Increased Hepatic Insulin Sensitivity Together with Decreased Hepatic Triglyceride Stores in Hormone-Sensitive Lipase-Deficient Mice

PETER J. VOSHOL, GUENTER HAEMMERLE, D. MARGRIET OUWENS, ROBERT ZIMMERMANN, RUDOLF ZECHNER, BAS TEUSINK, J. ANTONIE MAASSEN, LOUIS M. HAVEKES, AND JOHANNES A. ROMIJN

The Netherlands Organization for Applied Scientific Research–Prevention and Health (P.J.V., B.T., L.M.H.), Division of Vascular Biology, and Leiden University Medical Centre (P.J.V., J.A.R.), Department of Endocrinology and Metabolic Diseases, Leiden, The Netherlands; Institute of Molecular Biology, Biochemistry and Microbiology (G.H., R.Zi., R.Ze.), University of Graz, Graz, Austria; Leiden University Medical Centre (D.M.O., J.A.M.), Department of Molecular Cell Biology, and Departments of Cardiology and General Internal Medicine (L.M.H.), Leiden, The Netherlands

Hormone-sensitive lipase (HSL) is a major enzyme for triglyceride (TG) lipolysis in adipose tissue. In HSL-knockout mice, plasma free fatty acid and TG levels are low, associated with low liver TG content. Because a decreased hepatic insulin sensitivity has been reported to be associated with high liver TG levels, our aim was to determine whether a hepatic TG content lower than normal, as observed in HSL-knockout mice, leads to increased hepatic insulin sensitivity. Therefore, hyperinsulenic clamp experiments in combination with d-3H-glucose were used. Furthermore, hepatic insulin receptor and phosphorylated protein kinase B (PKB-P)/akt were analyzed by Western blotting. No significant differences were observed in insulin-mediated whole-body glucose uptake between HSL-knockout and control mice. Interestingly, hepatic insulin sensitivity of HSL-knockout mice was increased, because insulin caused a greater reduction in endogenous glucose production (71% compared with 91% in control mice; P < 0.05), despite decreased plasma adiponectin levels. PKB/akt phosphorylation and phosphatidylinositol-3-kinase activity was significantly higher in livers of HSL-knockout mice after insulin stimulation. In HSL-knockout mice, reduced hepatic TG stores result in an increased suppressive effect of insulin on hepatic glucose production, in line with an increased hepatic PKB-P/akt and phosphatidylinositol-3 kinase activity. Thus, hepatic insulin sensitivity is indeed increased after reducing hepatic TG stores below normal. (Endocrinology 144: 3456–3462, 2003)

**Materials and Methods**

**Animals**

Male, 3- to 4-month-old HSL knockout [HSL(−/−)] and wild-type mice littermates, on a mixed background, were taken from the breeding colony at the University of Graz (Graz, Austria) (3, 4). Mice were kept in a temperature- and humidity-controlled environment and had free access to standard lab chow and water. Daily food intake was monitored during a 3-d period, and no difference was observed between both genotypes. All animals used during the experiments received humane care, and all experiments were approved by the animal ethics committee from The Netherlands Organization for Applied Scientific Research–Prevention and Health (Leiden, The Netherlands).

**Fasted plasma parameters**

After an overnight fast, blood samples of 150 μl were taken in paraoxon-coated capillaries, to prevent lipolysis (7), via tail bleeding. Plasma glyceride(s).
was collected by centrifugation, and total plasma glucose, FFA, TG, and total cholesterol were determined via commercially available kits (Sigma Diagnostics, St. Louis, MO; Roche Molecular Biochemicals GmbH, Mannheim, Germany; and Wako Chemicals GmbH, Neuss, Germany) according to the manufacturers’ instructions. Plasma insulin was measured byRIA, using rat insulin standards (Sensitive Rat Insulin Assay, Linco Research Inc., St. Charles, MO; 100% cross-reaction with mouse and human insulin). Plasma adiponectin was measured using a mouse-specific RIA (Mouse Adiponectin RIA Kit, Linco Research Inc.).

