Defective natriuretic peptide receptor signaling in skeletal muscle links obesity to type 2 diabetes

Marine Coué1,2, Pierre-Marie Badin1,2, Isabelle K. Vila1,2, Claire Laurens1,2, Katie Louche1,2, Marie-Adeline Marquès1,2, Virginie Bourlier1,2, Etienne Mousiel1,2, Geneviève Tavernier1,2, Arild C. Rustan3, Jose E. Galgani4, Denis R. Joanisse5, Steven R. Smith6, Dominique Langin1,2,7, and Cedric Moro1,2

1INSERM, UMR1048, Obesity Research Laboratory, Institute of Metabolic and Cardiovascular Diseases, Toulouse, France; 2University of Toulouse, UMR1048, Paul Sabatier University, France; 3Department of Pharmaceutical Biosciences, School of Pharmacy, University of Oslo, Norway; 4School of Medicine, Pontificia Universidad Católica de Chile, Santiago, Chile; 5Department of Kinesiology, Centre de Recherche de l’Institut Universitaire de Cardiologie et de Pneumologie de Québec, Laval, Canada; 6Translational Research Institute for Metabolism and Diabetes, Florida Hospital, Sanford-Burnham Medical Research Institute, Orlando, Florida, USA; 7Toulouse University Hospitals, Department of Clinical Biochemistry, Toulouse, France

Corresponding author: Cedric Moro, Ph.D.

Inserm UMR1048, Institute of Metabolic and Cardiovascular Diseases, Obesity Research Laboratory, CHU Rangueil, BP84225, 1 avenue Jean Poulhès, 31432 Toulouse cedex 4, France; Phone: +33 561 32 5626; Fax: +33 561 32 5623; E-mail: Cedric.Moro@inserm.fr

Main Text Word count: 4862

Abstract Word count: 199

Number of figures: 8

Supplementary data
Abstract

Circulating natriuretic peptide (NP) levels are reduced in obesity and predict the risk of type 2 diabetes (T2D). Since skeletal muscle was recently shown as a key target tissue of NP, we aimed to investigate muscle NP receptor (NPR) expression in the context of obesity and T2D. Muscle NPRA correlated positively with whole-body insulin sensitivity in humans, and was strikingly down-regulated in obese subjects and recovered in response to diet-induced weight loss. In addition, muscle NP clearance receptor (NPRC) increased in individuals with impaired glucose tolerance and T2D. Similar results were found in obese diabetic mice. Although no acute effect of brain-NP (BNP) on insulin sensitivity was observed in lean mice, chronic BNP infusion improved blood glucose control and insulin sensitivity in skeletal muscle of obese and diabetic mice. This occurred in parallel of a reduced lipotoxic pressure in skeletal muscle due to an up-regulation of lipid oxidative capacity. In addition, chronic NP treatment in human primary myotubes increased lipid oxidation in a PGC1α-dependent manner and reduced palmitate-induced lipotoxicity. Collectively, our data show that activation of NPRA signaling in skeletal muscle is important for the maintenance of long-term insulin sensitivity and has the potential to treat obesity-related metabolic disorders.
Introduction

Obesity is a major risk factor of type 2 diabetes (T2D) and cardiovascular diseases (1, 2). Although multiple hypotheses have been proposed, the link between obesity and the risk of T2D is still poorly understood. Over the last decade, several large cohort studies reported an inverse association between plasma natriuretic peptide (NP) levels and body mass index (3, 4), and the risk of T2D (5, 6). Therefore, dysregulation of the NP system referred as the "NP handicap", might be an important factor in the initiation and progression of metabolic dysfunction, making NP potential candidates linking obesity and T2D (7-10).

NP including atrial-NP (ANP) and brain-NP (BNP) are mainly known as heart hormones secreted in response to cardiac overload and mechanical stretch in order to regulate blood volume and pressure (11, 12). ANP and BNP classically bind to a biologically active receptor-A (NPRA) that promotes cGMP signaling (13). They are also quickly cleared from the circulation and degraded through NP clearance receptor (NPRC). The NPRA-to-NPRC ratio therefore controls the biological activity of NP at the target tissue level (14).

Besides their well-documented role in the cardiovascular system, several studies revealed a metabolic role of NP (15, 16). Pioneering studies demonstrated a potent lipolytic role of these peptides in human adipose tissue (17, 18), while more recent studies indicated they may play a role in the "browning" of human white fat cells (19) as well as in favoring fat oxidative capacity in human skeletal muscle cells (20). The underlying mechanism involves activation of cGMP signaling, induction of PGC1α (peroxisome proliferator-activated receptor gamma coactivator-1α) and enhancement of mitochondrial respiration. Together these studies argue for an important role of NP in the regulation of whole-body energy metabolism.

The lipolytic effect of NP is absent in mice naturally expressing high levels of NPRC in adipose tissue (19, 21). However mice overexpressing BNP are protected from diet-induced obesity and insulin resistance, which suggests that the protective effect of NP is achieved by targeting other metabolic tissues such as skeletal muscles (22). We therefore hypothesized that a down-regulation of NPRA and/or an up-regulation of NPRC in skeletal muscle could
contribute to the “NP handicap” and provide a novel pathophysiological and mechanistic link between obesity and T2D.

In the current study, through a comprehensive set of experiments in humans, mouse models of obesity and T2D, and human primary skeletal muscle cells, we demonstrated a pathophysiological link between obesity-induced insulin resistance and T2D and defective skeletal muscle NPR signaling. In addition, increasing circulating BNP levels in diabetic and high-fat diet-fed mice improved blood glucose control and insulin sensitivity. These effects were accompanied by improved muscle insulin signaling resulting from reduced lipotoxic lipid pressure and elevated lipid oxidative capacity.
Research Design and Methods

