Hepatic steatosis : metabolic consequences

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Chapter 3

Hepatic Glucose Production is More Sensitive to Insulin-mediated Inhibition than Hepatic VLDL-triglyceride Production

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Abstract

Insulin is an important inhibitor of both hepatic glucose output and hepatic VLDL-triglyceride (VLDL-TG) production. We investigated whether both processes are equally sensitive to insulin-mediated inhibition. To test this, we used euglycemic clamp studies with four increasing plasma concentrations of insulin in wild type C57Bl/6 mice. By extrapolation we estimated that half-maximal inhibition of hepatic glucose output and hepatic VLDL-TG production by insulin were obtained at plasma insulin levels of ~ 3.6 and ~ 6.8 ng/mL, respectively. In the same experiments, we measured that half-maximal decrease of plasma free fatty acid levels and half-maximal stimulation of peripheral glucose uptake were reached at plasma insulin levels of ~ 3.0 and ~ 6.0 ng/mL, respectively. We conclude that, in comparison to insulin sensitivity of hepatic glucose output, peripheral glucose uptake and hepatic VLDL-TG production are less sensitive to insulin.

Introduction

The liver is a very important regulator in the homeostasis of both glucose and lipid metabolism. Not only does the liver control the storage, production and secretion of glucose, it also produces and secretes very-low density lipoproteins (VLDL) and takes up VLDL-remnants, low density lipoproteins (LDL) and albumin-bound fatty acids (FA). Insulin inhibits both hepatic glucose and VLDL-TG production. It is not known, however, whether both processes are equally sensitive to insulin-mediated inhibition.

Hepatic glucose output (HGO) is determined by the rate of hepatic glycogen breakdown, which is regulated by glucose-6-phosphatase (G6Pase), and by the rate of hepatic gluconeogenesis, which is regulated by phosphoenolpyruvate carboxykinase (PEPCK). In the fed state insulin inhibits HGO via inhibition of these two key regulatory enzymes.[1-3] Insulin also stimulates glucose uptake by peripheral tissues, such as muscle and adipose tissue. In these tissues, insulin stimulates translocation of the glucose transporter-4 (Glut-4) mediating uptake of glucose.[4] Previous studies have documented different dose-response effects of insulin on the HGO and peripheral glucose uptake (PGU). Rizza *et al*.[5] showed that HGO is more sensitive to inhibition by insulin than peripheral glucose uptake is to stimulation by insulin.

Hepatic VLDL-TG production is commonly assumed to be primarily a substrate-driven process[6], but insulin also plays an important role in the regulation of this VLDL-TG production. Insulin can inhibit the hepatic VLDL-TG production via direct and indirect mechanisms. The exact mechanism remains unclear, but it is thought that insulin can directly accelerate the degradation of apoB which is necessary for VLDL-TG secretion.[7] An indirect effect of insulin is suggested to work via inhibition of hormone sensitive lipase (HSL) in adipose tissue, leading to decreased plasma levels of FA and thus, decreased flux of FA from the adipose tissue to the liver.[8] However, in a study in humans a metabolic relationship between insulin-mediated suppression of FA release from adipose tissue and FA flux to the liver on one hand, and the rate of hepatic VLDL-TG production on the other hand was not observed.[9] A study by Lewis *et al*.[10] showed that in normal individuals the acute inhibition of VLDL-TG production by insulin in vivo was only partly due to the suppression of plasma FA, and may also be due to an FA-independent process.

We investigated in wild type C57Bl/6 mice, whether HGO and hepatic VLDL-TG production are equally sensitive to insulin-mediated inhibition using the hyperinsulinemic euglycemic clamp technique[11] which was adapted to mice as described previously by our group.[12,13] We found that the HGO is much more sensitive to insulin-mediated inhibition than hepatic VLDL-TG production.

Materials and Methods

Animals

For our experiments we used 12-week old male C57Bl/6 mice that were housed under standard conditions. The mice were fed a standard mouse/rat chow diet (Hope Farms, Woerden, Netherlands) and water *ad libitum*. Mice were fasted for 2 h before the experiments and randomly assigned to respective groups which were infused with different amounts of insulin. Per group 5 to 6 animals were used. All animal experiments were approved by the Animal Ethics Committee from our institute.

