PKA Regulatory Subunits in Human Adipose Tissue: Decreased R2B Expression and Activity in Adipocytes from Obese Subjects

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ABSTRACT

Objective. In human adipocytes the cAMP-dependent pathway mediates signals originating from beta-adrenergic activation, thus playing a key role in the regulation of important metabolic processes, i.e. lipolysis and thermogenesis. Cyclic AMP effects are mainly mediated by protein kinase A (PKA), whose R2B regulatory isoform is the most expressed in mouse adipose tissue, where it protects against diet-induced obesity and fatty-liver development. Aim of the study was to investigate possible differences in R2B expression, PKA activity and lipolysis in adipose tissues from obese and non-obese subjects.

Research Design and Methods. The expression of the different PKA regulatory subunits were evaluated by immunohistochemistry, western blot and real-time PCR in subcutaneous and visceral adipose tissue samples from 20 non-obese and 67 obese patients. PKA activity and glycerol release were evaluated in total protein extract and adipocytes isolated from fresh tissue samples, respectively.

Results. Expression techniques showed that R2B was the most abundant regulatory protein, both at mRNA and protein level. Interestingly, R2B mRNA levels were significantly lower in both subcutaneous and visceral adipose tissues from obese than non-obese patients, and negatively correlated with BMI, waist circumference, insulin levels and HOMA-IR. Moreover, both basal and stimulated PKA activity and glycerol release were significantly lower in visceral adipose tissue from obese patients than non-obese subjects.

Conclusions. Our results first indicate that in human adipose tissue there are important BMI-related differences in R2B expression and PKA activation, which might be included among the multiple determinants involved in the different lipolytic response to beta-adrenergic activation in obesity.
Cyclic AMP is implicated in the regulation of a variety of cell functions that are, at least in part, related to protein phosphorylation through the activation of protein kinase A (PKA). In addition to the control of differentiated functions, such as motility, secretion, metabolism, differentiation, synaptic transmission and ion channel activities, cAMP inhibits or stimulates cell proliferation depending on the cell type. In human adipocytes the cAMP-dependent pathway mediates signals originating from the activation of beta-adrenergic receptors, thus playing a key role in the regulation of important metabolic processes, such as lipolysis and thermogenesis. Cyclic AMP effects are mainly mediated by PKA, a tetrameric enzyme composed by two catalytic subunits associated with two regulatory subunits. There are four different regulatory subunit genes and proteins (R1A, R1B, R2A, R2B) expressed with a tissue-specific pattern and exerting distinct roles in cell differentiation and growth control (1). Dramatic changes in the proportion of the two PKA regulatory subunits, R1 and R2, occur during ontogenic development, differentiation processes and neoplastic transformation, indicating distinct roles for these isoenzymes in cell homeostasis and growth control (2,3). In the past few years many studies seem to indicate that signalling via PKA plays an important role in regulating metabolism and body weight (4). In particular, the R2B isoform has been demonstrated to be, in mice, the most expressed in three tissues known to regulate energy homeostasis, that is brown adipose tissue, white adipose tissue, and brain (4,5). In general, the activation of the holoenzyme PKA in fat is now thought to decrease obesity, as demonstrated in both genetically obese (ob/ob) (6,7) and diet-induced obese mice (8). As far as the R2B subunit is concerned, studies in mice lacking this specific PKA subunit, have revealed an unexpected role for this protein in regulating energy balance (5). R2B knockout mice (RIIß−) remain remarkably lean even when challenged with a high-fat diet (5). These animals have increased metabolic activity, manifested by increases in body temperature, uncoupling protein 1 concentration, and lipid hydrolysis. Biochemical studies have shown that loss of R2B is compensated by increased R1A regulatory subunit, which is more sensitive to cAMP activation and results in a net increase in basal PKA activity (9).

In contrast to this increasing knowledge in mice, very little is known about the differential role played by the different PKA regulatory subunits in humans. Two studies from the same group have described lower R2B levels in adipose tissues from 10 normal weight women affected with polycystic ovary syndrome (PCOS) when compared to 13 matched control women, and associated this event with lipolytic catecholamine resistance and insulin resistance in these patients (10,11). We present the first study evaluating the relative expression of the different PKA isoforms in a large series of human adipose tissues. Our results indicate important BMI-related differences in R2B expression and PKA activation, which might play a role in the different lipolytic response to beta-adrenergic activation in obese and non-obese subjects.

