Insulin/Glucose Induces Natriuretic Peptide Clearance Receptor in Human Adipocytes: a Metabolic Link With the Cardiac Natriuretic Pathway

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ABSTRACT

Cardiac natriuretic peptides (NP) are involved in cardio-renal regulation and in lipolysis. The NP activity is largely dependent on the ratio between the signaling receptor NPRA and the clearance receptor NPRC. Lipolysis increases when NPRC is reduced by starving or very-low calorie diet. On the contrary, insulin is an anti-lipolytic hormone that increases sodium-retention, suggesting a possible functional link with NP. We examined the insulin-mediated regulation of NP receptors in differentiated human adipocytes and tested the association of NP receptors expression in visceral adipose tissue (VAT) with metabolic profiles of patients undergoing renal surgery. Differentiated human adipocytes from VAT and Simpson-Golabi-Behmel Syndrome (SGBS) adipocyte cell line were treated with insulin in the presence of high glucose or low-glucose media to study NP receptors and insulin/glucose regulated pathways. Fasting blood samples and VAT samples were taken from patients in the day of renal surgery. We observed a potent insulin-mediated and glucose-dependent up-regulation of NPRC, through the PI3K pathway, associated with lower lipolysis in differentiated adipocytes. No effect was observed on NPRA. Low-glucose medium, utilized to simulate in vivo starving conditions, hampered the insulin effect on NPRC through modulation of insulin/glucose regulated pathways, allowing ANP to induced lipolysis and thermogenic genes. An expression ratio in favor of NPRC in adipose tissue was associated with higher fasting insulinemia, HOMA-IR and atherogenic lipid levels. Insulin/glucose dependent NPRC induction in adipocytes might be a key factor linking hyperinsulinemia, metabolic syndrome and higher blood pressure by reducing NP effects on adipocytes.
KEY WORDS natriuretic peptide receptors, insulin, insulin-resistance, insulin-induced genes, lipolysis, human adipocytes
INTRODUCTION

Atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) are cardiac natriuretic peptides (NP), true hormones well known for their renal, endocrine and cardiovascular activities, reducing sodium reabsorption and arterial blood pressure and exerting an overall protective function on heart, kidney and the vascular tree (16). These effects are mainly mediated by the second messenger cGMP. Other physiological functions exerted through a cGMP-dependent pathway (3, 35) have been described including activation of lipolysis (34), mitochondriogenesis (21, 2) and thermogenic program with potency similar to catecholamines (5).

Two distinct NP receptors modulate the final response to cardiac NP: the cGMP-signaling receptor NPRA and the clearance receptor NPRC. The cellular and systemic effects of NP largely depend upon the ratio of the clearance receptor NPRC to the signaling receptor NPRA, both abundantly expressed in human adipose tissue and adipocytes (29). In non-primate species, such as rodents, the NPRC is so abundant that the lipolytic activity of NP is very difficult to demonstrate (36). On the contrary, in mice lacking NPRC, the natriuretic peptide system is clearly more active (19) and mice are leaner and have smaller, brownish adipocytes (5). In NPRC-deficient mice, the ANP half-life is prolonged and ANP effects are enhanced (19). Indeed, most of the available data indicate that the total NPRC expression in the body is the main negative modulator of the circulating NP levels and, at cellular level, NPRC is a main silencer of their biological effects (32). NP are not only 'cleared' from circulation by NPRC, but they are also degraded very quickly by neutral endopeptidase (NEP) and both are increased in obesity (11).
We have found that starving sharply decreases NPRC expression in white and brown rat adipose tissue (31) and that a low-calorie diet in obese hypertensive patients strongly potentiates the clinical and biological effects of infused ANP (11). Hypocaloric diet enhances ANP-induced lipolysis in humans whereas obesity is associated with lower circulating levels of NP (29).

Moreover, while physical exercise acutely increases ANP release in obese patients, hypocaloric diet induces weight loss primarily affecting NPRC expression (14). ANP-induced lipolysis appears to be more sensitive to obesity than catecholamine probably due to an altered adipocyte NPRC/NPRA expression ratio in obesity (28). Indeed, recent studies reported also a link between obesity/type 2 diabetes and NP receptors mRNA levels in both muscle and subcutaneous adipose tissue, suggesting that NPRA and NPRC expression are impaired in obesity not only in adipose but also in skeletal muscle (24, 21, 10, 28).

In 1994, Endre et al. showed that euglycemic hyperinsulinemic clamp with 2 hours of insulin infusion produced a reduction of serum ANP together with a reduction of sodium excretion in normotensive and hypertensive men (12). These data suggested that insulin could promote sodium retention and higher blood pressure through an interaction with the NP system. Insulin inhibits catecholamine-induced lipolysis mainly via phosphodiesterase 3B (PDE3B) activation which subsequently leads to cAMP degradation and deactivation of PKA (9) but does not seem to have a direct antilipolytic effect through an inhibition of the cGMP-protein kinase G (cGMP/GK-I) dependent lipolytic pathway (22, 23).

