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Access to gram scale amounts of functional globular adiponectin from *E. coli* inclusion bodies by alkaline-shock solubilization

John T. Heiker [a], Nora Klöting [b], Matthias Blüher [b], Annette G. Beck-Sickinger [a,*]

[a] Institute of Biochemistry, Faculty of Biosciences, Pharmacy and Psychology, Leipzig University, Brüderstrasse 34, 04103 Leipzig, Germany
[b] Department of Medicine, University of Leipzig, Leipzig, Germany

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ABSTRACT

The adipose tissue derived protein adiponectin exerts anti-diabetic, anti-inflammatory and anti-atherosclerotic effects. Adiponectin serum concentrations are in the microgram per milliliter range in healthy humans and inversely correlate with obesity and metabolic disorders. Accordingly, raising circulating adiponectin levels by direct administration may be an intriguing strategy in the treatment of obesity-related metabolic disorders. However production of large amounts of recombinant adiponectin protein is a primary obstacle so far.

Here, we report a novel method for large amount production of globular adiponectin from *E. coli* inclusion bodies utilizing an alkaline-shock solubilization method without chaotropic agents followed by precipitation of the readily renaturing protein. Precipitation of the mildly solubilized protein capitalizes on advantages of inclusion body formation. This approach of inclusion body protein recovery provides access to gram scale amounts of globular adiponectin with standard laboratory equipment avoiding vast dilution or dialysis steps to neutralize the pH and renature the protein, thus saving chemicals and time. The precipitated protein is readily renaturing in buffer, is of adequate purity without a chromatography step and shows biological activity in cultured MCF7 cells and significantly lowered blood glucose levels in mice with streptozotocin induced type 1 diabetes.

1. Introduction

Adipose tissue as an endocrine organ secretes a wide variety of bioactive proteins and lipids (adipokines). Adiponectin is one of the most abundant adipokines. Since its identification by four independent research groups in the mid 1990s [1–4] adiponectin has emerged as a key player in the regulation of insulin sensitivity, inflammation and energy homeostasis [5–7]. Adiponectin plasma concentrations are inversely correlated to obesity and its related disorders including type 2 diabetes, steatosis hepatis, atherosclerosis and cardiovascular disease [8–10], suggesting adiponectin as a promising candidate for drug development and treatment of obesity-related metabolic disorders.

Adiponectin circulates in microgram levels per milliliter in the circulation and full-length adiponectin (fAd) forms a wide range of complexes from low, medium to high molecular weight oligomers (LMW, MMW and HMW) and appears to be involved in different and tissue specific signaling pathways [11]. Serum concentrations of the proteolytic globular adiponectin fragment (gAd) are significantly lower, but gAd seems to be the more potent signaling molecule compared to the full-length protein in animal studies [5,12].

Two receptors have been described as adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2). AdipoR1 shows high affinity for gAd and a reduced affinity for fAd whereas AdipoR2 exerts medium affinity for both variants [13]. The receptors contain seven transmembrane domains but are structurally and functionally distinct from G-protein coupled receptors (GPCRs). We recently confirmed the inverse membrane orientation of AdipoRs in the plasma membrane compared to GPCRs with an intracellular N-terminus and an extracellular C-terminus [14] and reported protein kinase CK 2 as interaction partner of the AdipoR1 [15].

Despite the ongoing discussion which form of recombinant adiponectin (HMW, MMW or LMW) should be used for therapeutic administration, bacterially expressed recombinant gAd exerts a great therapeutic potential. Fruebis et al. [6] demonstrated increased β-oxidation in muscle tissue after acute gAd treatment which resulted in sustained weight loss in high fat/sucrose fed mice. In addition, gAd expressed in yeast significantly lowered blood glucose levels in mice with STZ-induced type 1 diabetes (T1D) and promoted free fatty acid clearance in mice with dyslipidemia [16].

Abbreviations: ACC, acetyl-CoA carboxylase; AdipoR, adiponectin receptor; fAd, full-length adiponectin; gAd, globular adiponectin; HMW, MMW, LMW, high, medium, low molecular weight.

* Corresponding author. Fax: +49 341 9736909.
E-mail address: beck-sickinger@uni-leipzig.de (A.G. Beck-Sickinger).

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Recently, chronic treatment with the trimeric fraction of recombinant gAd was shown to improve glucose tolerance and completely reversed insulin resistance in mice with high-fat diet induced diabetes [17].

However the major drawback for a therapeutic application of adiponectin remains the lack of an easy, cost efficient production of functional protein. This could be overcome by a novel strategy for adiponectin production. Here, we report the alkaline-shock method to solubilize inclusion body derived gAd without chaotropic agents which yields gram amounts of pure, folded and biologically active protein.

2. Materials and methods

2.1. Plasmids, antibodies and chemicals

Globular adiponectin was cloned into pET15b bacterial expression vector (Novagen). Protease inhibitor cocktail was from Sigma–Aldrich. ACC and phospho-ACC specific antibodies were from Cell Signalling Technologies (Danver, MA).

2.2. Bacterial strains

Escherichia coli strain DH5α was used as a host for plasmid constructions and *E. coli* BL21(DE3)pLysS_RARE was used for bacterial expression of His_6-tagged protein.

