Original Article

The Autophagy-Lysosomal Pathway Is Involved in TAG Degradation in the Liver: the Effect of High-Sucrose and High-Fat Diet

(lysosomal lipase / high-fat diet / high-sucrose diet / steatosis / rat)

M. CAHOVÁ, H. DAŇKOVÁ, E. PÁLENÍČKOVÁ, Z. PAPÁČKOVÁ, L. KAZDOVÁ

Department of Metabolism and Diabetes, Centre of Experimental Medicine, Institute for Clinical and Experimental Medicine, Prague, Czech Republic

Abstract. This study was designed to test the role of liver lipases in the degradation of liver triacylglycerols (TAG) and to determine the effect of dietary induced TAG accumulation in the liver on regulation of their lipolysis. Male Wistar rats were administered high-fat or high-sucrose diet for two weeks. Individual lipases (HL; TGH; LAL) were identified according to their different pH optimum. Administration of both diets resulted in liver TAG accumulation (HFD >>> HSD). The only lipase capable to hydrolyse intracellular TAG was LAL. On standard diet, LAL activity towards both endogenous and exogenous substrates was up-regulated in fasting and down-regulated in fed state. The intensity of autophagy determined according to the LC3-II/LC3-I protein ratio followed a similar pattern. HFD led to an increase of this ratio, elevation of LAL activity in phagolysosomal fraction and abolishment of fasting/fed-dependent differences. LAL activity significantly correlated with ketogenesis in all groups ($r = 0.86$; $P < 0.01$). In the HFD group, we determined the enhanced release of lysosomal enzymes (glucuronidase, LAL) into the cytosol. Dgat-1 expression was up-regulated in HFD- and HSD-fed groups, which indicates increased FFA esterification. We demonstrated that LAL is a dominant enzyme involved in degradation of intracellular TAG in the liver and its translocation into the fraction of active (auto)phagolysosomes is stimulated by diet-induced TAG accumulation. Autophagy is stimulated under the same conditions as LAL and may represent the mechanism ensuring the substrate-enzyme contact in autophagolysosomes. In fatty liver, destabilization of (auto)phagolysosomes may contribute to their susceptibility to further stress factors.

Introduction

Non-alcoholic fatty liver disease (NAFLD) defined as fat accumulation in the liver exceeding 5–10% of wet weight (Mensink et al., 2008) is alarmingly spreading around the Western world. It clusters with other disorders associated with metabolic syndrome and increases the risk of development of insulin resistance, type 2 diabetes, cardiovascular and liver diseases. Recent data indicate that dietary induced liver triacylglycerol (TAG) accumulation induces hepatic insulin resistance (IR) before the onset of whole body IR (Samuel et al., 2004). Hepatic steatosis also represents a serious risk factor for further development of progressive stages of liver injury. There are several conditions contributing to the development of fatty liver, i.e. impaired very-low-density lipoprotein (VLDL) secretion or increased supply of FFA or remnant chylomicrons from circulation, either as a result of the increased supply of dietary lipids or as the consequence of increased free fatty acid (FFA) release from adipose tissue. The FFA entry into hepatocytes is not actively regulated and depends only on FFA concentration in the serum. Nevertheless, the TAG liver content is not merely a function of FFA serum levels but the induction of steatosis is the result of concerted action of several intrahepatic mechanisms. Changes in the FFA endogenous utilization, increased *de novo* FFA synthesis, changes in the secretion of VLDL and genetic predispositions rank among the most important factors. In the past, substantial progress was made in identifying the molecular and physiologic changes that cause hepatic steatosis.

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Corresponding author: Monika Cahová, Centre of Experimental Medicine, Institute for Clinical and Experimental Medicine, Vídeňská 1958/9, 140 21 Prague 4, Czech Republic. Phone: (+420) 261 365 366; e-mail: monika.cahova@ikem.cz

Abbreviations: DAG – diacylglycerol, Dgat-1 – diacylglycerol-acyl transferase 1, FA – fatty acid, FFA – free fatty acid, HFD – high-fat diet, HL – hepatic lipase, HSD – high-sucrose diet, IR – insulin resistance, LAL – lysosomal lipase, LD – lipid droplets, NAFLD – non-alcoholic fatty liver disease; SD – standard diet, TAG – triacylglycerols, TGH – triacylglycerol hydrolase, VLDL – very-low-density lipoproteins.

Nevertheless, one important feature of intrahepatocyte fatty acid metabolism – the regulation of breakdown of intracellular TAG – is still poorly understood.

Several groups using different experimental approaches have quantitatively determined that the TAG droplets are not a metabolically inactive storage pool but undergo an intensive cycle of lipolysis followed by re-esterification within hepatocytes (Yang et al., 1995; Lankester et al., 1998). Approx. 70 % of exogenous FFA taken up by the hepatocyte are not directly utilized for VLDL secretion but enter the intracellular storage pool as TAG (Gibbons at Wiggins, 1995). The necessary condition for TAG further utilization is their breakdown catalysed by one of the hepatic lipases. Three main distinct lipolytic activities were identified in the liver. Lysosomal acid lipase (LAL) is an intracellular enzyme with pH optimum 4–5 located in the lysosomes (EC 3.1.1.13) (Vavřínková and Mosinger, 1971). Triacylglycerol hydrolase (TGH) belongs to the carboxylesterase family of enzymes (EC 3.1.1.1), is most active at neutral pH (optimum 7–8) and is associated with endoplasmic reticulum (Lehner et al., 1997). Hepatic lipase (HL) (EC 3.1.1.3) is a heparin-releasable enzyme with alkaline optimum (pH = 9.5) and is associated with the exterior face of the plasma membrane of hepatocytes and liver sinusoidal cells (Assmann et al.,1973). In spite of the fact that these enzymes were described long ago, their exact role in physiological TAG mobilization and in the development of hepatic steatosis is still imperfectly understood and the results obtained are sometimes contradictory.

Also the intracellular localization of the TAG degradation process is still controversial. Previous reports suggested that TAG breakdown is associated with endoplasmic reticulum (Lehner et al. 1999; Dolinsky et al., 2004). An alternative to this concept was published recently by Singh et al. (2009), who described a previously unknown function for autophagy in degradation of intracellular lipid droplets – macrolipophagy. According to their report, the degradation of lipid droplets occurs in the cytoplasm, particularly in autophagolysosomes. The lysosomal degradation pathway, autophagy, is the main physiological pathway for the degradation of intracellular macromolecules such as proteins, organelles, etc., and provides metabolic substrates during the period of starvation. Very little information is available concerning the role of macrolipophagy in NAFLD and associated disorders.