Clinical studies and human subjects

Muscle biopsy samples from lean, obese with normal glucose tolerance, obese with impaired glucose tolerance and obese with type 2 diabetes subjects were obtained from three independent clinical studies. Study 1 included nine young lean and nine young obese subjects (Figure 1A-D) (23). Study 2 included four middle-aged obese subjects with type 2 diabetes, and six with impaired glucose tolerance at baseline and in response to 12-weeks of calorie restriction to induce weight loss and improve metabolic health (Figure 1E-F) (24). Study 3 included twenty one subjects with normal glucose tolerance but a wide range of body fat (Supplemental Figure 1) (25). The clinical characteristics of the subjects are summarized in Supplemental Table 1. All volunteers gave written informed consent and the protocol was approved by an institutional review board. Studies were performed according to the latest version of the Declaration of Helsinki and the Current International Conference on Harmonization guidelines. Samples of vastus lateralis weighing 60–100 mg were obtained by muscle biopsy using the Bergström technique, blotted, cleaned, and snap-frozen in liquid nitrogen (26). Insulin sensitivity was measured by hyperinsulinemic euglycemic clamp after an overnight fast (27). An intravenous catheter was placed in an antecubital vein for infusion of glucose and insulin during the clamp. A second catheter was placed retrograde in a dorsal vein of the contralateral hand for blood withdrawal. The hand was placed in a plastic heated box at ~60°C for arterialization of venous blood. Three blood samples were drawn before the initiation of insulin and glucose for the clamp and during the last 30 minutes of the clamp. A primed infusion of regular insulin (80 mU.min⁻¹.m⁻²) was initiated and continued for 2h. Plasma glucose was clamped at 90 mg/dl in all participants. Arterialized plasma glucose was measured at 5 min intervals and a variable infusion of exogenous glucose (20% solution) is given to maintain plasma glucose concentration. Plasma glucose was analyzed with a Yellow Springs Instrument (YSI) 2300 STAT Glucose Analyzer (Yellow Springs Instruments Incorporated, Yellow Springs, OH), and plasma insulin was measured using an ultrasensitive ELISA kit (ALPCO Diagnostics, Salem, New Hampshire). Glucose disposal rate was
adjusted by kilograms of fat-free mass. Body composition (considering a 3-compartments
model) was determined using a total body Dual-Energy X-ray Absorptiometer (DPX,
Software 3.6, Lunar Radiation Corp., Madison, WI).

**Mice and diets**

Five-week-old male diabetic-prone, obese db/db mice of the C57BL/KsJ-lept\(^{+}\)-lept\(^{db}\) strain
with their non-diabetic lean littermates control db/+ were used. For high fat diet studies, we
used regular C57BL/6J male mice (Janvier laboratories). The mice were housed in a
pathogen-free barrier facility (12h light/dark cycle) with *ad libitum* access to water and food.
After weaning, db/db and db/+ mice were fed a normal chow diet (A04, SAFE Diets) for 4
weeks. C57BL/6J mice were fed for 16 weeks either a normal chow diet (10% energy as fat,
Research Diets D12450J; Inc, New Brunswick, New Jersey) or high fat diet (HFD) containing
60% Kcal from fat (Research Diets D12492; Inc, New Brunswick, New Jersey). All
experimental procedures were approved by a local institutional animal care and use
committee and performed according to INSERM guidelines for the care and use of laboratory
animals.

**BNP infusion studies**

Mice were randomly assigned to receive a saline vehicle (NaCl 0.9%) and/or chronic
rat/mouse BNP1-32 (B9901, Sigma-Aldrich) at a rate of 5 ng/kg/min or 10 ng/kg/min.
Treatments were chronically administered intraperitoneally with mini-osmotic pumps (Alzet,
model 1004; Cupertino, CA, USA) (28). Mini-pumps were placed after 12 weeks of HFD and
treatment was administered for 4 weeks in C57BL/6J mice and at 6 weeks of age in db/db
mice. Body weight was measured weekly and body composition was assessed by
quantitative nuclear magnetic resonance imaging (EchoMRI 3-in-1 system; Echo Medical
Systems).

**Glucose and Insulin Tolerance Tests**
Six hour-fasted mice were injected intraperitoneally with a bolus of D-glucose at 2g/kg (Sigma-Aldrich, Saint-Quentin Fallavier, France) and insulin 0.5U/kg (Insuman Rapid, Sanofi Aventis, France) for glucose and insulin tolerance tests respectively (GTT and ITT) (29). Blood glucose levels were monitored from the tip of the tail with a glucometer (Accucheck; Roche, Meylan, France) at 0, 15, 30, 45, 60, and 90 minutes after injection. Radiolabeled glucose tolerance tests were performed as previously described (29).

Blood analyses and tissue collection

After an overnight fast, mice were decapitated and blood collected into tubes containing EDTA and protease inhibitors. Organs and tissues were rapidly excised and snap frozen in liquid nitrogen before being stored at -80°C. Blood glucose was assayed using the glucose oxidase technique (Biomérieux, Paris, France), and plasma insulin was measured using an ultrasensitive ELISA kit (ALPCO Diagnostics, Salem, New Hampshire). Plasma BNP was measured using the RayBio BNP Enzyme Immunoassay Kit (RayBiotech, Inc., Norcross, Georgia, USA). HbA1c and fructosamines were determined using a PENTRA 400 multi-analyzer.

Human skeletal muscle cell culture

Satellite cells from rectus abdominis biopsies of healthy subjects with normal glucose tolerance (age 34.3 ± 2.5 years, BMI 26.0 ± 1.4 kg/m², fasting glucose 5.0 ± 0.2 mM) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS and growth factors (human epidermal growth factor, BSA, dexamethasone, gentamycin, fungizone, fetuin) as previously described (23, 30). Myotubes were differentiated up to 5 days and were treated with 100 nM human ANP (A1663, Sigma-Aldrich) or BNP (B5900, Sigma-Aldrich) every day for the last 3 days.

Determination of FA metabolism
Pulse-chase experiments to determine lipolytic flux and oleate incorporation into total lipids, TAGs, DAGs by thin-layer chromatography were performed as previously described (31). Incorporation rates were normalized to total protein content in each well. Palmitate oxidation rates was measured as described previously (25).

**Lipid intermediates determination**

Triacylglycerol and diacylglycerol content were measured by gas chromatography, ceramide and sphingomyelin species by HPLC-MS after total lipid extraction as previously described for mouse and human muscle tissues (25, 29).

**Western blot analysis**

Soleus and gastrocnemius skeletal muscles, and white and brown adipose tissues, and myotubes were homogenized in a buffer containing 50 mM HEPES, pH 7.4, 2 mM EDTA, 150 mM NaCl, 30 mM NaPO₄, 10 mM NaF, 1% Triton X-100, 10 µl/ml protease inhibitor (Sigma-Aldrich), 10 µl/ml phosphatase I inhibitor (Sigma-Aldrich), 10 µl/ml phosphatase II inhibitor (Sigma-Aldrich), and 1.5 mg/ml benzamidine HCl. Tissue homogenates were centrifuged for 25 min at 15,000 g, and supernatants were stored at -80°C. Solubilized proteins (30-40 µg) were run on a 4-20% SDS-PAGE (Biorad), transferred onto nitrocellulose membrane (Hybond ECL, Amersham Biosciences) and blotted with the following primary antibodies: NPRA (Abcam), NPRC (Sigma-Aldrich), Akt (Cell Signaling Technology [CST] Inc., Beverly, MA), phospho-Akt Ser473 (CST), p-Thr180/Tyr182-p38 MAPK (CST), p38 MAPK (CST), HSL (CST), phospho-HSL Ser660 (CST) and phospho-HSL Ser565 (CST). Subsequently, immunoreactive proteins were blotted with secondary HRP-coupled antibodies and revealed by enhanced chemiluminescence reagent (SuperSignal West Dura or SuperSignal West Femto; Thermo Scientific), visualized using the ChemiDoc MP Imaging System and data analyzed using the Image Lab 4.1 version software (Bio-Rad Laboratories, Hercules, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (CST) and α-tubulin
(Sigma-Aldrich) were used as internal controls for skeletal muscle and myotubes, while β-actin (CST) was used as internal control for adipose tissues.

