Natriuretic peptides promote glucose uptake in a cGMP-dependent manner in human adipocytes

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Robust associations between low plasma level of natriuretic peptides (NP) and increased risk of type 2 diabetes (T2D) have been recently reported in humans. Adipose tissue (AT) is a known target of NP. However it is unknown whether NP signalling in human AT relates to insulin sensitivity and modulates glucose metabolism. We here show in two European cohorts that the NP receptor guanylyl cyclase-A (GC-A) expression in subcutaneous AT was down-regulated as a function of obesity grade while adipose NP clearance receptor (NPRC) was up-regulated. Adipose GC-A mRNA level was down-regulated in prediabetes and T2D, and negatively correlated with HOMA-IR and fasting blood glucose. We show for the first time that NP promote glucose uptake in a dose-dependent manner. This effect is reduced in adipocytes of obese individuals. NP activate mammalian target of rapamycin complex 1/2 (mTORC1/2) and Akt signalling. These effects were totally abrogated by inhibition of cGMP-dependent protein kinase and mTORC1/2 by rapamycin. We further show that NP treatment favoured glucose oxidation and de nova lipogenesis independently of significant gene regulation. Collectively, our data support a role for NP in blood glucose control and insulin sensitivity by increasing glucose uptake in human adipocytes. This effect is partly blunted in obesity.

Atrial- and B-type Natriuretic Peptides (NP), ANP and BNP respectively, are well-known cardiovascular hormones produced by the right atria of the heart in response to mechanical stretch. They signal through the guanylyl cyclase-A (GC-A), a transmembrane receptor exhibiting guanylyl cyclase activity1–4. ANP and BNP can also bind to a clearance receptor named NPRC that sequesters, internalizes and degrades the peptides4. Over the last decade, NP have emerged as potent metabolic hormones as recently discussed5–8. NP were first identified as potent lipolytic hormones in human adipocytes9. They signal through the second messenger cGMP and downstream activation of a cGMP-dependent protein kinase-I (PRKGI)10. NP have subsequently been shown to modulate adipokine secretion11, and the browning of white fat cells12.

Several cohort and community-based studies have reported a strong association between plasma NP levels and obesity. In 2004, Thomas Wang and coworkers showed an inverse relationship between plasma NP levels and body mass index (BMI)13, findings which were then confirmed in several independent studies14,15. Kahn et al. later demonstrated an inverse relationship between plasma NP levels, insulin resistance and fasting blood glucose16. More recently, at least three prospective studies demonstrated a robust association between baseline plasma NP concentrations and the incidence of new onset type 2 diabetes (T2D)17–19. However a causal link between reduced plasma NP levels in obesity, insulin resistance and T2D has not yet been demonstrated. Thus it is so far unclear how NP may influence blood glucose control in humans.

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Considering that adipose tissue is a major target organ of NP20–22 and that glucose metabolism in adipocytes is a determinant of whole-body insulin sensitivity23,24, we hypothesized that NP signalling in adipose tissue could be a determinant of insulin sensitivity and blood glucose control. The aim of the present study was: 1) to explore the relationships between adipose NP receptor expression and obesity, insulin resistance and T2D, and 2) to determine the impact of NP on glucose metabolism in human adipocytes. Our data demonstrate a robust link between adipose GC-A expression and insulin sensitivity, and highlight a novel biological pathway in human adipocytes by which NP promote glucose uptake and metabolism in a cGMP-dependent manner.