Glucose turnover studies

After an overnight fast, glucose turnover studies were performed as described earlier (8). In short, animals were anesthetized (0.5 ml/kg Hypnorm, Janssen Pharmaceutica, Beerse, Belgium; and 12.5 mg/g midazolam, Genthon BV, Nijmegen, The Netherlands), and an infusion needle was placed in one of the tail veins, after which basal glucose parameters were determined by infusion of [3-3H]-glucose (0.6 μCi/kg/min, Amersham Biosciences, Little Chalfont, UK) alone during a 2-h continuous infusion to achieve steady state levels. After basal glucose turnover rate was determined, a bolus of insulin (100 μU/kg; Actrapid, Novo Nordisk, Charlots, France) was given, and a hyperinsulinemic clamp was started with the following continuous infusion of insulin (3.5 μU/kg/min) and [3-3H]-glucose (0.6 μCi/kg/min). Blood samples (<1 μl) were taken every 10 min (tail bleeding) to monitor plasma glucose levels (Freestyle, TheraSense, Disetronic Medical Systems BV, Vianen, The Netherlands). A variable infusion of 12.5% 2-deoxy-glucose (in PBS) solution was started at time 0 and adjusted to maintain blood glucose at approximately 7.0 mM. When steady state glucose levels were reached (1 h after start of the insulin infusion), blood samples were taken every 20 min for 1 h to determine insulin-stimulated glucose turnover. The total amount of blood withdrawn during the experiment was approximately 200 μl. To estimate the insulin-stimulated glucose transport activity in individual tissues, 2-deoxy-d-[6-3H]-glucose (2-[3H]DCG; Amersham Biosciences) was administered as a bolus (2 μCi) 45 min before the end of the clamps. Blood samples were taken 5, 25, and 45 min after bolus injection to determine plasma 3H-glucose, 3H2O, and 2-[3H]DG specific activities. After the last blood sample, mice were killed and liver, skeletal muscle (hind limb), and adipose tissue (reproductive fatpad) were taken for analysis.

Tissue homogenates

Tissue samples were homogenized (~10% wet weight/vol) in PBS. Total TG, diacylglycerol, and cholesterol content in these homogenates were determined after lipid extraction as described previously (9, 10). For determination of tissue 2-[3H]-DG uptake, tissues were homogenized (~10%) in water, boiled, and subjected to ion-exchange column to separate 2-DG-6-P from 2-DG, as previously described (11, 12).

Calculations

Total plasma 3H-glucose radioactivity was determined in 10-μl plasma and in supernatants after trichloric acid (20%) precipitation and water evaporation to eliminate tritiated water. Under steady state conditions for plasma glucose concentrations, the rate of glucose disappearance equals the rate of glucose appearance (Ra; i.e. endogenous glucose production plus exogenous d-glucose infusion). Ra glucose was calculated as the ratio of the rate of infusion of [3-3H]-glucose (disintegrations per minute/minute) and the steady state specific activity of plasma 3H-glucose (disintegrations per minute per micromole of glucose). Endogenous glucose production was calculated as the difference between Ra and the infusion rate of exogenous 3H-glucose. 

Northern blot analysis

Total RNA was isolated from white adipose tissue using the TRI Reagent procedure according to manufacturer’s protocol (Molecular Research Center, Karlsruhe, Germany). Specific mRNAs were detected using standard Northern blotting techniques with 10 μg total RNA. Probes for specific hybridization were generated using random priming. Northern blots were visualized by exposure to a phosphor imager screen (Aptioech, Freiburg, Germany) and analyzed using ImageQuant software (Amersham Biosciences).

Primer and probes

cDNA probes for Northern blot analysis of adiponectin were prepared by RT-PCR by use of primer to cDNA mRNA. The PCR primers used to generate an adiponectin (ACRP30) specific probe were as follows: forward, 5′-GTGACACAGGAGATGTGGGA-3′; reverse, 5′-GAGTCGTGACGCTTACCTGC-3′.

