance is related to obesity [87, 93–95]. Leptin deficiency in human subjects is associated with morbid obesity with insulin resistance, indicating the physiological role of leptin in both animal models and humans [96, 97]. Leptin is implicated in a number of manifestations seen in obese animal models [91, 98–101], especially obesity-related hypertension [99], abnormal reproduction [98], bone changes [100], and Cushing syndrome [102]. Leptin is also produced by human placenta [103] and choriodecidual tumors [104].

Generation of Tg mice overexpressing leptin

To explore the clinical implications of leptin *in vivo*, we generated leptin-Tg mice displaying elevated plasma leptin concentrations comparable to those seen in obese subjects [105]. A fusion gene comprised of the human SAP promoter upstream of the mouse leptin cDNA coding sequences was designed to target hormone expression to the liver [43, 106]. Overexpression of leptin in the liver resulted in the complete disappearance of both white and brown adipose tissues in mice [105]. Such a phenotype did not occur when transgene expression was targeted to adipose tissue, the endogenous site of leptin production, using adipocyte-specific promoters [107]. The hyperlepti-

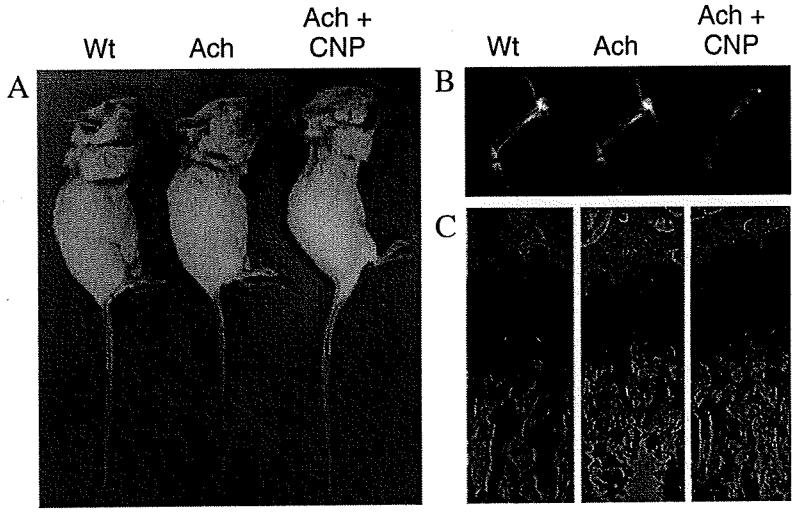

Fig. 2 Rescue of Ach mice by administration of synthetic CNP. Three-week-old female wild-type (*Wt*) or Ach mice were continuously administered CNP intravenously. The gross appearances (**a**), soft X-ray pictures of femurs (**b**), and histological pictures of tibial growth plates stained with safranin-O and hematoxylin and eosin (**c**) are shown for wild-type mice treated with vehicle (*left*), Ach mice treated with vehicle (*middle*), and Ach mice treated with 1 μg/kg per minute CNP (*right*) after a 4-week administration period. *Scale bar* in **c**, 50 μm

nemia seen in these transgenic "skinny" mice provides a unique experimental system in which the long-term effects of leptin are investigated in vivo [98–101, 105, 108, 109]. Skinny mice exhibit augmented glucose metabolism and increased insulin sensitivity of both skeletal muscle and liver [105], supporting the concept that leptin acts as an antidiabetic hormone in vivo [110–112]. These studies suggest the potential usefulness for leptin treatment of diabetes and obesity.

Crossbreeding of transgenic skinny mice with A-ZIP/F-1 mice, a mouse model of severe lipoatrophic diabetes

Generalized lipodystrophy, caused by a systemic deficiency of adipose tissue, is characterized by severe insulin resistance and hypertriglyceridemia [113]. A form of diabetes, called lipoatrophic diabetes, eventually develops, although the precise mechanism by which this paucity of fat results in diabetes has remained to be elucidated. Plasma leptin concentrations are markedly reduced or absent in patients with lipoatrophic diabetes and in rodent models of this disease [114–117]. Given leptin's antidiabetic action, leptin deficiency may play a role in the pathogenesis of lipoatrophic diabetes; thus, leptin may be a drug for lipoatrophic diabetes.

A mouse model of severe lipoatrophic diabetes (A-ZIP/F-1) was generated by expressing in adipose tissue a protein that inactivates basic-zipper transcription factors [116]. To assess the pathophysiological role and therapeutic potential of leptin in lipoatrophic diabetes, we crossed transgenic skinny (LepTg/+) and A-ZIP/F-1 (A-ZIPTg/+) mice to produce double transgenic mice (LepTg/+:A-ZIPTg/+) virtually lacking adipose tissue and expressing approximately tenfold higher levels of leptin than normal controls [118]. LepTg/+:A-ZIPTg/+ mice were hypophagic in comparison to A-ZIPTg/+ mice and exhibited decreased hepatic steatosis. Glucose and insulin tolerance tests displayed increased insulin sensitivity and normal glucose tolerance in LepTg/+:A-ZIPTg/+ mice, which was comparable to LepTg/+ mice. Pair-feeding experiments demonstrated that the effects of leptin were not solely due to decreased food intake. Leptin also helped to prevent diabetic nephropathy in generalized lipoatrophic diabetes mice [101]. These results demonstrate that leptin can improve insulin resistance and diabetic manifestations in a mouse model of severe systemic lipodystrophy, indicating that leptin is therapeutically useful in the treatment of lipoatrophic diabetes [118].

Leptin replacement therapy in Japanese patients with generalized lipodystrophy

We previously reported a novel homozygous mutation of *MC4R* in a Japanese woman with severe obesity (body mass index (BMI) 62 kg/m^2) [119]. *MC4R* mutations have been identified at a relatively high frequency (3–4%) in morbidly obese patients in Europe; all of the mutations reported to date occur in an autosomal-dominant fashion, with the exception of a single unique pedigree in the UK. [120, 121]. Although both parents were heterozygous for the mutation, neither exhibited such a severe obese phenotype (BMI 27 and 26 kg/m^2, respectively, which are preobese according to WHO criteria). As genetic backgrounds and lifestyles vary significantly between European and Asian countries, it is necessary to examine the effect of lifestyle on the phenotypes resulting from genetic mutations and on treatment efficacy in each country.

