CLINICAL STUDY

Effect of acute hyperinsulinaemia with and without angiotensin II type 1 receptor blockade on resistin and adiponectin concentrations and expressions in healthy subjects

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Abstract

Objective: The potential insulin-sensitizing function of angiotensin II type 1 receptor blockade (ARB) with regard to selected adipokines is not fully explained so far. Our study aimed to explore the influence of acute hyperinsulinaemia and acutely induced ARB on resistin and adiponectin concentrations and expressions in healthy subjects.

Design and methods: Plasma adipokines were measured: 1) at 0, 30 and 240 min of hyperinsulinaemic (1 mU/kg per min) euglycaemic (5 mmol/l) clamp (HEC), and 2) during HEC after acute ARB (losartan 200 mg; AT-HEC) using the same protocol, in eight healthy subjects. Needle biopsy of abdominal s.c. fat was performed at 0, 30 and 240 min of both clamps to assess the adipokines’ expressions.

Results: Comparing the glucose disposals of HEC and AT-HEC, no difference in insulin sensitivity was found. Plasma resistin increased equally during HEC and AT-HEC (P < 0.05). The expression of resistin in s.c. fat increased during HEC (P < 0.05), while no significant changes in expression were observed during AT-HEC. Plasma levels of adiponectin did not change during both clamps. Adiponectin expression increased during HEC (P < 0.05), while it did not change during AT-HEC.

Conclusions: In healthy subjects, acute hyperinsulinaemia is associated with an increase in plasma resistin independently of ARB, while plasma adiponectin is not influenced by insulin or ARB. The expressions of both resistin and adiponectin in s.c. adipose tissue are stimulated by acute hyperinsulinaemia, whereas losartan attenuates their insulin-stimulated expressions. This suggests a potential effect of losartan on adipokines’ expression.

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Introduction

Resistin is an adipocyte-secreted peptide hormone that has been proposed to link obesity with insulin resistance. It was identified as a factor impairing glucose homeostasis and inducing hepatic insulin resistance in mice (1). Recent studies in rodent models and humans show contradictory results (2–6). Mouse resistin is predominantly expressed in white adipose tissue, whereas human resistin expression in adipocytes is significantly lower and there are other important sources of resistin, e.g. mononuclear cells, endothelial, vascular smooth cells (2) or islets of Langerhans (7). The receptor for resistin, its signalling pathway, target tissues and its biological effect and regulation in human physiology have not been clearly identified up to now.

Adiponectin is an insulin-sensitizing adipokine specifically and abundantly expressed in adipose tissue (8, 9). It is present in the circulation at high concentrations in the form of different multimeric complexes, among which the high-molecular weight (HMW) multimers exert the predominant action. Two adiponectin receptors, AdipoR1 and AdipoR2, have been identified that up-regulate AMP-activated protein kinase activity. Adiponectin directly increases hepatic insulin sensitivity, promotes fuel oxidation in skeletal muscle and decreases vascular inflammation. Hypoadiponectinaemia is known to be consistently related to insulin resistance, obesity, type 2 diabetes, coronary heart disease, hypertension and atherosclerosis based on both experimental and clinical studies (10, 11). Up-regulation of adiponectin is a partial cause of the insulin-sensitizing action of thiazolidinediones.

Evidence has accumulated that insulin resistance may be improved by interrupting renin–angiotensin system (RAS). Angiotensin-converting enzyme (ACE) inhibitors and angiotensin II type 1 receptor antagonists are able to interfere with the adverse metabolic effects of angiotensin II on insulin signalling, tissue blood flow, oxidative stress, sympathetic activity or
adipogenesis (12, 13). The antidiabetic properties of ACE inhibitors may be largely mediated through increases in bradykinin levels, nitric oxide and the GLUT4 glucose transporter (13).

The effect of angiotensin II type 1 receptor blockade (ARB) on glucose metabolism and insulin resistance remains controversial, and the mechanisms are not fully understood. Experimental and in vitro studies showed that ARB may improve insulin sensitivity via decreasing adipocyte size (14), promotion of adipocyte differentiation and preventing ectopic lipid deposition (15). Several randomized, placebo-controlled studies using the HEC technique have found little positive or no effect of ARB on insulin action in rodents (16, 17) and humans (18, 19). Clinical trials using ARB have provided indirect support for the possibility that ARB per se might improve insulin sensitivity and decrease incidence of type 2 diabetes (LIFE, CHARM and VALUE) (20).

