Insulin/glucose induces natriuretic peptide clearance receptor in human adipocytes: a metabolic link with the cardiac natriuretic pathway


In 1994, Endre et al. showed that euglycemic hyperinsulinemia clamp with 2 h 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 phospho-

ATRIAL NATRIURETIC PEPTIDE (ANP) and B-type natriuretic peptide 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), mitochon- drosis (2, 21), and thermogenic program with potency similar to catecholamines (5).

Two distinct NP receptors mediate 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 on the ratio of the clearance receptor NPRC to the signaling receptor (NPRC/NPRA), both abundantly expressed in human adipose tissue and adipocytes (29). In nonprimate 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 brown adipocytes (5). In NPRC-deficient mice, the NP half-life is prolonged, and NP 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 the 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, 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 poten-
tiates 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-in-
duced lipolysis appears to be more sensitive to obesity than catecholamine probably due to an altered adipocyte NPRC-NPRA expression ratio (NPRC/NPRA) in obesity (28). Indeed, recent studies reported also a link between obesity/type 2 diabetes and NP receptor mRNA levels in both muscle and subcutaneous adipose tissue, suggesting that NPRC and NPRC expression is 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 h 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 phosho-
diesterase 3B activation, which subsequently leads to cAMP degradation and deactivation of protein kinase A (9) but does not seem to have a direct antilipolytic effect through an inhibition of the cGMP-protein kinase G-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 with 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). An increased expression of NPRC in human monocytes (but not in macrophages) after simultaneous high glucose and insulin infusion was also demonstrated (26).

Adipose tissue RNA comes mostly from nonadipocyte 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 the NP system as well as for glucose-regulated gene analysis, whereas human VAT-derived primary preadipocytes are the more closely related model to 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 receptor expression in VAT and available clinical metabolic parameters of patients undergoing renal surgery.

**MATERIALS AND METHODS**

**Reagents and Antibodies**

Insulin, dexamethasone, isobutyl methylxanthine, triiodothyronine, transferrin, and wortmannin were obtained from Sigma-Aldrich (St. Louis, Missouri, MO). Antisera against NPRC was a kind gift from William Gower at the James A. Haley Veterans Affairs Medical Center (Tampa, FL). GAPDH antibody (catalog no. SC25778) and secondary antibody antirabbit (catalog no. SC2054) were from Santa Cruz Biotechnology. Super signal West Femto Maximum Sensitivity secondary antibody antirabbit (catalog no. SC2054) were from Santa Cruz Biotechnology. GAPDH antibody (catalog no. SC25778) and William Gower at the James A. Haley Veterans Affairs Medical Center (Tampa, FL). Antisera against NPRC was a kind gift from William Gower at the James A. Haley Veterans Affairs Medical Center (Tampa, FL). GAPDH antibody (catalog no. SC25778) and secondary antibody antirabbit (catalog no. SC2054) were from Santa Cruz Biotechnology. Super signal West Femto Maximum Sensitivity Substrate was from Thermo Scientific (Rockford, IL).

**Adipocyte Cell Culture**

SGBS Cells from SGBS were grown in growth medium (DMEM-F-12 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 (Fig. 1). Thus, on the 7th day the cells were washed and deprived of insulin for 5 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 1 μM (Fig. 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-F-12 (17.5 mM glucose, osmolality between 290 and 330 mosmol/kgH2O) + 10 nM insulin for 6 h; phase 2: DMEM-F-12 (17.5 mM glucose) + 100 nM insulin for 6 h followed by overnight (12–14 h) incubation with DMEM low-glucose medium (5.5 mM glucose) instead of DMEM-F-12; and phase 3: DMEM-F-12 (17.5 mM glucose, osmolality 300–340 mosmol/kgH2O) + 100 nM insulin for 6 h followed by overnight (12–14 h) incubation with low-glucose medium (5.5 mM glucose) instead of DMEM-F-12 + 100 nM insulin for 6 h.

To assess whether the 70% reduction of glucose concentration in culture media simulates starving conditions, 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-α (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 vs. insulin on lipolysis and antilipolysis pathways, SGBS cells were treated as described above and with 100 nM ANP for 6 h. RNA was then 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 implicated in mitochondrial biogenesis.