Results

Adipose NP receptors expression is altered in obesity and type 2 diabetes. GC-A and NPRC gene expression was investigated in human adipose tissue biopsy samples from Cohort 1. We observed a gradual down-regulation of adipose GC-A mRNA levels as a function of the obesity grade, with the lowest expression levels in subjects with BMI >40 kg/m² (Fig. 1A). In contrast, adipose NPRC mRNA levels were progressively higher as a function of BMI and were nearly doubled in subjects with BMI >40 kg.m⁻² (Fig. 1B). Thus the ratio of GC-A-to-NPRC gene expression was significantly reduced by 39% for BMI between 30 and 35 kg.m⁻², and by 63% for BMI >40 kg.m⁻² (Fig. 1C). We also found a significant decrease of adipose GC-A expression in prediabetic (Pre-D, defined as impaired fasting glucose or glucose tolerance) and type 2 diabetic subjects (T2D) compared to individuals with normal glucose tolerance (NGT) (Fig. 1D), while no change was observed for NPRC (Fig. 1E). This association was independent of BMI as the average BMI was comparable between groups (NGT: 34.0 ± 0.3, Pre-D: 34.2 ± 0.4, T2D: 34.5 ± 1.3 kg.m⁻²). Adipose GC-A mRNA level was also reduced with increasing quartiles of HOMA-IR, demonstrating that the most insulin resistant individuals have the lowest adipose GC-A gene expression (Supplemental Fig. 1A), while no significant change was observed for NPRC (Supplemental Fig. 1B). In multivariate regression analyses, adipose GC-A levels correlated negatively with HOMA-IR (r = −0.20, p = 0.008), even after adjustment for BMI (β = −0.123, padj = 0.031). Finally, we observed a significant negative correlation (r = −0.26, p < 0.0001) between adipose GC-A gene expression and fasting blood glucose at baseline that was independent of BMI (β = −0.229, padj < 0.0001) (Fig. 1F). These data demonstrate a strong link between adipose NPR expression, obesity and blood glucose control.
Adipose GC-A expression relates to insulin sensitivity. The findings in cohort 1 were validated in cohort 2 showing a reduced adipose GC-A expression (Fig. 2A), increased NPRC expression (Fig. 2B) and reduced GC-A-to-NPRC ratio (Fig. 2C) in obese compared to lean individuals. Moreover, a strong inverse relationship between adipose GC-A expression and HOMA-IR (Fig. 2D) and de novo lipogenesis measured in isolated adipocytes (Fig. 2E). ***p < 0.0001 vs. lean.

Table 1. Correlations between adipose GC-A gene expression and HOMA-IR, and adipose GLUT4 and MLXIPL (ChREBP) gene expression after adjustment for BMI.
Natriuretic peptides promote glucose uptake in human adipocytes. We therefore directly assessed the effect of ANP on glucose uptake in human isolated adipocytes obtained from surgical samples. ANP dose-dependently activated basal glucose uptake with a 1.6 fold increase at doses of 10 and 100 nM, and a 2.2 fold increase at the highest dose of 1 µM (Fig. 3A). Of note, ANP promoted glucose uptake at submaximal insulin concentrations of 1 and 10 nM (Supplemental Fig. 3A). Importantly, ANP-mediated glucose uptake was impaired in isolated adipocytes from obese subjects when compared to lean (Fig. 3B). A similar effect was observed for insulin (Supplemental Fig. 3B). To investigate the underlying molecular mechanisms, we next switched to an established human adipocyte cell model system, i.e. human multipotent adipose-derived stem cells (hMADS)25. These cells express all the components of the NPR signalling pathway evidenced by the fact that GC-A mRNA emerged early in the time-course of adipocyte differentiation (day 3) and remained at steadily high levels till the end of the differentiation process (day 13) (Supplemental Fig. 4). NPRC and PRKGI were expressed at lower levels throughout the time-course of differentiation (Supplemental Fig. 4). Similar to the findings obtained in freshly isolated human adipocytes, ANP (Supplemental Fig. 5A) and BNP (Fig. 3C) induced a dose-dependent increase in glucose uptake that resulted in a 1.5 and 2 fold maximal activation in differentiated hMADS cells. In comparison, the maximal insulin-induced glucose uptake was about 2.5 fold (Supplemental Fig. 5B). ANP and BNP stimulated glucose uptake significantly starting from concentrations of 100 nM and upwards displaying EC₅₀ of 0.24 and 0.53 µM, respectively. It is worth mentioning at this stage that no significant effect of the specific NPRC agonist cANP₄₋₂₃ on glucose uptake could be noticed (data not shown). We next used BNP, that is more stable than ANP, for all subsequent experiments. Interestingly, BNP-induced glucose uptake was blunted in presence of (Rp)-8-pCPT-cGMPS 100 µM (PKG inhibitor, PKGi) in differentiated hMADS adipocytes (n = 8). *p < 0.05, **p < 0.01, ***p < 0.0001 vs. 0 or control.

