Brief Communication

MOLECULAR METABOLISM

Divergent effects of a designer natriuretic peptide CD-NP in the regulation of adipose tissue and metabolism

Anja Glöde [1,3], Jennifer Naumann [1], Thorsten Gnad [1], Valentina Cannone [2], Ana Kilic [1], John C. Burnett Jr. [2], Alexander Pfeifer [1,3,*]

ABSTRACT

Objective: Obesity is defined as an abnormal increase in white adipose tissue (WAT) and is a major risk factor for type 2 diabetes and cardiovascular disease. Brown adipose tissue (BAT) dissipates energy and correlates with leanness. Natriuretic peptides have been shown to be beneficial for brown adipocyte differentiation and browning of WAT.
Methods: Here, we investigated the effects of an optimized designer natriuretic peptide (CD-NP) on murine adipose tissues *in vitro* and *in vivo*.
Results: In murine brown and white adipocytes, CD-NP activated cGMP production, promoted adipogenesis, and increased thermogenic markers. Consequently, mice treated for 10 days with CD-NP exhibited increased "browning" of WAT. To study CD-NP effects on diet-induced obesity (DIO), we delivered CD-NP for 12 weeks. Although CD-NP reduced inflammation in WAT, CD-NP treated DIO mice exhibited a significant increase in body mass, worsened glucose tolerance, and hepatic steatosis. Long-term CD-NP treatment resulted in an increased expression of the NP scavenging receptor (NPR-C) and decreased lipolytic activity.
Conclusions: NP effects differed depending on the duration of treatment raising questions about the rational of natriuretic peptide treatment in obese patients.

Keywords Natriuretic peptides; cGMP; Adipocytes; Browning; Diet-induced obesity

1. INTRODUCTION

There is an urgent need for novel treatments of obesity and diabetes. In obesity, excessive nutritional energy is stored in white adipose tissue (WAT), which also acts as an endocrine organ releasing adipokines. In contrast to WAT, brown adipose tissue (BAT) dissipates energy for the production of heat (thermogenesis) [1—5]. Metabolically active BAT is present in adult humans, and the amount of BAT inversely correlates with body mass index (BMI) [6—9]. When fully stimulated, BAT can burn large amounts of calories [10]. Thus, BAT might be a target for novel anti-obesity therapies. In animal models, different pharmacological stimuli, e.g. β_3-adrenergic agonists, promote the appearance of brown-like adipocytes — also known as beige or brown-in-white (brite) cells — within WAT, a process called "browning" [1,2]. Classical brown adipocytes (BA) and brown-like cells express uncoupling protein-1 (UCP-1), which is responsible for thermogenesis and can promote energy expenditure (EE) [11]. Besides cold exposure and the β_3-adrenergic pathway, the cyclic GMP (cGMP) signaling pathway is also capable of inducing WAT browning [12,13].
The natriuretic peptide (NP) family consists of atrial NP (ANP), the B-type NP (BNP), and C-type NP (CNP) [14—17]. NPs bind to natriuretic peptide receptors (NPR), thereby inducing the production of second messenger cGMP [15,18,19]. ANP and BNP preferentially bind to NPR-A (also known as guanylate cyclase A; GC-A) while CNP binds mainly to NPR-B (GC-B) [15,16]. CNP and its major receptor NPR-B are both widely expressed, and the major phenotype of mice deficient in CNP or NPR-B is connected with skeletal growth anomalies as both CNP and NPR-B KO mice are dwarfs [16].
NPs are removed from circulation by clearance receptor (NPR type C, NPR-C) or by the neutral endopeptidase neprilysin (NEP) [20—22], and the ratio of NPR-C versus NPR-A/B can affect NP signaling/effectiveness [23]. Interestingly, NPR ratios are altered in adipose tissues of genetic mouse models of obesity [24,25]. Moreover, NPs have metabolic effects like promotion of lipolysis in human adipocytes [26,27]. Importantly, obesity, diabetes, and the metabolic syndrome are all characterized by reduced levels of circulating NPs [28,29]. Conversely, the ANP genetic variant rs5068 represents a unique "human genetic model" characterized by a life-time exposure to moderately elevated circulating levels of ANP and BNP [30]. The carriers of rs5068 have a reduced risk for cardiovascular disease [31] and also have lower odds of obesity and metabolic syndrome [30,32].
The physiological function of NPs in "classical" BAs as well as NPR expression during brown and white adipocyte differentiation so far has not been studied. In the present study, we addressed these questions

[1]Institute of Pharmacology and Toxicology, University Hospital, University of Bonn, Bonn, Germany [2]Cardiorenal Research Laboratory, Department of Cardiovascular Diseases, Mayo Clinic, Rochester, USA [3]Bonn International Graduate School of Drug Sciences BIGSDrugS, University of Bonn, Bonn, Germany

*Corresponding author. Institute of Pharmacology and Toxicology, University of Bonn, 53127 Bonn, Germany. E-mail: alexander.pfeifer@uni-bonn.de (A. Pfeifer).

Received October 27, 2016 • Revision received December 20, 2016 • Accepted December 29, 2016 • Available online 4 January 2017

http://dx.doi.org/10.1016/j.molmet.2016.12.010

using an optimized designer NP (CD-NP, or Cenderitide). CD-NP is a chimeric designer peptide that contains parts of CNP and *Dendroaspis* NP and activates both NPR-A and B [33]. Moreover, CD-NP is more resistant to NEP degradation than endogenous NPs and is presently in clinical trials for heart failure [34]. As CD-NP had beneficial effects on BA differentiation and induced UCP-1 in white adipocytes, we analyzed the effect of long-term CD-NP in the context of diet-induced obesity (DIO). Although BNP and CNP have been suggested as novel modulators of EE [27,35], NPs have so far not been applied long-term in the context of DIO, and it is not clear whether they could be used for anti-obesity treatment. In addition, no previous studies have investigated the metabolic effect of a combined activation of NPR-A and NPR-B.

2. MATERIAL AND METHODS

2.1. Isolation and differentiation of BA

The stromal vascular fraction (SVF) of interscapular BAT was isolated from newborn wildtype pubs as described before [36]. The dissected tissue was digested using collagenase II (Worthington). Cells were immortalized using Simian Virus 40 (SV40) large T-antigen under control of the phosphoglycerate kinase (PGK) promoter and expanded in growth medium (Dulbecco's modified Eagle's medium (DMEM Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 100 IU penicillin and streptomycin). For differentiation of mature BAs, 180,000 preadipocytes/6-well were seeded in growth medium (d-4). After 48 h, the medium was changed to differentiation medium (d-2, DM) containing 20 nM insulin and 1 nM triiodothyronine. Adipogenesis was then induced with induction medium (d = 0, IM, DM supplemented with 0.5 mM IBMX and 1 mM Dexamethasone) for 48 h. Until day 7, the adipocytes maintained DM, which was replenished every second day. Where indicated, cells were treated from day −2 until day +7 with 200 μM 8-pCPT-cGMP (Biolog), 100 nM ANP (Bachem), 100 nM BNP (Anaspec), 100 nM CNP (Bachem), or 100 nM CD-NP (kindly provided by Prof. Burnett, Mayo Clinic). For all preadipocyte experiments, the cells were used on day 0, and for mature adipocyte experiments, cells were used on day 7.

2.2. White adipocyte isolation and differentiation

The SVF of inguinal WAT was isolated from 8 to 12 week old wildtype mice. The tissue was divided by cutting and incubated for 30 min in digestion buffer (DMEM containing 0.5% BSA and 0.15% collagenase II). Digestion was stopped by adding the same volume of DMEM + 10% FBS (GM) for 10 min, and the tissues was centrifuged at 700 g for 10 min. The pellet was then resuspended in GM, filtered, and seeded on a T175 flask until 90% of confluency. For differentiation, 180,000 cells/6-well were seeded in GM until 100% confluency. After two more days, the differentiation was induced by adding induction medium for two days (DMEM supplemented with 5% FBS, 1% P/S, 1 μM dexamethasone, 0.5 mM IBMX, 1 nM triiodothyronine, 1 μM D-biotin, 17 μM pantothenate, L-ascorbate (50 μg/ml), 1 μM rosiglitazone, 0.172 mM insulin), and for 5 more days, the cells were maintained in maintenance media (DMEM supplemented with 5% FBS, 1% P/S, 1 nM Triiodothyronine, 0.172 mM insulin, 1 μM D-biotin, 17 μM pantothenate, L-ascorbate (50 μg/ml)).

For browning experiments, the differentiation was performed with 80 μg/ml metformin instead of 1 μM rosiglitazone; therefore, the cells were kept in induction medium for 6 days and then kept for 5 more days in maintenance medium. Where indicated, cells were treated from day 0 until day +7 with 200 μM 8-pCPT-cGMP, 100 nM ANP, 100 nM BNP, 100 nM CNP, or 100 nM CD-NP. For all preadipocyte experiments, the cells were used on day 0, and for mature adipocyte experiments, cells were used on day 6 (differentiation with rosiglitazone) or on day 11 (differentiated with metformin).

2.3. Oil Red O staining

Mature BA and WA were fixed in PBS containing 4% paraformaldehyde. After washing with PBS, the cells were incubated with Oil Red O (Sigma) solution (3 mg/ml in 60% isopropyl alcohol) for 3 h at room temperature, washed with water, and visualized under a microscope.

2.4. TG measurement *in vitro*

Mature BA were washed and frozen with TX lysis buffer (150 mM NaCl, 0.05% Triton X-100, 10 nM tris—HCL (pH 8)). After thawing, cells were sonicated, resuspended, and Triglyceride reagent (Sigma Aldrich) was added. The mixture was incubated for 5 min at 37 °C. Absorption at 450 nm was measured against water and a glycerol standard. Protein content was determined using Bradford method The TG content was calculated and normalized to protein content of the sample.