The aim of this study was to determine the role of three liver lipases (TGH, HL, LAL) under different physiological situations (fasted vs. fed animals) and after different dietary manipulations (high-sucrose or high-fat diet) with respect to their possible role in the degradation of intracellular TAG in the liver.

Material and Methods

Animals and experimental protocol

Male Wistar rats were kept in a temperature-controlled room at 12:12-h light-dark cycle. Animals had free access to drinking water and diet if not stated otherwise. All experiments were performed in agreement with the Animal Protection Law of the Czech Republic 311/1997, which is in compliance with European Community Council recommendations for the use of laboratory animals 86/609/ECC, and were approved by the ethical committee of the Institute the Clinical and Experimental Medicine.

Starting at age of 3 months (b. wt. 300 ± 20 g), all animals were fed either high-sucrose diet (HSD: 70 cal % as sucrose; 20 cal % as protein, 10 cal % as fat), high-fat diet (HFD: 70 cal % as saturated fat, 20 cal % as protein, 10 cal % as carbohydrate) or standard laboratory chow diet (SD) for 2 weeks (N = 14 animals per group). The groups labelled "fed" had free access to the diet until decapitation (10–11 a.m.), the groups designated as "fasted" were deprived of food for the last 24 h (N = 7 animals in each subgroup). A separate experiment was designed to assess the contribution of Kupffer cells to the lysosomal lipase activity determined in whole liver homogenate. The rats were fed SD or HFD according to the above-described protocol. Kupffer cells were depleted using gadolinium chloride ($GdCl_3$) as described previously (Krohn et al., 2009). $GdCl_3$ was applied i.v. in three doses (10 mg/kg each dose) 48 h, 24 h and 2 h prior to the beginning of the experiment.

Preparation of subcellular fractions

Lysosomes and phagolysosomes represent a heterogeneous population of organelles sedimenting in a wide range of relative centrifugation force. Active (auto)phagolysosmes have a lower density than the small, inactive lysosomes, allowing their separation by differential centrifugation (Seglen and Solheim, 1985). Twenty-percent (wt/vol) homogenate was prepared by homogenization of liver tissue in 0.25 M sucrose; 0.001 M EDTA, pH = 7.4; heparin 7 IU/ml, 1 mM PMSF, leupeptin 10 μg/ml, aprotinin 10 μg/ml by Teflon pestle homogenizer. Crude impurities were removed by brief centrifugation at 850 *g*. Fat cake and all traces of fat remaining on the tube walls were carefully removed in order to prevent contamination of the homogenate. An aliquot of the homogenate was kept at 4 °C until lipase assay (max. 2 h), the rest was centrifuged for 10 000 *g* 20 min 4 °C and the resulting pellet and supernatant were separated. The supernatant preferentially contains the less dense lysosomes with higher TAG content ("(auto)phagolysosomes"), the pellet is formed by more dense particles ("dense lysosomes"). The cytosolic fraction was obtained after centrifugation of the initial homogenate at 100 000 *g*.

Assay of triacylglycerol lipase activity using exogenous substrate

The use of exogenous substrate enables determination of maximal releasable enzyme activity using a standard amount of the substrate. The reaction medium for all lipase assays was prepared identically except for the buffer used. ^{3}H triolein (92.5 kBq) in toluene was

added to100 μM of cold triolein and 100 μM lecithin in chloroform, and solvents were evaporated under a stream of nitrogen. Three-percent FFA-free BSA was dissolved in 0.1 M buffer (acetate buffer pH = 4.5 for LAL, TRIS buffer pH = 8.0 for TGH or glycine buffer pH = 9.5 for HL), 0.15M NaCl. The whole mixture was emulgated by sonication in Hielscher sonicator UP200S (Hielscher Ultrasonic GmbH, Teltow, Germany) 20 min continuously and incubated with the homogenate or subcellular fractions for 60 min at 30 °C in a shaking water bath. The released fatty acids were extracted according to Belfrage and Vaughan (1969) and counted for radioactivity.

Assay of triglyceride lipase activity using endogenous substrate

This approach takes advantage of the coordinated changes in the intracellular localization of LAL and its intracellular substrate. The liver homogenate was prepared as described above under iso-osmotic conditions that prevent disruption of lysosomes. The lysis of lysosomes was induced only after separation of fractions during the assay. Twenty-percent homogenate was mixed 1 : 1 with 0.2 M acetate buffer and incubated for 60 min in 30 °C in shaking water bath. The reaction mixture was extracted in chloroform-methanol and phases were separated by 1 M NaCl. Aliquots of lower chloroform phase were separated by TLC. The lipid subclasses (FFA, DAG) were visualized by Coomassie blue staining (Sigma-Aldrich, St. Louis, MO) and quantified using Quantity One software (Bio-Rad Laboratories, Hercules, CA). Palmitic acid, 1.2-dioleylglycerol and triolein of known concentrations were used as standards.

Real-time RT-PCR

The samples of liver tissue were dissected immediately after decapitation and frozen in liquid nitrogen. Total RNA was extracted from tissue samples using Trizol reagent (Invitrogen, Carlsbad, CA) according to a standard protocol as described previously (Brinkhof et al., 2006). A DNAase step was included to avoid possible DNA contamination. A standard amount of total RNA (1600 ng) was used to synthesize first-strand cDNA (High Capacity RNA-to-cDNA kit, Applied Biosystems, Foster City, CA). No template control was included to test for contamination. RT-PCR amplification mixtures (25 μl) contained 1 μl template cDNA, SYBR Green master mix buffer (Quanti-Tect, Qiagen, Hilden) and 400 nM (10 pmol/reaction) forward and reverse primers. Reactions were run in an Applera 7300H Fast Real-Time PCR detector (Applied Biosystems). The results were analysed by SDS software vs2.3 (Applied Biosystems). *DGAT1* primers were designed using Primer3 software (left primer: TGATGGCTCAGGTCCCACTG; right primer: GTTAGGGAGGCTGGCCTTTG).

Incubation of liver slices in vitro

Production of β-hydroxybutyrate from liver slices *in vitro* was measured in the absence of exogenous FFA. Liver slices (width approx. 1 mm) were quickly dissected and incubated for 1 h in Krebs Ringer bicarbonate buffer (Sigma-Aldrich) with 5 mmol/l glucose, 2% bovine serum albumin, gaseous phase 95% O_2 and 5% CO_2. All incubations were carried out at 37 °C in sealed vials in a shaking water bath. The aliquots of the incubation medium were stored frozen until further analysis.