Four-month leptin replacement therapy has been reported to improve glucose and lipid metabolism in lipodystrophy patients in the USA [122]. To elucidate the efficacy, safety, and mechanisms underlying leptin replacement therapy in Asian patients with generalized lipodystrophy, we treated seven Japanese patients, two acquired and five congenital types, with physiological replacement dose of leptin [123, 124]. Leptin replacement therapy dramatically improved fasting glucose (mean±SE, 172±20 to120±12 mg/dl, $P<0.05$) and triglyceride (mean ± SE, 700±272 to 260±98 mg/dl, $P<0.05$) levels within 1 week. Leptin replacement reduced insulin resistance, as demonstrated by the euglycemic clamp method. Improvement of

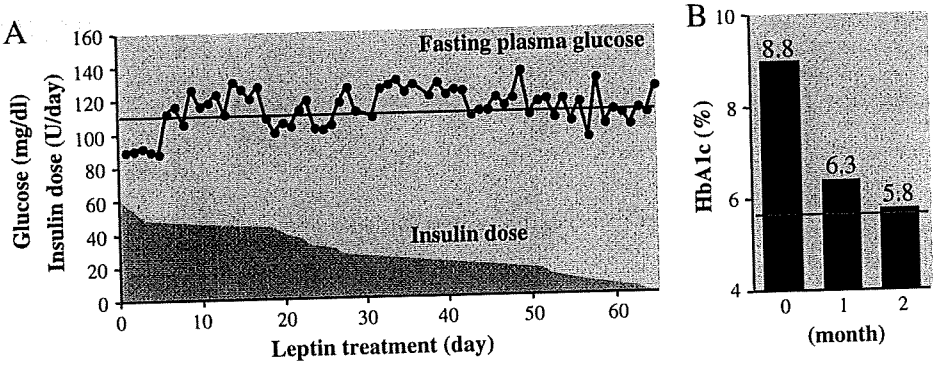

Fig. 3 **a** Daily insulin doses and fasting plasma glucose levels and **b** HbA1c levels during the first 2 months of leptin therapy in a 19-year-old male patient with congenital generalized lipodystrophy (Seipin gene mutant)

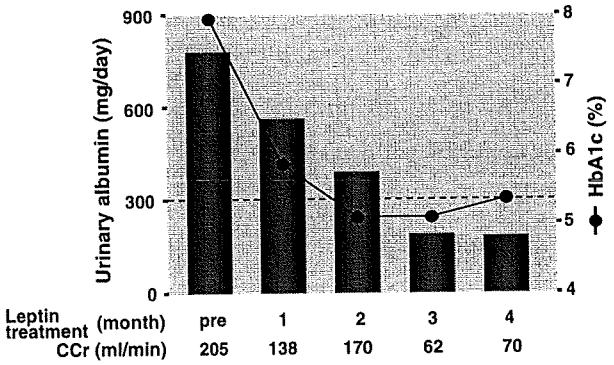

Fig. 4 Time course of daily urinary albumin secretion, creatinine clearance, and HbA1c levels during leptin treatment of a 16-year-old female patient with acquired generalized lipodystrophy

fatty liver was also confirmed by changes in computed tomography (CT) attenuation, and liver volume was calculated by CT imaging. By 4 months, six of seven patients were able to discontinue all antidiabetic drugs, including insulin (Fig. 3). The decreased fasting plasma glucose levels, triglyceride levels, and liver volumes in all seven patients were well maintained throughout the therapy period with no adverse effects. The longest period of leptin replacement therapy has now extended beyond 7 years.

Leptin treatment was also effective at combating diabetic complications. The macroalbuminuria seen in two patients regressed to microalbuminuria, while microalbuminuria in two additional patients normalized. The creatinine clearance of patients with glomerular hyperfiltration decreased with improved glucose tolerance (Fig. 4), which was consistent with previous findings in the lipoatrophic diabetes model mice [101].

We also examined the effect of leptin therapy on a 16-year-old girl with severe hypertriglyceridemia who suffered from repeated episodes of acute pancreatitis (Fig. 5). After the initiation of leptin therapy, her triglyceride levels normalized; she did not have any additional episodes of acute pancreatitis (Fig. 5). These results clearly demonstrate the safety and efficacy of the long-term leptin replacement therapy in patients with generalized lipodystrophy. While these results are impressive, it is important to remember that the efficacy of leptin replacement therapy in patients from Japan, a country in which the prevalence of obesity is relatively low, is excellent.

Leptin therapy for more prevalent forms of diabetes

To assess the therapeutic potential for leptin treatment in insulin-deficient diabetes, we generated diabetic animals by treating wild-type and LepTg/+ mice with a relatively low dose of streptozotocin (STZ 180 g/g body weight) [125]. Plasma insulin concentrations were reduced (<0.10 ng/ml), resulting in severe hyperglycemia in both wild-type and LepTg/+ mice 2 weeks after STZ treatment. LepTg/+ mice were more sensitive to exogenously administered insulin than wild-type mice; STZ-treated LepTg/+ mice became normoglycemic at doses of insulin that did not improve the hyperglycemia in STZ-treated wild-type mice. To clarify if combination therapy with leptin and insulin is beneficial for insulin-deficient diabetes, we also examined the effect of chronic coadministration of leptin and insulin in STZ-treated wild-type mice. We demonstrated that subthreshold doses of insulin, which do not affect glucose homeostasis, are effective at improving diabetes in STZ-treated wild-type mice in combination with leptin. These results indicate that leptin therapy may be used as an adjunct for insulin therapy in insulin-deficient diabetes.

We also investigated the therapeutic usefulness of leptin in a mouse model of type 2 diabetes mellitus with increased adiposity [126], generated using a combination of a low-dose STZ (120-g/g body weight) and a high-fat diet (HFD, 45% of energy as fat; STZ/HFD). In STZ/HFD mice, continuous infusion of leptin (20-ng/g body weight per hour) reduced food intake and body weight gain and improved glucose and lipid metabolism with enhanced insulin sensitivity. Leptin therapy also decreased the triglyceride content of both the liver and skeletal muscle.

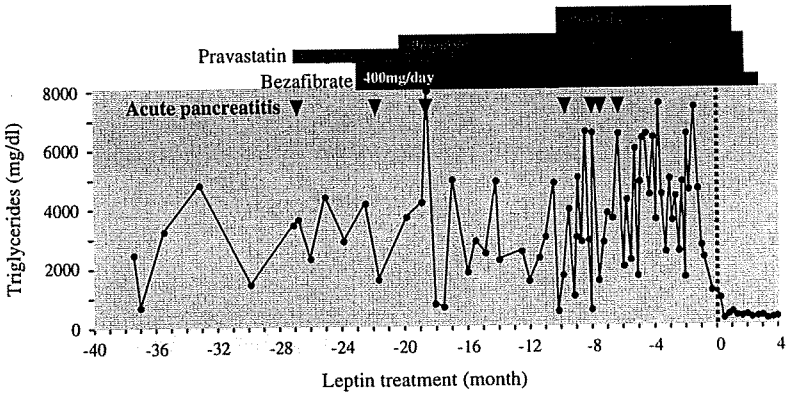

Fig. 5 Fasting serum triglyceride levels, doses of lipid-lowering drugs, and episodes of acute pancreatitis (red inverted triangle) before and after leptin therapy in a 16-year-old girl with acquired generalized lipodystrophy

These results indicate a beneficial effect of leptin therapy for type 2 diabetes mellitus with increased adiposity, which corresponds to a BMI in the range of 25–30 kg/m^2 [126].