2.5. Radioimmunoassay

Brown preadipocytes (d0) were treated with 100 nM NPs (ANP, BNP, CNP, CD-NP) for 5 min in GM (supplemented with 20 nM HEPES, 0.1% BSA and 0.5 mM IBMX). The reaction was stopped with ice cold 70% Ethanol and frozen at −80 °C, thawed, and scraped. After centrifugation (3000 g, 5 min, 4 °C), the protein content was determined using Bradford method, and the supernatants were dried in a speed vacuum concentrator. The pellets were resuspended in sodium acetate buffer (50 mM, pH 6.0) and acetylated. cGMP contents were quantified using a competitive RIA cGMP kit (Perkin—Elmer, Boston, MA, USA).

2.6. Proliferation assay

1000 white preadipocytes/well were seeded in a 96 well plate in GM and stimulated every day with 100 nM CD-NP. The proliferation was determined by adding presto blue for 2 h and then measuring the fluorescence (560 nm Excitation—590 nM Emission).

2.7. RNA isolation and real-time RT-PCR (qPCR)

RNA was isolated from WA and BA using Trizol method (InnuSolv Analytik Jena AG). RNA from fat tissue was isolated using RNeasy lipid tissue kid (Qiagen). cDNA was synthesized from 0.5 μg RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche). qPCR was performed with PowerSYBR-Green PCR master mix (Applied Biosystems) or SYBR-Green (Roche, Life Technologies) using a HT7900 instrument (Applied Biosystems). The results were calculated using relative quantification methods with HPRT (hypoxanthine guanine phosphoribosyl transferase). Primer sequences are listed in Table 1

2.8. Western blot analysis

BA and WA protein lysates were prepared with lysis buffer (50 mM Tris, pH 7.5, 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mM EDTA, 0.1 mM EGTA). Protein was isolated from fat tissue in a buffer containing 50 mM Tris (pH 7.5) 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 1% SDS, 0.1 mM EDTA, each supplemented with complete protease inhibitor cocktail (Roche), 1 mM Na_3VO_4, and 10 mM NaF. Protein contents were determined by the Bradford method. Western blotting was performed as described previously [36]. The following antibodies were used: antibodies against aP2 (Santa Cruz Biotechnology); antibody against UCP-1 (Sigma Aldrich) and antibody against tubulin (DianovaUpstate). Secondary horseradish peroxidase-linked antibodies against goat (Pierce), mouse (Dianova), and rabbit (Cell Signaling) were

Table 1 — Primer sequences used to detect gene expression levels in murine samples.

Gene	Forward	Reverse
PowerSYBR-Green		
NPR-A	AGT ACG CCA ACA ACC TGG AG	AAG AGC TGT AAA GCC CAC GA
NPR-B	TCA TGA CAG CCC ATG GGA AA	GGT GAC AAT GCA GAT GTT GG
NPR-C	TCC ATG GAG GTG AAA AGT TCT GTT	CCC CAT CCT TCT TGC TGT AGC
UCP-1	TAAGCCGGCTGAGATCTTGT	GGCCTCTACGACTCAGTCCA
F4/80	CTT TGG CTA TGG GCT TCC AGT C	GCA AGG AGG ACA GAG TTT ATC GTG
IFNγ	ACA CTG CAT CTT GGC TTT GC	CAT GTC ACC ATC CTT TTG CCA G
CCL3	AAG TCT TCT CAG CGC CAT ATG	AAG ACT CTC AGG CAT TCA GTT CCA GGT C
CCL7	TCC ACA TGC TGC TAT GTC AAG	TTT CAT GTC TAA GTA TGC TAT AGC C
IL6	CCA GAG ATA CAA AGA AAT GAT GG	ACT CCA GAA GAC CAG AGG AAA T
HPRT	CCC AGC GTC GTG ATT AGC GAT G	TTC AGT CCT GTC CAT AAT CAG TC
SYBR-Green		
PGC-1a	GCA CAC ACC GCA ATT CTC CCT TGT A	ACG CTG TCC CAT GAG GTA TTG ACC A
PRDM16	CAGCACGGTGAAGCCATTC	GCGTGCATCCGCTTGTG
Glut4	GCTGAGCTGAAGGATGAGAAACGGA	CAACATACTGGAAACCCATGCCGAC
HPRT	ACA TTG TGG CCC TCT GTG TGC TCA	CTG GCA ACA TCA ACA GGA CTC CTC GT

used. All bands were quantified by densitometric analysis with Image J software.

2.9. Animal studies

Wildtype mice C57BJ/6 were purchased from Charles River. For the short-term study, 10 week old male mice were injected daily intraperitoneally with either saline or CD-NP (0.1 μg/kg body weight). For the histological analysis of control mice injected for 10 days with saline were part of a bigger cohort [37]; the sections/animals have not been published previously.

For the long-term study, 5 week-old male mice were implanted with mini-osmotic pumps (Alzet model 1004) and subsequently fed a HFD (60% calories from fat, Ssniff D12492) or normal diet (ND) (Ssniff D12450B) for 12 weeks. The pumps were replaced every 4 weeks in the back region of the mice. CD-NP (0.25 mg/day/kg), or saline was continuously released with a pumping rate of 0.11 μl/h. Mice were maintained on a daily cycle of 12 h (06:00 to 18:00) light and 12 h darkness (18:00 to 6:00) and were allowed free access to chow and water.

During the studies, mice were weighed weekly. All studies were conducted according to the German animal welfare law and permitted by the Landesamt für Natur, Umwelt und Verbraucherschutz, Nordrhein-Westfalen, Germany.

2.10. Body composition

Body composition was determined using a NMR device Minispec (Bruker Corporation).

2.11. Blood pressure

Blood pressure was measured using a Doppler system, kindly provided by PD Dr. med. Oliver Dewald, University Bonn. The mice were placed on a heat pad and anesthetized with 4% isoflurane, during the procedure kept on 1.5% isoflurane (Abbott). The tail cuff was placed on their tail vein and the Doppler signal was determined acoustically. After pumping the cuff, the systolic values were identified when the Doppler signal came back and diastolic values when the signal was completely replenished. For each mouse, at least 3 measurements were performed.

2.12. Glucose tolerance test

Mice were fasted for 5 h and then injected intraperitoneally with 8 μl/g body weight of a glucose solution (2.5 g/ml glucose in 0.9% saline). Blood glucose was measured at indicated time points post injection. The tail vein was punctured, and blood was analyzed using AccuChek (Aviva Nano) analyzer and dipsticks (Roche).

2.13. Indirect calorimetry

Individual oxygen consumption (VO_2) and CO_2 production (VCO_2) as well as food intake and motility were measured using a Phenomaster device (TSE systems) at ambient temperature for 120 s every 18 min for 24 h.

2.14. Immunohistochemistry

Adipose tissue was fixed in PBS containing 4% paraformaldehyde for 48 h and dehydrated using increasing ethanol content baths. Tissue was embedded in paraffin and cut into 5 μM sections, which were blocked with 2% normal goat serum-TBS (Tris-buffered saline) for 30 min RT. Immunohistochemical stainings were performed with primary antibody (UCP-1 1:100, Sigma Aldrich) overnight. Secondary antibody-conjugated with horseradish peroxidase (Santa Cruz Biotechnology) was applied for 1 h at RT and sections were visualized using DAB substrate (Vector Laboratories). Nuclei were stained with hematoxylin.

2.15. *Ex vivo* lipolysis

Adipose tissue was minced and incubated with lipolysis medium (Life Technologies) supplemented with 2% fatty acid free BSA (Sigma Aldrich) for 2 h at 37 °C. The media were collected, incubated with free glycerol reagent (Sigma Aldrich) for 5 min at 37 °C, and the absorption was measured at 540 nm. Glycerol release was calculated with glycerol standard and normalized to wet weight of the tissue.

2.16. Tissue TG level

Livers were weighed and transferred to TGx lysis buffer (as mentioned above). Tissue was cut, sonicated, and resuspended. The solution was used to measure the TG content by using the Triglyceride reagent (Sigma Aldrich) following the manufacturer's instructions and normalized to wet liver weight.

2.17. Plasma parameters

Plasma levels of adiponectin (AdipoGen), insulin, and leptin (Crystal Chem Inc.) were measured using a commercially available ELISA kit following the manufacturer's instructions.

2.18. Statistics

Single comparisons were analyzed using two tailed Student's t-test. Multiple comparisons were analyzed using analysis of variance (ANOVA) with Newman–Keuls post-hoc test. Values below 0.05 were considered statistically significant. Analyses were performed using GraphPad Prism 5 software. All data are presented as mean ± s.e.m.

3. RESULTS

3.1. Effects of NP treatment on adipocyte differentiation *in vitro*

First, we assessed the expression of all members of the NPR family (NPR-A, NPR-B and NPR-C) in murine BAs (Figure 1A). Expression of NPR-B in brown preadipocytes was 24.7-fold higher than NPR-A (Figure 1A). In mature BAs, the clearance receptor NPR-C was the