Extraction, separation and immunodetection of LC3

Twenty-percent liver homogenate was prepared as described above and kept frozen at -50 °C until analysis. The samples were lysed by 2% SDS at 100 °C and the proteins were immediately separated by SDS-PAGE (15% gel). LC3 in the lysates was recognized using rabbit polyclonal to LC3A/B (Abcam, Cambridge, UK). LC3-I and LC3-II were distinguished by difference in molecular weight (18 and 16 kDa, respectively).

Biochemical analysis

The TAG content in the liver was determined in chloroform extract after the extraction according to Folch et al. (1957). FFA, insulin, triglyceride and glucose serum content and β-hydroxybutyrate production were determined using commercially available kits (FFA: FFA half micro test, Roche Diagnostics GmbH, Mannheim, Germany; triglycerides and glucose: Pliva-Lachema, Brno, Czech Republic; glucuronidase: Sigma-Aldrich; insulin: Mercodia, Uppsala, Sweden; β-hydroxybutyrate: RanBut, Randox Laboratories Limited, Country Antrim, UK).

Chemicals

All materials were reagent grade. ^{3}H-triolein was purchased from Amersham Biosciences (Amersham, Little Chalfont, UK), FFA-free bovine serum albumin (fraction V) was purchased from Serva (Heidelberg, Germany), palmitic acid and triolein from Fluka (Buchs, Switzerland), all other chemicals were purchased from Sigma Czech Republic (Prague, Czech Republic).

Statistical analysis

Data are presented as mean ± S.E.M. Statistical analysis was performed using Kruskal-Wallis test with multiple comparisons (N = 7). Differences were considered statistically significant at the level of $P < 0.05$. The correlations between LAL activity and hepatic TAG content or LAL activity and serum β-hydroxybutyrate concentration were evaluated using Spearman's correlation coefficient.

Results

Characteristics of experimental groups

The animals fed the SD, HSD or HFD did not differ in their final body weight, but the relative weight of epididymal fat pads was higher in HSD and HFD groups. Both diets induced fasting hyperinsulinaemia, but the

Table 1. Characteristics of experimental groups

	SD		HSD		HFD	
	fasted	**fed**	**fasted**	**fed**	**fasted**	**fed**
weight (g)	-	290 ± 10	-	295 ± 15	-	299 ± 12
adiposity index	1.00 ± 0.04	1.24 ± 0.04*	1.40 ± 0.05^{x}			
glycaemia (mmol/l)	4.60 ± 0.10	7.90 ± 0.30	4.50 ± 0.05	8.90 ± 0.10	5.40 ± 0.05$^{\&}$	7.80 ± 0.10
insulinaemia (pmol/l)	56 ± 15	135 ± 21	125 ± 10$^{\#}$	281 ± 32$^{@}$	115 ± 12$^{\&}$	127 ± 18
serum TAG (mmol/l)	0.70 ± 0.05	1.10 ± 0.08	1.00 ± 0.10$^{\#}$	1.60 ± 0.10$^{@}$	0.65 ± 0.08	0.85 ± 0.09^{+}
serum FFA (mmol/l)	0.70 ± 0.05	0.40 ± 0.02	1.00 ± 0.09$^{\#}$	1.30 ± 0.10$^{@}$	0.60 ± 0.08	0.45 ± 0.07
serum β-hydroxybutyrate (μmol/l)	1.67 ± 0.05	0.05 ± 0.01	2.47 ± 0.14$^{\#}$	0.07 ± 0.01	3.20 ± 0.25$^{\&}$	0.28 ± 0.05^{+}

Data are given as mean ± S.E.M., N = 7. The adiposity index is expressed as the relative weight of epididymal adipose tissue per 100 g of total body weight. * SD vs. HSD $P < 0.05$; ^{x} SD vs. HFD $P < 0.05$; $^{\#}$ HSD fasted vs. SD fasted $P < 0.05$; $^{\&}$ HFD fasted vs. SD fasted $P < 0.05$; $^{@}$ HSD fed vs. SD fed $P < 0.05$; $^{+}$ HFD fed vs. SD fed $P < 0.05$.

elevated fasting glycaemia was found only in the HFD group. The effect of HSD and HFD on serum TAG levels was the opposite – HSD increased fed triglyceridaemia by 45 % compared with standard diet, while HFD decreased the serum TAG content by 25 %. The changes in serum FFA content followed a similar trend as TAG. Both diets significantly increased the ketogenesis in fasting, but only HFD led to increased production of β-hydroxybutyrate in the fed state. The data are shown in Table 1.

Table 2. Liver triacylglycerol content

diet	**fasted (μmol/g)**	**fed (μmol/g)**
SD	4.1 ± 0.8	3.2 ± 0,3
HSD	6.6 ± 0.6$^{\#}$	4.4 ± 0.2$^{\bullet,@}$
HFD	14.7 ± 1.1$^{\&}$	17.1 ± 0.4$^{\bullet,+}$

Data are expressed in μmol triacylglycerol/g wet weight as mean ± S.E.M., N = 7. $^{\bullet}$ fasted vs. fed $P < 0.05$; $^{\#}$ HSD fasted vs. SD fasted $P < 0.05$; $^{\&}$ HFD fasted vs. SD fasted $P < 0.05$; $^{@}$ HSD fed vs. SD fed $P < 0.05$; $^{+}$ HFD fed vs. SD fed $P < 0.05$.

Triacylglycerol content in the liver

Both diets resulted in TAG accumulation in the liver. The effect of HSD was less pronounced (fasted +60 %, fed +37 %) compared with HFD (fasted + 258 %, fed + 434 %). In SD and HFD groups, the liver TAG content was higher in fasted compared with fed animals. In the HFD group this relationship was reversed, the TAG content was higher in fed than in fasted liver (Table 2).

