

## Autophagy-Lysosomal Pathway Is Involved in Lipid Degradation in Rat Liver

V. ŠKOP<sup>1</sup>, M. CAHOVÁ<sup>2</sup>, Z. PAPÁČKOVÁ<sup>2</sup>, E. PÁLENÍČKOVÁ<sup>2</sup>, H. DAŇKOVÁ<sup>2</sup>, M. BARANOWSKI<sup>3</sup>, P. ZABIELSKI<sup>3</sup>, J. ŽDYCHOVÁ<sup>2</sup>, J. ZÍDKOVÁ<sup>1</sup>, L. KAZDOVÁ<sup>2</sup>

<sup>1</sup>Department of Biochemistry and Microbiology, Institute of Chemical Technology, Prague, Czech Republic, <sup>2</sup>Department of Metabolism and Diabetes, Institute for Clinical and Experimental Medicine, Prague, Czech Republic, <sup>3</sup>Medical University of Białystok, Białystok, Poland

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### Summary

We present data supporting the hypothesis that the lysosomal-autophagy pathway is involved in the degradation of intracellular triacylglycerols in the liver. In primary hepatocytes cultivated in the absence of exogenous fatty acids (FFA), both inhibition of autophagy flux (asparagine) or lysosomal activity (chloroquine) decreased secretion of VLDL (very low density lipoproteins) and formation of FFA oxidative products while the stimulation of autophagy by rapamycin increased some of these parameters. Effect of rapamycin was completely abolished by inactivation of lysosomes. Similarly, when autophagic activity was influenced by cultivating the hepatocytes in "starving" (amino-acid poor medium) or "fed" (serum-supplemented medium) conditions, VLDL secretion and FFA oxidation mirrored the changes in autophagy being higher in starvation and lower in fed state. Autophagy inhibition as well as lysosomal inactivation depressed FFA and DAG (diacylglycerol) formation in liver slices *in vitro*. *In vivo*, intensity of lysosomal lipid degradation depends on the formation of autophagolysosomes, i.e. structures bringing the substrate for degradation and lysosomal enzymes into contact. We demonstrated that lysosomal lipase (LAL) activity in liver autophagolysosomal fraction was up-regulated in fasting and down-regulated in fed state together with the increased translocation of LAL and LAMP2 proteins from lysosomal pool to this fraction. Changes in autophagy intensity (LC3-II/LC3-I ratio) followed a similar pattern.

### Key words

Autophagy • Lysosomes • Lipolysis • Hepatocyte • Lysosomal lipase

### Corresponding author

Monika Cahova, Institute for Clinical and Experimental Medicine, Department of Metabolism and Diabetes, Videnska 1958/9, 140 21 Prague 4, Czech Republic. Fax: +420 26136 3027. E-mail: monika.cahova@ikem.cz

### Introduction

The liver has an enormous capacity to store excessive fat in the form of lipid droplets. Growing evidence indicates that lipid droplets in the hepatocytes are not the physiologically inactive particles but that the liver is a site of a continuous lipolysis/reesterification cycle of stored TAG (triacylglycerol). Most of the FFA transported into hepatocytes are rapidly esterified and must be released prior any further utilization, i.e. for oxidation or VLDL particles assembly, by lipolysis (Zechner *et al.* 2005, Lankester *et al.* 1998, Yang *et al.* 1995). Thus, the lipolysis is an important part of hepatocellular TAG metabolism, but little is known about the lipases responsible for controlling hepatic TAG hydrolysis or about how this process is regulated. Several lipases have been identified in rat liver. Triacylglycerol hydrolase (TGH, EC 3.1.1.1) is expressed in the liver as well as in the heart, kidney, small intestine and adipose tissue and localizes to the endoplasmic reticulum and lipid droplets (Dolinsky *et al.* 2004). Adipose triglyceride lipase (ATGL, alias PNPLA2, desnutrin, FP17548, TTS-2.2) is expressed predominantly in adipose tissue but its expression in the liver is relatively low (Haemmerle *et al.* 2006).

Lipolytic activity in a low pH range (4.5-5) in the liver was first demonstrated by Vavrinkova and Mosinger (1965). The lipase responsible for this phenomenon is of lysosomal origin (lysosomal lipase, LAL, EC 3.1.1.13). Its involvement in endogenous TAG degradation was first demonstrated by Debeer *et al.* (1979) who showed on isolated hepatocytes that lysosomotropic agents (chloroquine or  $\text{NH}_4\text{Cl}$ ) that inactivate lysosomal enzymes *via* elevation of intralysosomal pH significantly inhibited ketogenesis from endogenous (intracellular TAG) but not exogenous (added oleate) sources. Furthermore, Kalopissis and colleagues demonstrated in the same model that in the absence of exogenous fatty acids chloroquine not only reduced ketogenesis but also attenuated VLDL secretion (Francone *et al.* 1992). The essential role of LAL in TAG hydrolysis in the liver was confirmed in a LAL null mice model. *Lal*  $^{-/-}$  mice exhibited massive TAG and cholesterol storage in the adult liver, adrenal glands and small intestine, severe hepatosplenomegaly and shortened life span (Du *et al.* 2001). This phenotype could be reversed by LAL cDNA adenoviral transfection (Du *et al.* 2002)

The main objection made against LAL involvement in the degradation of intracellular lipid droplets was the different intracellular localisation of lipolytic enzyme (LAL sequestered in lysosomes) and substrate (lipid droplets in cytoplasm). Only recently did Singh *et al.* (2009) bring evidence supporting the hypothesis 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 carry the cargo to the final degradation in lysosomes. The intensity of autophagy is closely linked with the nutrient sensing being up-regulated in starvation and suppressed in nutrient excess. The regulatory and functional similarities between autophagy and lipolysis indicate that autophagy may contribute to the TAG breakdown in the liver.