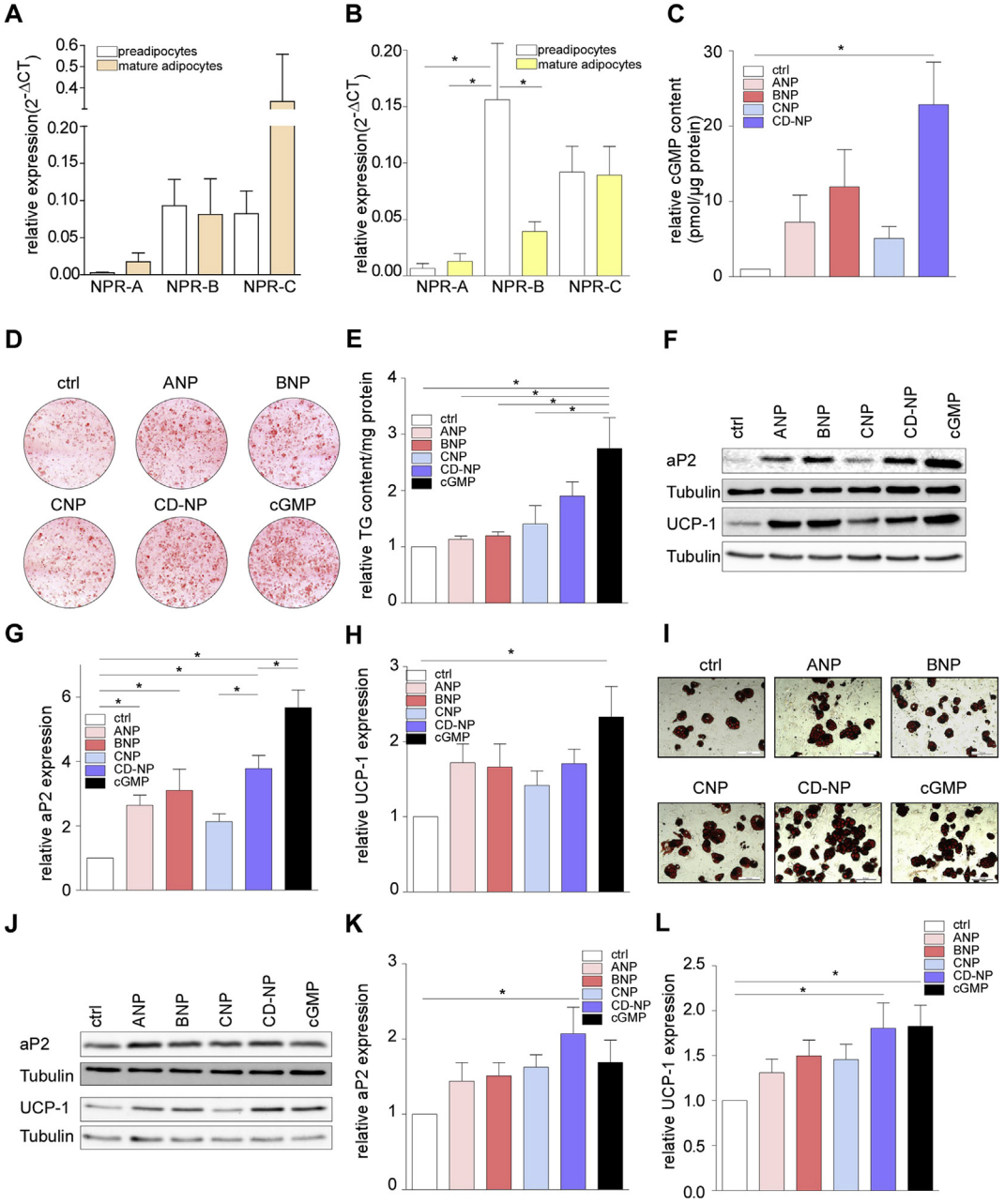

Figure 1: The effects of natriuretic peptides in murine brown and white adipocytes *in vitro*. **(A)** Relative mRNA expression of NPR-A, NPR-B, and NPR-C in pre- and mature brown adipocytes; n = 7 independent experiments. **(B)** Relative mRNA expression of NPR-A, NPR-B and NPR-C in pre- and mature white adipocytes; n = 7 independent experiments. **(C)** Relative cGMP content in brown preadipocytes after 5 min treatment with 100 nM ANP, BNP, CNP or CD-NP; n = 4–5 independent experiments. **(D)** Oil Red O stain of BA differentiated in the presence and absence of NPs (100 nM) and 8-pCPT-cGMP (200 µM). **(E)** Triglyceride content of BA differentiated in the presence and absence of NPs and cGMP. **(F–H)** Representative immunoblots **(F)** and quantification of aP2 **(G)** (n = 6 independent experiments) and UCP-1 **(H)** (n = 7–8 independent experiments) of BA differentiated in the presence and absence of NPs (100 nM) and 8-pCPT-cGMP (200 µM). **(I)** Oil Red O stain of WA differentiated in the presence and absence of NPs (100 nM) and 8-pCPT-cGMP (200 µM). **(J–L)** Representative immunoblots **(J)** and quantification of aP2 **(K)** (n = 5 independent experiments) and UCP-1 **(L)** (n = 10 independent experiments) after differentiating WA in the presence and absence of NPs (100 nM) and 8-pCPT-cGMP (200 µM). *$P < 0.05$. Error bars, s.e.m.

most highly expressed NPR (Figure 1A). Although, white preadipocytes expressed higher levels (1.6-fold) of NPR-B mRNA than brown preadipocytes (Figure 1A and B), NPR-B levels fell by 75% during white adipogenesis (Figure 1B). In contrast to BAs, NPR-C expression was unchanged during WA differentiation (Figure 1A and B). The ratio of NPR-C versus NPR-A and NPR-B is an important determinant of the effectiveness of NP/cGMP signaling. NPR-A/NPR-C ratios were <0.75 in both BA and WA as well as in preadipocytes (Figure S1A and B). NPR-B/NPR-C ratios were >0.75 in brown and white preadipocytes but decreased in mature adipocytes (Figure S1A and B).

To analyze the effectiveness of individual NPs, we measured cGMP levels in brown preadipocytes. Of the NPs tested, CD-NP had the strongest effect increasing cGMP levels 23-fold (22.85 ± 5.65; versus ANP 7.25 ± 3.58; BNP 11.93 ± 5.0; CNP 5.1 ± 1.56) compared to untreated control (Figure 1C).

To study the effect of CD-NP on differentiation, brown preadipocytes were differentiated using our established differentiation protocol [36]. Oil Red O staining of BA and measurement of triglyceride (TG) content revealed increased lipid accumulation after incubation with NPs; CD-NP had the strongest effects (Figure 1D and E). In line, chronic treatment with NPs increased protein expression of the adipogenic marker aP2 and the thermogenic marker UCP-1. Again, CD-NP had a pronounced effect (Figure 1F–H).

Next, we investigated the effect of NPs on murine WA differentiation. Incubation with CD-NP had no significant effect on proliferation of preadipocytes isolated from WAT (Figure S1C). Chronic treatment of WA with CD-NP or other, natural NPs increased lipid storage (Figure 1I) and significantly upregulated protein levels of aP2 and of UCP-1 (Figure 1J–L). Moreover, acute treatment with CD-NP caused an increase in UCP-1, PGC-1α, and PRDM16 mRNA expression, indicating browning of WAs (Figure S1D–F).

Taken together, both brown and white murine adipocytes express all three NPRs with NPR-C being the most highly expressed receptor. CD-NP generates intracellular cGMP more efficiently than endogenous CNP resulting in increased brown and white adipocyte differentiation, as well as browning of WAs.

3.2. *In vivo* effects of CD-NP

To study the effects of CD-NP *in vivo*, we fed mice a normal chow diet and treated them with CD-NP for 10 days. Histological analysis revealed multilocular, UCP-1-positive cells reminiscent of brown-like adipocytes within inguinal, subcutaneous WAT (WATi) depots of CD-NP treated mice (Figure S2A). Thus, CD-NP can stimulate browning of WATi within 10 days, similar to 7 day BNP treatment [27].

We next studied whether CD-NP treatment might prevent DIO in mice. To this end, we fed mice a high fat diet (60% of calories from fat, HFD) for 12 weeks in the presence of CD-NP (0.25 mg/kg/day, HFD/CD-NP) or NaCl (HFD/NaCl) as control using mini-osmotic pumps. These animals were compared to mice fed a matched control diet (13% of calories from fat, ND) and treated with CD-NP (ND/CD-NP) or NaCl (ND/NaCl). Due to the known effect of NPs on blood pressure, we also checked whether CD-NP treatment influenced it. Blood pressure was not affected during DIO or by long-term treatment with CD-NP (Figure S2B).

Unexpectedly, CD-NP-treated mice on HFD (HFD/CD-NP) showed a significantly increased body weight gain starting from week 7 compared to HFD controls (HFD/NaCl) (Figure 2A). CD-NP treatment for 12 weeks also increased weight in mice on ND (ND/CD-NP) as compared to the ND controls (ND/NaCl) (Figure 2A). Moreover, CD-NP caused an increase in body length irrespective of diet by 1.1-fold (Figure S2C), which could be explained by the stimulatory effect of the CNP-part of CD-NP and NPR-B on linear bone growth [38]. Tibia length was significantly increased in both HFD/CD-NP and ND/CD-NP mice compared to the respective controls (Figure S2D). However, tibia weight was not significantly changed (Figure S2E).

Analysis of body composition showed a significant increase (1.27-fold) of fat mass in HFD/CD-NP treated mice compared to HFD/NaCl (Figure 2B). Fat mass was also increased 1.2-fold, albeit not significantly, in ND/CD-NP versus ND/NaCl (Figure 2B). The weights of all individual fat depots analyzed were significantly increased in HFD/CD-NP compared to HFD/NaCl mice: BAT, WATi, and gonadal WAT (WATg) were enlarged 1.19, 1.42- and 1.59-fold, respectively (Figure 2C–E). Consistent with changes in body weight, plasma levels of leptin were significantly increased in HFD/CD-NP mice compared to HFD/NaCl (Figure 2F). Basal glucose levels of HFD/CD-NP mice were significantly higher (1.27-fold) compared to HFD/NaCl, whereas basal glucose levels of ND/CD-NP were not changed (Figure 2G). Also, insulin plasma levels were increased by 1.3-fold in HFD/CD-NP treated mice (compared to HFD/NaCl), albeit not significantly (Figure 2H). In line with these data, mice exposed to CD-NP treatment and fed a HFD, showed significantly decreased glucose tolerance (Figure 2I and Figure S2F). In addition, HFD/CD-NP mice stored a significantly higher amount of TG in liver in comparison to mice only fed a HFD/NaCl (Figure 2J).

To sum up, CD-NP treatment during a HFD for 12 weeks increased body weight and worsened the diabetic phenotype as well as ectopic fat storage in the liver.

3.3. Metabolic analysis

Next, we assessed the metabolic status after CD-NP treatment for 12 weeks. In 6 out of the 24 time points analyzed, HFD/CD-NP treated mice had higher EE than HFD/NaCl mice (Figure 3A). However, comparison of mice with the same body weight (Figure 3B) revealed no significant difference in EE.

Food intake was not significantly different among the groups (Figure 3C). Motility of HFD/CD-NP was reduced compared to HFD/NaCl mice, albeit not significantly (Figure 3D).

