Regulation of leptin, adiponectin and acylation-stimulating protein by hyperinsulinaemia and hyperglycaemia in vivo in healthy lean young men

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Abstract

Aim. – Both type 1 and 2 diabetes are associated with differential regulation of leptin, adiponectin and ASP. Our aim was to examine whether or not acute hyperinsulinaemia and/or hyperglycaemia per se have differential regulation of these hormones in healthy subjects.

Methods. – We examined changes in leptin, adiponectin and ASP concentrations and subcutaneous white adipose tissue mRNA expression with 3-hour hyperinsulinaemic (HI, n = 10), hyperglycaemic (HG, n = 7) and hyperinsulinaemic-hyperglycaemic (HGHI, n = 8) clamps in healthy lean young men. As somatostatin was used for the HG and HGHI clamps, a control somatostatin clamp was carried out (n = 4). Changes in the expression of HKII and p85α Pi3K were examined as positive controls for the induction of gene expression by the insulin pathway.

Results. – HI, HG and HGHI clamps increased expression of HKII and p85α Pi3K while somatostatin did not. The HI clamp decreased serum adiponectin (−15%, P < 0.001) and increased serum leptin (+11%, P = 0.031), while the HG clamp reduced serum leptin (−20%, P = 0.003). The HGHI clamp increased serum ASP (+21%, P = 0.047) and expression of C3 (+26%, P = 0.018) and leptin (+50%, P = 0.024). Interestingly, the control somatostatin clamp suppressed both serum leptin (−17%, P = 0.043) and adiponectin (−7%, P = 0.020).

Conclusion. – HG and/or HI per se regulated the concentrations and expression of leptin, adiponectin and ASP in healthy lean young men, suggesting a contribution to dysregulation of these hormones in diabetes.

Résumé


Objectif. – Les concentrations plasmatiques de leptine, d’adiponectine et d’ASP sont fortement modifiées, à la fois dans le diabète de type 1 et le diabète de type 2. L’objectif de cette étude était de vérifier si l’hyperinsulinémie et/ou l’hyperglycémie aiguë pouvaient être des acteurs importants de la régulation de ces hormones chez l’homme.

Méthodologie. – Nous avons étudié les modifications des concentrations plasmatiques de leptine, d’adiponectine et d’ASP, ainsi que l’expression de leur ARNm dans le tissu adipeux sous-cutané abdominal, au cours de clamps hyperinsulinémiques (HI, n = 10), hyperglycémiques (HG, n = 7) et hyperinsulinémiques hyperglycémiques (HGHI, n = 8) d’une durée de trois heures. Afin de tenir compte de l’effet de la somatostatine utilisée pour les clamps HG et HGHI, un clamp témoin à perfusion de somatostatine seule a été réalisé chez quatre sujets. Les clamps ont été réalisés chez des hommes jeunes et en bonne santé. Les variations de l’expression génique de l’hexokinase II (HKII) et de la sous-unité p85α de la Pi3kinase (p85α Pi3K) ont été utilisées comme témoins positifs de l’action de l’insuline dans le tissu adipeux.

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The objectives of this study of healthy young men were to examine the acute in vivo roles of HG, HI and the two in combination in the regulation of:

- plasma concentrations of leptin, adiponectin and ASP;
- WAT mRNA expression of leptin, adiponectin and C3.

The effect of HI in the present study population has been reported earlier [12], so here we report the effects of HG, with or without HI, in the same population. We also examined, under the same experimental conditions, the WAT mRNA expression of two genes involved in insulin signaling and activity that we, as well as others, have previously reported to be under the control of insulin [13–15]. The genes hexokinase II (HKII) and the regulatory subunit p85α of phosphatidylinositol-3 kinase (Pi3K) have been used as positive controls for the induction of WAT gene expression by the insulin pathway in this population.