We therefore hypothesize that insulin/glucose attenuate lipolysis in adipocytes by inducing NPRC expression, a pathway leading to triglyceride accumulation and adipocyte hypertrophy, a known insulin-resistance condition.
Indeed, in ob/ob mice, which represent a hyperinsulinemic model, NPRA level was lower and
NPRC mRNA level was higher in adipose tissue compared to lean control mice (24). Recently it
was shown, in murine 3T3-L1 adipocytes, that insulin appears to be a major regulator of the NP
receptors, reducing NPRA and enhancing NPRC expression through the phosphatidylinositol 3-
kinase (PI3K) pathway (24). Similar results were obtained with subcutaneous adipose tissue
(SAT) of individuals undergoing euglycemic and hyperglycemic hyperinsulinemic clamp (26). It
was also demonstrated an increased expression of NPRC in human monocytes (but not in
macrophages) after simultaneous high glucose and insulin infusion (26).

Adipose tissue RNA comes mostly from non-adipocyte cell types rich in NPRC whereas
the effect of insulin on NP receptor expression has never been studied in isolated human
adipocytes obtained from visceral adipose tissue (VAT). Therefore, the main aim of our study
was to investigate the effect of insulin on NPRA and NPRC expression in primary cultures of
visceral human adipocytes, obtained from VAT of patients undergoing nephrectomy, as well as
in a differentiated human adipocyte cell line obtained from Simpson-Golabi-Behmel syndrome
(SGBS) (13). SGBS is a well reproducible in vitro model of human adipocytes to test insulin and
NP system as well as for glucose-regulated genes analysis, whereas human VAT-derived
primary preadipocytes is the more closely related model to \textit{in vivo} visceral adipocytes,
maintaining patient-specific variability without the interferences of leukocytes, progenitor cells
and vascular-stromal cells present in the whole adipose tissue.

Insulin-mediated regulation of NP receptors was also assessed using low-glucose culture
media to simulate in-vivo starving conditions. Moreover, we tested the association between the
pattern of NP receptors expression in VAT and available clinical metabolic parameters of
patients undergoing renal surgery.
GLOSSARY

ANP: atrial natriuretic peptide
ATGL: adipose triglyceride lipase
BNP: B-type natriuretic peptide
cAMP: cyclic adenosine monophosphate
cGMP/GK-I: cGMP-dependent protein kinase I
ChREBP-α: carbohydrate-responsive-element binding protein alpha
ChREBP-β: carbohydrate-responsive-element binding protein beta
CYTO C: cytochrome c
GLUT4: glucose transporter type 4
HSL: hormone sensitive lipase
LXR-α: liver X receptor alpha
NP: cardiac natriuretic peptides
NPRA: natriuretic peptide receptor A
NPRC: natriuretic peptide clearance receptor
PGC-1α: peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K: phosphatidylinositol 3-kinase
PKA: protein kinase A
SAT: subcutaneous adipose tissue
SGBS: cell line obtained from Simpson-Golabi-Behmel syndrome
SGBS: Simpson-Golabi-Behmel syndrome
SREBP-1c: sterol regulatory element binding protein 1c
UCP1: uncoupling protein 1
VAT: visceral adipose tissue
MATERIALS AND METHODS

Reagents and antibodies

Insulin, dexamethasone, isobutylmethylxanthine, tri-iodothyronine, transferrin and wortmannin were obtained from Sigma–Aldrich (St. Louis, Missouri, USA). Antisera against NPRC was a kind gift from William Gower at the James A. Haley VA Medical Center, Tampa, Florida, USA. GAPDH antibody (cat #SC25778) and secondary antibody anti-rabbit (cat #SC2054) were from Santa Cruz Biotech; California, USA. Super signal West Femto Maximum Sensitivity Substrate was from Thermo scientific, Rockford, Illinois, USA.

Adipocyte cells culture

Simpson-Golabi-Behmel syndrome cell line (SGBS)

Cells from Simpson-Golabi-Behmel syndrome cell line (SGBS) were grown in growth medium (DMEM/F12 with 10% fetal calf serum). When adipocytes reached 85-90% confluence, they were differentiated in differentiation medium, that included insulin, as previously described (13). To test the acute effect of insulin on NP receptors, we removed insulin from differentiation media at day 7 instead of day 12 (Figure 1). Thus, on the seventh day the cells were washed and deprived of insulin for five days, and then were treated or not with 100 nM wortmannin, a specific and potent PI3K inhibitor (37, 38), for 30 min before adding 100 nM of insulin. This dose was chosen on the basis of the dose-response experiments from 50 to 1µM (Figure 2).

Another set of experiments was performed to simulate in vivo “starving” conditions. Adipocytes were differentiated as described above and then were studied under three different conditions:
- Phase 1: DMEM F12 (glucose 17.5 mM, osmolality between 290-330 mOsm) + insulin 100 nM for 6 hrs
- Phase 2: DMEM/F12 (glucose 17.5 mM) + insulin 100 nM for 6 hrs followed by over-night (12-14 hrs) incubation with DMEM low glucose medium (glucose 5.5 mM) instead of DMED/F12;
- Phase 3: DMEM/F12 (glucose 17.5 mM, osmolality 300-340 mOsm) + insulin 100 nM for 6 hrs followed by over-night (12-14 hrs) incubation with low glucose medium (glucose 5.5 mM) instead of DMED/F12 + insulin 100 nM for 6 hrs.

