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

Running title: NP/cGMP/cGK cascades prevent obesity

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

Research Design and Methods: We used three types of genetically engineered mice: BNP transgenic (BNP-Tg), cGK-Tg, and 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 NP 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 chow, and surprisingly, giant mitochondria were densely packed in the skeletal muscle. Both mice showed an increase in muscle mitochondrial content and fat oxidation through up-regulation of PGC-1α and PPARδ. The functional NP-receptors, GCA and GCB, were down-regulated by feeding high-fat diet; while GCA+/- mice showed increases in body weight and glucose intolerance when fed on high-fat diet. NP 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.
Natriuretic peptides (NP), consisting of atrial, brain and C-type natriuretic peptides (ANP, BNP and CNP, respectively), have been characterized as cardiac or vascular hormones which reduce vascular tone and circulating blood volume [1]. NP can stimulate at least two types of biologically active receptors, guanylyl cyclase-A (GCA) and guanylyl cyclase-B (GCB), which act as membrane-bound guanylyl cyclases to synthesize intracellular cGMP. NP exerts their biological effects through GC-mediated synthesis of cGMP, and subsequent activation of cGMP-dependent protein kinase-I (cGK), which constitute the common signal transduction pathway for nitric oxide (NO). On the other hand, type-C natriuretic peptide receptor (C-receptor) is indicated a role as a clearance receptor, which binds and incorporates NP into cytoplasm, and inactivates them.

We and others have demonstrated that the intravenous infusion of ANP or BNP into the patients with heart failure reduces cardiac pre- and after-load, and results in beneficial hemodynamic function, so that they are widely used for the treatment of congestive heart failure [2]. Recently, we have elucidated new roles for NP in the promotion of neo-vascularization in ischemic tissues and introduced therapeutic application of NP 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, NP, have been indicated to act on the cardio-vascular and cartilage-bone systems. Recent reports have suggested that NP 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 natriuretic peptide levels compared to those with normal weight [8]. Lower plasma natriuretic peptide levels were also associated with the development of insulin resistance and metabolic syndrome, even after adjustment for body mass index [9]. These findings indicate that the activation of NP/cGK cascades can regulate lipid metabolism in human, so that reduce susceptibility to obesity and the metabolic syndrome.

In a previous report, 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 ROS production [10]. 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 over-express BNP specifically in the liver, and with BNP plasma levels 100 times higher than the physiological condition [11]. The other two types are cGK transgenic mice (cGK-Tg) with a chicken beta-actin promoter combined with cytomegalovirus immediate-early enhancers (CAG promoter) which over-express 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.
RESULTS

BNP-transgenic mice attenuate diet-induced obesity and insulin resistance. To examine the effects of NP 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 chow tended to decrease compared to that of their littermate Wt mice (4.8% reduction at 18 weeks old, n=18 per group, p=0.06, Figure 1A). When fed on 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 Wt controls: 38.9±1.0g for the former and 43.0±0.9g for the latter (n=10, p<0.01; Figure 1A). The reduction in body weight of the transgenic mice fed on high-fat diet could be macroscopically observed (Figure 1B). Food intake (kcal/day) was not significantly different between BNP-Tg and Wt mice whether on standard chow or high-fat diet, despite the difference in body weight (Figure 1C).

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

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 high-fat diet was significantly lower (26% reduction, n=6, P<0.01; Figure 1E) than that of Wt. The relative reduction ratio was similar for both subcutaneous and visceral fats (21% reduction for subcutaneous fat and 29% for visceral). The high-fat fed BNP-Tg mice had less surgically harvested epididymal and visceral fats than the Wt mice (Figure 1F), which is a compatible finding with the CT-based fat quantification. On the other hand, the lean body mass showed no significant difference (26.6±1.3g in BNP-Tg mice fed on high-fat diet and 27.2±1.2g in Wt).

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

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BNP-Tg mice, in addition to the reduction in adipose tissue.