**RESEARCH DESIGN AND METHODS**

*Patients, adipose tissues and cell cultures.* The study included 87 human subcutaneous and visceral adipose tissue samples from 67 obese patients (BMI>30 kg/m²) and 20 age- and sex-matched non-obese subjects (BMI<30 kg/m²), collected during bariatric surgery and abdominal surgical procedure for benign disease (elective cholecystectomy), respectively. The subjects were all Caucasians and, beside the surgery indication, were all...
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apparently healthy, with no history of alcohol overconsumption. No patient had overt diabetes mellitus, lipodystrophia and nobody was under anti-hypertensive treatment with beta-blockers. Height, weight, waist circumference, systolic and diastolic blood pressures were measured the day before surgery. A fasting morning blood sample was obtained from all patients for measurement of glucose and insulin that were analyzed by the Hospital’s routine chemistry laboratory. Insulin resistance degree was determined using the homeostasis model assessment (HOMA-IR = Fasting Insulin (mU/l) × Fasting Glucose (mmol/l)/22.5). The study was approved by the local Ethical Committee of Fondazione Ospedale Maggiore Policlinico IRCCS, Milan. All subjects gave their informed consent before participation.

Small sample fragments were fixed for immunohistochemistry (IHC), part of the tissues was digested with Collagenase type II (Sigma-Aldrich, Milan, Italy) for the isolation of adipocytes, while the remaining tissue was quickly frozen for subsequent molecular analysis. Adipocytes separation from pre-adipocytes and other cell types was based on cellular dimension and density. Briefly, adipose tissue biopsies (0.4-1.5 g) were digested in an albumin solution containing collagenase (DMEM F12 containing Hapes 15mM, Biotin 33μM, FBS 10%, antibiotics, all reagents from Sigma-Aldrich, Milan, Italy) at 37°C for two hours. The digested tissue was passed on a 100 μm filter (nylon cell strainer, BD Transduction Laboratories, Lexington, UK) to eliminate undigested material. The obtained cell suspension was centrifugated (1000 rpm, 15 minutes). After this centrifugation step, pre-adipocytes are pelleted at the bottom of the tube, while adipocytes are found on the surface as a ring due to their low density. At this step, the adipocyte ring is transferred to a new tube and isolated adipocytes are ready to be used for subsequent analysis. Pelleted pre-adipocytes are resuspended in culture medium (DMEM F12 containing Hapes 15mM, Biotin 33μM, FBS 10%, antibiotics) and passed on a 40 μm filter to eliminate eventually remaining adipocytes and other cell types on the basis of their different diameter. After filtration the cell suspension is centrifugated (1000 rpm, 15 minutes) and pelleted pre-adipocytes are resuspended in culture media and used for subsequent experiments.

Immunohistochemistry. Adipose tissue samples were processed for IHC, as previously reported (10). Specific monoclonal antibodies for PKA R1A, PKA R2A, and PKA R2B were used under the conditions specified by the manufacturer (BD Transduction Laboratories, Lexington, UK). Antigen-antibody detection was performed using the DAKO ChemMate En Vision detection kit (DAKO A/S, Glostrup, Denmark) according to the manufacturer’s instructions. Sections were stained with 3,3’-diaminobenzidine substrate, counterstained with Meyer hematoxylin and slides prepared for light microscopy examination, as previously reported (12). Negative controls were obtained by occulting the primary antibody or by using an unrelated mouse monoclonal antibody.

Western blot. Western blotting was performed as previously described (12) on total protein extracts from 40 visceral adipose tissue samples (20 from non-obese and 20 from obese subjects). Briefly, total proteins extracted from adipose tissue samples were quantified using the BCA assay protein kit (Pierce, Rockford, IL), and 20μg were resolved by 10% SDS-PAGE and transferred onto nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). PKA R1A, PKA R2B and PKA R2A were detected using the same specific monoclonal antibodies used for IHC, detected by chemiluminescent method and the resulting bands evaluated by imaging densitometer (BioRad GS-670) (12). Experiments were repeated at least twice.
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mRNA quantitative analysis. Total RNA was isolated from tissue specimens using a commercial kit (Qiagen Rneasy mini kit) according to the manufacturer’s instructions and 200 ng RNA was reverse transcribed (Superscript Invitrogen S.R.L., Italy). PKA R1A, PKA R2A and PKA R2B mRNA levels were evaluated in adipose tissue samples by real time quantitative RT-PCR based on TaqMan methodology, using a ready-to-use assay (identification numbers Hs00267597_m1, Hs00177760_m1 and Hs00176966_m1, respectively; Assay-on-Demand™ Gene Expression Products, Applied Biosystems), according to the manufacturer’s instructions, and the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster city, CA, USA). PKA R1A, R2A and R2B mRNA expression were determined applying the ΔCt method, as previously described (12,13). To normalize the amount of total RNA added to each reaction mixture we quantified as internal RNA control the GUSB mRNA (14). The level of CD68 mRNA was also evaluated to quantify blood contamination and inflammatory infiltration. Similar results were obtained with or without normalization with CD68 mRNA levels, thus indicating absent or poor contamination in all samples.