Figure 3. Natriuretic peptides promote glucose uptake in a cGMP-dependent manner in human adipocytes. (A) Dose-response effect of ANP on 2-deoxyglucose uptake in human isolated adipocytes (n = 13). (B) Dose-response effect of ANP on 2-deoxyglucose uptake in human isolated adipocytes from lean (n = 14) versus overweight/obese subjects (n = 9). *p = 0.06 vs. lean. (C) Dose-response effect of BNP on 2-deoxyglucose uptake in differentiated hMADS adipocytes (n = 8). (D) BNP (100 nM)-mediated glucose uptake in absence or presence of (Rp)-8-pCPT-cGMPS 100 µM (PKG inhibitor, PKGi) in differentiated hMADS adipocytes (n = 8). *p < 0.05, **p < 0.01, ***p < 0.0001 vs. 0 or control.
Natriuretic peptides activate Akt-signalling in human adipocytes. Glucose uptake in human adipocyte is mediated by the glucose transporter GLUT4 in response to insulin through activation of the IRS1-P13K-Akt-signalling pathway\(^{27,28}\). Short-term treatment with BNP induced a time-dependent activation of Akt Ser473 phosphorylation, nearing 1.23 fold at 20 min and 3.7 fold at 60 min (\(p < 0.0001\)) (Fig. 4A,B). This effect was completely abolished by the cGK inhibitor (Rp)-8-pCPT-cGMPS. Similarly, BNP induced a 2-fold induction of Akt Thr308 phosphorylation that was totally abrogated by cGK inhibition (Fig. 4A–C). BNP-induced Akt activation was further associated with a downstream activation of AS160, a GTPase involved in GLUT4 translocation to the plasma membrane. As for Akt, BNP-mediated phosphorylation of AS160 was totally abrogated by the addition of a pharmacological inhibitor of cGK (Fig. 4A–D). We also confirmed previous findings\(^{12}\) showing that BNP treatment induces p38 MAPK (3.2 fold, \(p < 0.0001\)) in a cGK-dependent manner (Supplemental Fig. 6).

Natriuretic peptides activate mTOR-signalling in human adipocytes. Akt has been shown to be phosphorylated at Ser473 by mTOR complex 2 (mTORC2)\(^{29}\). Consistent with this, BNP-mediated phosphorylation of Akt was associated with increased mTOR (Fig. 5A,B), reduced Raptor (Fig. 5A–C), and increased Rictor (Fig. 5A–D) phosphorylation, indicating an activation of the mTORC1 and mTORC2 complexes respectively. Importantly, we further show that pharmacological inhibition of mTOR by rapamycin totally abrogates BNP-mediated glucose uptake (Supplemental Fig. 7A) and Akt Ser473 phosphorylation (Supplemental Fig. 7B).

In summary, our data demonstrate that NP-mediated glucose uptake requires mTORC1/2 to induce Akt signalling in human adipocytes.

Natriuretic peptides enhance glucose metabolism in human adipocytes. We finally investigated the fate of glucose taken up by adipocytes in response to NP. Acute BNP treatment (3 h) induced both glucose oxidation (+19% versus control, \(p < 0.05\)) (Fig. 6A), and glucose incorporation into glycerol (+33% versus control, \(p < 0.05\)) (Fig. 6B) or fatty acids (+78% versus control, \(p < 0.05\)) (Fig. 6C) in hMADS adipocytes. No significant change in mRNA levels of prototypical de novo lipogenic genes such as MLXIPL (ChREBP), ACC1, FASN, and ELOVL6 was observed in response to 6 h treatment with ANP or BNP (Supplemental Fig. 8). No significant

Figure 4. Natriuretic peptides activate Akt-signalling in a cGMP-dependent manner in hMADS adipocytes. Representative blots (A) and quantitative bar graphs of Akt Ser473, phosphorylation relative to total Akt (B), Akt Thr308 phosphorylation relative to total Akt (C), AS160 Thr642 phosphorylation relative to total AS160 (D), in response to 20 min and 60 min treatment with BNP 100 nM in absence or presence of (Rp)-8-pCPT-cGMPS 100\(\mu\)M (PKG inhibitor, PKGi). *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.0001\) vs. control (n = 6).
change in GLUT1 and GLUT4 mRNA levels were observed as well (data not shown). BNP also induced about 2-fold the incorporation of glucose into fatty acids in human isolated adipocytes (Fig. 6D).