It has been shown that treatment with losartan does not influence plasma leptin levels (21) and that RAS blockade (22) or losartan (23) increases adiponectin levels, but the effect of losartan on expressions of adipokines in human adipose tissue under conditions of hyperinsulinaemia has not been studied yet.

The aims of our study were twofold: to determine resistin and adiponectin plasma concentrations together with their expressions in abdominal s.c. adipose tissue and also to test their responses to acutely induced hyperinsulinaemia with and without acute ARB in healthy subjects.

Subjects and methods

Subjects

We examined eight healthy men who had normal glucose tolerance (confirmed by an oral glucose tolerance test), blood pressure and serum lipids. Only male subjects participated because of the variable insulin sensitivity in women according to the menstrual cycle and with respect to sexual dimorphism of plasma adiponectin levels. Clinical and physiological characteristics of the study group are summarized in Table 1. The subjects were not taking any drugs and none had a family history of diabetes. They were euthyroid and none had a concomitant disease. Informed consent was obtained from all the individuals after the purpose, nature and potential risks of the study had been explained. The study was approved by the local ethics committee.

Study protocol

The subjects were examined on an outpatient basis, after overnight fasting with only tap water allowed ad libitum. They were instructed to adhere to their ordinary lifestyle, avoid any changes in food intake or alcohol consumption and to refrain from strenuous physical activity for a period of 24 h before the experiment. The subjects underwent two HECs at least 4 weeks apart, both taking 4 h to complete, in random order:

**Study 1** The HEC was conducted as previously described (24). Briefly, a Teflon cannula (Venflon: Viggo, Helsingborg, Sweden) was inserted into an antecubital vein for the infusion of all test substances. A second cannula was inserted retrogradely into a wrist vein for blood sampling and the hand was placed in a heated (65 °C) box in order to achieve venous blood arterIALIZATION. A primed continuous insulin infusion (1 mU/kg per min of Actrapid HM (NovoNordisk, Copenhagen, Denmark) dissolved in 0.9% NaCl) was administered to acutely raise and maintain the plasma concentrations of insulin. Plasma glucose concentrations during the clamp were maintained at the 5 mmol/l level by continuous infusion of 15% glucose. To prevent hypokalaemia during insulin infusion, potassium chloride was added to the 15% glucose infusion (30 mmol/l). To assess plasma levels of selected adipokines, blood samples were taken at 0, 30 and 240 min of the clamp study.

Needle biopsy of abdominal s.c. adipose tissue was performed at 0, 30 and 240 min of the clamp. Under local anaesthesia (1% prinecaïn in a field block pattern), an incision (3–4 mm) was made through the skin at the lower abdomen and an s.c. fat specimen (300 mg) was obtained by needle aspiration. The samples were washed in 0.9% natrium chloride solution, immediately frozen by liquid nitrogen and stored at −80 °C until used for RNA extraction.

**Study 2** An identical clamp study was performed after acute ARB (AT-HEC). Losartan 2×100 mg was given per os prior to the study (8 h and immediately prior to the clamp) and a hyperinsulinaemic (1 mU/kg per min) euglycaemic (5 mmol/l) clamp was conducted as described previously. Blood samples and adipose tissue biopsies were collected at 0, 30 and 240 min of the clamp.
clamp to determine plasma concentrations and expressions of selected adipokines.

**Analytical methods**

Plasma concentrations of glucose were measured using the Beckman analyzer (Beckman Instruments Inc., Fullerton, CA, USA) with the glucose oxidase method. Immunoreactive insulin (IRI) was determined by the RIA method using an IMMUNOTECH Insulin IRMA kit (IMMUNOTECH AS, Prague, Czech Republic) with an analytical sensitivity 0.5 μIU/ml, and intra-assay and inter-assay coefficients of variation below or equal to 4.3 and 3.4% respectively. Glycosylated haemoglobin was measured by HPLC method using the Variant II HbA1c Program (Bio-Rad Laboratories GmbH), and for calibration, a method approved by International Federation of Clinical Chemistry and Laboratory Medicine was used (25). Plasma concentrations of resistin were measured according to manufacturer’s instructions by Human Resistin ELISA kit (BioVendor Lab. Med. Inc., Brno, Czech Republic; the detection limit was 0.033 ng/ml and intra-assay and inter-assay coefficients of variation are 2.8–3.4 and 5.1–6.9% respectively). For analysis of total adiponectin plasma levels, Human Adiponectin ELISA kit was used (BioVendor Lab. Med. Inc.; the detection limit was 210 ng/ml and intra-assay and inter-assay coefficients of variation are 6.4–7.0 and 7.3–8.2% respectively).