2.3. Protein expression

Escherichia coli Bl21(DE3)plysS_RARE were transformed with the plasmid gAd_pET15b and grown on solid LB/ampicillin (100 μg/mL) plates at 37 °C overnight. A single colony was selected to grow a 50 mL starter culture overnight at 37 °C. The starter culture was centrifuged at 1500*g* for 5 min and the cell pellet was resuspended in 10 mL LB. The starter culture was inoculated in 1.5 L LB/ampicillin (100 μg/mL) and incubated at 37 °C with shaking until the OD_{600} reached 0.6. Expression of recombinant gAd was induced by addition of IPTG to a final concentration of 1 mM and expression was continued for 6 h at 37 °C. Finally cells were harvested and collected by centrifugation. The cell pellet was resuspended in lysis buffer (25 mM Tris, 500 mM NaCl, 5 mM $MgCl_2$, pH 8, containing lyophilized DnaseI) and lysis was performed in five freeze and thaw cycles using liquid nitrogen and a 30 °C water bath. Bacterial lysates were centrifuged at 18,000*g* at 4 °C for 45 min and the pellet was stored at −70 °C.

2.4. Inclusion body treatment

The pellet (~3 g wet pellet) was resuspended in ~5 volumes of buffer containing 25 mM Tris, 500 mM NaCl, 2 M urea, 0.25% Triton X-100, pH 8.0, incubated for 1 h at RT and centrifuged at 18,000*g* for 45 min at 4 °C. This was repeated twice and a third and fourth time without Triton X-100 to remove detergent. The washed pellet was subjected to three solubilization steps. Inclusion bodies were resuspended in buffer (20 mM Tris, 500 mM NaCl, 8 M urea, 5 mM β-mercaptoethanol, pH 8), incubated for 1 h at RT and centrifuged at 18,000*g* for 45 min at 4 °C.

2.5. Alkaline-shock solubilization and subsequent protein precipitation of pure recombinant gAd

The remaining pellet (~1 g wet pellet) was resuspended in 10 mL of buffer (20 mM Tris, pH 8) and the pH was increased to 12.5 using 1 M NaOH resulting in complete solubilization of the inclusion bodies within seconds. The clear solution was centrifuged for 15 min at 18,000*g* and the supernatant was filtered through a 0.45 μm sterile filter. Protein was precipitated by addition of ~5 volumes of acetone and precipitated protein was collected by centrifugation. The pellet was washed once with acetone, spun down and air dried for storage at −70 °C. Precipitated protein readily dissolved in distillated water or buffer (10 mM Tris, 150 mM NaCl, pH 7.5) up to concentrations of 100 mg/mL. Endotoxin removal for recombinant gAd used in *in vivo* experiments was performed using an ActiClean Etox column (Sterogene) following the manufacturers' instructions.

2.6. SDS–PAGE and Western blot

Samples were run on 15% SDS gels and stained with Coomassie Brilliant Blue R250 (Sigma–Aldrich) or silver stained (Fermentas). SDS gels (7%) of cell lysates (30 μg/lane) were transferred onto PVDF membrane (HyPond, GE Healthcare), blocked with protein free blocking solution (Pierce). After respective antibody incubation blots were developed using ECL-substrate (Pierce) and G:BOX ChemieXL camera (Syngene). Bands were quantified using GeneSnap software (Syngene) and values are presented as means ± SEM of three independent experiments performed. Statistical evaluation of the data was done using one-way analysis of variance (ANOVA) with the Graph-Pad Prism program (Graph-Pad, Inc.). Statistical significance is indicated as follows: $^{*}P < 0.01$.

2.7. Protein concentration determination

Protein concentrations were determined with Bio-Rad Protein Assay (Bio-Rad) using BSA as a standard.

2.8. MALDI-TOF/TOF mass spectrometry

Recombinant protein was analyzed by MALDI-TOF mass spectrometry using a MALDI-TOF/TOF UltraflexIII (Bruker Daltonics) in linear mode. Spectra were calibrated using a mixture of recombinant cytochrome *c* and myoglobin from horse heart (both Fluka) as standard. Tryptic in solution digests were analyzed by MALDI-TOF MS and MSMS. Peak lists of the tryptic peptide masses were generated and searched against the National Center for Biotechnology Information non-redundant database using the Mascot search engine (Matrix Science, London, UK; http://www.matrixscience.com) in order to identify the proteins. For database searches the following parameters were used – species: *Homo sapiens*, tryptic digestion with a maximum of one missed cleavage site, monoisotopic masses; variable modification: methionine residues oxidized; mass tolerance MS: 50 ppm; mass tolerance MSMS: 0.25 Da.

2.9. Circular dichroism spectroscopy

CD spectra were recorded using a JASCO model J720 spectropolarimeter over 250–185 nm at 20 °C in a N_2 atmosphere. Protein solution was measured at a concentration of 5 μM in water. Each measurement was repeated three times using a thermo stable sample cell with a path of 0.2 cm and following parameters: response time of 4 s, scan speed of 20 nm/min, sensitivity of 10 mdeg, step resolution of 0.2 nm, and bandwidth of 2 nm. The CD spectrum of the solvent was subtracted from the CD spectra of the protein solutions to eliminate the interference from solvent and optical equipment.

High-frequency noise was reduced by means of a low-path Fourier transform filter. The ellipticity was expressed as the mean residue weight ellipticity $[\theta]$NRW in deg cm^2 $dmol^{-1}$.