Biochimica et Biophysica Acta 1822 (2012) 1161–1168

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

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

Transcriptional changes in OXPHOS complex I deficiency are related to anti-oxidant pathways and could explain the disturbed calcium homeostasis

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ARTICLE INFO

Article history:
Received 15 August 2011
Received in revised form 20 September 2011
Accepted 11 October 2011
Available online 17 October 2011

Keywords:
Gene expression
Complex I deficiency
Mitochondrion
Oxidative stress
Selenoprotein
Nrf2

ABSTRACT

Defective complex I (CI) is the most common type of oxidative phosphorylation disease, with an incidence of 1 in 5000 live births. Here, whole genome expression profiling of fibroblasts from CI deficient patients was performed to gain insight into the cell pathological mechanism. Our results suggest that patient fibroblasts responded to oxidative stress by Nrf2-mediated induction of the glutathione antioxidant system and Gadd45-mediated activation of the DNA damage response pathway. Furthermore, the observed reduced expression of selenoproteins, might explain the disturbed calcium homeostasis previously described for the patient fibroblasts and might be linked to endoplasmic reticulum stress. These results suggest that both glutathione and selenium metabolism are potentially therapeutic targets in CI deficiency.

1. Introduction

Mitochondria produce most of the cellular ATP through the process of oxidative phosphorylation (OXPHOS). The OXPHOS system is comprised of four multisubunit electron transport chain (ETC) complexes (complexes I–IV) and the F_0/F_1-ATP-synthase (complex V). Complex I (CI) is the largest of the OXPHOS complexes and one of the entry points of electrons into the ETC. CI deficiency is the most frequently encountered defect in mitochondrial energy metabolism [1] and is associated with *e.g.* Leigh and LHON disease, fatal infantile acidosis, neonatal cardiomyopathy with lactic acidosis, leucodystrophy with macrocep.haly and hepatopathy with renal tubulopathy [2, 3]. Although children usually have a normal prenatal development, symptoms start occurring during their first year of life after which the disease deteriorates rapidly and may become fatal [4].

Disease-causing mutations have been described in both the mitochondrial DNA (mtDNA)-encoded (*ND1* to *ND6*, and *ND4L* genes) and nuclear DNA-encoded structural CI subunits (*e.g.* NDUFS1, NDUFS2, NDUFS4, NDUFS7, NDUFS8) and CI assembly factors (*e.g.* B17.2L, NDUFA12L, C20ORF7) [5–26]. The cellular consequences of CI deficiency have been extensively studied in patient fibroblasts. CI deficiency leads to a slightly depolarized mitochondrial membrane potential [27], increased reactive oxygen species (ROS) levels [28], increased NAD(P)H levels [29], changes in mitochondrial morphology [30] and disturbed calcium homeostasis [31, 32]. However, increased ROS production and mitochondrial fragmentation was not always detectable [33]. A first study investigating transcriptional responses in CI deficient fibroblasts cultured with glucose and galactose using a mitochondria-targeted microarray detected the induction of metallothioneins and heat shock proteins and the decreased expression of mtDNA-encoded transcripts [34]. To get an unbiased overview of the underlying pathological processes (not restricted to mitochondria) in CI deficiency, we performed whole genome gene expression and additional pathway analysis in fibroblasts of a homogeneous group of CI deficient patients with a defect in one of the nuclear encoded structural subunits. The CI deficiency might not stress the cell to such an extent that relevant disease-associated gene expression changes can be picked up. Therefore, fibroblasts were deprived of glucose to stimulate energy production through oxidative phosphorylation. In galactose medium, the flow of galactose to glucose-1-phosphate is very slow which obliges cells to obtain ATP from mitochondrial oxidation of pyruvate and glutamine [35].

