Original Paper

Lysophosphatidic Acid Inhibits Insulin Signaling in Primary Rat Hepatocytes via the LPA₃ Receptor Subtype and is Increased in Obesity

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Key Words

Lysophosphatidic acid • Insulin signaling • Adipose tissue • Autotaxin • Hepatic insulin resistance • LPA₃ receptor subtype

Abstract

Background/Aims: Obesity is a main risk factor for the development of hepatic insulin resistance and it is accompanied by adipocyte hypertrophy and an elevated expression of different adipokines such as autotaxin (ATX). ATX converts lysophosphatidylcholine to lysophosphatidic acid (LPA) and acts as the main producer of extracellular LPA. This bioactive lipid regulates a broad range of physiological and pathological responses by activation of LPA receptors (LPA₁₋₆). **Methods:** The activation of phosphatidylinositol 3-kinases (PI₃K) signaling (Akt and GSK-3β) was analyzed via western blotting in primary rat hepatocytes. Incorporation of glucose into glycogen was measured by using radio labeled glucose. Real-time PCR analysis and pharmacological modulation of LPA receptors were performed. Human plasma LPA levels of obese (BMI > 30, n = 18) and normal weight individuals (BMI 18.5–25, n = 14) were analyzed by liquid chromatography tandem-mass spectrometry (LC-MS/MS). **Results:** Pretreatment of primary hepatocytes with LPA resulted in an inhibition of insulin-mediated Gsk expression, PI₃K activation and glycogen synthesis. Pharmacological approaches revealed that the LPA₃-receptor subtype is responsible for the inhibitory effect of LPA on insulin signaling. Moreover, human plasma LPA concentrations (16:0 LPA) of obese participants (BMI > 30) are significantly elevated in comparison to normal weight individuals (BMI 18.5–25). **Conclusion:** LPA is able to interrupt insulin signaling in primary rat hepatocytes via the LPA₃ receptor subtype. Moreover, the bioactive lipid LPA (16:0) is increased in obesity.

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Introduction

Hepatic insulin resistance is a major mediator for the progression of type 2 diabetes mellitus (T2DM). This pathological condition is manifested by blunted insulin ability to induce glucose utilization in hepatocytes and to suppress hepatic gluconeogenesis, which leads to the development of hyperglycemia. Obesity and adipocyte hypertrophy are associated with hepatic insulin resistance; however the molecular mechanisms responsible for these interactions have been poorly understood [1, 2]. Adipose tissue regulates insulin sensitivity and energy homeostasis of peripheral tissues via the secretion of adipokines. The first adipokine to be discovered was leptin, which promotes metabolic health by increasing fat oxidation in muscle cells and modulates several metabolic regulators such as fibroblast growth factor 21 [3]. More recently, the ectoenzyme autotaxin (ATX) was discovered converting lysophosphatidylcholine (LPC) into lysophosphatidic acid (LPA) [4]. LPA (1-acyl 2-hydroxy-*sn*-glycerol-3-phosphate) are bioactive lipid mediators that act as extracellular signaling molecules by binding at least 6 specific LPA receptors (LPA₁₋₆) [5]. The length of the acyl chain and its degree of saturation depend on the precursor molecule. Between the different species, LPA 18:1 and LPA 16:0 are the most abundant lysophosphatidic acids in the circulation [6]. In addition to the well-established effects of LPA in physiological functions such as cell proliferation, migration, differentiation and maintaining fluid homeostasis, a growing body of evidence suggests that ATX/LPA is involved in pathological conditions [7-9]. Recent studies demonstrated that ATX is aberrantly expressed in obesity, which leads to insulin resistance and impaired glucose homeostasis.

Furthermore, administration of LPA to obese prediabetic glucose-intolerant mice diminished insulin secretion and induced a hyperglycemic condition. Moreover, these studies also indicated that LPA plasma levels were increased in the high fat diet fed mice compared to controls. Inhibition of LPA signaling improved insulin tolerance, glycogen storage, and glucose use in these mice, possibly by enhanced pancreatic secretion of insulin [10-12]. However, the molecular mechanisms of LPA signaling on insulin sensitivity are not well characterized.