The aim of our study was to address the issue concerning the contribution of autophagy-lysosomal pathway to intracellular lipid degradation in the liver. We determined the effect of autophagy inhibition/stimulation alone or in combination with lysosomal inactivation in the formation of lipid degradation products in primary hepatocytes and liver slices incubated *in vitro*. We further assessed the correlation between the effect of starvation on both autophagy intensity and lysosomal lipid degradation in the liver *in vivo*.

## Material and Methods

### *Animals and experimental protocol*

Male Wistar rats (AnLab, Prague; b.wt.  $300\pm 20$  g) were kept in temperature-controlled room at 12:12-h light-dark cycle. Animals had free access to drinking water and standard chow-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 Principles of Laboratory Animal Care (NIH Guide to the Care and Use of Laboratory Animals, 8<sup>th</sup> edition, 2011) and were approved by the ethical committee of the Institute for Clinical and Experimental Medicine. Animals designed as “starved” were deprived of food 24 hrs prior the experiment, “fed” animals had free access to food.

### *Preparation and cultivation of primary hepatocytes*

The cells were prepared as described before (Rousar *et al.* 2009). Briefly, rats were narcotized, the abdomen was opened and heparin was injected into *v. cava inferior*. Cannula was fixed in *v. portae* and the liver was then perfused by solution without  $\text{Ca}^{2+}$  and collagenase (137 mM NaCl; 5.4 mM KCl; 0.4 mM  $\text{MgSO}_4$ ; 0.35 mM  $\text{Na}_2\text{HPO}_4$ ; 0.45 mM  $\text{KH}_2\text{PO}_4$ ; 26 mM  $\text{NaHCO}_3$ ; 0.5 mM EGTA; pH=7.4). Next, the liver was removed from the rat and perfused by solution with  $\text{Ca}^{2+}$  and collagenase (Sigma) (137 mM NaCl; 5.4 mM KCl; 0.4 mM  $\text{MgSO}_4$ ; 1 mM HEPES; 4 mM  $\text{CaCl}_2$ ; 0.25 g/l collagenase; pH 7.4) for 15 min. The liver was placed into Krebs-Henseleit medium and the tissue was disintegrated by slow shaking. Cell suspension was filtered through gauze and washed three times. Isolated hepatocytes were suspended in Williams' medium E supplemented with 10 % fetal bovine serum (FBS) and plated in collagen-coated 25 cm<sup>2</sup> T-flask, at the density of  $4\times 10^6$  cells per flask. The viability of the hepatocytes was >86 % determined according to the Trypan Blue exclusion. After adherence, the medium was changed and the cells were incubated overnight under conditions promoting TAG accumulation in Williams' medium E supplemented with 0.6 mM oleic acid conjugated to bovine serum albumin (Sigma), 10 % FBS, 100 nM dexamethasone, 2 mM glutamine, 1 mM pyruvate, 10 mM lactate, 80 mU/ml insulin (Humulin R, Eli Lilly). During this “pulse” period, the newly synthesized TAG were labeled by <sup>14</sup>C-oleate (S.A.1  $\times 10^6$  dpm/  $\mu\text{mol}$ ). After 18 hrs, the medium was removed and replaced with non-radioactive medium (“chase”). During this period the

cells were kept either in starving (amino acid-poor Williams' medium E supplemented only with 1 mM pyruvate) or in fed conditions (the same medium supplemented with 10 % FBS, 20 mM glutamine, 10 mM lactate, 80 mU/ml insulin). The cells were incubated for further 6 hrs in the medium alone or in the presence of autophagy flux inhibitors asparagine 20 mM, autophagy stimulator rapamycin 100 nM or inhibitor of lysosomal activity chloroquine 500  $\mu$ M (Berg *et al.* 1998).

#### *Determination of lipid degradation products in primary hepatocytes*

At the end of the chase period, the incubation medium was collected; the cells were quickly frozen by immersion of the cultivation flask into liquid nitrogen and then stored in  $-40^{\circ}\text{C}$  until the analysis. For the determination of TCA (tricarboxylic acid cycle) intermediates content, the hepatocytes were homogenised by sonication (2 x 30 sec, Hielsler ultrasonic homogeniser UP200S, Teltow, Germany) in 150 mM NaCl, extracted by petroleum ether and the radioactivity remaining in the water fraction was counted. According to Kawamura (Kawamura *et al.* 1981) this fraction represents mostly TCA cycle intermediates (>80 %) and minor part are amino acids derived from FFA *via* TCA cycle (<20 %). The  $^{14}\text{C}$  oleate incorporation into VLDL was determined in chloroform extract of the incubation medium. The conversion of  $^{14}\text{C}$  oleate into secreted water-soluble oxidation products (i.e. predominantly ketone bodies) was assessed according to the radioactivity remaining in the aqueous fraction of incubation medium after chloroform extraction.

#### *Determination of FFA and DAG production in liver slices in vitro*

Another set of experiments was designed to assess the effect of autophagy inhibition or lysosomal inactivation on FFA and DAG production in liver slices *ex vivo*. Precisely cut liver slices (150 $\pm$ 8 mg; approx. 1 mm width) were incubated in Krebs Ringer phosphate medium (2 ml) supplemented with 0.01 M KCN for 2 hours. As KCN prevents oxidation of released FFA their accumulation in the tissue serves as the indicator of lipolysis. At the end of the incubation period, the tissue was frozen in liquid nitrogen and stored in  $-80^{\circ}\text{C}$  until the analysis. The linearity of the assay was tested up 3 hours of incubation. Samples were grinded in aluminum mortar precooled in liquid nitrogen. Lipids were extracted by Folch method (Folch *et al.* 1957). The

fractions of diacylglycerols (DAG) and nonesterified fatty acids (FFA) were separated by thin-layer chromatography (TLC) according to Roemen and van der Vusse (1985). Authentic lipid class standards were spotted on the outside lanes of the TLC plates to enable localization of sample lipid fractions. The gel bands corresponding to the standards were scrapped from the plates, transferred into fresh tubes and then transmethylated in 14 % methanolic boron trifluoride (Sigma) at  $100^{\circ}\text{C}$  for either 2 (FFA) or 10 (DAG) minutes. The content of resulting fatty acid methyl esters was determined by means of gas-liquid chromatography as previously described in detail (Nawrocki *et al.* 2004). Heptadecanoic acid and 1,2-diheptadecanoin (Larodan Fine Chemicals) were used as internal standards.