Our previous and ongoing studies utilizing transgenic skinny mice and other animal models have demonstrated the pleiotropic actions of leptin in the regulation of energy homeostasis and food intake [98–101, 105, 108, 109] and its clinical usefulness as a therapy for multiple conditions, particularly diabetes mellitus [108, 118, 124, 125]. Tg skinny mouse may be a useful model to study the long-term effects of leptin therapy in vivo and to evaluate the clinical implications of leptin therapy.

Conclusions

Currently, the primary targets of our ongoing translational research of CNP and leptin are achondroplasia and lipoatrophic diabetes, respectively. Demonstration of the efficacy of CNP therapy for achondroplasia and leptin replacement therapy for lipoatrophic diabetes has relied heavily on basic and preclinical studies using excellent animal models. Although lipoatrophic diabetes is a rare disease in humans, the safety and efficacy of leptin replacement therapy for patients with lipoatrophic diabetes have been well established. Achondroplasia, while also a rare disease in humans, may be effectively managed with CNP therapy.

It has been possible to establish the safety and efficacy of these hormones in rare human diseases through studies that began with excellent animal models. These studies provided us with novel treatments for common human diseases, which were explored as adjacent to or in extension of these rare human diseases, as seen in the study of hypertension. Research on the SHR animal model and study of a relatively rare cause of hypertension, renovascular hypertension, led to more detailed studies on the blockade of renin–angiotensin system, bringing research forward to the current widespread field of cardiovascular disorders in translational research. These lessons teach us the importance of the breakthroughs using animal models and rare human diseases.

Conflict of interest statement The authors declare that they have no conflict of interests.

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ORIGINAL ARTICLE

Natriuretic Peptides/cGMP/cGMP-Dependent Protein Kinase Cascades Promote Muscle Mitochondrial Biogenesis and Prevent Obesity

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OBJECTIVE—Natriuretic peptides (NPs) have been characterized as vascular hormones that regulate vascular tone via guanylyl cyclase (GC), cyclic GMP (cGMP), and cGMP-dependent protein kinase (cGK). Recent clinical studies have shown that plasma NP levels were lower in subjects with the metabolic syndrome. The present study was conducted to elucidate the roles for NP/cGK cascades in energy metabolism.

RESEARCH DESIGN AND METHODS—We used three types of genetically engineered mice: brain NP (BNP) transgenic (BNP-Tg), cGK-Tg, and guanylyl cyclase-A (GCA) heterozygous knockout ($GCA^{+/-}$) mice and analyzed the metabolic consequences of chronic activation of NP/cGK cascades in vivo. We also examined the effect of NPs in cultured myocytes.

RESULTS—BNP-Tg mice fed on high-fat diet were protected against diet-induced obesity and insulin resistance, and cGK-Tg mice had reduced body weight even on standard diet; surprisingly, giant mitochondria were densely packed in the skeletal muscle. Both mice showed an increase in muscle mitochondrial content and fat oxidation through upregulation of peroxisome proliferator–activated receptor (PPAR)-γ coactivator (PGC)-1α and PPARδ. The functional NP receptors, GCA and guanylyl cyclase-B, were downregulated by feeding a high-fat diet, while $GCA^{+/-}$ mice showed increases in body weight and glucose intolerance when fed a high-fat diet. NPs directly increased the expression of PGC-1α and PPARδ and mitochondrial content in cultured myocytes.

CONCLUSIONS—The findings together suggest that NP/cGK cascades can promote muscle mitochondrial biogenesis and fat oxidation, as to prevent obesity and glucose intolerance. The vascular hormone, NP, would contribute to coordinated regulation of oxygen supply and consumption. ***Diabetes* 58: 2880–2892, 2009**

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Natriuretic peptides (NPs), consisting of atrial, brain, and C-type NPs (ANP, BNP, and CNP, respectively), have been characterized as cardiac or vascular hormones that reduce vascular tone and circulating blood volume (1). NPs can stimulate at least two types of biologically active receptors, guanylyl cyclase-A (GCA) and guanylyl cyclase-B (GCB), which act as membrane-bound GCs to synthesis intracellular cGMP. NPs exert their biological effects through GC-mediated synthesis of cyclic GMP (cGMP) and subsequent activation of cGMP-dependent protein kinase (cGK)-I, which constitute the common signal transduction pathway for nitric oxide (NO). On the other hand, type C NP receptor (C-receptor) is indicated as having a role as a clearance receptor, which binds and incorporates NPs into cytoplasm and inactivates them.

We and others (2) have demonstrated that the intravenous infusion of ANP or BNP into patients with heart failure reduces cardiac pre- and post-load and results in beneficial hemodynamic function; therefore, they are widely used for the treatment of congestive heart failure. Recently, we have elucidated new roles for NPs in the promotion of neovascularization in ischemic tissues and introduced a therapeutic application of NPs for patients with peripheral artery occlusive diseases (3,4). Meanwhile, CNP is shown to stimulate endochondral bone formation through GCB-dependent signal pathways, and its therapeutic application to human achondroplasia is expected (5).

In these ways, the cardiac hormones, NPs, have been indicated to act on the cardiovascular and cartilage-bone systems. Recent reports have suggested that NPs may also affect cultured human adipocytes and exert lipolytic action (6), which is associated with cGK-mediated activation of hormone-sensitive lipase (HSL) (7). In addition, obese individuals in the cohorts of the Framingham Heart Study were found to hold considerably lower plasma NP levels than those with normal weight (8). Lower plasma NP levels were also associated with the development of insulin resistance and metabolic syndrome, even after adjustment for BMI (9). These findings indicate that the activation of NP/cGK cascades can regulate lipid metabolism in humans to reduce susceptibility to obesity and the metabolic syndrome.

In a previous report (10), we have shown that cGMP can regulate mitochondrial content and function in C2C12 myotubular cells by altering the expressions of genes involved in mitochondrial biogenesis and reactive oxygen

species production. In the present study, we analyzed three types of genetically engineered mice to elucidate the metabolic consequences of chronic activation of NP/cGK cascades in vivo. One type is BNP transgenic mice (BNP-Tg) with serum amyloid P (SAP) promoter, which overexpress BNP specifically in the liver and have BNP plasma levels 100 times higher than the physiological condition (11). The other two types are cGK transgenic mice (cGK-Tg) with a chicken β-actin promoter combined with cytomegalovirus immediate-early enhancers (CAG promoter), which overexpress human cGK-I ubiquitously (3), and GCA heterozygous knockout ($GCA^{+/-}$) mice (12). The findings of the present study demonstrate significant roles for NP/cGK cascades in mitochondrial biogenesis, fat oxidation, and oxygen consumption, indicating that an activation of the cascades would be therapeutically beneficial for the treatment of obesity, insulin resistance, fatty liver, and the metabolic syndrome.

