Postprandial triglyceride-rich lipoproteins induce hepatic insulin resistance in HepG2 cells independently of their receptor-mediated cellular uptake

Tobias Tatarczyk, Christian Ciardi, Andreas Niederwanger, Michael Kranebitter, Josef R. Patsch, Michael T. Pedrini

To cite this version:
Tobias Tatarczyk, Christian Ciardi, Andreas Niederwanger, Michael Kranebitter, Josef R. Patsch, et al.. Postprandial triglyceride-rich lipoproteins induce hepatic insulin resistance in HepG2 cells independently of their receptor-mediated cellular uptake. Molecular and Cellular Endocrinology, Elsevier, 2011, 343 (1-2), pp.71. 10.1016/j.mce.2011.06.008 . hal-00723607

HAL Id: hal-00723607
https://hal.archives-ouvertes.fr/hal-00723607
Submitted on 11 Aug 2012

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
Postprandial triglyceride-rich lipoproteins induce hepatic insulin resistance in HepG2 cells independently of their receptor-mediated cellular uptake

Tobias Tatarczyk, Christian Ciardi, Andreas Niederwanger, Michael Kranebitter, Josef R. Patsch, Michael T. Pedrini

PII: S0303-7207(11)00316-9
DOI: 10.1016/j.mce.2011.06.008
Reference: MCE 7892

To appear in: Molecular and Cellular Endocrinology

Received Date: 4 March 2011
Revised Date: 8 June 2011
Accepted Date: 9 June 2011

Please cite this article as: Tatarczyk, T., Ciardi, C., Niederwanger, A., Kranebitter, M., Patsch, J.R., Pedrini, M.T., Postprandial triglyceride-rich lipoproteins induce hepatic insulin resistance in HepG2 cells independently of their receptor-mediated cellular uptake, Molecular and Cellular Endocrinology (2011), doi: 10.1016/j.mce.2011.06.008

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
Postprandial triglyceride-rich lipoproteins induce hepatic insulin resistance in HepG2 cells independently of their receptor-mediated cellular uptake

Tobias Tatarczyk, Christian Ciardi, Andreas Niederwanger, Michael Kranebitter, Josef R. Patsch, Michael T. Pedrini

Department of Internal Medicine I, Medical University of Innsbruck, Anichstrasse 35, A-6020 Innsbruck, Austria

Corresponding author: Michael T. Pedrini, MD, Department of Internal Medicine I, Medical University of Innsbruck, Anichstrasse 35, A-6020 Innsbruck, Austria, Tel.: +43-512-504 23326, Fax: +43-512-504 28539, E-mail: michael.pedrini@i-med.ac.at
ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is associated with hepatic insulin resistance with the molecular basis of this association being not well understood. Here we studied the effect of hepatic triglyceride accumulation induced by postprandial triglyceride-rich lipoproteins (TGRL) on hepatic insulin sensitivity in HepG2 cells. Incubation of HepG2 cells with purified TGRL particles induced hepatocellular triglyceride accumulation paralleled by diminished insulin-stimulated glycogen content and glycogen synthase activity. Accordingly, insulin-induced inhibition of glycogen synthase phosphorylation as well as insulin-induced GSK-3 and AKT phosphorylation were reduced by TGRL. The effects of TGRL were dependent on the presence of apolipoproteins and more pronounced for denser TGRL. Moreover, TGRL effects required the presence of heparan sulfate-proteoglycans on the cell membrane and lipase activity but were independent of the cellular uptake of TGRL particles by receptors of the LDL receptor family. We suggest postprandial lipemia to be an important factor in the pathogenesis of NAFLD.

Keywords: glucose metabolism; hepatic insulin resistance; insulin signalling; liver steatosis; postprandial lipemia

Abbreviations: BMI body mass index, DAPI 4’,6-diamidino-2-phenylindole, DMEM dulbeccos minimal essential media, FCS fetal calf serum, GS glycogen synthase, GSK-3 glycogen synthase kinase 3, HL hepatic lipase, HOMA-IR homeostasis model assessment of insulin resistance, HSPG heparan sulfate proteoglycans, LPL lipoprotein lipase, LRP LDL-receptor-related protein, NAFLD non-alcoholic fatty liver disease, PBS phosphate buffered saline, RAP receptor-associated protein, ROS reactive oxygen species, Sf Svedberg flotation rate, TGRL triglyceride-rich lipoproteins, THL tetrahydrolipstatin
1. INTRODUCTION

Type 2 diabetes is frequently associated with non-alcoholic fatty liver disease (NAFLD) which is characterized by hepatic fat accumulation and insulin resistance. Insulin resistance has been shown to promote hepatic fat accumulation through various mechanisms (Chen et al. 1987; Ginsberg 2006). Whether hepatic fat accumulation, in turn, may be a direct cause of insulin resistance, in particular of hepatic insulin resistance, is still a matter of debate (Boden, 1997; Marchesini et al. 2005). One possible mechanism by which hepatic fat may induce hepatic insulin resistance is the development of oxidative stress (Videla 2009).

