CLINICAL STUDY

Studies of insulin resistance in patients with clinical and subclinical hypothyroidism

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Abstract

Objective: Although clinical hypothyroidism (HO) is associated with insulin resistance, there is no information on insulin action in subclinical hypothyroidism (SHO).
Design and methods: To investigate this, we assessed the sensitivity of glucose metabolism to insulin both *in vivo* (by an oral glucose tolerance test) and *in vitro* (by measuring insulin-stimulated rates of glucose transport in isolated monocytes with flow cytometry) in 21 euthyroid subjects (EU), 12 patients with HO, and 13 patients with SHO.
Results: All three groups had comparable plasma glucose levels, with the HO and SHO having higher plasma insulin than the EU ($P < 0.05$). Homeostasis model assessment index was increased in HO (1.97 ± 0.22) and SHO (1.99 ± 0.13) versus EU (1.27 ± 0.16, $P < 0.05$), while Matsuda index was decreased in HO (3.89 ± 0.36) and SHO (4.26 ± 0.48) versus EU (7.76 ± 0.87, $P < 0.001$), suggesting insulin resistance in both fasting and post-glucose state. At 100 µU/ml insulin: i) GLUT4 levels on the monocyte plasma membrane were decreased in both HO (215 ± 19 mean fluorescence intensity, MFI) and SHO (218 ± 24 MFI) versus EU (270 ± 25 MFI, $P = 0.03$ and 0.04 respectively), and ii) glucose transport rates in monocytes from HO (481 ± 30 MFI) and SHO (462 ± 19 MFI) were decreased versus EU (571 ± 15 MFI, $P = 0.04$ and 0.004 respectively).
Conclusions: In patients with HO and SHO: i) insulin resistance was comparable; ii) insulin-stimulated rates of glucose transport in isolated monocytes were decreased due to impaired translocation of GLUT4 glucose transporters on the plasma membrane; iii) these findings could justify the increased risk for insulin resistance-associated disorders, such as cardiovascular disease, observed in patients with HO or SHO.

European Journal of Endocrinology **160** 785–790

Introduction

Clinical hypothyroidism (HO) is an insulin-resistant state (1–4). Studies *in vivo*, in hypothyroid patients (1, 2) and *in vitro*, in tissues isolated from hypothyroid rats (3, 4) have established that this is due to defects in the ability of insulin to increase glucose utilization in peripheral tissues, mainly muscle.

Subclinical hypothyroidism (SHO) is defined as an elevated plasma TSH level in the presence of normal plasma thyroid hormone values (FT_3, FT_4). In this condition, data regarding insulin effects on glucose metabolism are contradictory: insulin sensitivity in the fasting state (assessed with homeostasis model assessment (HOMA) index) has been found to be either normal (5, 6) or decreased (7). However, in patients with SHO, fasting hyperinsulinemia has been reported (7, 8). Moreover, in such patients, disorders having insulin resistance as a common pathogenic denominator (such as dyslipidemia and cardiovascular disease) have recently been identified (9).

This study was undertaken in patients with HO and SHO to examine the sensitivity of glucose metabolism to insulin both *in vivo* (by an oral glucose tolerance test, OGTT) and *in vitro* (by measuring insulin-stimulated rates of glucose transport in isolated monocytes).

Material and methods

Subjects

The groups that participated in the study were: i) patients with HO, ii) patients with SHO, and

iii) the euthyroid subjects (EU). There was no statistically significant difference of either body mass index (BMI) or age between EU and HO, SHO groups ($P > 0.05$, with repeated-measures ANOVA). The characteristics and the hormonal data of the groups are presented in Table 1. All subjects were submitted to dual X-ray absorptiometry for the determination of the percentage of the whole body fat mass (Hologic QDR, Bedford, MA, USA). None of the subjects was receiving any treatment or had a family history of type 2 diabetes. The protocol was approved by the hospital ethics committee and all subjects gave informed consent.

Study protocol

Subjects were admitted to the hospital at 0800 h after an overnight fast and received an OGTT (75 g glucose). All groups of participants were on a free diet. Blood samples were drawn before the administration of glucose (at -30 and 0 min) and at 15- to 60-min intervals for 300 min thereafter, and used for measurements of glucose (Yellow Springs Instrument, Yellow Springs, OH, USA) and insulin (RIA, Linco Research, St Charles, MO, USA). Prolactin levels were determined at 0 min with chemiluminescence's method (Roche Diagnostics, GmbH). Free fatty acids (FFA) at 0 min were also measured (FFA, Roche Diagnostics).