#### *Preparation of lysosomal and phagolysosomal fractions*

Lysosomes and autophagolysosomes were separated according to their density by differential centrifugation (Seglen *et al.* 1985). The liver homogenate (20 % wt/vol) 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  $\mu\text{g/ml}$ , aprotinin 10  $\mu\text{g/ml}$  by teflon pestle homogenizer. The 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  $4^{\circ}\text{C}$  until lipase assay (max. 2 hour), the rest was centrifuged for 10 000 g 20 min  $4^{\circ}\text{C}$  and the resulting pellet and supernatant were separated. The supernatant preferentially contains the less dense lysosomes with higher TAG content ("autophagolysosomes", PhL), the pellet is formed by more dense particles.

#### *Determination of autophagy flux*

The most frequently used autophagy marker is the quantification of microtubule-associated protein 1 light chain 3 (LC3). LC3 is initially synthesized in an unprocessed form, proLC3, which is converted into proteolytically processed form lacking amino acids from the C-terminus, LC3-I, and is finally modified into a phosphatidylethanolamine-conjugated form, LC3-II. LC3-II is the only protein marker that is reliably associated with phagophores, sealed autophagosomes and mature autophagosomes/autolysosomes (Rubinsztein 2009). Nevertheless, LC3-II is localized both in the luminal and cytosolic site of the autophagosome and undergoes rapid degradation within the lysosome.

Consequently, a “snapshot” measurement of LC3-II content or LC3-II: LC3-I ratio is not sufficient for the determination of autophagy flux as the increased LC3-II may be the consequence of either its increased formation as well as its attenuated degradation. Similarly, the low LC3-II expression may indicate its low formation as well as its increased degradation due to the increased lysosomal activity. In our study, we estimated the autophagy flux by inferring LC3-II turnover by western blot in the presence and absence of lysosomal degradation (Klionsky *et al.* 2007). The hepatocytes were incubated either alone or in the presence of 100  $\mu$ M chloroquine, a lysosomotropic agent that increases intralysosomal pH and thus completely blocks the activity of lysosomal hydrolases. The autophagy flux is proportional to the rise in LC3-II content in the treated vs. untreated sample.

#### *Separation and immunodetection of proteins*

Homogenate, PhL and dense lysosomal fraction were prepared as described above. Proteins in particular fraction were separated by SDS-PAGE and the protein of interest was recognized by specific antibody (LAL: mouse monoclonal to lysosomal acid lipase; LAMP2: rabbit polyclonal to LAMP2, Abcam, Cambridge, UK). LC3-II content was determined in the 20 % liver homogenate lysed by 2 % SDS at 100 °C. LC3 in the lysates was recognized with rabbit polyclonal to LC3A/B (Abcam, Cambridge, UK). LC3-I and LC3-II were distinguished by difference in molecular weight (18 and 16 kDa). Despite the increased molecular weight than LC3-I, LC3-II migrates more rapidly in SDS-PAGE compared to LC3-I, likely due to higher hydrophobicity associated with the phosphatidylethanolamine group (Barth 2010). The loading control was performed using rabbit polyclonal antibody to beta actin (Abcam, Cambridge, UK). The bands were visualized using chemiluminescence and quantified using FUJI LAS-3000 imager (FUJI FILM, Japan) and Quantity One software (BioRad, Hercules, CA).

#### *Assay of lysosomal lipase activity*

The optimal conditions for the lipase assay (pH optimum, substrate concentration, reaction temperature and linear range of the assay) were determined as described previously (Cahova 2011). 4 % liver homogenate or lysosomal sub-fractions prepared from the fresh tissue under iso-osmotic conditions (250 mM sucrose) were used for the assay. The reaction medium

(92.5 kBq  $^3$ H triolein, 100  $\mu$ M cold triolein, 110  $\mu$ M lecithin, 0.15 M NaCl) was emulsified by sonication (Hielsler ultrasonic homogeniser UP200S, Teltow, Germany) in 0.1 M acetate buffer (pH=4.5) containing 6 % FFA-free BSA. The assay itself was performed under hypo-osmotic conditions in order to ensure the release of the enzyme sequestered within the lysosomes. The substrate was incubated with the enzyme (liver homogenate or subcellular fractions) for 60 min at 30 °C. The released fatty acids were extracted according to Belfrage and Vaughan (Belfrage *et al.* 1969) and counted for radioactivity.