The sources of fatty acids stored in liver as triglycerides are diverse and the quantitative contribution of various fatty acid sources to NAFLD in humans is not well defined. In the postabsorptive state, fatty acids mainly derive from adipose tissue. In the postprandial state, de novo lipogenesis, spillover of non-esterified fatty acids (NEFA) after lipoprotein lipase (LPL)-mediated lipolysis of intestinally derived chylomicrons and hepatic uptake of the resulting triglyceride-depleted remnant particles (Barrows & Parks 2006) are the major sources of hepatic triglycerides. Using magnetic resonance spectroscopy, it could be demonstrated that postprandial triglycerides are rapidly incorporated into the hepatic triglyceride pool in both normal and diabetic subjects (Ravikumar et al. 2005). The liver has a central role in chylomicron remnant catabolism (Cooper 1997) which is underlined by the observation that defenestration of the sinusoidal endothelium and, consequently, an abolishment of the livers capability to eliminate chylomicron remnants from the circulation is closely correlated with massive hypertriglyceridemia in animal models (Cogger et al. 2006).

Studies have shown that a large portion of chylomicron remnants are bound to heparan sulfate proteoglycans (HSPG) on the endothelial wall of peripheral capillary beds where their triglyceride portion undergoes initial LPL-mediated lipolysis resulting in an increase in chylomicron remnant density (Karpe et al. 2007). With an increase in density, the affinity of
chylomicron remnants to adhere to the endothelial wall decreases (Karpe et al. 1997). As a consequence, chylomicron remnants re-enter plasma and eventually reach the space of Disse after having reached a size corresponding to a Svedberg flotation rate (S_f) of up to 400 allowing them to pass the 100-200 nm sized pores in the protecting endothelium (Cogger et al. 2004). During the process of lipolysis, chylomicron remnants become enriched with Apo E (Batal et al. 2000) which has been shown to bind to LPL (Huff et al. 1997), hepatic lipase (HL) (Krapp et al. 1996) and LDL-receptor-related protein (LRP), a receptor mediating hepatic chylomicron remnant clearance (Beisiegel et al. 1989; Herz et al. 1991). LPL and HL also possess LRP-binding properties of their own (Krapp et al. 1996, Olivecrona & Lookene 1997), further enhancing the affinity of chylomicron remnants towards the LRP.

In the present work, we sought to analyze the effects of hepatic triglyceride accumulation induced by postprandial triglyceride-rich lipoproteins (TGRL) with a S_f < 400, i.e. chylomicron remnants and VLDL/VLDL remnants, on several parameters of hepatic insulin sensitivity using the human hepatoblastoma-derived cell line HepG2. Our decision to study postprandial lipoproteins was based on the following observations suggesting that the postprandial state plays an important role in the development of NAFLD, hepatic insulin resistance and skeletal muscle insulin resistance. Firstly, we have previously shown that insulin sensitivity is impaired during the state of postprandial lipemia in healthy men (Pedrini et al. 2006) and, moreover, that postprandial TGRL particles cause insulin resistance in cultured skeletal muscle cells (Pedrini et al. 2005). Secondly, postprandial lipids represent a substantial source of hepatic triglycerides in vivo in humans (Barrows & Parks 2006; Ravikumar, 2005), yet have been incompletely studied as a potential factor in the pathogenesis of NAFLD and hepatic insulin resistance. Thirdly, in Westernized societies, a large part of the day or even of life is spent in the state of postprandial lipemia.
2. MATERIALS AND METHODS

2.1. Materials

The HepG2 cell line was obtained from ATCC (Manassas, VA, USA); DMEM and amyloglucosidase were purchased from Sigma (St. Louis, MO, USA), FCS from PromoCell (Heidelberg, Germany) and fatty acid-/insulin-free BSA from Valeant Pharmaceuticals (Bryan, OH, USA). Sepharose 2B, [14C]-UDP G and the ECL kit were purchased from Amersham Biosciences (Buckinghamshire, UK), and the 4–15% linear-gradient mini gels from Biorad (Hercules, CA, USA). The anti-GSK-3 (anti-glycogen synthase kinase 3) antibody was obtained from Upstate (Charlottesville, VA, USA) and the LPL antibody from Abcam (Cambridge, UK); anti-phospho-GSK-3/β-ser21/9, anti-phospho-Akt-ser473 and anti-Akt, anti-GS (anti glycogen synthase) and anti-phospho-GS antibodies were from Cell Signaling Technology (Beverly, MA, USA). The receptor-associated protein (RAP) fusion protein was obtained from Progen (Heidelberg, Germany), Tetrahydrolipstatin (Orlistat®) from Roche (Hertfordshire, UK) and purified LPL from Sigma.

2.2. Cell culture

HepG2 cells were stored in liquid nitrogen and cultured to confluence using low glucose DMEM containing 10% FCS.

2.3. Lipoprotein isolation

TGRL were isolated from healthy male individuals 3 hours after ingestion of a standardized fatty meal (Patsch et al. 1983) by zonal ultracentrifugation as previously described (Pedrini et al. 2005). The mean age of our study subjects was 35.1±3.5 years, the mean body mass index (BMI) was 21.4±1.52 kg/m² and the mean homeostasis model assessment of insulin resistance
(HOMA-IR) was 0.75±0.10. Informed consent was obtained from all donors. Three fractions were obtained by pooling appropriate volume contents of the rotor: firstly, a lipoprotein fraction with a $S_f$ of 20-400 corresponding mainly to VLDL/VLDL remnants and chylomicron remnants; secondly, a fraction with a $S_f$ of 20-60 corresponding mainly to VLDL remnants and small dense chylomicron remnants and, thirdly, another fraction with a $S_f$ of 60-400 corresponding mainly to VLDL and more buoyant chylomicron remnants. Prior to the experiments, lipoproteins were extensively dialyzed against phosphate buffered saline (PBS).