Real-time qRT-PCR

Total from tissues and primary myotubes were processed for RNA extraction using the RNeasy RNA mini kit (Qiagen GmbH, Hilden, Germany). After reverse transcription of total RNA (1 µg), samples were analyzed on a StepOnePLus real-time PCR system (Applied Biosystems). All primers were obtained from Applied Biosystems and presented in Supplemental Table 5. All expression data were normalized by the $2^{\Delta\Delta C_t}$ method using 18S as internal control.

Statistics

Statistical analyses were performed using GraphPad Prism 5.0 for Windows (GraphPad Software Inc.). Normal distribution and homogeneity of variance of the data were tested using Shapiro-Wilk and F tests, respectively. One-way ANOVA followed by Tukey’s post-hoc tests and Student’s t-tests were performed to determine differences between groups, interventions and treatments. Two-way ANOVA followed by Bonferonni’s post hoc tests were applied when appropriate. Linear regression was performed after log transformation of nonparametric data. The false discovery rate for multiple testing was controlled by the Benjamini-Hochberg procedure with $p_{adj}$ values $\leq 0.05$ as threshold. All values in figures and tables are presented as mean $\pm$ SEM. Statistical significance was set at $P < 0.05$. 
Results

Muscle NPRA and NPRC proteins relate to insulin sensitivity in humans

Muscle NPRA protein expression was investigated in human vastus lateralis biopsies of healthy volunteers with varying degree of body fat and insulin sensitivity. We observed that muscle NPRA protein was inversely related to body fat (Figure 1A and Supplemental Figure 1A), BMI, fasting insulin, indices of insulin resistance (Supplemental Table 2). In addition, muscle NPRA correlated positively with whole-body insulin sensitivity measured by euglycemic hyperinsulinemic clamp (Figure 1B) and the insulin sensitivity index (Supplemental Figure 1B), and negatively with total muscle saturated ceramide content (Figure 1C). Importantly, muscle NPRA protein content was significantly reduced (~65%) in obese subjects when compared to age-matched lean subjects (Figure 1D). Conversely, muscle NPRA protein was up-regulated (1.8 fold) together with insulin sensitivity (+37%, 5.4±0.6 vs. 7.4±1.1 mg/min/kg for pre- and post-calorie restriction respectively, p=0.03) in obese subjects with impaired glucose tolerance in response to diet-induced weight loss (Figure 1E). Finally, muscle NPRC protein content was unchanged in obese versus lean individuals with normal glucose tolerance (0.41±0.08 vs. 0.29±0.07 A.U., NS) but increased significantly in obese individuals with impaired glucose tolerance (IGT) and T2D (Figure 1F). The ratio of NPRA-to-NPRC protein was significantly reduced in obese vs. lean subjects (2.1±0.3 vs. 3.6±0.2 A.U. respectively, p=0.0005) and increased in obese subjects in response to calorie restriction (0.38±0.16 vs. 0.14±0.04 A.U. respectively, p=0.08). Together this suggests that skeletal muscle NPR expression relates to insulin sensitivity in humans and is altered in obesity and T2D.

Impaired NPRA expression in skeletal muscle and fat of diet-induced obese mice

Since both skeletal muscle and adipose tissue are known as key target tissues of NP, both in humans and mice, we further examined NPR expression in metabolic tissues of chow-fed versus HFD-fed mice. In line with human data, we found a significant down-regulation of NPRA protein in skeletal muscle (Figure 2A and D), as well as in white (Figure
2B and D) and brown fat (Figure 2C and D) of HFD-fed mice. No significant change in NPRC protein content was found in skeletal muscle and brown fat, while NPRC protein decreased significantly in white fat (0.48±0.08 vs. 0.14±0.04 A.U. for chow and HFD respectively, p<0.05). Plasma BNP levels were unchanged in HFD-fed mice compared to chow-fed mice (Figure 2E). Collectively, as in humans, our data indicate a reduced NPRA expression in skeletal muscle of obese mice.

Chronic BNP infusion protects against HFD-mediated obesity and glucose intolerance

Since muscle NPRA is associated with insulin sensitivity in humans, we assessed the effect of acute and chronic BNP infusion on glucose tolerance and insulin sensitivity in chow-fed and HFD-fed mice. Acute i.p. BNP injection did not affect fasting blood glucose levels over a time-course of 30 min (Supplemental Figure 2A), and had no effect on glucose excursion during an i.p. GTT (Supplemental Figure 2B). No effect of acute BNP injection was also seen on glucose disposal in skeletal muscle (Supplemental Figure 2C). We further assessed the influence of acute NP treatment on basal and insulin-stimulated glucose uptake in human primary skeletal muscle cells. No effect of increasing doses of ANP and BNP on glucose uptake was observed (Supplemental Figure 3). From these data we concluded that NPRA signaling does not acutely modulate glucose uptake in skeletal muscle.

Based on a previous study (32), we next infused BNP at a dose of 5 ng/kg/min which raised plasma BNP levels by ~40% (data not shown). BNP-treated mice had a similar body weight (Figure 3A) and body composition (Figure 3B) after saline and BNP treatment. Chronic BNP treatment significantly reduced fasting blood glucose levels in mice fed a HFD for 8 or 12 weeks (Figure 3C). Lower blood glucose in the fasting state was also accompanied by improved glucose tolerance (Figure 3D) in face of no change in fasting and peak insulin at 15 min during the intraperitoneal glucose tolerance test (GTT) (Figure 3E). In conclusion, while acute BNP treatment has no effect on insulin sensitivity, chronic BNP treatment improves glucose tolerance in HFD-fed mice.
Impaired NPR signaling in skeletal muscle of obese diabetic mice contributes to the “NP handicap”

We next examined NPR expression in metabolic tissues from leptin receptor-deficient mice (db/db) that become spontaneously obese and T2D by the age of 8 weeks. In line with data in human skeletal muscle and HFD-fed mice, NPRA protein was down-regulated in white (Figure 4B-D) and brown fat (Figure 4C-D) of db/db mice compared to control db/+ mice. In agreement with data in IGT/T2D individuals (Figure 1E), we noted a remarkable up-regulation of NPRC in skeletal muscle (Figure 4A-E), as well as in white (Figure 4B-E) and brown (Figure 4C-E) fat of db/db mice. Overall the NPRA-to-NPRC protein ratio was markedly down-regulated in muscle and fat of db/db mice (Figure 4F), and associated with dramatically lower levels of plasma BNP in db/db mice (-80%, p<0.05) (Figure 4G). This was also associated with a remarkable down-regulation of p38 MAPK phosphorylation in skeletal muscle of db/db mice (-55%, p<0.001) (Supplemental Figure 4). Importantly, muscle NPRC was negatively correlated with plasma BNP levels (Supplemental Table 3). These changes in NPR signaling and plasma NP characterized a “NP handicap” of db/db mice. No association was found between white and brown fat NPRC protein and plasma BNP levels (data not shown). However, muscle NPRC was positively related to fasting blood glucose, insulin and HbA1c (Supplemental Table 4), again suggesting a link between defective skeletal muscle NPR signaling and impaired glucose control. Collectively, these data suggest that obesity and T2D are accompanied by profound changes in NPR expression and signaling in skeletal muscle which may contribute to reduced plasma BNP levels.