In the fasting state, insulin resistance was estimated by the HOMA (fasting glucose $\times$ fasting insulin/22.5) (10), while in the post-glucose state, insulin sensitivity was estimated by the Matsuda index (10 000/SQRT (mean glucose$_{(0-120)} \times$ mean insulin$_{(0-120)} \times$ fasting glucose $\times$ fasting insulin)) (11).

At -30 min, 20 ml blood was drawn for the isolation of mononuclear cells in order to assess: a) GLUT3 and GLUT4 glucose transporter levels on the monocyte plasma membrane, in the presence of insulin, and b) insulin-stimulated rates of glucose transport.

Table 1 The characteristics, the hormonal data, and the indices of insulin resistance of the groups involved in the study.

	EU	**HO**	**SHO**
Age (years)	40 ± 3	45 ± 2	49 ± 3
Body mass index (kg/m^2)	25 ± 1.2	26 ± 0.7	26 ± 0.9
FT$_3$ (pg/ml)	2.99 ± 0.12	0.71 ± 0.26	2.67 ± 0.10
FT$_4$ (ng/dl)	1.23 ± 0.03	0.23 ± 0.09	1.04 ± 0.03
TSH (μU/ml)	1.71 ± 0.38	75.28 ± 10	8.92 ± 1.31
HOMA index	1.27 ± 0.16	$1.97 \pm 0.22^*$	$1.99 \pm 0.13^*$
Matsuda index	7.76 ± 0.87	$3.89 \pm 0.36^\dagger$	$4.26 \pm 0.48^\dagger$
Fat (%)	32 ± 3	34 ± 1.4	33.7 ± 3
Free fatty acids (FFA)	460 ± 25	430 ± 30	445 ± 20
n	21	12	13

$^*P < 0.05$, $^\dagger P < 0.001$.

Effect of insulin on GLUT expression and 6-[N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl)-amino]-6-deoxyglucose (NBDG) uptake: flow cytometry analysis

Blood was diluted $1 \div 1$ (v/v) with PBS (w/o Ca^{2+}, Mg^{2+}), placed on Histopaque-1077 ($1 \div 2$, v/v) and centrifuged at 400 **g** for 30 min at 22 °C, to isolate the 'buffy' coat (mononuclear inter-phase layer). Mononuclear cells were then resuspended in PBS and washed twice (150 g, 10 min at 22 °C). Erythrocytes were lysed with BD Pharm Lyse (lysing reagent, BD Biosciences Pharmigen, San Jose, CA, USA).

The mononuclear cells were aliquoted at the desired concentration (1×10^6 cells/ml) and incubated for 60 min, at 22 °C, in a buffer (NaCl 140 mM, HEPES 20 mM, KCl 5 mM, MgSO$_4$ 2.5 mM, glucose 5.5 mM (pH 7.4)), containing different concentrations of insulin (Sigma Diagnostics). Termination of incubation was achieved with the addition of cytochalasin-B (10 μM; Sigma Diagnostics).

Insulin exerts its action, on a cellular level, by a numerous steps intracellular mechanism, the insulin signaling pathway. Regarding glucose transport, the final step of insulin signaling is the enrichment of plasma membrane with GLUT3 and GLUT4 isoforms. Surface glucose transporter isoforms were determined after staining the cells with anti-GLUT antisera. The antibodies used were mouse anti-GLUT3 (RnD Systems, Minneapolis, MN, USA) and rabbit anti-GLUT4 (0.1 mg/ml, polyclonal antibody, Millipore Corporation, Billerica MA, USA); each corresponded to the exofacial loop of the human GLUT. Since the antibodies were not fluorochrome conjugated, they were labeled with the Zenon Alexa Fluor 488 Rabbit IgG labeling kit or Zenon Alexa Fluor 488 Mouse IgG$_{2b}$ (Invitrogen). Cells were incubated for 30 min with Alexa Fluor 488-conjugated immunoglobulin in a ratio 1×10^6 cells/1 μg immunoglobulin under mild constant shaking. The monocyte fraction was simultaneously stained with anti-CD14-PE MAB (BD Biosciences). After the incubation with the antisera and one wash with PBS, cells were fixed with 0.1% (w/v) paraformaldehyde. A histogram of log green fluorescence of each GLUT isoform was used for the determination of the mean fluorescence intensity (MFI, results expressed in arbitrary units of MFI) of each sample. Two-color flow cytometric analysis was performed on a BD FACSCalibur 4 color flow cytometer (BD Biosciences).