2.4. Analysis of lipoprotein fatty acid composition

The fatty acid composition of postprandial TGRL was analyzed by gas chromatography according to the method described by Kang & Wang 2005. Using known standards, we were able to identify 98% of the area under the gas chromatography curve of fatty acids extracted from lipoproteins. The postprandial lipoproteins used for our experiments were composed of 33% palmitic acid, 21% oleic acid, 11% stearic acid, 7% arachidoic acid and 4% linoleic acid.

2.5. Analysis of glycogen content

After incubation with TGRL, incubation media was replaced by serum-free DMEM containing 0.25% BSA. Cells were then incubated without or with 100 nmol/l insulin for 3 hours at 37°C. Subsequently, cells were analyzed for glycogen content as previously described (Pedrini et al. 2005). For the experiments using RAP, which blocks binding of ligands to receptors of the LDL family, this protein was added to the incubation media at a concentration of 1 µmol/l five minutes prior to the addition of TGRL. Tetrahydrolipstatin (THL), a potent lipase inhibitor, was prepared as described elsewhere (Krebs et al. 2000) and co-incubated at a concentration of 250 µg/l with lipoproteins.
2.6. NEFA determination

NEFA levels in the culture media were determined using a commercial kit from WAKO (Germany) with a Cobas MIRA analyzer.

2.7. Determination of hepatocellular triglyceride content

Cells were collected in trypsin, centrifuged at 3000xg for 10 minutes and extensively washed with PBS containing 500 U/ml Heparin to remove any excess triglyceride on the cell surface. After another centrifugation step, the pellet was lysed using 0.1 mol/l NaOH and a brief sonication step. The supernatant was analyzed for triglyceride content using the TG kit from Roche.

2.8. Glycogen synthase activity

After incubation of cells without or with 100 nmol/l insulin for 30 minutes at 37°C, glycogen synthase activity was determined by a method recently established in our laboratory (Niederwanger et al. 2005).

2.9. Western blot analysis

After incubation with TGRL, cells were incubated without or with 100 nmol/l insulin for 5 minutes. All subsequent steps for Western blot analysis were performed as previously described (Pedrini et al. 2005). The dilution of the glycogen synthase, the phospho-glycogen synthase and the LPL antibodies, respectively, was 1:1.000. The dilution of the secondary antibody (goat anti rabbit) was 1:20.000.

2.10. Statistical analysis

Data are expressed as arbitrary units. Therefore, for all experiments, the control condition, i.e. the condition in the absence of any stimulating agent, was set to one, and the other conditions
of the experiment were expressed as n-fold of this reference condition. Statistics were done by calculation of significances to the reference condition with a one-sampled test and calculation of significances among other conditions with an analysis of variances. Subsequently, significances were corrected for multiple testing using the Bonferroni procedure. All values were expressed as means±SD, and significance was accepted as p being less than 0.05.

3. RESULTS

3.1. Controls for cytotoxicity and cell viability
To test whether TGRL have toxic effects on HepG2 cells, we measured lactate dehydrogenase (LDH) in the incubation media at the beginning and end of all lipoprotein incubations. We did not observe any rise in LDH concentration upon TGRL incubation. In addition, possible TGRL-induced apoptosis was excluded using 4',6-diamidino-2-phenylindole (DAPI) staining of the cells. Cell viability after treatment with TGRL was assessed by trypan blue exclusion. Viability was found to be equal to that in non-TGRL-treated cells. For all these assays, H₂O₂ (0.3% for 20 minutes) was used as a positive control.

3.2. Reduction of glycogen content and glycogen synthase activity by TGRL
First, we performed a series of concentration-response and time course experiments to determine the concentration of TGRL triglycerides in the incubation media and the length of incubation time for all following experiments using the analysis of insulin-induced glycogen content, a sensitive parameter for insulin sensitivity. In the concentration-response experiments, we started at a concentration of 40 mg/dl lipoprotein triglycerides, which we previously demonstrated to induce insulin resistance in L6 skeletal muscle cells (Pedrini et al.
2005) and compared it to 80 mg/dl. Overnight incubation with TGRL at a triglyceride concentration of 80 mg/dl significantly reduced insulin-stimulated glycogen content (Fig. 1A). Next, we studied the effect of TGRL on glycogen content as a function of incubation time. TGRL at a triglyceride concentration of 80 mg/dl were added to the incubation media and incubated for 3, 6 and 18 hours. As shown in Fig. 1B, a significant reduction of insulin-induced glycogen content was reached with overnight incubation, i.e. after 18 hours. Hence, a dose of 80 mg/dl lipoprotein triglycerides and overnight incubations were chosen for all following experiments.

Next, we studied the effect of TGRL on insulin-stimulated glycogen synthase activity. The significant insulin stimulation of glycogen synthase activity was lost when cells were incubated in the presence of TGRL (Fig. 1C).

3.3. Effect of TGRL on the phosphorylation of glycogen synthase, GSK-3 and AKT

In accordance with the observations on glycogen synthase activity, we found the insulin-induced inhibition of glycogen synthase phosphorylation to be abolished in the presence of TGRL (Fig. 2A). In agreement with the changes noted for glycogen synthase phosphorylation, insulin-mediated phosphorylation of GSK-3 α at serine 21 and GSK-3 β at serine 9 was significantly impaired by TGRL (Fig. 2B). Accordingly, the phosphorylation of AKT at serine 473, a signalling step upstream of GSK-3, was also impaired by TGRL (Fig. 2C).

