Available online at www.sciencedirect.com
SCIENCE DIRECT®
Analytical Biochemistry 355 (2006) 29–38
ANALYTICAL BIOCHEMISTRY
www.elsevier.com/locate/yabio

Micro method for determination of nonesterified fatty acid in whole blood obtained by fingertip puncture

Jesper S. Hansen [a], Jens K. Villadsen [a], Michael Gaster [b], Nils J. Færgeman [a], Jens Knudsen [a,*]

[a] Department of Biochemistry and Molecular Biology, University of Southern Denmark, DK-5230 Odense M, Denmark
[b] Department of Medical Endocrinology, Odense University Hospital, DK-5000 Odense C, Denmark

Received 28 December 2005
Available online 19 May 2006

Abstract

Diagnostic tools for early identification of subjects at high risk for type 2 diabetes and other obesity-related disorders are important in prevention of these diseases. Nonesterified fatty acids (NEFAs) have been suggested to serve as a prediagnostic marker of diabetes and obesity-related disorders. In the current study, we developed a sensitive and reproducible micro method for quantification of NEFA in less than 10 μl whole blood. The method involves only two steps: (i) conversion of NEFA to fatty acid acyl-coenzyme A (acyl-CoA) esters using an acyl-CoA synthetase and (ii) quantification of the formed acyl-CoA esters with a fluorescent biosensor based on bovine acyl-CoA binding protein (ACBP). Lys50 of ACBP was mutagenized to a cysteine residue that was covalently modified with 6-bromoacetyl-2-dimethylaminonaphthalene to make a fluorescent acyl-CoA indicator (FACI-50). FACI-50 exhibits high fluorescence emission yield with maximum at 490 nm in the presence of CoA when excited at 387 nm. The addition of palmitoyl-CoA to a CoA-saturated FACI-50 lowered fluorescence emission by eightfold. Ethanol extract from 1 μl whole blood was incubated with ATP, CoA, and FACI-50. Following background fluorescence reading, NEFAs were converted to acyl-CoA by the acyl-CoA synthetase and the NEFA content was calculated from fluorescence emission changes using palmitic acid as external standard. The FACI-50 NEFA method was compared with two commercially available methods for quantification of NEFA.

Keywords: Diagnostics; Type 2 diabetes; Nonesterified fatty acids; Acyl-CoA binding protein

Plasma nonesterified fatty acid (NEFA)[1] levels are tightly controlled by the rate of adipose tissue lipolysis and uptake and reesterification in the liver (for a review, see Ref. [1]) and are little affected by the immediate consumption of fat [2]. In addition to being an important energy source, circulating blood NEFAs exert a pleiotropic regulatory function on pancreatic β-cell function and skeletal and smooth muscle glucose metabolism [3], and they regulate the transcription of a number of genes encoding proteins relevant to adipocyte differentiation and other aspects of lipid metabolism [4–7]. Acute elevations of NEFA levels sustain basal insulin secretion during fasting and are essential to maintaining a normal insulin secretory response to glucose [1]. Long-term elevated NEFA levels, often associated with obesity, lead to accumulation of neutral lipids, ceramides, diacylglycerol (DAG), and fatty acid acyl-coenzyme A (acyl-CoA) esters in peripheral tissues [8]. The accumulation of these lipids in various tissues has been associated with pancreatic β-cell malfunction [8], induction of insulin resistance in skeletal muscle [9–11], and development of insulin resistance and type 2 diabetes (T2D). The fasting NEFA level can predict conversion from normal

* Corresponding author. Fax: +45 6550 2467.
E-mail address: jkk@bmb.sdu.dk (J. Knudsen).

[1] *Abbreviations used:* NEFA, nonesterified fatty acids; DAG, diacylglycerol; acyl-CoA, fatty acid acyl-coenzyme A; T2D, type 2 diabetes; IGT, impaired glucose tolerance; ACS, acyl-CoA synthetase; FACI-50, fluorescent acyl-CoA indicator; badan, 6-bromoacetyl-2-dimethylaminonaphthalene; ACBP, acyl-CoA binding protein; *Tb*ACS4, *Trypanosoma brucei* acyl-CoA synthetase 4; LB, Luria broth; Amp, ampicillin; DTT, dithiothreitol; Ni-NTA, Ni^{2+}-nitrilo-triacetate; DMDCS, dimethyldichlorosilane; BSA, bovine serum albumin.