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

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 the skeletal muscle, which are the major sites for energy expenditure. Quantitative PCR analysis demonstrated significant increase in the mitochondrial DNA copy number in the skeletal muscle of high-fat fed BNP-Tg mice (Figure 2G); however, the increase in the brown adipose tissue was weak (Figure 2G), in accordance with unchanged rectal temperature (Figure 2C). In conjunction with these findings, the expressions of the genes encoding PGC-1α and UCP1, which are known to mediate mitochondrial biogenesis and thermogenesis, respectively, were not significantly up-regulated in the brown adipose tissue of the Tg mice (Figure 2H). While in the skeletal muscle, the expressions of the genes encoding PGC-1α and PPARδ, which is known to participate in fat oxidation and energy expenditure, were up-regulated in high-fat fed BNP-Tg mice (Figure 2H).

**The expressions of natriuretic peptide receptors are regulated by feeding condition.**

GCA-knockdown mice are susceptible to diet-induced obesity and glucose intolerance. The results of the study shown in Figure 3 are described in Supplement 2 in the online appendix, in order to keep within the word limit.

**cGK-transgenic mice are lean and insulin-sensitive even on standard chow.** To determine the effect of cGK-I, a major down-stream effector of NP/GC/cGMP cascades, on body weight and on glucose and lipid metabolism, we examined the cGK-Tg mice with ubiquitously over-expressing human cGK-I. The cGK-Tg mice showed a significant reduction in body weight compared with Wt mice, even on standard chow: 27.6±0.4g for cGK-Tg and 32.0±0.6g for Wt mice (at 18 weeks old on standard chow, n=8, p<0.01; Figure 4A). Moreover, high-fat diet induced weight gain was attenuated in the Tg mice and the reduction in body weight eventually reached more than 20%. At 18 weeks of age, their body weight was 35.7±0.4g and that of Wt mice was 43.9±0.6g (after 8 weeks of high-fat feeding, n=8, p<0.01; Figure 4A,B). The daily food intake (kcal/day) was not noticeably different for cGK-Tg and Wt mice, while it showed a significant increase in cGK-Tg mice when it was adjusted for body weight (kcal/day/g BW) (Figure 4C).

The blood glucose levels were
cGK-transgenic 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 chow and on the high-fat diet (n=6, P<0.01; Figure 5A). Mean oxygen consumption for 24 hours of Wt mice on standard chow was 63.5±0.3 (ml/min/kg BW) and that of cGK-Tg was 71.7±0.4. The value of Wt on high-fat diet decreased to 43.6±0.2 (ml/min/kg BW) and that of cGK-Tg was 54.1±0.5 (Figure 5B). The increase in oxygen consumption in cGK-Tg mice was accompanied with a significantly higher rectal temperature (Figure 5C). The respiratory quotient showed a significant reduction in cGK-Tg mice (n=6, P<0.01, Figure 5D), and the reduction was prominent in the light phase (Data not shown), alike to the case of BNP-Tg mice (Figure 2D). Fat oxidation was increased in cGK-Tg mice both when on standard chow and on high-fat diet (n=6, P<0.01, Figure 5E). Mean fat oxidation for 24 hours of high-fat fed cGK-Tg mice was 22.8±0.5 (mg/min/kg BW) and that of Wt was 16.8±0.3.

Quantitative PCR analysis revealed increased mitochondrial DNA copy number both in the brown adipose tissue and the skeletal muscle of cGK-Tg mice, both on standard chow and high-fat diet (Figure 5F). In conjunction with these findings, the expressions of the genes encoding PGC-1α and UCP1 were up-regulated in the brown adipose tissue of cGK-Tg mice (Figure 5G). In the skeletal muscle, expressions of the genes encoding PGC-1α and PPARδ were up-regulated in cGK-Tg mice both when on standard chow and high-fat diet, associated with the increase in their down-stream target genes involved in mitochondrial oxidative function and fatty acid catabolism (Figure 5H). In addition to PGC-1α and PPARδ, the expression of ATPsyn, COX and CPT1b was significantly enhanced in the skeletal muscle.
of cGK-Tg mice, compared to Wt, both when on standard chow and high-fat diet; while UCP3, FATP and ACO were up-regulated when on the high-fat diet only (Figure 5H). The expression of PPARα in the skeletal muscle of cGK-Tg mice was not significantly different from Wt mice, although high-fat diet increased the expression (data not shown). Giant mitochondria were densely packed in the skeletal muscle of high-fat fed cGK-Tg mice when it was observed by means of an electron microscope (Figure 5I).