RESEARCH DESIGN AND METHODS

RESEARCH DESIGN AND METHODS are shown in supplement 1 in the online appendix (13–16) (available at http://diabetes.diabetesjournals.org/cgi/content/full/db09-0393/DC1).

RESULTS

BNP-Tg mice attenuate diet-induced obesity and insulin resistance. To examine the effects of NPs on body weight and on glucose and lipid metabolism, BNP-Tg mice were given a high-fat (60 kcal% fat) diet. The body weight of BNP-Tg mice on standard diet tended to decrease compared with that of their littermate wild-type mice (4.8% reduction at 18 weeks old, $n = 18$ per group, $P = 0.06$) (Fig. 1*A*). When fed a high-fat diet from the age of 10 weeks, on the other hand, the weight of the BNP-Tg mice at 18 weeks old was significantly lower than that of the wild-type controls (38.9 ± 1.0 g for the former and 43.0 ± 0.9 g for the latter; $n = 10$, $P < 0.01$) (Fig. 1*A*). The reduction in body weight of the transgenic mice fed a high-fat diet could be macroscopically observed (Fig. 1*B*). Food intake (kcal/day) was not significantly different between BNP-Tg and wild-type mice whether on standard or high-fat diet, despite the difference in body weight (Fig. 1*C*).

The blood glucose and insulin levels were identical for BNP-Tg and wild-type mice on standard diet, both during ad libitum feeding and fasting. However, these levels were significantly lower in BNP-Tg mice during ad libitum feeding of the high-fat diet (Table 1). Blood glucose levels were also lower in BNP-Tg mice fed a high-fat diet after administration of glucose or insulin (Fig. 1*D*). On the other hand, serum triglyceride and fatty acid levels did not show any significant differences between the two groups, both when feeding ad libitum and fasting (Table 1). The greater increase in the serum fatty acid level after 24-h fasting was observed in BNP-Tg mice (Table 1). Urinary excretion of catecholamines (epinephrine and norepinephrine) was similar between the two groups (Table 1).

To estimate the fat weight in mice, we used computed tomography (CT) and scanned the whole body of mice. The high-fat diet produced a substantial increase in the adipose tissue in both the subcutaneous and visceral area. The total fat weight of BNP-Tg mice fed on a high-fat diet was significantly lower (26% reduction, $n = 6$, $P < 0.01$) (Fig. 1*E*) than that of wild-type mice. The relative reduction ratio was similar for both subcutaneous and visceral fat (21% reduction for subcutaneous and 29% for visceral fat). The high-fat–fed BNP-Tg mice had less surgically harvested epididymal and visceral fats than the wild-type mice (Fig. 1*F*), which is a compatible finding with the CT-based fat quantification. On the other hand, lean body mass showed no significant difference (26.6 ± 1.3 g in BNP-Tg mice fed a high-fat diet and 27.2 ± 1.2 g in wild-type mice).

To further study the effects of NPs on diet-induced lipid accumulation, we examined adipose tissue, liver, and skeletal muscle of BNP-Tg mice fed a high-fat diet for a comparison with those of wild-type mice. We found that the histologically examined adipocytes in the epididymal fat were smaller in BNP-Tg mice (Fig. 1*G*). In support of this finding, serum leptin was found to decrease and adiponectin to increase in BNP-Tg mice fed a high-fat diet (Fig. 1*H*). The liver of wild-type mice had a whitish appearance, while that of high-fat–fed BNP-Tg mice was reddish and lower in weight (1.4 ± 0.1 g in high-fat–fed BNP-Tg mice and 1.7 ± 0.1 g in wild-type mice, $n = 6$, $P < 0.05$) (Fig. 1*I*). Oil red O staining and triglyceride measurements of the liver confirmed that diet-induced lipid accumulation was significantly attenuated in high-fat–fed BNP-Tg mice (30% decrease in triglyceride concentration, $P < 0.05$, $n = 12$) (Fig. 1*J*), and a similar attenuation of lipid accumulation was observed in the skeletal muscle (27% reduction, $n = 12$, $P < 0.05$) (Fig. 1*K*). These findings indicate that diet-induced ectopic fat accumulation was reduced in BNP-Tg mice in addition to the reduction in adipose tissue.

High-fat–fed BNP-Tg mice exhibit higher oxygen consumption and fat oxidation. The respiratory gas analysis demonstrated that BNP-Tg mice on a high-fat diet consumed more oxygen than wild-type mice ($n = 6$, $P < 0.01$) (Fig. 2*A*). Mean oxygen consumption for 24 h of BNP-Tg mice on standard diet was 64.3 ± 0.9 ml · min^{-1} · kg body wt^{-1}, and that of wild-type mice was 62.6 ± 0.9 ml · min^{-1} · kg body wt^{-1}. The value of wild-type mice on high-fat diet decreased to 51.8 ± 0.7 ml · min^{-1} · kg body wt^{-1}, and that of BNP-Tg was 58.7 ± 0.5 ml · min^{-1} · kg body wt^{-1}. The value was significantly higher than that of wild-type mice ($n = 6$, $P < 0.01$) (Fig. 2*B*). The rectal temperature was similar between BNP-Tg and wild-type mice, whether on standard diet or high-fat diet (Fig. 2*C*). The respiratory quotient showed a significant reduction in high-fat–fed BNP-Tg mice, especially during the daytime (Fig. 2*D*). The mean respiratory quotient for 24 h of high-fat–fed BNP-Tg mice was 0.80 ± 0.02, and that of wild-type mice was 0.81 ± 0.01 ($n = 6$, $P < 0.05$). In line with the reduction in respiratory quotient, mean fat oxidation for 24 h of high-fat–fed BNP-Tg mice, estimated from the results of the respiratory gas analysis, was increased to 18.5 ± 0.2 ml · min^{-1} · kg body wt^{-1}, while that of wild-type mice was 16.8 ± 0.2 ml · min^{-1} · kg body wt^{-1} ($n = 6$, $P < 0.01$) (Fig. 2*E*). The increase in fat oxidation in BNP-Tg mice could be augmented by fasting (Fig. 2*F*).