Hyperinsulinemic euglycemic clamp

The clamp protocol was adapted from previously published studies performed by our group.[12,13] Food was withdrawn at 7 A.M. and at 9 A.M. the mice were anaesthetized with a combination of acetylpromazine (Vetranquil, Sanofi Santé Nutrition Animale, Libourne Cedex, France), midazolam (Dormicum, Roche, Woerden, Netherlands) and fentanyl (Fentanyl, Janssen-Cilag, Tilburg, Netherlands). An infusion needle was placed into the tail vein and basal glucose turnover rates were determined by infusion of D-[3-^{3}H]-glucose (0.6 µCi/kg.min, Amersham Biosciences, Little Chalfont, UK) alone during 45 minutes to achieve steady-state levels. After 45 and 60 minutes of infusion blood samples (60 µL) were drawn from the tip of the tail into chilled capillary tubes (Hawksley and Sons Limited, West Sussex, UK) coated with paraoxan (diethyl p-nitrophenyl phosphate, Sigma, St Louis, USA) to prevent *ex vivo* lipolysis.[14] These capillaries were kept on ice and spun for 5 min at 13.000 rpm to isolate the plasma which was snap-frozen in liquid nitrogen and stored at -20°C until analysis. After the basal period a hyperinsulinemic clamp was started with the continuous infusion of a combination of D-[3-^{3}H]glucose (0.6 µCi·kg^{-1}·min^{-1}) and insulin at the respective 4 concentrations (3.5, 7, 14 or 28 mU·h^{-1}). To maintain euglycemic blood glucose levels, exogenous glucose was infused via an adjustable infusion of a 20% D-glucose solution in phosphate-buffered saline (PBS). A blood sample (<5 µL) was taken every

10 min to monitor blood glucose (Freestyle, Disetronic Medical Systems BV, Vianen, Netherlands). When steady state blood glucose levels were reached, two blood samples (60 µL) were taken with 15 min intervals to measure hyperinsulinemic parameters of peripheral glucose uptake and HGO. After the last blood sample, Triton was injected which completely blocks lipolysis of plasma triglycerides (TG).[15] Plasma TG were measured before injection of Triton and at 30, 60 and 90 min after injection and related to the body mass of the mice. Hepatic TG production was calculated from the slope of the curve and expressed as $\mu mol \cdot h^{-1} \cdot kg\ bodyweight^{-1}$. The clamp experiments lasted approximately 4 h.

Plasma parameter analyses

Plasma glucose was measured using the glucose hexokinase method (Instruchemie, Delfzijl, Netherlands). FA and TG were determined using commercially available kits (#315 and #310-A Sigma GPO-Trinder kit, St. Louis, MA, USA) according to the manufacturer's instructions. Plasma insulin concentrations were measured by ELISA (ALPCO Diagnostics, Windham, NH, USA). To measure plasma [^{3}H]glucose, trichloroacetic acid (final concentration 10%) was added to 7.5 µL plasma to precipitate proteins using centrifugation. The supernatant was dried to remove water and resuspended in milliQ. The samples were counted by scintillation counting (Packard Instruments, Dowers Grove, IL, USA).

Calculations

Under steady-state conditions for plasma glucose concentrations, the rate of glucose disappearance equals the rate of glucose appearance. The latter ($\mu mol \cdot min^{-1} \cdot kg^{-1}$) was calculated during the basal period and under steady-state clamp conditions as the rate of tracer infusion (dpm/min) divided by the plasma specific activity of [^{3}H]glucose (dpm/µmol). The ratio was corrected for body weight. Hyperinsulinemic HGO was calculated as the difference between the tracer-derived rate of glucose appearance and the glucose infusion rate.

Statistical analysis

Results are presented as means ± SE for 5 animals per group. Differences between experimental groups were determined by the Mann-Whitney U test. The means per group were tested for linear trend (P_{trend}) with increasing insulin levels. The level of

statistical significance of the differences was set at $P < 0.05$. Analyses were performed using SPSS 12.0 for Windows software (SPSS, Chicago, USA) and Prism 4.0 (GraphPad).

Results

Plasma glucose and insulin levels and glucose infusion rates during the clamp analyses

Plasma glucose levels during the basal and hyperinsulinemic clamp period were not different between the groups (Table 1). At basal level plasma insulin levels were not different between the groups, averaging at ~1.4 ng/mL. At hyperinsulinemic conditions, steady state plasma insulin concentrations in the respective groups averaged at 2.4, 3.6, 9.3 and 22.4 ng/mL with increasing insulin infusion rates. In addition, to maintain euglycemia during the respective insulin infusion rates, glucose infusion rate (GIR) increased concomitantly, as expected (Table 1; $P_{trend} < 0.01$).