**Natriuretic peptides directly increase the expression of PGC-1α and PPARδ, and mitochondrial content in cultured myocytes.** The results of the study shown in Figure 6A and 6B are described in Supplement 2, in order to keep within the word limit.

Schematic representation of the suggested roles for NP/cGK cascades, as described in the present and previous studies, is shown in Figure 6C.

**DISCUSSION**

The findings in this study demonstrate that the activation of NP/cGK cascades augments mitochondrial biogenesis and fat oxidation in mice through up-regulation of PGC-1α and PPARδ in the skeletal muscle, thus conferring resistance to obesity and glucose intolerance. BNP-Tg mice fed on high-fat diet were protected from obesity and insulin resistance; while, cGK-Tg mice were lean even on standard chow and showed increased insulin sensitivity, and surprisingly, giant mitochondria were densely packed in the skeletal muscle. Both types of mice showed a reduction in fat tissue and excessive lipid accumulation in the liver and skeletal muscle, in accordance with an increase in fat oxidation. Functional NP receptors were up-regulated during fasting, whereas they were down-regulated by ad-lib feeding or high-fat challenge; while whole body knock-down of the functional receptor, GCA, led to promotion of obesity in mice. These findings together with those that demonstrated NP-induced lipolysis lead us to propose the concept that NP/cGK cascades play significant roles in lipid catabolism during fasting or chronic caloric restriction, in addition to their well-known roles in the cardiovascular system, such as in the attenuation of hypertension and congestive heart failure (Figure 6C) [7,8,17].

Genetic over-expression of NP/cGK in mice attenuated diet-induced obesity and insulin resistance, associated with the increase in muscle mitochondrial content and fat oxidation. We identified the dual up-regulation of PGC-1α and PPARδ in the skeletal muscle of the Tg mice as a molecular basis of the increase in mitochondria, and confirmed the direct increase in the expression of PGC-1α and PPARδ, and mitochondrial content by both ANP and BNP through the experiments using cultured myocytes. PGC-1α is a transcriptional co-factor that acts as a master regulator of mitochondrial biogenesis [18]. PPARδ is a transcription factor that binds with PGC-1α and enhances mitochondrial biogenesis and lipid catabolism [19]. Activation of PPARδ in mice by the treatment with the PPARδ-agonist GW501516 or over-expression of a constitutively active form of PPARδ were shown to increase muscle mitochondrial content and fatty acid oxidation, and thus to prevent diet-induced obesity and glucose intolerance [20, 21]. However, mice with muscle-specific over-expression of wild-type PPARδ did not exhibit such a prominent phenotype [22]. Muscle-specific over-expression of PGC-1α has been reported not to prevent diet-induced obesity and insulin resistance in mice, although there was an increase in mitochondrial content [23]. These results indicate that the coordinated increase in the expression of PGC-1α and PPARδ, which was observed in the skeletal muscle of BNP- and cGK-Tg mice, led to synergistic
effects that were beneficial for ameliorating diet-induced obesity and insulin resistance. The expression of PGC-1α and PPARδ in muscle is augmented by exercise via exercise-sensitive molecules such as AMP-activated protein kinase (AMPK) [18,19]. The increases in circulating NP and cGMP levels during exercise have been demonstrated in previous reports [24,25]. Therefore, we hypothesize that NP/cGMP/cGK cascades can interact with the exercise-sensitive molecules in muscle and increase the expression of PGC-1α and PPARδ.