The specificity of the different GLUT-antisera was evaluated by staining cells with isotype controls suitable for each antisera and the blockage of Fc-receptors prior to staining, as previously described in detail (12).

For the glucose transport experiments, the tracer used to monitor glucose flux in monocytes was NBDG (Invitrogen). Cells were suspended to the above-mentioned buffer, at the same concentration. Flow cytometric analysis was initiated immediately after

the addition of NBDG (final concentration 30 μM) and insulin. The uptake of the fluorescent probe was recorded as MFI during a 500-s interval, when the reaction reached a plateau (12).

Statistical analysis

Grouped data are expressed as mean±S.E.M. 6-NBDG uptake is presented as an increase over baseline (MFI of cells prior to the addition of the fluorescent analog). Insulin dose–response curves were analyzed with repeated-measures ANOVA. Comparison between groups was performed with one-way ANOVA, with Bonferroni's post test. Spearman's rank correlation was used to assess the relationship between thyroid hormone levels and insulin sensitivity indices.

Results

Fasting and postprandial plasma insulin levels were increased in patients with HO and SHO versus EU. Plasma glucose levels in patients with HO and SHO were similar to those in EU (Fig. 1). Increased prolactin levels
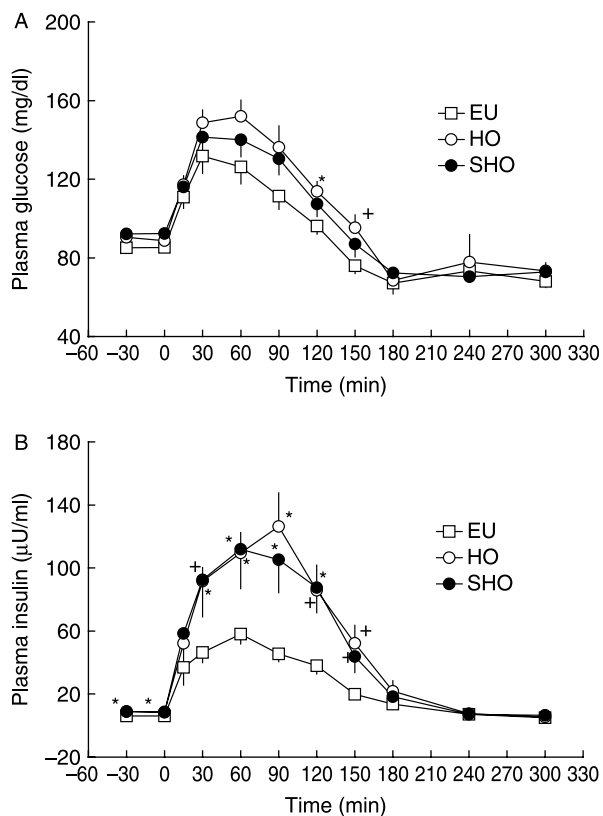
Figure 1 (A) Plasma glucose and (B) insulin levels in euthyroid subjects (EU), and patients with clinical (HO) and subclinical hypothyroidism (SHO) after an OGTT; $^+P<0.05$, $^*P<0.01$ versus EU.

have been associated with the manifestation of insulin resistance (13). Fasting prolactin levels in patients with HO and SHO had no statistically significant differences compared with those in EU (7.37 ± 1.09 and 11.5 ± 1.98 vs 9.48 ± 0.98 ng/ml respectively, $P>0.1$).

HOMA index reflects the insulin resistance in the fasting state (mainly insulin resistance in the liver) while Matsuda index reflects insulin sensitivity in the postprandial state (mainly insulin sensitivity in the peripheral tissues).

HOMA index was increased in patients with HO (1.97 ± 0.22) and SHO (1.99 ± 0.13) versus EU (1.27 ± 0.16, $P<0.05$).

Matsuda index was decreased in patients with HO (3.89 ± 0.36) and SHO (4.26 ± 0.48) versus EU (7.76 ± 0.87, $P<0.001$).

Matsuda index in all subjects correlated positively with both FT_3 and FT_4 levels ($r=0.41$, $P=0.04$).