Discussion

Despite the robust inverse link between plasma NP levels, obesity and the incidence of T2D, no study has so far provided data showing a direct mechanistic link between adipose tissue NP signalling and glucose homeostasis. Herein, we provide some evidence that NP receptor expression in adipose tissue is tightly related to blood glucose control and insulin sensitivity in different European populations. We further demonstrate a novel biological role of NP in human adipocytes where they directly promote glucose uptake and de novo lipogenesis in fat cells through a cGMP-dependent pathway (Fig. 6E). Overall, our data suggest that NP signalling in human adipocytes is an important determinant of insulin sensitivity that is altered in obesity and T2D.

Previous studies have established that adipose tissue is a key target organ of NP. In this study, we took advantage of a large adipose tissue collection from cohort 1 to investigate NPR gene expression in the context of obesity and T2D. Due to the large sample size, we observed a very strong negative correlation between adipose GC-A gene expression and insulin resistance, suggesting that low adipose GC-A expression relates to low insulin sensitivity. In contrast, adipose NPRC mRNA levels were not associated with insulin resistance. Recent studies indicate that adipose glucose transporter-4 (GLUT4) and carbohydrate-responsive element binding protein (ChREBP, encoded by MLXIPL) are major determinants of adipocyte and whole-body insulin sensitivity. In the current study, we found very robust relationships between adipose GC-A, HOMA-IR, GLUT4 and MLXIPL mRNA levels in two independent cohorts. These relationships remained significant also after statistical adjustment for BMI in cohort 2, indicating that adipose GC-A behaves as a determinant of whole-body insulin sensitivity independently of body weight. In addition, we observed major differences in adipose tissue NPR expression in relation to degree of obesity and diabetic status. Due to paucity of tissue sample it was not possible to also measure protein expression. However previous studies in human adipose tissue suggest a close relationship. Thus, in agreement with recent studies reporting on the mRNA and protein levels of GC-A and NPRC in obese and non-obese subjects and mice, GC-A expression was negatively and NPRC positively associated with BMI. Moreover, GC-A mRNA levels were lower in individuals with prediabetes and T2D compared with NGT subjects, an observation that fits well with previously reported animal studies in high-fat diet-fed and db/db mice.
Our current data are also in line with a recent study from Kovacova et al., demonstrating a down-regulation of the GC-A-to-NPRC ratio in obese versus lean individuals as well as a strong link with whole-body insulin sensitivity.

In light of the tight relationship observed between adipose GC-A, de novo lipogenesis and the lipogenic genes GLUT4 and MLXIPL, we next studied the effect of NP on glucose uptake in human adipocytes. We could first show that ANP dose-dependently activated glucose uptake in human isolated adipocytes. Although both the cGMP and the cAMP pathways promote lipolysis and browning of white adipocytes, they display contrasting effects with respect to glucose uptake which is inhibited by activating the cAMP-signalling pathway. Catecholamines inhibit glucose uptake by inducing lipolysis and facilitating the dissociation of the mTORC1/2 complex. In the current study, we provide evidence of a NP-mediated glucose uptake in adipocytes, which is independent of insulin and requires downstream cGMP-signalling since pharmacological inhibition of cGK totally abrogates NP-mediated glucose uptake. Although cGMP has been shown to mediate glucose uptake in skeletal muscle, this is the first study reporting that activation of cGMP-signalling by NP promotes glucose uptake in human adipocytes.