3.4. Reduced inflammation after CD-NP treatment

Fat depot size is regulated by lipid uptake and lipolysis, the breakdown of lipids, and release of free fatty acids. To investigate lipolysis, we measured glycerol release in adipose tissue explants (*ex vivo*). Basal glycerol release in WAT explants of HFD/CD-NP treated mice was reduced by 72% compared to HFD/NaCl (Figure 4A), albeit this effect was not significant. Although norepinephrine (NE) caused an increase in lipolysis rate in fat depots of HFD/CD-NP mice, it was nevertheless 64% lower than in NE-stimulated control tissues (HFD/NaCl). ND/CD-NP WAT exhibited a 60% lower basal glycerol release compared to ND/NaCl (Figure 4A). To identify a potential reason for these differences in lipolysis, we focused on NPR-C and differences in NPR expression ratios. Interestingly, the ratio of NPR-A/NPR-C was reduced by 42% (Figure 4B) and the NPR-B/NPR-C ratio was reduced by 18% in HFD/CD-NP compared to HFD/NaCl mice (Figure 4C). We also observed a reduction in NPR-A/B versus NPR-C ratios in ND/CD-NP mice (Figure 4B and C). The major reason for this change in NPR ratios was a significant increase in NPR-C expression, whereas NPR-A and -B did not change significantly (Figure S3A–C).

Since it has been shown that reduced lipolysis can decrease accumulation of adipose tissue macrophages (ATM) and inflammation [39], we measured ATM (F4/80) and inflammation markers (IFNγ and IL-6) in WAT. Expression of F4/80 levels was significantly reduced in WAT of HFD/CD-NP compared to HFD/NaCl mice by 45% (Figure 4D). CD-NP treatment of ND mice also reduced F4/80 expression by 33%

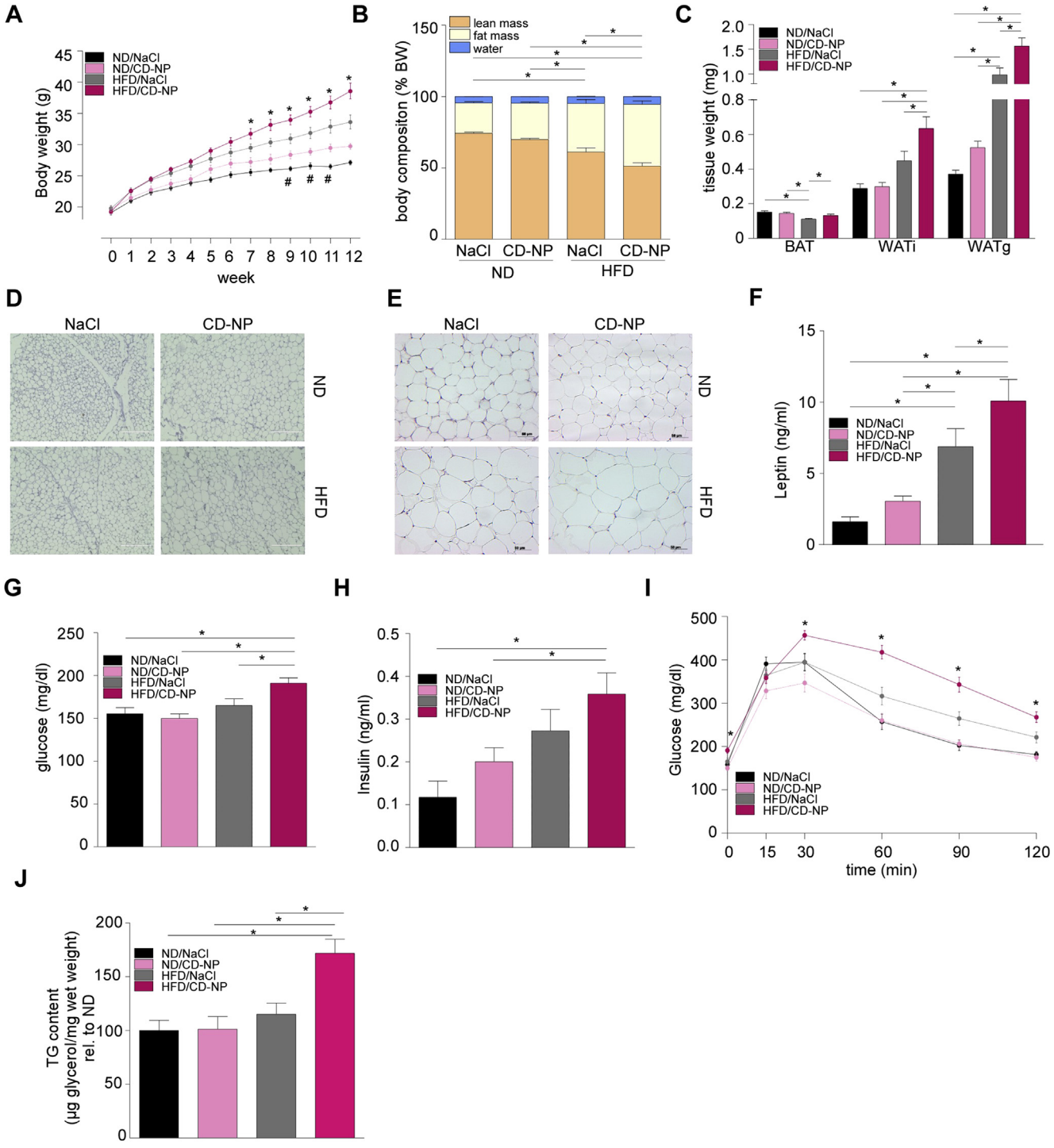

Figure 2: Effects on CD-NP on diet-induced obesity. (A–C) Body-weight **(A),** body composition (fat mass, lean mass, water content), **(B)** and fat tissue weight of BAT, WATi, and WATg **(C)** of mice treated with and without CD-NP and fed a ND or HFD for 12 weeks. **(D)** Representative hematoxylin staining of WATi sections from mice treated with or without CD-NP receiving a HFD, scale bar 200 µM. **(E)** Representative hematoxylin staining of WATg from mice treated with or without CD-NP on ND or HFD, scale bar 50 µM. **(F–J)** Leptin **(F)**, basal glucose **(G)** and basal insulin **(H)** plasma levels in mice fed a ND or HFD and treated with or without CD-NP. **(I)** Glucose tolerance test performed after 12 weeks of mice treated with and without CD-NP and fed ND or HFD. **(J)** Triglyceride content in liver of mice after 12 weeks on ND or HFD and treatment with and without CD-NP; 9–10 mice per group for all analysis. *, #$P < 0.05$. Error bars, s.e.m. # ND/NaCl versus ND/CD-NP; * HFD/NaCl versus HFD/CD-NP.

compared to controls (ND/NaCl) (Figure 4D). Moreover, IFNγ expression was reduced in WAT of HFD/CD-NP by 52% and in ND/CD-NP by 31% compared to the respective controls (Figure 4D). Similarly, levels of pro-inflammatory cytokine Il-6 were reduced by 56% and 28% of HFD/CD-NP and ND/CD-NP mice, compared to NaCl-treated controls, respectively (Figure 4E). Expression of the chemokines CCL3 and CCL7 was significantly reduced in HFD/CD-NP mice compared to HFD/NaCl (Figure 4E). Since reduced expression of pro-inflammatory cytokines

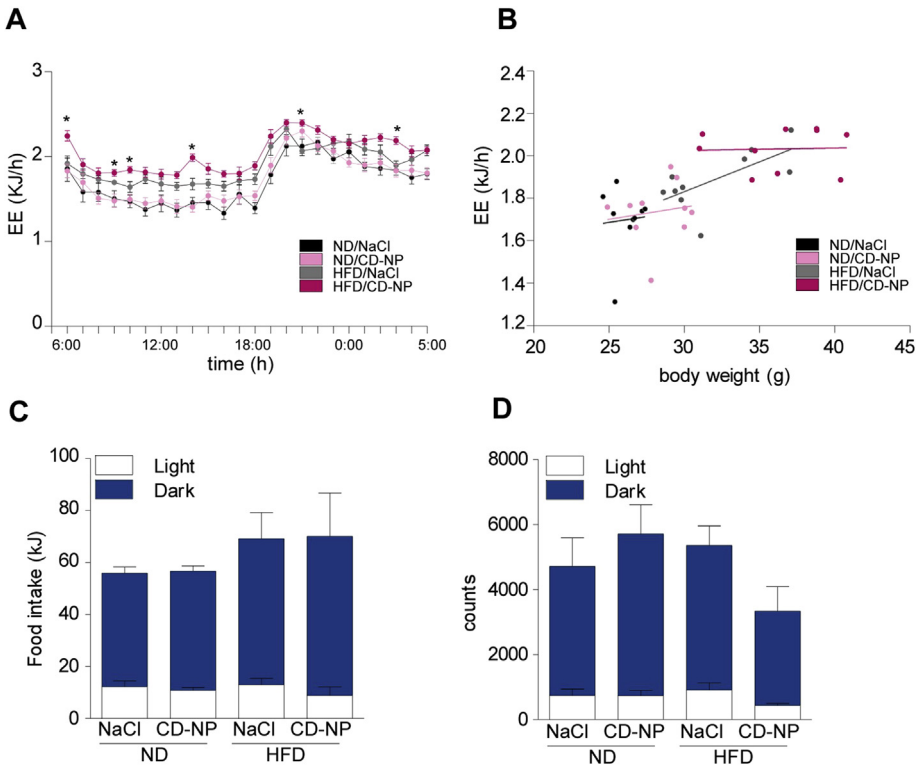

Figure 3: Metabolic effects of CD-NP. (A, B) Mean energy expenditure (EE) over 24 h **(A)** of mice fed a ND or HFD and treated with CD-NP or vehicle (NaCl) and EE in relation to the body-weight **(B)**. **(C, D)** Food intake **(C)** and motility **(D)** during day and night phase. 9–10 mice per group for all analyses. *, #$P < 0.05$. Error bars, s.e.m. # ND/NaCl versus ND/CD-NP; * HFD/NaCl versus HFD/CD-NP.

was not able to protect CD-NP-treated mice from glucose intolerance, we analyzed if CD-NP influenced Glut4 expression, the most important mediator of glucose clearance in adipose tissue [40,41]. Importantly, Glut4 has been shown to be downregulated in adipocytes by macrophage-derived pro-inflammatory cytokines [42]. CD-NP-treatment of mice on HFD significantly downregulated Glut4 expression by 52% compared to control animals (Figure 4F).