In monocytes from EU, when insulin was increased from 0 to 100 μU/ml, GLUT4 and GLUT3 isoforms on the plasma membrane increased by 39% and 34% respectively ($P<0.001$ for both). The respective increases for patients with HO were 19% ($P=0.0016$) and 15% ($P=0.012$), while for patients with SHO they were 23% ($P=0.005$) and 20% ($P=0.0037$; Fig. 2).

At 100 μU/ml insulin, GLUT4 levels on the monocyte plasma membrane were decreased in patients with HO and SHO versus EU ($P<0.05$ for both; Fig. 2).

In monocytes from EU, increases in insulin from 0 to 100 μU/ml were associated with a 48% increase in 6-NBDG transport ($P<0.0001$). The respective increases for patients with HO and SHO were 12.57% ($P<0.0001$) and 12.6% ($P<0.0001$).
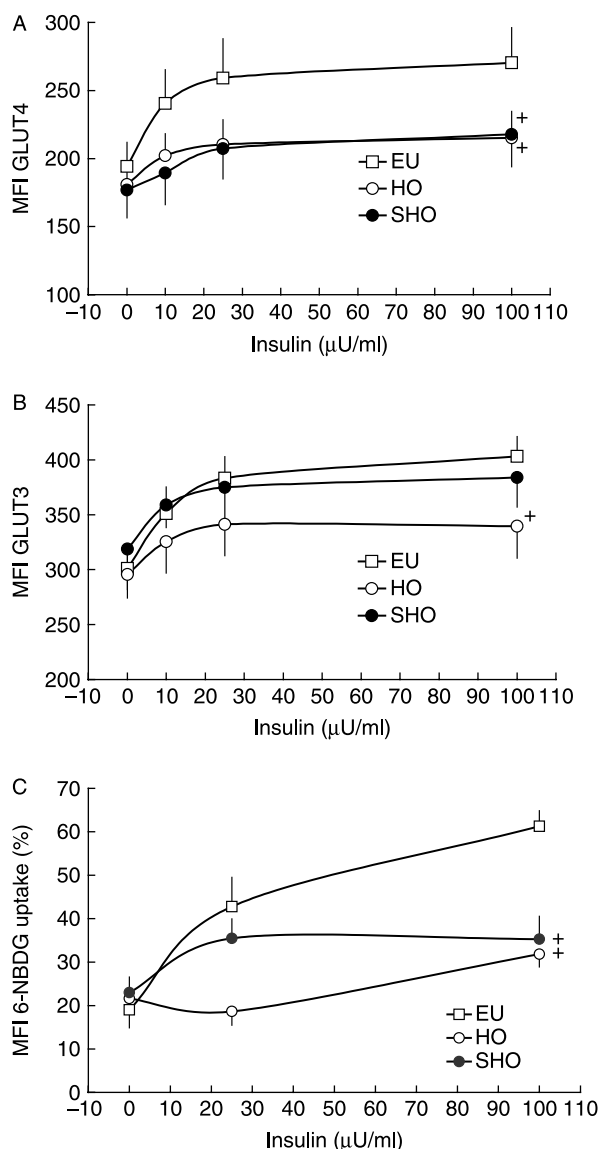
In monocytes from EU, the 6-NBDG uptake (increases from baseline) in the presence of 0, 25, and 100 μU/ml insulin was increased by 19, 43, and 62% respectively ($P<0.005$; Fig. 2). The respective increases for HO were 22, 19, and 32% ($P<0.2$ ns), while for SHO were 23, 35, and 35% ($P<0.036$; Fig. 2).

At 100 μU/ml insulin, 6-NBDG transport rates were decreased in patients with HO and SHO versus EU ($P<0.05$ for both; Fig. 2).

Discussion

Our results demonstrate the presence of insulin resistance not only in HO but also in SHO. Increased HOMA index and decreased Matsuda index in both groups suggest that insulin resistance is present in both fasting and post-glucose state. These findings are consistent with the recent studies reporting an increased cardiovascular risk in these conditions (14–16).

