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Research report

Skin fibroblast model to study an impaired glutathione synthesis: Consequences of a genetic polymorphism on the proteome

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ABSTRACT

An impaired glutathione (GSH) synthesis was observed in several multifactorial diseases, including schizophrenia and myocardial infarction. Genetic studies revealed an association between schizophrenia and a GAG trinucleotide repeat (TNR) polymorphism in the catalytic subunit (GCLC) of the glutamate cysteine ligase (GCL). Disease-associated genotypes of this polymorphism correlated with a decrease in GCLC protein expression, GCL activity and GSH content. To clarify consequences of a decreased GCL activity at the proteome level, three schizophrenia patients and three controls have been selected based on the GCLC GAG TNR polymorphism. Fibroblast cultures were obtained by skin biopsy and were challenged with *tert*-butylhydroquinone (t-BHQ), a substance known to induce oxidative stress. Proteome changes were analyzed by two dimensional gel electrophoresis (2-DE) and results revealed 10 spots that were upregulated in patients following t-BHQ treatment, but not in controls. Nine corresponding proteins could be identified by MALDI mass spectrometry and these proteins are involved in various cellular functions, including energy metabolism, oxidative stress response, and cytoskeletal reorganization. In conclusion, skin fibroblasts of subjects with an impaired GSH synthesis showed an altered proteome reaction in response to oxidative stress. Furthermore, the study corroborates the use of fibroblasts as an additional mean to study vulnerability factors of psychiatric diseases.

1. Introduction

Glutathione (GSH) metabolism related genes have been shown to be associated with various diseases, including myocardial infarction, diabetes, lung diseases, and schizophrenia [16,34,20,5,24]. Measurements of GSH synthesis in skin fibroblast cultures from schizophrenia patients, as compared to control subjects, revealed a compromised GSH synthesis at the level of the enzyme glutamate cysteine ligase (GCL) [16]. GSH is synthesized by two consecutive reactions, catalyzed by the enzymes GCL and GSH synthetase (GSS). GCL is the rate limiting enzyme and consists of a catalytic (GCLC) and a modifier (GCLM) subunit [25]. GCLC contains a functional GAG trinucleotide repeat (TNR) polymorphism in the 5′untranslated region with either 7, 8, or 9 GAG repeats [36,35]. Genetic analysis has shown an association between this GCLC GAG TNR polymorphism and schizophrenia. In parallel, skin fibroblast cultures with disease-associated GCLC GAG TNR genotypes (high-risk: 7/8, 8/8, 8/9 and 9/9) had decreased GCLC protein expression, decreased GCL activity, and decreased GSH contents, as compared to cultures with genotypes that were present more often in control subjects (low-risk: 7/7 and 7/9) [16].

The aim of the present study was to examine, whether an impaired GSH synthesis, due to the GCLC GAG TNR polymorphism, would have consequences on the regulation of the proteome. We assumed that any deficit in GSH synthesis would be more pronounced under conditions of oxidative stress. Thus, we cultivated skin fibroblasts from three control subjects with genotype 7/7 and three patients with disease-associated genotypes (7/8, 8/9 and 9/9), and we challenged skin fibroblasts with *tert*-butylhydroquinone (t-BHQ), a substance known to induce oxidative stress [33,30]. We then analyzed total protein extracts from cell cultures under baseline (untreated) and t-BHQ treated conditions by 2D gel electrophoresis (2-DE), we compared proteomes in response to t-BHQ treatment and in relation to the GCLC GAG TNR polymorphism, and finally, we identified altered spots by MALDI mass spectrometry.

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2. Materials and methods

2.1. Subjects

Patients were diagnosed using DSM-IV criteria, and controls were all of caucasian origin, and assessed by the Diagnostic Interview for Genetic studies. Two female and one male of each, patients and controls were matched for age and sex, with mean age and standard deviation of 37 ± 17 years for patients and of 38 ± 15 years respectively for controls. All subjects were recruited with fully informed written consent according to ethical guidelines of the Lausanne University.

2.2. Genotyping

DNA was purified from blood samples using NucleonBACC3 system (Amersham International, Little Chalfont, UK). DNA concentrations were quantified using PicoGreen kit (Invitrogen, Basel, Switzerland). GCLC GAG TNR polymorphism was assessed as originally described [36] with the following modifications: briefly, PCR amplification was performed in reaction mixtures of 25 μl containing 40 ng of each primer, 200 μM of each NTP and 0.3 U EuroTaq DNA polymerase with its buffer (Euroclone, UK). Temperature cycling of the PCR was as originally described. PCR products were separated on an 8% polyacrylamide gel (6 cm × 8 cm) for 5 h with 40 V and stained using SilverXpress Silver Staining Kit (Invitrogen, Basel, Switzerland).