The insulin signaling cascade is initiated by binding of insulin to the insulin receptor (IR) followed by an autophosphorylation on several intracellular tyrosine residues on the IR and the subsequent phosphorylation of insulin receptor substrates (IRS). One major branch of intracellular insulin signaling is the activation of phosphatidylinositol 3-kinases (PI₃K)/Akt pathway, which mediates most of metabolic actions of insulin specially the regulation of glucose homeostasis. Stimulation of the PI₃K/Akt pathway directly induces the phosphorylation and subsequent inactivation of glycogen synthase kinase-3 β (GSK-3 β) which in turn activates glycogen synthase. Moreover, PI₃K/Akt activation is involved in the modulation of *glucokinase* (*Gck*) expression and hence activity. Both, enhanced Gck activity and inactivation of GSK-3 β in response to insulin contribute to glycogen synthesis [13]. To examine the role of LPA on hepatic insulin signaling, primary rat hepatocytes were used and insulin-mediated *Gck* expression, PI₃K activation and glycogen synthesis as markers for insulin sensitivity were examined in the presence of LPA. Our data clearly indicate that the treatment of hepatocytes with LPA impaired insulin-mediated signaling via the LPA₃ receptor subtype. Indeed, the ATX/LPA pathway in adipose tissue has recently been implicated in obesity and insulin resistance in animal models and moreover an increase of circulating ATX has been shown in older humans with obesity. However, the role of circulating LPA in humans remains unclear. In this study we have shown that plasma concentrations of a specific LPA species is elevated in obese humans compared to normal weight individuals. Taken together, ATX-mediated LPA production, and LPA₃ signaling seems to be implicated in obesity associated hepatic insulin resistance and the instigation of abnormal glucose utilization. These data suggest that the inhibition of LPA₃ signaling in obese or diabetic patients may be a possible treatment option for these disorders.

Materials and Methods

Materials

Narcocleen was purchased from Merial GmbH (Hallbergmoos, Germany). Percoll™ and D-[U-¹⁴C] glucose were obtained from GE Healthcare (Freiburg, Germany). LPA (18:1, 17:0 and 16:0), VPC-12249 ((S)-phosphoric acid mono-[3-(4-benzyloxy-phenyl)-2-octadec-9-enoylamino-propyl] ester) and (2S)-OMPT (1-oleoyl-2-methyl-sn-glycero-3-phosphothionate) were purchased from Avanti Polar Lipids (Alabaster, AL). Monoclonal rabbit anti-phospho Akt (Ser⁴⁷³) antibody, monoclonal anti-total Akt antibody, monoclonal rabbit anti-phospho GSK-3β (Ser⁹) antibody, secondary anti rabbit IgG HRP linked antibody as well as LumiGLO reagent and peroxide chemiluminescent substrate were from Cell Signaling Technology (Frankfurt a. M., Germany). Polyclonal rabbit anti-Gck (H-88) antibody was purchased from Santa Cruz Biotechnology (Dallas, TX). Anti-β-actin HRP linked antibody was obtained from abcam (Cambridge, UK). Primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany). NCS was purchased from Biochrom (Berlin, Germany). All other chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany).

Animal Experiments

Male Wistar rats (200-300 g) were obtained from Charles River (Sulzfeld, Germany) and were kept on a 12 h day/night rhythm with free access to water and a standard 1326 rat diet from Altromin (Lippe, Germany). All animal experiments have been conducted according to relevant national and international guidelines and were performed with permission of the state animal welfare committee [A-01-14-Uni].

Preparation and cultivation of primary rat hepatocytes

Density gradient purified hepatocytes were prepared as described previously [14]. The cells were plated on 35-mm diameter culture plates (1 × 10⁶ cells/plate) in M199 containing 1% (v/v) antibiotics (10 U/μg penicillin, 10 μg/μL streptomycin), 100 nM dexamethasone, 0.5 nM insulin and 4% (v/v) NCS. After initial 4 h incubation for the attachment of the cells to the substratum, the medium was changed to Williams' medium E 1% (v/v) antibiotics (10 U/μg penicillin, 10 μg/μL streptomycin), 100 nM dexamethasone and 0.5 nM insulin without NCS. Experimental treatments were performed after 44 h of culture in Williams' medium E containing 1% (v/v) antibiotics and 100 nM dexamethasone.