To assess whether the 70% reduction of glucose concentration in culture media simulates starving condition, we tested the gene expression of a cluster of insulin/glucose induced genes involved in lipogenesis. Particularly we analyzed the major insulin-responsive glucose transporter type 4 (GLUT4), liver X receptor alpha (LXR-α) together with the two major transcription factors involved in the lipogenesis pathway, sterol regulatory element binding protein 1c (SREBP-1c) and carbohydrate-responsive-element binding protein α (ChREBP-α) and β (ChREBP-β).

Moreover to evaluate the opposite effects of NP versus insulin on lipolysis and anti-lipolysis pathway, SGBS cells were treated as described above and with ANP 100 nM for 6 hrs. RNA was than analyzed to test the main genes involved in the lipolysis pathway such as hormone sensitive lipase (HSL), adipose triglyceride lipase (ATGL) and the main thermogenic genes involved in the process of adaptive thermogenesis in brown adipocytes such as mitochondrial uncoupling protein 1 (UCP1), the transcriptional coregulator PPARγ coactivator-1α (PGC-1α), and cytochrome c (CYTO C) implied in mitochondrial biogenesis.

Primary visceral adipocytes culture
Visceral adipose samples (2–3 g) obtained from patients undergoing radical nephrectomy for localized clear cell renal carcinoma (without any evidence of local or metastatic cancer spread: T1/T2, N0, M0) at the “Ospedali Riuniti” University Hospital of Ancona, Italy, were cut into small pieces and digested with collagenase type I to obtain the stromal vascular fraction (SVF). Adipocyte differentiation was obtained as previously described (30). Treatment with insulin, wortmannin and starving condition were done as described above for SGBS cells.

Patients

Another set of human visceral adipose tissue samples (n=34) were obtained from patients undergoing radical nephrectomy for localized clear cell renal carcinoma (without any evidence of local or metastatic cancer spread: T1/T2, N0, M0) at the “Ospedali Riuniti” University Hospital of Ancona, Italy. In the morning, after an overnight fasting, blood was drawn for lipid profile as well as glucose and insulin assays. Anthropometrics, clinical and humoral parameters are shown in Table 1. Insulin resistance was evaluated by homeostasis model assessment (HOMA-IR) formula: fasting glucose (mmol/L) x fasting insulin (microU/ml) /22.5. All women were in menopause. The study was conducted in accordance with the guidelines proposed in The Declaration of Helsinki and the local Ethics Committee approved the study protocol. All patients gave written informed consent for the collection of clinical data and tissue samples.

RNA isolation and gene expression analysis

Total RNA was extracted using Trizol (Invitrogen) and RNA reverse transcription of 2 μg was performed with High-Capacity cDNA reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Warrington, UK). All gene expression experiments in SGBS and primary VAT
adipocytes cultures were analyzed with Syber Select Master mix (Applied Biosystems Darmstadt, Germany). Each single gene expression experiment was performed in triplicate. Differences in total RNA or different efficiency of cDNA synthesis among samples were normalized using human GAPDH expression.

Lipolysis

SGBS adipocytes were differentiated with insulin for seven days and treated at day 12 as described above (see Phase 1) with insulin (100nM) or ANP (100nM) or both. Moreover a set of SGBS differentiated adipocytes were treated as described for Phase 2 [DMEM/F12 (glucose 17.5 mM) + insulin 100nM for 6hrs followed by over-night (12-14 hrs) incubation with low glucose medium (glucose 5.5 mM) instead of DMED/F12] and than treated with ANP 100 nM for 6hrs (see Phase 3). Extracted RNA was used to test gene expression levels of HSL, ATGL. Free Glycerol Determination Kit (Sigma-Aldrich) was used to measure free glycerol released in the cultured medium. Glycerol concentrations were determined by comparison with a standard curve. Intra-assay and inter-assay CV were less than 6% and 9.1%, respectively.

Western blotting

Treated cells were lysed and sonicated in an appropriate buffer as previously described (5). Protein concentrations were determined using the Bradford Assay (Biorad) and 50 μg of total proteins was resolved in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a PVDF membrane (Immobilon P, Millipore), and probed overnight at 4°C with specific NPRC primary antibodies. NPRC antibody specificity was tested using samples from NPRC deficient mice NP, as previously described (5). Secondary antisera against
rabbit IgG conjugated with peroxidase was used for specific protein detection. NPRC protein was visualized using an enhanced chemiluminescent substrate (SuperSignal West Femto Maximum Sensitivity Substrate, Pierce) and were measured in comparison with GAPDH (Santa Cruz Biotech). Image acquisition was performed on a Chemidoc (Biorad) and analyzed using Quantity One software. In some cases membranes were ‘stripped’ by incubation in a buffer (0.76 g Tris, 2 g SDS, 700 μl β-mercaptoethanol in 100 ml) at 37°C for 45 min in order to be subsequently probed with additional antibodies.

Microscopy and image analysis

A Nikon ECLIPSE Ti-S microscope was used for image analysis. Images were captured with a Nikon digital sight Model C-SHG1 camera. Random fields for each sample were analyzed in each experiment to verify the effect of the Insulin (100 nM) treatment. Oil red O staining (SIGMA) for lipid droplets was used to evaluate adipocyte differentiation.