Dose-response effects of insulin on peripheral glucose uptake and hepatic glucose output

We observed no differences in basal peripheral glucose uptake between the groups (Table 2). During the hyperinsulinemic period insulin dose-dependently stimulated peripheral glucose uptake when compared to the respective basal levels, ($P_{trend} < 0.01$). Similarly, basal HGO did not differ between the groups, whereas HGO was dose-dependently inhibited by insulin during the hyperinsulinemic conditions ($P_{trend} < 0.01$).

Dose-response effects of insulin on plasma FA levels and hepatic VLDL-TG production

The decrease in plasma FA levels was determined as a measure of insulin sensitivity of adipose tissue lipolysis. Upon infusion of insulin plasma FA levels decreased dose-dependently ($P_{trend} < 0.01$; Table 3). To measure the effect of insulin infusion on hepatic VLDL-TG production at the end of the hyperinsulinemic period, all groups of mice were injected with Triton WR1339 to completely block plasma VLDL-TG lipolysis.[15] Table 3 presents that insulin infusion leads to a dose-dependent decrease in hepatic VLDL-TG production ($P_{trend} < 0.01$; Table 3).

Table 1. Plasma levels of glucose and insulin and glucose infusion rates.

Insulin Infusion ($mU \cdot h^{-1}$)	Bodyweight (g)	Plasma Glucose (mM)		Plasma Insulin (ng/mL)		GIR * ($\mu mol \cdot min^{-1} \cdot kg^{-1}$)
		Basal	*Hyper*	*Basal*	*Hyper **	
0	28.3 ± 0.9	7.5 ± 0.5	N.A.	1.1 ± 0.2	N.A.	0 ± 0
3.5	25.5 ± 0.5	7.9 ± 0.6	10.1 ±2.1	1.5 ± 0.4	2.4 ± 0.3#	13 ± 6
7	23.9 ± 0.7	8.9 ± 0.5	8.1 ± 1.3	1.1 ± 0.3	3.6 ± 0.5#	32 ± 11
14	26.0 ± 0.7	8.2 ± 0.3	8.2 ± 1.1	1.4 ± 0.3	9.3 ± 1.0#	104 ± 37#
28	27.2 ± 1.4	8.1 ± 0.5	7.3 ± 0.9	1.7 ± 0.4	22.4 ± 4.3#	152 ± 16#

Body weight was measured at the beginning of the experiment. Plasma glucose and insulin levels were measured during the basal and during the hyperinsulinemic (Hyper) period. GIR is the glucose infusion rate necessary to maintain euglycemia during hyperinsulinemia. Values represent means ± SE. (# $P < 0.05$ compared to basal group; * $P_{trend} < 0.01$; n=5-6 mice per group)

Table 2. Effects of insulin infusion on peripheral glucose uptake and hepatic glucose output.

Insulin Infusion ($mU \cdot h^{-1}$)	PGU ($\mu mol \cdot min^{-1} \cdot kg^{-1}$)		% of basal (%)*	HGO ($\mu mol \cdot min^{-1} \cdot kg^{-1}$)		% of basal (%)*
	Basal	*Hyper **		*Basal*	*Hyper **	
0	58.8±9.4	N.A.	N.A.	58.8 ±9.4	N.A.	N.A.
3.5	64.6 ±6.3	57.2 ±4.4	90 ±7.3	64.6 ±6.3	44.7 ±6.0	69 ±6.9
7	74.8 ±11.1	79.0 ±11.1	107 ±12.9	74.8 ±11.1	45.3 ±11.7	60 ±12.5
14	75.9 ±5.3	152.0 ±14.9#	202 ±19.1#	75.9 ±5.3	28.9 ±12.2#	39 ±17.3#
28	61.4 ±7.0	136.4 ±17.0#	221 ±8.1#	61.4 ±7.0	12.5 ±7.9#	21 ±13.8#

During the clamp experiment whole-body glucose uptake (PGU) and hepatic glucose output (HGO) were measured under basal and under hyperinsulinemic conditions. Values represent means ± SE. (# $P < 0.05$ compared to basal group; * $P_{trend} < 0.01$; n=5-6 mice per group)

Table 3. Effects of insulin infusion on plasma fatty acid levels and hepatic VLDL-TG production.