Quantitative real Time PCR

To investigate mRNA expression of LPA receptors unstimulated rat hepatocytes were used. To analyze the role of LPA on *Gck* and *SREBP-1c* expression primary rat hepatocytes were incubated for 5 h with different concentration of LPA (16:0 and 18:1) and subsequently stimulated with 10 nM insulin for 2 h. Total RNA was isolated using the total RNA isolation system (Roboklon, Berlin, Germany). Reverse transcriptase reaction was carried out using the FermentasAid™ first strand cDNA synthesis kit (Fermentas GmbH, St. Leon-Rot, Germany) according to the instruction of the provider. 50 ng cDNA solution was subjected to quantitative real-time PCR using a LightCycler480 and the SYBR-Green PCR master mix (Roche Diagnostics, Applied Science, Mannheim, Germany). β-Actin was used as normalization control. The thermal cycle profile used was denaturing for 10 s at 95°C, annealing primers for 20 s at 57°C, and extending the primers for 30 s at 72°C. The PCR amplification was performed at 45 cycle with monitoring fluorescence.

The following primers were used for rat: *Gck* (forward 5'- GCC GTG CCT GTG AAA GCG TGTC -3' and reverse 5'- CCA CCC GTA GCA GCA GAA TAG GTC -3'), *LPA₁* (forward 5'- TGT GCT GGG TGC CTT TAT TGT -3' and reverse 5'-AAG GTG GCG CTC ATC TCTTT-3'), *LPA₂* (forward 5'-AGA ATG GCC ACC CAC TGATG-3' and reverse 5'- ACA GTT ACA GCA CCA CGGAG -3'), *LPA₃* (forward 5'- TCC TCT CTG GCC CCG ATTTA -3' and reverse 5'- CAC ACC ACG AAG GCT CCTAA -3'), *LPA₄* (forward 5'- AAG TGC GAG TTG CCC GTTTA -3' and reverse 5'- ATT CCT CGA ATG AGT GCC CAAAA -3'), *LPA₅* (forward 5'- CCA AGC ACA GGT CTC CACTT -3' and reverse 5'- AGA GCA TGG CTT TCA CCTCC -3'), *LPA₆* (forward 5'- AAC ACG GAA TTG GCC GTTTG -3' and reverse 5'- GAA AGA AAA CTG CAG GCGCA -3'), β-actin (forward 5'- CCC TAA GGC CAA CCG TGA AAA GATG -3' and reverse 5'- AGG TCC CGG CCA GCC AGG TCCAG -3') and *SREBP-1c* (forward 5'- ACG ACG GAG CCA TGG ATTG -3', and reverse 5'- TTT GAT TCC AGG CCC AGGGG -3').

D-[U-¹⁴C] glucose incorporation into glycogen

Glycogen synthesis was assessed by measuring D-[U-¹⁴C] glucose incorporation into glycogen as recently described [15]. Briefly, cultured hepatocytes were preincubated for 5 h with 10 μM LPA 18:1, LPA

16:0, (2S)-OMPT in M199 medium containing 1% penicillin/streptomycin and 100 nM dexamethasone. Subsequently the medium was replaced by medium containing 1 Ci/mL D-[U-¹⁴C] glucose and after 30 min hepatocytes were stimulated with 10 nM insulin for 2 h. Glycogen was precipitated and the amount of D-[U-¹⁴C] glycogen was determined by scintillation counting.

Immunoblotting

Cultured rat hepatocytes were preincubated with Williams' medium E containing 1% (v/v) antibiotics and 100 nM dexamethasone for 4 h and then stimulated with 10 μM LPA 18:1 and LPA 16:0 for the indicated time periods. Subsequently, cells were treated with 10 nM insulin for 10 min, washed with ice-cold PBS and frozen in liquid nitrogen. Western blot analysis was performed as recently described [16]. Briefly, cells were lysed in RIPA buffer and lysates were centrifuged and boiled in SDS sample buffer. After separation by SDS-PAGE, gels were blotted onto PVDF-membranes and blocked with nonfat dry milk. Membranes were incubated with the primary antibodies followed by an incubation with secondary anti rabbit IgG HRP linked antibodies. Detection was performed with LumiGLO according to the manufacturer's protocol using a ChemiDoc XRS+system (Bio-Rad Laboratories GmbH, Muenchen, Germany). Values of the phosphorylated proteins were normalized to the corresponding total protein level.