BNP-Tg mice gained less body weight than the controls when they were fed on high-fat diet. The same tendency was observed even when on standard chow; however, the magnitude of the change was not so prominent. We interpret the results as the Tg mice escaped from diet-induced obesity by increasing muscle mitochondrial content and fat oxidation. The elevation of serum adiponectin levels observed in the high-fat fed BNP-Tg mice, associated with reduced adiposity, might be contributing to the attenuation of insulin resistance [26,27]. Apparent changes in food intake and catecholamine kinetics were not observed.

In cGK-Tg mice, the increase in mitochondria was evident even on standard chow, resulting in the Tg mice being leaner and more insulin sensitive than BNP-Tg mice. Our data suggested that one reason for the difference in the magnitude of the altered phenotypes between the BNP- and cGK-Tg mice was caused by the variations of the target tissues. In the skeletal muscle, similar changes in gene expression were evident in the two lines of Tg mice, including up-regulation of PGC-1α and PPARδ. In the brown adipose tissue, on the other hand, the increase in the expression level of PGC-1α and its downstream effector UCP1 was significant in cGK-Tg mice, associated with higher body temperature; however, these changes were not so prominent in BNP-Tg mice. We observed that the functional receptors for NP, GCA and GCB, were abundantly expressed in the brown adipose tissue; however, the clearance receptor (C-receptor) was also rich in the tissue. Therefore, the functional receptor to C-receptor ratio, which is suggested to correlate with the biological action of NP, was lower in the brown adipose tissue than skeletal muscle. We speculate that the abundant expression of C-receptor and the lower ratio of the functional receptors reduced the effect of NP in the brown adipose tissue.

High fat-fed GCA-heterozygous KO mice accumulated more fat mass and were glucose intolerant compared to Wt mice. This phenotype is the opposite to that of BNP- or cGK-Tg mice, so that the effects of NP in terms of the prevention of diet-induced obesity and insulin resistance seemed to be mediated at least in part by GCA. We were unable to determine whether GCB also participated because GCB-KO mice were not available. The NP receptors were regulated by dietary conditions; that is, GCA and GCB were up-regulated by fasting and down-regulated during high-fat feeding. On the other hand, the C-receptor showed opposite kinetics. Therefore, the ratio of functional receptors to C-receptor was lowered by feeding, or during chronic high-fat diet; conversely, fasting increased relative amount of the functional receptors. These results indicate that high-fat diet can contribute to the development of diet-induced obesity and insulin resistance at least partly by down-regulation of GCA and up-regulation of the C-receptor. The notion that the kinetics of NP-receptor expression depend on dietary conditions, such as high-fat diet or fasting, is consistent with earlier findings of others. The GCA to C-receptor ratio was shown to be lowered in obese people than in non-obese [28]. Natriuresis is known to be increased after several hours of fasting, despite the fact that sodium intake is decreased [29]. The up-
regulation of functional NP-receptors in renal tubular cells by fasting and subsequent augmentation of natriuresis might account for the well-known phenomenon of "fasting-induced natriuresis". Taken together, the dietary regulation of the receptor expressions indicates that the signal transduction through NP/cGK cascades, which is augmented by starvation, might have a role in fasting-induced fat oxidation under physiological conditions.

Augmented production of NP caused by congestive heart failure plays important roles in compensating for the volume expansion and subsequent decrease in oxygen supply by exerting vaso-dilating, natriuretic and neurohumoral-modulating actions [30]. This compensatory effect in the cardiovascular system has been thought to be the primary role for NP/cGK cascades [31]. In the present study, we reveal the new roles for the cascades on mitochondrial biogenesis and fat oxidation through up-regulation of PGC-1α and PPARδ. The results of the present study are in line with earlier publications from Lafontan’s group which have demonstrated the importance of the NP/cGK system in the regulation of adipose tissue lipolysis [6,7,32,33]. Their results and ours together support the view that NP/cGK cascades have significant roles in lipid catabolism. Moreover, the increase in circulating NP and cGMP levels during exercise has been demonstrated in previous reports [24,25]. Therefore, NP/cGMP/cGK cascades might have significant roles in metabolic adaptations in response to exercise, including mitochondrial biogenesis and fat oxidation.

