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

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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. β3-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 β3-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 nephrilysin (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...
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 NRP-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 NRP-A and NRP-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 pups 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 day until day 7 with 200 μM 8-p-CTP-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 were 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 2 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 mM triiodothyronine, 1 μM D-biotin, 17 μM pantothenate, L-ascorbate (50 μM/mL), 1 μM Rosiglitazone, 0.172 μM insulin), and for 5 more days, the cells were maintained in maintenance media (DMEM supplemented with 5% FBS, 1% P/S, 1 mM Triiodothyronine, 0.172 μM insulin, 1 μM D-biotin, 17 μM pantothenate, L-ascorbate (50 μM/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-p-CTP-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 mM 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 RNaseasy 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 EDTA), 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 Na3VO4, 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 p2 (Santa Cruz Biotechnology); antibody against UCP-1 (Sigma Aldrich) and antibody against tubulin (Dia-novaUpstate). Secondary horseradish peroxidase-linked antibodies against goat (Pierce), mouse (Dianova), and rabbit (Cell Signaling) were
used. All bands were quantified by densitometric analysis with Image J software.

2.9. Animal studies
Wildtype mice C57Bl/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 mg/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, Snniff D12450B) for 12 weeks. The pumps were replaced every 4 weeks in mice. CD-NP (0.25 mg/day/kg), or saline was continuously released with a pumping rate of 0.11 ml/h. 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 (Abbott). The tail cuff was placed on 1.5% 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 (VO2) and CO2 production (VCO2) 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 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. Êvivo 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 6 h 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) and UCP-1 (H) (n = 6 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).

Next, we investigated 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 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 fat 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 3A–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%
compared to controls (ND/NaCl) (Figure 4D). Moreover, IFN-γ 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...
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 WAT as well as the increased adiponectin levels indicates that CD-NP treatment reduces inflammation in WAT.

4. DISCUSSION

Cyclic GMP regulates a variety of important physiological processes ranging from cardiovascular 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/enseymes (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 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...
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 mg/kg/h) than we did in our study (10.4 mg/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 epidydimal 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 NPR-A/NPR-C 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 WAT 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. Li 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 Gi 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 Glu4, 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 Glu4 expression. In contrast, WAT from CD-NP-treated obese mice have significantly lower expression of Glu4 compared to control animals.

NPN 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. [79], 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 and drafted the manuscript. A.P. supervised experiments and wrote the manuscript.

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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.

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