#### *Statistical analysis*

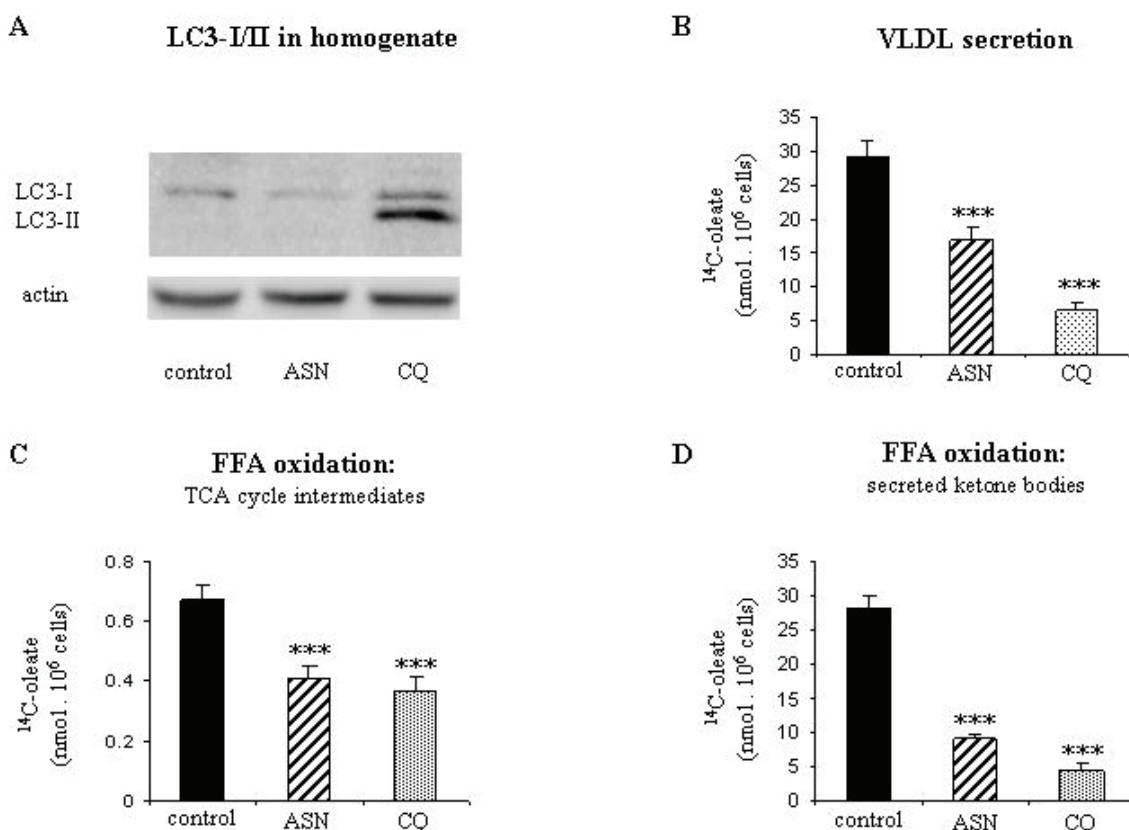
Data are presented as mean  $\pm$  SEM. Statistical analysis was performed using Kruskal-Wallis test with multiple comparisons (n=5-7). Differences were considered statistically significant at the level of  $p < 0.05$ . The correlation between FFA and DAG content in *ex vivo* incubated liver slices was evaluated using Spearman's correlation coefficient.

## **Results**

#### *Effect of autophagy and lysosomal inhibition on the utilization of lipids-derived FFA in hepatocytes*

The aim of the following experiment was to evaluate the effect of either autophagy inhibition or lysosomal inactivation in the formation of VLDL or FFA oxidation products under the situation when the intracellular TAG's are the only source for FFA-utilizing metabolic pathways. The experiment was carried out on hepatocytes cultivated in “starved” conditions, i.e. in the situation when autophagy is promoted. It was based on the rationale that if autophagy-lysosomal pathway is a significant contributor to intracellular FFA pool than the inhibition of either autophagy flux (by asparagine) or lysosomal activity (by chloroquine) would decrease the formation of lipid degradation-derived products. As shown in Fig. 1A, chloroquine effectively blocked the LC3-II degradation in autophagolysosomes. The accumulation of LC3-II in chloroquine-treated samples confirmed that the autophagy flux was preserved in our experimental setting, but that the final step, i.e. lysosomal degradation, was inhibited.

The FFA released from intracellular TAG that are not re-esterified back can either be utilized for VLDL formation or transported into mitochondria for oxidation in order to produce acetyl-CoA. Acetyl-CoA can enter



**Fig. 1.** Effect of autophagy inhibition and lysosomal inactivation on the utilization of lipids-derived FFA in starved hepatocytes. **A:** LC3-I/II content in homogenate; **B:**  $^{14}\text{C}$ -oleate incorporation into secreted TAG; **C:**  $^{14}\text{C}$ -oleate incorporation into TCA cycle intermediates; **D:**  $^{14}\text{C}$ -oleate incorporation into secreted ketone bodies. The hepatocytes were pre-labeled with  $^{14}\text{C}$ -oleate overnight and then incubated in serum- and amino acid-free medium for 6 hours without radioactive label. During this period the hepatocytes were left in the medium alone (control) or in the presence of autophagy flux inhibitor asparagine (ASN, 20 mM) or in the presence of lysosomotropic agent chloroquine (CQ, 100  $\mu\text{M}$ ) that prevent the degradation of material internalized in lysosomes.  $^{14}\text{C}$ -oleate incorporation into secreted TAG was determined as the radioactivity recovered in the chloroform extract of the incubation medium. TCA intermediates formation was assessed according to the  $^{14}\text{C}$ -oleate incorporation into intracellular water-soluble fraction. Ketone bodies production was determined as the radioactivity recovered in the aqueous fraction of the incubation medium. Data are given as mean  $\pm$  S.E.M from three independent experiments. \*\*\*  $p < 0.001$  vs. control

several metabolic pathways – it can pass through the TCA cycle and proceed to the oxidation in mitochondrial respiratory chain or, to a lesser extent, be converted into amino acids (Kawamura *et al.* 1981) or may be used for ketone body production. The effect of anti-autophagic and lysosomotropic treatment on FFA availability for further metabolic utilization is demonstrated in Fig. 1B-D.  $^{14}\text{C}$ -oleate released from intracellular lipids was nearly equally used for VLDL synthesis and for ketogenesis. TCA intermediates represented only approx 1 % of  $^{14}\text{C}$ -oleate derived radioactivity. The inhibition of autophagy flux by asparagine as well as the inactivation of lysosomes similarly decreased the availability of  $^{14}\text{C}$ -oleate for all these pathways. Taken together, we showed that both the inhibition of autophagy and the inactivation of lysosomes had significant and comparable effect in the formation of lipid degradation-dependent