Chronic BNP infusion improves blood glucose control in obese diabetic mice

We next studied the influence of chronic (4 weeks) BNP infusion on blood glucose control in db/db mice. BNP was infused at a dose of 10 ng/kg/min to induce a nearly two fold increase in plasma BNP levels with the goal of rescuing the “NP handicap”. Despite no change in body weight (Figure 5A) and composition (Figure 5B), BNP-treated db/db mice displayed significantly improved blood glucose control by reducing fasting plasma glucose (-
21%) (Figure 5C) and HbA1c (-17%) (Figure 5D). This improved blood glucose control occurred in absence of noticeable changes in fasting insulin (Figure 5E). In addition insulin tolerance (Figure 5F) and insulin responsiveness (AIC ITT, area above the curve during the ITT, +36%, p=0.08) were improved in BNP-treated mice. In summary, chronic BNP treatment improves blood glucose control and peripheral insulin sensitivity in obese diabetic mice independently of changes in body weight, thus suggesting direct effect of NP on metabolic organs.

Enhanced insulin signaling, reduced lipotoxicity and increased lipid oxidative capacity in skeletal muscle of BNP-treated mice

We next studied the mechanism by which chronic BNP treatment improved blood glucose control and muscle insulin sensitivity in both HFD-fed and db/db mice. Insulin sensitivity is inhibited by the accumulation of toxic lipids such as diacylglycerols (DAG) and ceramides in skeletal muscle and liver (33, 34). No significant change in total DAG and ceramides was found in liver of BNP-treated db/db (Supplemental Figure 5A-B) and HFD-fed mice (Supplemental Figure 6A-B). No change as well in mRNA levels of genes involved in fat oxidation and glucose metabolism were observed BNP infusion in liver of db/db (Supplemental Figure 5C) and HFD-fed mice (Supplemental Figure 6C). Similarly, no change in the expression level of classical thermogenic genes in brown and white fat depots was observed in BNP-treated db/db (Supplemental Figure 7) and HFD-fed mice (Supplemental Figure 8). No change in Ucp1 mRNA levels was noted as well in inguinal white adipose tissue (data not shown). However, we observed a muscle-autonomous improvement of insulin-mediated Akt (46%, p=0.02) and p38MAPK phosphorylation (278%, p=0.06) (Figure 6A), which was paralleled by a reduced content of total and main species (data not shown) of ceramides (-17%) (Figure 6B) as well as total and main species (data not shown) of sphingomyelin (-19%) (Figure 6C) in skeletal muscle of BNP-treated HFD-fed mice, as well as a reduced content of total ceramides in db/db mice (52.4±4.4 vs. 40.0±5.1 ng/µg protein for db/+ and db/db mice respectively, p<0.05). The content of total and subspecies of
Diacylglycerols was also reduced in BNP-treated HFD-fed mice (ANOVA p<0.05) (Figure 6D). This lower lipotoxic pressure was paralleled by an up-regulation of muscle palmitate oxidation rate (+46%) (Figure 6E), and of PGC1α mRNA levels in HFD-fed mice (Figure 6F) and in db/db mice (+32%, p=0.08). Collectively, the data indicate that chronic BNP treatment improves insulin sensitivity in skeletal muscle by reducing lipotoxicity and up-regulating fat oxidative capacity in a PGC1α-dependent manner in obese and diabetic mice.

**Chronic NP treatment reduces lipotoxicity and enhances lipid oxidative capacity in human primary myotubes**

We previously demonstrated a functional NPR signaling in human primary myotubes (20). Because NP are known to activate lipolysis in human adipocytes (35, 36), we here studied the acute effect of NP treatment on lipid metabolism. Acute treatment of myotubes with BNP did not influence lipid storage, endogenous TAG-derived fatty acid (FA) release (i.e. lipolysis) (Supplemental Figure 9A), and endogenous TAG-derived FA oxidation (Supplemental Figure 9B). We further tested whether NP could activate one of the rate-limiting enzymes of lipolysis. Acute BNP treatment of human myotubes did not influence hormone-sensitive lipase phosphorylation neither on the activating Ser660 residue (Supplemental Figure 9C) nor on the inhibitory Ser565 residue (Supplemental Figure 9D). In contrast, chronic treatment with NP for 3 days robustly reduced total lipid accumulation, total TAG and DAG content (one-way ANOVA p<0.001) (Figure 7A-C). In line with ex vivo muscle data in mice (Figure 6), reduced lipid accumulation was concomitant with an up-regulation of palmitate oxidation rate (+27% and +19% respectively for ANP and BNP treatment) (Figure 7D), and a significant induction of PGC1α gene expression (Figure 7E), which was independent of PPARδ activation (Figure 7F). No change in myogenic gene expression and differentiation of myoblasts into myotubes was observed in response to chronic NP treatment (data not shown). Based on the findings that muscle NPRA protein relates inversely with saturated ceramides content in human skeletal muscle (Figure 1C) and that chronic BNP treatment reduces ceramide content in skeletal muscle of HFD-fed mice (Figure 6B), we
assessed the influence of chronic NP treatment on ceramide content in human primary myotubes. No significant effect of NP treatment on the content of total ceramides and various ceramide species (Supplemental Figure 10) was noticed in the basal condition with FA-free BSA treatment. When myotubes were challenged overnight with 500 µM of palmitate/BSA to induce ceramide production (2.7 fold, p=0.001), we observed a significant decrease of about 30% in total and various ceramide species analyzed in response to chronic ANP and BNP treatment (Figure 7G). In summary, chronic NP treatment protects against lipotoxicity by up-regulating lipid oxidative capacity in human primary myotubes.
**Discussion**

Although longitudinal prospective studies evoked that high baseline levels of plasma NP confer a reduced risk of developing T2D (5, 6), no study so far had demonstrated a mechanistic link between NP biological activity and T2D. We believe our data provide the first evidence that NPRA signaling in skeletal muscle is necessary for the maintenance of long-term insulin sensitivity by regulating lipid oxidative capacity and metabolism (Figure 8). Our data show for the first time that muscle NPRA signaling is impaired in the context of obesity and glucose intolerance in humans and mice. We also provide evidences that up-regulation of NPRC in muscle tissue can contribute to the “NP handicap” observed in T2D.