PKA activity. PKA activity was measured, as previously reported (12), using a non-radioactive PKA kinase activity assay kit (Stressgen, Victoria BC, Canada) in 1 μg of protein extracts from human subcutaneous and visceral adipose tissue samples (20 non-obese and 20 obese subjects). The assay is based on a solid phase enzyme-linked immuno-absorbent assay (ELISA) that utilizes a specific synthetic peptide as a substrate for PKA (kempeptide) and a polyclonal antibody that recognizes the phosphorylated form of the substrate. PKA activity reflects the enzymatic activity after stimulation with 5 μM cAMP, free PKA activity represents basal activity, in the absence of cAMP stimulation, and total PKA activity is calculated as the difference between cAMP-stimulated PKA and the PKA inhibited by the protein kinase inhibitor PKI (5μM). Data obtained from different assays where normalized by including the same control sample in each experiment.

Glycerol release. Adipocytes were isolated from 40 visceral and subcutaneous human fresh adipose tissue samples (20 obese and 20 non-obese patients) and seeded in 96 well plates. The amount of material used for glycerol release evaluation was determined on digested tissue weight and the results were then normalized for DNA quantification. Glycerol release in the medium (Assay Buffer) was measured using a commercial kit (Adipolysis assay kit, Chemicon) according to the manufacturer’s instructions. Briefly, after 2h incubation at 37°C in the Assay Buffer (Hank’s balanced salt solution, 2% BSA) with or without the addition of isoproterenol 1μM or 8-Cl-cAMP 100μM, Glycerol Reagent Solution was added to a cell-free aliquot of the incubation medium. Glycerol released to the medium is phosphorylated by ATP by glycerol kinase. Glycerol-1-phosphate (G-1-P) is then oxidized by glycerol phosphate oxidase with the production of H₂O₂, that reacts with dyes contained in the reagent solution producing a quinoeimine dye which shows an absorbance maximum at 540 nm. The increase in the absorbance at 540 nm is directly proportional to glycerol concentration of the sample.

Statistical analysis. Variables that were not normally distributed were log transformed. Paired t-test and 2 way ANOVA were used to compare the expression levels of PKA regulatory subunits mRNA in subcutaneous and visceral adipose tissue where indicated, and two sample t-tests to examine the differences between obese and non-obese subjects. Pearson's correlation analyses were used to evaluate bivariate relationships (14). Partial correlation was used to explore if
correlations between PKA R2B expression and insulin resistance were independent of obesity. Data are expressed as mean±s.d. A P-value<0.05 was considered statistically significant. All analyses were performed using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA).

RESULTS
PKA regulatory subunits expression in human subcutaneous and visceral adipose tissues. Immunohistochemistry performed on human adipose tissue samples from both non-obese and obese subjects included in the study showed a strong immunopositivity for PKA R2B, while the other PKA regulatory subunits analysed (R2A and R1A) were either absent or expressed at very low levels (Figure 1A).

Western-blot analysis was performed on total protein extracts from 40 visceral adipose tissue samples (20 from non-obese and 20 from obese subjects). This approach confirmed the prevalence of PKA R2B subunit over R1A and R2A subunits, that were either absent or detected at extremely low levels (Figure 1C). Moreover, quantification of the resulting bands showed that PKA R2B protein was significantly lower in adipose tissues from obese patients compared to those from non-obese patients (P<0.01, Figure 1B, 1C).

