SUPPLEMENT 1

RESEARCH DESIGN AND METHODS

Animal studies. All animal experiments were performed in accordance with the ethical guidelines of Kyoto University and approved by the institutional review board. The generation of BNP-Tg, cGK-Tg and GCA-KO mice used in this study has been described in previous reports [3,11,12]. In brief, for the BNP-Tg mice, a fusion gene composed of the SAP promoter and mouse BNP coding sequences was generated so that the hormone expression targets the liver and is secreted into the circulating blood. We used line #55 of the Tg mice with a plasma BNP concentration of 1.8±1.1 pmol/ml at 4 months of age, which is approximately 100 times higher than the physiological concentration. For the cGK-Tg mice, cDNA encoding human cGK-Iα was fused to the CAG promoter for ubiquitous over-expression of cGK in the Tg mice. We then used quantitative PCR analysis to confirm that the expression of cGK in the skeletal muscle, liver and white adipose tissue was approximately 10 times higher in the Tg mice than in the wild type (Wt) mice. From the age of 10 weeks, the mice were maintained on standard chow (12kcal%-fat, 3.8kcal/g: F-2; Funahashi Farm, Chiba, Japan) or a high-fat diet (60kcal%-fat, 5.2kcal/g: D12492; Research Diets Inc., New Brunswick, NJ) unless indicated otherwise. The mice were kept under specific pathogen-free (SPF) conditions with a 14-h light/10-h dark cycle at 23°C. To explore the metabolic effects of chronic activation of NP/cGK cascades in vivo, we compared the phenotypes of the genetically engineered mice and their Wt littermates.

Physiological and biochemical analysis of mice. The body weight of the mice was measured by means of an electronic scale, beginning at 6 weeks of age. After the mice had become accustomed to individual housing, food quantities were measured daily to determine the food intake (g/day) of an individual mouse. Blood samples were collected from the tail or the retro-orbital vein, and the blood glucose level was determined with the glucose oxidase method (Glutest Sensor; Sanwa Kagaku, Kyoto, Japan), and the serum insulin level with an insulin measurement ELISA kit (Morinaga, Tokyo, Japan). Serum triglyceride and non-esterized fatty acid (NEFA) were measured with enzymatic methods (Triglyceride E-test and NEFA C-test, respectively; Wako, Osaka, Japan), and serum leptin and adiponectin levels with ELISA kits (Morinaga and Otsuka Pharmaceutical, Tokushima, Japan, respectively). For the glucose tolerance test, mice were injected with 1.0 mg/g-BW glucose intra-peritoneally after overnight fasting; and for the insulin tolerance test, they were injected with 0.75 mU/g-BW human regular insulin (Novolin R; Novo Nordisk, Bagsvaerd, Denmark) intra-peritoneally after 6 hours fasting. Urinary epinephrine and norepinephrine excretions were determined with an ELISA (SRL, Tokyo, Japan), and blood pressure was measured with the tail-cuff method. (Softron, Tokyo, Japan). Analyses and blood sampling were performed when the mice were 18~20 weeks old, unless indicated otherwise.

CT analysis of mice. The weight of fat in the whole body was determined with computed tomography (CT) (LaTheta; Aloka Co., Ltd., Tokyo). CT scanning of the entire body was performed at 5-mm intervals, and the fat tissue was automatically identified by the density of the CT images. The weight of fat was then calculated by multiplying the fat volume for the whole body (obtained by integration of the fat area identified on each image) by the fat gravity (0.90 g/cm^3).

Histological analysis of mice. Mice aged 20~22 weeks were sacrificed and their epididymal fat and liver were fixed in 4% formaldehyde. Paraffin-embedded sections with a thickness of 5 μm were stained with hematoxylin and eosin, or Oil-red O, and examined by light microscopy (BX40F4; Olympus, Tokyo, Japan). Mean adipocyte size in BNP-Tg mice was measured in eight fields per mouse by means of image analyzing software (Scion Image; Scion Corporation, Frederick, MD)..

The size of the epididymal fat pad adipocytes in cGK-Tg mice was determined with a Coulter counter and a multi-channel particle analyzer (Multisizer II; Coulter Electronics, Fullerton, CA), as described previously [13]. In brief, the fat pads (approximately 100 mg, pooled from four mice in each group) were fixed with osmium tetroxide, passed through a 250μm nylon filter to remove fibrous elements, and the diameter of a total of 10,000 cells was analyzed. Electron microscopy was performed by a standard method using a transmission electron microscope (JEM-2000EX, JEOL Ltd, Tokyo, Japan).