The effect of HSD and HFD on lipase activities in liver homogenate: exogenous substrate

In the liver homogenate, we were able to distinguish three lipolytic activities with distinct pH optimum *in vitro* using exogenous triolein substrate: TGH (pH = 8.0), HL (pH = 9.5) and LAL (pH = 4.5) (Fig. 1). TGH was responsible for production of approx. 10 % of the

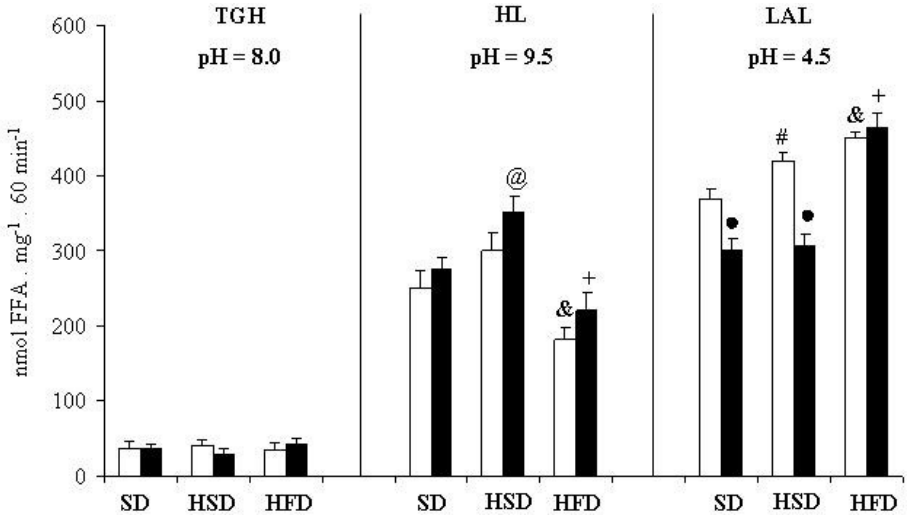

Fig. 1. Liver lipase activities measured as the release of fatty acids from exogenous substrate: the effect of HSD and HFD
Four-percent liver homogenate was incubated in appropriate buffer with 100 μM cold triolein labelled by ^{3}H-triolein and the release of ^{3}H-FFA was determined. LAL activity was measured at pH 4.5 (0.1 M acetate buffer); TGH activity was measured at pH = 8.0 (0.1 M Tris buffer); HL activity was measured at pH = 9.5 (0.1 M glycin-HCl buffer).
Values represent means ± S.E.M. of 7 animals. $^{\bullet}$ fed vs. fasted $P < 0.05$; $^{\#}$ HSD fasted vs. SD fasted $P < 0.05$; $^{@}$ HSD fed vs. SD fed $P < 0.05$; $^{\&}$ HFD fasted vs. SD fasted $P < 0.05$; $^{+}$ HFD fed vs. SD fed $P < 0.05$. □ fasted animals ■ fed animals.

sum of FFA released by all three lipases and its activity was not affected by any dietary manipulation or by the fasted/fed state. HL activity accounted for approx. 40 % of total FFA production. HSD increased the activity of this enzyme (HL) only in fed animals, while HFD diet led to a significant decrease of its activity independently of the nutritional status. LAL exhibited the highest activity among all three lipases tested. On SD, the activity of LAL in the homogenate was elevated in fasting and significantly lower in fed animals. Both diets increased the activity of LAL in fasting, but only HFD resulted in the elevation of LAL activity in the fed animals. In the HFD group the difference between fasted and fed state was blunted.

We determined the correlation between lipase activities and hepatic TAG content (possible source of substrate) and between lipase activities and serum β-hydroxybutyrate (possible end product of intrahepatic lipid metabolism). The LAL activity strongly correlated with ketogenesis ($r = 0.86$; $P < 0.01$) and with hepatic TAG content (fed: $r = 0.97$; $P < 0.001$; fasted: $r = 0.65$; $P < 0.05$). We found no correlation in the case of TGH and HL (data not shown).

The effect of HFD on lipase activities in liver homogenate: endogenous substrate

A separate set of experiments was designed in order to identify the roles of HL, TGH and LAL in the breakdown of endogenous TAG stores. Animals were fed either SD or HFD and production of FFA and DAG from liver homogenate at pH = 4.4; 8.0 and 9.5 during 60 min incubation was determined without any addition of exogenous substrate. These experimental conditions ensured that intracellular TAG were the only available source of substrate. We found no detectable amount of fatty acids released at pH = 8.0 and 9.5 (data not shown). The results obtained at pH = 4.5 are shown in Fig. 2. We were able to identify two main metabolites – FFA and DAG. In the SD group, the LAL activity pattern was similar as those observed with the exogenous substrate – LAL was activated in fasting and significantly less active in fed animals. In the HFD group, the fasting LAL activity was comparable with those in the SD group but in contrast to the latter, the LAL activity in the fed state remained elevated. The net production of DAG during incubation was found only in the HFD group.

The effect of HSD and HFD on lysosomal lipase intracellular distribution

Within the cell, LAL can degrade the substrate only when it co-localizes with the substrate (TAG droplets) in activated (i.e. acidified) (auto)phagolysosomes. Centrifugation of the liver homogenate at 10 000 *g* allowed us to separate the fraction containing dense lysosomes and the fraction containing (auto)phagolysosomes. The enzyme activity in individual fractions was determined with a standard amount of ^{3}H-triolein. We found that the type of the diet significantly affects the portion of LAL activity detected in the (auto)phagolysosomal fraction (Fig 3A). In the SD group, LAL activity in this fraction is significantly higher in fasting compared with fed state. In the HSD-fed group, this pattern of LAL activity regulation (up-regulation in fasting, down-regulation in the fed state) is preserved with an elevation found in fasted animals. HFD led to a significant increase of LAL activity, and no difference between fasted and fed animals