Statistical analysis

Results are presented as mean ± SEM unless otherwise indicated. Data were analyzed using two-tailed Student’s t-test or one-way ANOVA, followed by post hoc Newman-Keuls tests when F was significant. A nonparametric test for two related samples (Wilcoxon’s signed ranks test) was used to identify differences between each treated group and controls, differences between more than 2 groups were analyzed by analysis of variance and post hoc Bonferroni-Holm test. Pearson correlation coefficient was used to assess the association between NPRC/NPRA ratio, an index of reciprocal expression of NP receptors, and clinical parameters. Multiple linear regression analysis was done to define the variables that may affect HOMA-IR and NPRC/NPRA ratio.
correlation. SPSS 11.0 software was used for statistical analysis (SPSS Inc., Chicago, Illinois, USA) and a $P < 0.05$ was considered significant.
RESULTS

In-vitro regulation of NP receptors

To study the effect of insulin on NP receptors in human adipocytes, we used two different adipocyte models: a primary culture from human VAT and the SGBS adipocyte cell line. First, we accurately tested the experimental conditions: adipocytes (both VAT-derived and from SGBS cells) reached the same elevated differentiation grade with insulin for 7 or 12 days (Figure 1). There were no differences in NP receptors gene expression between adipocytes differentiated with insulin for 7 or 12 days (Figure 1).

On the base on these data, we removed insulin on day 7th from the differentiation media to study the acute effect of insulin on NP receptors expression. Insulin dose-response analysis revealed that NPRA did not significantly change after insulin treatment whereas NPRC increased in a dose-dependent manner (from 50nM to 1μM; Figure 2). Therefore, our experiments were carried out using only 7 days of insulin to differentiate preadipocytes and than 100nM of insulin for 6 hrs to asses acute effects at day 12.

As shown in Figure 3A and 3B, NPRA gene expression did not significantly change after insulin treatment in both adipocyte models. In contrast, there was about two-fold enhancement of NPRC expression after 6 hours of insulin treatment in both SGBS and primary cultures (p=0.0056 and p=0.0231, respectively; Figure 3C and 3D). Of note, this regulation was completely blocked by wortmannin (Figure 3C and 3D), which is a specific covalent inhibitor of PI3K, the kinase involved both in the stimulation of glucose-transport and in the anti-lipolytic effect of insulin (25, 18).

Western blotting analysis confirmed the ability of insulin to increase NPRC expression and the effect of wortmannin to completely suppress the action of insulin on NPRC expression (Figure
3E and 3F), but unfortunately NPRA western could not be performed due to lack of sufficient quality antisera. Moreover, these results do not clarify whether the increased steady-state levels of NPRC were due to an effect on mRNA stability rather than an increased gene transcription, even if the final effect on NPRC protein was the increased expression of this receptor.

In another set of experiments we used low-glucose culture media to simulate in vivo starving condition (Figure 4). Adipocytes were cultured as described above and treated for 6 hrs with insulin (100nM, experiment Phase 1). To simulate starving condition, after the insulin treatment experiment (Phase 1), we substituted the media usually used (DMEM/F12 with glucose 17.5 mM) with low-glucose media (DMEM-low glucose 5.5 mM for experiment Phase 2 of Figure 4).

After overnight incubation (between 12 and 14 hours) with low-glucose media, adipocytes were treated again with insulin (100nM, Phase 3 of experiment in Figure 4). In both adipocyte cell models, NPRC expression in adipocytes in Phase 2 significantly decreased to low levels when compared to insulin-treated cells in DMEM F12 (Figure 4A and 2B; p<0.001, p<0.001 for SGBS and primary cells, respectively). Moreover, the low level of NPRC obtained with low-glucose incubation was still present even after insulin stimulation in low-glucose media (Phase 3 of Figure 4 A and 4B; p<0.001, p<0.001, SGBS and primary cells, respectively).

These data suggest the existence of a “nutritional signaling” depending mainly on extracellular glucose concentration. When insulin was not coupled with glucose (as shown in Figure 4 with low glucose concentration), the induction of NPRC gene expression was not observed. NPRA gene expression did not significantly change after low-glucose cell condition (Figure 4C, 4D).

To investigate on the possibility of a “nutritional signaling”, we tested whether insulin regulates the main gene effector of insulin/glucose regulated pathway depending on glucose
concentrations. GLUT4, the major insulin glucose responsive transporter, was induced by insulin but this effect was blocked by the presence of low glucose (Figure 5A). Of note SREBP-1c, a key gene involved in insulin-induced lipogenesis, behaved similarly, and was up-regulated by insulin and blocked by low glucose (Figure 5B). Expression of LXR α was modestly increased by insulin even in the presence of low-glucose (Figure 5C). ChREBP-α was well expressed in SGBS cells independently by insulin treatment. The low-glucose conditions significantly reduced ChREBP-α gene expression levels (Figure 5D). In contrast ChREBP-β, that requires GLUT4 in adipose tissue (15), was stimulated by insulin and suppressed by low-glucose (Figure 5E). Gene expression analysis of this set of genes involved in the regulation of lipogenesis suggests that glucose concentrations in cell media drive adipocytes versus lipogenesis or lipolysis pathway. Indeed, the reduction of glucose in the media blocked also the insulin induction of NPRC.