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doi:10.1016/j.bbadis.2011.10.009

2. Material and methods

2.1. Fibroblast cell lines

Fibroblasts were derived from skin biopsies of five patients homozygous or compound heterozygous for nuclear complex I mutations and five controls. The groups were matched for age and sex. All patients have been described previously (#8807 see [26], #7898 see [24], #5175 see [15], #6613 see [30], and #8382 see [25]). Table 1 provides an overview of the subjects and physiological parameters in the patient cell lines [4, 28, 29, 32]. The age- and sex-matched control group consisted of four male subjects, ages 6 months (mo), 1 year (y) 7 mo, 1 y 6 mo, and 3 y 6 mo respectively and one female subject of 1 y 11 mo.

Fibroblasts were routinely cultured in medium 199 (Gibco, Paisley, UK) supplemented with 10% fetal calf serum and penicillin/streptomycin (respectively 100 U/ml and 100 μg/ml). To stimulate energy production by oxidative phosphorylation, fibroblasts were cultured in galactose medium for 48 h. Galactose medium consisted of DMEM without glucose, without pyruvate and with 4 mM L-glutamine (Invitrogen, Paisley, UK), 5.5 mM D-galactose (Sigma, Zwijndrecht, Netherlands), 1 mM uridine (Acros, Geel, BE), 10% dialyzed fetal calf serum (Invitrogen) and penicillin/streptomycin. Glucose medium was identical to galactose medium except that D-glucose was present at 5.5 mM instead of galactose. The stability of the mtDNA was preserved during the course of the experiment in all cell lines under both conditions as no mtDNA deletions or group/treatment specific differences in mtDNA copy number (Supplementary Table 1) could be detected.

2.2. Microarray procedure

Total RNA was isolated using the TRIzol reagent (Invitrogen) and purified with the RNeasy clean-up kit (Qiagen, Hilden, DE). RNA quantity and purity were determined spectrophotometrically using the Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE, USA). RNA integrity was assessed by determining the RNA 28S/18S ratio using the Bioanalyser 2100 (Agilent Technologies, Santa Clara, CA, USA). Fibroblast RNA (150 ng) was reverse transcribed into cDNA and amplified in a two-round amplification reaction according to the manufacturer's protocol (Affymetrix, Santa Clara, CA, USA). A mixture of cDNA and added hybridization controls was hybridized on Affymetrix HG-U133 Plus 2.0 chips, followed by staining and washing steps in the GeneChip fluidics station 400 (Affymetrix) according to the manufacturer's procedures. To assess the raw probe signal intensities, chips were scanned using the GeneChip scanner 3000 (Affymetrix).

2.3. Microarray data analysis

Images of the Human Genome U133 Plus 2.0 arrays were quantified with GCOS software (Affymetrix). The microarray data reported in this manuscript have been deposited in NCBI Gene expression omnibus (GEO), accession number GSE27041. The chip description file (CDF) used for the analysis was an update created and freely distributed by the microarray lab of the university of Michigan (http://brainarray.mbni.med.umich.edu; [36]) based on Ensembl (version 10). A more detailed description of this analysis is shown in the supplementary data. Briefly, the genes were analyzed using Gaussian linear regression including the hybridization and labeling spikes, age, sex, passage, copy number, and medium. The inference criterion used for comparing the models is their ability to predict the observed data, i.e. models are compared directly through their minimized minus log-likelihood. When the numbers of parameters in models differ, they are penalized by adding the number of estimated parameters, a form of the Akaike information criterion (AIC) [37]. For each gene, the group was then added to the model. Then, the group-medium interaction was also added to the model. The gene under consideration was found to be differentially expressed if the AIC of either of these two models decreased compared to the model containing no group effect at all. The genes analyzed and fold changes were loaded into the Pathvisio (version 2.0.8) [38] software package to evaluate the transcripts in relation to known biological processes. The gene database version "Hs_Derby_20090509" was used. Only gene-transcripts with either their average intensities for the control and patient groups above 150 or average intensities for one of these groups above 300 and a 10% up or down regulation fold change were used to obtain a ranked list of pathways with differentially expressed genes. Pathvisio software was used to select the pathways with a Z score larger than 1.96 and thus containing relatively high numbers of differentially expressed genes.