Quantification of LPA species in human plasma using liquid chromatography tandem-mass spectrometry (LC-MS/MS)

Plasma samples (100 μL) were thawed on ice prior to addition of 1 pmol of 1-heptadecanoyl lysophosphatidic acid (17:0 LPA) as internal standard. Then, 1 mL 30 mM citric acid /40 mM sodium hydrogen phosphate buffer (pH 4.0), 2 mL 1-butanol and 1 mL water-saturated 1-butanol were added to the samples. Lipid extraction was carried out by extensive mixing (10 min at 1,500 rpm) followed by centrifugation for 5 min at 2,300 x g (4°C). The upper organic phases were transferred to new sample tubes and, subsequently, evaporated to dryness under reduced pressure. The dried residues were reconstituted in 50 μL methanol, thoroughly vortexed (5 min at 1,500 rpm) and centrifuged for 5 min at 2,300 x g (4°C). The supernatants were subjected to LC-MS/MS analysis. In parallel to the plasma samples, a matrix-matched external calibration was performed. For that, 100 μL 4.5% fatty acid-free bovine serum albumin in PBS (pH 7.4), spiked with 1-palmitoyl lysophosphatidic acid (16:0 LPA) and 1-oleoyl lysophosphatidic acid (18:1 LPA) in the range of 0-50 nM as well as constantly 10 nM 17:0 LPA were treated as described for plasma samples. Analyses were conducted with an Agilent 1260 Infinity LC system coupled to an Agilent 6490 triple quadrupole-mass spectrometer (Agilent, Waldbronn, Germany) interfaced with an electrospray ion source operating in the negative ion mode (ESI-). Chromatographic separation of LPA species was carried out using an Agilent Poroshell 120 EC-C8 column (2.7 μm, 3.0 x 150 mm). Aqueous ammonium acetate (50 μM) and methanol/acetonitrile 9:1 (v:v) were used as eluents A and B, respectively. Samples (10 μL) were injected into a mobile phase consisting of 88% eluent B. Analytes were eluted from the column, which was tempered at 30°C, with a 15-min linear gradient to 100 % eluent B at a flow rate of 0.5 mL/min. The total run time for one analysis was 26 min, including re-equilibration of the LC system. The following ion source parameters were determined after repeated injection of a standard solution containing 16:0 and 18:1 LPA using the Source Optimizer tool of the Agilent MassHunter Workstation Software (Version B.06.00): drying gas temperature = 280°C, drying gas flow = 11 L/min of nitrogen, sheath gas temperature = 400°C, sheath gas flow = 10 L/min of nitrogen, nebulizer pressure = 10 psi, capillary voltage = 4500 V, nozzle voltage = 2000 V. The optimized ion funnel parameters were: high pressure RF voltage = 130 V and low pressure RF voltage = 160 V. Quantification of five LPA species (16:0, 18:0, 18:1, 20:4 and 22:6), normalized to the internal standard 17:0 LPA, by means of external calibration was carried out using the multiple reaction monitoring (MRM) approach. Deprotonated molecular ions [M-H]⁻ were selected as precursor ions (*m/z* 409.2, 423.3, 435.3, 437.3, 457.2 and 481.2 for 16:0, 17:0, 18:1, 18:0, 20:4 and 22:6 LPA) and two characteristic fragmentations, [M-H]⁻ > *m/z* 153.0 (quantifier) and [M-H]⁻ > *m/z* 79.0 (qualifier), were recorded for each LPA species. The optimized collision energies for the quantifier MRM transitions, which were determined using the Optimizer tool of the MassHunter Software, were 20 eV (18:1, 18:0, 20:4 and 22:6 LPA) or 24 eV (16:0 and 17:0 LPA), respectively. The dwell time for all 12 MRM transitions recorded was 100 ms.

LPA profile in human plasma samples

The recruitment of patients was carried out at one of the rehabilitation centers of Brandenburg (Klinik am See, Rüdersdorf, Germany). Approval of the protocol was obtained by the ethic review board of

Brandenburg [ethic number 9/2016]. Inclusion criteria were patients aged between 40-65 years with a BMI of 18-25 in the control group ($n = 14$) and a BMI of ≥ 30 ($n = 18$) in the verification group. Exclusion criteria were operated patients (< 6 weeks prior to blood sampling) and diabetics. Plasma samples were collected and the quantification of LPA was performed via LC-MS/MS as described.

Statistical Analyses

Data are presented as the mean $\pm$ SEM of results of ≥ 3 independent experiments. Statistical analyses (t tests) were performed using GraphPad Prism 6.0 software (GraphPad Software, La Jolla, CA). * $p < 0.05$, ** $p < 0.01$ *** and $p < 0.001$ indicate a statistically significant difference vs. control experiments.

