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ORIGINAL ARTICLE

Reduced expression of adipose triglyceride lipase decreases arachidonic acid release and prostacyclin secretion in human aortic endothelial cells

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ABSTRACT

Background: Vascular endothelial cells represent an important source of arachidonic acid (AA)-derived mediators involved in the generation of anti- or proatherogenic environments. Evidence emerged (in mast cells), that in addition to phospholipases, neutral lipid hydrolases as adipose triglyceride lipase (ATGL) also participate in this process.

Objective: To examine the impact of ATGL on AA-release from cellular phospholipids (PL) and on prostacyclin secretion in human aortic endothelial cells (HAEC).

Methods and results: siRNA-mediated silencing of ATGL promoted lipid droplet formation and TG accumulation in HAEC (nile red stain). ATGL knockdown decreased the basal and A23187 (calcium ionophore)-induced release of ^{14}C-AA from (^{14}C-AA-labeled) HAEC. In A23187-stimulated ATGL silenced cells, this was accompanied by a decreased content of ^{14}C-AA in cellular PL and a decreased secretion of prostacyclin (determined by 6-keto PGF1α EIA).

Conclusions: In vascular endothelial cells, the efficiency of stimulus-induced AA release and prostacyclin secretion is dependent on ATGL.

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Introduction

The efficiency of prostanoid production is largely dependent on the activity of biosynthetic enzymes and the availability of arachidonic acid (AA) in cellular glycerolipid pools (Scott *et al.* 1982). Stimulation of cells by various agonists leads to the release of AA from membrane phospholipids (PL) by phospholipase A2 (PLA2) enzymes, primarily Ca^{2+}-dependent cytosolic PLA2IVA (cPLA2). While a portion of the released AA is converted into various eicosanoids, the remaining free AA is reacylated into PL and triglycerides (TG) (Triggiani *et al.* 1994).

As found initially in adipose tissue, adipose triglyceride lipase (ATGL) catalyses the first, rate-limiting step in the hydrolysis of TG stored within intracellular lipid droplets, yielding free fatty acid and diacylglycerol (DG) (Zimmermann *et al.* 2004). ATGL deficiency in mice is associated with massive lipid accumulation in tissues, severe skeletal- and cardiomyopathy and premature death, primarily due to heart failure (Haemmerle *et al.* 2006). Humans with mutations in the ATGL gene suffer from neutral lipid storage disease (NLSD) characterised by severe myopathy and massive TG accumulation in most tissues (Fischer *et al.* 2007). Lately, ATGL gained attention in the context of inflammation, when the lipase was described to regulate eicosanoid production in activated human mast cells and neutrophils (Dichlberger *et al.* 2014, Schreiber and Zechner 2014, Schlager *et al.* 2015).

Importantly, also a functional role in endothelial cells (ATGL deficient mice exhibit severe endothelial dysfunction) (Schrammel *et al.* 2014) and in the pathogenesis of atherosclerosis was attributed to ATGL. Decreased expression of ATGL in the obese insulin-resistant state (Jocken *et al.* 2007, Steinberg *et al.* 2007) (a pathological condition accompanied with increased incidence of atherosclerosis), as well as increased expression of adhesion molecules in ATGL depleted human aortic endothelial cells (HAEC), indicates a significant role of ATGL in *endothelial* inflammatory processes as well (Inoue *et al.* 2011).

Based on the fact that provision of AA is a basic prerequisite for the biogenesis of many pro- and anti-inflammatory mediators and on the observation that stimulus-induced AA release from cellular PL is often dependent on TG-derived AA (Triggiani *et al.* 1994), we aimed to examine the role of ATGL in human endothelial cells – focusing on stimulus-induced AA release and on prostacyclin secretion (as a representative anti-inflammatory prostanoid).

Materials and methods

Cell culture

Human primary aortic endothelial cells were obtained from Lonza (Cologne, Germany) and maintained in endothelial cell

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Supplemental data for this article can be accessed here.

growth medium [EGM-MV Bullet Kit = EBM medium + growth supplements + FCS (Lonza)] supplemented with 50 IU/ml penicillin, and 50 μg/ml streptomycin. Cells were cultured in gelatine-coated dishes at 37 °C in a 5% CO_2 humidified atmosphere and were used for experiments from passage 5 to 10.