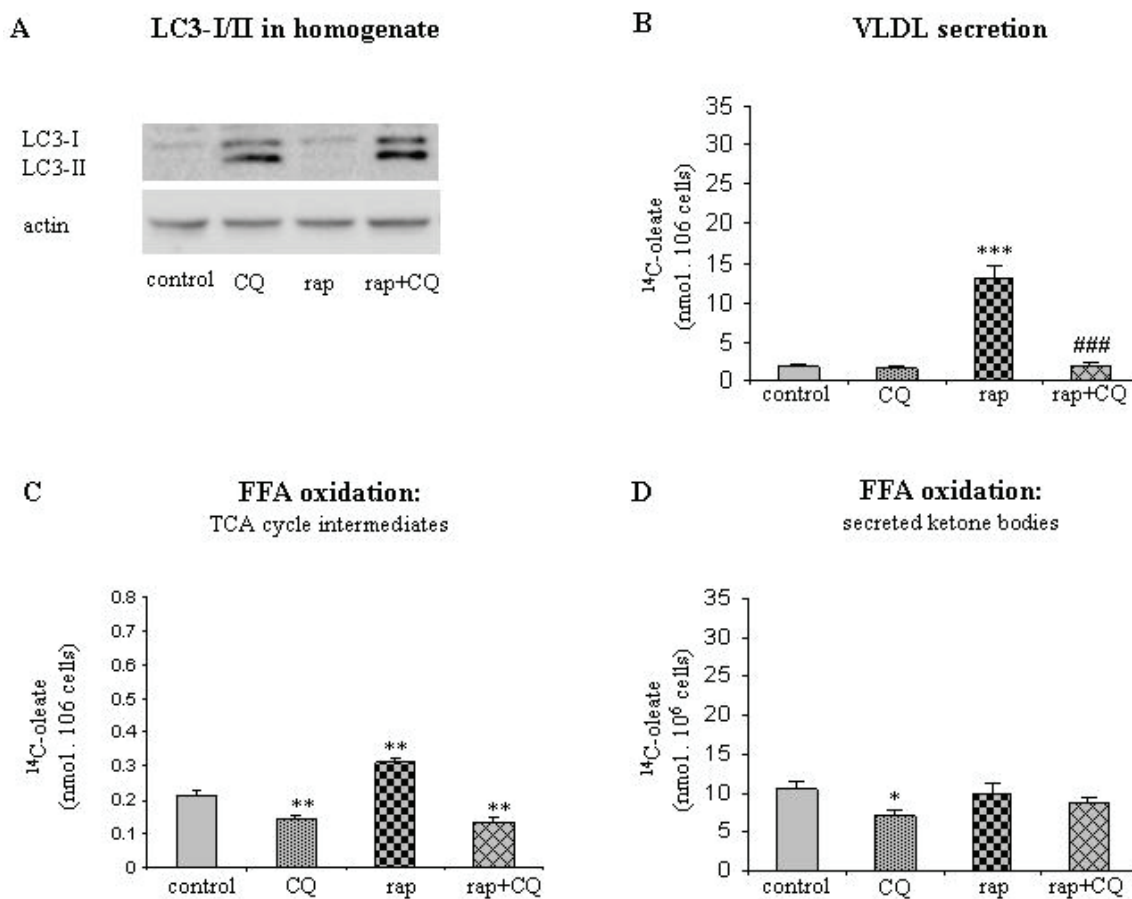
products. These data indicate that the autophagy lysosomal pathway is one of the pathways involved in the degradation of intracellular TAG.

#### *Effect of autophagy stimulation in combination with lysosomal inhibition on the utilization of lipids-derived FFA in hepatocytes*

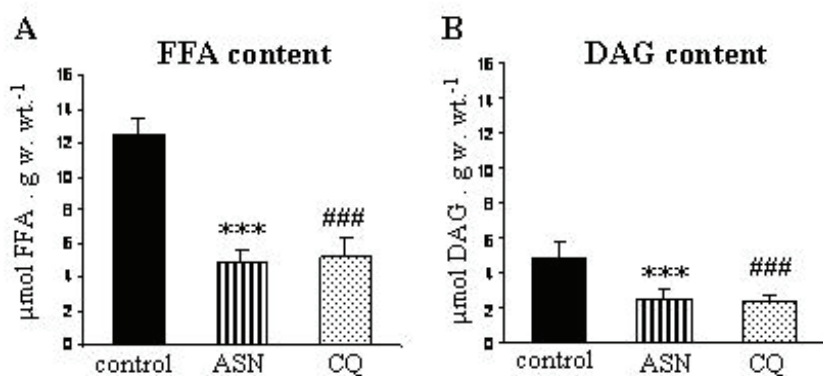
A different set of experiments was undertaken in order to answer the question whether the experimentally induced autophagy stimulation may increase the lipid degradation and FFA availability in hepatocytes and whether this effect could be prevented by the inhibition of lysosomal degradation. The hepatocytes pre-labeled with  $^{14}\text{C}$ -oleate were incubated in a “fed” condition in order to depress the autophagy intensity. Part of the hepatocytes were treated either with rapamycin, that counteracts the inhibitory effect of amino acids thereby stimulating

autophagy, or by a combination of rapamycin and lysosmotropic agent chloroquine. The results are shown in Fig. 2. The positive effect of rapamycin on autophagy intensity was confirmed by the increase of LC3-II content in the presence of rapamycin and chloroquine compared with chloroquine-only treated cells (Fig. 2A). We found no effect of the rapamycin itself on LC3-II formation and we ascribe this finding to the increased LC3-II turnover due to the stimulated autophagy. Rapamycin treatment led to a significant increase of  $^{14}\text{C}$ -oleate content in secreted TAG (Fig. 2B). The addition of chloroquine to rapamycin-treated hepatocytes completely abolished this effect and attenuated VLDL secretion from control hepatocytes as well. We found only subtle effect of rapamycin in the TCA cycle