2.3. Cell cultures and treatment

Fibroblast cultures were established from skin biopsies taken after written informed consent of the patients and of controls. Cells were grown with DMEM medium (Invitrogen, Basel, Switzerland) (0.5 l medium completed with 10 ml Ultroser-G serum (BioSepra, Cergy, France), 5 ml penicillin/streptomycin (Invitrogen, Basel, Switzerland), 5 ml sodium pyruvate (100 mM)) at 37 °C in a humidified atmosphere containing 5% CO_2/95% air. t-BHQ was dissolved in DMSO to a final concentration of 0.05%. Human fibroblasts near confluence after five passages were treated with 50 μM t-BHQ, or the vehicle alone for 18 h.

2.4. Western-blot analysis

Cells were scraped with cold lysis buffer (2.37 ml scraping buffer (Tris–HCl 50 mM, pH 7.2; NaCl 150 mM; NaF 10 mM; EDTA 2 mM; EGTA 2 mM; Triton X-100 1%); 100 μl complete (Roche Diagnostics, Basel Switzerland; 1 tablet/2 ml H_2O); including 25 μl PMSF 100 mM and 2.5 μl DTT 1 mM). Lysates were homogenized 10 times with a 25-gauge needle, and cellular debris was cleared by centrifugation (14,000 rpm, 14 min, 4 °C). Protein concentration was determined by BCA protein assay (Pierce, Rockford, IL). Equal amounts of protein (40 μg) were dissolved in 5× Laemmli sample buffer, separated by 12% SDS-PAGE, and electro-transferred to a PVDF membrane. Membranes were blocked (BSA 1%/milk powder 3%) overnight at 4 °C. Following incubation with primary and secondary antibodies, protein was visualized by using the ECL detection system (Amersham International) and protein abundance was quantified by densitometry analysis (Multi Genius, Bio Imaging System, Syngene, Cambridge, UK). The results were normalized to α-tubulin, corrected and reported as relative values. Anti-human α-tubulin antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz Biotechnology, Germany). Anti-human GCLC antibody was obtained from Dr. Portia Vliet (Department of Environmental Health at the University of Washington, Seattle).

2.5. GCL activity

Fibroblast cells were trypsinized and pelleted. The pellet was taken up into PBS and frozen. GSH content and GCL-activity was determined in protein extracts from fibroblast cultures, with a fluorescence-based microtiter plate assay according to White et al. [37], with the following modifications. Cell pellets (corresponding approximately to 1 mg protein) were resuspended in 500 μl PBS and sonicated on ice. The sonicated solutions were centrifuged (14,000 × *g* at 4 °C, 15 min), and the supernatants were transferred to 8 wells (50 μl each) of microtiter plates with a round bottom (Nunc, Apogent, Denmark). Half of the 8 samples were treated with 5 μl BSO (final concentration 100 μM) for 15 min at 37 °C, the other four wells were treated at the same time with 5 μl water. Before the reaction initiation, 50 μl of the reaction mix (400 mM Tris pH 8.0, 40 mM ATP, 20 mM L-glutamic acid, 2.0 mM EDTA, 20 mM sodium borate, 2 mM L-serine, 40 mM $MgCl_2$) were added to the samples and prewarmed for 5 min at 37 °C. The GCL reaction was started with 50 μl of 2 mM cysteine (dissolved in 20 mM Tris pH 8.0, 1.0 mM EDTA, 250 mM D+ sucrose, 2 mM L-serine, 20 mM sodium borate). After 45 min, the reaction was stopped by precipitation of the proteins with 50 μl sulfo-salicylic acid (200 mM) and centrifugation (1200 × *g*, 5 min). 20-μl aliquots of supernatant from each well of the reaction plate were transferred to a 96-well plate designed for fluorescence detection (96 Well EIA/RIA Plate with flat bottom, Corning Incorporate, USA). The fluorescence reaction was initiated by adding 180 μl of 2,3-naphthalenedicarboxyaldehyde (NDA, Fluka, Switzerland) derivatization solution (50 mM Tris, pH 10, 0.5N NaOH, and 10 mM NDA in Me_2SO, v/v/v 1.4/0.2/0.2). Following incubation (30 min at room temperature) the fluorescence intensity was measured (485 ex/530 em) with a fluorescence plate reader (FluoroCount microplate reader, PerkinElmer, Switzerland). Wells containing BSO had a blocked GCL activity and represented GSH contents. GCL activity was determined as the difference between the unblocked and the BSO treated wells per min and per mg protein. Protein concentration was determined by BCA protein assay.

2.6. Sample preparation for 2-DE

Skin fibroblasts from four petri dishes (10 cm) were pooled and homogenized with 1:10, w/v sample buffer (5 M urea, 2 M thiourea, 35 mM Tris, 4% CHAPS, 1% dithioerythritol (DTE)) followed by protein determination (Bio-Rad, Reinach, Switzerland), obtaining between 1.2 and 1.5 mg total protein.