Hepatic insulin-signaling protein levels

For analysis of proteins involved in the insulin-signaling pathway, Western blotting was performed for insulin receptor and PKB-B. For this purpose, six HSL-knockout and wild-type mice were killed 10 min after an ip injection of insulin (50 U/kg body weight) or PBS as a control (15). Liver and muscle tissues were snap-frozen in liquid nitrogen, and parts of these tissues were homogenized in RIPA buffer (30 mM Tris (pH 7.5), 1 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, 0.5% deoxycholate, 1 mM/liter sodium orthovanadate, 10 mM/liter sodium fluoride) containing protease inhibitors (Complete, Roche Molecular Biochemicals). Extracts were cleared by centrifugation (4°C), and protein content in the supernatant was measured using a BCA kit (Pierce, Rockford, IL). Proteins (25 μg/lane) were separated by SDS-PAGE on an 8% gel and blotted on polyvinylidene difluoride-membrane (Millipore, Bedford, MA). Filters were blocked in Tris-buffered saline containing 0.25% Tween 20 and 5% nonfat dried milk (Protiﬁlar, Nutricia, Cuijk, The Netherlands) (16) and incubated overnight with a phospho-Akt (Ser473) antibody (Cell Signaling Technology, Westburg BV, Leusden, The Netherlands) or antiinsulin receptor β-subunit (Transduction Laboratories, Devon, UK) (16). Antitotal PKB (a kind gift of Dr. B. Burgering, University of Utrecht, Utrecht, The Netherlands) was used as a control protein. After extensive washing in Tris-buffered saline containing 0.25% Tween-20, bound antibodies were detected using horseradish peroxidase-conjugated goat-antirabbit IgG (Promega, Madison, WI) in a 1:5000 dilution, followed by visualization by enhanced chemiluminescence. Blots were quantitated on a Lumimager (Roche Molecular Biochemicals), using LumiAnalyzer software, and protein intensities were normalized against total PKB intensities.

Insulin receptor substrate 1 (IRS1)-dependent PI3-kinase activity was analyzed as described previously (16, 17). In short, liver homogenates were immunoprecipitated with anti-IRS1 K6 (16). PI and 32P-yATP were added in the reaction, and radioactive PI3-phosphate was detected with thin layer chromatography (15).

Statistical analysis

Results are presented as mean ± sn values for the number of animals indicated. Differences between the experimental groups were determined by Mann-Whitney U test (18). The level of statistical significance of the differences was set at P < 0.05. Analyses were performed using SPSS 11.0 for Windows software (SPSS, Inc., Chicago, IL).

Results

Basal plasma parameters

Table 1 shows plasma levels of TG, FFA, cholesterol, glucose, and insulin in HSL-knockout mice and controls after an overnight fast. Plasma TG levels were decreased by approximately 70%, whereas FFA levels showed a decrease of approximately 40%. Plasma cholesterol levels did not differ between the two genotypes. Plasma glucose concentrations were increased by 55% in HSL-knockout mice compared with controls. Interestingly, fasted insulin levels were significantly lower (~63%) in HSL-knockout mice when compared with their control littermates.
Glucose production and glucose uptake

Basal and insulin-stimulated whole-body glucose uptake and hepatic glucose production were measured in HSL-knockout and control animals. Figure 1 shows that we achieved steady state specific activities of $^3$H-glucose during basal and hyperinsulinemic clamp conditions. Under basal, overnight-fasted conditions, there were no differences between HSL-knockout and control mice in whole-body glucose uptake (Fig. 2A). Table 2 shows that there were no differences between HSL-knockout and wild-type animals in plasma glucose, insulin, and FFA concentrations during the hyperinsulinemic clamp study. Moreover, during hyperinsulinemic conditions whole-body glucose uptake did not differ between the two genotypes (Fig. 2B). For both wild-type and HSL-knockout mice, hepatic glucose production was suppressed during hyperinsulinemic conditions (Fig. 3A). Interestingly, hepatic glucose production was suppressed significantly greater in HSL-knockout mice during insulin-mediated conditions than in control mice ($-71\%$ vs. $31\%$; $P = 0.001$; Fig. 3B). We observed no differences in insulin-mediated glucose uptake in both muscle and adipose tissue between the two genotypes (Table 3).