2. Study design and methods

Subjects were recruited by advertising leaflets distributed around the University of Montreal. Inclusion criteria were:

- men aged 18–30 years;
- those having healthy weight (BMI = 18–25 kg/m²);
- those taking less than 2 hours of structured exercise per week;
- availability to participate in the study.

Upon medical and biochemical examinations, the exclusion criteria were:

- being a smoker;
- an abnormal serum glucose (> 6.1 mM), triglycerides (> 2.3 mM) and total cholesterol (> 5.2 mM);
- familial or personal history of diabetes, obesity, dyslipidaemia or hypertension;
- taking any medication that can affect metabolism.

Twenty lean, healthy, young Caucasian men participated in the study. Ten subjects underwent the HI clamp [12], eight underwent the HG clamp and eight underwent the HGHI clamp. Five subjects repeated the HG and HGHI clamps to examine the additive effect of HI on HG in a crossover design with a washout period of at least two months. One subject in the HG clamp group received only half the required somatostatin infusion dose and was not included in the analysis. The study was conducted in a postabsorptive state after a 12-hour fast, and all subjects were instructed not to engage in any physical activity for 48 hours prior to the study. Subjects gave their written consent after being informed of the nature, purpose and possible risks of the study.
The study was approved by the ethics committee of the university of Montreal.

2.1. WAT biopsies

Fasting preclamp blood samples were collected followed by a subcutaneous abdominal WAT biopsy obtained from one side, at periumbilical level, by needle biopsy under local anaesthesia (Xylocaine® 20 mg/mL, AstraZeneca, Mississauga, Ontario) [12,16]. A postclamp W AT sample was collected at the end of the 3-hour clamp from the other side, at periumbilical level, during which serum glucose, insulin and somatostatin infusion rates were maintained at the target levels for each clamp. Tissue samples were immediately frozen in liquid nitrogen and then stored at −80°C for later extraction of mRNA.

2.2. Clamp techniques

The HI clamp was carried out on 10 subjects as previously reported [12,17]. Briefly, plasma insulin was increased to a plateau concentration using primed constant exogenous insulin infusion at 75 mU/m²/min. Plasma glucose was maintained at plateau concentration using primed constant exogenous insulin infusion at ples were immediately frozen in liquid nitrogen and then stored were maintained at the target levels for each clamp. Tissue sam- ing which serum glucose, insulin and somatostatin infusion rates the 3-hour clamp from the other side, at periumbilical level, dur-

2.3. Serum parameters

Fasting glucose concentrations were measured with a glucose analyzer (Beckman Glucose Analyzer, Mississauga, ON). Serum total and LDL cholesterol were measured by an automated analyzer (Beckman-Coulter, Brea, CA) and serum non esterified fatty acids (NEFA) by a commercial enzymatic colorimetric kit (WAKO Chemicals, Richmond, VA). Serum insulin, C-peptide, adiponectin and leptin levels were measured with a commercial human radioimmunoassay kit (Linco Research, Saint Charles, MO, USA). Serum ASP was assayed by an in-house ELISA described in detail elsewhere [20]. Serum leptin, adiponectin, ASP, NEFA and C-peptide were not measured for three subjects in the HI clamp group because of an inadequate sample.

2.4. White adipose tissue mRNA expression levels

Total RNA was extracted from frozen WAT samples using the RNeasy total RNA minikit (Qiagen, Courtaboeuf, France). First-strand cDNA was synthesized from 250 ng of the total RNA in the presence of 100 U of Superscript II (Invitrogen, Ergan, France) using both random hexamer and oligo (dT) primers (Promega, Charbonnières, France). Real-time PCR was performed using a LightCycler (Roche Diagnostics, Meylan, France) in a final volume of 20 μL, containing 5 μL of a 100-fold dilution of the RT reaction and 15 μL of reaction buffer from the FastStart DNA Master SYBR Green kit (Roche Diagnostics), with 3 mM of MgCl₂ and the specific primers. After amplification, melting curve analysis was performed to verify the specificity of the reactions. For quantification, a standard curve was systematically generated with six different amounts (range: 150–30,000 molecules per tube) of cDNA of the human target mRNA cloned in the pGEM plasmid (Promega). Analysis was performed using the LightCycler software (Roche Diagnostics). The sequences for leptin, adiponectin, C3, HKII and p85α PI3K have already been published [12,16,17]. The expression of target genes was normalized according to the reference gene hypoxan-
thine phosphoribosyl-transferase (HPRT). The HPRT primer sequences were 5’-TTG-CTG-ACC-TGC-TGG-ATT-AC-3’ (forward) and 5’-AGT-GAG-ATC-ATC-TCC-AC-3’ (reverse).

