Biochimie 95 (2013) 1403–1410

Contents lists available at SciVerse ScienceDirect

Biochimie

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

Research paper

Natural mutations lead to enhanced proteasomal degradation of human Ncb5or, a novel flavoheme reductase

Fanni S. Kálmán, Beáta Lizák, Szilvia K. Nagy, Tamás Mészáros, Veronika Zámbó, József Mandl, Miklós Csala*, Éva Kereszturi

Department of Medical Chemistry, Molecular Biology and Pathobiochemistry, Semmelweis University, POB 260, 1444 Budapest, Hungary

ARTICLE INFO

Article history:
Received 4 September 2012
Accepted 8 March 2013
Available online 19 March 2013

Keywords:
Missense mutation
Proteasome
Desaturase
Endoplasmic reticulum
Diabetes

ABSTRACT

NADH cytochrome b_5 oxidoreductase (Ncb5or) protects β-cells against oxidative stress and lipotoxicity. The predominant phenotype of lean Ncb5or-null mouse is insulin-dependent diabetes due to β-cell death. This suggests the putative role of NCB5OR polymorphism in human diabetes. Therefore, we aimed to investigate the effect of natural missense mutations on the expression of human NCB5OR. Protein and mRNA levels of five non-synonymous coding variants were analyzed in transfected HEK293 and HepG2 cells. Although the mRNA levels were only slightly affected by the mutations, the amount of Ncb5or protein was largely reduced upon two Glu to Gly replacements in the third exon (p.E87G, p.E93G). These two mutations remarkably and synergistically shortened the half-life of Ncb5or and their effect could be attenuated by proteasome inhibitors. Our results strongly indicate that p.E87G, p.E93G mutations lead to enhanced proteasomal degradation due to manifest conformational alterations in the *b*5 domain. These data provide first evidence for natural mutations in NCB5OR gene resulting in decreased protein levels and hence having potential implications in human pathology.

1. Introduction

Cytochrome b_5 reductase (*b*5R) and cytochrome b_5 (*b*5) are key enzymes in fatty acid desaturation in the endoplasmic reticulum (ER) of human and animal cells. The recently discovered NADH cytochrome b_5 oxidoreductase (Ncb5or) is a soluble natural fusion protein containing both *b*5R-like and *b*5-like domains [1]. The former contains FAD prosthetic group and binds NAD(P)H while the latter contains heme in a unique environment [2]. Ncb5or is widely expressed in human tissues; nevertheless, the highest mRNA levels were found in heart, skeletal muscle, kidneys and pancreas [3].

Ncb5or was shown to be localized in the ER and suggested to transfer electrons to unidentified substrates [4]; however, its exact role has not been clarified yet. Progressive loss of white adipose tissue in Ncb5or knock-out mice is accompanied by a pronounced reduction in the ratios of Δ^9 desaturated to saturated fatty acids in the liver [5], which supports the role of Ncb5or in fatty acid desaturation. It also seems to be involved in maintaining the redox status in β-cells because Ncb5or-deficient pancreatic β-cells have increased susceptibility to the oxidant streptozotocin [6]. In line with this assumption, increased FFA accumulation and catabolism are associated with oxidative stress in Ncb5or-deficient hepatocytes [7].

There is mounting evidence that the ER is a target of palmitate-induced lipotoxicity in pancreatic β-cells [8] and this mechanism seems to contribute to β-cell apoptosis in type 2 diabetes [9–11]. ER stress and the unfolded protein response (UPR) develop more rapidly and severely in Ncb5or-deficient hepatocytes than in wild-type cells when exposed to palmitate [12]. Sensitization to lipotoxicity might underlie the progressive loss of β-cell mass and development of insulin-deficient diabetes observed in Ncb5or-null mice [5,13,14]. This is further supported by the phenotype similarity between the Ncb5or-null mouse and two mouse models of ER stress in the pancreatic β-cells [15–17].

The obvious relationship between Ncb5or and β-cell survival in animal models raises the possible role of human NCB5OR mutations in insulin-dependent diabetes and urges association analyzes and functional *in vitro* studies in this field. The present work aimed

Abbreviations: Ncb5or, NADH cytochrome b_5 oxidoreductase; ER, endoplasmic reticulum; FFA, free fatty acid.