To further investigate the mechanism for the increase in oxygen consumption of high-fat–fed BNP-Tg mice, we checked mitochondrial DNA copy number in the brown adipose tissue and skeletal muscle, which are the major sites for energy expenditure. Quantitative PCR analysis demonstrated a significant increase in the mitochondrial DNA copy number in the skeletal muscle of high-fat–fed BNP-Tg mice (Fig. 2*G*); however, the increase in the brown adipose tissue was weak (Fig. 2*G*), in accordance with unchanged rectal temperature (Fig. 2*C*). In conjunction with these findings, the expressions of the genes encoding peroxisome proliferator–activated receptor (PPAR)-γ coactivator (PGC)-1α and un-

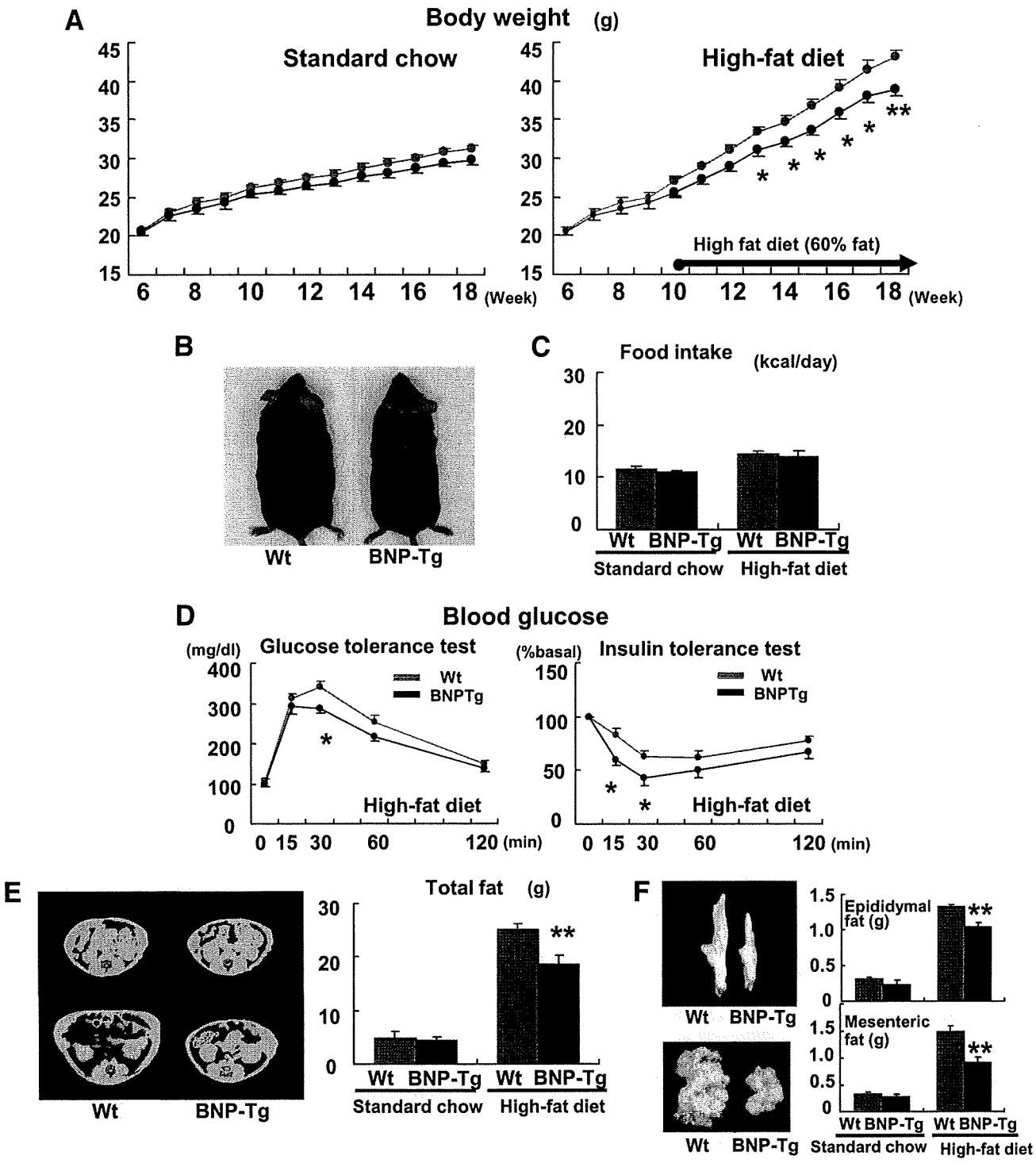

FIG. 1. BNP-Tg mice are protected against diet-induced obesity and insulin resistance. Wild-type and BNP-Tg mice were given high-fat (60 kcal% fat) diet from the age of 10 weeks. *A*: Body weight of the BNP-Tg mice on standard diet (*left panel*) and a high-fat diet (*right panel*) (n = 18 per group on standard diet and n = 10 on high-fat diet). ▨, wild type; ■, BNT-Tg. *B*: Macroscopic appearance of a wild-type (Wt) and a BNP-Tg mouse fed on high-fat diet at 18 weeks of age. *C*: Food intake on standard diet and high-fat diet (n = 6). *D*: Blood glucose levels determined with the glucose and insulin tolerance tests (n = 8). ▨, wild type; ■, BNT-Tg. *E*: CT images obtained at kidney level of a wild-type (Wt) and a BNP-Tg mouse on standard diet (*upper panel*) and high-fat diet (*lower panel*). Subcutaneous fat (yellow), abdominal fat (red), and muscular region (blue) were distinguished. Total fat weight was estimated from the images (n = 6). *F*: Macroscopic appearances and weights of epididymal fat (*upper panel*) and mesenteric fat (*lower panel*). *G*: Microscopic analysis with hematoxylin and eosin staining of epididymal fat in high-fat–fed mice (*left panels*). Scale bar, 100 μm. Adipocyte size of epididymal fat in high-fat–fed mice was estimated from the histological analysis (*right panel*) (n = 8). *H*: Serum leptin and adiponectin levels (n = 8). *I*: Macroscopic appearance of the liver of the mice fed on high-fat diet (*upper panel*). Microscopic images of the liver stained with Oil red O (*lower panel*). Scale bar, 100 μm. *J* and *K*: Triglyceride concentrations in the liver (*J*) and the quadriceps (*K*) (n = 12). *P < 0.05; **P < 0.01 vs. wild-type (Wt) mice on the same feeding condition. (A high-quality color digital representation of this figure is available in the online issue.)