2.5. Statistical analyses

Data are expressed as means ± SEM. The effect of each clamp was examined by two-tailed paired t-test. Differences between the clamps in different subjects were analyzed by repeated-measures two-way ANOVA (HI versus HG and HGHI, somatostatin versus HG and HGHI). Differences between the HG and HGHI clamps in the same five subjects were analyzed by two-way repeated-measures ANOVA. Intraindividual coefficients of variation (CV) in the five repeated subjects in the HG and HGHI clamp groups were calculated as the standard deviation (S.D.) of two measurements divided by the average of the two measurements and multiplied by 100 (the two measurements were at least two months apart). Similarly, interindividual CV among the 20 subjects of the study were calculated as the CV of five repeated subjects in the HG and HGHI clamps in the same five subjects were analyzed by two-way repeated-measures ANOVA. Intraindividual coefficients of variation (CV) in the five repeated subjects in the HG and HGHI clamp groups were calculated as the standard deviation (S.D.) of two measurements divided by the average of the two measurements and multiplied by 100 (the two measurements were at least two months apart). Similarly, interindividual CV among the 20 subjects of the study were calculated as the S.D. of 20 measurements divided by the average of the 20 measurements and multiplied by 100. Statistical significance was set at $P \leq 0.05$.

3. Results

Twenty young men, aged 23.1 ± 2.4 years, were recruited into the study, and their baseline characteristics are presented in Table 1. All subjects were within the normal healthy range for weight and serum metabolic parameters, and there were no significant differences between the subjects in the four clamp groups in baseline anthropometric measures. There was a more than 20-fold increase in serum insulin with no change in serum glucose or C-peptide, while serum NEFA were increased (Table 2). During the HI clamp, there was a more than 20-fold increase in serum insulin with no change in serum glucose or C-peptide, while serum NEFA was reduced by $-83.6 ± 8.6\% \ (P=0.041)$ (Figs. 1 and 2). The HI clamp induced about a 1.5-fold increase in the expression of WAT-derived proteins have already been described[12], and are mentioned briefly here for comparison with the HG, HGHI and somatostatin clamps (Table 2). During the HI clamp, there was a more than 20-fold increase in serum insulin with no change in serum glucose or C-peptide, while serum NEFA was reduced by $-83.6 ± 8.6\% \ (P=0.041)$ (Figs. 1 and 2). The HI clamp induced about a 1.5-fold increase in the expression of both $P_{3}K$ and $HKII$ (Fig. 2) while expression of the reference gene HPRT was not affected. Hyperinsulinaemia increased leptin and decreased adiponectin concentrations without affecting their expression (Figs. 3 and 4). Neither serum ASP nor the