Insulin promotes glucose uptake in skeletal muscle cells and adipocytes through activation of the phosphatidylinositol-3-kinase/Akt pathway leading to GLUT4 translocation to the plasma membrane. We here demonstrate that BNP treatment in hMADS adipocytes induces Akt phosphorylation at both Thr308 and Ser473 residues. Again this effect appears to be mediated by cGMP since pharmacological blockade of...
cGK completely abrogated BNP-mediated Akt phosphorylation. Since Akt phosphorylation at Ser473 requires mTORC2 activation, we further show that BNP treatment was able to induce mTORC2 through phosphorylation of mTOR and Rictor. We also show that NP signalling induces mTORC1 by modulating both mTOR and Raptor. It is possible that cGK-I directly phosphorylates mTOR and Raptor, since cAMP-dependent protein kinase was recently shown to do so. We could next demonstrate that pharmacological inhibition of mTORC1/2 by rapamycin totally suppresses NP-mediated Akt phosphorylation at Ser473 and glucose uptake. Altogether, this suggests that cGK-I may indirectly modulate Akt signalling through mTORC1/2. Our data are overall consistent with previous work showing the critical role of mTORC1/2 in the control of glucose metabolism in adipocytes. Our data are also in line with recent data from the Collins’ group showing that enhanced NP signalling by deleting Npr3 specifically in mouse adipose tissue is associated with higher phosphorylation of Akt at Ser473 and Thr308. Interestingly, BNP-mediated Akt activation was accompanied by an elevated phosphorylation of the Rab GTPase-activating protein AS160 (also termed TBC1D4), which coordinates GLUT4 translocation to the plasma membrane in adipocytes and myocytes.

Considering the tight relationship observed between adipose GC-A and MLXIPL mRNA levels, we examined the fate of the glucose taken up by adipocytes in response to NP. We could demonstrate that about one quarter of the glucose taken up by the adipocyte was directed toward glucose oxidation while the remaining three quarters were incorporated into the glycerol backbone for triglyceride synthesis. Of interest, a quantitatively minor fraction of glucose (less than 1%) served for de novo production of fatty acids, i.e. de novo lipogenesis. Thus activation of NP signalling in adipocytes enhances de novo lipogenesis. No significant changes in prototypical genes of de novo lipogenesis were observed after NP treatment indicating that NP-mediated de novo lipogenesis likely results from NP-mediated glucose uptake independently of the transcriptional activity of the glucose-regulated transcription factor ChREBP.

In light of the potent lipolytic effect of NP previously observed in human adipocytes, it may seem paradoxical at first glance that NP can also trigger glucose uptake. Although speculative, this could reflect some sort of futile cycle by which NP firstly enhance glucose uptake and de novo lipogenesis, and then promote triglyceride breakdown through lipolysis and fatty acid oxidation. The effect of NP on glucose uptake is also consistent with their browning effect in white adipocytes as glucose is an important substrate for brown adipocytes, and this futile cycle lipogenesis/lipolysis could provide an additional energy dissipating process in beige/brown adipocytes. Indeed, futile cycling between de novo lipogenesis and fatty acid oxidation has been shown to be induced in adipose tissues during cold exposure or stimulation with a β-adrenergic agonist as recently discussed. High rates of de novo lipogenesis are observed in brown adipocytes during cold exposure supplying approximately 30% of oxidized FA required for heat production. Such a dual effect on glucose uptake and lipolysis has also been observed in rat adipocytes treated with β-sitosterol.

We obviously acknowledge a number of limitations in the present work: 1) We were not able to measure GC-A and NPRC protein expression, to corroborate mRNA data, as no sample was prepared and/or biopsy material left for this type of analysis in both clinical cohorts. 2) We did not fully elucidate the precise signalling pathway by which NP promote glucose uptake. However we can also emphasize the strengths of the current study: 1) Gene expression data and associations with surrogates of insulin sensitivity were observed in a large sample size from two independent cohorts. 2) The effect of NP on glucose uptake was consistently observed in isolated human mature adipocytes and cultured hMADS adipocytes. 3) Our data are in line with a recent study indicating that enhancing NP signalling specifically in adipose tissue improves insulin signalling in white and brown fat, systemic insulin sensitivity and glucose tolerance.

