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Studies of insulin resistance in patients with clinical and subclinical hypothyroidism

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ABSTRACT

Objective: Although clinical hypothyroidism is associated with insulin resistance, there's no information on insulin action in subclinical hypothyroidism.

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 clinical hypothyroidism (HO) and 13 patients with subclinical hypothyroidism (SHO).

Results: All three groups had comparable plasma glucose levels, with the HO and SHO having higher plasma insulin than the EU ($p<0.05$). HOMA index was increased in HO (1.97±0.22) and SHO (1.99±0.13) versus EU (1.27±0.16, $p<0.05$), whilst 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 postglucose state. At 100μU/ml insulin: 1) 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 2) 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: 1) insulin resistance was comparable; 2) insulin-stimulated rates of glucose transport in isolated monocytes were decreased due to impaired translocation of GLUT4 glucose transporters on the plasma membrane; 3) These findings could justify the increased risk for insulin resistance-associated disorders, such as cardiovascular disease, observed in patients with HO or SHO.

INTRODUCTION

Clinical hypothyroidism 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 is defined as an elevated plasma thyroid-stimulating hormone (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 HOMA index) has been found either normal (5, 6) or decreased (7). However, in patients with subclinical hypothyroidism, 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 clinical and subclinical hypothyroidism 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 participated in the study were: 1) the group of patients with clinical hypothyroidism, 2) the group of patients with subclinical hypothyroidism and 3) the group of the euthyroid subjects. 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 analysis of variance). The characteristics and the hormonal

data of the groups are presented in Table 1. All subjects were submitted to Dual X-Ray Absorptionmetry (DEXA) 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 08:00 h after an overnight fast and received an OGTT (75g 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 0min with chemiluminescence's method (Roche Diagnostics, GmbH, Mannheim, Germany). Free fatty acids at 0min were also measured (FFA, RocheDiagnostics, Mannheim, Germany).

In the fasting state, insulin resistance was estimated by the homeostasis model assessment index [HOMA, (Fasting glucose*Fasting insulin/22.5)] (10), while in the post-glucose state, insulin sensitivity was estimated by the Matsuda index [10000/SQRT (Mean $glucose_{(0-120)}$*Mean $insulin_{(0-120)}$*Fasting glucose*Fasting insulin)] (11) .

At -30 min, 20ml of 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.

Effect of insulin on GLUT expression and NBDG uptake - Flow cytometry analysis

Blood was diluted 1÷1 (v/v) with phosphate buffer saline (PBS, w/o Ca^{2+}, Mg^{2+}), placed on Histopaque-1077 (1÷2, v/v) and centrifuged at 400g for 30min at 22^{o}C, to isolate the "buffy"coat (mononuclear inter-phase layer). Mononuclear cells were then resuspended in PBS and washed twice (150g, 10 min at 22^{o}C). Erythrocytes were lysed with BD Pharm Lyse™ (lysing reagent, BD Biosciences Pharmigen, SJ, California, USA).

The mononuclear cells were aliquoted at the desired concentration ($1x10^6$ cells/ml) and incubated for 60min, at 22^{0}C, in a buffer (NaCl 140mM, HEPES 20mM, KCl 5mM, $MgSO_4$ 2.5mM, glucose 5.5mM, pH 7.4), containing different concentrations of insulin (Sigma Diagnostics, St. Louis, MO, USA). Termination of incubation was achieved with the addition of cytochalasin-B (10μM) (Sigma Diagnostics, Missouri, USA).

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, USA) and rabbit anti-GLUT4 (0.1mg/ml, polyclonal antibody, Millipore Corporation, Massachusetts, 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, Carlsbad, California,USA). Cells were incubated for 30min with Alexa Fluor® 488-conjugated immunoglobulin in a ratio

$1x10^6$cells/1μg of immunoglobulin under mild constant shaking. The monocyte fraction was simultaneously stained with anti-CD14-PE monoclonal antibody (BD Biosciences, SJ, California, USA). 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 (results expressed in Arbitrary Units of Mean Fluorescence Intensity, MFI) of each sample. Two-colour flow cytometric analysis was performed on a BD FACSCalibur 4 colour flow cytometer (BDBiosciences, SJ,California USA).

