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# Adipose triglyceride lipase activity is inhibited by long-chain acyl-coenzyme A☆

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ABSTRACT

Adipose triglyceride lipase (ATGL) is required for efficient mobilization of triglyceride (TG) stores in adipose tissue and non-adipose tissues. Therefore, ATGL strongly determines the availability of fatty acids for metabolic reactions. ATGL activity is regulated by a complex network of lipolytic and anti-lipolytic hormones. These signals control enzyme expression and the interaction of ATGL with the regulatory proteins CGI-58 and G0S2. Up to date, it was unknown whether ATGL activity is also controlled by lipid intermediates generated during lipolysis. Here we show that ATGL activity is inhibited by long-chain acyl-CoAs in a non-competitive manner, similar as previously shown for hormone-sensitive lipase (HSL), the rate-limiting enzyme for diglyceride breakdown in adipose tissue. ATGL activity is only marginally inhibited by medium-chain acyl-CoAs, diglycerides, monoglycerides, and free fatty acids. Immunoprecipitation assays revealed that acyl-CoAs do not disrupt the protein–protein interaction of ATGL and its co-activator CGI-58. Furthermore, inhibition of ATGL is independent of the presence of CGI-58 and occurs directly at the N-terminal patatin-like phospholipase domain of the enzyme. In conclusion, our results suggest that inhibition of the major lipolytic enzymes ATGL and HSL by long-chain acyl-CoAs could represent an effective feedback mechanism controlling lipolysis and protecting cells from lipotoxic concentrations of fatty acids and fatty acid-derived lipid metabolites.



## 1. Introduction

Adipose triglyceride lipase (ATGL, also referred to as patatin-like phospholipase domain containing 2 [PNPLA2] or desnutrin [1]) performs the first step in triglyceride (TG) hydrolysis generating diglyceride (DG) and free fatty acids (FFAs) [2]. Consequently, the enzyme controls the availability of FFAs, which may serve as energy substrates, precursors for other lipids, and lipid signaling molecules. This central function has a major impact on overall energy metabolism and becomes evident in fasted ATGL-deficient mice (ATGL-ko). In this mouse model, the lack of sufficient FFAs for energy conversion promotes the usage of glucose for energy conversion [3]. As a consequence, short fasting periods or moderate exercise leads to rapid consumption of glycogen stores. Fasting for more than 6 h results in hypoglycemia, hypometabolism, and hypothermia [3,4].

ATGL activity is regulated by a complex network of hormones which control enzyme expression and the interaction of the enzyme with the regulatory proteins. ATGL is stimulated by the presence of an activator protein as observed for other TG lipases, such as pancreatic lipase or lipoprotein lipase. The activator of ATGL is termed comparative gene identification-58 (CGI-58) [or alpha/beta-hydrolase domain containing 5 (ABHD5)] [5]. Currently, the molecular mechanism on how CGI-58 stimulates ATGL activity is unknown [6]. However, loss of either ATGL or CGI-58 function causes systemic TG accumulation in humans and mice. This inherited disorder is known as Neutral Lipid Storage Disease (NLSD) [7]. A second regulatory protein of ATGL is G0/G1 switch gene-2 (G0S2). This protein was originally described to be required to commit cells to enter the G1 phase of the cell cycle [8]. Recent evidence suggests that G0S2 specifically inhibits ATGL activity in rodents and humans [9,10]. Both G0S2 and CGI-58 have been shown to interact with ATGL. Furthermore, they are present on lipid droplets and regulated by metabolic hormones. G0S2 appears to be regulated primarily on the expression level. The antilipolytic hormone insulin increases G0S2 expression in 3T3-L1 adipocytes, whereas activation of lipolysis by fasting, β-adrenergic agonists, and tumor necrosis factor-α has the opposite effect [9,11]. In contrast to G0S2, fasting and β-adrenergic stimulation have minor effects on CGI-58 protein expression in adipose tissue. This co-activator protein is regulated primarily by its reversible interaction with the lipid droplet coating protein perilipin 1 [12]. In non-activated adipocytes, CGI-58 is bound to perilipin 1 and lipolysis is low. Upon lipolytic stimulation by β-adrenergic agonists, perilipin 1 gets phosphorylated by protein kinase A leading to the release of CGI-58 which is