Last but not least, increasing NP levels in obese and diabetic mice, with the goal to rescue the “NP handicap” and so a normal NPRA signaling tissue response, markedly improves blood glucose control and insulin sensitivity in skeletal muscle.

We first observed a significant positive association between muscle NPRA protein and insulin sensitivity measured by clamp in humans, at a dose that mainly reflects skeletal muscle insulin sensitivity. This observation is consistent with the negative association that we found between muscle NPRA and body fat, and between muscle NPRA and muscle total saturated ceramide content, two factors negatively influencing whole-body and muscle insulin sensitivity (33, 34). To our knowledge this is the first study reporting an association between skeletal muscle NPRA signaling and insulin sensitivity. This indicates that besides plasma NP levels, NPR signaling in skeletal muscle may influence insulin sensitivity. Additionally, muscle NPRA protein was dramatically down-regulated in obese individuals while increased in response to diet-induced weight loss and related improvement in insulin sensitivity. Although the biological factors modulating muscle NPRA protein content were not investigated in the current study, the data suggest that muscle NPRA behaves as a determinant of insulin sensitivity. Moreover, up-regulation of muscle NPRC as glucose tolerance deteriorates in obese subjects with impaired glucose tolerance and T2D can further repress biological activation of muscle NPRA and contribute to the “NP handicap” on the long-term. Considering that muscle mass represents up to 40% of total body weight, even a
moderate increase in muscle NPRC expression could largely reduce plasma NP levels by an increased rate of clearance. Muscle NPRC might be induced by high blood insulin levels in obese subjects as glucose tolerance worsens independently of blood glucose concentrations as previously shown in adipose tissue (37). Although obese control and IGT/T2D were not age-matched, increased expression of NPRC in skeletal muscle appeared independent of age since no correlation between age and muscle NPRC protein was found. Importantly, these findings in human muscle were largely replicated in obese diabetic mice. NPRC protein content was increased in skeletal muscle, white fat, and brown fat of obese diabetic mice, but only muscle NPRC protein negatively correlated with plasma BNP levels, reflecting that an increased plasma BNP clearance by the muscle can contribute to the “NP handicap” observed in these mice. Our data are in line with other studies demonstrating that elevated NPRC mRNA levels in white fat relates to metabolic dysfunction in mice and humans (22, 38, 39). Our data also provide a mechanistic understanding of the tight link observed between the NP handicap and insulin resistance independently of obesity in humans (40). The “NP handicap” concept is supported by the fact that the half-life of NP in the blood circulation is substantially increased in NPRC knockout mice and the biological activity of NP significantly increased in target tissues (41). Importantly, the altered NPRA-to-NPRC protein ratio in skeletal muscle was accompanied by a marked alteration of p38 MAPK phosphorylation in db/db vs. db/+ mice, thus indicating a potential signaling defect, considering that p38 MAPK is recognized as a canonical downstream molecular effector of the NPR signaling pathway (19).

Despite the observed link between muscle NPRA and insulin sensitivity, acute injection of BNP had no impact on fasting blood glucose, glucose tolerance and muscle insulin sensitivity in mice. Furthermore, no acute effect of NP on glucose uptake was observed in human primary myotubes. These findings are in agreement with at least one human study reporting no acute effect of BNP on insulin sensitivity and insulin secretion (42). Altogether these data indicate that NP signaling does not acutely modulate skeletal muscle glucose uptake in vivo. We therefore performed chronic BNP infusion studies in HFD-fed and
obese diabetic db/db mice to assess the long-term influence of BNP treatment on blood glucose control and insulin sensitivity. BNP was preferred for infusion studies as it has a higher half-life than ANP (14). Strikingly, chronic BNP infusion, at doses mimicking a physiological increase of the peptide and targeted to rescue the “BNP handicap” and/or a normal tissue NPRA signaling response, improved very significantly blood glucose control in both mouse models of obesity-induced glucose intolerance and T2D. We observed over 20% reduction in fasting blood glucose levels as well as over 15% decrease in HbA1c which is clinically meaningful and strongly reduces the risk of T2D complications (43). Reduced blood glucose levels during fasting and upon oral glucose challenge occurred in absence of changes in blood insulin levels indicating an improved metabolic clearance of glucose and insulin sensitivity. These findings are in agreement with other studies showing that increasing plasma BNP levels either pharmacologically (32) and/or genetically (22) improves glucose tolerance in obese mice. Preliminary evidence from our laboratory indicates that ANP knockout mice are insulin resistant under normal chow diet compared to their wild-type littermate (data not shown), again arguing for a direct physiological link between NP signaling and insulin sensitivity.

Improved blood glucose control and insulin sensitivity were independent of significant changes in total DAG and ceramides in liver, neither with noticeable changes in expression level of key metabolic genes in liver and thermogenic genes in white and brown fat of BNP-treated db/db and HFD-fed mice. However BNP-treated obese mice had an increased insulin-mediated Akt activation in skeletal muscle. Because Akt activation and phosphorylation is inhibited by lipotoxic lipids such as ceramides and DAG (33, 34), we measured ceramides and DAG in skeletal muscle. In agreement with the negative correlation found in humans between muscle NPRA and ceramides content, we found a reduced level of total and main species of ceramides as well as and main species of sphingomyelin in muscle of both HFD-fed and db/db mice chronically treated with BNP. Ceramides inhibit Akt activation and are produced de novo from saturated fatty acids and from sphingomyelin degradation (33). We also observed reduced muscle total DAG levels in BNP-treated mice.
Interestingly, the reduced muscle lipotoxic lipid level was accompanied by a significant up-regulation of muscle fat oxidative capacity and PGC1α gene expression. To demonstrate that elevated lipid oxidative capacity can reduce lipid accumulation, we chronically treated human primary myotubes with NP and showed increased palmitate oxidation rates and robustly reduced total lipid, TAG, and DAG accumulation. Chronic NP treatment also prevented palmitate-induced ceramide production in human primary myotubes. Although the precise mechanism was not investigated, it is likely that NP treatment reduces de novo ceramide production by increasing palmitate oxidation. We also show that NP-mediated elevated lipid oxidation involved the induction of PGC1α which was independent of PPARδ activation. We and others previously described a cGMP-dependent induction of PGC1α gene expression by NP in white fat and skeletal muscle cells (19, 20). PPARδ can be activated by lipid ligands derived from endogenous TAG lipolysis (31, 44). In contrast to what has been shown in human fat cell (35, 36), acute NP treatment of human primary myotubes did not influence the rate of lipolysis and TAG-derived FA oxidation or HSL phosphorylation at key regulatory sites.