Several other vascular hormones that regulate vascular tone, including catecholamines, angiotensin II and nitric oxide (NO), are also implicated in the regulation of cellular metabolism and subsequent change in body weight and glucose tolerance [34-38]. In this context, vascular hormones seem to lie at the crossroad of cardiovascular and metabolic diseases. In a recent report, the number of risk factors acting as diagnostic markers of metabolic syndrome is shown to inversely correlate with plasma concentrations of NP [9]. The clinical data and the findings of the present study collectively indicate that insufficient activation of NP/cGK cascades can be a causative factor of both obesity and hypertension. We speculate that the state of over-nutrition would down-regulate functional NP-receptors, as to promote both obesity and hypertension. Furthermore, we believe that physiological responses of vascular hormones, in accordance with blood pressure, circulating fluid volume and oxygen demand, would result in coordinated regulation of oxygen supply and consumption; and the coordination is especially important for the adaptive responses to exercise.

To summarize, the results of the present study indicate that NP/cGK cascades can promote muscle mitochondrial biogenesis and fat oxidation through up-regulation of PGC-1α and PPARδ, as to prevent obesity and glucose intolerance. The results were compatible with our recent report which showed that cGMP can increase mitochondrial content and function in cultured myocytes [10]. The pharmaceutical interventions that activate NP/cGMP/cGK cascades seem to be potentially beneficial for the treatment of obesity, fatty liver and glucose intolerance.

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Authors Contributions. Kazutoshi Miyashita designed the experiments, maintained the mice, performed the experiments, analyzed the data and wrote the...
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paper. Hiroshi Itoh directed the study, contributed to discussion and edited the manuscript. Hirokazu Tsujimoto supported the study with superb technical assistances and contributed to discussion. Naohisa Tamura, Yasutomo Fukunaga, Masakatsu Sone, Kenichi Yamahara, Daisuke Taura, Megumi Inuzuka and Takuhro Sonoyama contributed to discussion. Kauwa Nakao contributed to discussion and encouraged the authors.

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Competing Interest. The authors declare no conflicts of interest.

GenBank accession number. PGC-1α, NM_008904; PPARδ, NM_011145; UCP1, NM_009463; ATPsyn, NM_007505; COX, NM_009464; FATP, NM_011977; ACO, NM_015729; CPT1b, NM_00948
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Table I: Physical and metabolic parameters of high-fat fed BNP-Tg mice

| Parameter       | Condition          | Wt       | BNP-Tg    |
|-----------------|--------------------|----------|----------|
| Body weight     | (g)                | 18 weeks old | 43.0±0.9 | 38.9±0.9 ** |
| Glucose         | (mg/dl)            | Ad-lib feeding | 230.2±10.5 | 196.7±16.5 * |
|                 |                    | 24 hour fasting | 135.9±7.9 | 104.0±10.2 * |
| Insulin         | (ng/dl)            | Ad-lib feeding | 9.87±1.9  | 5.25±1.1 *  |
|                 |                    | 24 hour fasting | 1.05±0.2  | 0.86±0.1   |
| Triglyceride    | (mg/dl)            | Ad-lib fed  | 131.9±13.2 | 126.7±15.6 |
|                 |                    | 24 hour fasting | 90.9±7.9  | 92.6±8.8   |
| Fatty acid      | (mEq/L)            | Ad-lib feeding | 1.28±0.10 | 1.08±0.09  |
|                 |                    | 24 hour 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<0.05, ** P<0.01 compared to Wt.