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now available for ATGL activation. In addition, ATGL activity is influenced by other members of lipid droplet coat proteins of the perilipin (PAT) family. Perilipin 2 has been shown to reduce the lipid droplet association of ATGL [13]. Recent data also suggest that perilipin 5 interacts with ATGL and inhibits its activity [14–17].

Up to date, it was unknown whether ATGL activity or its interaction with regulatory proteins is controlled by lipid metabolites arising during lipolysis. Here we show that ATGL is directly inhibited by long-chain acyl-CoA via a non-competitive mechanism.

## 2. Materials and methods

### 2.1. Materials

Acyl-CoA with various fatty acid chain lengths and triolein were obtained from Sigma-Aldrich (Taufkirchen, Germany). Radiolabeled [9,10(N)-$^3$H]triolein was obtained from PerkinElmer Life Sciences and hexadecyl-CoA was obtained from Avanti Polar Lipids.

### 2.2. Expression of recombinant proteins

For expression of murine ATGL and CGI-58 in *Escherichia coli*, sequences containing the complete open reading frame of murine ATGL and murine CGI-58 were amplified from cDNA by PCR using Phusion ™ High Fidelity DNA Polymerase (Finnzymes, Espoo, Finland). Respective primers were designed to create 5′ and 3′ restriction endonuclease cleavage sites (underlined) for subsequent cloning strategies:

mATGL_fw: 5′-TCGGTACC ATGTTCCCGAGGGAGACCAA-3′

mATGL_rv: 5′-ACCTCGAG TCAGCAAGGCGGGAGGC-3′
mCGI-58_fw: 5′-GGGGATCC CAAAGCGATGGCGGCGG-3′
mCGI-58_rv: 5′-CTGATATC TCAGTCTACTGTGTGGCAGATCTCC-3′.

PCR products were inserted into the target vector pASK-IBA5plus (IBA, Goettingen, Germany) and transformed into *E. coli* (strain XL-1 and BL-21 for ATGL and CGI-58, respectively). Protein expression was induced by adding 200 ng/ml anhydro-tetracycline. Cells were harvested 3 h after induction. Expression of strep-tagged proteins was detected by Western blot analysis using mouse anti-Strep-tag II antibody (1:5000 dilution; IBA, Goettingen, Germany) as primary antibody and HRP-linked sheep-anti mouse antibody, (1:10,000; GE Healthcare Amersham, Buckinghamshire, UK) as secondary antibody.

Transient transfection of Monkey embryonic kidney cells (COS-7, ATCC CRL-1651) with pcDNA4/HisMax coding for His-tagged ATGL, HSL, or ß-galactosidase (LacZ) was performed with Metafectene™ (Biontex GmbH) as described [2]. Expression of His-tagged proteins was detected using anti-His monoclonal antibody (6xHis, BD Biosciences) and a horseradish peroxidase-conjugated anti-mouse antibody (GE Healthcare) as secondary antibody.

### 2.3. Preparation of cell and tissue extracts

*E. coli* and COS-7 cells were disrupted by sonication resuspended in lysis buffer in lysis buffer (0.25 M sucrose, 1 mM dithiothreitol, 1 mM EDTA, 20 μg/ml leupeptine, 2 μg/ml antipain, 1 μg/ml pepstatin, pH 7.0). Lysates of E. *coli* were centrifuged at 15,000 ×*g* at 4 °C for 20 min. For the preparation of COS-7 cell extracts, nuclei and unbroken cells were removed by centrifugation at 1000 ×*g* at 4 °C for 5 min. Supernatants were collected and used for activity assays. The specific activity of these lysates ranged from 100 to 400 nmol/h·mg depending on the expression levels of recombinant proteins.