The transcriptional level of PKA R subunits was analyzed by real-time RT-PCR in total RNA extracted from all the adipose tissue samples included in the study. This analysis confirmed that in all samples PKA R2B was the predominant PKA regulatory subunit also at the mRNA level, while PKA R1A and particularly PKA R2A were expressed at lower levels. However, mRNA levels of the three regulatory subunits were significantly lower in obese subjects than in non-obese subjects (1A: P<0.00005; 2A: P<0.01; 2B: P<0.0001). In particular, the reduced expression of PKA R2B and R1A subunits mRNA in obese versus non-obese subjects was strongly significant in both subcutaneous (2B: P<0.001; 1A: P<0.001) and visceral (2B: P<0.01; 1A: P<0.05) adipose tissue samples, while PKA R2A expression was extremely low in all samples, with a tendency to be lower in those from obese patients (P=0.06) (Figure 2). When comparing the expression level of PKA R in visceral versus subcutaneous adipose tissue, no difference in PKA R expression was observed in samples from non-obese subjects, while visceral adipocytes from obese patients expressed higher levels of PKA R2B and R1A when compared with the corresponding subcutaneous adipose tissue (PKA 1A and PKA 2B: 2.54±1.5 and 3.67±0.9 in subcutaneous vs 4.82±1.8 and 8.72±2.2 in visceral adipose tissue, p=0.013 and p=0.019, respectively).

Correlation between PKA R2B mRNA levels and clinical parameters. The main clinical data of obese and non-obese patients included in this study are summarised in Table 1. We correlated the expression of PKA regulatory subunits with clinical parameters. In particular, following the demonstration of low R2B protein levels in patients with PCOS (10,11), a syndrome characterized by insulin resistance, we searched for correlations between PKA regulatory subunits expression and insulin resistance, as well as HOMA-IR as a surrogate marker of insulin resistance. Statistical analysis demonstrated that PKA R2B expression was strongly negatively correlated to BMI, waist circumference, insulin levels and HOMA-IR (subcutaneous: r=-0.280, r=-0.296, r=-0.269, r=-0.255, respectively; P<0.01; visceral: r=-0.298, r=-0.267, r=-0.240, r=-0.245, respectively; P<0.01), these last two correlations being independent of BMI (Figure 3). No correlation was observed with age and/or the presence or the absence of menopause, and no difference was detected between males and females in the two groups (obese and non-obese).
PKA and lipolytic activity in human subcutaneous and visceral adipose tissues. PKA activity was evaluated in 1 μg total protein extracts from both visceral and subcutaneous adipose tissue samples of 20 non-obese and 20 obese subjects. Basal PKA activity was reduced in visceral and subcutaneous protein samples from adipocytes of obese subjects compared to what observed in non-obese subjects. However, this difference was statistically significant only for visceral adipose tissue (visceral: p=0.017; subcutaneous: p=0.464).

In all the samples analyzed enzymatic PKA activity was significantly stimulated by cAMP treatment. The same results were obtained after treatment with 8-Cl-cAMP, a cAMP analogue able to selectively activate PKA type 2 (2), while the addition of the PKA inhibitor PKI (5 μM) significantly reduced cAMP-stimulated activity, as expected (Figure 4). Interestingly, the cAMP- and 8-Cl-cAMP-induced stimulation of PKA activity in proteins from visceral adipose tissue of obese patients was significantly lower than that observed in samples from non-obese patients, while the difference did not reach statistical significance in subcutaneous adipose tissues (Figure 4).

Lipolytic activity was measured by evaluating the quantity of glycerol released by adipocytes in culture medium. Glycerol release was measured both at basal level and after stimulation of beta-adrenergic receptor by isoproterenol or after stimulation of PKA R2B by 8-Cl-cAMP. The stimulation of cAMP pathway by isoproterenol or 8-Cl-cAMP was able to induce an increase in glycerol release both in visceral and subcutaneous adipocytes from obese and non-obese subjects (P <0.05 vs basal). However, glycerol release was lower in adipocytes from obese subjects, compared to non-obese subjects, both at basal level and after treatment with isoproterenol or 8-Cl-cAMP, these data being statistically significant only in visceral tissue (Figure 5). Finally, no difference in basal glycerol release between subcutaneous and visceral adipocytes was observed, while the effects of cAMP and 8-Cl-cAMP on lipolysis were significantly higher in visceral than subcutaneous adipose tissue from non-obese subjects (p<0.05).