Finally, we analyzed levels of the key adipokine adiponectin [43], which, in contrast to leptin, are reduced in HFD fed mice [44]. We observed a significant (1.7 fold) increase of adiponectin plasma levels in HFD/CD-NP compared to HFD/NaCl (Figure 4G). Interestingly, increased adiponectin levels have also been found in metabolically healthy obese individuals [43]. Both the reduced macrophage recruitment in WATi as well as the increased adiponectin levels indicates that CD-NP treatment reduces inflammation in WATi.

4. DISCUSSION

Cyclic GMP regulates a variety of important physiological processes ranging from cardio vascular function to renal function and bone growth [45–47]. Most recently, a role of cGMP signaling as a key regulator of adipogenesis in metabolism and its potential in anti-obesity therapies has emerged [1,48].

Importantly, cGMP is generated through two pathways, which include activation of soluble guanylate cyclase (sGC) via nitric oxide or via NPs acting on NPRs [16,49,50].

Both cGMP producing pathways/enzymes (sGC, as well as NPRs) are expressed in adipocytes [12,27,36,51]. However, the main source of cGMP in adipose tissue and how cGMP production can be efficiently stimulated with pharmacological substances/stimulators was still not clear.

Activation of the cGMP pathway enhances differentiation in both brown and white adipocytes, as well as induces browning of WAs *in vitro* [12,13,27]. Accordingly, stimulation of cGMP production might be a way to enhance whole body EE [13,27,52].

Since the exact role of NPR-B in regulating adipogenesis (*in vitro*) and whole-body metabolism (*in vivo*) is not known, we investigated the role of NP/NPR signaling in BA and whether pharmacological activation of NPR-A and NPR-B through CD-NP could be metabolically beneficial in DIO.

CD-NP (cenderitide) is a chimeric natriuretic peptide (NP) that targets both NPR-A and -B [33]. CD-NP was shown to have more potent effects in cardiac fibroblasts than BNP or CNP [53], less hypotensive properties when compared with BNP [53], and to be more resistant to degradation by NEP than native NPs [54].

In the current study, CD-NP induced the strongest increase in cGMP levels of all the NPs studied in brown preadipocytes. Stimulation with CD-NP during differentiation increased TG content in BA as well as adipogenic (aP2) and thermogenic (UCP-1) markers. We also demonstrated that chronic treatment of WA with CD-NP enhanced differentiation. Importantly, acute treatment with CD-NP leads to browning of mature WA with upregulation of UCP-1, PGC1-α and PRDM16 *in vitro*. Thus, CD-NP proved *in vitro* to be a robust activator of cGMP with positive actions on BA and WA.

NP signaling is regulated by the complex interplay between the cGMP-producing NPRs (i.e. NPR-A and B) and the scavenging clearance receptor NPR-C. In accordance with previous studies, we found that WA highly express NPR-C [27,29,45], while NPR-A is expressed at the

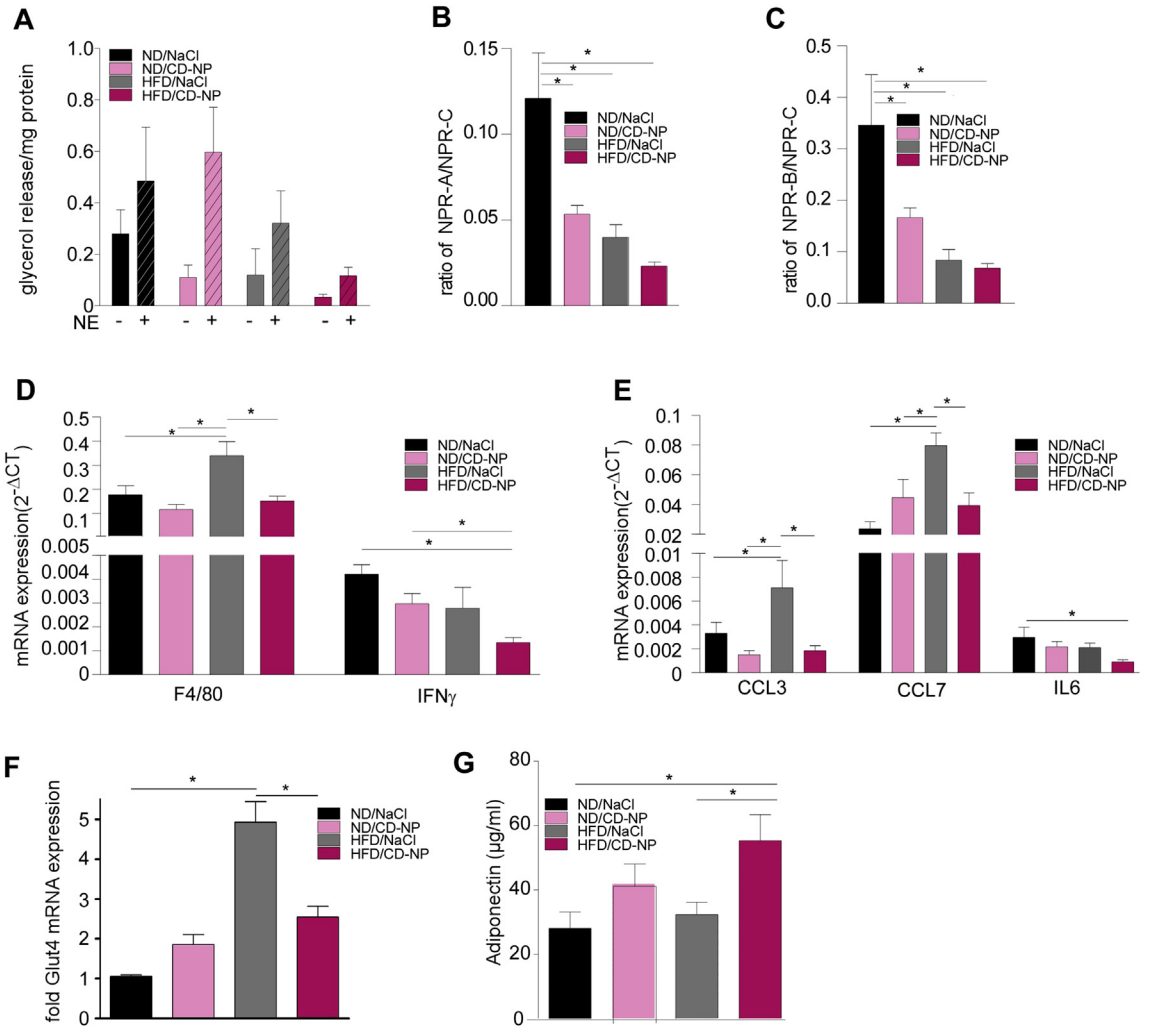

Figure 4: Effect of CD-NP on adipose tissue lipolysis and inflammation. (A) Basal and NE-induced *ex vivo* lipolysis of WATi explants from mice treated with and without CD-NP and fed a ND or HFD. **(B)** Ratio of mRNA expression of NPR-A versus NPR-C in WATi of mice fed ND or HFD for 12 weeks and with or without CD-NP treatment. **(C)** Ratio of mRNA expression of NPR-B versus NPR-C in WATi of mice fed ND or HFD for 12 weeks and with or without CD-NP treatment. **(D, E)** mRNA expression of macrophage and inflammation markers F4/80 and IFNγ **(D)**, proinflammatory markers CCL3, CCL7, IL6, **(E)** and glucose transporter Glut4 **(F)** in WATi mice fed ND or HFD for 12 weeks and with or without CD-NP treatment. **(F)** Adiponectin plasma levels in mice fed a ND or HFD and treated with or without CD-NP. 9–10 mice per group for all experiments. $*P < 0.05$. Error bars, s.e.m.

lowest level. In BA, NPR-B is the most highly expressed cGMP-producing NPR that is — in contrast to WA — not down-regulated during differentiation.

For the *in vivo* studies, we first tested CD-NP effects over a ten day period and found browning of WATi, which is in line with the findings of Bordicchia et al., who showed that treatment of mice with BNP for 7 days increases oxygen consumption, EE, and expression of thermogenic markers in WATi [27].

For the first time, we extended NP therapy in mice beyond 10 days in the setting of DIO, applying dual activation of NPR-A and NPR-B with CD-NP for three months. Unexpectedly, CD-NP treatment for 12 weeks in the presence of HFD resulted in a paradoxical phenotype with expansion of all fat depots and an increased body weight, which was significantly pronounced at the end of the HFD. Furthermore, there were no signs of browning.

In accordance with the increased obesity of the HFD/CD-NP mice, we found increased insulin and basal glucose levels, impaired glucose tolerance, as well as increased hepatosteatosis. Thus, long-term CD-NP treatment worsened DIO with negative metabolic consequences. This is in stark contrast to the protective effect of increased cGMP signaling during DIO observed after treatment with pharmacological stimulators of sGC [13] or PDE5 inhibitor [52].