3.4. Increase of intracellular triglyceride content by TGRL incubation

To study whether the changes in glucose metabolism and insulin signalling were paralleled by hepatocellular triglyceride accumulation, HepG2 cells were incubated with TGRL for 18 hours. As shown in Fig. 3A, incubation of HepG2 cells with postprandial lipoproteins led to a concentration-dependent increase in the intracellular triglyceride content (2.31±0.25 fold with 40 mg/dl and 2.61±0.67 fold with 80 mg/dl TGRL triglycerides compared to controls).
3.5. Specificity of the TGRL effects

To assess whether the observed effects were specific for triglycerides assembled in intact lipoprotein particles, we conducted control experiments using an apolipoprotein-free lipid emulsion (Intralipid®). Intralipid® was purified by extensive dialysis and matched for triglycerides in TGRL particles.

As shown in Fig. 3B, Intralipid® induced an increase in the intracellular triglyceride content. However, this increase with Intralipid® incubation was 77±25% lower compared to the increase with TGRL incubation. Accordingly, Intralipid® reduced insulin-stimulated glycogen content to 76±33% of control, whereas TGRL reduced insulin-stimulated glycogen content significantly to 47±23% (Fig. 3C).

3.6. Denser TGRL have more pronounced effects on intracellular triglyceride accumulation and reduction of glycogen content

To test whether TGRL size plays a role in their uptake and capability to induce insulin resistance, we pooled respective rotor effluent volumes to obtain one fraction of TGRL with S of 20-60 corresponding to very dense chylomicron remnants and VLDL remnants, and another fraction of TGRL with flotation rates 60-400 corresponding mainly to VLDL and more buoyant chylomicron remnants (Shepherd & Packard 1987). For the following experiments, the two lipoprotein fractions were normalized to their triglyceride content. As shown in Fig. 4A, denser TGRL induced an increased intracellular triglyceride content compared to the more buoyant TGRL fraction. As with intracellular triglyceride accumulation, denser lipoproteins had more pronounced effects on the reduction of insulin-stimulated glycogen content (Fig. 4B).

As the presence of lipoprotein lipase (LPL) has been shown to be a prerequisite for triglyceride accumulation in HepG2 cells, we determined whether TGRL contain LPL and
whether there is a difference between the \( S_f 20-60 \) and the \( S_f 60-400 \) fractions. When controlling for total protein content, denser TGRL particles contained considerably more LPL than more buoyant ones (Fig. 4C).

3.7. **HSPG are required for TGRL effects**

Binding of TGRL to cell membrane bound HSPG has been shown to play a crucial role in hepatic removal of postprandial TGRL (Cooper 1997; Huff et al. 1997). To elucidate the role of cell-membrane bound HSPG in TGRL-induced hepatic insulin resistance, cells were treated with heparinase prior to the addition of TGRL, thereby cleaving the glycosaminoglycan side-chains of the HSPG. Heparinase treatment reduced the effect of an overnight incubation with TGRL on insulin-stimulated glycogen content by 54%, rendering the TGRL effect insignificant (Fig. 5A).

3.8. **TGRL effects on glycogen content are not receptor-mediated**

LDL receptor-related protein (LRP) has been shown to be an important component of the hepatic removal process of TGRL. To investigate whether the effects of TGRL on hepatic insulin sensitivity were receptor-mediated, we co-incubated cells with TGRL and receptor-associated protein (RAP), known to block binding of ligands to receptors of the LDL family (Herz et al. 1991; Medh et al. 1995). RAP at a concentration of 1 \( \mu \text{mol/l} \) was added to the incubation media 5 minutes prior to the addition of TGRL and then co-incubated with TGRL. As shown in Fig. 5B, RAP did not significantly alter TGRL effects on insulin sensitivity in HepG2 cells, whereas in rat L6 myocytes, used as a positive control, RAP abolished the TRGL effect on glycogen content (Fig. 5C).

3.9. **TGRL effects require the activity of lipases**
To assess if possible triglyceride hydrolysis with resulting NEFA release is a prerequisite for the observed TGRL effects, we co-incubated TGRL with the potent lipase inhibitor Tetrahydroplipstatin (THL) at a concentration of 250 µg/l and studied the TGRL-induced effect on glycogen content. This concentration of THL has been previously shown to completely inhibit the activity of 8 ng/ml LPL (Huff et al. 1997). Addition of THL completely abolished the TGRL effects on hepatic insulin sensitivity (Fig. 5D). In accordance, NEFA levels in incubation media significantly correlated with intracellular triglyceride accumulation \((r^2=0.633, p<0.001)\) (Fig. 5E).

4. DISCUSSION

Patients diagnosed with NAFLD have been shown to exhibit prolonged and exaggerated phases of postprandial lipemia as well as decreased insulin sensitivity compared to healthy controls (Musso et al. 2003). In our study, we elucidated several factors linking hepatic triglyceride accumulation induced by postprandial lipoproteins to hepatic insulin resistance. Our data on glucose metabolism and insulin signalling show internally consistent results with TGRL particles reducing insulin effects in a dose- and time-dependent fashion. The effects on these processes were accompanied by hepatocellular triglyceride accumulation indicating that liver steatosis may be a direct cause of hepatic insulin resistance. One mechanism in development of hepatic insulin resistance through hepatic fat may be increased rates of mitochondrial fatty acid beta-oxidation, a process known to lead to enhanced reactive oxygen species (ROS) production (Houstis et al. 2006).