Si-RNA mediated knockdown of ATGL

To create ATGL knockdown cells and respective controls, cells were transfected with siRNA against ATGL (Qiagen, Hilden, Germany) or predesigned control siRNAs (Qiagen, AllStars Negative Control siRNA) and PrimeFect-siRNA transfection reagent (Lonza, Cologne, Germany). In brief, one day after seeding (50,000 cells/12-well), the medium was switched to 500 μl serum-free medium. Two microlitres of PrimeFect was diluted in 101 μl of dilution buffer and incubated for 15 min. Thereafter, 15 μl of the corresponding siRNA (1 μM) was added to the PrimeFect mixture and incubated for further 15 min. The lipid–RNA complexes were applied to the cell supernatant for 3 h, followed by the addition of serum-containing medium on top. Cells were used for experiments 48 h after siRNA transfection.

Quantitative real-time PCR (qRT-PCR)

RNA isolation and qRT-PCR were performed using ATGL (Primer Assay QT00019754) and beta-2-microglobulin (Primer Assay QT01665006) primers, exactly as described (Riederer *et al.* 2010).

Western blotting

Western blots were performed as described (Riederer *et al.* 2010). Briefly, HAEC transfected with ATGL siRNA or negative control siRNA, were washed with PBS and collected in 50 μl/well loading buffer [20% (w/v) glycerol, 5% (w/v) SDS, 0.15% (w/v) bromophenol blue, 63 mmol/l Tris–HCl, pH 6.8, and 5% (v/v) β-mercaptoethanol] followed by boiling for 10 min. Forty microliters of the lysate were subjected to each lane and analysed by SDS-PAGE (10% gel) and subsequent immunoblotting using an ATGL-specific antibody (Cell Signaling Technology, Beverly, MA) and a HRP-labelled anti-rabbit secondary antibody. Protein signals were detected by an enhanced chemiluminescent substrate for detection of HRP (SuperSignal West Pico or Femto, Thermo Scientific, Rockford, IL). Blots were re-probed with actin antibody (Oncogene).

^{14}C-AA release

HAEC were transfected with siRNA and labelled with ^{14}C-AA (4 μM, spec. activity 58 mCi/mmol) in complete medium for 20 h. Unbound ^{14}C-AA was removed by excessive washing in PBS supplemented with 1% BSA. Following incubation in serum-free medium for 7 h, cells were incubated with or without A23187 (1 μM) in the presence of 0.03% BSA for 10 min. Cell media were collected, spun to remove cells and immediately frozen at −70 °C until extraction. Cells were washed with PBS and lysed in 0.3 M NaOH/0.1% SDS. The amounts of ^{14}C-AA released into medium were detected by thin-layer chromatography, quantified by densitometry and normalised to total cellular radioactivity measured by scintillation counting of cell lysates exactly as described. Results of this relative quantification are described in arbitrary units (AU) (Riederer *et al.* 2010).

Quantification of ^{14}C-PL, and -TG by thin layer chromatography (TLC)

For the determination of the ^{14}C-AA content in total PL and TG, HAEC were labelled as described above. Cell layers were extracted with two volumes of hexane/isopropanol (3:2, v/v), evaporated in the SpeedVac and redissolved in chloroform before application onto TLC plates. For separation of total ^{14}C-PL and ^{14}C-TG, hexane–diethylether–glacial acetic acid (70:29:1; v/v/v) was used as the mobile phase. The signals corresponding to ^{14}C-PL, and -TG, were visualised upon exposure of the TLC plates to a tritium screen (GE Healthcare, Little Chalfont, UK) on the STORM imager. Relative quantification was performed by densitometric volume report analysis and results were normalised to total cellular radioactivity (measured by scintillation counting of cell lysates) and expressed as AU.

Nile red stain

ATGL-silenced or control HAEC were incubated with Nile Red (100 ng/ml) for 15 min. Lipid staining was evaluated by fluorescence microscopy.

Lipid extraction and mass spectrometry of TG

The cell layer of one 12-well (150,000 cells) was extracted according to Bligh and Dyer (1959) and dried under a stream of nitrogen. Acquisition of TG species was performed on a LTQ-FT in FT full scan mode at a resolution of 200,000 (Fauland *et al.* 2011). TG were quantified by using the Lipid Data Analyzer (Hartler *et al.* 2011), and shown as results relative to the total amount of selected molecules per 150,000 cells – depicted as AU.