Mouse gonadal WAT was homogenized in lysis buffer (~1 ml/fat pad) using an Ultra Turrax Homogenizer (Fisher Scientific, Waltham, MA). The homogenate was centrifuged at 20,000 ×*g* at 4 °C for 1 h. The interphase was collected and used for activity assays.

### 2.4. Assay for TG hydrolase activity

The substrate for the measurement of TG hydrolase activity was prepared as described previously with minor modifications [2]. Briefly, triolein and [9,10-$^3$H]triolein (10 μCi/ml) were emulsified in the presence of phosphatidylcholine/phosphatidylinositol using a sonicator (Virsonic 475, Virtis, Gardiner, NJ) and adjusted to 2.5% BSA (FFA free). The final substrate concentration was 1.67 μmol/ml triolein and 0.15 mg/ml PC/PI (3:1). For kinetic investigations, the TG substrate was diluted to the indicated concentrations after sonication. Activity assays were performed using 0.1 ml of cell lysates and 0.1 ml substrate in a water bath at 37 °C for 20 min. The reaction was terminated by adding 3.25 ml of methanol/chloroform/heptane (10:9:7) and 1 ml of 0.1 M potassium carbonate, 0.1 M boric acid, pH 10.5. After centrifugation at 800 ×*g* for 20 min, the radioactivity in 1 ml of the upper phase was determined by liquid scintillation counting.

### 2.5. Protein interaction of ATGL and CGI-58

Cos-7 cells were co-transfected with Flag-tagged CGI-58 and His-tagged ATGL. After 3 h of binding, FLAG-beads were washed and incubated for 20 min with indicated concentrations of acyl-CoAs at 37 °C. Subsequently, beads were washed 3-times with lysis buffer, proteins were eluted by boiling in SDS-containing sample puffer, and probes were subjected to Western blot analysis using FLAG- and His-tag specific antibodies (Monoclonal mouse ANTI-FLAG® M2-Peroxidase (HRP) antibody, Sigma, A8592; Monoclonal mouse ANTI-HIS antibody, GE Healthcare, 27-4710-01) and sheep anti-mouse IgG (HRP-linked, GE Healthcare; NA931) as secondary antibody.

### 2.6. Protein determinations

Protein concentrations of cell lysates were determined by Bio-Rad protein assay kit according to manufacturer's instructions (Bio-Rad, Hercules, CA) using BSA as standard.

### 2.7. Statistical analysis

Data are presented as mean ± S.D. Statistical significance was determined by the Student's unpaired *t*-test (two-tailed). Group differences were considered significant for $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

## 3. Results

### 3.1. ATGL is inhibited by oleoyl-CoA

Inhibition of ATGL activity by lipid intermediates was first investigated in lysates of COS-7 cells expressing His-tagged ATGL and CGI-58. Lysates containing approximately equimolar concentrations of ATGL and CGI-58 (Fig. 1A) were incubated with a radiolabeled triolein substrate in the absence (control) or presence of various lipid metabolites. At a concentration of 50 μM, ATGL activity was almost completely inhibited in the presence of oleoyl-CoA. In comparison, oleic acid had little effect and free CoA, (OA), monoolein (MO), and diolein (DO) did not affect enzyme activity (Fig. 1B). ATGL was also inhibited by a thioether analog of palmitoyl-CoA (hexadecyl-CoA, Fig. 1B) suggesting that protein acylation is not required for inactivation.

Addition of oleoyl-CoA led to inactivation of ATGL activity with an $IC_{50}$ value of 33 μM (Fig. 1C).

It is important to note that all assays have been performed in the presence of excess BSA (360 μM) which harbors high affinity sites for acyl-CoA [18]. When BSA was omitted from the reaction, we observed an almost complete inhibition of ATGL activity implicating that the enzyme requires an FFA acceptor for full activity. In the presence of low amounts of BSA (3.6 μM), the enzyme retained ~50% of its activity.



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