2-DE. This method was described previously [11]: for first dimension separation, ReadyStrip IPG Strip pH 3.0–10, 11 cm long (Bio-Rad, Reinach, Switzerland) were rehydrated with 500 μg of proteins adjusted overnight in 0.350 mL rehydration buffer (8 M urea, 2% CHAPS, 15 mM DTT, 0.2% Bio-Lytes pH 3–10, 0.001% bromophenol blue) [31]. The strips were then covered with mineral oil and the proteins were separated for 98,000 V/h at 20 °C [29]. For the second dimension, strips were first incubated in equilibration buffer (6 M urea, 0.375 M Tris–HCl pH 8.8, 2% SDS, 20% glycerol, v/v), 130 mM DTT, 0.001% bromophenol blue for 15 min and a second buffer (135 mM iodoacetamide, 0.001% bromophenol blue) for 15 min. The proteins were then separated on a 9–16% acrylamide gradient gel prepared from a stock solution: (30% acrylamide, 0.8% N,N′-methylene-bis-acrylamide) 25% Tris–HCl, 1.5 M pH 8.8, 0.1% SDS, 0.03% Temed ultrapure water, and 0.5% APS 10%. Migration was performed with 25 mA/gel in Laemmli buffer (25 mM Tris, 0.192 M glycine, 0.1% SDS) at 20 °C for 6–7 h.

2.7. Image acquisition and analysis

Proteins were visualized by Coomassie brilliant blue staining. Gels were scanned using a transmissive, flatbed scanner (UMAX) and analyzed using Melanie 2D gel analysis software (Melanie version 4, GENEBIO, Geneva, Switzerland). Following background subtraction and volume normalization on entire gels, signal intensity was quantified and controlled by 3D view representation. Moreover, average gels were created for each group to assist comparison and reduce within group variations. Protein spots that have been expressed more potently (mean > 1.5 times) in t-BHQ treated cells from patients compared to controls were excised for identification by mass spectrometry.

2.8. Protein identification by tandem mass spectrometry

Excised Coomassie blue stained spots were distained and identified by the Protein Analysis Facility of the University of Lausanne (www.unil.ch/paf). An in-gel proteolytic cleavage was performed by the robotic workstation Investigator ProGest (Genomic Solutions, Ann Arbor, Michigan). The instrument washes the gel pieces, performs reduction and alkylation, adds trypsin (Promega, Madison, WI, USA), and finally extracts the peptides from each gel piece in 30% (v/v) acetonitrile, 0.5% formic acid. The peptide extracts were then dried by centrifugal evaporation and subsequently resuspended in 4 μl of matrix (4 mg/ml alpha-cyanohydroxy cinnamic acid in 40% acetonitrile). One microliter of the obtained samples were deposed on a MALDI plate, rapidly dried and analyzed on a 4700 Proteomics Analyzer MALDI-TOF/TOF instrument (Applied Biosystems, Framingham, MA, USA). Spectra were acquired for peptide mass fingerprinting (PMF) in the mass range 800–2300 *m*/*z*, combined with MS/MS on the 10 most intense peptide signals for those spots whose identification was below threshold by PMF (spot 6). All the spectra were used for analysis. For spectra compare supplementary figures. Peak lists were exported with the "Peak to Mascot" tools included in the version 2 of the 4000 Series Explorer software (Applied Biosystems, Framingham, MA, USA). The software extracted centroided, deisotoped MS peaklists with the following parameters: mass range 830–2300 Da, maximal peak density 100 peaks per 200 Da, minimum signal to noise ratio of 2.0, minimal area 10, maximum peaks per spectrum 200. For the MS/MS analysis of spot 6 the spectra were exported in the mass range from 60 Da to the mass of the precursor minus 20 Da. The peak density was at maximum 100 peaks per 200 Da, the signal to noise and area threshold were as above. Database searches were performed with MASCOT 2.0.04 software (http://www.matrixscience.com). 68497 sequences were present in the database after taxonomy filter (Homo sapiens). The identification parameters were set as follows: database, release 11.5 of UNIPROT (SWISSPROT + TrEMBL, July 20th, 2007); species, human; enzyme, trypsin; mass tolerance, 50 ppm for PMF spectra and 0.5 Da for MS/MS spectra; missed cleavages, 1 allowed; carbamidomethylation was specified as a fixed modification for cysteine and methionine oxidation was allowed as a variable modification. The statistical threshold for protein identification by MASCOT was 61 for PMF and 41 for MS/MS.

Proteins with common sets of peptides were grouped together by MASCOT according to the principle of parsimony. Whenever more than one sequence matched exactly the same set of peptides, the database entry with the highest score was chosen to be reported in Table 2. However if that entry appeared to be a fragment