To find out which component of TGRL particles is responsible for the observed effects, we used Intralipid®, a lipid emulsion closely resembling TGRL particles except for the lack of apolipoproteins. With Intralipid®, we observed a less pronounced effect on intracellular
triglyceride accumulation compared to TGRL suggesting that triglycerides not assembled in lipoproteins are capable of causing comparable effects to TGRL, but apolipoproteins are required to achieve maximal effects. This may be due to the well known effects of apolipoproteins on the binding properties of TGRL and various enzymatic processes. The decreased intracellular triglyceride accumulation with Intralipid® as compared to TGRL particles was associated with reduced effects on glycogen content, underlining the concept of a causal relationship between hepatic triglyceride content and hepatic insulin resistance. Comparing TGRL of different densities, we found denser TGRL particles with a $S_f$ of 20-60 to be more effective in intracellular lipid accumulation and in reducing glycogen content. One potential explanation for these observations comes from our experiments showing that denser TGRL particles contain more LPL, thus, enhancing the affinity of TGRL remnants towards HSPG and LRP.

Our experiments with heparinase pre-treatment of HepG2 cells show that binding of chylomicon remnants to HSPG is a prerequisite for internalization as HSPG mediate TGRL adherence to the cell surface where they are brought together with HL which is also bound to HSPG (Ji et al. 1997). Therefore, our results obtained by HSPG cleavage may be caused by lack of adherence of TGRL to the cell surface as well as removal of HSPG-bound HL from the cell membrane.

From our experiments with THL, we conclude that triglyceride hydrolization by HL is another crucial prerequisite for intracellular triglyceride accumulation. After hydrolization, the resulting NEFA pass the cellular membrane and are re-esterified to di- and triglycerides (Guo et al. 2006). In agreement, the addition of RAP to the incubation media blocking the LRP (Willnow et al. 1994) had no effect on TGRL-induced insulin resistance suggesting that the effects of TGRL on insulin sensitivity are independent of receptor-mediated uptake of the triglyceride-depleted remnant particles. Various studies suggest chylomicon remnant uptake to be LRP-mediated, but these studies were mainly performed with $^{125}$I labelled lipoproteins
whereby only a minor fraction of the tracer is found in the lipid fraction of the lipoproteins (Krapp et al. 1996). Our study suggests the lipid fraction to be solely responsible for the effects observed. Further support for independence of TGRL effects of their receptor-mediated uptake comes from a recent report showing that HSPG are involved in hepatic triglyceride clearance independently of receptors of the LDL family (MacArthur et al. 2007) as well as from previous studies demonstrating that the inhibition of the LRP does not exert any effect on triglyceride accumulation derived from VLDL isolated from hypertriglyceridemic subjects (Cianflone et al. 1996).

Interestingly, a previous study investigating the effect of TGRL on insulin-stimulated glycogen synthesis could not find any effect of TGRL on insulin-dependent glycogen metabolism (Rinninger et al. 1986). Methodological differences could represent a possible explanation for this discrepancy including the assay used to determine insulin-stimulated glycogen metabolism. In their study, incorporation of radiolabeled glucose into glycogen was measured, thus, only accounting for glycogen synthesis, but not for differences in glycogenolysis, which, however, has been shown to play a pivotal role in changes in hepatic insulin-dependent glycogen metabolism during postprandial lipemia (Stingl et al. 2001; Boden et al. 2002). In our study, we used the determination of total glycogen content taking into account both glycogen synthesis and glycogenolysis and, therefore, yielding larger effect sizes. Accordingly, in our study, the changes induced by insulin and TGRL on glycogen synthase activity and phosphorylation were rather small compared to the changes in glycogen content.

We are aware that our results ought to be interpreted with caution since, inherent to all cell culture models of diseases, the effects observed in vitro may be diminished or even divergent if other tissues are present. On the other hand, in vivo studies conducted to demonstrate that postprandial TGRL cause hepatic insulin resistance involve metabolic alterations not
restricted to liver, such as an acute decrease in skeletal muscle insulin sensitivity or secondary hyperglycemia and hyperinsulinemia ((Itani et al. 2002; Ide et al. 2004) making conclusions in regard to TGRL as the causative agent very difficult. Therefore, we strongly believe that our data are of significance since we were able to show conclusively that TGRL induce hepatic insulin resistance using a cell line which is well established and widely accepted for studying lipoprotein metabolism in human hepatocytes.

In conclusion, our experiments support the concept that liver steatosis may be a direct cause of hepatic insulin resistance. Our data indicate that the hepatic accumulation of triglycerides through TGRL particles requires prior hydrolysis of TGRL triglycerides at the cell membrane, a process dependent on the presence of HL and HSPG. LRP-mediated uptake of the resulting remnant particles is not required for intracellular triglyceride accumulation and the development of hepatic insulin resistance since these remnants consist mainly of apolipoproteins and cholesteryl esters, and are presumably devoid of sufficient amounts of triglycerides to impair insulin sensitivity.

Together with our previous studies on TGRL-induced muscle insulin resistance and on insulin resistance during postprandial lipemia (Pedrini et al. 2005; Pedrini et al. 2006), this study may be interpreted to suggest that the almost persistent phases of postprandial lipemia in many Westernized societies play an important role not only in the development of NAFLD and its associated hepatic insulin resistance, but also in the development of whole body insulin resistance and the metabolic syndrome.