Additional experiments with SGBS adipocytes, were set to verify the different modulation of hormone sensitive lipases (HSL) under conditions of ANP-enhanced lipolysis or insulin-induced anti-lipolysis. ANP is a well known activator of the lipolytic pathway and, as expected, we found a 3-fold induction of HSL (p<0.001, Figure 6A) after ANP treatment. In contrast, adipocytes treated with ANP and insulin (Figure 6A) showed a basal level of HSL gene expression, suggesting a balancing between lipolytic and anti-lipolytic modulation. Moreover, when low glucose was added and high-glucose was removed after 6 hrs of insulin treatment (as described for the Phase 2 of the previous set of experiments) we observed that ANP induced an even higher expression of HSL (almost 5-fold, p<0.001), suggesting again that the effect of insulin is linked to the presence of glucose (Fig 6A). ATGL gene expression was also activated by ANP (1.5–fold) and slightly reduced by insulin (Fig 6B). Insulin pre-treatment followed by over-night low-
glucose media incubation before ANP, on the contrary, reduced ATGL (Fig 6B), maybe reflecting a different regulation from HSL (4).

Lipolysis data, obtained by the measurements of glycerol release into cell-media, were in line with HSL and NPRC regulation. Glycerol release was increased by ANP and it was blocked by insulin-induced NPRC but not in starving-like conditions with low glucose (Fig 6C).

We also examined whether ANP could increase expression of UCP1, PGC-1α, CytoC in SGBS cells and if NPRC induction by insulin could block these ANP abilities. ANP significantly increased UCP1, PGC-1α, CytoC (Fig 6D, 6E, 6F), the thermogenesis/mitochondriogenesis set of genes, as we previously demonstrated in other adipocyte models (5). Of note in these sets of experiments we also demonstrated that the ANP ability to induce markers of adipocyte “browning” was blocked by insulin. When insulin-mediated NPRC induction was blocked by low glucose media, ANP was again able to significantly reactivate UCP1, PGC-1α with a similar trend for Cyto C (Fig 6D, 6E, 6F).

NPRC/NPRA ratio and clinical metabolic parameters

Thirty-four samples of perirenal VAT were collected from consecutive patients undergoing nephrectomy at the Clinical of Urology, University Hospital of Ancona. Table 1 reports the available clinical parameters of these patients. Of note, since these patients were hospitalized for nephrectomy, we could collect only few selected clinical parameters (fasting plasma insulin, glucose and lipid profile) and we didn’t have any chance to investigate in further details their clinical profile for our study.

In order to better investigate the potential metabolic role of NPRC, we analyzed the relationship between NPRC/NPRA gene expression in VAT and clinical metabolic parameters available,
Unfortunately because of limited amount of tissue samples we were not able to detect NPRC protein levels. As shown in Figure 7A, we observed that a higher NPRC/NPRA ratio was associated with increased total serum cholesterol ($r=0.4997$, $p=0.0347$). A similar direct association was shown with LDL-cholesterol ($r=0.5170$, $p=0.0280$; Fig 7B) and non-HDL cholesterol ($r=0.5397$, $p=0.0208$; Fig 7C). No significant correlation was found with triglycerides ($r=0.2437$, $p=0.329$, data not shown). On the contrary, considering fasting insulinemia ($r=0.430$, $p=0.0199$; Fig 7D), an even stronger association was found. Fasting insulinemia correlated positively with NPRC/NPRA expression ratio in VAT also after adjustment for age, gender and BMI. Importantly, we also observed that higher NPRC/NPRA ratio was associated with increasing HOMA-IR ($r=0.4611$, $p=0.0118$; Fig 7E). Because gender, age, waist and BMI are known to affect insulin resistance, multiple regression analysis was performed to examine whether the observed association between HOMA-IR and NPRC/NPRA was independent of these confounding variables. The result indicated that the positive association between HOMA-IR and NPRC/NPRA ratio was significant and independent of age, gender, BMI and waist ($\beta$ coefficient = 0.544; $r^2 = 0.571$; $p=0.015$). Overall these results suggest that high insulin levels, even in the context of increased insulin resistance, might excessively stimulate NPRC expression in VAT. Conversely, higher NPRC gene expression might contribute to lower lipolysis, triglyceride accumulation and enlarged adipocytes size, favoring insulin resistance and lipid abnormalities.