Respiratory gas analysis of mice. O_2 consumption and CO_2 production were determined by means of indirect calorimetry (RL-600; Arco System, Kashiwa, Japan), as described previously [14]. In brief, room air was pumped through the chamber of an independently housed mouse at a rate of 1.0 L/min. The expired air was then analyzed by mass spectrometry for quantification of the O_2 and CO_2 concentrations. The respiratory quotient and fat oxidation were calculated from the O_2 consumption and CO_2 production by using equations described elsewhere [15].

Quantitative PCR analysis and determination of mitochondrial DNA copy number. Quantitative PCR analysis was performed according to the manufacturer's instructions. Total RNA was extracted with the aid of RNeasy mini kit (Qiagen, Tokyo, Japan) from the skeletal muscle (quadriceps), brown adipose tissue, white adipose tissue (epididymal fat), or cultured cells. RNA samples were reverse transcribed (ExScript RT reagent kit; Takara Bio, Otsu, Japan) and the amount of gene expression was determined by means of quantitative PCR reactions (Applied Biosystems 7500 Real-Time PCR System; Applied Biosystems, Foster City, CA) in the presence of a fluorescent dye (SYBR Premix Ex Taq; Takare Bio). The relative quantity of mRNA was calculated after normalization to beta-actin mRNA. Details for the primers (Takara-Bio) used in this study are available on the manufacturer's homepage (http://www.takara-bio.com).

Mitochondrial DNA copy number was determined by quantitative PCR, using specific primers for mitochondrial DNA encoded 16S rRNA gene and nuclear DNA encoded hexokinase 2 gene, as described previously [16]. Results were estimated from the difference in threshold cycle values (delta-Ct) for the mitochondrial gene and the diploid nuclear gene.

Cell experiments. C2C12 cells (RIKEN BioResource Center, Tsukuba, Japan) were grown to over-confluence, fully differentiated and treated with or without indicated agents: 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, or cell permeable cGMP analogue, 8-Br-cGMP (cG) 10^{-4} mol/L. RNA were extracted from the cells after 8 hours of the treatment for the examination of the gene expression of PGC-1α and PPARδ by quantitative PCR analysis. Mitochondrial mass was determined after 48 hours of the treatment

Probes, Eugene, OR), following the same procedures as described previously [10]

Statistical Analysis. All values were expressed as means±S.E and comparisons between two groups were performed with Student's t test. When more than two groups were compared, analysis of variance (ANOVA) was used to evaluate significant differences among groups, and if significant differences were confirmed, each difference was further examined by means of Fisher's protected least significant difference (PLSD) method. Two-way ANOVA was performed in Supplemental Table 1 and 2. Probability was considered to be statistically significant at $P< 0.05$

SUPPLEMENT 2. Results of the studies that were shown in Figure 3 and Figure 6.

Figure 3 **The expressions of natriuretic peptide receptors are regulated by feeding condition.** To elucidate the physiological regulation of signal transduction through NP/cGK cascades in response to feeding condition, we examined the expressions of NP-receptors by means of quantitative PCR analysis. Both GCA and GCB were up-regulated after 24-hour fasting and the expression levels became approximately 2 times higher in the tissues examined compared to their level during ad-lib feeding (Figure 3A), while that of C-receptor was down-regulated in response to fasting (Figure 3A). In contrast, GCA and GCB were down-regulated after high-fat feeding for 10 weeks, and C-receptor was up-regulated (Figure 3A). The results of two-way ANOVA of the effects of high-fat diet and those of fasting on the expressions of NP-receptors were shown in Supplemental Table 2.

GCA-knockdown mice are susceptible to diet-induced obesity and glucose intolerance. GCA-homozygous knock-out (GCA-/-) mice are known to be hypertensive and easily incur heart failure, meaning that these characteristics could affect the metabolic condition of the mice. We therefore used GCA-heterozygous knockout (GCA+/-) mice, whose blood pressure and circulatory dynamics are almost unchanged, to study the physiological roles for GCA in the regulation of metabolism. We found that heterozygous deficiency of GCA increased susceptibility to high-fat induced obesity, so that the body weight of high-fat-fed GCA+/- mice increased significantly compared to that of Wt mice (Figure 3B), even though food intake was similar for Wt and GCA+/- mice (Figure 3C). The blood glucose levels after administration of glucose were significantly higher in GCA+/- mice on the high-fat diet (Figure 3D). The total fat weight estimated from the CT analysis showed significant increases in high-fat fed GCA+/- mice (Figure 3E), in parallel with the increase in body weight. These findings demonstrate that the phenotype of GCA+/- mice was the opposite to that of BNP-Tg mice in regard to diet-induced obesity and glucose intolerance.