2.4. Quantitative PCR

Differentially expressed genes were validated by real-time quantitative PCR (qPCR) with the same RNA samples used for the microarrays. Primers were designed using the NCBI Primer-BLAST tool (NCBI home page; http://www.ncbi.nlm.nih.gov/). cDNA was prepared from 1 μg of RNA in a standard reverse transcriptase reaction. PCR was performed in a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using SensiMixPlus SYBR (Quantace, Finchley, UK). Cycling conditions were: an initial step of 2 min at 50 °C, activation of the polymerase at 95 °C for 10 min, and 40 cycles of 15 s at 95 °C followed by 1 min at 60 °C. The TATA-box binding protein (TBP) gene was used as an internal reference. Genes for which QPCR was performed and the primers used for amplification are listed in Supplementary Table 2. Results were analyzed using Gaussian linear regression, similar to the microarray analysis. The housekeeping gene (TBP), age, sex, passage, copy number, rin, and medium were included during the analysis. The AIC was used to assess whether there was a difference between the controls and patients (group effect). All statistical analyses presented were performed using the freely available program R [39] and the publicly available library 'growth'[40].

Table 1
Patient characteristics and physiological cell line parameters.

Cell line	Group	Age	Sex	Affected subunit and mutation	CI	HEt	CM-H_2DCF	GSH	GSSG	ER_{Ca}	$[Ca]_C$ peak	$[Ca]_M$ peak
8328	Patient	1 y	M	NDUFS1-211delE/V288A	**24**	N/A	N/A	N/A	N/A	N/A	N/A	N/A
8807	Patient	7 mo	F	NDUFS2–D445N	**26**	**191**	**244**	N/A	N/A	**82**	**91**	**87**
7898	Patient	<7 mo	M	NDUFS4–R106X	**36**	**174**	**187**	18.8	0.587	**77**	**84**	**79**
5175	Patient	3 y	M	NDUFS7–V122M	**68**	**151**	**212**	21.3	0.630	**73**	**80**	**76**
6613	Patient	<1 mo	M	NDUFS8–R94C	**18**	**222**	**275**	28.5	1.017	**85**	**91**	**89**

Physiological parameters have been published previously [4, 28, 29, 32]; values in bold are significantly different from control (see respective publications); mutations are given at the protein level; CI = residual CI activity (% of lowest control); HEt = rate of ethidium formation as a measure of ROS levels (% of control); CM-DCF = rate of 5-[and −6]-chloromethyl-2′,7′-dichlorofluorescein formation as a measure of ROS levels (% of control); GSH = reduced glutathione levels (average control value 26.1 ± 1.8 nmol/mg protein); GSSG = oxidized glutathione levels (average control value 0.793 ± 0.033 nmol/mg protein); ER_{Ca} = resting calcium content of the endoplasmic reticulum (% of control); $[Ca]_C$ peak = bradykinin(Bk)-induced peak in crease in cytosolic free Ca^{2+} concentration (% of control); $[Ca]_M$ peak = Bk-induced peak increase in mitochondrial free Ca^{2+} concentration (% of control); in previous publications, cell lines 8807, 7898 and 6613 correspond to cell lines 7276, 5260 and 6603 respectively; m = male, f = female, N/A = not available.

2.5. Western blot analysis

Protein expression was monitored by Western blot analysis of 12% SDS-PAGE gels loaded with 40 μg of whole cell extract. The following primary antibodies were used: Complex I (NDUFA9; Mitosciences, Oregon, USA), HMOX1 (Abcam, Cambridge, UK), GCLM (Sigma), GSR (Santa Cruz, Heidelberg, DE), and TrxR1 (Santa Cruz). Complex II (70 kDa Fp; Mitosciences) was used as a loading control. Secondary antibodies used were polyclonal goat anti-mouse IgG/HRP (Dako, DK) and immunoPure goat anti-rabbit IgG/peroxidase (Pierce Biotechnology, Rockford, IL, USA). Detection of the signal was performed using ECL Western Blotting Substrate (Thermo Scientific, Amsterdam, NL) following the manufacturer's instructions.