Table II: Physical and metabolic parameters of cGK-Tg mice fed on standard chow or high-fat diet.

| Parameter       | Condition          | Standard chow | High-fat diet |
|-----------------|--------------------|---------------|---------------|
|                 |                    | Wt            | cGK-Tg        | Wt            | cGK-Tg        |
| Body weight     | (g)                | 18 weeks old  | 32.0±0.6      | 27.6±0.4 **   | 43.9±1.2      | 35.7±1.4 **   |
| Glucose         | (mg/dl)            | Ad-lib feeding | 160.7±6.0    | 137.7±5.1 **  | 233.5±17.1    | 174.0±7.7 **  |
|                 |                    | 24 hour fasting | 93.5±4.7     | 78.8±2.3 *    | 113.5±7.6     | 89.8±3.7 *    |
| Insulin         | (ng/dl)            | Ad-lib feeding | 1.4±0.2      | 0.8±0.2 *     | 9.1±1.0       | 3.5±0.6 **    |
|                 |                    | 24 hour fasting | 0.26±0.05    | 0.23±0.03     | 0.95±0.22     | 0.41±0.05 *   |
| Triglyceride    | (mg/dl)            | Ad-lib feeding | 103.3±10.1   | 89.2±7.0      | 134.9±11.5    | 134.9±15.8   |
|                 |                    | 24 hour fasting | 84.3±6.8     | 81.6±6.9      | 112.8±9.5     | 103.4±11.6   |
| Fatty acid      | (mEq/L)            | Ad-lib feeding | 1.04±0.05    | 0.84±0.10     | 1.32±0.18     | 1.08±0.11    |
|                 |                    | 24 hour fasting | 1.69±0.09    | 1.86±0.08     | 1.66±0.09     | 2.22±0.08 ** |
| Epinephrine     | (ng/day)           | Urinary excretion | 48.2±9.3     | 52.7±12.8     | 49.6±6.3      | 40.5±6.1     |
| Norepinephrine  | (ng/day)           | Urinary excretion | 312.3±62.2   | 312.3±97.4    | 547.7±62.2    | 570.3±47.6   |

* P<0.05, ** P<0.01 compared to Wt on the same feeding condition
NP/cGMP/cGK cascades prevent obesity

FIGURE LEGENDS

Figure 1: BNP-Tg mice are protected against diet-induced obesity and insulin resistance. Wt 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 chow (left panel) and a high-fat diet (right panel) (n=18 per group on standard chow and n=10 on high-fat diet). [B] Macroscopic appearance of a Wt and a BNP-Tg mouse fed on high-fat diet at 18 weeks of age. [C] Food intake on standard chow and high-fat diet (n=6). [D] Blood glucose levels determined with the glucose and insulin tolerance tests (n=8). [E] CT images obtained at kidney level of a Wt and a BNP-Tg mouse on standard chow (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, 100µm. 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, 100µm. [J,K] Triglyceride concentrations in the liver (J) and the quadriceps (K) (n=12). * P<0.05, ** P<0.01 compared to Wt mice on the same feeding condition.

Figure 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 chow (left panel) and high-fat diet (right panel) (n=6). [B] Mean oxygen consumption for 24 hours on standard chow or high-fat diet (n=6). [C] Rectal temperature on standard chow or high-fat diet (n=6). [D] Respiratory quotient of high-fat fed mice (n=6). [E] Mean fat oxidation estimated from the respiratory gas analysis for 24 hours of high-fat fed mice (n=6). [F] Fat oxidation before and during fasting starting from midnight. [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). The values were standardized to those for the control (Wt mice fed on standard chow) in either group. * P<0.05 ** P<0.01 compared to Wt on the same feeding condition.

Figure 3: The expressions of natriuretic peptide 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 Wt mice and quantitative PCR analysis for the expressions of natriuretic peptide receptors was performed. Wt and GCA-heterozygous knockout mice (GCA+/-) mice 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 Wt mice for indicated feeding condition (n=8). The values represent the expression levels of each gene compared to that of beta-actin determined by quantitative-PCR analysis. Statistical analysis was performed in order to evaluate the effects of fasting and high-fat diet. * P<0.05 ** P<0.01 compared to ad-lib eating on the same diet. # P<0.05 ## P<0.01 compared to standard chow on the same eating condition (ad-lib 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. [B] Body weight of Wt and
GCA+/− mice fed on standard chow (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). [E] Total fat weight estimated from the CT images (n=6). * P<0.05, ** P<0.01 compared to Wt on the same feeding condition. GCA+/−, GCA-heterozygous knockout mice.