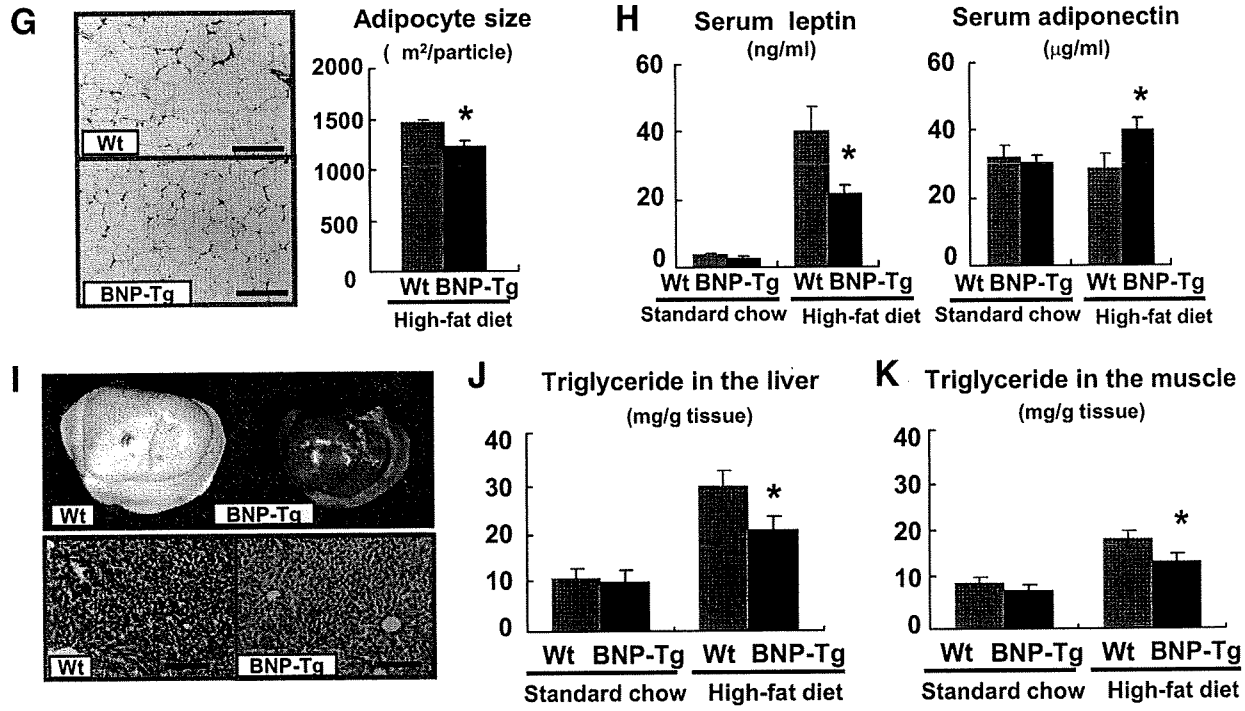

FIG. 1. Continued.

coupling protein (UCP) 1, which are known to mediate mitochondrial biogenesis and thermogenesis, respectively, were not significantly upregulated in the brown adipose tissue of the Tg mice (Fig. 2*H*); whereas in the skeletal muscle, the expressions of the genes encoding PGC-1α and PPARδ, which are known to participate in fat oxidation and energy expenditure, were upregulated in high-fat–fed BNP-Tg mice (Fig. 2*H*).

The expressions of NP receptors are regulated by feeding condition, and GCA knockdown mice are susceptible to diet-induced obesity and glucose intolerance. The results of the study shown in Fig. 3 are described in supplement 2 in the online appendix.

TABLE 1
Physical and metabolic parameters of high-fat–fed BNP-Tg mice

	Wild type	BNP-Tg
Body weight (g)		
18 weeks old	43.0 ± 0.9	38.9 ± 0.9*
Glucose (mg/dl)		
Ad libitum feeding	230.2 ± 10.5	196.7 ± 16.5†
24-h fasting	135.9 ± 7.9	104.0 ± 10.2†
Insulin (ng/dl)		
Ad libitum feeding	9.87 ± 1.9	5.25 ± 1.1†
24-h fasting	1.05 ± 0.2	0.86 ± 0.1
Triglyceride (mg/dl)		
Ad libitum feeding	131.9 ± 13.2	126.7 ± 15.6
24-h fasting	90.9 ± 7.9	92.6 ± 8.8
Fatty acid (mEq/l)		
Ad libitum feeding	1.28 ± 0.10	1.08 ± 0.09
24-h fasting	1.70 ± 0.17	1.75 ± 0.21
Epinephrine (ng/day)		
Urinary excretion	26.5 ± 2.9	28.0 ± 4.2
Norepinephrine (ng/day)		
Urinary excretion	358.0 ± 15.1	349.0 ± 52.8

*$P < 0.01$, †$P < 0.05$, compared with wild type.

cGK-Tg mice are lean and insulin sensitive even on standard diet. To determine the effect of cGK-I, a major downstream effector of NP/GC/cGMP cascades, on body weight and on glucose and lipid metabolism, we examined the cGK-Tg mice with ubiquitously overexpressing human cGK-I. The cGK-Tg mice showed a significant reduction in body weight compared with wild-type mice, even on standard diet (27.6 ± 0.4 g for cGK-Tg and 32.0 ± 0.6 g for wild-type mice, at 18 weeks old on standard diet, $n = 8$, $P < 0.01$) (Fig. 4*A*). Moreover, high-fat diet–induced weight gain was attenuated in the Tg mice, and the reduction in body weight eventually reached >20%. At 18 weeks of age, their body weight was 35.7 ± 0.4 g, and that of wild-type mice was 43.9 ± 0.6 g (after 8 weeks of high-fat feeding, $n = 8$, $P < 0.01$) (Fig. 4*A* and *B*). The daily food intake (kcal/day) was not noticeably different for cGK-Tg and wild-type mice, while it showed a significant increase in cGK-Tg mice when it was adjusted for body weight (kcal · day^{-1} · g body wt^{-1}) (Fig. 4*C*).

The blood glucose levels were significantly lower in cGK-Tg mice, both during ad libitum feeding and fasting and on standard diet and high-fat diet (Table 2). The decrease was accompanied by significantly lower insulin levels (Table 2), except for the value for fasting while fed on standard diet. After administration of glucose or insulin, the difference in glucose levels between cGK-Tg and wild-type mice became more prominent (Fig. 4*D*). Serum triglyceride and fatty acid levels were similar for cGK-Tg and wild-type mice, both during ad libitum feeding and fasting, except for the significant increase in fatty acid levels in high-fat–fed cGK-Tg mice during fasting, when compared with that of wild-type mice (Table 2). Urinary excretion of the catecholamines (epinephrine and norepinephrine) was similar for wild-type and cGK-Tg mice (Table 2).