In summary, our data suggest an important role of NP in regulating adipocyte glucose metabolism and insulin sensitivity in humans. This occurs through GC-A in a cGMP-dependent manner in parallel of the insulin pathway. Our clinical data also argue that adipose GC-A expression is tightly associated with whole-body insulin sensitivity. Future studies should investigate the causal link between the NP/GC-A system and T2D before considering GC-A activation as a potential target to improve blood glucose control and insulin sensitivity.

**Methods**

Details for cultures of hMADS adipocytes can be found in Supplementary Material.

**Clinical studies and human subjects.**

**Cohort 1.** The samples investigated in this paper were collected from 2006 to 2007 during the DiOGenes study, a pan-European randomized trial, which was approved by the ethics committees of each of the 8 European centers participating to the program (NCT00390637). The DiOGenes project investigated the effects of diets with different content of protein and glycemic index on weight-loss maintenance and metabolic and cardiovascular risk factors after an 8-week calorie restriction phase, in obese/overweight individuals. Written informed consent was obtained from each patient according to the local ethics committee of the participating countries as previously described. The samples investigated in this paper were collected from 2006 to 2007 during the DiOGenes study, a pan-European randomized trial, which was approved by the ethics committees of each of the 8 European centers participating to the program (NCT00390637). The DiOGenes project investigated the effects of diets with different content of protein and glycemic index on weight-loss maintenance and metabolic and cardiovascular risk factors after an 8-week calorie restriction phase, in obese/overweight individuals. Written informed consent was obtained from each patient according to the local ethics committee of the participating countries as previously described.

Healthy overweight (body mass index (BMI) ≥ 27 kg/m²) individuals, aged <65 years were eligible for the study. Exclusion criteria were BMI >45 kg/m², liver or kidney diseases, cardiovascular diseases, diabetes mellitus type 1, special diets/eating disorders, systemic infections/chronic diseases, cancer within the last 10 years, weight change >3 kg within the previous 3 months, and other clinical disorders or use of prescription medication that might interfere with the outcome of the study.

A detailed description of inclusion and exclusion criteria has been published previously. BMI was calculated by dividing weight in kilograms by the square of height in meters. A detailed description of the DiOGenes intervention trial and main outcomes can be found in the core publication. Briefly, among 1209 individuals screened, 932 entered a baseline clinical investigation day including anthropometric measures (height, weight, waist circumference, body composition), blood pressure measurements, fasting blood sampling, and subcutaneous
adipose tissue biopsies were performed (at baseline and at the end of each phase). All procedures were standard-
ized between the 8 study centers across Europe.

Cohort 2. Cohort 2 comprised 30 obese (BMI >30kg/m²) otherwise healthy and 26 non-obese (BMI <30kg/m²)
healthy women that have been described in detail previously52. All were pre-menopausal and free of continuous
medication. They were investigated in the morning after an overnight fast in the midst of their menstrual cycle.
A venous blood sample was obtained for measurements of glucose and insulin and the values were used to calculate
HOMA-IR53. An abdominal subcutaneous adipose tissue biopsy was obtained by needle aspiration as described54.

Gene microarrays. From adipose tissue biopsy total RNA in Cohort 2, biotinylated complementary RNA
was analyzed using the GeneChip Human Gene 1.0 ST Array (Affymetrix Inc., Santa Clara, CA). Slides were
washed, stained, scanned and analyzed using standardized protocols (Affymetrix Inc.) as described previously52.
Data are deposited at the National Center for Biotechnology Information Gene Expression Omnibus (GEO;
http://ncbi.nlm.nih.gov/geo) under the accession number GSE25402.