### Table 1

<table>
<thead>
<tr>
<th>Baseline characteristics</th>
<th>Average ± SEM</th>
</tr>
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<tbody>
<tr>
<td>Age (years)</td>
<td>23.1 ± 0.5</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>69.0 ± 1.5</td>
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<td>BMI (kg/m²)</td>
<td>22.3 ± 0.3</td>
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<tr>
<td>Serum concentrations</td>
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<tr>
<td>Glucose (mM)</td>
<td>5.0 ± 0.1</td>
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<tr>
<td>Insulin (pM)</td>
<td>66.9 ± 6.8</td>
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<tr>
<td>NEFA (mM)</td>
<td>0.78 ± 0.09</td>
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<tr>
<td>Total cholesterol (mM)</td>
<td>4.01 ± 0.17</td>
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<tr>
<td>LDL cholesterol (mM)</td>
<td>2.12 ± 0.13</td>
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<tr>
<td>Leptin (ng/mL)</td>
<td>2.81 ± 0.53</td>
</tr>
<tr>
<td>Adiponectin (µg/mL)</td>
<td>9.64 ± 0.90</td>
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<td>ASP (nM)</td>
<td>25.1 ± 2.4</td>
</tr>
</tbody>
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### Table 2

<table>
<thead>
<tr>
<th>Percentage changes</th>
<th>HI (n=10)</th>
<th>HG (n=7)</th>
<th>HGHI (n=8)</th>
<th>Somatostatin (n=4)</th>
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</thead>
<tbody>
<tr>
<td>Serum concentrations</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>6.6 ± 4.2</td>
<td>92.4 ± 9.3***</td>
<td>90.3 ± 6.7***</td>
<td>41.8 ± 10.5*</td>
</tr>
<tr>
<td>Insulin</td>
<td>2128 ± 579***</td>
<td>67.1 ± 17.6**</td>
<td>1847 ± 330***</td>
<td>-46.1 ± 4.0**</td>
</tr>
<tr>
<td>C-peptide</td>
<td>-26.4 ± 24.8</td>
<td>29.1 ± 27.4</td>
<td>-17.7 ± 17.4</td>
<td>-73.2 ± 6.3**</td>
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<tr>
<td>NEFA</td>
<td>-83.6 ± 8.6*</td>
<td>-60.5 ± 7.9**</td>
<td>-38.3 ± 5.0**</td>
<td>56.2 ± 11.8*</td>
</tr>
<tr>
<td>Leptin</td>
<td>10.8 ± 4.0*</td>
<td>-20.2 ± 3.9**</td>
<td>5.9 ± 5.3</td>
<td>-17.1 ± 4.7*</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>-15.0 ± 2.3***</td>
<td>-2.0 ± 4.0</td>
<td>-6.7 ± 3.2</td>
<td>-6.8 ± 1.2*</td>
</tr>
<tr>
<td>ASP</td>
<td>-14.7 ± 12.8</td>
<td>-0.6 ± 4.5</td>
<td>21.1 ± 10.2*</td>
<td>-4.7 ± 15.8</td>
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<tr>
<td>WAT mRNA expression levels</td>
<td></td>
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<tr>
<td>Leptin/HPRT</td>
<td>48.3 ± 20.4</td>
<td>3.5 ± 13.0</td>
<td>56.8 ± 20.5*</td>
<td>39.2 ± 39.6</td>
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<tr>
<td>Adiponectin/HPRT</td>
<td>6.3 ± 12.0</td>
<td>1.5 ± 10.5</td>
<td>4.6 ± 7.1</td>
<td>38.7 ± 42.7</td>
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<tr>
<td>C3/HPRT</td>
<td>6.4 ± 3.5</td>
<td>2.2 ± 8.2</td>
<td>29.4 ± 9.3*</td>
<td>11.7 ± 26.4</td>
</tr>
<tr>
<td>P85a P3K/HPRT</td>
<td>150.2 ± 62.6*</td>
<td>46.1 ± 13.0*</td>
<td>35.8 ± 17.2*</td>
<td>-5.6 ± 20.9</td>
</tr>
<tr>
<td>HKII/HPRT</td>
<td>146.7 ± 45.5**</td>
<td>48.7 ± 21.8</td>
<td>112.1 ± 12.2**</td>
<td>11.7 ± 32.6</td>
</tr>
</tbody>
</table>

$* P \leq 0.05; ** P \leq 0.01; *** P \leq 0.001$ (between pre- and postclamp levels measured by paired t-test).
expression of its precursor protein C3 was affected. Also, there was no correlation between postclamp leptin, adiponectin and ASP concentrations, and their corresponding mRNA levels.