**Primary visceral adipocyte culture.** Visceral adipose samples (2–3 g) 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, and with any evidence of local or metastatic cancer spread: T1/T2, N0, M0) at the Ospedali Riuniti University Hospital of Ancona. In the morning, after an overnight fast, blood was drawn for lipid profile as well as glucose and insulin assays. Anthropometrics and clinical and humoral parameters are shown in Table 1. Insulin resistance was evaluated by homeostasis model assessment (HOMA-IR) formula: fasting glucose (mmol/l) × fasting insulin (μIU/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 H9262 g was performed with a High-Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Applied Biosystems, Warrington, UK). All gene expression experiments in SGBS and primary VAT adipocyte 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 7 days and treated at day 12 as described above (see phase 1) with insulin (100 nM), ANP (100 nM), or both. Moreover, a set of SGBS-differentiated adipocytes was treated as described for phase 2 [DMEM-F-12 (17.5 mM glucose) + 100 nM insulin for 6 h followed by overnight (12–14 h) incubation with low-glucose medium (5.5 mM glucose) instead of DMEM-F-12] and then treated with 100 nM ANP for 6 h (see phase 3). Extracted RNA was used to test gene expression levels of HSL and ATGL. The 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 interassay coefficients of variation were <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 (Bio-Rad), and 50 µg of total proteins were...
Table 1. Clinical characteristics and fasting blood chemistry of patients

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<thead>
<tr>
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<th>n</th>
<th>Means ± SE</th>
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<tr>
<td>Gender (male/female)</td>
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<td>19/15</td>
</tr>
<tr>
<td>Age, yr</td>
<td>34</td>
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<tr>
<td>BMI, kg/m²</td>
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<tr>
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<tr>
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<td>HDL cholesterol, mg/dl</td>
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<tr>
<td>Non-HDL cholesterol, mg/dl</td>
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<td>147.70 ± 10.9</td>
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Data are means ± SE; n, numbers of available clinical laboratory data. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoproteins; LDL, low-density lipoprotein.

resolved in 12% 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, 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 was measured compared with GAPDH (Santa Cruz Biotechnology). Image acquisition was performed on a Chemidoc (Bio-Rad) and analyzed using Quantity One software. In some cases membranes were “stripped” by incubation in a buffer (0.76 g Tris, 2 g SDS, and 700 μl mercaptoethanol in 100 ml) at 37°C for 45 min 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-SH1 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 means ± SE 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 two groups were analyzed by ANOVA and post hoc Bonferroni-Holm test. Pearson’s correlation coefficient was used to assess the association between the NPRC/NPRA, 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 correlation. SPSS 11.0 software was used for statistical analysis (SPSS, Chicago, IL), 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 (Fig. 1). There were no differences in NP receptor gene expression between adipocytes differentiated with insulin for 7 or 12 days (Fig. 1).

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

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

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 (Fig. 3, E and F), 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 conditions (Fig. 4). Adipocytes were cultured as described above and treated for 6 h with insulin (100 nM, experiment phase 1). To simulate starving conditions, after the insulin treatment experiment (phase 1), we substituted the media usually used (DMEM-F-12 with 17.5 mM glucose) with low-glucose media (DMEM-5.5 mM low glucose for experiment phase 2 of Fig. 4).

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

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 Fig. 4 with low glucose concentration), the induction of NPRC gene expression was not observed. NPRA gene expression did not
To investigate the possibility of a nutritional signaling, we tested whether insulin regulates the main gene effector of the 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 (Fig. 5A). Of note SREBP-1c, a key gene involved in insulin-induced lipogenesis, behaved similarly and was upregulated by insulin and blocked by low glucose (Fig. 5B). Expression of LXR-α was modestly increased by insulin even in the presence of low glucose (Fig. 5C). ChREBP-α was well expressed in SGBS cells independent of insulin treatment. The low-glucose conditions significantly reduced ChREBP-α gene expression levels (Fig. 5D). In contrast, ChREBP-β, which requires GLUT4 in adipose tissue (15), was stimulated by insulin and suppressed by low glucose (Fig. 5E). Gene expression analysis of this set of genes in adipocytes and human visceral adipocytes (B and 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, 100 nM insulin; w, 100 nM wortmannin; ins + w, pretreatment with wortmannin before insulin stimulation. Data are expressed as means ± SE. *P < 0.05 and **P < 0.01 vs. untreated controls.