Measurement of 6-keto-PGF1α

Following incubation in serum-free medium for 7 h, siRNA transfected cells were incubated with or without A23187 (1 μM) in the presence of 0.03% BSA for 10 min. Cell media were collected, spun to remove cells and immediately frozen at −70 °C until extraction. 6-Keto PGF1α was measured in cell culture media by an EIA kit (Cayman, Ann Arbor, MI) according to the manufacturer's protocol and was normalised to cellular protein content (BCA, Pierce, Rockford, IL).

Statistical analysis

Cell culture experiments were performed at least three times and values are expressed as mean ± STD. Data were analysed by Student's *t*-test or one-way analysis of variance and the Bonferroni *post hoc* test. Group differences were considered significant for $p < .05$ (*).

Results

ATGL knockdown increases TG content in HAEC

To address the role of ATGL in endothelial inflammatory processes, ATGL was silenced in HAEC by transfection with siRNA. Compared with control HAEC (transfected with negative control siRNA), ATGL silenced HAEC exhibited markedly decreased both ATGL mRNA (Figure 1(A)) and protein levels (Figure 1(A) inset). This was accompanied by a massive accumulation of Nile-red stained lipid droplets (Figure 1(B)) and an increase in total TG content (Figure 1(C)).

ATGL knockdown decreases ^{14}C-AA release in HAEC

To investigate the capability of AA release, ^{14}C-*AA* labelled HAEC were tested under basal conditions as well as after 10 min stimulation with the calcium ionophore A23187. As shown in Figure 2(A), basal as well as A23187-induced ^{14}C-AA release was significantly lower in ATGL silenced, compared with control cells.

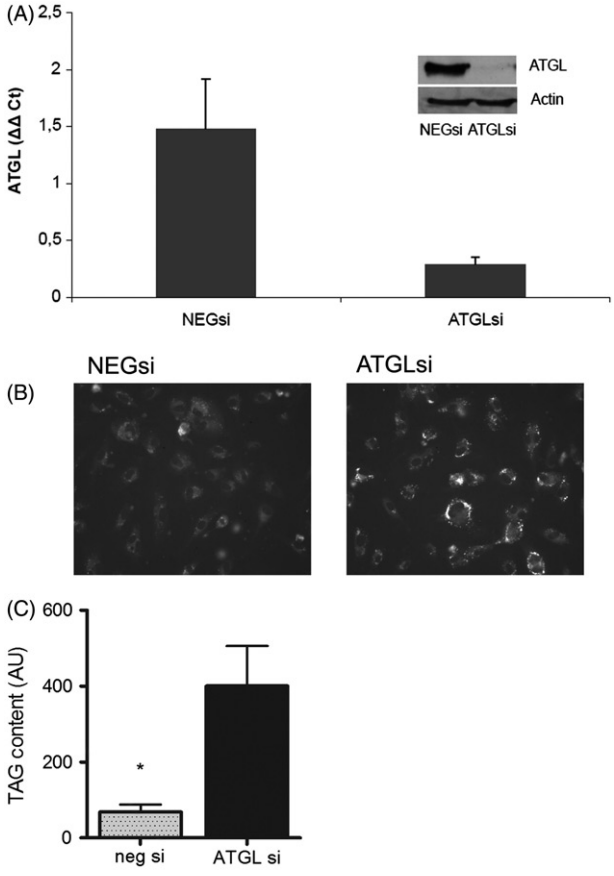

Figure 1. ATGL knockdown increases lipid droplets and TG content in HAEC. (A) ATGL silencing efficiency: Forty eight hours after transfection of HAEC with ATGL siRNA (ATGLsi) or negative control siRNA (NEGsi) the silencing efficiency was determined by qRT-PCR and Western blotting (inset). (B) Lipid accumulation in cells described in (A) was visualised by fluorescence microscopy upon staining with Nile Red. (C) The TG content of cell layers described in (A) was determined by mass spectrometry. Results were obtained by relative quantification using the Lipid Data Analyzer software (relative to the total amount of selected molecules/150,000 cells) and are expressed as arbitrary units (AU) of mean ± STD.