3.2. Hyperglycaemic euinsulinaemic (HG, n = 7) clamp

The HG clamp induced a doubling of serum glucose (baseline: 5.1 ± 0.1 versus postclamp: 9.8 ± 0.4 mM; average increase: 4.7 ± 0.5 mM, 92.3 ± 9.3%, P < 0.0001; [Fig. 1]). Serum insulin was, however, slightly increased during this clamp (baseline: 67.1 ± 2.4 versus postclamp: 111.3 ± 10.9 pM, 67 ± 17.6%, P = 0.008), but only to levels that were still within the normal fasting range (36–179 pM). Moreover, the increase in serum insulin was significantly less than its increase during the HI and HGHI clamps (P < 0.001 for both; [Fig. 1]). Serum C-peptide levels did not differ from baseline (Fig. 2), indicating inhibition of a glucose-induced rise in endogenous insulin.

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*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

secretion by somatostatin. Therefore, increased insulinaemia suggests that the exogenous insulin dose used to maintain euinsulinaemia may have been overestimated. Serum NEFA levels were suppressed (baseline: 0.71 ± 0.06 versus postclamp: 0.27 ± 0.05 mM, −60.5 ± 7.8%, \( P = 0.001 \); [Fig. 2]). Expression of \( \text{Pi3K} \) was increased (baseline: 10.2 ± 1.3 versus postclamp: 14.4 ± 1.8, +46.1 ± 13.0%, \( P = 0.015 \)) while that of \( \text{HKII} \) was unaffected (Fig. 2). Expression of the reference gene HPRT also did not change (baseline: 1.09 ± 0.42 versus postclamp: 1.06 ± 0.45).

Serum leptin was suppressed by the HG clamp (baseline: 1.81 ± 0.45 versus postclamp: 1.52 ± 0.48 ng/mL, −20.2 ± 3.9%, \( P = 0.003 \); [Fig. 3]) with no change in its expression (Fig. 4). The concentrations and expression of ASP and adiponectin also remained unaltered. There was no correlation between hormone concentration and its corresponding mRNA expression at the end of the clamp. The average percent changes induced by the HG clamp in comparison to the other clamps are summarized in Table 2.

### 3.3. Hyperglycaemic-hyperinsulinaemic (HGHI) clamp

Serum insulin increased 18-fold (baseline: 64.9 ± 7.4 versus postclamp: 1107.5 ± 64.8 pM, +1847 ± 330%, \( P < 0.001 \)) while serum glucose increased around twofold (baseline: 5.3 ± 0.1 versus postclamp: 10.0 ± 0.4 mM; average increase: 4.8 ± 0.4 mM, +90.3 ± 6.7%, \( P < 0.001 \); [Fig. 1]), results that were similar to those obtained during the HI and HG clamps, respectively. As in the HG clamp, serum C-peptide did not differ from baseline, indicating inhibition of a glucose-induced rise in endogenous insulin secretion, while serum NEFA was suppressed by −38.3 ± 5.0% (baseline: 0.71 ± 0.07 versus postclamp: 0.42 ± 0.03, \( P = 0.002 \); [Fig. 3]). There was increased expression of both \( \text{Pi3K} \) (baseline: 9.43 ± 0.80 versus postclamp...
12.55 ± 1.67, +35.8 ± 17.2%, P = 0.042) and HKII (baseline: 14.73 ± 1.96 versus postclamp: 30.69 ± 3.46, +112.1 ± 12.2%, P = 0.001; [Fig. 3]), although expression of the reference gene HPRT was unaffected by the HGH clamp (baseline: 1.41 ± 0.40 versus postclamp: 1.30 ± 0.38).