Our findings provide the first evidence that NPRA signaling in skeletal muscle is pivotal for the maintenance of long-term insulin sensitivity by regulating lipid oxidative capacity through a PGC1α-dependent pathway. We also provide strong evidence that NPR signaling in skeletal muscle relates to insulin sensitivity and is disrupted in obese and diabetic humans and mice. Increasing plasma BNP levels in obese diabetic mice remarkably improves blood glucose control and could prove a novel therapeutic avenue to alleviate obesity-related insulin resistance.

Acknowledgements
MC, PMB, IKV, CL, KL, VB, MAM, EM, GT, ACR, JEG, DRJ, SRS, DL and CM, researched data, edited the manuscript, MC and CM wrote the manuscript. Dr. Cedric Moro is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. The authors
have no conflict of interest to disclose. The authors are very grateful to Pr. Max Lafontan
(I2MC, Toulouse) for helpful discussions and critical reading of the manuscript. We thank
Justine Bertrand-Michel and Aude Dupuy (Lipidomic Core Facility, INSERM, UMR1048, part
of Toulouse Metatoul Platform) for lipidomic analysis, advice, and technical assistance; and
the Anexplo Mouse Phenotyping and Animal Care Facility cores. This study was supported
by grants from the National Research Agency ANR-12-JSV1-0010-01 and Société
Francophone du Diabète (CM), Fondecyt 11090007-Chile (JEG). DL is a member of Institut
Universitaire de France.
References


Figures Legend

**Figure 1.** Skeletal muscle NPR expression relates to insulin sensitivity in humans.
Correlation between *vastus lateralis* NPRA protein expression and (A) percent body fat, (B) glucose disposal rate measured by euglycemic hyperinsulinemic clamp and (C) muscle saturated ceramides content (n=15-20). (D) NPRA protein levels in skeletal muscle of lean and obese subjects and (E) in obese subjects pre- and post-calorie restriction (CR). (F) NPRC protein levels in skeletal muscle of obese subjects with normal glucose tolerance (NGT) and with impaired glucose tolerance and type 2 diabetes (IGT/T2D). **p<0.01 vs. lean; £p=0.06 vs. pre-CR; *p<0.05 vs. NGT (n=6-10 per group).

**Figure 2.** Defective NPR expression in metabolic tissues of diet-induced obese mice.
Representative blots of NPRA and NPRC proteins in (A) skeletal muscle, (B) epidydimal white adipose tissue (EWAT), and (C) brown adipose tissue (BAT) of chow-fed and HFD-fed mice. (D) Quantitative bar graph of NPRA protein and (E) overnight fasting plasma BNP levels in chow and HFD-fed mice. *p<0.05 versus chow-fed mice (n=8-10 per group).

**Figure 3.** Chronic BNP infusion protects from HFD-induced obesity and glucose intolerance.
C57BL/6J mice were treated for 4 weeks with saline (0.9% NaCl) or with BNP (5 ng/kg/min) via mini-osmotic pumps after 12 weeks of HFD. (A) Follow-up of body weight during HFD and after mini-pump were placed. (B) Body composition at the end of treatment in saline- and BNP-treated obese mice. (C) Overnight fasting blood glucose in BNP-treated mice after 8 and 12 weeks of HFD. (D) Time-course of blood glucose levels during an i.p. glucose tolerance test and corresponding AUC. (E) Plasma insulin after a 6h fast (0 min) and 15 min after glucose bolus injection. *p<0.05, **p<0.01 vs. saline (n=8-10).

**Figure 4.** Defective NPR signaling in metabolic tissues of obese diabetic mice.
Representative blots of NPRA and NPRC proteins in (A) skeletal muscle, (B) EWAT, and (C) BAT of db/db and db/+ mice. (D) Quantitative bar graph of NPRA, (E) NPRC protein, (F) of the NPRA-to-NPRC protein ratio, and (G) of overnight fasting plasma BNP levels in db/db and db/+ mice. *p<0.05, **p<0.01 versus db/+ mice (n=8-10 per group).

**Figure 5. Chronic BNP infusion improves blood glucose control in obese diabetic mice.**

5 weeks old db/db mice were chronically treated for 4 weeks with saline (0.9% NaCl) or with BNP (10 ng/kg/min) via mini-osmotic pump. (A) Follow-up of body weight over 4 weeks of treatment with saline or BNP. (B) Body composition at the end of treatment. (C) Overnight fasting blood glucose, (D) HbA1c, and (E) overnight fasting insulin were measured after 4 weeks of BNP treatment. (F) Time-course of blood glucose levels during an intraperitoneal insulin tolerance test, and corresponding area under the curve (AUC) after 4 weeks of treatment. *p<0.05; **p<0.01; ***p<0.001 vs. saline-treated db/db mice (n=8-10).

**Figure 6. Muscle-autonomous improvement of insulin signaling and reduced lipotoxicity in skeletal muscle of BNP-treated obese mice.**

(A) Extensor digitorum longus muscles were incubated ex vivo in absence (-) or presence of 100 nM of insulin (+) and phosphorylated and total Akt and p38 MAPK were measured by western blot. (B) Total ceramides, (C) total sphingomyelin, (D) Total and diacylglycerols sub-species content, (E) ex vivo palmitate oxidation rate, and (F) PGC1α gene expression in skeletal muscle of HFD-fed mice treated with saline and BNP 5 ng/kg/min. *p<0.05 versus saline (n=8-10).

**Figure 7. Chronic NP treatment reduces lipotoxicity and increases lipid oxidative capacity in human primary myotubes.**

(A) Total lipid accumulation, (B) TAG and (C) DAG content were determined with [1-14C]oleate after 3-days chronic treatment with 100 nM of ANP and BNP in human
differentiated myotubes. (D) Total palmitate oxidation rate was also measured in response to chronic ANP and BNP treatment. (E) PGC1α gene expression in response to 3-days treatment with ANP and BNP, and (F) in presence or absence of 500 nM of the selective PPARδ antagonist GSK0660. *p<0.05 vs. basal; $p<0.05$ vs. GSK0660 (n=4-10). (G) Ceramide species content in human primary myotubes in basal condition (BSA), and in response to overnight treatment with 500 µM of palmitate/BSA (Palm) in control myotubes and in response to 3-days treatment with ANP or BNP. **p<0.01 vs. control BSA; *p<0.05; **p<0.01 vs. control palm (n=4).

**Figure 8. Model linking natriuretic peptide signaling in skeletal muscle and insulin sensitivity.**

In lean individuals, NPRA activation by circulating NP induces PGC1α expression in a cGMP-dependent manner that leads to increased fat oxidation rates and low levels of lipotoxic DAGs and ceramides (CER) which maintain a normal insulin responsiveness in skeletal muscle. Defective NPR signaling in skeletal muscle during obesity contributes to reduced fat oxidative capacity, increased lipotoxicity and insulin resistance. Up-regulation of NPRC in skeletal muscle as glucose tolerance deteriorates with obesity further inhibits the biological activation of NPRA by circulating NP and reduce their circulating levels.
Figure 1

(A) Body fat (%) vs. Muscle NPRA protein (A.U.)