A positive correlation was found between PKA R2B mRNA expression and basal PKA activity and isoproterenol-stimulated glycerol release in visceral fat (r=0.430, r=0.398, respectively; P<0.01) (Figure 6).

DISCUSSION

Obesity is rapidly increasing in industrialized countries and, with very few exceptions, the molecular mechanisms underlying the predisposition to become obese are largely unknown. In both white and brown adipocytes cAMP and the cAMP-dependent protein kinase A (PKA) are key regulators of lipolysis, the most important process determining fat storage or mobilization (4). Data present in the literature to date indicate that, at least in mice, among the different subunits constituting the PKA holoenzyme, PKA R2B is the key regulatory subunit in adipose tissue (4,5,9,15-20). Interestingly, R2B expression is limited to a few tissues known to regulate energy homeostasis, i.e. the white and brown adipose tissue and the brain. Studies in R2B knockout mice revealed an unexpected role for this protein in regulating energy balance, since these animals remain remarkably lean even when challenged with a high-fat diet (5).

Despite this increasing amount of data on murine adipose tissue, and although many groups, including ours, have clearly demonstrated distinct and cell-specific roles for the different PKA isoenzymes in the regulation of growth control (1,2,12,13,21), very little is known about the differential role played by the different PKA regulatory subunits in human fat tissue. In this study, we evaluated the possible differences in R2B...
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expression, PKA activity and lipolysis in subcutaneous and visceral adipose tissue samples from a large number of obese and non-obese subjects. Immunohistochemistry demonstrated a strong immunoreactivity for the R2B protein in all fat tissues included in our study, while the other PKA regulatory subunits analysed (R2A and R1A) were expressed at very low levels. This was true in visceral as well as in subcutaneous adipose tissue and similar data were obtained by western blot analysis. This observation first demonstrates that PKA regulatory subunits expression in human adipose tissue is similar to what previously observed in murine adipocytes, and supports the hypothesis that PKA R2B expression is specific for adipose tissue and few other cell types (5,9).

Western blot analysis, that better allows the quantification of protein expression than immunohistochemistry, showed that PKA R2B protein expression was dramatically reduced in adipose tissues from obese patients compared to those from non-obese subjects. This observation lead us to investigate the pattern of expression also at the mRNA level. In contrast to what observed at the protein level, mRNA of the 3 subunits was detected in all the adipose samples analyzed, R2B being the most represented regulatory subunit. Nevertheless, while R2A mRNA was detected at very low levels, PKA R1A seems to be actively transcribed in all adipose tissues. The discrepancy between R1A mRNA and protein expression suggests the existence of high rate of protein instability and degradation, as already demonstrated for this subunit by us and others in other cell systems (12,22). Interestingly and similarly to western blot data, mRNA levels of the three regulatory subunits were significantly reduced in obese subjects if compared with non-obese subjects. In particular, the difference in the expression of PKA R2B and R1A subunits mRNA between obese and non-obese subjects was strongly significant in both subcutaneous and visceral adipose tissue samples while the difference in PKA R2A expression while the difference in PKA R2A expression did not reach statistical significance. In agreement with the restricted expression of R1B gene (23), this transcript was absent in subcutaneous and visceral adipose tissues from obese and non-obese subjects (personal observations).

We then tried to correlate the expression of PKA regulatory subunits with clinical parameters, particularly insulin levels and HOMA-IR. We chose HOMA-IR as a surrogate marker of insulin resistance, since an oral glucose tolerance test was indicated and performed only in obese patients. Indeed, PKA R2B expression was negatively correlated to BMI and waist circumference, a clinical parameter strongly associated with visceral obesity. Moreover, R2B mRNA levels were also negatively correlated with insulin levels and HOMA-IR, these correlations being independent of BMI. These findings are in agreement with the only data present in the literature on PKA expression and function in humans that indicate low levels of PKA R2B in normal weight women affected with polycystic ovary syndrome (PCOS) when compared with matched control women, this defect being associated with lipolytic catecholamine resistance and insulin resistance in these patients (10,11). Taking into account that PRKAR2B promoter has SP-1 transcription factor binding sites, it is tempting to speculate that insulin might act on these sites to positively regulate PRKAR2B transcription (24).