Insulin Infusion ($mU \cdot h^{-1}$)	FA * (mM)	% of basal (%)*	HVP * ($\mu mol \cdot h^{-1} \cdot kg^{-1}$)	% of basal (%)*
0	0.69 ± 0.06	100	159.6 ± 9.0	100
3.5	0.63 ± 0.05	91 ± 9	189.5 ± 19.5	119 ± 12
7	0.37 ± 0.05#	54 ± 9#	158.1 ± 17.6	99 ± 11
14	0.24 ± 0.03#	35 ± 4#	90.3 ± 6.0#	57 ± 4#
28	0.25 ± 0.02#	36 ± 3#	83.3 ± 7.7#	52 ± 5#

After the clamp experiment plasma fatty acid levels (FA) and hepatic VLDL-TG production-HVP) rate were measured under basal and under hyperinsulinemic conditions. Values represent means ± SE. (#$P < 0.05$ compared to basal group; * $P_{trend} < 0.01$; n=5-6 mice per group)

Table 4. Plasma insulin levels at half-maximal effect.

Parameter	Plasma insulin level (ng/mL)
FA	3.0
HGO	3.6
PGU	6.4
HVP	6.8

The half-maximal effect of insulin was determined for each parameter during hyperinsulinemic clamp studies. We estimated the half-maximal effect by extrapolation from the curves using the numbers presented in Table 2 and 3. FA = plasma FA, HGO = hepatic glucose output, PGU = peripheral glucose uptake, HVP = hepatic VLDL-TG production.

Comparison of peripheral glucose uptake, plasma FA decrease, HGO and hepatic VLDL-TG production regarding insulin sensitivity

Taken together, the data presented in Tables 2 and 3 clearly indicate that increasing plasma concentrations of insulin lead to a dose-dependent increase in peripheral glucose uptake (Figure 1A) and a dose-dependent decrease in adipose tissue FA

release (Figure 1B). Simultaneously, we measured in the same animals that in the liver the HGO (Figure 1C) and hepatic VLDL-TG production were inhibited dose-dependently (Figure 1D). For comparison of the dose-response characteristics of each of these effects of insulin, we estimated by extrapolation the insulin concentrations at which the half-maximal inhibitory or stimulatory effect was reached for these respective parameters (Table 4). It is obvious, that in the periphery FA release from adipose tissue is more sensitive to plasma insulin than peripheral glucose uptake. In the liver, HGO is more sensitive to plasma insulin levels than hepatic VLDL-TG production.