Results

LPA impairs insulin-induced PI_3K activation in hepatocytes

The PI_3K /Akt signaling cascade plays a crucial role in insulin-mediated glucose homeostasis in hepatocytes. To evaluate the role of LPA on insulin signaling the phosphorylation of Akt was analyzed via western blotting. As expected the treatment of hepatocytes with insulin induced a significant increase in Akt-phosphorylation. Indeed, pretreatment of cells with the most prominent LPA species, namely LPA 16:0 and LPA 18:1, reduced insulin-mediated Akt-phosphorylation significantly (Fig. 1A). As presented in Fig. 1B, LPA was able to interrupt insulin-induced Akt-phosphorylation in a time-dependent manner with a maximum after 15 min and a significant reduction of almost 50%.

LPA inhibits insulin-stimulated *Gck* and *SREBP-1c* expression

Gck is the rate limiting enzyme in hepatic glucose utilization and provides the substrate for glycogen synthesis and glycolysis via the phosphorylation of glucose to glucose-6-phosphate. It is well known that insulin induces *Gck* mRNA expression by upregulating the transcription factor *SREBP-1c* and the activation of PI_3K signaling. To investigate the role of LPA on insulin-induced *Gck* mRNA expression, primary rat hepatocytes were treated with different concentrations of LPA for 5 h and subsequently stimulated with insulin for 2 h.

Fig. 1. Effect of LPA on insulin-mediated Akt-phosphorylation in primary rat hepatocytes. Primary rat hepatocytes were preincubated with either LPA 16:0 or 18:1 (10 μ M) for 15 min followed by insulin stimulation (10 nM) for 10 min (A). Cells were treated with LPA 16:0 for the indicated time periods followed by insulin stimulation (10 nM) for 10 min (B). The phosphorylation of Akt (Ser 473) was determined via western blot and normalized to total Akt. Insulin-induced Akt-phosphorylation was set to 100%. Values are means $\pm$ SEM of at least four independent experiments. * $p < 0.05$ and ** $p < 0.01$ indicate a statistically significant difference.

Fig. 2. Effect of LPA on insulin-induced *Gck* and *SREBP-1c* mRNA expression. Primary rat hepatocytes were pretreated with the indicated concentrations of LPA 18:1 (A) and 16:0 (B, C) for 5 h. Then insulin (10 nM) was added for 2 h to the medium. Quantitative real time-PCR analysis of *Gck* and *SREBP-1c* mRNA expression was performed using β -actin as reference gene. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ indicate a statistically significant difference.

An approximately 10-fold increase in *Gck* mRNA expression was visible when cells were stimulated with insulin (Fig. 2). Pretreatment of cells with LPA reduced insulin-induced *Gck* expression in a concentration dependent manner. A significant inhibition of insulin-mediated *Gck* expression was detected with a concentration of 0.001 μ M of LPA 16:0 whereas a maximal inhibitory effect was visible when cells were treated with 10 μ M LPA (Fig. 2A, B). To proof whether *Gck* is also reduced on the protein level, western blot analyses were performed. Indeed, in congruence with the mRNA expression data, LPA was able to inhibit insulin-induced *Gck* protein expression (Fig. 3A).

As presented in Fig. 2C, treatment of hepatocytes with insulin induced an elevation of *SREBP-1c* mRNA expression. In analogy with the inhibitory effect of LPA on insulin-mediated *Gck* expression, the stimulation with LPA almost completely prevented insulin-induced *SREBP-1c* expression.

LPA reduces insulin-mediated hepatic glycogen synthesis

An elevated hepatic glycogen synthesis is the physiological outcome of insulin-mediated PI_3K activation via phosphorylation of GSK-3 β . To analyze the role of LPA on insulin-induced glycogen synthesis the phosphorylation of GSK-3 β (Ser 9) was determined. As presented in Fig. 3B, an approximately 2-fold increase of GSK-3 β -phosphorylation occurred in response to a stimulation with insulin. However, when cells were pretreated with LPA, insulin lost its ability to phosphorylate and inactivate GSK-3 β , which is in agreement with the inhibitory effect of LPA on Akt activation (Fig. 3B).

To further investigate the role of LPA on insulin-mediated glycogen synthesis the incorporation of D-[U- ^{14}C] glucose into glycogen was measured. As expected, the stimulation of hepatocytes with insulin was connected with an increased glycogen synthesis. In congruence with the inhibitory effect of LPA on insulin signaling, pretreatment of cells with LPA impaired insulin-mediated glycogen synthesis significantly by more than 50% (Fig. 3C).

Role of LPA receptor subtypes on hepatic insulin signaling

LPA acts extracellularly via binding and activation of 6 LPA receptor subtypes (LPA $_{1-6}$). Real-time PCR showed that 4 LPA receptors, namely LPA $_1$, LPA $_2$, LPA $_3$ and LPA $_6$, are present in primary rat hepatocytes with the highest expression of the LPA $_3$ receptor subtype (Fig. 4).

