Biochemical and Biophysical Research Communications xxx (2017) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Newly developed selective immunoinactivation assay revealed reduction in adipose triglyceride lipase activity in peripheral leucocytes from patients with idiopathic triglyceride deposit cardiomyovasculopathy

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ARTICLE INFO

Article history:
Received 8 November 2017
Accepted 10 November 2017
Available online xxx

Keywords:
Adipose triglyceride lipase
Leucocytes
Selective immunoinactivation assay
Triglyceride
Triglyceride deposit cardiomyovasculopathy

ABSTRACT

Triglyceride deposit cardiomyovasculopathy (TGCV) is a rare and newly identified disease among patients requiring cardiac transplantation. TGCV is characterized by cardiomyocyte steatosis and triglyceride (TG)-deposit atherosclerosis, resulting from the abnormal intracellular metabolism of TG. TGCV is classified into primary and idiopathic types. Primary TGCV carries ultra-rare genetic mutations in the adipose triglyceride lipase (ATGL), a rate-liming enzyme that hydrolyzes intracellular TG in adipose and non-adipose tissues. Idiopathic TGCV, first identified among autopsied individuals with diabetes mellitus (DM) with severe heart diseases, shows no ATGL mutations and its causes and underlying mechanisms are still unknown. TGCV is difficult to diagnose in daily clinics, thereby demanding feasible diagnostic procedures. We aimed to develop an assay to measure ATGL activity using peripheral leucocytes. Human his6-ATGL was expressed in COS1 cells, purified to homogeneity, and used to raise a polyclonal antibody neutralizing TG-hydrolyzing activity of ATGL. We developed a selective immunoinactivation assay (SIIA) for the quantitation of ATGL activity in cell lysates of leucocytes by the antibody neutralizing ATGL activities. ATGL activity was measured in 13 idiopathic TGCV patients, with two patients with primary TGCV as the negative control. Healthy (non-DM) and DM controls without heart diseases were also subjected. The developed SIIA assay revealed significant reduction in ATGL activity in leucocytes from patients with idiopathic TGCV who did not carry ATGL mutations as compared with non-DM and DM controls. Thus, ATGL in leucocytes may be an important biomarker for the diagnosis of TGCV and our assay may provide insights into pathophysiology and elucidate the underlying mechanism of TGCV and related disorders.

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https://doi.org/10.1016/j.bbrc.2017.11.070
0006-291X/

Abbreviations

ATGL	adipose triglyceride lipase
BSA	bovine serum albumin
DM	Diabetes mellitus
FFA	free fatty acid
HTGL	hepatic triglyceride lipase
LPL	lipoprotein lipase
PCR	polymerase chain reaction
PL	phospholipid
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SIIA	selective immunoinactivation assay
TG	triglyceride
TGCV	triglyceride deposit cardiomyovasculopathy

1. Introduction

Triglyceride deposit cardiomyovasculopathy (TGCV) was discovered as a rare and intractable heart disease among patients requiring cardiac transplantation in 2008 [1–3]. TGCV is characterized by massive accumulation of TG in the myocardium [4] and coronary arteries [5]. A known primary cause for TGCV is mutations in *PNPLA2* gene encoding adipose triglyceride lipase (ATGL) [6], an enzyme that catalyzes the first step in the hydrolysis of intracellular TG. This enzyme, originally identified in the adipose tissue, mobilizes long-chain fatty acids [7] in coordination with hormone-sensitive lipase. Gene disruption of *PNPLA2* in mice [8] showed massive cardiomyocyte steatosis and premature cardiac death, as observed in primary TGCV patients with ATGL mutations [9].

In our retrospective postmortem analyses, myocardial and coronary TG contents were biochemically measured in autopsied specimens from individuals who had died of intractable heart diseases. This study revealed individuals with another type of TGCV [10] and the following characteristics: 1) ATGL expression in their autopsied specimens was detectable; 2) no apparent skeletal myopathy, a major co-morbidity of primary TGCV, in clinicopathological information; 3) such individuals might be prevalent in diabetic population. As these profiles were different from primary TGCV with genetic ATGL deficiency, we designated them as idiopathic TGCV [11]. Furthermore, our animal experiments demonstrated an association between reduced myocardial expression of both ATGL mRNA and protein and cardiomyocyte steatosis in a diabetic model, *db/db* mice [12].