No significant correlation with BMI, waist circumference, glycosylate hemoglobin and fasting glucose concentrations were observed (data not shown). We were not able to show any correlation with systolic blood pressure ($r=0.116$, $p=0.532$) or diastolic blood pressure ($r=0.152$, $P=0.413$), likely because we did not have information from 24-hour ambulatory blood pressure
monitoring (ABPM) but only from clinical BP measurement on the day of surgery.
DISCUSSION

We demonstrated that insulin, via the PI3K pathway, is an important stimulator of NPRC expression in human differentiated adipocytes (primary human visceral adipocytes and adipocytes from the SGBS cell line). We showed that adipocytes treated with insulin increased their NPRC mRNA and protein levels without significant changes in NPRA. NPRC is a receptor known to reduce NP cellular responses and NP plasma levels. Moreover, when adipocytes were in a fasting-like cell culture conditions (obtained by reducing glucose concentration in cell media from the usual 17 mM to 5.5 mM), we observed that NPRC was reduced to basal levels, suggesting a “nutritional signaling” in NPRC regulation. If insulin signaling is coupled with reduced glucose entry, the induction of NPRC gene expression does not occur in human adipocytes.

Gene expression analysis of the main genes involved in insulin/glucose regulated pathways showed that a 70% reduction of glucose concentration in cell media appears to be able to suppress the effect of insulin on GLUT4, CHREBP-β and SREBP-α and CHREBP-α in our cell model. In SGBS adipocytes, LXR-α was well expressed and its expression seems unaffected by cell culture conditions probably because LXR-α is required to induce glucose-induced gene pathway (27, 8, 15). In our study we just uncovered some aspect of the nutritional regulation of NPRC but further, in-depth analysis of glucose insulin/glucose regulated pathway in human adipocytes is needed.

The first observation of relative NPRC upregulation in humans was described in obese patients with hypertension (11). In conditions such as human obesity where insulin is abundant and reaches many peaks after snacks, meals or sweet beverages, it might attenuates the lipolytic and
thermogenic effects of NP, promoting lipogenesis in adipocytes by upregulating NPRC gene expression.

A link between NP system and the metabolic profile is also supported by previous studies in humans and rodents (29, 36). Cannone et al., reported that higher ANP circulating levels were associated with a favorable cardiometabolic profile (6, 7). Indeed, the analysis of the ANP genetic variant 5068, revealed that the presence of the minor allele is associated with higher ANP levels, lower BMI, lower prevalence of obesity, lower waist circumference and lower blood pressure but higher level of HDL-cholesterol (6, 7). A previous association study with an NPRC variant had found lower abdominal obesity and lower risk of developing central obesity at follow up (33).

Our previous analysis of mice lacking NPRC showed that these mice had a lean phenotype with a significantly reduced fat mass (5). Despite the normal circulating levels of NP, likely dependent on cardiac secretion “resetting”, the absence of NPRC determines longer half-lives for these cardiac hormones and enhances their biological effects on blood pressure and on target cells like adipocytes (19), thus explaining the “browning” of adipocytes and the activation of heat-dissipating fat-burning (5). In the present study, we confirmed the ability of ANP to induce markers of the “browning” of human adipocytes consisting in the activation of the main thermogenic genes such as UCP1 and PGC-1α as well as cytochrome c, this last one being a marker of mitochondrial biogenesis. We have shown, for the first time, that insulin/glucose were also able to counteract the ANP-induced thermogenic-pathway through the induction of NPRC: when insulin-mediated NPRC induction was blocked by low-glucose, ANP was able to significantly reactivate UCP1 and PGC-1α (Figure 6). These data indicate that if insulin is not coupled with glucose, NPRC expression is not induced and consequently ANP is able again to
enhanced lipolysis as well as thermogenic genes. Our results suggest that higher insulin levels, together with higher glucose concentration, as in the prediabetes of the metabolic syndrome, induce NPRC expression in human adipocytes blocking the NP ability to stimulate lipolysis and the thermogenic pathway.

Therefore, insulin appears to be a key hormone in the crosstalk of glucose/lipid metabolism with the NP system activity. Insulin downregulates NPRA and upregulates NPRC in murine 3T3-L1 differentiated adipocyte (24) and down-regulation of NPRC with increased NPRA expression was observed in insulin-deficient mice (24). Indeed, the relative ratio of NPRA to NPRC mRNA level was lower in SAT of patients with type 2 diabetes compared with subject with normal glucose tolerance or impaired glucose metabolism (17). Patients with type 2 diabetes treated with pioglitazone, a PPARγ agonist, shown a significant reduction of NPRC with a tendency toward increased NPRA gene expression in SAT (17).

Here, we observed that human adipocytes stimulated with ANP increased expression of genes involved in lipolysis (HSL and ATGL). In contrast, when ANP and insulin are in costimulation these lipolytic genes are expressed at basal levels, confirming the opposite effect of ANP and insulin on lipolysis. Only in starving-like cell conditions lipolysis and HSL are up-regulated by ANP independently by the presence of insulin in the media (Figure 6), suggesting a functional physiological role of this regulation in energy/lipid metabolism. Differences between ATGL and HSL reactivation, after incubation in low-glucose media, is probably due to the different pathways that regulate these enzyme activity: AMP-activated kinase for ATGL (1) and protein kinase A together with protein kinase G for HSL (39), subtending similar but distinct roles of these two lipases, with HSL well-known to be more hormone-regulated (4).
A detailed study of cardiovascular, renal and metabolic clinical parameters could not be completed in the patients enrolled in our study as explained in the method section. Two other limitations to take into consideration are: 1) we were unable to measure circulating NP; 2) the patients studied had renal cell carcinoma although in the T1/T2, N0, M0 stage. Despite this limitation and with the limited clinical data available, we found a direct association of VAT NPRC/NPRA ratio with fasting insulinemia as well as with HOMA-IR. It is reasonably to think that higher insulin levels, despite a relative increase in total body insulin resistance, induced higher NPRC/NPRA ratio in VAT of these patients, an increased ratio that favor reduced lipolysis and increased lipogenesis. This unbalancing between lipolysis and lipogenesis is believed to be the main determinant of the increasing adipocyte size, a condition known to be associated with worsening of insulin resistance, configuring a vicious-circle.