Figure 6 **Natriuretic peptides directly increase the expression of PGC-1α and PPARδ, and mitochondrial content in cultured myocytes.** To confirm direct effects of NP on the gene expressions and mitochondrial content in C2C12 myocytes, the cells were treated with or without 10^{-11}～$^{-9}$ mol/L ANP or BNP for 8 hours for the analysis of gene expressions or 48 hours for that of mitochondrial content. The expression of PGC-1α and PPARδ was dose dependently increased by the treatment with either ANP or BNP (Figure 6A). The effects of the NPs were almost completely nullified by the cGMP antagonist, Rp-8-br-cGMP (Rp) 10^{-4} mol/L (Figure 6A). On the other hand, treatment with the membrane permeable cGMP analogue, 8-Br-cGMP (cG) 10^{-4} mol/L, strongly increased the expressions of the two genes (Figure 6A). Mitochondrial mass, estimated with the aid of a mitochondria-specific fluorescent probe, showed a dose-dependent increase (20% and 23% increase at 10^{-9} mol/l ANP and BNP, respectively; n=12, $p<0.05$ and 0.01; Fig. 6B) in the NP treated groups. In a similar manner with the case of gene expressions, the cGMP antagonist, Rp-8-br-cGMP, nullified the effect of NPs, and the cGMP analogue strongly increased the mitochondrial content (Figure 6B).

Table S1: Two-way ANOVA of the effects of high-fat diet, those of genetic manipulation, and the interaction between them throughout the study.

Figure	Parameter	Genetic manipulation	Effects of High-fat duet	P-value	Effects of Genetic manipulation	P-value	Interaction
1C	Food intake (kcal/day)	BNP-Tg	25% increase	<u>≤0.01</u>	8% decrease	0.45	0.98
1E	Total fat	BNP-Tg	358% increase	<u>≤0.01</u>	23% decrease	<u>0.01</u>	<u>0.03</u>
1F	Epididymal fat	BNP-Tg	327% increase	<u>≤0.01</u>	20% decrease	<u>≤0.01</u>	0.07
1F	Mesenteric fat	BNP-Tg	282% increase	<u>≤0.01</u>	34% decrease	<u>0.01</u>	<u>0.03</u>
1H	Leptin	BNP-Tg	935% increase	<u>≤0.01</u>	47% decrease	<u>0.01</u>	<u>0.03</u>
1H	Adiponectin	BNP-Tg	15% increase	0.39	22% increase	0.19	0.09
1J	Triglyceride in liver	BNP-Tg	138% increase	<u>≤0.01</u>	23% decrease	<u>0.05</u>	0.07
1K	Triglyceride in muscle	BNP-Tg	91% increase	<u>≤0.01</u>	22% decrease	0.07	0.27
2B	Oxygen consumption	BNP-Tg	13% decrease	<u>≤0.01</u>	8% increase	<u>≤0.01</u>	0.08
2C	Rectal temperature	BNP-Tg	1% increase	<u>0.02</u>	0% increase	0.26	0.76
2G	Mitochondrial DNA in BAT	BNP-Tg	12% decrease	0.27	8% increase	0.56	0.84
2G	Mitochondrial DNA in muscle	BNP-Tg	8% decrease	0.49	31% increase	<u>0.03</u>	0.61
2H	PGC1α expression in BAT	BNP-Tg	22% increase	0.21	11% increase	0.57	0.96
2H	UCP1 expression in BAT	BNP-Tg	28% increase	<u>≤0.01</u>	7% increase	0.48	0.27
2H	PGC1α expression in muscle	BNP-Tg	17% increase	<u>0.01</u>	20% increase	<u>≤0.01</u>	0.45
2H	PPARδ expression in muscle	BNP-Tg	38% increase	<u>≤0.01</u>	22% increase	<u>0.03</u>	0.36
4C	Food intake (kcal/day/BW)	cGK-Tg	4% decrease	0.08	12% increase	<u>≤0.01</u>	0.11
4E	Total fat	cGK-Tg	415% increase	<u>≤0.01</u>	48% decrease	<u>≤0.01</u>	0.20
4I	Triglyceride in liver	cGK-Tg	163% increase	<u>≤0.01</u>	26% decrease	<u>0.03</u>	0.12
4J	Triglyceride in muscle	cGK-Tg	100% increase	<u>≤0.01</u>	46% decrease	<u>≤0.01</u>	0.25
5B	Oxygen consumption	cGK-Tg	28% decrease	<u>≤0.01</u>	17% increase	<u>≤0.01</u>	0.69
5C	Rectal temperature	cGK-Tg	1% increase	<u>0.03</u>	1% increase	<u>≤0.01</u>	0.97
5D	Respiratory quotient	cGK-Tg	21% decrease	<u>≤0.01</u>	3% decrease	<u>≤0.01</u>	0.69
5E	Fat oxidation	cGK-Tg	267% increase	<u>≤0.01</u>	43% increase	<u>≤0.01</u>	0.06
5F	Mitochondrial DNA in BAT	cGK-Tg	11% decrease	0.20	51% increase	<u>≤0.01</u>	0.90
5F	Mitochondrial DNA in muscle	cGK-Tg	8% decrease	0.35	58% increase	<u>≤0.01</u>	0.61
5G	PGC1α expression in BAT	cGK-Tg	47% increase	<u>≤0.01</u>	64% increase	<u>≤0.01</u>	0.17
5G	UCP1 expression in BAT	cGK-Tg	32% increase	<u>0.02</u>	78% increase	<u>≤0.01</u>	0.20
5H	PGC1α expression in muscle	cGK-Tg	22% increase	0.22	64% increase	<u>≤0.01</u>	0.84
5H	ATPsyn expression in muscle	cGK-Tg	2% decrease	0.58	34% increase	<u>≤0.01</u>	0.46
5H	COX expression in muscle	cGK-Tg	12% decrease	0.09	28% increase	<u>≤0.01</u>	0.89
5H	UCP3 expression in muscle	cGK-Tg	97% increase	<u>≤0.01</u>	63% increase	<u>≤0.01</u>	<u>0.01</u>
5H	PPARδ expression in muscle	cGK-Tg	38% increase	<u>0.05</u>	102% increase	<u>≤0.01</u>	0.27
5H	FATP expression in muscle	cGK-Tg	164% increase	<u>≤0.01</u>	68% increase	<u>0.01</u>	0.06
5H	ACO expression in muscle	cGK-Tg	47% increase	<u>0.02</u>	88% increase	<u>0.02</u>	0.19
5H	CPT1b expression in muscle	cGK-Tg	48% increase	<u>≤0.01</u>	55% increase	<u>≤0.01</u>	0.15