In agreement with our results, previous studies in patients (1, 2, 17) or rats (3, 4) with overt hypothyroidism have shown the presence of insulin resistance due to impaired glucose disposal in peripheral tissues in

Figure 2 Plasma membrane levels of (A) GLUT4, (B) GLUT3, and (C) 6-NBDG transport rates in isolated monocytes in the presence of physiological concentrations of insulin in euthyroid subjects (EU), and patients with clinical (HO) and subclinical hypothyroidism (SHO); $^{+}P<0.05$ versus euthyroid. 6-NBDG uptake is presented as an increase over baseline (MFI of cells prior to 6-NBDG addition).

concentrations and, in the presence of insulin, rapidly increase their rates of glucose disposal (12, 20, 21). Moreover, monocytes express all GLUT isoforms found in muscle and adipose tissue; and the increases in glucose transport in response to insulin in these cells correspond well with those observed in tissues quantitatively important for glucose disposal (12). In our study, insulin-stimulated glucose transport in monocytes from patients with HO and SHO was found to be decreased due to impaired translocation of GLUT4 glucose transporters on the plasma membrane. If these findings in monocytes reflect respective changes in peripheral tissues, our results suggest impairment in insulin-stimulated rates of glucose disposal in muscle and adipose tissue in patients with HO and SHO, which is accounted for by impaired translocation of GLUT4 transporters on the cell surface.

A possible pathogenetic mechanism involved in insulin resistance in hypothyroidism is the decreased blood flow in the peripheral tissues (2). Although this parameter was not studied in the present study, it might still apply to SHO (22).

An interesting observation is the positive correlation between thyroid hormones and the Matsuda index, suggesting that the lower the thyroid hormone levels in plasma, the lower the sensitivity of tissues to insulin. This could explain the insulin resistance found in patients with HO and SHO in our study. It is known that T_3 and insulin have a synergistic role in glucose homeostasis, since these hormones possess similar action sites in the regulation of glucose metabolism, at both cellular and molecular levels (23). It could therefore be hypothesized that a reduced intracellular content of T_3 could lead to an impaired insulin stimulated glucose disposal. Interestingly, even subtle decreases in the levels of thyroid hormones within the physiological range have been shown to correlate inversely with the HOMA index (24).

In our study, both overt (HO) and SHO exhibited comparable levels of insulin resistance. This corresponds well with the studies showing that patients with mild thyroid failure and even subjects with high normal serum TSH values have evidence of comparable atherogenic factors, such as endothelial dysfunction manifested by flow-mediated endothelial-dependent vasodilatation (22) and high serum cholesterol levels (25). Moreover, the fact that insulin resistance was similar in patients with overt (HO) and SHO indicates that thyroid hormones levels *per se* may not be entirely responsible for the manifestation of this phenomenon. Future research should be focused on the insulin signaling cascade and the plausible association of impairment of phosphorylation pattern of signaling molecules (such as insulin receptor substrate 1) with decreased glucose uptake.

The strength of this study is our finding that HO and SHO showed comparable levels of insulin resistance. Therefore, screening and treatment for SHO may be

response to insulin. This is the first report showing that patients with SHO have insulin resistance, which is comparable with that of patients with HO. However, the insulin resistance observed does not seem to be clinically relevant in terms of significant hyperglycemia, due possibly to a compensatory decrease in hepatic glucose output as a result of hyperinsulinemia (18, 19).

Monocytes provide an easily accessible and reliable model for metabolic studies. These cells have insulin receptors that quickly respond to changes in insulin

warranted due to its adverse effects on glucose metabolism. Moreover, the strength of this study is the combination of the flow cytometric method with easily accessible cells such as monocytes. Flow cytometry requires a very small amount of blood, does not involve time-consuming steps, is characterized by an easiness of handling and is based on the immunological transaction of antigen–antibody, which guarantees specificity and accuracy of measurement. On the other hand, our study has several limitations: i) monocytes are not the main target tissue for insulin such as liver, skeletal muscle or adipose tissue; ii) insulin action was evaluated by studying the increment of surface GLUT3 and GLUT4 glucose transporters; it would be useful to investigate whether hypothyroidism has caused impairments in the insulin signaling cascade; iii) the uptake of glucose was studied on a whole body basis by OGTT; this approach does not provide data on insulin-stimulated glucose uptake in the liver or peripheral tissues (skeletal muscle and adipose tissue).

In conclusion, our study showed that patients with SHO have insulin resistance that is comparable with that of the patients with HO. These findings could justify the increased risk for insulin resistance-associated disorders, such as cardiovascular disease, observed in patients with HO and SHO.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

Acknowledgements

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