The functional impact of the differential expression of PKA regulatory subunits in adipose tissues from non-obese and obese patients was assessed by evaluating PKA activity and glycerol release in the same samples. Although both basal and cAMP stimulated PKA activity were lower in visceral and subcutaneous protein samples from obese subjects, compared to what observed in samples from non-obese patients,
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these differences were statistically significant only in visceral adipose tissue. Interestingly, a similarly reduced PKA activity in adipose tissue from obese patients was observed after stimulation with a cAMP analogue able to selectively activate R2 subunits (8-Cl-cAMP), suggesting that the low levels of PKA R2B mRNA and protein detected in these tissues are indeed associated with a lower PKA activity. Similarly, glycerol release measured at basal level and after stimulation of beta-adrenergic receptor by isoproterenol was significantly reduced in visceral adipocytes from obese patients compared to non-obese subjects. Stimulation with the R2-selective analogue 8-Cl-cAMP induced a significant increase in lipolysis in samples from both obese and non-obese subjects, this increase being significantly lower in visceral adipose tissue from obese patients. Taken together these data further support the correlation between Real-Time PCR and western blot data and the activation of the cAMP/PKA pathway and lipolysis. At the moment, these data do not allow any speculation on the role played in this circuit by AKAPs, specific anchor proteins that recruit the PKA holoenzyme close to its substrate/effecter proteins, thus directing and modulating PKA activation.

Our data seem to be partially at variance with the data in PCOS patients previously reported, in whom the decreased expression of R2B in visceral fat cells obtained from non-obese PCOS patients was associated with a two-fold increase in R1A and in catalytic subunit and a consequent twofold increase in catecholamine-induced adipocyte lipolysis (10), while the reduction of R2B in subcutaneous fat was associated with a reduced lipolytic responsiveness and sensitivity, mostly due to decreased expression of beta 2-adrenergic receptor (11). However, in the present study PKA activity and glycerol release were investigated by selectively activating R2 subunits, thus providing novel evidence for a specific functional impact of R2B loss in adipocytes, not related to adrenocceptor down-regulation.

In mice, the loss of R2B revealed an unexpected role for this protein in regulating energy balance (5). In particular, the absence of this protein is compensated by an increase in the R1A subunit, which is more sensitive to cAMP activation and results in a net increase in basal PKA activity, leading to increase in metabolic activity, body temperature and lipid hydrolysis (5,9). In humans, our work indicates that the reduction of R2B that accompanies obesity is not associated with an increase in R1A. Nevertheless, it seems likely that the higher R2B levels found in non-obese subjects may account, alone, for the PKA activity required for lipolysis activation in physiological conditions.

The regulation of human fat cell lipolysis is complex and, in many ways, species unique. Catecholamines influence lipolysis through four different adrenoceptor subtypes, and there are profound regional variations in adipocyte lipolysis leading to more release of fatty acids from the visceral than subcutaneous adipose tissue during hormone stimulation (25-27). These regional variations in lipolysis have been demonstrated to be further enhanced in obesity and polycystic ovarian syndrome, and are of importance for dyslipidaemia, hyperinsulinaemia and glucose intolerance in these conditions. In particular, a decreased lipolytic effect of catecholamines in adipose tissue has been repeatedly demonstrated in obesity and has been suggested as a cause of excess accumulation of body fat (28,29). Our data on lipolysis are, at least in part, in contrast with the prevalent idea that adipocytes from visceral fat depots are thought to be hyperlipolytic. Nevertheless, relatively few studies have directly compared lipolysis in visceral vs subcutaneous adipocytes or adipose tissues, with not unanimous findings. Moreover, although several determinants, such as polymorphisms
of beta-adrenergic receptor and its level of expression (30,31) and/or reduced expression and activity of adipocyte hormone-sensitive and triglyceride lipases (32,33) have been proposed as responsible for the lipolytic catecholamine resistance in obesity, the mechanisms of this defect have not been fully understood. We now propose that differences in PKA expression and function, that interestingly were particularly evident in the visceral adipose tissue, may be included among the complex and multiple players which cause fat accumulation or mobilization by reducing catecholamine signalling.

In conclusion, our study indicates that, as in mice, R2B is the key PKA regulatory subunit in human adipose tissue, both in obese and normal subjects, with no significant differences between visceral and subcutaneous adipose tissue. Moreover, R2B was dramatically reduced in adipocytes from obese patients, with a significant negative correlation between R2B expression levels and BMI, waist circumference, insulin levels, and HOMA-IR. Accordingly, both basal and cAMP-stimulated PKA activity and glycerol release were significantly lower in adipocytes from obese patients in comparison with those from non-obese subjects. Our results indicate important BMI-related differences in R2B expression and PKA activation, which might be included among the multiple determinants involved in the different lipolytic response to beta-adrenergic activation in obese and non-obese subjects.