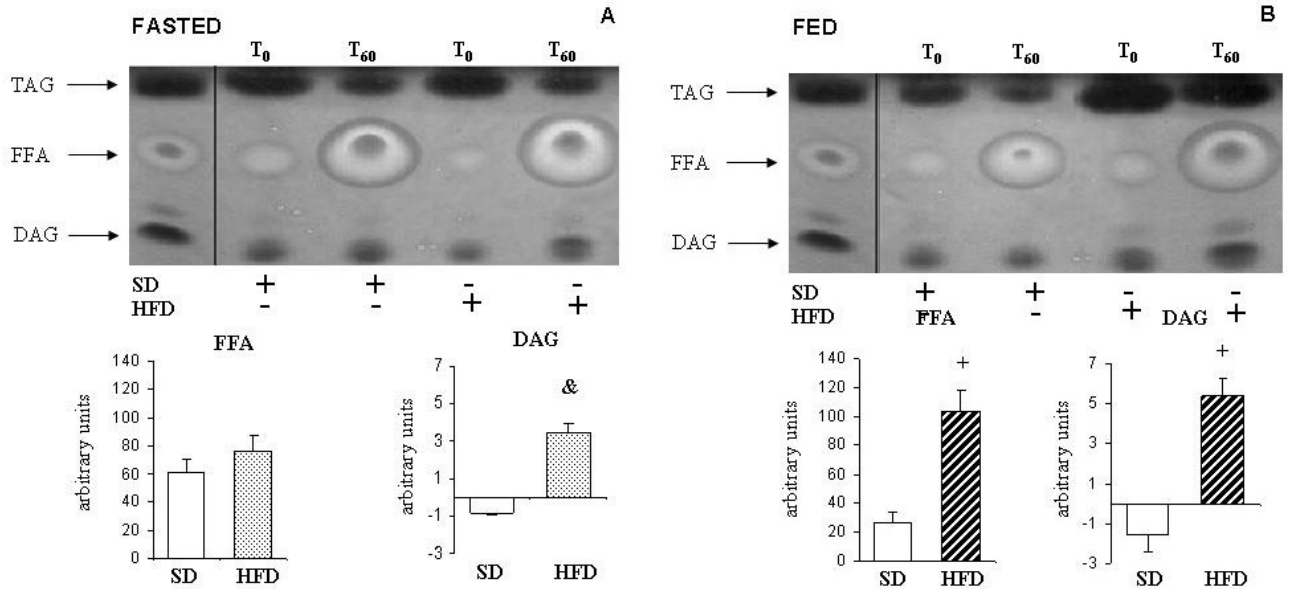

Fig. 2. Production of fatty acids and diacylglycerol from endogenous substrate in fasted (A) and fed (B) state
A typical result of TLC separation is shown in the upper part of the figure. Ten-percent liver homogenate was incubated for 60 min at pH 4.5 and the released FFA and DAG were determined by TLC and visualized by Coomassie blue staining. T_0: state at the beginning of incubation, T_{60}: state at the end. Bars represent the difference T_{60} - T_0. Values represent means ± S.E.M. of 7 animals. $^{\&}$ HFD fasted vs. SD fasted $P < 0.05$; $^{+}$ HFD fed vs. SD fed $P < 0.05$.

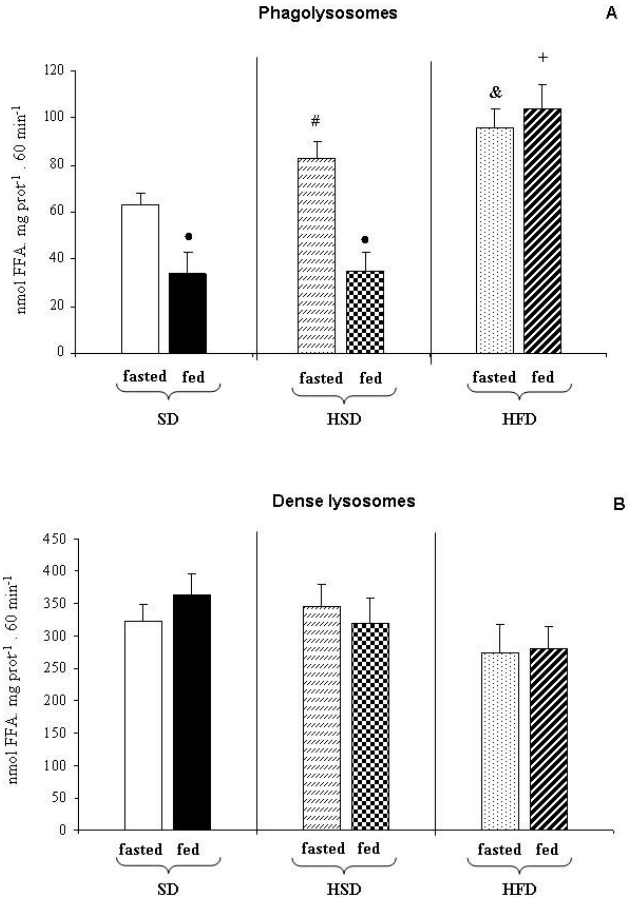

Fig. 3. Effect of HSD and HFD on the distribution of LAL activity between fractions of (auto)phagolysosomes (A) and dense lysosomes (B) prepared from rat liver homogenates

The subcelullar fractions prepared from fresh liver homogenate were incubated with emulgated ^{3}H-triolein and LAL activity was measured as the release of fatty acids at pH = 4.5. Values represent means ± S.E.M. of 7 animals. • fed vs. fasted $P < 0.05$; # HSD fasted vs. SD fasted $P < 0.05$; & HFD fasted vs. SD fasted $P < 0.05$; + HFD fed vs. SD fed $P < 0.05$.

was found. The changes in (auto)phagolysosomal LAL activity were partly mirrored by the corresponding fluctuations in the fraction of dense lysosomes (Fig 3B).

The effect of HSD and HFD on the intensity of autophagy

The ratio of lipidated, membrane-bound (LC3-II) to the cytosolic (LC3-I) form of LC3 protein is considered to be an indicator of the intensity of autophagy. As shown in Fig. 4, the LC3-II/LC3-I ratio was elevated in the SD group after starvation and low in fed condition. HSD had no significant effect either on the LC3-II amount or on the LC3-II/LC3-I ratio. The HFD diet affected LC-II formation in fed and starved animals differently. In the fed animals, HFD administration resulted in approximately three-fold elevation of the LC3-II/LC3-I ratio compared with the SD group. LC3-II formation in fasting was not affected by the diet. Consequently, the effect of the prandial status was completely reversed by high-fat feeding being higher in the fed than in the fasted animals.

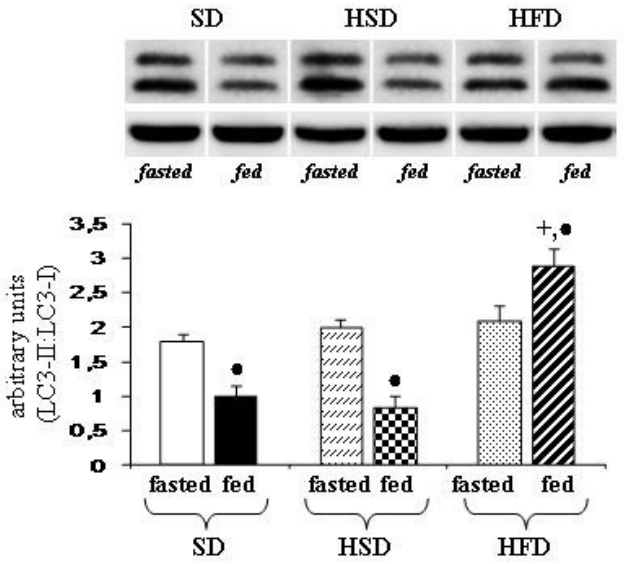

Fig. 4. Effect of HSD and HFD on autophagy in the liver

The intensity of autophagy was determined according to the LC3-II/LC3-I ratio in phagolysosomal fraction. Representative Western blots are shown above the graph. Values represent means ± S.E.M. of 7 animals. • fed vs. fasted $P < 0.05$; + HFD fed vs. SD fed $P < 0.05$.