Additional experiments with SGBS adipocytes were set to verify the different modulation of HSL under conditions of ANP-enhanced lipolysis or insulin-induced antilipolysis. ANP is a well known activator of the lipolytic pathway, and, as expected, we found a threefold induction of HSL (P < 0.001; Fig. 6A) after ANP treatment. In contrast, adipocytes treated with ANP and insulin (Fig. 6A) showed a basal level of HSL gene expression, suggesting a balancing between lipolytic and antilipolytic modulation. Moreover, when low glucose was added and high-glucose was removed after 6 h of insulin treatment (as described for 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 pretreatment followed by overnight 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 in 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, and cytochrome c in SGBS cells and if NPRC induction by insulin could block these ANP abilities. ANP significantly increased UCP1, PGC-1α, and cytochrome c (Fig. 6, D, E, and F), 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 and PGC-1α with a similar trend for cytochrome c (Fig. 6, D, E, and F).
NPRC/NPRA and Clinical Metabolic Parameters

Thirty-four samples of perirenal VAT were collected from consecutive patients undergoing nephrectomy at the Clinic 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 did not have any chance to investigate in further detail their clinical profile for our study.

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 the limited amount of tissue samples, we were not able to detect NPRC protein levels. As shown in Fig. 7A, we observed that a higher NPRC/NPRA was associated with increased total serum cholesterol ($r = 0.4997, P = 0.0347$). A similar direct association was shown with low-density lipoprotein (LDL) cholesterol ($r = 0.5170, P = 0.0280$; Fig. 7B) and non-high-density lipoprotein (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 the NPRC/NPRA expression in VAT also after adjustment for age, gender, and body mass index (BMI). Importantly, we also observed that higher NPRC/NPRA 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 the NPRC/NPRA was significant and independent of age, gender, BMI, and waist (β-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 adipocyte size, favoring insulin resistance and lipid abnormalities.

No significant correlation with BMI, waist circumference, glycosylate hemoglobin, and fasting glucose concentrations was 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-h ambulatory blood pressure monitoring but only from clinical blood pressure 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 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, SREBP-1c, 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 the 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 the 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 attenuate the lipolytic and thermogenic effects of NP, promoting lipogenesis in adipocytes by upregulating NPRC gene expression.

A link between the 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 a higher level of HDL cholesterol (6, 7). A previous association study with an NPRC variant 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 of 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 (Fig. 6). These data indicate that, if insulin is not coupled with glucose, NPRC expression is not induced, and consequently ANP is able again to enhance 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 cross talk of glucose/lipid metabolism with the NP system activity.
Insulin downregulates NPRA and upregulates NPRC in murine 3T3-L1 differentiated adipocytes (24), and downregulation 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 subjects with normal glucose tolerance or impaired glucose metabolism (17). Patients with type 2 diabetes treated with pioglitazone, a PPARγ agonist, showed 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, ATGL) and glycerol release (Fig. 6). Moreover, adipocytes treated with ANP for 6 h after insulin treatment showed increased expression of thermogenic and mitochondrial biogenesis genes (UCP1, PGC-1α, CYTO C) compared to untreated controls. Data are expressed as means ± SE. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. untreated controls.
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 are lipolysis and HSL upregulated by ANP independent of the presence of insulin in the media (Fig. 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, are probably due to the different pathways that regulate these enzyme activities: 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 MATERIALS AND METHODS. Two other limitations to take into consideration are: 1) we were unable to measure circulating NP; and 2) the patients studied had renal cell carcinoma although in the T1/T2, N0, and M0 stages. Despite this limitation and with the limited clinical data available, we found a direct association of the VAT NPRC/NPRA with fasting insulinemia and with HOMA-IR. It is reasonable to think that higher insulin levels, despite a relative increase in total body insulin resistance, induced a higher NPRC/NPRA in VAT of these patients, an increased ratio that favors 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 nondiabetic subjects (26). An insulin/glucose-mediated upregulation 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 increased blood pressure.
In our patients we also found that the NPRC/NPRA was directly associated with higher total cholesterol, LDL cholesterol, and, most importantly, with non-HDL cholesterol (Fig. 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 attention. Our results suggest that elevated insulin/glucose levels might impair the biological action of NP in human adipocytes through the upregulation of NPRC expression. The resulting reduced lipolysis and uncoupled “fat-burning”-dependent energy expenditure in adipose tissue might contribute to the onset of the metabolic syndrome and hypertension. Our finding sheds a new light on insulin interaction with the natriuretic peptide system in human adipocytes, an interaction that helps 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.

**ACKNOWLEDGMENTS**

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**GRANTS**

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**DISCLOSURES**

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

**AUTHOR CONTRIBUTIONS**

M.B. and R.S. conception and design of research; M.B., M.C., and M. Polito editing and revised manuscript; M.B., M.C., M. Pavan, D.M., M. Polito, M.W., V.C., J.C.B.J., P.D.-F., and R.S. interpreted results of experiments; M.B. prepared figures; M.B., M.C., and M. Polito, and R.S. drafted manuscript; M.B., M.C., D.M., M. Polito, and R.S. reviewed and approved final version of manuscript.

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