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 6-[N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl)amino]-6-deoxyglucose (NBDG, Invitrogen, Carlsbad, California, USA). 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 30μM) and insulin. The uptake of the fluorescent probe was recorded as MFI during a 500sec interval, when the reaction reached a plateau (12).

Statistical analysis

Grouped data are expressed as mean±SE. 6-NBDG uptake is presented as an increase over baseline (MFI of cells prior to the addition of the fluorescent analogue). Insulin dose-response curves were analyzed with repeated-measures analysis of variance (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 clinical and subclinical hypothyroidism versus euthyroid subjects. Plasma glucose levels in patients with clinical and subclinical hypothyroidism were similar to those in euthyroid subjects (**Figure 1**). Increased prolactin levels have been associated with the manifestation of insulin resistance (13). Fasting prolactin levels in patients with clinical and subclinical hypothyroidism had no statistically significant differences compared to those in euthyroid subjects (7.37 ± 1.09 and 11.5 ± 1.98 versus 9.48 ± 0.98ng/ml, respectively, $p>0.1$).

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

HOMA index was increased in patients with clinical (1.97±0.22) and subclinical hypothyroidism (1.99±0.13) versus euthyroid subjects (1.27±0.16, $p<0.05$).

Matsuda index was decreased in patients with clinical (3.89±0.36) and subclinical hypothyroidism (4.26±0.48) versus euthyroid subjects (7.76±0.87, $p<0.001$).

Matsuda index in all subjects correlated positively with both FT3 and FT4 levels ($r= 0.41$, $p=0.04$).

In monocytes from euthyroid subjects, when insulin was increased from 0 to 100μU/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

clinical hypothyroidism were 19% (p=0.0016) and 15% (p=0.012), whilst for patients with subclinical hypothyroidism were 23% (p=0.005) and 20% (p=0.0037) (**Figure 2**).

At 100μU/ml insulin, GLUT4 levels on the monocyte plasma membrane were decreased in patients with clinical and subclinical hypothyroidism versus euthyroid subjects (p<0.05 for both) (**Figure 2**).

In monocytes from euthyroid subjects, increases in insulin from 0 to 100μU/ml were associated with a 48% increase in 6-NBDG transport (p<0.0001). The respective increases for patients with clinical and subclinical hypothyroidism were 12.57% (p<0.0001) and 12.6% (p<0.0001).

In monocytes from euthyroid subjects the 6-NBDG uptake (increases from baseline) in the presence of 0, 25 and 100 μU/ml of insulin was increased by 19, 43 and 62% respectively (p<0.005) (**Figure 2**). The respective increases for clinical hypothyroidism were 22, 19 and 32% (p<0.2 ns), whilst for subclinical hypothyroidism were 23, 35 and 35% (p<0.036) (**Figure 2**).

At 100μU/ml insulin, 6-NBDG transport rates were decreased in patients with clinical and subclinical hypothyroidism versus euthyroid subjects (p<0.05 for both) (**Figure 2**).

DISCUSSION

Our results demonstrate the presence of insulin resistance not only in clinical but also in subclinical hypothyroidism. Increased HOMA index and decreased Matsuda index in both groups suggest that insulin resistance is present in both fasting and postglucose state. These findings are consistent with 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 response to insulin. This is the first report showing that patients with subclinical hypothyroidism have insulin resistance which is comparable to that of patients with clinical hypothyroidism. 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 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 clinical and subclinical hypothyroidism 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 clinical and subclinical hypothyroidism, 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 subclinical hypothyroidism (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 clinical and subclinical hypothyroidism in our study. It is known that T3 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 level (23). It could therefore be hypothesized that a reduced intracellular content of T3 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 and subclinical hypothyroidism exhibited comparable levels of insulin resistance. This corresponds well with studies showing that patients with mild thyroid failure and even subjects with high normal serum thyroid stimulating hormone (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 and subclinical hypothyroidism 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 clinical and subclinical hypothyroidism showed comparable levels of insulin resistance. Therefore, screening and treatment for subclinical hypothyroidism may be 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 it 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: (1) monocytes are not the main target tissue for insulin as liver, skeletal muscle or adipose tissue; (2) 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; (3) 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 subclinical hypothyroidism have insulin resistance which is comparable to that of patients with clinical hypothyroidism. These findings could justify the increased risk for insulin resistance-associated disorders, such as cardiovascular disease, observed in patients with clinical or subclinical hypothyroidism.