Microfluidic card. Total RNA was extracted from adipose tissue biopsies and RT-qPCR was performed
using the Fluidigm BioMark System as described in55. Briefly, cDNA was prepared from 500 ng of total RNA and
diluted in water to 5 ng/μL (RNA equivalent). The reverse transcription step was checked using HPRRT
expression level using StepOnePlus (Applied Biosystems). A multiplexed preamplification process was performed on every
1.25 μL cDNA using 14 cycle cDNA preamplification step (95 °C for 15 sec and 60 °C 4 min) and Taqman PreAmp
Master Mix (Applied Biosystems) in a standard PCR thermocycler. Preamplified cDNA was diluted 1:5 in 10 mM
Tris, 1 mM EDTA (TE). Diluted cDNA (2.25 μL) was added to 2.5 μL Taqman Universal PCR Master Mix (Applied
Biosystems) and 0.25 μL GE Sample Loading Reagent (Fluidigm). In a separate tube, 3.5 μL of Taqman Assay
was added to 3.5 μL Sample Loading Reagent. Five μL cDNA samples were loaded into the sample inlet wells, and
5 μL assay samples were loaded into assay detector inlets. For each plate, 1 well was loaded with H2O as control for
each primer. The cap was primed and placed into the NanoFlex Integrated fluidic circuit controller where 8
nL of cDNA and 1 nL of Assay were mixed. Raw time PCR was on the BioMark System (Fluidigm). Raw data
obtained from the system’s software using the default global threshold setting (BioMark Real-time PCR Analysis
V2.1.1 Fluidigm) were checked using the graphical representation of the plate layout. PUM1, was found as the
most stable gene using the geNorm algorithm56, then raw Ct values were transformed to relative gene expression
using the 2ΔΔCt method using PUM1 mRNA level as reference.

Real-time qRT-PCR. Total RNA was from cultured hMADS cells was isolated in RNeasy Lysis Buffer ± mer-
captoethanol reagent (Qiagen GmbH, Hilden, Germany). The quantity of the RNA was determined on a
NanoDrop ND-1000 (Thermo Scientific, Rockford, IL, USA). Reverse transcriptase PCR was performed on
a GeneAmp PCR System 9700 using the Multiscribe Reverse Transcriptase method (Applied Biosystems,
Foster City, CA). Real-time quantitative PCR (qPCR) was performed to determine cDNA content. All prim-
ers were bought from Applied Biosystems. Primers used were: 18S (Taqman assay ID: Hs99999901_s1), ACC1
(Hs00167385_m1), EAS (Hs00188012_m1), ChREBP (Hs00975714_m1). ELOVL6, SYBR green primers, for-
ward: CCATCCAATGGATGCAGGAAAAC; reverse: CCAGAGCACTAATGGCTTCTCCT were purchased at
Eurogentec. qPCR was then performed on a StepOnePlus real-time PCR system (Applied Biosystems). For each
primer, a standard curve was made prior to mRNA quantification to assess the optimal total cDNA quantity. All
expression data were normalized by the 2−ΔΔCt method using 18S as internal control55,57.

Glucose uptake assay in isolated mature adipocytes. Fat cell suspensions were incubated without
(baseline) or with insulin or ANP for 45 min at 37 °C, pH 7.5 in 400 μL final volume of incubation medium (see
supplemental methods). Then, an isotopic dilution of 2-deoxy-D-[3H]glucose (2-DG) was added to reach 50
μCi/mL and 1,000,000 dpm in each assay tube that contained an average amount of fat cells equivalent to 19 ± 1 mg
lipids, and that was further incubated for an additional 10-min period as previously described49. After stopping
by addition of 100μM cytochalasin B, cell suspension aliquots were centrifuged through diisopropyl-phthalate
layer to separate the buoyant adipocytes from the medium allowing counting the intracellular radioactive 2-DG,
as an index of glucose uptake, which was expressed as nmol 2-DG internalized/100 mg cellular lipids/10 min, as
previously reported49.

Glucose uptake assay in hMADS adipocytes. The day before the assay, insulin was removed from culture
medium. After two washes with PBS, cells were incubated 50 min at 37 °C without or with various concentra-
tions of BNP (10−11 to 10−5 M), ANP (10−9 to 10−5 M) or insulin. Then, 125μM 2-deoxy-D-glucose and 0.4 μCi
2-DG per well were added for 10 min incubation. Culture plates were put on ice and rinsed with 10 mM glucose
in ice-cold PBS and then with ice-cold PBS. Cells were scraped in 0.05 N NaOH and 2-deoxy-D-glucose uptake
was measured by liquid scintillation counting of cell lysate. Data are expressed as nanomoles per minute and were
normalized per mg of protein49.