The effect of HSD and HFD on the release of lysosomal enzymes into the cytosol

Compared with SD fed animals, an elevated LAL (Fig. 5A) and glucuronidase (Fig. 5B) activity was found in the cytosol of both fasted and fed animals in the HFD group. HSD had no effect. This finding may indicate increased lysosomal fragility in the HFD group.

The effect of HSD and HFD on FFA esterification

The expression of *Dgat1* specific mRNA was determined by RT-PCR (Fig. 6). This enzyme catalyses the final step of TAG biosynthesis, i.e. translocation of the acyl moiety to DAG. HSD increased *Dgat1* expression both in fasted and fed animals. HFD led to significant up-regulation of *Dgat1* expression in the fed animals, but we found no differences between the HFD and SD groups in fasting.

The effect of HFD and HSD on ketogenesis in vitro

Liver slices harvested from the fasted animals from both HSD and HFD groups exhibited significantly higher β-hydroxybutyrate production into incubation medium as compared to SD-fast rats (HSD < HFD). When liver slices from fed animals were used for the incubation, ketone body production was potentiated only by HFD (Fig. 7). Our results confirmed that ketogenesis mirrors changes in the LAL activity, i.e. the nutritional

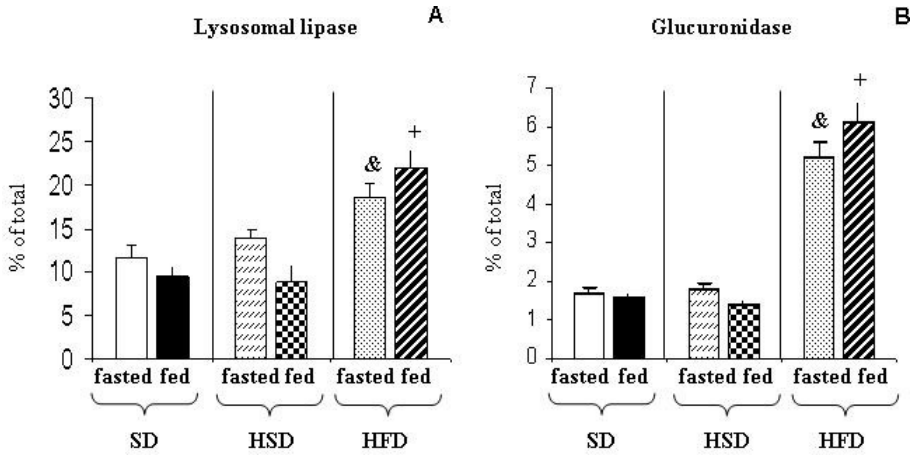

Fig. 5. Effect of HSD and HFD on the release of lysosomal enzymes into the cytosol
The LAL activity (A) and the glucuronidase activity (B) were determined in freshly prepared cytosolic fraction. The results are expressed as % of total enzyme activity released from homogenate used for preparation of the cytosolic fraction. Values represent means ± S.E.M. of 7 animals. & HFD fasted vs. SD fasted $P < 0.05$; + HFD fed vs. SD fed $P < 0.05$.

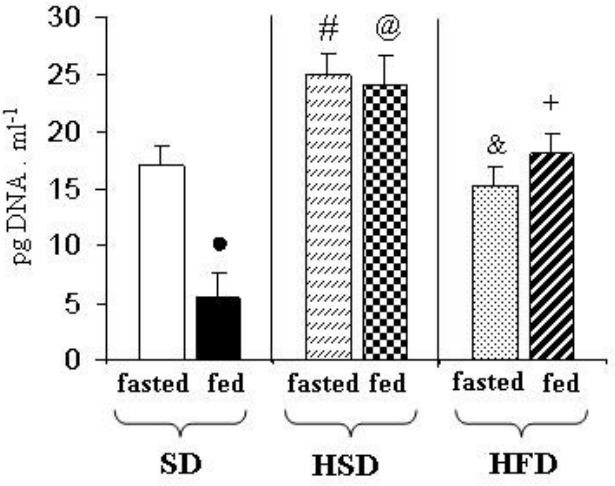

Fig. 6. Effect of HSD and HFD on *Dgat1* expression
Dgat1 mRNA expression was determined by RT-PCR. Values represent means ± S.E.M. of 7 animals. • fed vs. fasted $P < 0.05$; # HSD fasted vs. SD fasted $P < 0.05$; @ HSD fed vs. SD fed $P < 0.05$; & HFD fasted vs. SD fasted $P < 0.05$; + HFD fed vs. SD fed $P < 0.05$.

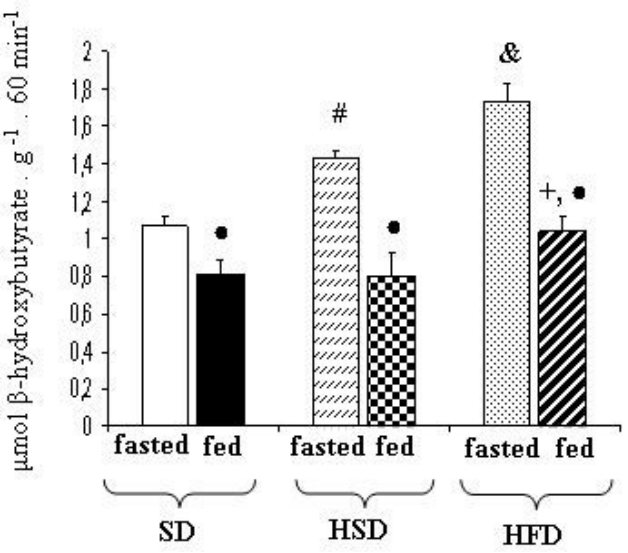

Fig. 7. Effect of HFD and HSD on the β-hydroxybutyrate production from liver slices *in vitro*
The liver slices harvested from fasted and fed animals of each group were incubated in KRB buffer for 60 min. Values represent means ± S.E.M. of 7 animals. • fed vs. fasted $P < 0.05$; # HSD fasted vs. SD fasted $P < 0.05$; & HFD fasted vs. SD fasted $P < 0.05$; + HFD fed vs. SD fed $P < 0.05$.

status-dependent regulation (stimulation in fasting/depression in the fed state) and the stimulatory effect of HFD.

The effect of gadolinium chloride treatment on lysosomal lipase activity

Gadolinium chloride was applied *in vivo* in three doses immediately prior to the experiment in order to deplete resident liver macrophages (Kupffer cells). As shown in Fig. 8, depletion of Kupffer cells led to an approx. 25% decrease of LAL activity determined in whole liver homogenates (Fig. 8A). In contrast, in the phagolysosomal fraction, no significant changes in the LAL activity due to the gadolinium treatment were detected either in the SD or in the HFD group (Fig. 8B).