Serum leptin was not altered by the HGH clamp, which was significantly different from its reduction during the HG clamp (P = 0.006, [Fig. 3]), confirming the counter-balancing stimulatory effect of HI, while leptin expression was increased (baseline: 10.02 ± 1.84 versus postclamp: 14.73 ± 2.51, +56.8 ± 20.5%, P = 0.024; [Fig. 4]). In addition, both ASP concentration (baseline: 29.9 ± 2.0 versus postclamp: 35.6 ± 2.5 mM, +21.1 ± 10.2%, P = 0.047; [Fig. 3]) and C3 expression (baseline: 10.47 ± 0.72 versus postclamp: 13.31 ± 0.93, +29.4 ± 9.3%, P = 0.018; [Fig. 4]) significantly increased whereas those of adiponectin did not (although the change in serum adiponectin in one subject appeared to be highly influential). There was no correlation between postclamp leptin, adiponectin and ASP concentrations and their corresponding mRNA expression. The average percent changes induced by the HGH clamp in comparison to the other clamps are summarized in Table 2.

3.4. Somatostatin clamp

To verify the possible confounding effects of somatostatin per se and correct for them in the analysis of the HG and HGH clamps, we carried out a control somatostatin clamp in four subjects. Somatostatin significantly suppressed both serum insulin (baseline: 82.9 ± 9.0 versus 44.6 ± 5.7 pM, −46.1 ± 4.0%, P = 0.005; [Fig. 1]) and C-peptide (baseline: 1.68 ± 0.11 versus postclamp: 0.43 ± 0.08 ng/mL, −73.2 ± 6.3%, P = 0.006; [Fig. 2]). As exogenous insulin was not infused in this clamp, a concomitant increase in both serum glucose (baseline: 5.0 ± 0.1 versus postclamp: 7.1 ± 0.6 mM; average increase: 2.1 ± 0.5 mM, +41.8 ± 10.5%, P = 0.030) and NEFA (baseline: 0.82 ± 0.02 versus postclamp: 1.28 ± 0.10 mM, +56.2 ± 11.9%, P = 0.018) was observed at the end of the procedure (Figs. 1 and 2). Of note, the increase in NEFA may be a direct effect of somatostatin, which inhibits lipogenesis in adipose tissue [21]. There was no change in either Pi3K or HKII expression (Fig. 2), and the expression of the reference gene HPRT was also unaffected (baseline: 1.90 ± 0.42 versus postclamp: 1.52 ± 0.66).

Somatostatin infusion per se suppressed serum leptin by −17.1 ± 4.7% (baseline: 3.49 ± 0.64 versus postclamp: 2.89 ± 0.59 ng/mL, P = 0.043) and serum adiponectin by −6.8 ± 1.2% (baseline: 9.30 ± 0.82 versus postclamp: 8.66 ± 0.75 µg/mL, P = 0.020; [Fig. 3]). The somatostatin clamp had no effect on serum ASP or expression of C3, leptin or adiponectin. The average percent changes induced by the somatostatin clamp in comparison to the other clamps are summarized in Table 2.

Intra- versus interindividual variability in measured parameters: we also determined the intraindividual (n = 5) and interindividual (n = 20) variability of the measured fasting parameters to examine the contribution of these two variation sources to the overall variability of the results. The average intraindividual CV of fasting serum levels of glucose was 5%, and 14% for insulin, 11% for C-peptide and 20% for NEFA, whereas those for gene expression of Pi3K and HKII were 13% and 39%, respectively. Moreover, the intraindividual CV for WAT hormones was: leptin concentrations = 30%; leptin gene = 48%; ASP concentration = 20%; C3 gene = 31%; adiponectin concentration = 12%; and adiponectin gene = 25%. However, there was no significant difference between the HG and HGH clamps in any of the repeated fasting measured parameters (by paired t-test).