Two previous studies assessed the effect of BNP treatment in murine obesity [24,25]. It is difficult to directly compare these previous studies with our data, because both Coue et al. [25] as well as Plante et al. [24] used BNP, which acts through NPR-A whereas CD-NP acts through NPR-A and NPR-B with similar potency [55]. Moreover, both groups used substantially lower doses of NP (0.6 µg/kg/h) than we did in our study (10.4 µg/kg/h). Coue et al. treated mice for four weeks *after* an initial 12 week HFD. Interestingly, they did not see a reduction of body weight or overall fat content but observed an improvement of the diabetic phenotype after treatment. Contrary to our data, they noted a downregulation of NPRA in BAT and epididymal WAT (eWAT) and of NPRC in eWAT. In addition, Coue et al. treated five week old db/db mice with BNP for 4 weeks. In contrast to the pharmacological model, but in line with our data, they found a strong upregulation of NPRC

concomitant with a decreased NPRA/NPRC ratio [25]. Although Coue et al. did not observe significant changes in body weight or fat/lean mass of the BNP treated db/db mice, they found beneficial effects on fasting glucose levels as well as an improved glucose tolerance. Plante et al. [24] also used db/db mice and observed significantly reduced body weight gain, improved plasma glucose levels and glucose tolerance after treatment with 0.6 μg/kg/h BNP for 12 weeks. Although whole-body energy expenditure was not analyzed, the appearance of UCP1-positive cells within white fat depots of BNP-treated db/db mice was observed, indicating browning of WAT [24]. Interestingly, in contrast to our data, Plante et al. saw a significantly increased food intake. Taken together, the differences in NPs, doses, and the obesity models used might explain the different outcomes of these and our studies.

Importantly, we observed that long-term CD-NP treatment resulted in a significantly higher NPR-C expression in WATi in both ND/CD-NP as well as HFD/CD-NP compared to the respective control mice. Not much is known about signaling pathways controlling NPR-C expression. Li et al. showed that knockdown of NPR-A leads to upregulation of NPR-C mRNA in vascular smooth muscle cells [56]. However, we did not see any changes in NPR-A mRNA levels with and without treatment on ND or HFD. Additionally, dexamethasone recently has been shown to upregulate NPR-C in podocytes [57], whereas ANP previously has been shown to regulate cortisol release from adrenal glands of guinea pigs [58]. Hence, CD-NP might have an effect on glucocorticoid/cortisol levels in mice. On the other hand, PPARγ agonism, (Pioglitazone) improves NPR ratio by downregulating NPR-C expression [59]. Also, Arejian et al. showed that nitric oxide can downregulate NPR-C expression in a cGMP-independent manner [60] and DAG/Ca2+/PKC signaling in HeLa cells downregulates NPR-C expression in a transcriptional and post-translational manner [61].

In humans, NPR-C expression is known to be increased in subcutaneous adipose tissue from obese as well as diabetic subjects [62–64], and changes in NPR-A and NPR-C expression in BAT and eWAT have been reported before in the context of chronic BNP treatment of DIO or db/db mice [24,25].

Increased NPR-C expression could scavenge both endogenous as well as exogenous NPs. In addition to the role of NPR-C in clearing NPs, previous studies in the cardiovascular system point toward a role of NPR-C in intracellular signaling and inhibition of the cAMP pathway [56,65,66]: CNP activated NPR-C, couples to G_i and inhibits adenylate cyclases, thereby diminishing cAMP production in cardiomyocytes [67]. In adipocytes, reduced cAMP levels would translate into decreased lipolysis, which would lead eventually to increased adipocyte size and adipose tissue mass. In accordance with this notion, we found decreased basal *ex vivo* lipolysis in the subcutaneous fat depots from ND/CD-NP and HFD/CD-NP treated mice. In contrast, the deletion of NPR-C leads to browning of WAT, reduced weight gain, and improved insulin sensitivity [59].

Although CD-NP worsened obesity, we were surprised to find increased adiponectin levels and reduced pro-inflammatory chemokine/cytokine expression in WAT. Adiponectin regulation may represent NPR-A activation with CD-NP [68]. Previous studies clearly demonstrate that the expression of adiponectin inversely correlates with BMI and body fat [69]. Furthermore, adiponectin expression has been described as a potent inhibitor of TNFα-induced monocytes adhesion and adhesion molecule expression [70]. Congruently, we demonstrated that CD-NP enhanced adiponectin expression while diminishing F4/80 expression. Moreover, mRNA expression of IFNγ, CCL3, CCL7, and IL-6 was reduced in WAT of HFD/CD-NP treated mice. Transgenic mice expressing ca. 3- to 4-fold more adiponectin than wild type controls, exhibited a significant increase in WAT weight and body-mass [71], which is reminiscent of mice treated long-term with CD-NP during HFD (HFD/CD-NP). However, CD-NP treated mice exhibited signs of insulin intolerance and disturbed glucose metabolism, which is in contrast to the adiponectin transgenic mice. This difference might be due to lower adiponectin levels in CD-NP treated mice compared to the transgenic mice or by other effects of CD-NP outside of the adipose organ. Reduced pro-inflammatory chemokine/cytokine expression in WAT could be a direct consequence of CD-NP action on macrophages (or other immune cells), leading to reduced macrophage infiltration into the tissue, reduced *in situ* proliferation or increased apoptosis of resident macrophages, with all processes potentially leading to reduced inflammation [41,72]. It has been shown that macrophages express all NPRs, and that ANP decreases LPS-induced iNOS expression and NO production in macrophages [73] via post-transcriptional mechanisms and Nf-κB signaling [74]. Also, NO-derived cGMP protects human macrophages from peroxynitrite-induced apoptosis [75]. However, nothing is known about NP effects on macrophage apoptosis. Interestingly, reduced inflammation after CD-NP treatment failed to protect mice from glucose intolerance. Chronic low-grade inflammation after high fat diet is dependent on cells of the immune system being recruited to WAT, where they secrete cytokines to modulate metabolism within WAT. Many of these cytokines are known to impair insulin action on different levels, i.e. insulin receptor autophosphorylation or inhibition Akt/PKB with subsequent impaired signal propagation [41,76]. To further study insulin tolerance in CD-NP-treated animals, we analyzed expression levels of Glut4, which is often downregulated by pro-inflammatory cytokines. Although CD-NP downregulates inflammatory markers as well as macrophage abundance, there is no beneficial effect on adipocyte tissue Glut4 expression. In contrast, WAT from CD-NP-treated obese mice have significantly lower expression of Glut4 compared to control animals.

CNP and NPR-B are also expressed in the CNS including the hypothalamus [35]; however, their role in brain is still not clear. CNP-deficiency in the whole body — except bone/cartilage — results in increased sympathetic nervous system outflow and subsequent stimulation of EE and increased expression of UCP-1 in BAT as well as decreased food intake [35]. All together, these mice exhibited a significant reduction in fat mass and body weight [35] indicating that CNP/NPR-B centrally inhibits EE and increases food intake. In contrast, intracerebroventricular injection of CNP had anorexigenic effects and decreased food intake [77]. Moreover, cerebral injection of CNP failed to significantly affect the thermogenic program in BAT thermogenesis [35]. Finally, mice with a brain specific-knockout of NPR-B gained less weight and exhibited significantly lower mesenteric fat and liver weights compared to WT mice on a HFD mainly because of reduced food intake without major changes in EE [78]. In the light of the study by Yamashita et al. [78], one must consider a potential role for central NPR-B as a pro-obesity/weight preserving mechanism, which could have been activated by CD-NP through activation of both NPR-A and NPR-B. However, we did not detect significant changes in food intake and EE at the end of the 12 week treatment with CD-NP.

The long-term safety of CD-NP (cenderitide) treatment in humans has not been fully investigated. A phase II study on cenderitide has been initiated in 14 patients with stable chronic heart failure to assess safety, tolerability, pharmacokinetics, and pharmacodynamics profiles. Other human studies with cenderitide are still recruiting or are ongoing, but so far there is no study involving obese patients. Recently completed studies of 12 weeks of subcutaneously delivered BNP to

patients with symptomatic and asymptomatic heart failure have been reported [79–81]. Compared to controls, treated subjects exhibited cGMP activation and improved cardiovascular and renal function. Unfortunately, metabolic profiles were not investigated.

5. CONCLUSION

In conclusion, our studies are the first to define both the short- and long-term actions of NP treatment using a dual NPR-A/NPR-B activator. Our findings indicate that the positive effects of NPs on metabolism (e.g. browning of WATi) are only transient and that long-term treatment with NPs might even exacerbate obesity in overweight or obese subjects when accompanied with excessive calorie intake. Further studies are clearly warranted to understand the complex mechanisms of NPs and their receptors in metabolic homeostasis.

AUTHOR CONTRIBUTIONS

A.G. performed most experiments, analyzed the data, and drafted the manuscript. J.N. performed experiments, analyzed data, and drafted the manuscript. T.G. analyzed data and gave input regarding metabolism. V.C. drafted the manuscript and provided expertise on metabolic properties of natriuretic peptides. A.K. performed experiments analyzed data and drafted the manuscript. J.C.B. planned experiments and drafted the manuscript. A.P. supervised experiments and wrote the manuscript.

ACKNOWLEDGEMENTS

We thank D. Hass and P. Zehner for expert technical assistance. We thank A. Balkow for her support with animal preparation. We thank the Federal Institute for Drugs and Medical Devices and BIGSDrugS for financial support.

CONFLICTS OF INTEREST

The authors declare that no conflict of interest exists.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2016.12.010.

REFERENCES

[1] Pfeifer, A., Hoffmann, L.S., 2015. Brown, beige, and white: the new color code of fat and its pharmacological implications. Annual Review of Pharmacology and Toxicology 55:207–227.

[2] Rosen, E.D., Spiegelman, B.M., 2014 Jan 16. What we talk about when we talk about fat. Cell 156(1–2):20–44.

[3] Cannon, B., Nedergaard, J., 2004 Jan. Brown adipose tissue: function and physiological significance. Physiological Reviews 84(1):277–359.

[4] Enerback, S., 2010 Apr 7. Human brown adipose tissue. Cell Metabolism 11(4):248–252.

[5] Kajimura, S., Seale, P., Spiegelman, B.M., 2010 Apr 7. Transcriptional control of brown fat development. Cell Metabolism 11(4):257–262.

[6] Cypess, A.M., Lehman, S., Williams, G., Tal, I., Rodman, D., Goldfine, A.B., et al., 2009 Apr 9. Identification and importance of brown adipose tissue in adult humans. The New England Journal of Medicine 360(15):1509–1517.