* Corresponding author. Tel./fax: +36 1 2662615.
E-mail addresses: kalmanfanni@yahoo.com (F.S. Kálmán), lizak.beata@med.semmelweis-univ.hu (B. Lizák), nagy.szilvia@med.semmelweis-univ.hu (S.K. Nagy), meszaros.tamas@med.semmelweis-univ.hu (T. Mészáros), zambo.veronika@med.semmelweis-univ.hu (V. Zámbó), mandl.jozsef@med.semmelweis-univ.hu (J. Mandl), csala.miklos@med.semmelweis-univ.hu (M. Csala), kereszturi.eva@med.semmelweis-univ.hu (É. Kereszturi).

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http://dx.doi.org/10.1016/j.biochi.2013.03.004

to study the effect of natural exonic missense mutations on the expression of human Ncb5or.

2. Materials and methods

2.1. In silico mutation analysis

Naturally occurring Ncb5or mutations were collected from NCBI SNP (http://www.ncbi.nlm.nih.gov/sites/entrez) and 1000 Genomes (http://www.1000genomes.org/) databases. The three-dimensional structure of human Ncb5or *b*5 domain was obtained from the PBD protein Data Bank (http://www.pdb.org/pdb/home/home.do; 3LF5). The 3D structure of Ncb5or mutants were generated by I-TASSER online prediction program (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). All images were rendered using DeepView/Swiss-Pdb Viewer version 4.0.2 (www.expasy.org/spdbv/).

2.2. Expression constructs and mutagenesis

NCB5OR cDNA produced from HepG2 cell mRNA was cloned into the pcDNA3.1- plasmid between the *Xho*I and *Hind*III restriction sites using 5′-AAA TTT **CTC GAG** GGG TTT GAA GAT GCT GAA C-3′ sense and 5′-AAA TTT **AAG CTT** GTT GAA TAA AGG ACA ATG ACA G-3′ antisense primers. Glu–Glu-tagged constructs [18] and the studied missense mutations were generated by overlap extension PCR mutagenesis [19]. All constructs were verified by sequencing.

2.3. Cell culture and transfection

Human embryonic kidney (HEK293) and hepatocellular carcinoma (HepG2) cells were chosen over pancreatic β-cells because they are easy to transfect and Ncb5or is expressed ubiquitously. Cells were cultured in 12-well plates (5×10^5 cells per well) in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS and antibiotics at 37 °C in a humidified atmosphere containing 5% CO_2. HEK293 and HepG2 cells were transfected with 2 μg pcDNA3.1-NCB5OR plasmid using 5 μL Lipofectamine 2000 (Invitrogen, Carlsbad, CA) or 5 μL ExGen 500 reagent (Fermentas) in 1 mL DMEM, respectively. The transfection efficiencies were tested under the same conditions by monitoring fluorescence emission from GFP-transfected cells, and they were quantified as the relative number of fluorescent vs. total cells (~80% for HEK293 and ~45% for HepG2). Ncb5or protein expression was detectable as early as 4 h post-transfection and persisted for at least three days. Maximal protein expression was usually achieved between 36 and 48 h post-transfection. NCB5OR-transfected cells were usually harvested and processed 36 h after transfection. For protein stability and proteasomal degradation assays, transfection medium was replaced after 12 h with 1 mL DMEM containing the translational inhibitor Cycloheximide (50 μg/mL) or the proteasome inhibitor MG132 (2 μM) or Lactacystin (5 μM) and the cells were incubated for 1, 2, 4 or 24 h. Non-transfected and GFP expression vector transfected cells were used as control in all experiments.

2.4. Preparation of cell lysates

Cells were washed twice with PBS and harvested in 100 μL reporter lysis buffer (Promega) by scraping and brief vortexing. Incubation (15 min, room temperature) of the lysates was followed by centrifuging in a benchtop centrifuge (5 min, max speed, 4 °C). Protein concentration of the supernatant was measured with Pierce® BCA Protein Assay Kit (Thermo Scientific).