Discussion

Most fatty acids entering the liver are esterified to TAG and their further utilization depends on intracellular TAG breakdown. In this study, we provided evidence that lipolysis of endogenous TAG is actually stimulated in the liver by dietary induced steatosis. Our results indicate that LAL is involved in lipolysis and mobilization of the stored TAG in liver cells and that the autophagy-lysosomal pathway is involved in the degradation of intracellular TAG. We further demonstrated that HFD blunted the physiological down-regulation of both au-

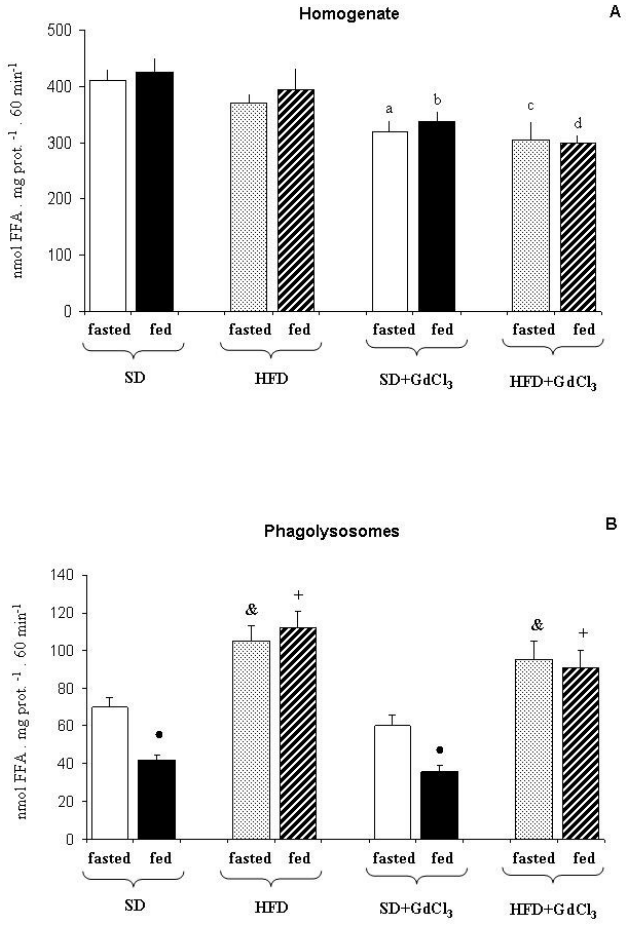

Fig. 8. Effect of Kupffer cell depletion on liver lysosomal lipase activity

The fresh 4% liver homogenate and phagolysosomal fraction prepared from the same homogenate were incubated with emulgated ^{3}H-triolein, and LAL activity was measured as the release of fatty acids at pH = 4.5. Values represent means ± S.E.M. of 7 animals. • fed vs. fasted $P < 0.05$; & HFD fasted vs. SD fasted $P < 0.05$; + HFD fed vs. SD fed $P < 0.05$; [a] SD+$GdCl_3$ fasted vs. SD fasted $P < 0.05$; [b] SD+$GdCl_3$ fed vs. SD fed $P < 0.05$; [c] HFD+$GdCl_3$ fasted vs. HFD fasted $P < 0.05$; [d] HFD+$GdCl_3$ fed vs. HFD fed $P < 0.05$.

tophagosome formation and LAL activity in the fed state. Expression of Dgat-1 was increased in both HSD and HFD groups, which indicates enhanced esterification of fatty acids. Finally, stimulation of lysosomal activity in the HFD group resulted in lysosomal destabilization measured as the release of lysosomal enzymes into the cytosol.

HSD and HFD had profoundly different effects on the development of hepatic steatosis. HSD led to elevation of serum FFA and to increased accumulation of liver TAG in fasting. On the other hand, these processes were compensated by accentuated oxidation of FFA originating from endogenous TAG (ketogenesis) in fasting and by enhanced postprandial VLDL output from the liver (Yamamoto et al., 1987). Taken together, the result was only a mild elevation of liver TAG in the HSD group. HFD actually led to lowering of TAG and FFA serum levels and this is what made questionable the increased uptake of FFA as the main cause of steatosis on this diet. Nevertheless, the hepatic TAG content rose in rats on HFD more than five times compared to SD and two or four times (fasted or fed animals, respectively) compared to the HSD group. In the light of these results, the only possible explanation of the significant accumulation of TAG in the liver and the normal TAG concentration in the serum in HFD-fed animals is the impaired output of VLDL. This effect of HFD has already been described by several authors (Francone et al., 1992; Oussadou et al. 1996).

As reported by Wiggins and Gibbons (1992), almost all FFA entering the hepatocyte are esterified and must be released from the TAG molecule prior to any utilization. It remains an open question which lipase(s) are involved in this process and whether manipulations leading to the accumulation of liver TAG influence its (their) activities.

It has been suggested that lipase mobilizing intracellularly stored TAG should be associated with endoplasmic reticulum (ER) in order to channel lipolytic products towards resynthesis to TAG at the site of VLDL assembly. These demands could be met by TGH with optimum at pH = 8 found nearly exclusively in the microsomal fraction. Nevertheless, TGH has high specific activity towards soluble short-chain triacylglycerol substrates and to esters, but much lower specificity towards insoluble TAG containing long-chain fatty acids – the specific activity of TGH towards tributyrin substrate was 240 μmol FFA/mg protein and only 0.2 μmol FFA/mg protein towards triolein substrate, i.e. 1000 times lower (Lehner et al., 1997). Recent results (Wei et al., 2007) documented that TGH may catalyse one of the important steps in the mobilization of lipids for lipoprotein assembly and secretion, but TGH activity has been reported to be associated preferentially with lipids found within the ER lumen (Gilham et al., 2003). This localization makes its contact with cytosolic lipid droplets rather complicated. Our results indicate that LAL is a dominant enzyme involved in the degradation of intracellular TAG stores. LAL was responsible for the lipolysis of a major part of the totally available exogenous substrate and this portion was further significantly increased in the HFD group. These findings are in accordance with those of Debeer et al. (1979), who measured the triacylglycerol lipase activity in liver homogenates from livers perfused with heparin prior to homogenization and found maximal activity at pH = 4.4. The essential role of LAL in the TAG hydrolysis was also demonstrated in LAL knock-out mice (*Lal*$^{-/-}$), which developed progressive hepatosplenomegaly and exhibited massive TAG accumulation in the liver (Du et al., 2001).