There was a larger inter- than intra-individual biovariability in all the fasting measured parameters. The interindividual CV of serum levels of glucose was 8%, and 46% for insulin, 26% for C-peptide and 45% for NEFA, while gene expression of Pi3K and HKII was 88% and 86%, respectively. Finally, the interindividual CV for leptin was 77 and 84% for leptin gene, 38% for ASP, 34% for C3 gene, 38% for adiponectin and 52% for adiponectin gene.

4. Discussion

To date, the concomitant contribution of HG and/or HI to the in vivo regulation of leptin, adiponectin and ASP has never been investigated in humans. For this reason, in this study of healthy young men, we examined whether or not HG and/or HI per se can regulate these hormones as this may help to explain their alteration in diabetes.

Certain limitations of our study should, however, be borne in mind when interpreting our results. The unexpected rise in insulin concentrations during the HG clamp, albeit within normal fasting values, should be considered in the interpretation of this clamp. More important, the study was carried out in healthy subjects and simulated acute, not chronic, conditions of HG and HI. Therefore, it should be recognized that the simple design of our study does not reflect the metabolic complexity of diabetes. Finally, there was a large interindividual biovariability in fasting serum concentrations of the WAT hormones, and more so in WAT gene expression. Therefore, the variability of the response to the four types of clamps is expected to mirror that of baseline and is likely to have affected the significance of the differences. Future in vivo studies examining the response of leptin, adiponectin and ASP in healthy subjects to various treatments needs to enroll a larger sample size to overcome this source of variability. On the other hand, WAT hormone gene expression and, even more so, its concentration were more reproducible among the same individuals, albeit using only two measurements and five subjects. Serum leptin was also previously reported to be reproducible among 20 lean and obese subjects with a CV of 20% (eight measurements per subject) [22]. A similar low biovariability in adiponectin concentrations was also reported in 10 healthy subjects (CV = 18.8% over a 30-day period) [23]. Thus, differences in responses to the HG and HGH clamps among the same subjects are less likely to have been affected by this source of variability.

We previously reported that HI clamps increased serum leptin in healthy lean young men [12]. In contrast, we report here that HG clamps decreased serum leptin, whereas HGH
clamps increased leptin expression without increasing its concentration. A couple of points appears to be conflicting and requires clarification. First, insulin may increase leptin concentration independent of any changes in its expression by acting at a post-translational level only, as previously suggested in studies in vitro of human adipose tissue [24]. In fact, increased serum leptin by HI clamp was, previously, reported using variable infusion rates and duration (37% increase over 6 hours in eight lean subjects [25] and 26 to 62% increase with 40 to 400 mU/m2/min insulin over 9 hours in 10 obese subjects [25,26]). However, levels of expression were not concomitantly examined in those studies. Furthermore, the decreased serum NEFA during the HI clamp is expected to stimulate leptin, as NEFA concentration during intralipid infusions in humans is negatively correlated with those of leptin [27]. Second, as somatostatin clamps reduced serum leptin in our study as in others [28], somatostatin per se may explain reduced leptin during the HG and HGHI clamps. In fact, somatostatin appears to have overwritten both stimulatory effects of hyperinsulinaemia and reduced NEFA, particularly in the HGHI clamp where leptin expression was increased. It is worth noting that, unlike the HGHI clamp, the HG clamp had no effect on leptin expression, indicating that the slightly increased insulin during this clamp had negligible effects. Taking all the clamps together, however, our data suggest that the frequently observed hyperleptinaemia in obesity [29], type 1 [11] and type 2 DM [3] may be, at least in part, an outcome of HI and HG per se, and independent of other complications of obesity and diabetes.