Pivovarova et al. showed similar results analyzing fasting insulin levels and NPRC expression in both visceral and subcutaneous adipose tissue of non-diabetic subjects (26). An insulin/glucose-mediated up-regulation of NPRC in human VAT could lead to a reduction of NP-mediated lipolysis/thermogenesis together with a systemic decrease in circulating levels of NP (), contributing to worsening of insulin resistance, metabolic syndrome and increasing blood pressure.

In our patients we also found that the NPRC/NPRA ratio was directly associated with higher total-cholesterol, LDL-cholesterol and, most importantly, with non-HDL cholesterol (Figure 7), suggesting that a modulation of NPRC and NPRA expression in adipocytes might also affect, directly or indirectly, lipoprotein metabolism.

Perspectives and Significance
The prevalence of obesity and metabolic syndrome is increasing worldwide and the search for key factors linking glucose and lipid dysmetabolism with higher blood pressure and cardiovascular damage is attracting increasing attentions. Our results suggest that elevated insulin/glucose levels might impair the biological action of NP in human adipocytes through the up-regulation of NPRC expression. The resulting reduced lipolysis and uncoupled “fat burning”-dependant energy expenditure in adipose tissue might contribute to the onset of the metabolic syndrome and hypertension. Our finding shed a new light on insulin interaction with the natriuretic peptide system in human adipocytes, an interaction that help understanding the association between increasing BMI, dysmetabolism, insulin resistance, sodium retention and hypertension.

Drugs blocking NPRC hold the promise of being the long-awaited “magic bullets” for hypertension and metabolic syndrome in the increasingly common people with central overweight and obesity.

**AUTHOR CONTRIBUTIONS**

M.B. and R.S. conceived, designed and wrote the manuscript; M.B., M.C. and M.P. performed experiments, analyzed data and prepared figures; M.W. managed the SGBS cells; D.M. and M.P. collected human adipose tissue samples; M.B. and R.S., drafted manuscript; M.B., R.S., V.C., P.D.F and J.B. edited and revised manuscript; M.B., M.C., M.P., M.W., D.M., M.P., V.C., P.D.F., J.B. and R.S. approved final version of manuscript.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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FIGURE LEGENDS

Figure 1.
Differentiation grade and NP receptors levels after 7 or 12 days of differentiation in DMEM/F12 with insulin (20nM).

A, D) Oil Red O staining for lipid droplets was used to evaluate adipocyte differentiation grade at day 7. B, E) NPRA mRNA levels in SGBS and VAT-derived adipocytes after 7 or 12 days with insulin. C, F) NPRC mRNA levels in SGBS and VAT-derived adipocytes after 7 or 12 days with insulin.
The data are expressed as mean ± SEM. Magnifications 20X and 40X.

Figure 2.
Effect of insulin on NPRC and NPRA in SGBS differentiated adipocytes.

A, B) Dose-dependent effect of insulin (from 50nM to 1μM) on NPRC and NPRA mRNA levels. SGBS were differentiated for 7 days with insulin and at day 12 were treated or not (0) with the indicated dosage of insulin for 6hrs.
The data are expressed as mean ± SEM. * P<0.05 versus untreated controls, *** P<0.001 versus untreated controls.

Figure 3.
Insulin induces NPRC in differentiated adipocytes from both SGBS and human VAT-derived adipocytes.
Pre-adipocytes were differentiated for 7 days with insulin and at day 12 were pretreated or not for 30 min with 100nM of wortmannin, a specific PI3K inhibitor, before adding 100nM of insulin. NPRA and NPRC mRNA levels were measured in differentiated adipocytes from SGBS (A, C) and human visceral adipocytes (B, D). SGBS (E) and primary adipocytes (F) were also treated as described above and samples were analyzed for protein levels by western blotting: C: untreated adipocytes; ins: insulin 100nM; w: wortmannin 100nM; ins+w: pretreatment with wortmannin before insulin stimulation.

The data are expressed as mean ± SEM. * P<0.05 versus untreated controls, ** P<0.01 versus untreated controls.

Figure 4.

Starving-like cell conditions: NPRC stimulation by insulin treatment is completely blocked by low glucose culture media.

Phase 1: adipocytes were treated in DMEM F12 (glucose 17.5 mM) with insulin (100 nM) for 6hrs; Phase 2: adipocytes were treated in DMEM F12 (glucose 17.5 mM) with insulin (100 nM) for 6hrs followed by over-night (12-14 hrs) incubation with DMEM low glucose medium (glucose 5.5 mM) instead of DMED/F12; Phase 3: cells in phase 2 were treated again with insulin (100 nM) for 6hrs.