2.5. RT-PCR and qPCR analysis

Total RNA was isolated from transfected cells and DNase digested using RNAqueous® - 4PCR kit (Ambion). 0.2–0.5 μg DNA-free RNA was reverse-transcribed with the SMART High Capacity RNA-to-cDNA Kit (Life Technologies), RT- reactions also were analyzed to rule out any possible effect of DNA contamination. NCB5OR sequence (267 nt) was amplified by PCR using 5′-ATG AAC TAA TGA GAG CAG CAG-3′ and 5′-TGG CAA TGG TGA CTA AAG AG-3′ primers, Qiagen HotStarTaq DNA-polymerase kit (Valencia, CA) at thermocycle conditions of 95 °C 15 min, 28 cycles of 94 °C 30 s, 50 °C 30 s and 72 °C 1 min and 72 °C 10 min final extension. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (261 nt fragment) was also amplified as a reference control using pseudogene-free amplification conditions, 5′-GTC CAC TGG CGT CTT CAC CA-3′ and 5′-GTG GCA GTG ATG GCA TGG AC-3′ primers [20]. Quantitative qPCR assay was performed in 15 μL final volume containing 3 μL cDNA, 1× ABI PCR master mix, 1× TaqMan® probes and primers (NCB5OR: Hs00953391_m1 and GAPDH: Hs99999905_m1; Life Technologies) using ABI 7300 Real-Time PCR System (Life Technologies). Denaturation at 95 °C, 10 min was followed by 40 cycles (95 °C, 15 s and 60 °C, 1 min). Reactions were performed in triplicate using RNase-free water as negative control. C_T-values were set in the exponential range of the amplification plots using the 7300 System Sequence Detection Software 1.3. Relative expression levels were expressed as $2^{-\Delta\Delta CT}$ where $\Delta\Delta C_T$ values correspond to the difference between the C_T-values of the target and the internal control genes.

2.6. Western blot analysis

Cell lysates (50 μg protein) were electrophoresed on 12% Tris-glycine minigels and transferred onto Immobilon-P membranes (Millipore, Billerica, MA). Primary and secondary antibodies were applied overnight at 4 °C and for 1 h at room temperature, respectively. Horseradish peroxidase (HRP)-conjugated goat polyclonal anti-Glu–Glu-tag (Abcam, Cambridge, MA; ab1267-100) and HRP-conjugated goat polyclonal anti-actin (Santa Cruz, sc-1616) antibodies were used at 1:2000 dilution. Ncb5or was detected with a goat polyclonal antibody raised against a peptide mapping near the N-terminus of human Ncb5or (Santa Cruz, Santa Cruz, CA; sc-68684), used at a dilution of 1:1,000, followed by HRP conjugated donkey polyclonal anti-goat IgG (Santa Cruz, sc-2020) at a dilution of 1:2000. HRP was detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce).

2.7. Pulse-chase experiment and immunoprecipitation

Protein half-lives were assessed in HEK293 cells 36 h after transfection with wild type or p.E87G mutant Ncb5or. Cells were incubated in methionine- and cysteine-free DMEM labeling medium (Invitrogen) for 45 min and pulse-labeled with 100 μCi ^{35}S-TransLabel (MP Biomedicals) for 1 h. The chase was initiated by removing the labeling media, washing the cells twice with ice cold PBS and adding DMEM containing excess unlabeled methionine and cysteine (2 mM each). At the indicated time points, cells were washed with PBS and lysed in 1 mL NP-40 lysis buffer (0.15 M NaCl, 10 mM Tris-HCl [pH 7.5], 1% Nonidet P-40 [NP-40], 0.2% deoxycholate). Ncb5or was immunoprecipitated by mixing cell lysates with mouse monoclonal antibody (Santa Cruz, sc-100529) and recovering immune-complexes with Protein-A Sepharose beads (Sigma). Beads were washed three times with NP-40 washing buffer (0.4 M NaCl, 10 mM Tris-HCl [pH 7.5], 1% Nonidet P-40 [NP-40], 0.2% deoxycholate), eluted with SDS gel loading buffer and analyzed on 10% SDS-PAGE. Gels were exposed to phosphoscreen for 24 h, and scanned by using phosphoimager (Typhoon). The