(B) Glucose disposal rate (mg/min/kg) vs. Muscle NPRA protein (A.U.)

(C) Saturated ceramides (ng/µg protein) vs. Muscle NPRA protein (A.U.)

(D) Western blots of NPRA and GAPDH in Lean and Obese groups.

(E) Western blots of NPRA and GAPDH in Pre-CR and Post-CR groups.

(F) Western blots of NPRC and α-Tubulin in NGT and IGT/T2D groups.

Correlation coefficients and p-values:

- A: \( r = -0.61 \), \( p_{adj} = 0.021 \)
- B: \( r = 0.51 \), \( p_{adj} = 0.03 \)
- C: \( r = -0.61 \), \( p_{adj} = 0.015 \)
Figure 2

A

<table>
<thead>
<tr>
<th></th>
<th>Muscle</th>
<th></th>
<th>EWAT</th>
<th></th>
<th>BAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPRA</td>
<td>Chow</td>
<td>HFD</td>
<td>NPRA</td>
<td>Chow</td>
<td>HFD</td>
</tr>
<tr>
<td>NPRC</td>
<td></td>
<td></td>
<td>NPRC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td></td>
<td>β-actin</td>
<td></td>
<td>β-actin</td>
</tr>
</tbody>
</table>

D

NPRA protein (relative to β-actin)

E

Plasma BNP (pg/ml)
Figure 3

A) Graph showing body weight (g) over time (weeks) for Saline and BNP treatments.

B) Bar graph comparing body composition (g) at 8 and 12 weeks for Saline and BNP treatments.

C) Bar graph showing fasting glucose (mg/dl) at 8 and 12 weeks for Saline and BNP treatments.

D) Graph showing blood glucose (mg/dl) over time (min) for Saline and BNP treatments.

E) Graph showing plasma insulin during GTT (ng/ml) at 0 and 15 min for Saline and BNP treatments.
Figure 4

A

<table>
<thead>
<tr>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>db/+ db/db</td>
</tr>
<tr>
<td>NPRA</td>
</tr>
<tr>
<td>NPRC</td>
</tr>
<tr>
<td>GAPDH</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>EWAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>db/+ db/db</td>
</tr>
<tr>
<td>NPRA</td>
</tr>
<tr>
<td>NPRC</td>
</tr>
<tr>
<td>β-actin</td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th>BAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>db/+ db/db</td>
</tr>
<tr>
<td>NPRA</td>
</tr>
<tr>
<td>NPRC</td>
</tr>
<tr>
<td>β-actin</td>
</tr>
</tbody>
</table>

D

NPRA protein (arbitrary units)

E

NPRA protein (arbitrary units)

F

NPRA-to-NPRC ratio (arbitrary units)

G

Plasma BNP (pg/ml)
Figure 5
Figure 6

A. Western blot analysis showing the expression levels of pSer473 Akt, Total Akt, pThr180 p38MAPK, and p38MAPK under different conditions.

B. Graph showing the effect of BNP on muscle ceramides (ng/µg protein).

C. Graph showing the effect of BNP on muscle sphingomyelin (ng/µg protein).

D. Graph showing the effect of BNP on muscle diacylglycerols (nmol/mg protein).

E. Graph showing the effect of BNP on muscle palmitate oxidation (nmol/h/mg protein).

F. Graph showing the effect of BNP on muscle PGC1α mRNA expression (fold change over control).
Figure 7
Figure 8
Supplemental Information for:

Defective natriuretic peptide receptor signaling in skeletal muscle links obesity to type 2 diabetes

Marine Coue, Pierre-Marie Badin, Isabelle K. Vila, Claire Laurens, Katie Louche, Marie-Adeline Marquès, Virginie Bourlier, Etienne Mouisel, Geneviève Tavernier, Arild C. Rustan, Jose E. Galgani, Denis R. Joanisse, Steven R. Smith, Dominique Langin, and Cedric Moro

Inventory of Supplemental Information:

1. Supplemental Tables : 5
2. Supplemental Figures : 10
### Supplemental Table 1. Clinical characteristics of the subjects.

<table>
<thead>
<tr>
<th></th>
<th>Lean</th>
<th>Obese</th>
<th>IGT/T2D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (male/female)</td>
<td>6/3</td>
<td>5/4</td>
<td>6/4</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>23.8±0.8</td>
<td>23.7±0.8</td>
<td>46.7±3.4</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>68.3±3.3</td>
<td>87.7±6.3b</td>
<td>103.3±4.1c</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.5±0.5</td>
<td>32.9±0.5c</td>
<td>34.3±1.2c</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>19.7±2.5</td>
<td>27.9±2.1a</td>
<td>30.1±1.4c</td>
</tr>
<tr>
<td>GDR (mg.min⁻¹.kg⁻¹ FFM)</td>
<td>9.4±0.7</td>
<td>7.0±0.4c</td>
<td>5.5±0.5c,d</td>
</tr>
</tbody>
</table>

Data are Mean ± SEM. BMI: body mass index; GDR: glucose disposal rate; FFM: fat-free mass. a,p<0.05, b,p<0.01, c,p<0.001 versus lean; d,p<0.05, *p<0.01 versus obese.
**Supplemental Table 2.** Correlation between muscle NPRA protein expression and biological variables in humans.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Human muscle NPRA</th>
<th>r</th>
<th>p value</th>
<th>p adj. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>-0.46</td>
<td>0.06</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>-0.46</td>
<td>0.05</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>-0.48</td>
<td>0.04</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>rQUICKI</td>
<td>0.63</td>
<td>0.005</td>
<td>0.025</td>
<td></td>
</tr>
<tr>
<td>Fasting Insulin</td>
<td>-0.52</td>
<td>0.03</td>
<td>0.07</td>
<td></td>
</tr>
</tbody>
</table>