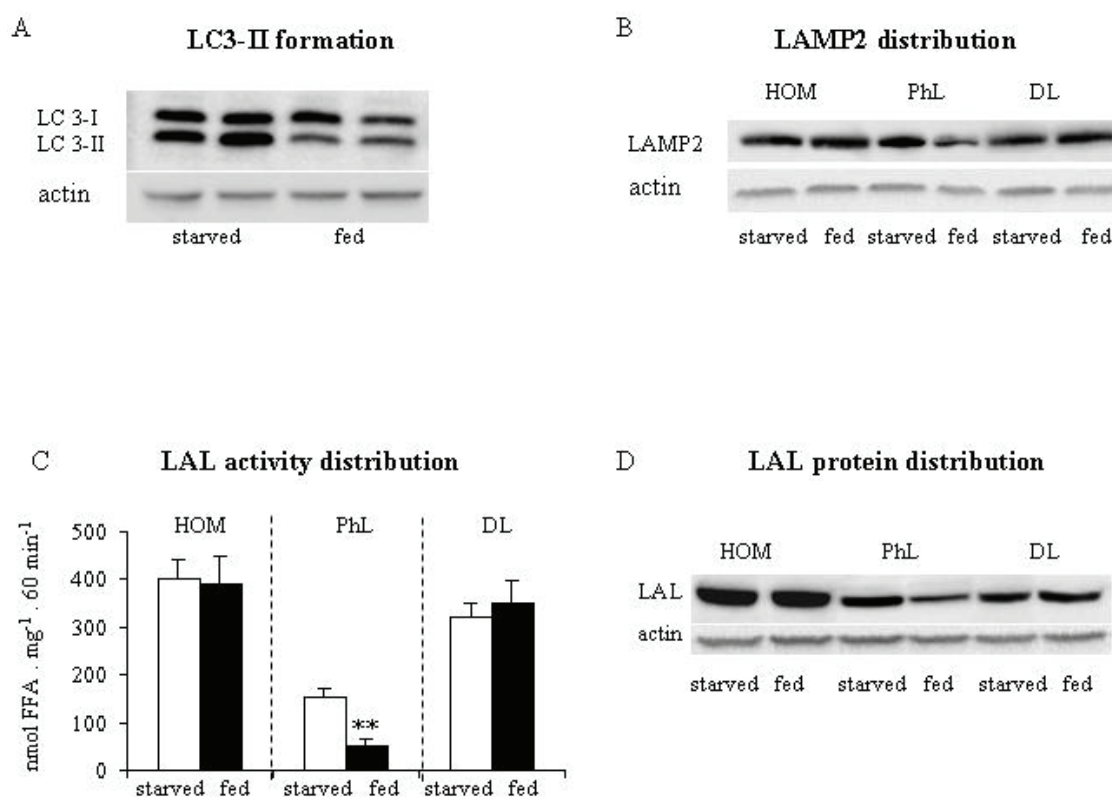
intermediates formation and no effect on ketogenesis (Fig. 2C,D). The  $^{14}\text{C}$ -oleate utilization for VLDL secretion or ketogenesis was significantly decreased in control “fed” hepatocytes (Fig. 2B,D) compared with control “starved” cells (Fig. 1B,D) which suggest the partial inhibition of lipid degradation under fed conditions. The conclusions derived from this experiment show that FFA availability in isolated hepatocytes is lower in the situation when autophagy is depressed (“fed” condition), that FFA availability for VLDL formation (and to a much smaller degree for FFA oxidation) could be increased by autophagy stimulation and that this effect is completely dependent on preserved activity of lysosomes.



**Fig. 2.** Effect of autophagy stimulation in combination with lysosomal inactivation on the utilization of lipids-derived FFA in fed hepatocytes. **A:** LC3-I/II content in homogenate; **B:**  $^{14}\text{C}$ -oleate incorporation into secreted TAG; **C:**  $^{14}\text{C}$ -oleate incorporation into TCA cycle intermediates; **D:**  $^{14}\text{C}$ -oleate incorporation into secreted ketone bodies. The hepatocytes were pre-labeled with  $^{14}\text{C}$ -oleate overnight and then incubated medium supplemented with 10 % FBS for 6 hours without radioactive label. During this period the hepatocytes were left in the medium alone (control) or in the presence of autophagy flux stimulator (rapamycin 100 nM) or in the presence of lysosmotropic agents (chloroquine 100  $\mu\text{M}$ ) that prevents the degradation of material internalized in lysosomes.  $^{14}\text{C}$ -oleate incorporation into secreted TAG was determined as the radioactivity recovered in the chlorophorm extract of the incubation medium. TCA intermediates formation was assessed according to the  $^{14}\text{C}$ -oleate incorporation into intracellular water-soluble fraction. Ketone bodies production was determined as the radioactivity recovered in the aqueous fraction of the incubation medium. Data are given as mean  $\pm$  S.E.M from three independent experiments. \*\*  $p < 0.01$  \*\*\*  $p < 0.001$  vs. control; ###  $p < 0.001$  CQ+ rap vs. rapamycin only treated cells.



**Fig. 3.** Effect of autophagy inhibition and lysosomal inactivation on FFA (A) and DAG (B) production in liver slices *in vitro*. Liver slices from fasted rats administered either SD diet were incubated under non-oxidative conditions (0.01 M KCN) in the KRF medium alone (control) or in the presence of autophagy flux inhibitor asparagine (ASN, 20 mM) or lysosomal activity inhibitor chloroquine (CQ, 100 µM) for 120 min. At the end of the incubation, the FFA and DAG content in the tissue was determined as described in Material and Methods. Data represent mean  $\pm$  S.E.M., n=6. \*\*\*p<0.001 asparagine-treated vs. control samples; ###p<0.001 CQ-treated vs. control samples.



**Fig. 4.** The effect of starvation on autophagolysosome formation and lipolytic activity *in vivo*. **A:** LC3-I and LC3-II protein expression in homogenate. **B:** LAMP2 expression in subcellular fractions. The PhL and DL fractions were prepared by differential centrifugation (see Material and Methods). **C:** LAL activity in homogenate and subcellular fractions. The activity of LAL was measured as the release of FFA from <sup>3</sup>H-triolein at pH=4.5. **D:** LAL protein expression in homogenate and subcellular fractions. Values represent means  $\pm$  S.E.M. of 7 animals. Open bars = starved animals; black bars = fed animals, \*\* p<0.01 fed vs. starved.