[7] Saito, M., Okamatsu-Ogura, Y., Matsushita, M., Watanabe, K., Yoneshiro, T., Nio-Kobayashi, J., et al., 2009 Jul. High incidence of metabolically active brown adipose tissue in healthy adult humans: effects of cold exposure and adiposity. Diabetes 58(7):1526–1531.

[8] Virtanen, K.A., Lidell, M.E., Orava, J., Heglind, M., Westergren, R., Niemi, T., et al., 2009 Apr 9. Functional brown adipose tissue in healthy adults. The New England Journal of Medicine 360(15):1518–1525.

[9] van Marken Lichtenbelt, W.D., Vanhommerig, J.W., Smulders, N.M., Drossaerts, J.M., Kemerink, G.J., Bouvy, N.D., et al., 2009 Apr 9. Cold-activated brown adipose tissue in healthy men. The New England Journal of Medicine 360(15):1500–1508.

[10] Bartelt, A., Bruns, O.T., Reimer, R., Hohenberg, H., Ittrich, H., Peldschus, K., et al., 2011 Feb. Brown adipose tissue activity controls triglyceride clearance. Nature Medicine 17(2):200–205.

[11] Nedergaard, J., Cannon, B., 2014 Sep 2. The browning of white adipose tissue: some burning issues. Cell Metabolism 20(3):396–407.

[12] Mitschke, M.M., Hoffmann, L.S., Gnad, T., Scholz, D., Kruithoff, K., Mayer, P., et al., 2013 Jan 9. Increased cGMP promotes healthy expansion and browning of white adipose tissue. FASEB Journal 27(4):1621–1630.

[13] Hoffmann, L.S., Etzrodt, J., Willkomm, L., Sanyal, A., Scheja, L., Fischer, A.W., et al., 2015. Stimulation of soluble guanylyl cyclase protects against obesity by recruiting brown adipose tissue. Nature Communications 6:7235.

[14] de Bold, A.J., Borenstein, H.B., Veress, A.T., Sonnenberg, H., 1981 Jan 5. A rapid and potent natriuretic response to intravenous injection of atrial myocardial extract in rats. Life Sciences 28(1):89–94.

[15] Kuhn, M., 2004 Mar. Molecular physiology of natriuretic peptide signalling. Basic Research in Cardiology 99(2):76–82.

[16] Potter, L.R., Abbey-Hosch, S., Dickey, D.M., 2006 Feb. Natriuretic peptides, their receptors, and cyclic guanosine monophosphate-dependent signaling functions. Endocrine Reviews 27(1):47–72.

[17] Boerrigter, G., Costello-Boerrigter, L.C., Burnett Jr., J.C., 2009 Oct. Natriuretic peptides in the diagnosis and management of chronic heart failure. Heart Failure Clinics 5(4):501–514.

[18] Song, D.L., Kohse, K.P., Murad, F., 1988 May 9. Brain natriuretic factor. Augmentation of cellular cyclic GMP, activation of particulate guanylate cyclase and receptor binding. FEBS Letters 232(1):125–129.

[19] Winquist, R.J., Faison, E.P., Waldman, S.A., Schwartz, K., Murad, F., Rapoport, R.M., 1984 Dec. Atrial natriuretic factor elicits an endothelium-independent relaxation and activates particulate guanylate cyclase in vascular smooth muscle. Proceedings of the National Academy of Sciences of the United States of America 81(23):7661–7664.

[20] Maack, T., 1992. Receptors of atrial natriuretic factor. Annual Review of Physiology 54:11–27.

[21] Suga, S., Nakao, K., Hosoda, K., Mukoyama, M., Ogawa, Y., Shirakami, G., et al., 1992 Jan. Receptor selectivity of natriuretic peptide family, atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide. Endocrinology 130(1):229–239.

[22] Kenny, A.J., Bourne, A., Ingram, J., 1993 Apr 1. Hydrolysis of human and pig brain natriuretic peptides, urodilatin, C-type natriuretic peptide and some C-receptor ligands by endopeptidase-24.11. Biochemical Journal 291(Pt 1):83–88.

[23] Ryden, M., Backdahl, J., Petrus, P., Thorell, A., Gao, H., Coue, M., et al., 2016 Apr. Impaired atrial natriuretic peptide-mediated lipolysis in obesity. International Journal of Obesity (London) 40(4):714–720.

[24] Plante, E., Menaouar, A., Danalache, B.A., Broderick, T.L., Jankowski, M., Gutkowska, J., 2014 Jun. Treatment with brain natriuretic peptide prevents the development of cardiac dysfunction in obese diabetic db/db mice. Diabetologia 57(6):1257–1267.

[25] Coue, M., Badin, P.M., Vila, I.K., Laurens, C., Louche, K., Marques, M.A., et al., 2015 Dec. Defective natriuretic peptide receptor signaling in skeletal muscle links obesity to type 2 diabetes. Diabetes 64(12):4033–4045.

[26] Sengenes, C., Berlan, M., De Glisezinski, I., Lafontan, M., Galitzky, J., 2000 Jul. Natriuretic peptides: a new lipolytic pathway in human adipocytes. FASEB Journal 14(10):1345–1351.

[27] Bordicchia, M., Liu, D., Amri, E.Z., Ailhaud, G., Dessi-Fulgheri, P., Zhang, C., et al., 2012 Mar 1. Cardiac natriuretic peptides act via p38 MAPK to induce the brown fat thermogenic program in mouse and human adipocytes. Journal of Clinical Investigation 122(3):1022–1036.

[28] Wang, T.J., Larson, M.G., Levy, D., Benjamin, E.J., Leip, E.P., Wilson, P.W., et al., 2004 Feb 10. Impact of obesity on plasma natriuretic peptide levels. Circulation 109(5):594–600.

[29] Sengenes, C., Zakaroff-Girard, A., Moulin, A., Berlan, M., Bouloumie, A., Lafontan, M., et al., 2002 Jul. Natriuretic peptide-dependent lipolysis in fat cells is a primate specificity. American Journal of Physiology Regulatory, Integrative and Comparative Physiology 283(1):R257–R265.

[30] Cannone, V., Boerrigter, G., Cataliotti, A., Costello-Boerrigter, L.C., Olson, T.M., McKie, P.M., et al., 2011 Aug 2. A genetic variant of the atrial natriuretic peptide gene is associated with cardiometabolic protection in the general community. Journal of the American College of Cardiology 58(6):629–636.

[31] Cannone, V., Huntley, B.K., Olson, T.M., Heublein, D.M., Scott, C.G., Bailey, K.R., et al., 2013 Nov. Atrial natriuretic peptide genetic variant rs5065 and risk for cardiovascular disease in the general community: a 9-year follow-up study. Hypertension 62(5):860–865.

[32] Cannone, V., Cefalu, A.B., Noto, D., Scott, C.G., Bailey, K.R., Cavera, G., et al., 2013 Sep. The atrial natriuretic peptide genetic variant rs5068 is associated with a favorable cardiometabolic phenotype in a Mediterranean population. Diabetes Care 36(9):2850–2856.

[33] Lee, C.Y., Huntley, B.K., McCormick, D.J., Ichiki, T., Sangaralingham, S.J., Lisy, O., et al., 2016 Apr. Cenderitide: structural requirements for the creation of a novel dual particulate guanylyl cyclase receptor agonist with renal-enhancing in vivo and ex vivo actions. European Heart Journal – Cardiovascular Pharmacotherapy 2(2):98–105.

[34] Lee, C.Y., Chen, H.H., Lisy, O., Swan, S., Cannon, C., Lieu, H.D., et al., 2009 Jun. Pharmacodynamics of a novel designer natriuretic peptide, CD-NP, in a first-in-human clinical trial in healthy subjects. Journal of Clinical Pharmacology 49(6):668–673.

[35] Inuzuka, M., Tamura, N., Yamada, N., Katsuura, G., Oyamada, N., Taura, D., et al., 2010 Aug. C-type natriuretic peptide as a new regulator of food intake and energy expenditure. Endocrinology 151(8):3633–3642.

[36] Haas, B., Mayer, P., Jennissen, K., Scholz, D., Diaz, M.B., Bloch, W., et al., 2009. Protein kinase g controls brown fat cell differentiation and mitochondrial biogenesis. Science Signalling 2(99):ra78.

[37] Gnad, T., Scheibler, S., von Kugelgen, I., Scheele, C., Kilic, A., Glode, A., et al., 2014 Dec 18. Adenosine activates brown adipose tissue and recruits beige adipocytes via A2A receptors. Nature 516(7531):395–399.

[38] Nakao, K., Osawa, K., Yasoda, A., Yamanaka, S., Fujii, T., Kondo, E., et al., 2015. The local CNP/GC-B system in growth plate is responsible for physiological endochondral bone growth. Science Report 5:10554.

[39] Kosteli, A., Sugaru, E., Haemmerle, G., Martin, J.F., Lei, J., Zechner, R., et al., 2010 Oct. Weight loss and lipolysis promote a dynamic immune response in murine adipose tissue. Journal of Clinical Investigation 120(10):3466–3479.

[40] Leto, D., Saltiel, A.R., 2012 May 23. Regulation of glucose transport by insulin: traffic control of GLUT4. Nature Reviews. Molecular Cell Biology 13(6):383–396.

[41] Samuel, V.T., Shulman, G.I., 2016 Jan. The pathogenesis of insulin resistance: integrating signaling pathways and substrate flux. Journal of Clinical Investigation 126(1):12–22.

[42] Lumeng, C.N., Deyoung, S.M., Saltiel, A.R., 2007 Jan. Macrophages block insulin action in adipocytes by altering expression of signaling and glucose transport proteins. American Journal of Physiology, Endocrinology and Metabolism 292(1):E166–E174.

[43] Scherer, P.E., 2016 Jun. The multifaceted roles of adipose tissue-therapeutic targets for diabetes and beyond: the 2015 Banting lecture. Diabetes 65(6):1452–1461.

[44] Barnea, M., Madar, Z., Froy, O., 2010 Feb. High-fat diet followed by fasting disrupts circadian expression of adiponectin signaling pathway in muscle and adipose tissue. Obesity (Silver Spring) 18(2):230–238.