BMI: body mass index; HOMA-IR: homeostasis model assessment of insulin resistance; revised QUICKI. r: Spearman correlation coefficients; non adjusted p value; p_adj. value: Benjamini-Hochberg false discovery rate considered statistically significant if ≤ 5%.
**Supplemental Table 3.** Correlation between log fasting plasma BNP and biological variables in db/+ and db/db mice.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Mouse Log [BNP]</th>
<th>r</th>
<th>p value</th>
<th>p adj. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td></td>
<td>-0.69</td>
<td>0.0007</td>
<td>0.0008</td>
</tr>
<tr>
<td>Fat Mass (%)</td>
<td></td>
<td>-0.79</td>
<td>&lt; 0.0001</td>
<td>0.0002</td>
</tr>
<tr>
<td>Fasting glucose</td>
<td></td>
<td>-0.76</td>
<td>&lt; 0.0001</td>
<td>0.0002</td>
</tr>
<tr>
<td>Fasting insulin</td>
<td></td>
<td>-0.85</td>
<td>&lt; 0.0001</td>
<td>0.0007</td>
</tr>
<tr>
<td>HbA1c</td>
<td></td>
<td>-0.70</td>
<td>0.0006</td>
<td>0.0008</td>
</tr>
<tr>
<td>Fructosamines</td>
<td></td>
<td>-0.82</td>
<td>&lt; 0.0001</td>
<td>0.0003</td>
</tr>
<tr>
<td>Muscle NPRC</td>
<td></td>
<td>-0.59</td>
<td>0.02</td>
<td>0.02</td>
</tr>
</tbody>
</table>

r: Spearman correlation coefficients; non adjusted p value; p adj. value: Benjamini-Hochberg false discovery rate considered statistically significant if ≤ 5%.
**Supplemental Table 4.** Correlation between muscle NPRC protein expression and biological variables in db/+ and db/db mice.

<table>
<thead>
<tr>
<th>Variables</th>
<th>r</th>
<th>p value</th>
<th>p adj. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting glucose</td>
<td>0.64</td>
<td>0.011</td>
<td>0.014</td>
</tr>
<tr>
<td>Fasting insulin</td>
<td>0.61</td>
<td>0.016</td>
<td>0.016</td>
</tr>
<tr>
<td>HbA1c</td>
<td>0.65</td>
<td>0.009</td>
<td>0.036</td>
</tr>
<tr>
<td>Fructosamines</td>
<td>0.65</td>
<td>0.012</td>
<td>0.024</td>
</tr>
</tbody>
</table>

HOMA-IR: homeostasis model assessment of insulin resistance. \( r \): Spearman correlation coefficients; non adjusted \( p \) value; \( p_{\text{adj.}} \) value: Benjamini-Hochberg false discovery rate considered statistically significant if \( \leq 5\% \).
Supplemental Table 5. List of mouse primer and probe sequences used for real-time qPCR

**Taqman chemistry:**

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Taqman Probe ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCP1</td>
<td>Mm01244861_m1</td>
</tr>
<tr>
<td>TFAM</td>
<td>Mm00447485_m1</td>
</tr>
<tr>
<td>GLUT1</td>
<td>Mm00441480_m1</td>
</tr>
<tr>
<td>GLUT2</td>
<td>Mm00446229_m1</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Mm00436615_m1</td>
</tr>
<tr>
<td>CPT1β</td>
<td>Mm00487200_m1</td>
</tr>
<tr>
<td>GYS1</td>
<td>Mm00472712_m1</td>
</tr>
<tr>
<td>PCK1</td>
<td>Mm00440636_m1</td>
</tr>
<tr>
<td>18S</td>
<td>Hs99999901_s1</td>
</tr>
</tbody>
</table>

**SYBR chemistry:**

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARα</td>
<td>AGTTCACGCATGTGAAGGCTG</td>
<td>TGTTCGGGTCTTCTTCTTCTGAATC</td>
</tr>
<tr>
<td>G6P</td>
<td>ACACCGACTACTACAGCAACAG</td>
<td>CCTCGAAAGATAGCAAGAGTAG</td>
</tr>
<tr>
<td>PGC1α</td>
<td>CTGTGTCACCACCACAAATCTTAT</td>
<td>TGTGTCGAGAAAAGGACCTTGA</td>
</tr>
</tbody>
</table>
Figure S1. Correlation between muscle NPRA protein and clinical variables in humans

Correlation between *vastus lateralis* NPRA protein expression, and (A) percent body fat (n=21), and (B) the McAuley insulin sensitivity index measured during an oral glucose tolerance test in human subjects with normal glucose tolerance (n=18).
Figure S2. Acute effect of BNP injection on insulin sensitivity

Fasted C57BL/6 mice fed standard chow diet were injected intraperitoneously with saline (0.9% NaCl) or with BNP (1 µg/kg) solution (arrow) and fasting blood glucose was measured (A) every 10 min in the basal state and (B) every 15 min during an i.p. GTT (n=11). (C) Tissue-specific glucose uptake was measured during a radiolabeled GTT with [2-\(^{3}\)H]deoxyglucose (n=6).
Figure S3. Effect of acute NP treatment on glucose uptake in human primary myotubes

Glucose uptake was measured in presence of 1, 10 and 50 µM of ANP or BNP, and 1 µM of insulin in human primary myotubes. *** p<0.001 vs. saline (n=6).
**Figure S4.** Representative blots and quantitative bar graph of p38MAPK phosphorylation relative to total p38MAPK and α-tubulin in gastrocnemius muscle of db/+ versus db/db mice. ***p<0.0001 vs. db/+ (n=8).
Figure S5. Chronic BNP treatment does not change lipid levels and gene expression in liver of db/db mice

(A) Total ceramides, (B) total diacylglycerols levels, and (C) mRNA levels of genes involved in fat oxidation and glucose metabolism in liver of saline- and BNP-treated db/db mice.
Figure S6. Chronic BNP treatment does not change lipid levels and gene expression in liver of HFD-fed mice

(A) Total ceramides, (B) total diacylglycerols levels, and (C) mRNA levels of genes involved in fat oxidation and glucose metabolism in liver of saline- and BNP-treated HFD-fed mice.
Figure S7. Expression of thermogenic and brown/beige gene markers in adipose tissues of db/db mice

*PGC1α, UCP1, TFAM, GLUT1* and GLUT4 mRNA levels in (A) BAT and (B) EWAT of db/db mice treated for 4 weeks with BNP (n=8-10).
Figure S8. Expression of thermogenic and brown/beige gene markers in adipose tissues of HFD-fed mice

(A) PGC1α, UCP1, TFAM, GLUT1 and GLUT4 mRNA levels in BAT and (B) UCP1, GLUT1 and GLUT4 gene expression in EWAT of HFD mice treated for 4 weeks with BNP (n=8-10).
Figure S9. Effect of acute BNP treatment on lipolysis in human primary myotubes

Time-course of (A) fatty acid (FA) release and (B) FA oxidation from endogenous pre-labeled TAG pools in response to 1, 3 or 6 hours BNP treatment in the presence or absence of triacsin C to block FA recycling into TAG pools. (C) HSL Ser660 and (D) HSL Ser565 phosphorylation were measured after 10, 30 and 60 min acute stimulation with 100 nM of BNP in human primary myotubes (n=3-5).
Figure S10. Effect of chronic NP treatment on basal ceramides content in human primary myotubes.

Ceramide species content in human primary myotubes in basal condition (BSA) in control myotubes and in response to 3-days treatment with 100 nM of ANP or BNP (n=4).