^{14}C-AA-PL content is decreased in ATGL silenced cells upon A23187 stimulation

To clarify the observed decrease in ^{14}C-AA release (Figure 2(A)), the levels of ^{14}C-AA were determined in the PL pool of the corresponding cell lysates, following a 20-h labelling with

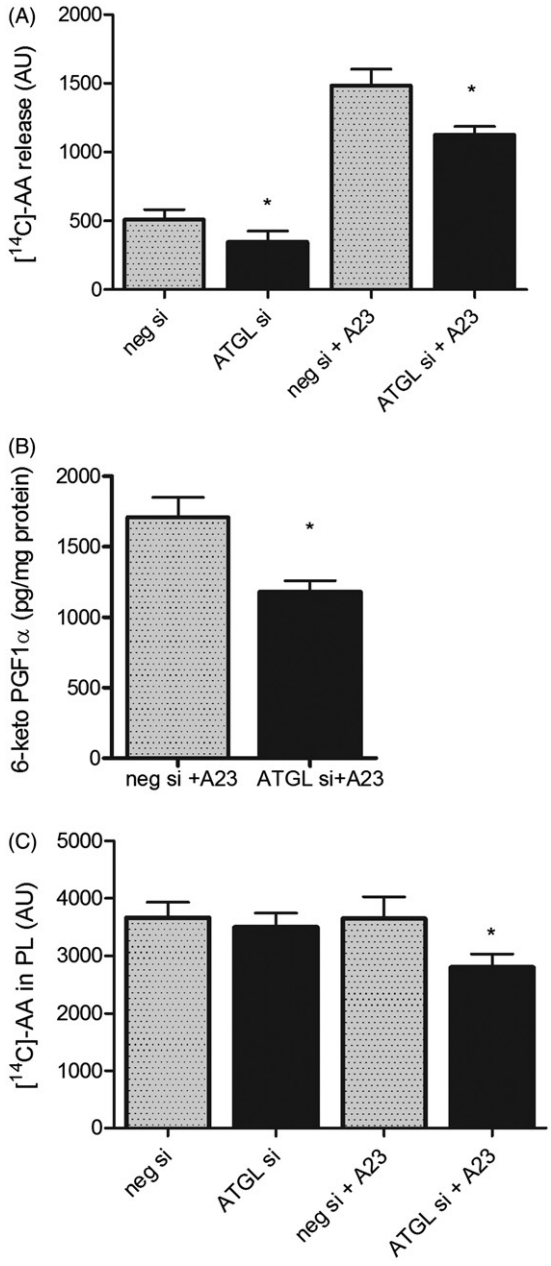

Figure 2. ^{14}C-AA release, ^{14}C-AA content in PL and 6-keto PGF1α secretion are decreased in ATGL silenced cells. (A) ^{14}C-AA release: After transfection with ATGL siRNA or negative control siRNA, HAEC were labelled with ^{14}C-AA for 20 h. After extensive washing, HAEC were further incubated in serum-free medium for 7 h, followed by incubation with or without A23187 in serum-free medium containing 0.03% BSA for 10 min. Lipid extracts of cell media were separated by TLC followed by densitometric quantification of ^{14}C-AA- spots. The amounts of ^{14}C-AA released into medium were normalised to total cellular radioactivity measured by scintillation counting of cell lysates and expressed as arbitrary units (AU). (C) ^{14}C-AA content in PL: Lipid extracts of cells treated as described in (A) were separated by TLC followed by densitometric quantification of ^{14}C-PL-spots. Results are mean ± STD. (B) 6-keto PGF1α-secretion: siRNA transfected HAEC were treated as in A (without AA-labelling). 6-Keto PGF1α was determined in cell culture supernatants by EIA and normalised to cellular protein content. Results are mean ± STD.

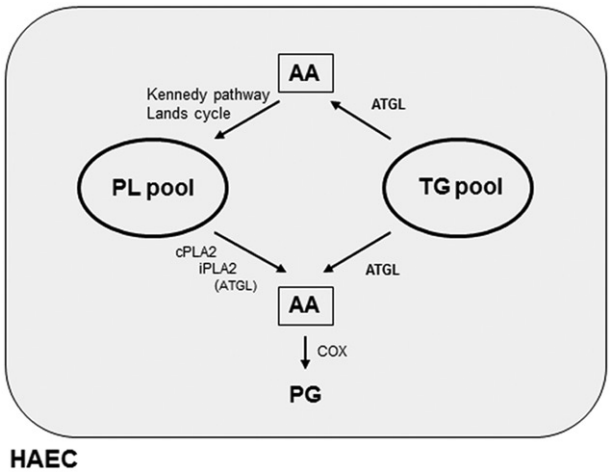

Figure 3. Schematic model of AA release in endothelial cells. In addition to the release of AA from PL stores (e.g. by the action of cPLA2, iPLA2, PLC, and *ATGL phospholipase*), our data provide evidence that AA is also released from TG pools (by ATGL) and might be directly metabolised to prostaglandins (by the action of COX enzymes). Alternatively, and especially evident upon stimulation with A23187, ATGL releases AA which is reincorporated into the PL pool (by the Kennedy pathway or the Lands cycle, respectively). Thus ATGL seems to be involved in the replenishment of the PL pool with AA.