In parallel with the reduction in body weight, fat tissues assessed by means of CT analysis were significantly reduced

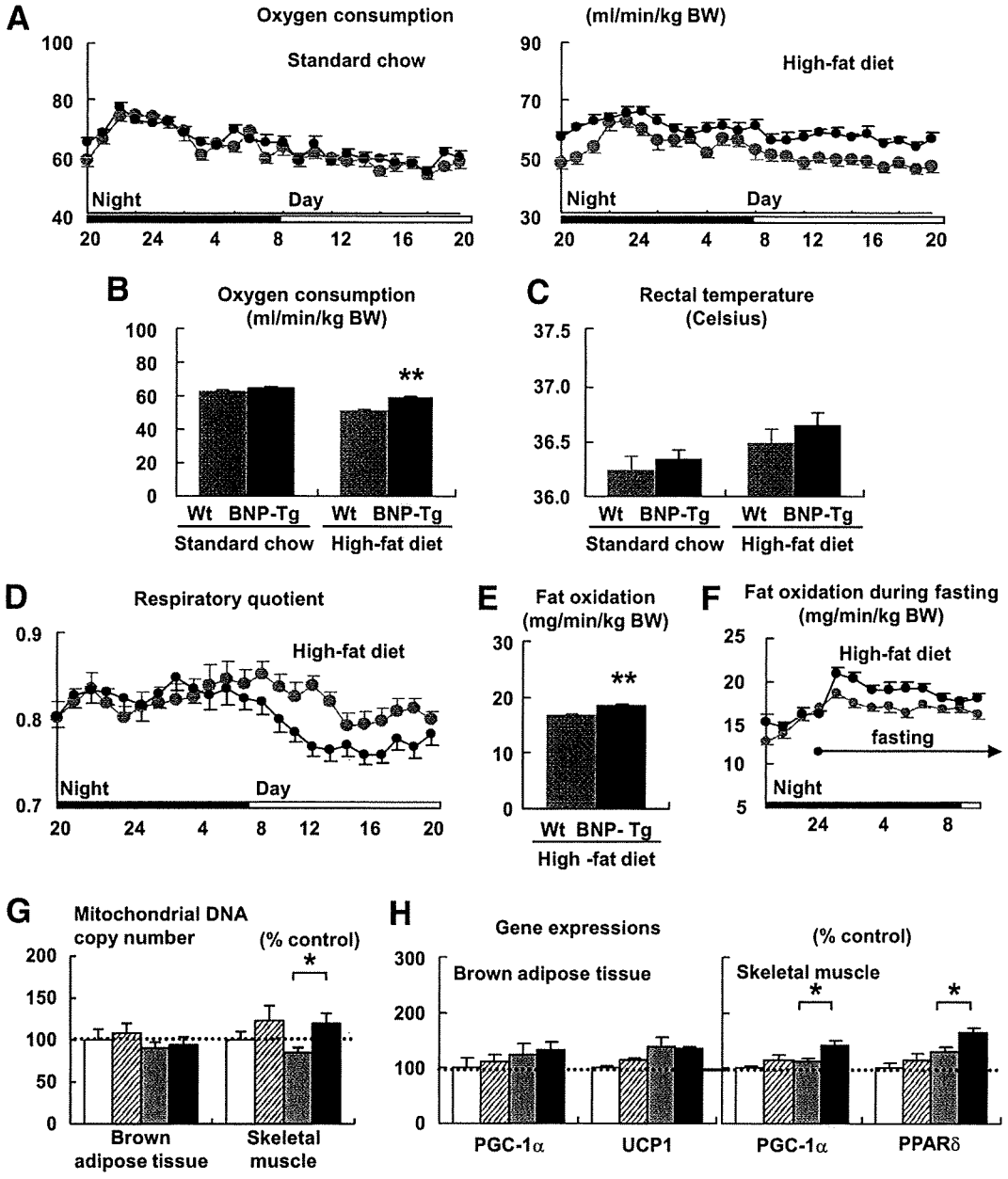

FIG. 2. High-fat–fed BNP-Tg mice exhibit higher oxygen consumption and fat oxidation in association with increased mitochondrial content in the skeletal muscle. Mice were subjected to respiratory gas analysis after fed on high-fat diet. Total DNA and RNA were extracted from the brown adipose tissue and the quadriceps, and quantitative PCR analysis was performed. *A*: Oxygen consumption on standard diet (*left panel*) and high-fat diet (*right panel*) (n = 6). ▤, wild type; ■, BNP-Tg. *B*: Mean oxygen consumption for 24 h on standard diet or high-fat diet (n = 6). *C*: Rectal temperature on standard diet or high-fat diet (n = 6). *D*: Respiratory quotient of high-fat–fed mice (n = 6). ▤, wild type; ■, BNP-Tg. *E*: Mean fat oxidation estimated from the respiratory gas analysis for 24 h of high-fat–fed mice. *F*: Fat oxidation before and during fasting starting from midnight. ▤, wild type; ■, BNP-Tg. *G*: Mitochondrial DNA copy number estimated from quantification of mitochondrial and nuclear genome (n = 8). *H*: Expressions of genes encoding PGC-1α and UCP1 in the brown adipose tissue and those for PGC-1α and PPARδ in the skeletal muscle (n = 8). Standard diet: □, wild type; ▨, BNP-Tg. High-fat diet: ▤, wild type; ■, BNP-Tg. The values were standardized to those for the control (wild-type [Wt] mice fed on standard diet) in either group. *P < 0.05; **P < 0.01 vs. wild type on the same feeding condition.

in cGK-Tg mice, both on standard and high-fat diet (Fig. 4*E*). Hematoxylin-eosin staining and the Coulter counter analysis showed that the adipocytes in the epididymal fat were smaller in cGK-Tg mice, with mean adipocyte diameters for wild-type and cGK-Tg mice of ~80 and 60 μm, respectively, when on standard diet (Fig. 4*F* and *G*). The high-fat diet produced an increase in the diameter to 130 μm in wild-type mice but to only 80 μm in the Tg mice (Fig. 4*G*). The liver of high-fat–fed cGK-Tg mice weighed less than that of wild-type mice (1.3 ± 0.1 g and 1.8 ± 0.1 g, n = 8, P < 0.01, respectively) and had reddish appearance (Fig. 4*H*). The hepatic triglyceride concentration of high-fat–fed cGK-Tg mice was significantly reduced when compared with wild-type mice (30% decrease at 20 weeks, n = 6, P < 0.05) (Fig. 4*I*). Muscular triglyceride concentration of cGK-Tg mice was also significantly lower, even on standard diet, as well as in high-fat–fed cGK-Tg mice (44% decrease in muscle triglyceride at 20 weeks, n = 6, P < 0.01) (Fig. 4*J*).