NPRA and NPRC mRNA levels were measured in differentiated adipocytes from SGBS (A, C) and human visceral adipocytes (B, D). The data are expressed as mean ± SEM. *** P<0.001 versus cells treated with 100nM of insulin in DMEM-F12 (17.5 mM of glucose).

Figure 5.
Insulin/glucose regulated genes in SGBS adipocytes treated with insulin in high or low-glucose culture media.

SGBS differentiated adipocytes were analyzed to evaluate gene expression of the insulin/glucose regulated genes involved in lipogenesis after insulin treatment (100nM) in high-glucose (17.5mM) or in low-glucose (5.5mM) culture media. A) Glucose transporter type 4 (GLUT4); B) sterol regulatory element binding protein 1c (SREBP-1c); C) liver X receptor-α (LXR-α); D) carbohydrate-responsive-element binding protein isoform α (ChREBP-α); E) carbohydrate-responsive-element binding protein isoform β (ChREBP-β). The data are expressed as mean ± SEM. ** P<0.01 and *** P<0.001 versus untreated cells cultured in DMEM-F12 (17.5 mM of glucose).

Figure 6.

Lypolitic genes expression, glycerol release and thermogenic/mitochondrial genes under lipolytic or anti-lipolytic conditions.

SGBS were differentiated for 7 days with insulin and at day 12 were untreated (Control), treated with 100 nM of insulin (+ insulin) or 100 nM ANP (+ ANP) or both (+ insulin + ANP). Moreover after insulin treatment the culture media were substituted with low-glucose media and after 12-14 hrs of incubation adipocytes were treated with ANP for 6 hrs (low-glucose between added insulin and ANP). Lypolitic genes/lypolisis: A) hormone sensitive lipase (HSL; B) adipose triglyceride lipase (ATGL); C) glycerol release were measured in the DMEM/F12 or DMEM low-glucose.

Adipocytes treated as described above were used to test the gene expression regulation of thermogenic and mitochondrial biogenesis genes (Thermogenic genes): D) mitochondrial
uncoupling protein 1 (UCP1); E) transcriptional coregulator PPARγ coactivator-1α (PGC-1α);

F) Cytochrome c (CYTO C).

The data are expressed as mean ± SEM.* P<0.05 versus untreated controls, ** P<0.01 versus untreated controls, *** P<0.001 versus untreated controls.

Figure 7.

Relationship between NPRC/NPRA ratio in human VAT and available clinical parameters of patients undergoing renal surgery.

Positive correlations between NPRC/NPRA expression ratio and total cholesterol (A), LDL cholesterol (B), non-HDL cholesterol (C) as well as with fasting insulinemia (D) and HOMA-IR (E) Pearson test and multiple regression analysis were used for statistical analysis. Pearson p values are reported.
Table 1.
Clinical characteristics and fasting blood chemistry of patients.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean±SE (n)</th>
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<tbody>
<tr>
<td>Gender (male/female)</td>
<td>19/15 (34)</td>
</tr>
<tr>
<td>Age (y)</td>
<td>68.69 ± 1.6 (34)</td>
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<tr>
<td>BMI (Kg/m²)</td>
<td>26.08 ± 0.7 (34)</td>
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<tr>
<td>WAIST (cm)</td>
<td>97.98 ± 2.0 (34)</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>140.10 ± 2.8 (31)</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>79.83 ± 2.0 (31)</td>
</tr>
<tr>
<td>Fasting Glucose (mg/dL)</td>
<td>90.71 ± 4.2 (30)</td>
</tr>
<tr>
<td>Fasting Insulinemia (microUI/ml)</td>
<td>7.32 ± 1.1 (29)</td>
</tr>
<tr>
<td>Triglyceride (mmol/L)</td>
<td>127.60 ± 10.8 (18)</td>
</tr>
<tr>
<td>Tot Chol (mg/dL)</td>
<td>188.10 ± 12.1 (18)</td>
</tr>
<tr>
<td>HDL Chol (mg/dL)</td>
<td>40.32 ± 2.9 (18)</td>
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<tr>
<td>LDL Chol (mg/dL)</td>
<td>122.20 ± 9.8 (18)</td>
</tr>
<tr>
<td>Non-HDL Chol (mg/dL)</td>
<td>147.70 ± 10.9 (18)</td>
</tr>
</tbody>
</table>

Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP diastolic blood pressure; MBP, mean blood pressure; Tot Chol, Total Cholesterol; HDL high-density lipoproteins; LDL, low-density lipoprotein. Data are mean and standard error (SE). n reported number of available clinical laboratory data.
Bordicchia et al. Figure 3

**A**

SGBS

**B**

Visceral adipocytes

**C**

**D**

**E**

**F**

NPRC

GAPDH
Figure 5

A. GLUT4 mRNA (fold over basal) with insulin (100nM) and low glucose

B. SREBP-1c mRNA (fold over basal) with insulin (100nM) and low glucose

C. LXRα mRNA (fold over basal) with insulin (100nM) and low glucose

D. ChREBP-α mRNA (fold over basal) with insulin (100nM) and low glucose

E. ChREBP-β mRNA (fold over basal) with insulin (100nM) and low glucose

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