Expression of adipokines was analysed by the real-time PCR (RT-PCR) using the following protocol:

1. The RNA was isolated from the liquid nitrogen frozen biopsy of the human fat tissue using the RNeasy Lipid Tissue Mini Kit and QIAzol Lysis Reagent (Qiagen). The starting amount of 100 mg tissue was excised from the biopsy, and homogenized in 1 ml QIAzol Lysis Reagent (guanidine thiocyanate–phenol solution) for 2 min. From the homogenate, the RNA was isolated by extraction on silica gel-based column, according to the kit handbook. Possible contamination of RNA with genomic DNA remains was taken off by DNase digestion (RNase-free DNase Set; Qiagen). This step is supposed to prevent any later DNA amplification. Beyond this, the denaturation curves were measured during each reaction and there was just one product present in all the measurements.

2. The cDNA was synthesized using a recombinant Omniscript Reverse Transcriptase (Qiagen), RNase inhibitor from human placenta (Sigma) and (d)T<sub>10</sub> oligonucleotides.

3. RT-PCR procedure itself was carried out on the DNA Engine Opticon 2 System (MJ Research, Waltham, MA, USA). HotStar Taq DNA polymerase and SYBR Green fluorescent dye (QuantTec SYBR Green PCR Kit; Qiagen) were used for the RT-PCR procedure. To eliminate the influence of primer dimers, negative controls were used. To account for differences in cDNA loading, the results were expressed relative to the expression of human cyclophillin (used as a reference gene). There is no evidence of insulin or ARB influence on cyclophillin, which is a house-keeping gene encoding a cytoskeleton protein. Primers used for RT-PCR are given in Table 2.

4. The data were processed by Q-gene 96 software.

**Calculations**

Insulin action was estimated as the glucose disposal (M) calculated during the last 30 min of the clamp as the rate of glucose infusion after correction for changes in glucose pool size and urinary glucose loss. Insulin sensitivity index M/I (glucose disposal normalized by plasma insulin during steady-state period) was calculated to correct for any variations in plasma insulin.

**Statistical analysis**

The data are expressed as mean ± S.E.M., unless provided otherwise. Steady-state periods of both clamps were compared using Wilcoxon’s paired test. The differences between time courses of both clamps were evaluated by repeated measures ANOVA model, including following factors and interactions: effects of treatment (HEC versus AT-HEC) and time (the effect of hyperinsulinaemia) as the within factors, subject factor (represents the interindividual variability of subjects) and treatment ! time interaction. The last term indicated if the shapes of the time profiles for HEC and AT-HEC were different or not. The differences between subgroups were evaluated

<table>
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<th>Table 2 Primes used for RT-PCR.</th>
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<tr>
<td><strong>Gene</strong></td>
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using least significant difference multiple comparisons. A statistical significance of $P < 0.05$ was chosen for both ANOVA testing and multiple comparisons. Due to non-Gaussian data distribution in all dependent variables, the data underwent power transformations to attain distributional symmetry and a constant variance in the data as well as in residuals. The non-homogeneities were detected using residual diagnostics. The experimental points with absolute values of studentized residual (after data transformation) > 3 were excluded from the analysis. The fraction of such points never exceeded 5% of the total number. Statistical software Statgraphics Plus v. 5.1 (Manugistics; Rockville, MD, USA) was used for the data analysis.

**Results**

Acute ARB had no impact on diuresis, systolic and diastolic blood pressure in our study group (data not shown).