Determination of glucose oxidation. The day before the assay, insulin was removed from culture
medium. Cells were preincubated with a glucose- and serum-free medium for 90 min then exposed to DMEM
low glucose (5.5 mM) supplemented with D-[U-14C]glucose (1 μCi/mL; PerkinElmer, Boston, MA) in the pres-
ence or absence of 100 nM BNP for 3 h. Following incubation, glucose oxidation rate was determined by meas-
uring [14C]CO2 by liquid scintillation counting as previously described57. Briefly, assayed medium is transferred
into a custom-made Teflon 48-well trapping plate. The plate is clamped and sealed, and perchloric acid is injected
through the perforations in the lid into the medium, which drives CO2 through the tunnel into an adjacent well,
where it is trapped in 1N NaOH. Following trapping, the total volume of NaOH is transferred into scintillation vials, and radioactivity is measured on a multipurpose scintillation counter (LS 6500; Beckman Coulter). All assays are performed in triplicates, and data are normalized to total protein content.

**Determination of glucose incorporation into glycerol and fatty acids.** To determine glucose carbon incorporation into fatty acid and glycerol, after the glucose oxidation as described above, adipocytes were collected in SDS 0.1% and total lipids were extracted in chloroform/methanol (2:1) according to Folch et al. After centrifugation, the organic phase was dried under nitrogen and hydrolyzed in 1 mL 0.25% NaOH in chloroform/methanol (1:1) for 1 h at 37 °C. The solution was neutralized with 500 mL 0.5 N HCl in methanol. FA and glycerol were separated by adding 1.7 mL chloroform, 860 mL water, and 1 mL chloroform/methanol (2:1). Incorporation of 14C into glycerol and FA was measured by liquid scintillation counting of upper and lower phases, respectively. Specific activity was counted and used to determine the quantity of incorporated glucose equivalent. Data were normalized to total cell protein content.

**Western blot.** Differentiated hMADS cell lysates were extracted, transferred onto nitrocellulose membranes and blotted with the following primary antibodies (all from Cell Signalling Technology Inc., Beverly, MA): phospho-Akt Ser473 (#4060), phospho-Akt Thr308 (#2965), Akt (#4691), phospho-IRS1 Tyr612 (#44816G), IRS1 (#3407), phospho-AS160 Thr642 (#4288), AS160 (#2670), phospho-p38 MAPK Thr180/Tyr182 (#9211), and p38 MAPK (#9212), phospho-Raptor Thr792 (#2083), Raptor (#1210), phospho-mTOR Ser2448 (#2971), Rictor (#2140), phospho-mTOR Ser2448 (#2971), mTOR (#2972). Immunoreactive proteins were detected by enhanced chemiluminescence reagent (SuperSignal West Dura or SuperSignal West Femto; Thermo Scientific), visualized using the ChemiDoc MP Imaging System and data analyzed using the Image Lab 4.1 version software (Bio-Rad Laboratories, Hercules, USA). α-tubulin (Sigma-Aldrich) was used as internal control.

**Statistical analyses.** All statistical analyses were performed using GraphPad Prism 5.0 for Windows (GraphPad Software Inc., San Diego, CA). Normal distribution and homogeneity of variance of the data were tested using Shapiro-Wilk and F tests, respectively. One-way ANOVA followed by Tukey’s post-hoc tests and Student’s t-tests were performed to determine differences between groups, interventions and treatments. Two-way ANOVA followed by Bonferonni’s post hoc tests were applied when appropriate. Linear regression was performed after log transformation of nonparametric data. The false discovery rate for multiple testing was controlled by ANOVA followed by Bonferonni’s post hoc tests were applied when appropriate. Linear regression was performed after log transformation of nonparametric data.

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Author Contributions
M.C., V.B., P.M., K.L., C.L., A.M., C.C., N.V., P.A., D.L., M.R. and C.M. researched data and edited the manuscript. M.C. and C.M. wrote the manuscript. Dr. Cedric Moro is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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