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REFERENCES
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### Table 1. Clinical data of obese and non-obese subjects included in this study

<table>
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<th>Obese</th>
<th>Non-obese</th>
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<tr>
<td>n.</td>
<td>67</td>
<td>20</td>
<td></td>
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<tr>
<td>Age (years)</td>
<td>46.4±11.7</td>
<td>41.9±10.2</td>
<td>NS</td>
</tr>
<tr>
<td>Sex (F:M)</td>
<td>53:14</td>
<td>14:6</td>
<td>NS</td>
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<tr>
<td>BMI (kg/m²)</td>
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<td>22.8±3.9</td>
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<tr>
<td>Waist circumference (cm)</td>
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<td>88.3±8.2</td>
<td>P&lt;0.001</td>
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<td>Systolic blood pressure (mm Hg)</td>
<td>143±18</td>
<td>122±21</td>
<td>NS</td>
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<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>94±13</td>
<td>83±9</td>
<td>NS</td>
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<td>Plasma insulin (mU/l)</td>
<td>18±10</td>
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<td>Plasma glucose (mmol/l)</td>
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<td>HOMA-IR</td>
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**Figure 1:** A. Immunostaining for the 3 PKA regulatory subunits (R1A, R2A and R2B) performed in a human subcutaneous adipose tissue, as representative example (upper panels). Similar data were obtained in the other 84 subcutaneous and visceral samples (from obese and non-obese subjects). All photomicrographs are shown at an original magnification of x25. B. PKA R2B protein representative western blot analysis performed in human subcutaneous adipose tissue samples from 4 obese and 4 non-obese subjects, as representative examples of the 40 samples analyzed. C. Immunoblots were measured by an imaging densitometer and the values expressed in arbitrary units. Data were normalized through GAPDH antibody hybridization.
Figure 2: PKA regulatory subunits (R1A, R2A, R2B) mRNA expression in human adipose tissue from obese and non-obese subjects as assessed by real-time PCR. Data are expressed as mRNA relative quantities. Overall, obese subjects express significantly lower levels of R1A and R2B subunits mRNA than non-obese subjects, and a similar expression pattern is observed both in visceral (upper panel) and subcutaneous (lower panel) adipose tissue samples. (adipose tissue from obese versus non-obese subjects: *: p<0.01; §: p<0.05; #: p<0.001).
Figure 3: Dot plots showing the negative correlations between BMI (upper panel) and HOMA-IR (lower panel) and PKA R2B mRNA expression in subcutaneous fat from 87 subjects ($r=-0.280$, $P=0.005$ and $r=-0.255$, $P=0.008$, respectively). Similar results were obtained when considering visceral fat.
Figure 4: PKA activity measured (RFU, relative fluorescence units) in cell extracts from visceral (upper panel) and subcutaneous (lower panel) adipose tissue samples from obese and non-obese patients. cAMP and 8-Cl-cAMP increased PKA activity both in obese and non-obese subjects adipocytes, this stimulation being inhibited by the PKA inhibitor PKI. Basal and stimulated PKA activity was significantly lower in visceral adipose tissue from obese patients than in samples from non-obese subjects. * p < 0.05 vs basal; # p < 0.05 vs cAMP stimulated; § p < 0.05 vs non-obese subjects.
**Figure 5:** Lypolitic activity (glycerol release, mg/ml) measured in adipocytes culture medium after 2h incubation with isoproterenol or 8-Cl-cAMP. Isoproterenol and 8-Cl-cAMP induced an increase in basal lipolytic activity in visceral (upper panel) and subcutaneous (lower panel) both in obese and non-obese subjects. Basal and stimulated lipolytic activity was significantly lower in visceral adipose tissue from obese patients than in samples from non-obese subjects. * p < 0.05 vs basal; § p < 0.05 vs non-obese.
Figure 6: Dot plots showing the positive correlations between basal PKA activity (upper panel) and isoproterenol-stimulated glycerol release (lower panel) and PKA R2B mRNA expression in visceral fat from 20 obese and 20 non-obese subjects ($r=0.430$, $P=0.009$ and $r=0.398$, $P=0.002$, respectively).