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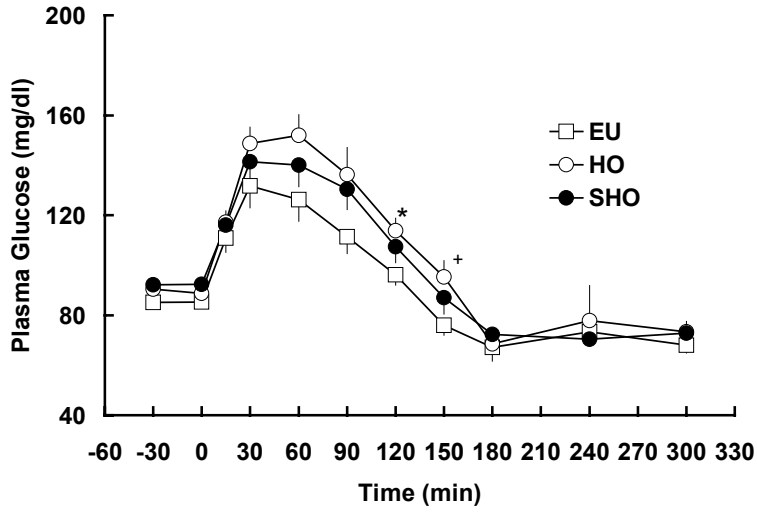
Figure 1: Plasma glucose (A) and insulin (B) levels in euthyroid subjects (EU), and patients with clinical (HO) and subclinical hypothyroidism (SHO) after an OGTT; + $p<0.05$, *$p<0.01$ vs. EU.

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

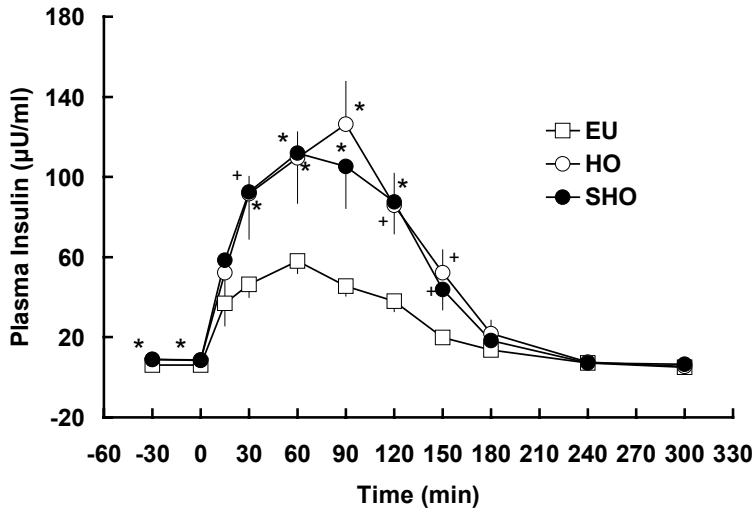
	EU	HO	SHO
Age (years)	40±3	45±2	49±3
Body Mass Index (Kg/m^2)	25±1.2	26±0.7	26±0.9
FT_3 (pg/ml)	2.99±0.12	0.71±0.26	2.67±0.10
FT_4 (ng/dl)	1.23±0.03	0.23±0.09	1.04±0.03
TSH (μU/ml)	1.71±0.38	75.28±10	8.92±1.31
HOMA index	1.27±0.16	1.97±0.22*	1.99±0.13*
Matsuda index	7.76±0.87	3.89±0.36**	4.26±0.48**
%Fat	32±3	34±1.4	33.7±3
Free Fatty Acids (FFA)	460±25	430±30	445±20
n	21	12	13

Table 1: The characteristics, the hormonal data and the indices of insulin resistance of the groups involved in the study. *p<0.05, **p<0.001