**Figure 4:** cGK-Tg mice are lean and insulin sensitive even on standard chow. Wt and cGK-Tg mice were given a high-fat (60 kcal%-fat) diet from the age of 10 weeks. [A] Body weight of cGK-Tg mice on standard chow (left panel) and high-fat diet (right panel) (n=8-10). [B] Macroscopic appearance of a Wt and a cGK-Tg mouse. [C] Food intake on standard chow and high-fat diet (upper panel, kcal/day, n=6), and the body weight (BW)-adjusted value (lower panel, kcal/day/g BW). [D] Blood glucose levels determined with the glucose and insulin tolerance tests for each genotype on standard chow and high-fat diet (n=8). [E] CT images obtained at kidney level of a Wt and a cGK-Tg mouse on standard chow (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] Microscopic analysis using hematoxylin and eosin staining of epididymal fats in high-fat fed mice. Scale bar, 100µm. [G] Distribution of adipocyte diameter determined with Coulter counter (n=8). [H] Macroscopic appearance of the liver in high-fat fed mice (upper panel). Microscopic images with Oil-red O staining of the liver (lower panel). Scale bar, 100µm. [I,J] Triglyceride concentration in the liver (I) and the quadriceps (J) (n=12). * P<0.05, ** P<0.01 compared to Wt on the same feeding condition.

**Figure 5:** cGK-transgenic mice exhibit giant mitochondria in the skeletal muscle, associated with higher oxygen consumption and fat oxidation. 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 chow (n=6). [B] Mean oxygen consumption for 24 hours on standard chow or high-fat diet. [C] Rectal temperature on standard chow or high-fat diet (n=12). [D] Mean respiratory quotient for 24 hours on standard chow or high-fat diet (n=6). [E] Mean fat oxidation estimated from the respiratory gas analysis for 24 hours on standard chow or high-fat diet (n=6). [F] Mitochondrial DNA copy number estimated from quantification of mitochondrial and nuclear genome (n=8). [G] Expressions of genes encoding PGC-1α and UCP1 in the brown adipose tissue. The values were standardized to those for the control (Wt mice fed on standard chow) in either group (n=12). [H] Expressions of the genes involved in mitochondrial regulation or fatty acid catabolism in the skeletal muscle (n=12). [I] Electron microscopic analysis of muscle mitochondria of high-fat fed cGK-Tg mice. PGC, PPAR gamma co-activator; ATPsyn, ATP synthase; COX, cytochrome c oxidase; UCP, uncoupling protein; PPAR, peroxisome proliferators-activated receptor; FATP, fatty acid transporter; ACO, acyl-CoA oxidase; CPT, carnitine palmitoyl transferase. * P<0.05, ** P<0.01 compared to Wt mice on the same feeding condition.

**Figure 6:** Natriuretic peptides directly increase the expression of PGC-1α and PPARδ, and mitochondrial content in cultured myocytes. C2C12 myocytes were stimulated with the indicated agents for 8 (A) or 48 (B) hours (ANP 10−11−9 mol/L or BNP 10−11−9 mol/L, with or without the cGMP antagonist, Rp-8-br-cGMP (Rp) 10−4 mol/L). Stimulation of cGK by 8-Br-cGMP (cG) 10−7 mol/L was also challenged. (n=12). [A] Gene expressions of PGC-1α and PPARδ in C2C12 cells when treated with ANP or BNP. [B] Mitochondrial mass in C2C12 cells quantified by use of MitoTracker Green, a fluorescent probe for mitochondria. * P<0.05, **
P<0.01 compared to control, # P<0.05, ## P<0.01 compared to the NP (10^{-9} \text{ mol/L}) treated group. Rp, Rp-8-br-cGMP (cGMP antagonist), cG, 8-Br-cGMP (membrane-permeable cGMP analogue). [C] Schematic representation of the suggested roles for NP/cGK cascades. Previous studies have shown the significant roles for NP/cGK cascades in the cardiovascular system that lead to resistance to ischemia, heart failure and hypertension. In the present study, NP/cGK cascades are suggested to promote muscle mitochondrial biogenesis and fat oxidation, as to prevent obesity, fatty liver and glucose intolerance.
NP/cGMP/cGK cascades prevent obesity