Acknowledgements: This study was supported by an Austrian-Science-Fund (FWF P17705-B05) to M. T. Pedrini.
**Duality of interest:** The authors report that there is no duality of interest associated with this manuscript.
Figure Legends

Figure 1: Effect of TGRL on insulin-stimulated glycogen content and glycogen synthase activity.

(A) Reduction of insulin-stimulated glycogen content by increasing TGRL concentrations. HepG2 cells were incubated overnight in the absence or presence of increasing concentrations of TGRL and subsequently incubated without or with 100 nmol/l insulin for 3 h and analyzed for glycogen content. TGRL concentrations corresponded to triglyceride concentrations of 40 and 80 mg/dl in the incubation media. Bars represent the means±SD for 3 experiments in triplicates and show relative values to the control condition in the absence of insulin. The numbers in the bars are the n-fold stimulation by insulin to the corresponding condition in the absence of insulin. Ins, insulin

(B) Reduction of insulin-stimulated glycogen content by TGRL over time. HepG2 cells were incubated in the absence or presence of TGRL at a triglyceride concentration of 80 mg/dl for indicated time periods and subsequently incubated without or with 100 nmol/l insulin for 3 h and analyzed for glycogen content. Bars represent the means±SD for 3 experiments in triplicates and show relative values to condition in the absence of insulin and TGRL. The numbers in the bars are the n-fold stimulation by insulin to the corresponding condition in the absence of insulin. Ins, insulin

(C) Reduction of glycogen synthase (GS) activity by TGRL. HepG2 cells were incubated overnight in the absence or presence of TGRL at a triglyceride concentration of 80 mg/dl. Glycogen synthase activity was analyzed after stimulation with 100 nmol/l insulin for 30 min. Bars represent the means±SD for 3 experiments in triplicates and show relative values to the control condition in the absence of insulin. The numbers in the bars are the n-fold stimulation by insulin to the corresponding condition in the absence of insulin. Ins, insulin
Figure 2: Effect of TGRL on the phosphorylation status of glycogen synthase (GS), GSK-3 and AKT.

HepG2 cells were incubated overnight in the absence or presence of TGRL at a triglyceride concentration of 80 mg/dl and then incubated without or with 100 nmol/l insulin for 5 min. Subsequently, cells were solubilised in lysis buffer and lysates were subjected to SDS-PAGE. Direct blotting was performed with an antibody to GS and phospho GS (A), an antibody to GSK-3α and β and phospho GSK-3α and β (B) and an antibody to AKT and phospho AKT (C). The bar graphs show the quantification of the phosphorylated proteins corrected for the content of the respective non-phosphorylated proteins. Bars represent the means±SD for 3 experiments in triplicates and show relative values to the control condition in the absence of insulin. The numbers in the bars are the n-fold stimulation by insulin to the corresponding condition in the absence of insulin. Ins, insulin.

Figure 3: Effect of TGRL and Intralipid® on intracellular triglyceride content and insulin-induced glycogen content.

(A) Increase of intracellular triglyceride content by TGRL incubation. HepG2 cells were incubated in the absence or presence of TGRL at a triglyceride concentration of 40 and 80 mg/dl overnight. Bars represent the means±SD for 3 experiments in triplicates and show relative values to the control condition.

(B) Comparison between overnight TGRL and Intralipid® incubations in regard to their effect on intracellular triglyceride content. HepG2 cells were incubated in the absence or presence of TGRL or Intralipid® at a triglyceride concentration of 80 mg/dl overnight and subsequently analyzed for intracellular triglyceride content. Bars represent the means±SD for 3 experiments in triplicates and show relative values to the control condition.
(C) Comparison between overnight TGRL and Intralipid® incubations in regard to their effect on insulin-induced glycogen content. HepG2 cells were incubated in the absence or presence of TGRL or Intralipid® at a triglyceride concentration of 80 mg/dl overnight and subsequently incubated without or with 100 nmol/l insulin for 3 h and analyzed for glycogen content. Bars represent the means±SD for 3 experiments in triplicates and show relative values to the control condition in the absence of insulin. The numbers in the bars are the n-fold stimulation by insulin to the corresponding condition in the absence of insulin. Ins, insulin

Figure 4: Comparison between TGRL fractions of S_f 20-60 and S_f 60-400 in regard to their LPL content and their effect on intracellular triglyceride content and insulin-induced glycogen content.

(A) Comparison between TGRL fractions of S_f 20-60 and S_f 60-400 in regard to their effect on intracellular triglyceride content. HepG2 cells were incubated in the absence or presence of TGRL (S_f 20-60 and S_f 60-400) at a triglyceride concentration of 80 mg/dl overnight and subsequently analyzed for intracellular triglyceride content. Bars represent the means±SD for 3 experiments in triplicates and show relative values to the control condition.

(B) Comparison between TGRL fractions of S_f 20-60 and S_f 60-400 in regard to their effect on insulin-induced glycogen content. HepG2 cells were incubated in the absence or presence of TGRL at a triglyceride concentration of 80 mg/dl overnight and subsequently incubated without or with 100 nmol/l insulin for 3 h and analyzed for glycogen content. Bars represent the means±SD for 3 experiments in triplicates and show relative values to the control condition in the absence of insulin. The numbers in the bars are the n-fold stimulation by insulin to the corresponding condition in the absence of insulin. Ins, insulin
(C) **Comparison between TGRL fractions of** $S_r$ **20-60 and** $S_r$ **60-400 in regard to their LPL content.** TGRL fractions were normalized for total protein content and subjected to SDS-PAGE. Direct blotting was performed with an antibody to LPL. The control sample was HPLC-purified LPL.