#### Effect of autophagy and lysosomal inhibition on lipid degradation products formation in liver slices *in vitro*

In order to verify the data obtained on primary hepatocytes we determined the effect of autophagy inhibition and lysosomal inactivation on the production of FFA and DAG in liver slices from fasted animals incubated in the presence of 0.01 M KCN. Under such conditions the oxidative utilization of FFA is completely

blocked but lysosomal lipolysis is unaffected and is linear for at least 4 hours (Mosinger *et al.* 1965). Consequently, the released FFA and DAG remain in the tissue. There was a linear correlation between DAG and FFA content ( $R^2=0.87$ ). As shown in Fig. 3 the formation of lipid degradation products, FFA and DAG, was significantly diminished by both autophagy flux inhibitor asparagine and by lysosomotropic agent chloroquine.



### *Effect of starvation on autophagolysosome formation and lipolytic activity in vivo*

The effect of starvation on autophagy, lysosomal translocation into autophagolysosomes and LAL activity and protein intracellular distribution was determined in the liver of rats administered SD who had either free access to food ("fed") or were deprived of food for 24 hours ("starved"). The autophagosome formation was estimated according to the LC3-II/LC3-I ratio. As shown in Fig. 4A, the LC3-II/LC3-I ratio was elevated in the SD group after starvation and low in the fed condition. In accordance with the hypothesis that autophagy pathway involves the conversion of lysosomes into autophagolysosomes and consequently the degradation of their cargo (lipid droplets), we found that starvation led to the increased abundance of lysosomal marker, LAMP2 protein, in autophagolysosomal fraction (Fig. 4B) but it had no effect on total LAMP2 expression. If our presumption that autophagy mediates the formation of activated autophagolysosomes containing LAL is correct, than the corresponding changes in its intracellular distribution should occur in response to starvation. LAL activity in particular subfractions (Fig. 4C) was determined using exogenous substrate ( $^3\text{H}$ -triolein). Starvation did not affect the total LAL activity in the whole-liver homogenate and in the fraction of dense lysosomes (DL) that represents the reserve pool of the enzyme which is not in contact with the substrate (lipid droplets) and thus probably inactive under physiological condition. In contrast, starvation significantly increased LAL activity associated with PhL fraction being 100 % higher in starved animals compared with the fed ones. These results corresponded well with the effect of starvation on the distribution of LAL protein among subcellular fractions (Fig. 4D). We found no effect of prandial status on LAL protein expression in homogenate and in DL fraction but a significant elevation of LAL protein amount in response to starvation was found in PhL fraction.

## **Discussion**

In this study we present evidence that the autophagy-lysosomal pathway contributes to the degradation of intracellular lipids in the liver. Our conclusions are based on following findings: 1) in primary hepatocytes, inhibition of autophagy as well as inhibition of lysosomal activity, decreased formation of lipid degradation-derived metabolites, i.e. TCA cycle

intermediates and ketone bodies, and VLDL secretion; 2) in the same model, stimulation of autophagy by rapamycin resulted in the increased VLDL production and this effect was completely abolished by the inactivation of lysosomes; 3) in liver slices incubated *in vitro*, both inhibition of autophagy and lysosomal inactivation prevented the FFA and DAG formation from intracellular lipids; 4) the autophagolysosome formation (determined as LC3-II/I ratio and LAMP2 translocation into PhL fraction) and the translocation of lysosomal lipase into autophagolysosomal fraction share the same prandial-dependent pattern of regulation in the liver of rats fed SD, i.e. stimulation in response to starvation and down-regulation in fed state.

The first hints suggesting the importance of autophagy in lipid metabolism came from Debeer *et al.* (1979) who demonstrated on isolated hepatocytes that the inhibition of lysosomal activity decreased not only protein degradation but that it inhibited also ketone body production. He suggested that autophagocytosis may be the likely mechanism of how the intracellular triglycerides may be taken up by the lysosomes. Thirty years later, Singh *et al.* (2009) using an electron microscopy approach, showed that lipid droplets or their parts are engulfed in autophagosome-like structures during starvation and are degraded in lysosomes *via* specific autophagy-related pathway, macrolipophagy. In contrast to their findings, Shibata *et al.* (2009) proposed an alternative role for autophagic machinery in lipid metabolism in hepatocytes. They suggested that LC3 is involved not only in lipid degradation but also in the process of lipid droplet formation in non-adipose tissues. Surprisingly both authors, using the same model, reported different effects of autophagy deficiency on TAG storage. Singh observed an increased TAG accumulation and lipid droplets content while Shibata reported decreased amount of lipid upon fasting in the same model of autophagy-deficient hepatocytes. This apparent contradiction clearly indicates some differences in experimental design – in fact, the experiments of Shibata were carried out on young, one month old mice while the data reported by Singh were obtained on 5-month old animals. Further studies are necessary in order to clarify this issue.

Our data supports the hypothesis of autophagy-mediated lipid degradation in the liver. In our experimental setting, the freshly isolated hepatocytes were pre-incubated with  $^{14}\text{C}$ -oleate overnight in order to label intracellular lipids on acyl moiety and the cells were then cultivated in the absence of any exogenous fatty



acids for 6 hrs. Under these conditions, the secreted VLDL and products of fatty acid oxidation had to be derived only from intracellular lipids and quantification of these products may serve as an indicator of lipolysis. As stated in Materials and Methods the lipid fraction either from cells or medium was extracted according to Folch (1957) and the individual lipid classes of intracellular lipids were not separated by thin layer chromatography. Nevertheless we suppose that most of the radioactivity detected in TCA, water-soluble fraction or VLDL are derived from TAG. Our assumption is based on the data published by Woodside *et al.* (1989) who analyzed the deposition of  $^{14}\text{C}$ -oleate into lipid fractions in isolated hepatocytes after 90 min incubation and demonstrated that most (88 %) of radioactivity was found in TAG. Francone *et al.* (1992) reported that incorporation of  $^{14}\text{C}$ -oleate after *in vivo* application into TAG, PL and cholesteryl esters (CE) is different in microsomes (consisting mostly of endoplasmic reticulum) and floating fat (consisting mostly of cytosolic lipid droplets). While in microsomes nearly half of the  $^{14}\text{C}$ -oleate was incorporated into PL, in floating fat 97.6 % of radioactivity was detected in TAG, 2.26 % in phospholipids (PL) fraction and 0.21 % in CE. Autolipophagy is supposed to be involved solely in the degradation of cytosolic lipid droplets in which the PL and CE labeling with  $^{14}\text{C}$ -oleate is rather insignificant.