0003-2697/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2006.04.054

glucose tolerance to impaired glucose tolerance (IGT) and conversion from IGT to T2D [12–14]. Therefore, the plasma NEFA concentration is a likely diagnostic marker for identification of individuals at high risk for development of T2D prior to the appearance of insulin resistance and insulin secretion defects [15]. Furthermore, an elevated fasting circulating NEFA concentration is an independent risk factor for sudden death in middle-aged men free of known cardiovascular disease [16]. An elevated circulating NEFA concentration has also been associated with increased risk for cardiovascular disease and been suggested to be directly involved in the induction of the atherosclerotic process [17,18]. High levels of circulating NEFA have been associated with a high frequency of ventricular arrhythmias and sudden death after myocardial infarction [16]. Finally, high fasting plasma NEFA concentration has exhibited a strong independent relation with cancer mortality in the Paris Prospective Study [19] and with some hormone-sensitive cancers such as breast cancer and endometrial cancer in obese postmenopausal women [20]. Therefore, reliable methods for the determination of blood NEFA levels are potentially important in providing early diagnoses of T2D, cancer, extent of myocardial infarction, and other obesity-related diseases.

Several enzymatic methods for quantification of NEFA in serum have been developed. Most of the methods depend on an initial enzymatic activation of fatty acids to acyl-CoA, followed by multiple enzyme-linked spectrophotometric color assays for quantification of the amount of acyl-CoA formed [21–28]. One common feature of the existing methods is that they cannot be used on whole blood and they require venous blood sampling and plasma. In addition, many of the methods have proved to be sensitive to blood components, including catecholamines, ascorbic acid, and bilirubin [27,29,30]. In the current work, we developed a new micro method for the determination of NEFA in a droplet of whole blood collected from fingertip puncture. The assay is also based on conversion of NEFA in a diluted blood sample by an acyl-CoA synthetase (ACS), whereas the enzymatic color quantification of the resulting acyl-CoA is replaced by the use of a new, acyl-CoA binding protein-based, high-affinity, fluorescent acyl-CoA indicator (FACI-50). The sensitivity, reproducibility, and performance of the developed method are compared with the performance of two existing commercial NEFA methods produced by Wako Chemicals and Roche (Boehringer, Mannheim, Germany).

Materials and methods

Reagents

6-Bromoacetyl-2-dimethylaminonaphthalene (badan) was purchased from Molecular Probes (Leiden, The Netherlands). CoA was obtained from ICN Biomedicals (Costa Mesa, CA, USA). The microtiter plates used were black 96-well polypropylene ones from In Vitro (Fredensborg, Denmark). Optiphase HiSafe 3 scintillation liquid was obtained from Perkin-Elmer (Boston, MA, USA). Vitrex end-to-end glass micropipettes used for blood collection were obtained from Modulohm (Herlev, Denmark). Screw-cap glass vials (1.5 ml) and glass inserts (300 μl) were purchased from In Vitro. FACI-50 was obtained from Biosensor (Årslev, Denmark). All other chemicals and solvents used were available from commercial sources.

Site-directed mutagenesis and protein expression and purification and badan labeling

Site-directed mutagenesis of recombinant bovine acyl-CoA binding protein (ACBP) was performed as described previously [31,32]. The primers used for amplification of mutant bovine ACBP were 5′-TGCGGTAAGGCTAA GTGGGACGCTTG upstream and 5′-GAAGTCCAACA TCCCGGGTCTTTC downstream. Protein expression, purification, and badan labeling of Lys50Cys–ACBP were carried out as described previously [31].

Extinction coefficient determination of FACI-50

The molar extinction coefficient of FACI-50 at 387 nm was determined in 200 mM Tris–HCl buffer (pH 7.2). The protein concentration was determined by quantitative amino acid analysis by Peter Højrup (Protein Research Group, Department of Biochemistry and Molecular Biology, University of Southern Denmark).

Determination of FACI-50 fluorescence emission specter

Determination of FACI-50 fluorescence emission specter of FACI-50 was performed on a Spex Fluorolog (Spex Industries, Edison, NJ, USA) with excitation at 387 nm and emission recorded from 400 to 600 nm. FACI-50 (1 μM) was dissolved in 10 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM KCl, and 1 mM Na_2HPO_4.

Expression and purification of TbACS4

The plasmid pET28a carrying the gene encoding His6-tagged *Trypanosoma brucei* acyl-CoA synthetase 4 (*Tb*ACS4) was a generous gift from Paul T. Englund (Department of Biological Chemistry, Johns Hopkins Medical School). Expression and purification were carried out essentially as described previously [33]. Briefly, the plasmid carrying His6-tagged *Tb*ACS4 was transformed into $CaCl_2$-competent TOP10 *Escherichia coli* cells (Invitrogen). Positive clones were identified on Luria broth (LB) plates supplemented with 50 μg/ml ampicillin (Amp), and a single clone was used to inoculate 5 ml LB + Amp, which was grown overnight at 37 °C (200 rpm). A new 200-ml LB + Amp overnight culture was made from this culture and used to inoculate 3 × 2 L LB medium + Amp in 5-L conical flasks to a final OD_{600} of 0.1. The culture was grown to $OD_{600} = 0.5$ at 37 °C,