Hepatic and muscle TG content

Lipid analysis showed a significant decrease in TG content in livers of HSL-knockout mice compared with control mice, whereas no significant difference in TG content could be observed in muscle tissue (Fig. 4). No differences were observed in liver diacylglycerol, with a small increase in cholesterol levels between HSL-knockout and control mice (data not shown).

---

**TABLE 1.** Plasma total cholesterol (TC), TG, FFA, glucose, and insulin concentrations in overnight fasted HSL-knockout and control (wild-type) mice

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Body weight (g)</th>
<th>TC (mM)</th>
<th>TG (mM)</th>
<th>FFA (mM)</th>
<th>Glucose (mM)</th>
<th>Insulin (pmol/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (n = 8)</td>
<td>24.3 ± 0.9</td>
<td>2.4 ± 0.5</td>
<td>1.4 ± 0.5</td>
<td>1.2 ± 0.2</td>
<td>2.9 ± 0.6</td>
<td>103 ± 64</td>
</tr>
<tr>
<td>HSL−/− (n = 7)</td>
<td>24.7 ± 1.3</td>
<td>2.9 ± 0.7</td>
<td>0.4 ± 0.1*</td>
<td>0.7 ± 0.1*</td>
<td>4.5 ± 0.6*</td>
<td>38 ± 16*</td>
</tr>
</tbody>
</table>

Values are represented as mean ± sd; the number of animals per group is indicated. * Statistical significant difference from wild-type mice; $P < 0.05$, as assessed by Mann-Whitney $U$ test.

**TABLE 2.** Concentrations of plasma glucose, insulin, and FFA during the hyperinsulinemic euglycemic clamp in control (wild-type) and HSL-knockout mice

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Glucose (mM)</th>
<th>Insulin (pmol/liter)</th>
<th>FFA (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (n = 8)</td>
<td>6.6 ± 0.4</td>
<td>800 ± 190</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>HSL−/− (n = 7)</td>
<td>7.2 ± 0.9</td>
<td>767 ± 276</td>
<td>0.6 ± 0.1</td>
</tr>
</tbody>
</table>

Values are represented as mean ± sd; the number of animals per group is indicated. No differences were found between both groups.

**Hepatic insulin receptor protein levels**

To obtain a molecular basis for the increase in hepatic insulin sensitivity, we analyzed insulin receptor protein levels and the induction of PKB S473 phosphorylation in insulin-mediated conditions in HSL-knockout and wild-type mice. Insulin receptor protein levels were increased in HSL−/− mice compared with wild-type mice (Fig. 5A). As shown in Fig. 5A, insulin-induced phosphorylation of PKB was also significantly increased in HSL-knockout mice. However, total PKB protein expression was unaltered between the two genotypes. In contrast, no differences were observed in muscle total PKB or PKB-P protein expression between the two genotypes (Fig. 5A). Insulin-induced IRS1-dependent PI3-kinase activity, a downstream event of PKB-phosphorylation, was significantly higher in livers of HSL-knockout mice compared with wild-type mice (Fig. 5B).
Values represent mean ± SD as nanomoles per milligram of protein for n = 6 (wild-type) and n = 6 (HSL−/-) animals per group, respectively. *, Statistical significance different from wild-type mice; P < 0.05, as assessed by Mann-Whitney U test.

### Table 3. Insulin-mediated glucose uptake (using [14C]2-deoxy-D-glucose) in muscle and adipose tissue during the hyperinsulinemic euglycemic clamp in control (wild-type) and HSL-knockout mice

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Muscle tissue (dpm/mg tissue/min)</th>
<th>Adipose tissue (dpm/mg tissue/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (n = 8)</td>
<td>284 ± 60</td>
<td>330 ± 55</td>
</tr>
<tr>
<td>HSL−/- (n = 7)</td>
<td>210 ± 50</td>
<td>220 ± 35</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SD; the number of animals per group is indicated. No differences were found between both groups.