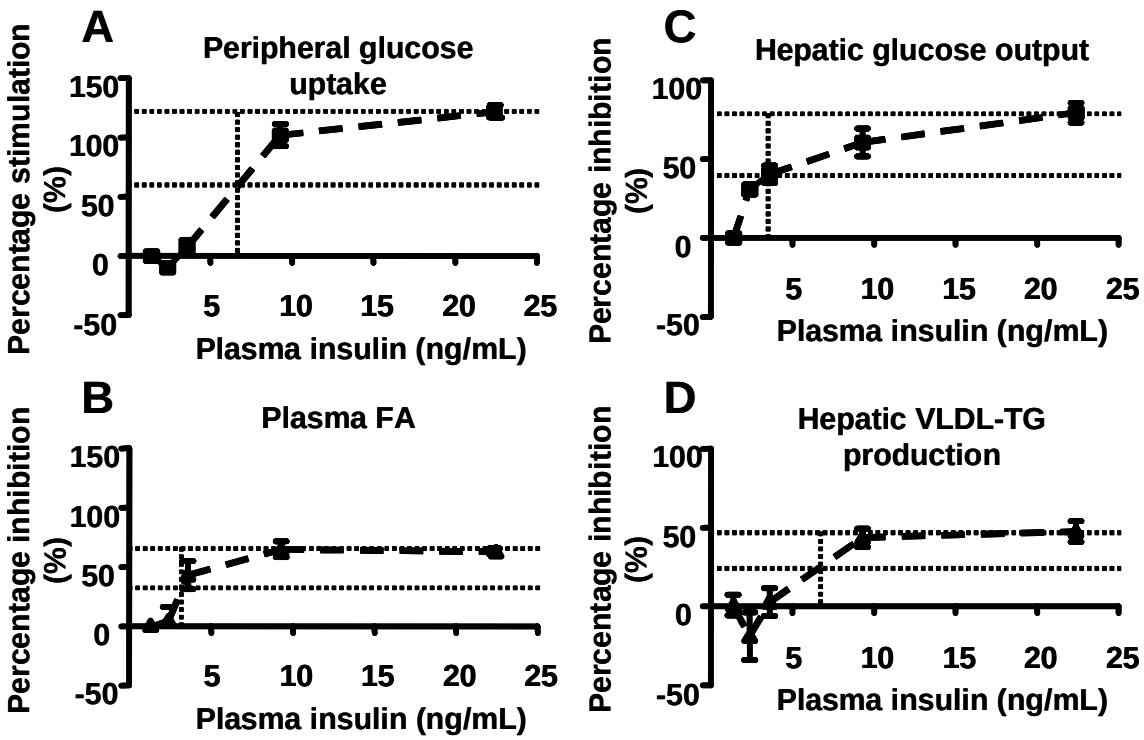

Figure 1. Hepatic glucose production is more sensitive to insulin-mediated inhibition than hepatic VLDL-TG production. During a hyperinsulinemic euglycemic clamp experiment with different plasma insulin concentrations per group we measured the stimulation of peripheral glucose uptake (**A**) and the decrease in plasma FA (**B**). Simultaneously we measured the insulin-mediated inhibition of hepatic glucose production (**C**) and of hepatic VLDL-TG production (**D**). The dotted lines indicate the maximal and half-maximal effect of insulin.

Discussion

Insulin inhibits both hepatic glucose output and VLDL-TG production. So far it was not known, whether both processes are equally sensitive to insulin-mediated inhibition. In the current study we addressed this question and found that the HGO is much more sensitive to insulin-mediated inhibition than hepatic VLDL-TG production.

Since in humans the liver is not readily accessible, mouse models are often used to investigate mechanisms of insulin resistance. The C57Black/6 mouse is a model that is sensitive to diet-induced obesity and insulin resistance.[16,17] Therefore we chose to use these mice for our studies of the glucose and lipid metabolism. In general there are three approaches to perform hyperinsulinemic clamp studies in mice *in vivo*. Some groups use free moving mice with preimplanted catheters [18], other groups use awake but restrained mice [19], and some groups use anesthetized mice.[12,13] Each approach has some limitations. In freely moving mice the effects of movement on the data of interest have to be taken into account. In restrained mice, the endocrine and neural effects of stress through restrainment will affect the data of interest. Finally, in anesthetized mice the effects of anesthetics on the parameters of interest have to be taken into account. Although formal studies comparing the three methods have not been published, it is clear from the publications that each approach is able to detect alterations in insulin effects induced by appropriate interventions. We performed the hyperinsulinemic euglycemic clamp experiments in anesthetized mice. Prior to the current study we compared different combinations of anesthetics. Using a combination of acetylpromazine, midazolam and fentanyl we observed no unwanted adverse effects of the anesthetics on glucose, lipid and insulin concentrations (den Boer *et al.* unpublished results). Nonetheless, we can not exclude the possibility that the exact dose-response relationships of insulin might be slightly different when one of the two other methods of hyperinsulinemic clamp experiments would have been used. However, our data on the relation between insulin concentrations and the parameters of glucose metabolism resemble those of previous studies.

By using this animal model we were able to measure the effect of insulin on HGO and hepatic VLDL-TG production, and in the same time also on peripheral glucose uptake and plasma FA levels, the latter as a measure for insulin sensitivity of adipose tissue lipolysis. Although an exact extrapolation for determination of half-maximal effect could not be made, Figure 1 shows that plasma FA levels, peripheral glucose uptake, HGO and hepatic VLDL-TG production differ in insulin sensitivity. By

comparison of the insulin levels at the half-maximal effect, we observed that HGO is more sensitive to insulin-mediated regulation than peripheral glucose uptake. This is in concordance with the study of Rizza *et al.*[5], who showed in humans that half-maximal suppression of HGO occurs at insulin levels of 29 µU/mL (~ 0.9 ng/mL), while half-maximal stimulation of peripheral glucose uptake occurs at 55 µU/mL (~ 1.8 ng/mL). Furthermore, the suppression of plasma FA appears to be much more sensitive to insulin than the stimulation of peripheral glucose uptake. In fact adipose tissue lipolysis and peripheral glucose uptake are two completely different processes. While lipolysis by HSL takes place in adipose tissue only, insulin-stimulated peripheral glucose uptake occurs both in adipose tissue and in muscle. Therefore, it is not possible to quantitatively compare these peripheral parameters regarding their regulation by insulin under these conditions.