Therefore, we aimed to develop an assay that measures the functional ATGL activity in order to further investigate the regulation and pathophysiological roles of human ATGL and cause(s) and mechanisms underlying idiopathic TGCV. We chose selective immunoinactivation strategies using a specific antibody to neutralize ATGL activity in peripheral leucocytes for following reasons: 1) drawing blood is non-invasive and feasible as compared with biopsy specimens from adipose tissues or heart; 2) genetic ATGL deficiency is characterized by the presence of lipid droplets in peripheral leucocytes [13], indicative of the significant role of ATGL in these cell types; 3) intracellular TG metabolism in leucocytes is likely to be regulated by enzymes including ATGL, similar to the regulation of plasma TG hydrolysis by enzymes such as lipoprotein lipase (LPL) and hepatic triglyceride lipase (HTGL) [14]; 4) we have previously established immunoinactivation assay (SIIA) system to measure plasma LPL and HTGL with corresponding antibodies [15,16] and successfully applied it to a clinical study [17].

In this study, using our newly developed SIIA, we investigated ATGL activities in peripheral leucocytes of patients with primary and idiopathic TGCV together with DM and non-DM controls without heart disease.

2. Materials and methods

2.1. Preparation of expression vectors with or without his6 tag

The human ATGL cDNA was previously cloned into pcDNA4/HisMax C vector containing his6 tag [18]. For the construction of the expression vector for human ATGL cDNA without his6 tag, the human ATGL cDNA was used as a template for polymerase chain reaction (PCR). The primers designed to create endonuclease cleavage sites (*Hin*dIII and *Xba*I sites are underlined) were as follows: forward primer, 5′-GTG AAGCTT*GAAAC*CATGTTTCCCCGCGA-GAAGAC-3' (No.649, the italic is a Kozak sequence) and reverse primer, 5′-TTATCTAGATCACAGCCCCAGGGCCCCGA-3' (No.635). PCR was carried out using KOD-Plus (Toyobo) and PCR fragments digested with *Hin*dIII and *Xba*I were subcloned into pRc/CMV2 without his6 tag (Invitrogen).

2.2. Cell culture and transfection with expression vectors, and solubilization with a detergent

The expression vectors with his6-ATGL cDNA or with ATGL cDNA which does not contain his6 tag were transfected into COS1 cells (JCRB 9082) with Lipofectamine^{R} 3000 (Invitrogen) and cells were cultured for 2 days. The cells were harvested, lysed, and sonicated on ice in buffer-A (50 mM Tris-HCl, pH 7.4, 1 M NaCl, 10% glycerol, 0.2% CHAPS, 5 mM benzamidine, and 0.02% NaN_3). The solubilized samples were centrifuged and supernatants were frozen in a liquid nitrogen bath for further experiments.

2.3. Measurement of TG lipase activity

Triglyceride lipase assay was performed using a substrate of triolein-phospholipid (PL) (TG/PL) particles containing tri[9,10-^{3}H] olein (Perkin Elmer) and bovine serum albumin (BSA) with a modification of the method described by Lass et al. [19]. The substrate of TG/PL particle was prepared by emulsifying 2 mM tri [9,10-^{3}H]olein (55.44 μCi/μmol) and 0.272 mM phosphatidylcholine/phosphatidylinositol (3: 1) in 100 mM Tris-HCl buffer, pH 7.4. The reaction mixture contained 0.2 M Tris-HCl (pH 7.4), 0.5% defatted BSA, 5 mM benzamidine, 0.15 M NaCl, 30 μL of the above TG/PL particles containing ^{3}H-triolein, and enzyme solution in a final volume of 300 μL. The reaction was started by the addition of the substrate and then the mixture was incubated for 30 min at 37 °C. The reaction was stopped by the addition of 3 mL of a heptane/chloroform/methanol (1.00: 1.25: 1.40, v/v) and 0.5 mL of 0.5 M NaOH as previously described [15]. The mixture was shaken for 5 min and centrifuged for 5 min at 1800 × g. The radioactivity of the upper phase (0.5 mL) containing [9, 10-^{3}H] oleic acid was determined by liquid scintillation counting. Enzyme activity was expressed as nmol of free fatty acid (FFA) released/h/mL of enzyme.

2.4. Purification of his6-ATGL with a HisTrap column

The his6-ATGL antigen was purified with HisTrap column (GE Healthcare) chromatography using AKTA-FPLC equipment (GE Healthcare). The cell lysate (40 mL) containing his6-ATGL was applied on the HisTrap column (20 mL) equilibrated with a HisTrap buffer (20 mM Tris-HCl, pH 7.4, 10% glycerol, 1 M NaCl, 0.1% CHAPS, and 0.02% NaN_3). The column was first washed with 200 mL HisTrap buffer and the absorbed proteins were eluted with a

Please cite this article in press as: A. Takagi, et al., Newly developed selective immunoinactivation assay revealed reduction in adipose triglyceride lipase activity in peripheral leucocytes from patients with idiopathic triglyceride deposit cardiomyovasculopathy, Biochemical and Biophysical Research Communications (2017), https://doi.org/10.1016/j.bbrc.2017.11.070