**Plasma adiponectin levels**

To exclude whether an increase in plasma adiponectin levels could explain the increased hepatic insulin sensitivity, we measured plasma adiponectin levels and determined adiponectin expression in adipose tissue of HSL-knockout mice and controls. Plasma adiponectin levels were approximately 60% decreased in HSL-knockout mice (Fig. 6A), concomitant with a decrease of approximately 70% of adiponectin expression (Fig. 6B) in white adipose tissue of these mice.

**Discussion**

Hydrolysis of TG in adipose tissue is the main mechanism regulating plasma FFA levels during fasting (19, 20). HSL is one of the most relevant enzymes involved in this process in adipose tissue (21, 22). In HSL-knockout mice after prolonged fasting, plasma FFA and TG levels as well as hepatic TG stores were significantly lower in HSL-knockout mice compared with wild-type mice (this study and Ref. 3). This low hepatic TG content is associated with increased insulin sensitivity with respect to the suppressive effect of insulin on hepatic glucose production. This increase in hepatic insulin sensitivity was associated with increased insulin receptor protein levels and increased activation of components of the insulin-signaling cascade.

The low hepatic content of TG in HSL-knockout mice can be explained by their low plasma FFA levels, because liver-specific FFA uptake is commonly assumed to be a concentration-driven process facilitated by specific membrane transporters (23). In accordance, we recently documented that plasma FFA are the main contributors to hepatic lipid storage in the fasted state (24). Mice with overexpression of muscle lipoprotein lipase are another model of altered distribution of TG in nonadipose tissue. These mice have decreased hepatic TG stores. In accordance with our current observation, these muscle lipoprotein lipase-overexpressing mice also exhibited an increase in hepatic insulin sensitivity in comparison with wild-type mice.

The inverse relationship between insulin sensitivity and hepatic TG content may be explained by alterations in gene expression by activation of nuclear transcription factors like peroxisome proliferator-activated receptors (PPARs) by intracellular TG and/or fatty-acyl intermediates (25). For instance, PPAR α-knockout mice are protected from hepatic insulin resistance induced by high-fat feeding (26). We cannot exclude the possibility that the decreased plasma FFA concentrations per se are, at least partly, responsible for the observed changes in hepatic insulin sensitivity; plasma FFA concentrations are inversely correlated with insulin sensitivity (27). However, during the hyperinsulinemic clamp, similar plasma FFA concentrations were observed in HSL-knockout and wild-type animals. This argues against a direct effect of plasma FFA during the hyperinsulinemic condition on hepatic insulin sensitivity. Plasma adiponectin has been shown to affect hepatic glucose production and hepatic insulin sensitivity (28). We were able to exclude the contribution of increased adiponectin levels to the observed increased hepatic insulin sensitivity in HSL-knockout mice; plasma adiponectin and adipose tissue expression were decreased during the hyperinsulinemic clamp in control (wild-type) and HSL-knockout mice.

**Fig. 3.** Hepatic glucose production under basal, overnight fast, and hyperinsulinemic (insulin) conditions (A), respectively, in wild-type (WT; white bars) and HSL-knockout mice (HSL−/−; black bars). Both the basal and hyperinsulinemic conditions were assessed in the same animal. B, Percentage of inhibition by insulin in both genotypes. Values represent mean ± SD as micromoles per minute per kilogram of body weight for n = 8 (WT) and n = 7 (HSL−/−) animals per group, respectively. *, Statistical significance different from wild-type mice; P < 0.05, as assessed by Mann-Whitney U test.

**Fig. 4.** TG content of liver and skeletal muscle from wild-type (WT; white bars) and HSL-knockout (HSL−/−; black bars) mice analysis after lipid extraction. Values represent mean ± SD as nanomoles per milligram of protein for n = 6 (WT) and n = 6 (HSL−/−) animals per group, respectively. *, Statistical significance different from wild-type mice; P < 0.05, as assessed by Mann-Whitney U test.
Finally, it is unlikely that our data are explained by our study design, i.e., the measurement of hepatic insulin sensitivity in anesthetized mice. We observed a considerable difference in hepatic insulin sensitivity, although both genotypes were subjected to the same protocol.