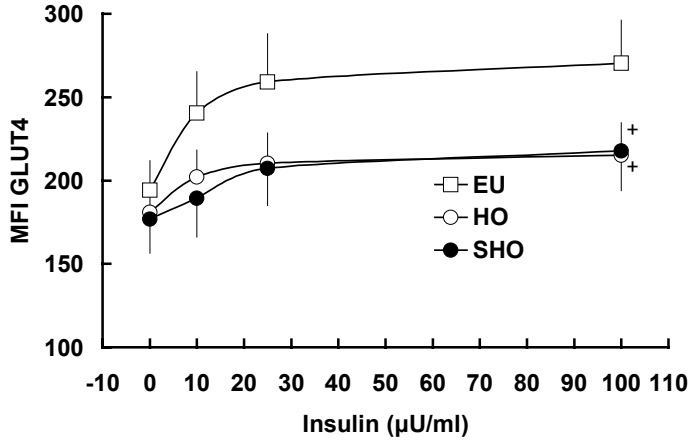
A


Plasma Glucose (mg/dl)
200
160
120
80
40
EU
HO
SHO
-60 -30 0 30 60 90 120 150 180 210 240 270 300 330
Time (min)

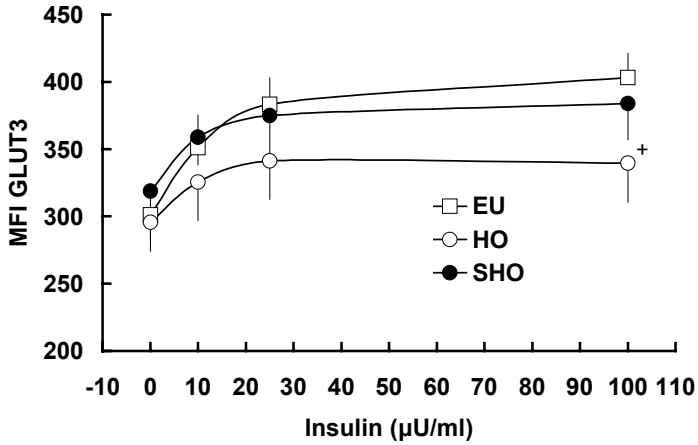
B


Plasma Insulin (μU/ml)
180
140
100
60
20
-20
EU
HO
SHO
-60 -30 0 30 60 90 120 150 180 210 240 270 300 330
Time (min)

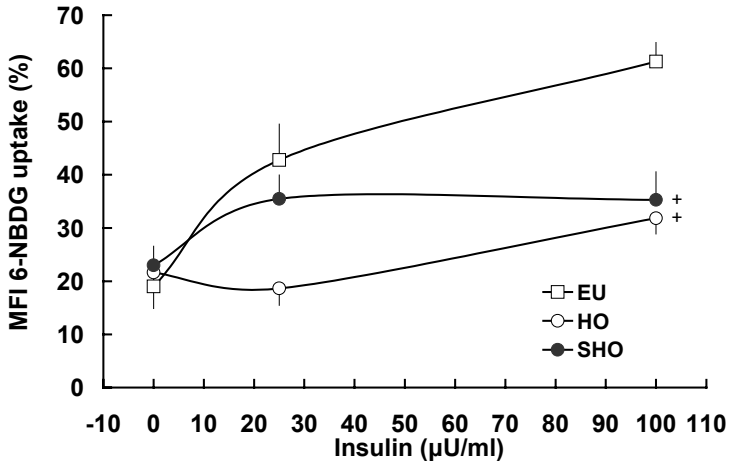
A


MFI GLUT4
300
250
200
150
100
-10 0 10 20 30 40 50 60 70 80 90 100 110
Insulin (µU/ml)
EU
HO
SHO
+
+

B


MFI GLUT3
450
400
350
300
250
200
-10 0 10 20 30 40 50 60 70 80 90 100 110
Insulin (µU/ml)
EU
HO
SHO
+

C


MFI 6-NBDG uptake (%)
70
60
50
40
30
20
10
0
-10 0 10 20 30 40 50 60 70 80 90 100 110
Insulin (µU/ml)
EU
HO
SHO
+
+