To proof whether the inhibitory effect of LPA on insulin signaling is mediated via LPA receptors, insulin-induced *Gck* expression was measured in the presence of LPA and the

Fig. 3. Effect of LPA on insulin-mediated Gck protein expression and glycogen synthesis. Primary rat hepatocytes were preincubated with LPA 16:0 for 12 h in the presence of LPA (10 μ M) and/or insulin (10 nM) as indicated (A). Cells were stimulated with LPA 16:0 for 15 min followed by an insulin stimulation (10 nM) for 10 min (B). Cells were preincubated with LPA either 16:0 or 18:1 (10 μ M) for 5 h followed by a stimulation with insulin (10 nM) for 2 h (B). The expression of Gck was determined via western blot and normalized to β -actin (A). The phosphorylation of GSK-3 β (Ser 9) was determined via western blot and normalized to β -actin (B). The incorporation of D-[U- 14 C] glucose into glycogen was determined as described in Materials and Methods (C). Insulin-induced GSK-3 β -phosphorylation and insulin-mediated glycogen synthesis were set to 100%. Values are means $\pm$ SEM of at least four independent experiments. * $p < 0.05$ and ** $p < 0.01$ indicate a statistically significant difference.

Fig. 4. LPA receptor expression in primary rat hepatocytes. Quantitative real-time PCR analysis of the LPA receptor subtypes LPA_1 , LPA_2 , LPA_3 , LPA_4 , LPA_5 and LPA_6 in rat hepatocytes was performed using β -actin as reference gene.

$LPA_{1/3}$ receptor antagonist VPC-12249. As presented in Fig. 5A, the inhibitory effect of LPA on insulin-mediated Gck expression was significantly reduced in the presence of VPC-12249 indicating that LPA_1 and/or LPA_3 may play a crucial role in the LPA-induced inhibition of insulin signaling.

To further characterize the role of these receptor subtypes on the insulin-cascade, (2S)-OMPT, a specific LPA_3 receptor agonist was used. Indeed, pretreatment with (2S)-OMPT resulted in an impaired insulin-induced Gck expression comparable to LPA. These data indicate that LPA_3 is the crucial LPA receptor subtype involved in the inhibitory effect of LPA on insulin signaling. Furthermore, to make sure that the inhibitory effect of LPA is initiated via the LPA_3 -receptor, the insulin-mediated glycogen synthesis was also measured in the presence of (2S)-OMPT. In accordance with the data of Gck expression, the incorporation of D-[U- 14 C] glucose into glycogen in response to insulin was significantly impaired when cells were preincubated with (2S)-OMPT (Fig. 5B).

Human plasma LPA levels are elevated in obese individuals

To further investigate the role of LPA as a mediator between obesity and insulin resistance, human plasma LPA levels in obese (BMI >30) and normal weight (BMI 18.5 - 25)

Fig. 5. Involvement of the LPA₃ receptor subtype on LPA-mediated attenuation of insulin signaling. Cells were preincubated with LPA 16:0, LPA 18:1 (10 μ M) $\pm$ VPC-12249 or with (2S)-OMPT for 5 h. Then insulin (10 nM) was added for 2 h to the medium. *Gck* mRNA expression (A) and glycogen synthesis (B) were determined as described. Insulin-induced *Gck* mRNA expression and glycogen synthesis were set to 100%. Values are means $\pm$ SEM of at least four independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ indicate a statistically significant difference.

Fig. 6. Plasma LPA concentration in relation to BMI in human samples. Quantification of LPA species in human plasma of normal weight (BMI 18.5-25, $n = 14$) and obese (BMI ≥ 30 , $n = 18$) individuals using LC-MS/MS. Data are expressed as the mean $\pm$ SEM per group. * $p < 0.05$ indicates a statistically significant difference.

individuals were measured via LC-MS/MS. As presented in Fig. 6, a significant enhancement of LPA 16:0 levels was detected in the obese group compared to individuals with normal body weight (27.70 ± 1.48 nM, $n=14$ vs. 33.67 ± 3.78 nM, $n = 18$).