**Figure 5:** Effect of heparinase pre-treatment, RAP pre-treatment and Tetrahydroplipstatin on insulin-stimulated glycogen content.

**A** Effect of heparinase pre-treatment of HepG2 cells on TGRL-induced impairment of insulin-stimulated glycogen content. Hep G2 cells were incubated in the absence or presence of TGRL at a triglyceride concentration of 80 mg/dl overnight, subsequently incubated without or with 100 nmol/l insulin for 3 h and analyzed for glycogen content. Where indicated, cells were treated with 3U/ml heparinase for 5 minutes prior to the addition of TGRL. Bars represent the means±SD for 3 experiments in triplicates and show relative values to the control condition in the absence of insulin. The numbers in the bars are the n-fold stimulation by insulin to the corresponding condition in the absence of insulin. Ins, insulin.

**B** Effect of RAP pre-treatment on TGRL-induced impairment of insulin-stimulated glycogen content in HepG2 cells. HepG2 cells were incubated in the absence or presence of TGRL at a triglyceride concentration of 80 mg/dl overnight, subsequently incubated with 100 nmol/l insulin for 3 h and analyzed for glycogen content. Where indicated, RAP was added at a concentration of 1 µmol/l to the incubation media 5 min prior to the addition of TGRL and then co-incubated with TGRL. Bars represent the means±SD for 3 experiments in triplicates and show relative values to the control condition in the absence of insulin. The numbers in the
bars are the n-fold stimulation by insulin to the corresponding condition in the absence of insulin. Ins, insulin

(C) Effect of RAP pre-treatment on TGRL-induced impairment of insulin-stimulated glycogen content in L6 myocytes. L6 myocytes were used as a positive control for the experiment shown in Fig. 5B. L6 cells were incubated in the absence or presence of TGRL at a triglyceride concentration of 80 mg/dl overnight, subsequently incubated with 100 nmol/l insulin for 3 h and analyzed for glycogen content. Where indicated, RAP was added at a concentration of 1 µmol/l to the incubation media 5 min prior to the addition of TGRL and then co-incubated with TGRL. Bars represent the means±SD for 3 experiments in triplicates and show relative values to the control condition in the absence of insulin. The numbers in the bars are the n-fold stimulation by insulin to the corresponding condition in the absence of insulin. Ins, insulin

(D) Effect of Tetrahydroplipstatin (THL) on TGRL-induced impairment of insulin-stimulated glycogen content. HepG2 cells were incubated with THL at a concentration of 250 µg/l in the absence or presence of TGRL at a triglyceride concentration of 80 mg/dl overnight, subsequently incubated with 100 nmol/l insulin for 3 h and analyzed for glycogen content. Bars represent the means±SD for 3 experiments in triplicates and show relative values to the control condition in the absence of insulin. The numbers in the bars are the n-fold stimulation by insulin to the corresponding condition in the absence of insulin. Ins, insulin

(E) Correlation between NEFA levels in the incubation media with intracellular triglyceride accumulation (n=23). HepG2 cells were incubated overnight without or with THL at a concentration of 250 µg/l in the presence of TGRL at a triglyceride concentration of 40 and 80 mg/dl. NEFA levels in the incubation media were correlated to intracellular triglyceride content using a general linear model.
References

Barrows, BR, Parks, EJ, 2006. Contributions of different fatty acid sources to very low-density lipoprotein-triacylglycerol in the fasted and fed states. J Clin Endocrinol Metab 91, 1446-1452

Batal, R, et al., 2000. Plasma kinetics of apoC-III and apoE in normolipidemic and hypertriglyceridemic subjects. Journal of lipid research 41, 706-718

Beisiegel, U, et al., 1989. The LDL-receptor-related protein, LRP, is an apolipoprotein E-binding protein. Nature 341, 162-164

Boden, G, 1997. Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. Diabetes 46, 3-10

Boden, G, et al., 2002. FFA cause hepatic insulin resistance by inhibiting insulin suppression of glycogenolysis. Am J Physiol Endocrinol Metab 283, E12-19

Chen, YD, et al., 1987. Resistance to insulin suppression of plasma free fatty acid concentrations and insulin stimulation of glucose uptake in noninsulin-dependent diabetes mellitus. J Clin Endocrinol Metab 64, 17-21

Cianflone, K, et al., 1996. Inhibition of lipoprotein lipase induced cholesterol ester accumulation in human hepatoma HepG2 cells. Atherosclerosis 120, 101-114
Cogger, VC, et al., 2004. The effects of oxidative stress on the liver sieve. J Hepatol 41, 370-376

Cogger, VC, et al., 2006. Hyperlipidemia and surfactants: The liver sieve is a link. Atherosclerosis 189(2), 273-81.