Figure 1

A. Body weight (g) over time for Wt and BNP-Tg on standard chow and high-fat diet.

B. Images of Wt and BNP-Tg mice on standard chow.

C. Food intake (kcal/day) comparison between Wt, BNP-Tg on standard chow and high-fat diet.

D. Glucose tolerance test and insulin tolerance test results for Wt and BNP-Tg on high-fat diet.

E. Imaging of fat distribution on standard chow and high-fat diet for Wt and BNP-Tg.

F. Total fat and epididymal fat content comparison between Wt, BNP-Tg on standard chow and high-fat diet.
NP/cGMP/cGK cascades prevent obesity

Figure 1

G

H

I

J

K

Adipocyte size
(cm²/particle)

Serum leptin
(ng/ml)

Serum adiponectin
(µg/ml)

Triglyceride in the liver
(mg/g tissue)

Triglyceride in the muscle
(mg/g tissue)

WT BNP-Tg
High-fat diet

WT BNP-Tg
Standard chow

WT BNP-Tg
Standard chow

WT BNP-Tg
High-fat diet

WT BNP-Tg
High-fat diet

WT BNP-Tg
Standard chow

WT BNP-Tg
Standard chow

WT BNP-Tg
High-fat diet

WT BNP-Tg
High-fat diet
NP/cGMP/cGK cascades prevent obesity

Figure 2

A. Oxygen consumption (ml/min/kg BW)

B. Oxygen consumption (ml/min/kg BW)

C. Rectal temperature (Celsius)

D. Respiratory quotient

E. Fat oxidation (mg/min/kg BW)

F. Fat oxidation during fasting (mg/min/kg BW)

G. Mitochondrial DNA copy number (% control)

H. Gene expressions (% control)

| Standard chow | Wt (control) | BNP-Tg | High-fat diet | Wt | BNP-Tg |
Figure 3

A. Gene expressions (arbitrary unit)

- Skeletal muscle
- Brown adipose tissue
- White adipose tissue

B. Body weight (g)

- Wt
- GCA+/ (High fat diet)
- GCA+/ standard chow
- High fat diet (45% fat)

C. Food intake (kcal/day)

- Wt
- GCA+/ High fat diet

D. Blood glucose (mg/dl)

- Glucose tolerance test
- Wt
- GCA+/ High-fat diet

E. Total fat (g)

- Wt
- GCA+/ High fat diet
Figure 4

NP/cGMP/cGK cascades prevent obesity
Figure 5

A: Oxygen consumption (ml/min/kg BW)

B: Oxygen consumption (ml/min/kg BW)

C: Rectal temperature (Celsius)

D: Respiratory quotient

E: Fat oxidation (mg/min/kg BW)

F: Mitochondrial DNA copy number (% control)

G: Gene expressions (% control)

H: Gene expressions (% control)
Figure 5

Figure 6

A Expressions in C2C12 myocytes (% control)

B Mitochondrial mass in C2C12 myocytes (% control)
Figure 6

NP/cGMP/cGK cascades prevent obesity

C

Insufficient Oxygen supply (Circulatory failure)

Decrease in Energy source (Starvation)

NP/cGMP/cGK cascades

Vascular wall
Angiogenesis
Vaso-dilation

Kidney
Natriuresis

Skeletal muscle
PGC-1α, PPARs
Increase in Mitochondria

Brown adipose tissue
PGC-1α, UCP1
Thermogenesis

Oxygen supply

Fat oxidation
Oxygen consumption

Coordinated regulation of oxygen supply and consumption

Resistance to Ischemia, Heart failure, and Hypertension

Resistance to Obesity, Fatty liver, and Diabetes