Discussion

Adipocyte hypertrophy and obesity are associated with hepatic insulin resistance which are main risk factors for the progression of T2DM [17]. However the molecular mechanisms responsible for the interaction between adipose tissue and the suppression of insulin action in hepatocytes are not fully understood. On the one hand, ectopic lipid accumulation in peripheral tissue in response to increased circulating free fatty acids due to an elevated lipolysis rate in adipose tissue is considered as an initiator for this pathological condition [17]. On the other hand, the generation and secretion of adipokines from adipose tissue are discussed as another conceivable mechanism involved in the interaction of obesity and insulin resistance. Therefore, the treatment of obesity with drugs such as metformin or the use of traditional medicine, which affects lipid homeostasis, may be beneficial to improve insulin resistance [18].

One of the adipocyte-secreted adipokines is the ectoenzyme ATX which converts LPC to the bioactive lipid mediator LPA. Indeed, ATX is the crucial enzyme accountable for extracellularly synthesized LPA [19]. There exist several studies indicating a crosstalk between the ATX/LPA axis and glucose homeostasis in obese individuals. Thus, Ferry et al. have demonstrated that ATX is released from adipocytes leading to the formation of LPA

and activates preadipocyte differentiation [10]. Furthermore, it has been shown that the expression of ATX is significantly increased in obese diabetic db/db mice compared to lean db/+ mice indicating a direct link between ATX and glucose metabolism. Adipocyte ATX expression strongly increases with adipogenesis as well as in individuals exhibiting T2DM associated with massive obesity [10, 20-22]. Moreover, enhanced levels of ATX strongly correlate with plasma LPA concentrations [23]. In the present work, we have shown that human plasma LPA 16:0 levels are elevated in obese (BMI > 30) compared to normal weight (BMI 18.5-25) individuals. These changes were mainly detected in LPA 16:0 species. A possible explanation for the significant enhancement of this lipid species could be elucidated by the specificity of ATX as LPC 16:0 is the preferred substrate for the ectoenzyme [24]. These findings are in congruence with the observation that LPC levels are reduced in diabetic patients confirming the fact that an elevation of plasma LPA is a result of an enhanced ATX expression and therefore an increased conversion of LPC to LPA [25]. In accordance with our study, Dusaulcy et al. have indicated that a high fed diet in mice leads not only to an increased body weight but also to enhanced plasma LPA concentrations due to an elevated ATX expression in adipose tissue. It should be mentioned that in this animal study only the total LPA levels have been determined, whereas the alterations of LPA levels were not assigned to a specific LPA species [21].

The molecular mechanisms involved in ATX-mediated LPA formation on insulin sensitivity of peripheral tissue are not well characterized. Owing to the fact that hepatic insulin resistance is an important mediator for the progression of hyperglycemia and T2DM, it was of great interest to examine the role of LPA on hepatic insulin signaling. The PI₃K/Akt pathway plays a distinguished role in the insulin cascade and its activation mainly contributes to hepatic glucose disposal. More specifically, we have demonstrated that LPA is able to reduce insulin-mediated Akt-phosphorylation in primary rat hepatocytes. An apparent contradictory role of LPA on Akt-signaling has been described. Thus, an activation of Akt after stimulation with LPA has been reported in several cell types such as ovarian and breast cancer cells. Even in the hepatic murine cell line AML-12 an activation of Akt has been detected after stimulation with 25 µM LPA [26-28]. On the contrary, an inhibitory effect of LPA on hepatic growth factor-mediated Akt-phosphorylation has been detected in human bronchial epithelial cells [29]. Our data demonstrate that incubation of hepatocytes with LPA alone had no significant effect on Akt-phosphorylation and the inhibitory effect is only detectable after insulin-mediated activation of Akt. Thus, the cell specific expression pattern of LPA receptors may be responsible for the contradictory effect of LPA on intracellular signaling pathways.

PI₃K/Akt modulates the expression and activity of Gck via the transcription factor SREBP-1c as well as the inactivation of GSK-3β [13]. Both, enhanced Gck activity, the rate limiting enzyme for glucose utilization, and inactivation of GSK-3β, the main enzyme for glycogen modulation, in response to insulin, contribute to an enhanced glycogen synthesis. Our study demonstrates that the inhibitory property of LPA on insulin-mediated Akt activation is accompanied by diminished insulin-mediated *Gck* and *SREBP-1c* expression, GSK-3β-phosphorylation and glycogen synthesis in primary hepatocytes leading to an impaired glucose tolerance. In agreement with these findings, Rancoule et al. demonstrated that exogenous injection of LPA in mice induced an acute failure in glucose tolerance. A reduced insulin secretion in response to LPA was discussed as a possible mechanism for the hyperglycemic condition after LPA supply [11]. Nevertheless, our results provide evidence that the effect of LPA on insulin signaling in hepatocytes is a considerable determinant that also contributes to an impaired glucose utilization after LPA injection. Further studies by the group of Saulnier-Blache indicate an improvement of glucose tolerance, when ATX is specifically knocked out in adipose tissue (FATX-KO). Despite being more sensitive to nutritional obesity, FATX-KO mice showed an ameliorated glucose utilization compared to wild type mice when fed with a high fat diet [12, 21]. These studies suggest also a possible role of LPA receptors on insulin-mediated glucose homeostasis as treatment of obese high fat diet fed mice with the LPA_{1/3} antagonist, Ki16425, improves their glucose and insulin