^{14}C-AA and a 10-min incubation under basal and A23187 stimulated conditions, respectively. While the ^{14}C-AA levels in PL were not affected by ATGL silencing under basal conditions, they were significantly decreased upon A23187-stimulation (in ATGL silenced but not in control cells) (Figure 2(C)).

6-Keto-PGF1α secretion is decreased in ATGL silenced HAEC

Addressing the role of ATGL in endothelial prostanoid production, 6-keto PGF1α secretion (an endothelial secretagogue and stable hydrolysis product of prostacyclin) was measured by EIA. Under basal conditions, values were beyond the detection limit. But upon stimulation with A23187, ATGL-silenced cells exhibited decreased secretion of 6-keto PGF1α (Figure 2(B)).

Discussion

Here we show for the first time that the efficiency of basal and stimulus-induced AA release in vascular endothelial cells is dependent on ATGL. Based on the current literature, ATGL is a single-compartment-acting enzyme that exerts its activity on TG in lipid droplets only. The observed ^{14}C-AA depletion in PL of A23187-stimulated ATGL knockdown cells points towards the role of ATGL and the cellular TG-pool in the replenishment of the PL-pool with AA. This is in line with previous pulse-chase studies in lung macrophages demonstrating a flux of AA from the TG- into the PL-pool (Triggiani *et al.* 1994).

Even the basal AA release was decreased in ATGL-silenced cells (Figure 2(A)), suggesting the involvement of ATGL in the remodelling of membrane PL (which might also or additionally be attributed to the minor phospholipase activity of ATGL (Notari *et al.* 2006, Kanno *et al.* 2013), or a decreased cPLA2 activity, which was not addressed in this study). In membrane PL remodelling processes, AA is cleaved from PL by primarily Ca^{2+}-independent group VI PLA2 (iPLA2) and reincorporated back into PL by acyltransferases and transacylases (Pérez-Chacón *et al.* 2009) (Figure 3).

As described previously, the TG-pool was found to serve as a re-acylation pool for AA released from cellular PL upon stimulation of cPLA2 (Triggiani *et al.* 1994). However, in our study, the ^{14}C-AA content in the TG-pool of A23187-stimulated control cells was not significantly increased, compared with unstimulated control cells (supplementary Figure 1). This discrepancy might be explained by the presence of BSA, a potent acceptor for released AA, in cell media in our experimental model, in contrast to BSA-free incubations described by Triggiani *et al.* (1994).

Considering co-localisation of ATGL and enzymes involved in eicosanoid production on lipid droplets (Brasaemle *et al.* 2004), our study highlights ATGL as a potential new player in the endothelial eicosanoid-synthesising machinery (Figure 3). In line with our findings in HAEC, ATGL likewise seems to be a missing link in the well-established interplay between TG- and PL-pools and the mobilisation of TG-associated AA for eicosanoid production in human inflammatory cells (Triggiani *et al.* 1994, Wan *et al.* 2007, Bozza *et al.* 2011, Dichlberger *et al.* 2011, Dichlberger *et al.* 2014).

Concerning the severe endothelial dysfunction discovered in ATGL deficient mice (Schrammel *et al.* 2014), the here described alteration of AA release and prostanoid production could also play a role in the dysbalance of endothelium-derived relaxing and contracting factors.

In the context of atherosclerosis, Inoue already showed that ATGL-knockdown leads to enhanced ICAM-1 expression and subsequently enhanced monocyte adhesion to HAEC (mediated by PKC-dependent activation of nuclear factor-kappa-B) (Inoue *et al.* 2011). As ICAM-1 upregulation is also known to be dependent on cPLA2 and eicosanoids (Hadad *et al.* 2011), and based on the here described novel role of ATGL in AA-release and prostacyclin secretion, an increased incidence of atherosclerosis in obese insulin-resistant patients (with decreased ATGL) (Jocken *et al.* 2007) might at least in part be attributed to altered eicosanoid production by vascular endothelial cells.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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