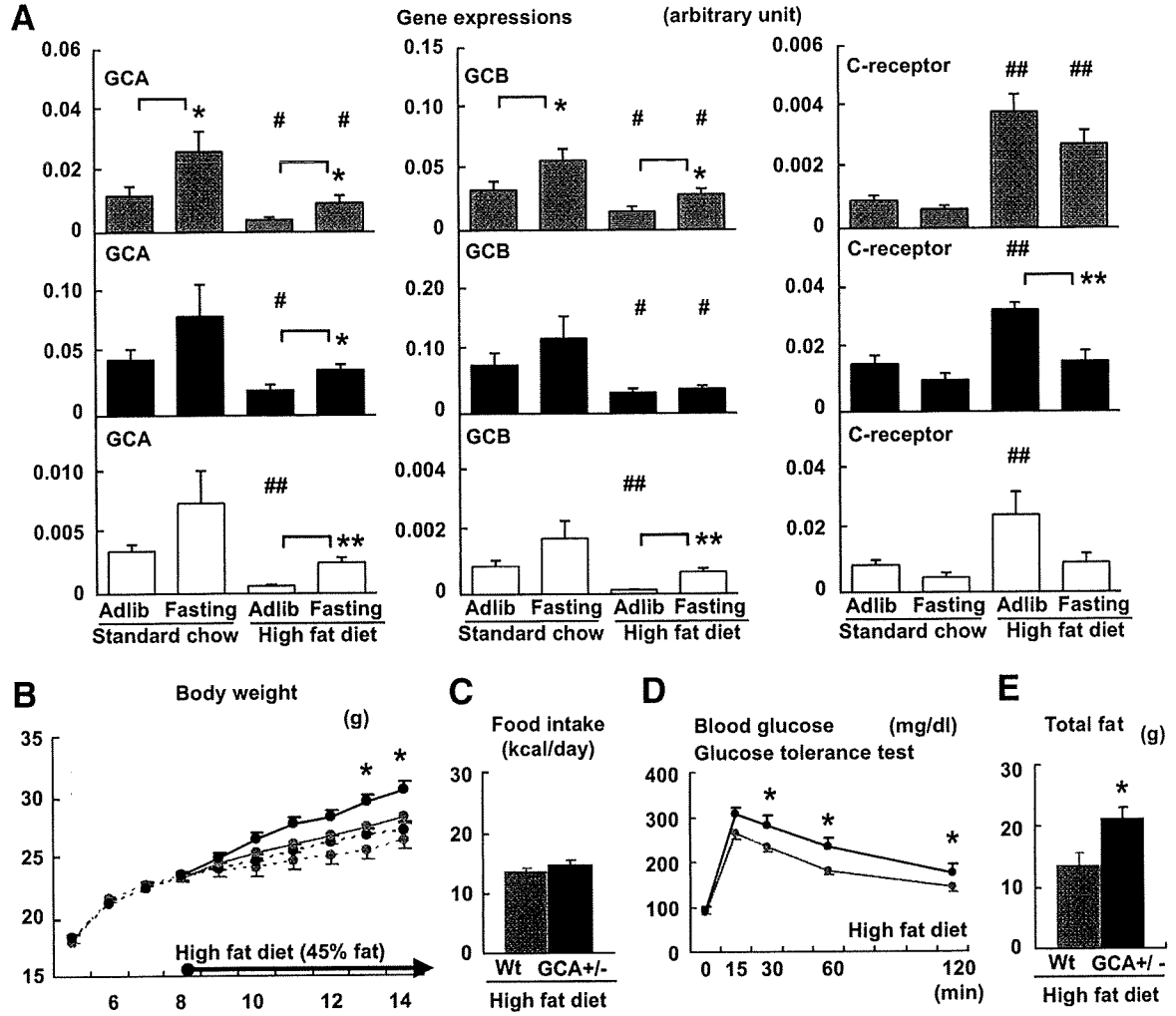

FIG. 3. The expressions of NP receptors are regulated by feeding condition, while GCA knockdown mice are susceptible to diet-induced obesity and glucose intolerance. Total RNA was extracted from the quadriceps of wild-type mice, and quantitative PCR analysis for the expressions of NP receptors was performed. Wild-type and GCA heterozygous knockout mice ($GCA^{+/-}$) were given a high-fat (45 kcal% fat) diet from the age of 8 weeks. *A*: Expressions of GCA, GCB, and C-receptor in the skeletal muscle, brown adipose tissue, and white adipose tissue of wild-type mice for indicated feeding condition ($n = 8$). The values represent the expression levels of each gene compared with that of β-actin determined by quantitative PCR analysis. Statistical analysis was performed to evaluate the effects of fasting and high-fat diet. $*P < 0.05$; $**P < 0.01$ vs. ad libitum eating on the same diet. $\#P < 0.05$; $\#\#P < 0.01$ vs. standard diet on the same eating condition (ad libitum or fasting). Further analysis to clarify whether there were interactions between the effect of fasting and that of high-fat diet was performed as shown in supplemental Table S2. ▤, skeletal muscle; ■, brown adipose tissue; □, white adipose tissue. *B*: Body weight of wild-type (Wt) and $GCA^{+/-}$ mice fed on standard diet (dotted lines) or high-fat diet (solid lines) after 8 weeks of age ($n = 8$). *C*: Food intake on high-fat diet ($n = 6$). *D*: Blood glucose levels determined with the glucose tolerance test ($n = 8$). ▤, wild type; ■, $GCA^{+/-}$. *E*: Total fat weight estimated from the CT images ($n = 6$). $*P < 0.05$; $**P < 0.01$ vs. wild type on the same feeding condition. $GCA^{+/-}$, GCA heterozygous knockout mice.

cGK-Tg mice exhibit giant mitochondria in the skeletal muscle, associated with higher oxygen consumption. The respiratory gas analysis demonstrated that the oxygen consumption increased in the cGK-Tg mice, both when on standard diet and on the high-fat diet ($n = 6$, $P < 0.01$) (Fig. 5*A*). Mean oxygen consumption for 24 h of wild-type mice on standard diet was 63.5 ± 0.3 ml · min^{-1} · kg body wt^{-1}, and that of cGK-Tg was 71.7 ± 0.4 ml · min^{-1} · kg body wt^{-1}. The value of wild-type mice on high-fat diet decreased to 43.6 ± 0.2ml · min^{-1} · kg body wt^{-1}, and that of cGK-Tg was 54.1 ± 0.5 ml · min^{-1} · kg body wt^{-1} (Fig. 5*B*). The increase in oxygen consumption in cGK-Tg mice was accompanied with a significantly higher rectal temperature (Fig. 5*C*). The respiratory quotient showed a significant reduction in cGK-Tg mice ($n = 6$, $P < 0.01$) (Fig. 5*D*), and the reduction was prominent in the light phase (data not shown), similar to the case of BNP-Tg mice (Fig. 2*D*). Fat oxidation was increased in cGK-Tg mice, both when on standard diet and on high-fat diet ($n = 6$, $P < 0.01$) (Fig. 5*E*). Mean fat oxidation for 24 h of high-fat–fed cGK-Tg mice was 22.8 ± 0.5 ml · min^{-1} · kg body wt^{-1}, and that of wild-type mice was 16.8 ± 0.3 ml · min^{-1} · kg body wt^{-1}.

Quantitative PCR analysis revealed increased mitochondrial DNA copy number in both the brown adipose tissue and the skeletal muscle of cGK-Tg mice, both on standard and high-fat diet (Fig. 5*F*). In conjunction with these findings, the expressions of the genes encoding PGC-1α and UCP1 were upregulated in the brown adipose tissue of cGK-Tg mice (Fig. 5*G*). In the skeletal muscle, expressions of the genes encoding PGC-1α and PPARδ were upregulated in cGK-Tg mice, both when on standard and high-fat diet, associated with the