During the steady-state periods of HEC versus AT-HEC, the clamps were comparable in terms of the mean plasma glucose concentrations ($4.68 \pm 0.32$ vs $4.75 \pm 0.20$ mmol/l) with coefficient of variation $3.37 \pm 0.55$ vs $3.42 \pm 1.87\%$. Although the mean IRI levels were different when comparing both the clamps ($65.11 \pm 7.53$ vs $75.28 \pm 6.95$ μIU/ml; $P < 0.05$), the parameters of insulin sensitivity did not significantly differ between HEC and AT-HEC, being expressed as the glucose disposal (M; $9.55 \pm 0.56$ vs $9.15 \pm 1.68$ mg/kg per min), as well as calculated as the insulin sensitivity index $M/I$ ($0.15 \pm 0.01$ vs $0.13 \pm 0.01$ mg/kg per min/μIU per ml).

Plasma concentrations of resistin during HEC and AT-HEC are shown in Fig. 1a. The ANOVA model indicated a significant increase in plasma resistin during both clamps. The time factor was significant ($P < 0.05$), while the treatment factor as well as the interaction between the factors time and treatment were not. Despite the multiple comparisons reaching significance only for the difference between basal value and 240 min for AT-HEC ($P < 0.05$), the time trend did not significantly differ between the clamps.

**Figure 1b** shows relative expressions of resistin. Here, the time effect, as well as the treatment effect, was not significant. On the other hand, the shapes of the expressions’ time profiles differed significantly between HEC and AT-HEC (treatment×time interaction; $P < 0.05$): resistin expression increased during HEC, as also documented by multiple comparisons (0 vs 240 min; $P < 0.05$), while during AT-HEC the expression did not change. Moreover, at 240 min of AT-HEC, resistin expression was significantly lower when compared with HEC ($P < 0.05$ by multiple comparisons).

In plasma adiponectin, no factor or interaction reached significance (Fig. 2a). In addition, the multiple comparisons did not show any significant differences between clamps for basal (0 min) or stimulated (240 min) values.

**Figure 2b** represents relative expressions of adiponectin. In this variable, both time and treatment effects were not significant. However, the shapes of the time profiles differed between HEC and AT-HEC (as documented by significant treatment×time interaction; $P < 0.05$). Adiponectin expression increased during HEC, as also confirmed by multiple comparisons (0 vs 240 min; $P < 0.05$), while it did not change during AT-HEC. Multiple comparisons found no significant differences between clamps for basal (0 min), as well as stimulated (240 min) values.

**Discussion**

Our study shows that in healthy subjects, acute hyperinsulinaemia induces an increase in resistin plasma concentration and stimulates the expression of resistin in s.c. adipose tissue, which is a novel observation *in vivo*. Our finding is consistent with
several studies in rodent models (2) or in vitro studies (26, 2). To the contrary, other groups showed the opposite in mice and 3T3-L1 adipocytes (2). Few studies have investigated the effect of insulin in vivo in humans; Heilbronn (27) observed an increase in serum resistin concentrations in response to supraphysiological doses of insulin (164 ± 5 mIU/l) in obese subjects with and without diabetes. However, clinical studies in humans do not show a consistent link between serum resistin and either insulin resistance or obesity (3–6).

In our study group, adiponectin plasma concentrations were not affected by hyperinsulinaemia, whereas its expression was stimulated by insulin. These findings are in agreement with recent evidence that circulating adiponectin per se is not a subject for acute regulation, but its expression seems to be regulated acutely (9), preceding thereby systemic changes. Consistent results in vivo were shown by Westerbacka et al. (28), who found an increased adiponectin expression in s.c. fat after 6 h of hyperinsulinaemia in insulin-sensitive subjects, which was not accompanied by any changes in serum levels of adiponectin. However, in the literature, a 7–8% decline of circulating adiponectin during hyperinsulinaemia in healthy men (29–31) is reported, which is more pronounced (16–20% decrease) when a twofold higher insulin infusion rate is used (32, 33). To the contrary, other groups did not find any changes in plasma adiponectin in response to insulin (34, 35), which is in agreement with our results. The source of such discrepancies might lie partly in diverse analyses that were used or different ethnic groups that were studied (there is a broad range of reported absolute values for serum adiponectin: 2–20 µg/ml). Another point that should be specified accurately is a potential confounding factor of haemodilution during clamp. To further distinguish the regulations in vivo, it will be necessary to measure the adiponectin isoforms selectively, since the HMW form is known to be the most active one (8) and it correlates better with glucose disposal (36).