Cooper, AD, 1997. Hepatic uptake of chylomicron remnants. Journal of lipid research 38, 2173-2192

Ginsberg, HN, 2006. Is the slippery slope from steatosis to steatohepatitis paved with triglyceride or cholesterol? Cell Metab 4, 179-181

Guo, W, et al., 2006. Fatty acid transport and metabolism in HepG2 cells. Am J Physiol Gastrointest Liver Physiol 290, G528-534

Herz, J, et al., 1991. 39-kDa protein modulates binding of ligands to low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor. J Biol Chem 266, 21232-21238

Houstis, N, et al., 2006. Reactive oxygen species have a causal role in multiple forms of insulin resistance. Nature 440, 944-948

Huff, MW, et al., 1997. Uptake of hypertriglyceridemic very low density lipoproteins and their remnants by HepG2 cells: the role of lipoprotein lipase, hepatic triglyceride lipase, and cell surface proteoglycans. Journal of lipid research 38, 1318-1333
Ide, T, et al., 2004. SREBPs suppress IRS-2-mediated insulin signalling in the liver. Nat Cell Biol 6, 351-357

Itani, SI, et al., 2002. Lipid-induced insulin resistance in human muscle is associated with changes in diacylglycerol, protein kinase C, and IkappaB-alpha. Diabetes 51, 2005-2011

Ji, ZS, et al., 1997. Heparan sulfate proteoglycans participate in hepatic lipase and apolipoprotein E-mediated binding and uptake of plasma lipoproteins, including high density lipoproteins. J Biol Chem 272, 31285-31292

Kang, JX, Wang, J, 2005. A simplified method for analysis of polyunsaturated fatty acids. BMC Biochem 6, 5

Karpe, F, et al., 1997. Chylomicron/chylomicron remnant turnover in humans: evidence for margination of chylomicrons and poor conversion of larger to smaller chylomicron remnants. Journal of lipid research 38, 949-961

Karpe, F, et al., 2007. Removal of triacylglycerols from chylomicrons and VLDL by capillary beds: the basis of lipoprotein remnant formation. Biochem Soc Trans 35, 472-476

Krapp, A, et al., 1996. Hepatic lipase mediates the uptake of chylomicrons and beta-VLDL into cells via the LDL receptor-related protein (LRP). Journal of lipid research 37, 926-936

Krebs, M, et al., 2000, Prevention of in vitro lipolysis by tetrahydrolipstatin. Clin Chem 46, 950-954
MacArthur, JM, et al., 2007. Liver heparan sulfate proteoglycans mediate clearance of triglyceride-rich lipoproteins independently of LDL receptor family members. J Clin Invest 117, 153-164

Marchesini, G, et al., 2005. Nonalcoholic fatty liver disease and the metabolic syndrome. Curr Opin Lipidol 16, 421-427

Medh, JD, et al., 1995. The 39-kDa receptor-associated protein modulates lipoprotein catabolism by binding to LDL receptors. J Biol Chem 270, 536-540

Musso, G, et al., 2003. Dietary habits and their relations to insulin resistance and postprandial lipemia in nonalcoholic steatohepatitis. Hepatology 37, 909-916

Niederwanger, A, et al., 2005, A gel filtration assay to determine glycogen synthase activity. J Chromatogr B Analyt Technol Biomed Life Sci 820, 143-145

Olivecrona, G, Lookene, A, 1997. Noncatalytic functions of lipoprotein lipase. Methods Enzymol 286, 102-116

Patsch, JR, et al., 1983. Inverse relationship between blood levels of high density lipoprotein subfraction 2 and magnitude of postprandial lipemia. Proc Natl Acad Sci U S A 80, 1449-1453

Pedrini, MT, et al., 2005. Human triglyceride-rich lipoproteins impair glucose metabolism and insulin signalling in L6 skeletal muscle cells independently of non-esterified fatty acid levels. Diabetologia 48, 756-766
Pedrini, MT, et al., 2006. Postprandial lipaemia induces an acute decrease of insulin sensitivity in healthy men independently of plasma NEFA levels. Diabetologia 49(7), 1612-1618

Ravikumar, B, et al., 2005. Real-time assessment of postprandial fat storage in liver and skeletal muscle in health and type 2 diabetes. Am J Physiol Endocrinol Metab 288, E789-797

Rinninger, F, et al., 1986. Lack of a lipoprotein-induced insulin resistance in hepatoma cells in culture. Diabetologia 29, 457-461

Shepherd, J, Packard, CJ, 1987. Metabolic heterogeneity in very low-density lipoproteins. Am Heart J 113, 503-508

Stingl, H, et al., 2001. Lipid-dependent control of hepatic glycogen stores in healthy humans. Diabetologia 44, 48-54

Videla, LA, 2009. Oxidative stress signalling underlying live disease and hepatoprotective mechanisms. World J Hepatol 1(1), 72-78

Willnow, TE, et al., 1994. Molecular dissection of ligand binding sites on the low density lipoprotein receptor-related protein. J Biol Chem 269, 15827-15832
Figure 1

A

Glycogen content
ary units

0

ins - +

Control

TGRL (40 mg/dl)

TGRL (80 mg/dl)

p = 0.057

p = 0.006

B

Glycogen content
ary units

0

Ins TGRL 0h 3h 6h 18h

p = 0.019

C

GS activity
ary units

0

Ins - +

Control

TGRL +

p = 0.027

n.s.

n.s.
Figure 3

A

Triglyceride content
arbitrary units

Control  TGRL 40  TGRL 80

B

Triglyceride content
arbitrary units

Control  TGRL  Intralipid®

C

Glycogen content
arbitrary units

ins  Control +  TGRL  Intralipid® +

p<0.001  p<0.001
p=0.001  p=0.041  p=0.007
p=0.034  n.s.
• The pathophysiological link between NAFLD and hepatic insulin resistance is unknown
• We studied the effect of postprandial lipoproteins on hepatic insulin sensitivity
• Postprandial lipoproteins cause liver steatosis and hepatic insulin resistance
• We characterize the underlying molecular mechanisms
• Postprandial lipoproteins are a link between NAFLD and hepatic insulin resistance