In HSL−/− mice, no differences were observed in insulin-mediated glucose uptake compared with wild-type mice. In accordance, we observed no differences in muscle TG content between HSL-knockout and wild-type mice, in accordance with the study by Haemmerle et al. (4) in the same mouse strain. The absence of a difference in muscle TG content in combination with the unchanged insulin-induced PKB/akt phosphorylation in HSL-knockout mice might in fact be responsible for the lack of enhanced whole-body glucose uptake despite lower plasma FFA concentrations.
In the current study, the inverse relationship between liver TG content and hepatic insulin sensitivity is supported by the increased phosphorylated PKB and IRS1-dependent PI3-kinase activity in HSL-knockout mice. Both proteins are important downstream targets of the insulin-signaling pathway regulating factors like phosphoenolpyruvate carboxykinase (29). At least two possible mechanisms may help to explain the inverse correlation between hepatic TG content and insulin sensitivity. Alterations in intracellular TG or fatty-acyl intermediates may alter gene expression levels (of components of the insulin-signaling cascade like the insulin receptor) by activation of nuclear transcription factors like PPARs (25). Alternatively, increased insulin receptor protein levels in the liver of HSL-knockout mice could be due to the low plasma insulin levels (Table 1). Lopez et al. (30) showed that chronic hypoinsulinemia leads to increased membrane-associated insulin receptor protein levels.

An interesting feature of the HSL−/− mice is the increase in basal glucose levels together with decreased insulin levels, which is in line with previous observations (3). However, basal hepatic glucose production was not significantly higher in HSL−/− mice. We hypothesize that, under normal conditions insulin regulates the glucose homeostasis, but when insulin can be provided in only low amounts, plasma glucose concentrations will rise to maintain homeostasis. This hypothesis is supported by Roduit et al. (31), who showed impaired insulin secretion in HSL-knockout mice, concomitant with increased pancreatic islet TG content.

In conclusion, impaired TG lipase activity in adipose tissue in HSL-knockout mice causes low plasma FFA and TG levels. The increased hepatic insulin sensitivity with regard to suppression of hepatic glucose production in HSL-knockout mice seems to be correlated to the decrease in hepatic TG content. This is related to increased protein levels of insulin receptor and downstream activated PKB/Akt phosphorylation and IRS1-dependent PI3-kinase activity. This observation extends the relevance of the inverse relationship between hepatic TG content and hepatic insulin sensitivity also to non-hepatic TG levels. The absence of a threshold in the inverse relationship between hepatic insulin sensitivity and hepatic TG stores may be of clinical relevance for the dose-response relationships of drugs, like thiazolidinediones, which decrease hepatic TG content and improve insulin sensitivity.

Acknowledgments

We thank Trea Streefland, Martin Muurling, and Andrea Kales for their excellent technical assistance.

Received November 13, 2002. Accepted April 22, 2003.

Address all correspondence and requests for reprints to: Peter J. Voshol, Ph.D., The Netherlands Organization for Applied Scientific Research-Prevention and Health, Division of Vascular Biology, Zernike-dreef 9, NL-2333 CK Leiden, The Netherlands. E-mail: pj.voshol@pg.tno.nl.

This work was supported by The Netherlands Organization for Applied Scientific Research (NOW Grants 903-39-194 and 903-39-291), the Netherlands Heart Foundation (NHS Grant 97,067), and the Dutch Diabetes Foundation (DFN 96,604). This study was conducted in the framework of the Leiden Center for Cardiovascular Research Leiden University Medical Centre-The Netherlands Organization for Applied Scientific Research.
27. Shulman GI 1999 Cellular mechanisms of insulin resistance in humans. Am J Cardiol 84: 3J–10J