In accordance with previously mentioned study (28), we report an increase in adiponectin mRNA in s.c. adipose tissue in response to insulin in vivo. A 24-hour insulin treatment induced an increase in adiponectin mRNA in isolated human visceral adipose tissue (37), but a reduction of adiponectin expression was detected in 3T3-L1 adipocytes (38). Although the secretion of adiponectin from visceral and s.c. depots appears comparable (39), the regulations may exhibit some depot specificity (9). It will be important to further elucidate the effect of insulin on adiponectin production in different fat depots and in different stages of insulin resistance.

Acute ARB did not change the parameters of insulin sensitivity in our study group, that can be defined as insulin sensitive. Interruption of angiotensin II signalling was reached by administration of losartan 2 × 100 mg total, which ensured linear pharmacokinetics within the biological period of the drug (2 h) and its active metabolite (6–9 h). However, the plasma levels may not mirror effective tissue concentrations, which are crucial for potential metabolic effects. Recently, it was proved that the active losartan metabolite EXP3179 also shows a partial PPARγ agonistic effect (40).

The effect of acute ARB on insulin sensitivity has not been studied in humans thus far; clinical trials using long-term administration of ARBs (other than telmisartan) with the HEC technique reported both increases (41, 22) and no effect (42, 43) on glucose disposal in insulin-resistant subjects. Hence, it is necessary to compare our findings in healthy controls with insulin-resistant subjects.

Independent of ARB, plasma concentrations of resistin increased during hyperinsulinaemia, and adiponectin plasma levels were not influenced. To the contrary, the insulin-stimulated expressions of resistin and adiponectin in s.c. adipose tissue were attenuated by losartan.

Our finding of increased plasma resistin in spite of decreased expression in s.c. fat implicates a role of another source of circulating resistin – a different fat depot or potential non-adipose sources (e.g. stromal vascular fraction of adipose tissue, macrophages or endothelium). Other studies in rodents and humans...
(2, 5) found increased expression of resistin in visceral fat depots when compared with s.c. at basal conditions, although the source of resistin was thought to be non-adipocytes. Nevertheless, the attenuation of insulin-stimulated resistin expression following acute ARB implies a positive role of losartan in endocrine activity of adipose tissue.

An increase in circulating adiponectin after 2-month treatment with losartan in hypertensive patients has been reported (23), and there are other experimental data showing enhanced adiponectin expression in response to long-term ARB (44, 45), possibly via PPARγ activation. All the experiments and clinical studies tested different ARB substances in different models of insulin resistance, and there is no evidence about the regulations in healthy subjects. We have observed that acute losartan administration attenuates the insulin-induced expression of adiponectin. This unexpected trend in adiponectin expression should be proven in long-term treatment to distinguish acute and long-term regulations in vivo. However, the limited sample size of our study should be considered as well. Further studies are needed to support the hypothesis that losartan exerts its positive effects on insulin sensitivity through other mechanisms than stimulation of adiponectin.

Recently, it was proven that the beneficial metabolic effect of ARB goes beyond simple interruption of RAS (12). Our results suggest potential effects of losartan on resistin and adiponectin expression that might influence further metabolic or proinflammatory factors.

Paradoxically, higher insulin plasma levels were detected in the steady-state period of AT-HEC when compared with HEC. There were no changes in body weight between the clamps, and therefore, the insulin infusion rates were comparable for both clamps. Higher insulin levels after losartan administration cannot be explained by involvement of insulin secretion, because angiotensin II receptor activation, not inhibition, was shown to stimulate insulin secretion (46). Impairment of insulin clearance in liver by ARB cannot be excluded, but there is no corresponding data available. However, the beneficial effects of losartan in our study were seen despite higher insulin levels.

We can conclude that in healthy subjects, acute hyperinsulinaemia stimulates an increase in plasma concentration and expression of resistin in s.c. adipose tissue. While the ARB does not modify the insulin-induced changes in plasma resistin, it attenuates the response of resistin expression in adipose tissue. Acute hyperinsulinaemia is associated with an increase in adiponectin expression, but not in its plasma levels. Losartan reduces the insulin-stimulated expression of adiponectin.

These findings have to be further investigated in larger cohorts in comparison with insulin-resistant subjects, by short- or long-term ARB administration, together with analysis of non-adipose sources of resistin.

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