Interaction: Interaction between the effects of high-fat diet and those of genetic manipulation

Underline in the P-value and interaction: values < 0.05

We performed the analysis in order to clarify whether the effects of the NP/cGK system were reversal of the effects of high-fat diet or simple pharmacological effects. As shown in the column on the far right, the interactions of them were weak. Therefore, we assume that the NP/cGK has pharmacological effects to increase mitochondria biogenesis and fat oxidation, regardless of the dietary condition, which are not due to reversal of the effects of high-fat diet.

Supplemental Table S2: Statistical analysis of the effects of high-fat diet and those of fasting on the expressions of NP-receptors, the results of which were shown in Figure 3A.

Figure		Effects of High-fat duet		Effects of Fasting		Interaction
	Parameter		P-value		P-value	
3A	GCA in muscle	66% decrease	<u>≤0.01</u>	130% increase	<u>0.01</u>	0.24
3A	GCA in BAT	55% decrease	<u>0.02</u>	79% increase	0.07	0.50
3A	GCA in WAT	74% decrease	<u>≤0.01</u>	207% increase	<u>0.04</u>	0.46
3A	GCB in muscle	51% decrease	<u>≤0.01</u>	82% increase	<u>≤0.01</u>	0.44
3A	GCB in BAT	62% decrease	<u>≤0.01</u>	36% increase	0.25	0.37
3A	GCB in WAT	73% decrease	<u>≤0.01</u>	266% increase	<u>0.02</u>	0.60
3A	C-receptor in muscle	332% increase	<u>≤0.01</u>	30% decrease	0.08	0.31
3A	C-receptor in BAT	86% increase	<u>≤0.01</u>	42% decrease	<u>≤0.01</u>	<u>0.04</u>
3A	C-receptor in WAT	140% increase	<u>0.02</u>	54% decrease	<u>0.02</u>	0.17

Interaction: Interaction between the effects of high-fat diet and those of fasting.

Underline in the p-value and interaction: $p<0.05$