The observation that hepatic VLDL-TG production is much less sensitive to the inhibitory effect of insulin than HGO suggests, that these two processes are regulated differentially. In the regulation of HGO insulin inhibits the forkhead box Other-1 (FoxO1) which binds to promoter regions of genes encoding the enzymes G6Pase and PEPCK[20], which are important regulators of glycolysis and gluconeogenesis respectively.[1-3] The molecular mechanism underlying the insulin-mediated suppression of hepatic VLDL-TG production is not completely clear. Studies have shown that insulin can inhibit the lipidation of pre-VLDL via inhibition of microsomal TG transfer protein (MTTP).[21,22] MTTP is the enzyme that catalyzes the fusion of the pre-VLDL with a lipid droplet, thereby rendering the pre-VLDL into a mature VLDL particle ready for secretion. In addition, *in vitro* studies have shown that insulin stimulates the degradation of apoB in hepatocytes.[23-25] Decreased intracellular apoB availability leads to a decreased hepatic VLDL-TG production. Furthermore, insulin is known to inhibit HSL in adipose tissue, leading to decreased plasma levels of FA and thus, to decreased flux of FA from adipose tissue to the liver, which will eventually decrease FA re-esterification into TG in hepatocytes.[8] It has indeed been shown, that in the presence of hyperinsulinemia the liver secretes less and smaller VLDL particles.[26] However, in a study in humans an association between insulin-mediated suppression of FA release from adipose tissue and FA flux to the liver on one hand, and the rate of hepatic VLDL-TG production (estimated from the mono-exponential slope of VLDL-TG [$^2H_5$]glycerol enrichment) on the other hand, was not observed.[9] Another semiquantitative study in humans also showed that in normal individuals the

acute inhibition of VLDL-TG production by insulin *in vivo* is only partly due to the suppression of plasma FA.[10] In accordance, in the current study, we could not find a significant correlation between decrease in plasma FA levels and decrease in hepatic VLDL-TG production during hyperinsulinemia. Apparently, plasma FA levels and FA availability to the liver *per se* do not determine hepatic VLDL-TG production. In accordance with this notion, we have previously shown that acute redirection of hepatic FA flux from β-oxidation to storage does not affect hepatic VLDL-TG production.[27] We suggest that under the conditions of our experiment insulin exerts direct effects on hepatic VLDL-TG production which are apparently of greater importance than the indirect effects via suppression of FA release from adipose tissue or FA availability in general, at least under the conditions of our experiments. We hypothesize that hepatic VLDL-TG production is inhibited by insulin via a combination of the three different mechanisms described above and may therefore be less sensitive to insulin.

Metabolic zonation may also be a factor involved in the difference in insulin sensitivity of HGO *versus* hepatic VLDL-TG production. Hepatic metabolic pathways are not uniformly distributed across the liver.[28] Within the liver acinus different zones exist. In the efferent perivenous zone more FA synthesis takes place and the activity of acetyl-CoA carboxylase is much higher than in the afferent periportal area. The perivenous zone also has a larger capacity to re-esterify exogenous FA into TG. Carbohydrate metabolism also differs between the two areas. Glucose uptake for glycogen synthesis mainly occurs in the perivenous zone, whereas the generation of glucose via glycogenolysis and gluconeogenesis occurs mainly periportally. Furthermore, although insulin receptor mRNA is homogenously distributed in the liver acinus, insulin receptor protein is mainly expressed in the perivenous area in rat liver.[29] How these differences in metabolic zonation may be reflected in differential regulation of HGO and hepatic VLDL-TG production by insulin is subject to speculation.

In summary, our study shows that HGO is much more sensitive to insulin-mediated inhibiton than hepatic VLDL-TG production. This is of major importance for the use of the golden standard of measuring insulin sensitivity: the hyperinsulinemic euglycemic clamp technique. A low insulin dose already suppresses HGO, while no effect on hepatic VLDL-TG production may be observed. Infusion of high insulin dosages may

lead to the overlooking of subtle differences in hepatic insulin sensitivity, especially with regard to the HGO.

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