Hyperinsulinaemia per se reduced serum adiponectin, results that were observed by others using similar (80 mU/m2/min, 16% increase [30]), but not lower (40 mU/m2/min [2]), insulin infusion rates. As with leptin, insulin-induced changes in adiponectin concentrations were independent of its expression. The lack of insulin effect on adiponectin expression in subcutaneous WAT was also recently reported in vivo in healthy women with 3-hour (but not 6-hour) clamps [31], although changes in adiponectin were not reported in this study. NEFA incubations were found to have an inhibitory effect on adiponectin secretion in murine adipocytes [32–34]. Nevertheless, adiponectin levels were reduced during the HI clamp, despite reduced NEFA concentrations. However, the stimulatory effect of reduced NEFA concentrations on adiponectin may have been cancelled out by the inhibitory effect of elevated insulin (2128%) on adiponectin. On the other hand, somatostatin decreased serum adiponectin, while both HG and HGHI clamps had no effect on serum adiponectin. Taking together the inhibitory effect of hyperinsulinaemia in the HGHI clamp and, to a lesser extent, in the HG clamp, and the inhibitory effect of somatostatin equally infused in both HGHI and HG clamps, we believe that HG may have had a counterbalancing stimulatory effect on serum adiponectin that led to unchanged adiponectin levels during the HGHI and HG clamps.

The novel finding that somatostatin infusion reduces serum leptin and adiponectin without affecting their expression may suggest a novel mechanism for the regulation of these hormones in humans. Despite reduced insulin concentrations with the somatostatin clamp, there was no effect on the expression of either Pit3K or HKII, suggesting a lack of insulin effect during this clamp. Therefore, somatostatin may be able to regulate leptin and adiponectin concentrations independently of the insulin-signaling pathway through perhaps a post-transcriptional mechanism. In fact, one of the most prominent effects of somatostatin is inhibition of the secretion of many other hormones such as insulin, insulin-like growth factor, glucagon, growth hormone, cortisol and GI hormones [35,36], many of which are also possible leptin regulators. Alternatively, somatostatin might be inhibiting leptin and adiponectin secretion indirectly through altering WAT metabolism, particularly given the findings that murine adipocytes have somatostatin receptors [36]. Both somatostatin infusion in humans [28] and somatostatin incubation with adipocytes [21] were found to exhibit anti-lipogenic/lipolytic effects. This is in line with our results of increased NEFA concentrations during the somatostatin clamp. The concentrations of NEFA during intralipid infusion were negatively correlated with those of leptin [27]. Similarly, the incubation of murine adipocytes with fatty acids inhibits the gene expression of adiponectin, and the secretion of leptin and adiponectin [32–34]. Therefore, it is plausible that elevated concentrations of NEFA during the somatostatin clamp may have had an inhibitory effect on leptin and adiponectin concentrations.

Finally, HI and HG in combination, but neither condition alone, increases serum ASP concentration and C3 expression. This is likely to be independent of reduced NEFA, as fatty acids do not affect ASP generation in human adipocytes [37], and is compatible with the previously published finding that an HI clamp does not regulate C3 expression in subcutaneous WAT in lean, obese or diabetic men [4]. Moreover, this is comparable to the elevated ASP concentration and C3 expression seen in non-diabetic obese subjects as well as in lean and obese diabetic patients [4,38]. However, somatostatin infusion did not affect serum ASP as it did serum leptin and adiponectin, despite taking similar origin from WAT. This may be related to the specific regulation of ASP production by WAT. Unlike leptin and adiponectin, ASP is not secreted directly by WAT, but is generated by the interaction of its precursor proteins C3, factors B and D (secreted by WAT) with carboxypeptidase (in human plasma) [39].

In conclusion, our novel data indicate that, while HI increases leptin and decreases adiponectin concentrations, HG may have a counterbalancing stimulatory effect on serum adiponectin. Moreover, a combination of HI and HG, but neither condition alone, increases ASP concentration as well as subcutaneous WAT expression of C3 and leptin. Finally, somatostatin decreases serum leptin and adiponectin without affecting their expression, which suggests a novel mechanism for the regulation of these hormones in vivo. Our findings of the regulation of leptin, adiponectin and ASP by HI and/or HG in healthy young men are compatible with the dysregulation of these hormones in obesity and diabetes.

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