When the hepatocytes were cultivated in amino acid-poor medium (i.e. under the autophagy-promoting conditions), we found that inhibition of autophagy flux by asparagine (Berg *et al.* 1998) decreased the formation of VLDL, TCA cycle intermediates and ketone bodies. A comparable effect was observed also when the autophagy was not influenced but the lysosomal activity was inhibited by chloroquine. Interestingly, Wiggins and Gibbons (1992) found no effect of chloroquine on TAG mobilization for VLDL secretion. To our opinion this discrepancy could be explained by differential experimental setting. In the study of Wiggins and Gibbons the hepatocytes were cultivated in the presence of amino acid mixture during the experimental phase (the composition and the concentrations were not described in detail). Amino acids are widely used inhibitors of autophagy and if the autophagy is down-regulated, the inhibition of lysosomal activity could have no additional effect. In accordance with this we also saw virtually no effect of chloroquine on VLDL secretion in fed hepatocytes as it will be discussed further. Furthermore, the dose of chloroquine used by Wiggins and Gibbons

was quite low (10  $\mu\text{M}$ ). In comparable studies focused on the manipulations of lysosomal degradation of either proteins or lipids the chloroquine concentrations administered *in vitro* vary from 0.1 mM (Francone *et al.* 1992) to 1 mM (Furuya *et al.* 2001).

In another experimental setting we tested the possibility of increasing the lipid degradation by stimulation of autophagy. The hepatocytes were incubated in the presence of exogenous proteins and amino acids in order to attenuate autophagy and in some of them autophagy was stimulated by rapamycin that attenuates amino acid-dependent inhibition of autophagy *via* mTOR/TORC1 signaling. The stimulation of autophagy significantly increased the formation VLDL and this effect was completely prevented by the inhibition of lysosomal activity. The autophagy stimulation by rapamycin did not elevate the ketone body production in this setting, i.e. under “fed” conditions. Ketogenesis has three highly regulated control points: 1. FFA supply (from the diet, from adipose tissue or from intrahepatic TAG), 2. mitochondrial fatty acids entry and 3. mitochondrial HM-CoA synthase. The second and third processes are down-regulated in a fed state and a simple increase of FFA availability cannot overcome this regulation. Consequently, the FFA surpluses are directed only into VLDL secretion (Fukao *et al.* 2004). Furthermore, the formation of lipid degradation-dependent products was significantly lower in hepatocytes cultivated in “fed” conditions (with serum) than in those incubated in “starved” conditions (without serum) which correlated with the autophagy intensity. Nevertheless, the inhibition of autophagy-lysosomal pathway did not abolish the lipid degradation completely which suggests that this pathway is an important but not the only mechanism involved in liver TAG metabolism.

The evidence obtained on primary hepatocytes were further confirmed by the analysis of the effect of autophagy/lysosomal inhibition on DAG and FFA production in liver slices incubated *in vitro* in the presence of KCN. Under these conditions, the lipolysis occurs but the further utilisation of lipid degradation products is completely blocked (Vavrinkova and Mosinger 1971). Similarly to primary hepatocytes, asparagine as well as chloroquine significantly decreased FFA and DAG formation. The FFA : DAG ratio was 2.46 which suggests that approx. one half of DAG undergoes further degradation. The DAG molecule may be further degraded by some other lipase (i.e. by TGH) or remodelled by transacylation (Sugiura *et al.* 1988). Similar

results were reported by Lankester on primary hepatocytes culture (Lankester *et al.* 1998). This data indicate that at least part of the TAG in the liver may be degraded in lysosomes and that the normal autophagy flux is necessary for this process.

*In vivo*, the regulation of autophagy is determined by its main function which consists in maintaining energy balance during the period of nutrient shortage. It has been shown that autophagy is increased during acute (24 hrs) starvation. The increased autophagy determines the increased delivery of intracellular material into autophagolysosomes and consequently, its decreased degradation and release of degradation products. In our study we proved this regulatory pattern in animals *in vivo*. In this group, fasting was associated with higher autophagy together with the increased translocation of LAL activity, LAL protein and LAMP2 lysosomal marker into autophagolysosomal fraction in fasted compared to fed animals. Nevertheless, the exact data directly showing the contribution of the autophagy-derived FFA to VLDL production or FFA oxidation *in vivo* are still lacking. In this context it is important to mention the finding of Du *et al.* (2001) who showed that LAL KO mice exhibit normal triglyceridemia. This rather surprising observation could be explained as the result of double defect in two metabolic pathways, both of them

associated with LAL – the VLDL production and LDL removal. This assumption is supported by the fact that LDL fraction is highly significantly enriched in agarose electrophoretogram of LAL KO mice serum. Unfortunately, VLDL production was not determined in this paper but we suppose that if the VLDL secretion was normal concomitantly with impaired LDL removal it would have resulted into significant hypertriglyceridemia that was not observed in this model.

In conclusion, we have demonstrated that the manipulation of either autophagy or lysosomal activity independently and comparably affects intracellular lipid degradation and lipid-degradation products formation in primary hepatocytes and liver slices incubated *in vitro*. Furthermore, we demonstrated that autophagy and lysosomal lipolysis are regulated similarly *in vivo*. Based on these findings we propose that at least part of the TAG in the liver is degraded in lysosomes and that the normal autophagy flux is necessary for this process.

### Conflict of Interest

There is no conflict of interest.

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