[45] Moro, C., Lafontan, M., 2013. Natriuretic peptides and cGMP signaling control of energy homeostasis. American Journal of Physiology. Heart Circulatory Physiology 304(3):H358–H368.

[46] Pfeifer, A., Kilic, A., Hoffmann, L.S., 2013 Oct. Regulation of metabolism by cGMP. Pharmacology & Therapeutics 140(1):81–91.

[47] Schlueter, N., de Sterke, A., Willmes, D.M., Spranger, J., Jordan, J., Birkenfeld, A.L., 2014 Oct. Metabolic actions of natriuretic peptides and therapeutic potential in the metabolic syndrome. Pharmacology & Therapeutics 144(1):12–27.

[48] Collins, S.A., 2014 Mar. heart-adipose tissue connection in the regulation of energy metabolism. Nature Reviews. Endocrinology 10(3):157–163.

[49] Koesling, D., Friebe, A., 1999. Soluble guanylyl cyclase: structure and regulation. Reviews of Physiology, Biochemistry and Pharmacology 135:41–65.

[50] Pfeifer, A., Ruth, P., Dostmann, W., Sausbier, M., Klatt, P., Hofmann, F., 1999. Structure and function of cGMP-dependent protein kinases. Reviews of Physiology, Biochemistry and Pharmacology 135:105–149.

[51] Miyashita, K., Itoh, H., Tsujimoto, H., Tamura, N., Fukunaga, Y., Sone, M., et al., 2009 Dec. Natriuretic peptides/cGMP/cGMP-dependent protein kinase cascades promote muscle mitochondrial biogenesis and prevent obesity. Diabetes 58(12):2880–2892.

[52] Ayala, J.E., Bracy, D.P., Julien, B.M., Rottman, J.N., Fueger, P.T., Wasserman, D.H., 2007 Apr. Chronic treatment with sildenafil improves energy balance and insulin action in high fat-fed conscious mice. Diabetes 56(4):1025–1033.

[53] Lisy, O., Huntley, B.K., McCormick, D.J., Kurlansky, P.A., Burnett Jr., J.C., 2008 Jul 1. Design, synthesis, and actions of a novel chimeric natriuretic peptide: CD-NP. Journal of the American College of Cardiology 52(1):60–68.

[54] Dickey, D.M., Potter, L.R., 2011 Jul. Dendroaspis natriuretic peptide and the designer natriuretic peptide, CD-NP, are resistant to proteolytic inactivation. Journal of Molecular and Cellular Cardiology 51(1):67–71.

[55] Martin, F.L., Sangaralingham, S.J., Huntley, B.K., McKie, P.M., Ichiki, T., Chen, H.H., et al., 2012. CD-NP: a novel engineered dual guanylyl cyclase activator with anti-fibrotic actions in the heart. PLoS One 7(12):e52422.

[56] Li, Y., Madiraju, P., Anand-Srivastava, M.B., 2012 Feb 1. Knockdown of natriuretic peptide receptor-A enhances receptor C expression and signalling in vascular smooth muscle cells. Cardiovascular Research 93(2):350–359.

[57] Lewko, B., Waszkiewicz, A., Maryn, A., Golos, M., Latawiec, E., Daca, A., et al., 2015 Nov. Dexamethasone-dependent modulation of cyclic GMP synthesis in podocytes. Molecular and Cellular Biochemistry 409(1-2):243–253.

[58] Raha, D., Tortorella, C., Neri, G., Prasad, A., Raza, B., Raskar, R., et al., 2006 Apr. Atrial natriuretic peptide enhances cortisol secretion from guinea-pig adrenal gland: evidence for an indirect paracrine mechanism probably involving the local release of medullary catecholamines. International Journal of Molecular Medicine 17(4):633–636.

[59] Kovacova, Z., Tharp, W.G., Liu, D., Wei, W., Xie, H., Collins, S., et al., 2016 Apr. Adipose tissue natriuretic peptide receptor expression is related to insulin sensitivity in obesity and diabetes. Obesity (Silver Spring) 24(4):820–828.

[60] Arejian, M., Li, Y., Anand-Srivastava, M.B., 2009 Jun. Nitric oxide attenuates the expression of natriuretic peptide receptor C and associated adenylyl cyclase signaling in aortic vascular smooth muscle cells: role of MAPK. American Journal of Physiology. Heart Circulatory Physiology 296(6):H1859–H1867.

[61] Yanaka, N., Akatsuka, H., Omori, K., 1997 Dec 01. Protein kinase C activation down-regulates natriuretic peptide receptor C expression via transcriptional and post-translational pathways. FEBS Letters 418(3):333–336.

[62] Dessi-Fulgheri, P., Sarzani, R., Tamburrini, P., Moraca, A., Espinosa, E., Cola, G., et al., 1997 Dec. Plasma atrial natriuretic peptide and natriuretic

peptide receptor gene expression in adipose tissue of normotensive and hypertensive obese patients. Journal of Hypertension 15(12 Pt 2):1695—1699.

[63] Pivovarova, O., Gogebakan, O., Kloting, N., Sparwasser, A., Weickert, M.O., Haddad, I., et al., 2012 May. Insulin up-regulates natriuretic peptide clearance receptor expression in the subcutaneous fat depot in obese subjects: a missing link between CVD risk and obesity? Journal of Clinical Endocrinology and Metabolism 97(5):E731—E739.

[64] Sarzani, R., Strazzullo, P., Salvi, F., Iacone, R., Pietrucci, F., Siani, A., et al., 2004 Feb. Natriuretic peptide clearance receptor alleles and susceptibility to abdominal adiposity. Obesity Research 12(2):351—356.

[65] Azer, J., Hua, R., Vella, K., Rose, R.A., 2012 Nov. Natriuretic peptides regulate heart rate and sinoatrial node function by activating multiple natriuretic peptide receptors. Journal of Molecular and Cellular Cardiology 53(5):715—724.

[66] Sciarretta, S., Marchitti, S., Bianchi, F., Moyes, A., Barbato, E., Di Castro, S., et al., 2013 May 10. C2238 atrial natriuretic peptide molecular variant is associated with endothelial damage and dysfunction through natriuretic peptide receptor C signaling. Circulation Research 112(10):1355—1364.

[67] Rose, R.A., Giles, W.R., 2008 Jan 15. Natriuretic peptide C receptor signalling in the heart and vasculature. Journal of Physiology 586(2):353—366.

[68] Birkenfeld, A.L., Boschmann, M., Engeli, S., Moro, C., Arafat, A.M., Luft, F.C., et al., 2012. Atrial natriuretic peptide and adiponectin interactions in man. PLoS One 7(8):e43238.

[69] Arita, Y., Kihara, S., Ouchi, N., Takahashi, M., Maeda, K., Miyagawa, J., et al., 1999 Apr 2. Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. Biochemical and Biophysical Research Communications 257(1):79—83.

[70] Ouchi, N., Parker, J.L., Lugus, J.J., Walsh, K., 2011 Feb. Adipokines in inflammation and metabolic disease. Nature Reviews Immunology 11(2):85—97.

[71] Kim, J.Y., van de Wall, E., Laplante, M., Azzara, A., Trujillo, M.E., Hofmann, S.M., et al., 2007 Sep. Obesity-associated improvements in metabolic profile through expansion of adipose tissue. Journal of Clinical Investigation 117(9):2621—2637.

[72] Odegaard, J.I., Chawla, A., 2015 Oct. Type 2 responses at the interface between immunity and fat metabolism. Current Opinion in Immunology 36:67—72.

[73] Kiemer, A.K., Vollmar, A.M., 1997 Oct. Effects of different natriuretic peptides on nitric oxide synthesis in macrophages. Endocrinology 138(10):4282—4290.

[74] Kiemer, A.K., Vollmar, A.M., 2001 Nov. The atrial natriuretic peptide regulates the production of inflammatory mediators in macrophages. Annals of the Rheumatic Diseases 60(Suppl. 3):iii68—iii70.

[75] Shaw, C.A., Webb, D.J., Rossi, A.G., Megson, I.L., 2009 May 07. Cyclic GMP protects human macrophages against peroxynitrite-induced apoptosis. Journal of Inflammation (London) 6:14.

[76] Kammoun, H.L., Kraakman, M.J., Febbraio, M.A., 2014 Mar. Adipose tissue inflammation in glucose metabolism. Reviews in Endocrine and Metabolic Disorders 15(1):31—44.

[77] Yamada-Goto, N., Katsuura, G., Ebihara, K., Inuzuka, M., Ochi, Y., Yamashita, Y., et al., 2013 May. Intracerebroventricular administration of C-type natriuretic peptide suppresses food intake via activation of the melanocortin system in mice. Diabetes 62(5):1500—1504.

[78] Yamashita, Y., Yamada-Goto, N., Katsuura, G., Ochi, Y., Kanai, Y., Miyazaki, Y., et al., 2016 Jul. Brain-specific natriuretic peptide receptor-B deletion attenuates high-fat diet-induced visceral and hepatic lipid deposition in mice. Peptides 81:38—50.

[79] McKie, P.M., Schirger, J.A., Benike, S.L., Harstad, L.K., Slusser, J.P., Hodge, D.O., et al., 2016 Apr. Chronic subcutaneous brain natriuretic peptide therapy in asymptomatic systolic heart failure. European Journal of Heart Failure 18(4):433—441.

[80] Wan, S.H., McKie, P.M., Schirger, J.A., Slusser, J.P., Hodge, D.O., Redfield, M.M., et al., 2016 Jul. Chronic peptide therapy with B-type natriuretic peptide in patients with pre-clinical diastolic dysfunction (stage B heart failure). JACC. Heart Failure 4(7):539—547.

[81] Chen, H.H., Glockner, J.F., Schirger, J.A., Cataliotti, A., Redfield, M.M., Burnett Jr., J.C., 2012 Dec 4. Novel protein therapeutics for systolic heart failure: chronic subcutaneous B-type natriuretic peptide. Journal of the American College of Cardiology 60(22):2305—2312.