Fig. 7. Hypothetical mechanism of ATX/LPA on insulin signaling. ATX is upregulated in obesity and induced extracellular LPA synthesis. LPA binds to the LPA₃-receptor subtype resulting in an inactivation of insulin-mediated Akt-phosphorylation. This crosstalk is connected to an attenuation of Gck expression and GSK-3 β activation. Physiologically, insulin-mediated glycogen synthesis is diminished in response to LPA suggesting that this bioactive lipid mediator may contribute to hepatic insulin resistance.

tolerance [12]. In agreement, our data provide evidence that LPA₃ stimulation attenuates insulin-dependent signaling in hepatocytes. Pharmacological inhibition of LPA_{1/3} by using VPC-12249 showed a significant improvement of insulin signaling in hepatocytes in the presence of LPA. To further elucidate the responsible LPA receptor subtype, insulin-mediated hepatic glycogen synthesis was measured in the presence of (2S)-OMPT, a specific LPA₃ receptor agonist. These data indicate that LPA₃ is the crucial receptor subtype involved in the inhibitory effect of LPA on insulin signaling. These findings reveal that modulation of LPA₃ could be considered as a new therapeutic strategy for obesity-induced insulin resistance. Moreover, Rancoule et al. have shown that treatment of high fat diet fed mice with Ki16425 increased glycogen content in hepatocytes which is also in agreement with our findings demonstrating the negative effect of LPA₃ in modulation of insulin-induced glycogen synthesis [11]. However, it should be mentioned that an opposite role of LPA on glucose mobilization has been reported in muscle and adipose tissue. Yea et al. demonstrated that LPA improves glucose uptake in myotubes and 3T3-L1 adipocytes by modulating GLUT4 translocation to the membrane in a G_{ai} dependent manner. Although these results suggest that LPA enhances glucose uptake in myotubes, measurement of LPA receptor expression revealed that the LPA₃ receptor subtype is not expressed in these L6 GLUT4myc myotubes. The different receptor profile may explain these contradictory results [30].

It is well known that LPA receptors belong to the family of G protein coupled receptors. Different subunits of G_α proteins can modulate intracellular signaling cascades. An activation of protein kinase C- θ or c-Jun N-terminal kinases and thereby a negative phosphorylation of IRS could be considered as a possible mechanism involved in LPA signaling [31–33]. Furthermore the activation of GSK-3 β and S6K can also impair intracellular insulin signaling via phosphorylation of IRS [34].

ATX/LPA is involved in a wide range of pathological conditions especially obesity related metabolic disorders; therefore ATX inhibitors have a broad therapeutic potential. ATX elicits its biological activity through its product LPA. The enlightenment of LPA signaling and identification of relevant LPA receptor subtypes could be considered as a much more effective therapeutic strategy for the treatment of metabolic diseases.

Taken together our findings indicate that LPA 16:0 is increased in obesity and may contribute to obesity-associated insulin resistance and in the progression of T2DM. LPA is able to interrupt insulin-induced Gck expression, PI₃K activation and glycogen synthesis in primary rat hepatocytes via the LPA₃ receptor subtype. Thus, a pharmacological intervention to LPA₃ signaling could be considered as a novel therapeutic target for the treatment of hepatic insulin resistance. A hypothetical mechanism how LPA may interrupt insulin signaling is presented in Fig. 7.

Abbreviations

ATX (autotaxin); Gck (glucokinase); GSK-3 β (glycogen synthase kinase-3 β , IR: insulin receptor); IRS (insulin receptor substrate); LC-MS/MS (liquid chromatography tandem-mass spectrometry); LPA (lysophosphatidic acid); LPC (lysophosphatidylcholine); PI₃K (phosphatidylinositol 3-kinase); SREBP-1c (sterol regulatory element binding protein-1c); T2DM (type 2 diabetes mellitus).

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Disclosure Statement

No conflict of interest to disclosure.

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