Production of fatty acids exclusively from an endogenous source of substrate (during incubation of 10% liver homogenate) was determined as this experimental setup might be closer to the real processes occurring *in vivo*. When using intracellular TAG as the exclusive

substrate for hydrolysis, the only lipolytic activity was detected at pH = 4.5. In contrast, the lipolytic activity towards endogenous substrate in pH range corresponding to TGH optimum (pH = 7–8) or HL (pH = 9.5) was very low. The activity of LAL in the (auto)phagolysosomal fraction positively correlates with the liver TAG content. Concomitantly occurring stimulation of lipolysis and accumulation of endogenous TAG after HFD administration seem to be contradictory. However, in hepatocytes a significant portion of FFA released from intracellular TAG (approx. 70%) is re-esterified (Gibbons et Wiggins, 1995). HFD impairs VLDL secretion (Francone et al., 1992) and most FFA re-enter the intracellular storage pool. The increased expression of Dgat-1, an enzyme catalysing the final step of triacylglycerol synthesis, in the HFD-fed group supports this idea. Thus, the increased lipolysis in the HFD group does not result in decreased liver TAG content but rather in higher TAG turnover.

As a lysosomal enzyme, LAL is synthesized in endoplasmic reticulum, transported into the trans-Golgi network and packed into vesicles termed primary lysosomes (Tanaka et al., 1990). Lysosomal enzymes can adopt the active form only after primary lysosomes fuse with autophagosomes or endosomes carrying the cargo destined for degradation and form (auto)phagolysosomes with acidic pH. Within the cell, most of LAL is present in inactive form. Singh et al. (2009) have recently found that lipid droplets in hepatocytes can enter the autophagic degradation pathway in the same manner as damaged proteins or organelles via formation of autophago(lipo)somes that further fuse with primary lysosomes. As the only known lysosomal enzyme with lipolytic activity is LAL and as it shares a similar regulatory pattern with autophagy (stimulation in response to starvation), we believe that our results are complementary to those of Singh et al. It is possible that the LAL activity determined in the (auto)phagolysosomal fraction quantitatively reflects the autophagosome formation.

LC3 protein is an important member of the autophagy metabolic pathway. During the formation of autophagosomes, the cytosolic form of LC3 protein (LC3-I) is conjugated to phosphatidylethanolamine (LC3-II). LC3-II is incorporated into the autophagosomal membrane and remains there until the stage of late autophagolysosome (Rubinsztein et al., 2009). The LC3-II/LC3-I ratio is considered to be an indicator of the intensity of autophagy. In the SD group, fasting condition was associated with the increase of LC3-II/LC3-I ratio (stimulated autophagy) while in the fed state this ratio decreased (depressed autophagy). In contrast, in the HFD group the LC3-II/LC3-I ratio in the fed state remained elevated and the intensity of autophagy estimated according to this parameter was stimulated and not prandially-dependent. Since part of LC3-II is processed by lysosomal hydrolases, the rise in LC3-II abundance may indicate either the increased rate of autophagosome formation or block in lysosomal degradation (Klionsky et al., 2007). However, concomitant stimulation of lysosomal lipolysis makes the second possibility rather improbable. We suggest that increased expression of the LC3-II protein in HFD groups indicates higher intensity of autophagy.

It is important to note that Singh et al. (2009) reported data indicating that formation of autophagosomes carrying a lipid cargo may be decreased in mice fed HFD for 16 weeks. Apart from the difference in the duration of HFD administration, they measured the LC3-II content in lipid droplets (LD) and found an inhibitory effect of the diet only in starved animals, while in the fed ones the LC3-II content in the LD fraction was higher in HFD compared with the SD group. This finding, i.e. the stimulatory effect of HFD manifesting itself in the fed state, is in accordance with our results.

Our data indicate that the activation of lysosomes, i.e. increased formation of (auto)phagolysosomes, is associated with destabilization of the (auto)phagosomal membrane and with release of lysosomal enzymes into the cytosol. The lysosomes contain a number of enzymes, e.g. proteases (cathepsins) that are able to damage subcellular organelles such as mitochondria (Li et al., 2008). We propose the hypothesis that not steatosis itself but rather the accelerated TAG lipolysis/re-esterification cycle accompanied by lysosomal destabilization may be one of the causes of NAFLD-associated liver injury.

LAL is present in a wide variety of cell types including hepatocytes (Debeer at al., 1979) and resident macrophages (Kupffer cells) (Du et al., 2001). Unfortunately, determination of the LAL activity in whole liver homogenates does not allow the exact determination of its source. To confirm that the described findings are valid for hepatocytes we performed the following experiment based on two presumptions: 1. hepatocytes are the only mammalian cells capable of FFA conversion to ketone bodies and 2. intracellular TAG are the only endogenous source of FFA for ketogenesis (McGarry et al., 1980). *In vitro* ketogenesis in liver slices incubated in the absence of exogenous FFA thus reflects the intrahepatocyte TAG breakdown. Our results confirmed that production of ketone bodies mirrors the changes in the LAL activity, i.e. the nutritional status-dependent regulation (stimulation in fasting/depression in the fed state) and the stimulatory effect of HFD. Another approach to the assessment of the Kupffer cells contribution to the LAL activity measured in homogenate is the employment of $GdCl_3$, which allows acute depletion of liver resident macrophages. The total LAL activity in the liver of $GdCl_3$-treated rats was 20–25 % lower compared with the untreated ones, but no significant effect was found in phagolysosomal fraction. Further, $GdCl_3$ treatment did not affect the nutritional status-dependent or dietary-induced changes of LAL activity. We conclude that these findings provide evidence that the changes in LAL activity reported in this study can be ascribed to changes occurring in hepatocytes.

In conclusion, we found that fat accumulation in the liver is associated with the increased lipolytic activity towards intracellular substrate and with higher produc-

tion of lipolytic products. LAL was identified as an important enzyme responsible for the breakdown of intracellular TAG. The increased LAL activity was associated with its translocation into the (auto)phagolysosomal fraction. Our data indicate that autophagy may represent the mechanism responsible for the transportation of the substrate (lipid droplets) to the site of degradation (autophagolysosomes). We further demonstrated that the increased lipolytic activity is accompanied by increased Dgat-1 expression in fatty liver, which may result in intensification of the lipolytic/re-esterification cycle in hepatocytes. Finally, our results showed that lysosomal activation is associated with destabilization of (auto)phagolysosomes, which may contribute to the increased susceptibility of fatty liver to further stress factors.

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