3. Results

Global gene expression profiles of fibroblast cell lines from five CI deficient patients with a mutation in a nuclear CI gene were characterized in glucose and galactose medium to identify processes involved in pathogenesis, novel biomarkers for CI deficiency and potential future targets for therapeutic interventions. In order to gain insight into the changes in essential cellular processes rather than single genes, genes were clustered based on cellular function and analyzed by pathway analysis using Pathvisio software [38].

3.1. Gene expression analysis

In total, disease state, culture condition and their interaction significantly altered the expression of 3279 genes. The disease state induced fold changes between 0.08 and 6.89 (of which 79% between 0.66 and 1.5 (<50% change)) in the glucose condition and between 0.08 and 12.18 (69% between 0.66 and 1.5) in the galactose condition. Furthermore, the different culture conditions caused gene expression changes of 0.32 to 2.91 (90% between 0.66 and 1.5) and 0.11 to 29.74 fold (46% between 0.66 and 1.5) in controls and patients respectively. Pathway analysis was performed to identify processes that were altered due to the cumulative effect of differentially expressed genes (Table 2 and Supplementary Table 3). The sum of the differentially expressed genes in a pathway can lead to increased expression, decreased expression or a miscellaneous effect (both inhibition and stimulation), which is indicated for each pathway.

3.1.1. Changes due to culture condition only

The metabolic switch due to glucose deprivation of the cells in galactose medium changed the expression of 2888 genes. This included genes with a larger/smaller fold change or an opposite fold change in patients compared with controls. If only genes were included that showed the same effect on galactose compared with glucose for both groups, 1304 genes were changed and these could be mapped to the pathways cholesterol biosynthesis (increased in galactose), proteasome degradation (miscellaneous effect) and prostaglandin synthesis and regulation (increased in galactose) (Fig. 1B).

3.1.2. Changes due to complex I deficiency

Between patients and controls, 1407 and 2335 genes were significantly changed in glucose and galactose, respectively, with an overlap of 1199 genes. Pathways that showed a significant change only in the normal glucose condition included the pentose phosphate

Table 2
Pathway analysis results.

	Patients versus controls glucose		Patients versus controls galactose		Galactose versus glucose identical in patients and controls		Galactose versus glucose different in patients and controls	
Pathway	% changed	Z score	% changed	Z score	% changed	Z score	% changed	Z score
Changes due to culture condition only								
Cholesterol biosynthesis	14.29	0.53	14.29	−0.32	**57.14**	**5.92**	0.00	−1.34
Proteasome degradation	13.33	0.85	25.00	1.53	**18.33**	**2.20**	15.00	0.89
Prostaglandin synthesis and regulation	13.33	0.60	20.00	0.36	**23.33**	**2.47**	6.67	−0.81
Changes due to disease, notable in normal condition								
Pentose phosphate pathway	**57.14**	**4.15**	42.86	1.76	28.57	1.65	14.29	0.24
Heme biosynthesis	**44.44**	**3.43**	33.33	1.25	22.22	1.24	0.00	−1.08
Alanine and aspartate metabolism	**33.33**	**2.69**	33.33	1.44	25.00	1.75	8.33	−0.33
Urea cycle and metabolism of amino groups	**26.32**	**2.36**	31.58	1.61	**31.58**	**3.17**	10.53	−0.12
Ganglio sphingolipid metabolism	**26.67**	**2.14**	33.33	1.61	**33.33**	**3.04**	6.67	−0.57
Adipogenesis	**15.70**	**2.10**	23.14	1.65	11.57	0.62	12.40	0.36
Changes due to disease, notable in normal and selective condition								
Senescence and autophagy	**22.58**	**4.07**	**27.96**	**2.68**	15.05	1.68	13.98	0.80
Selenium	**17.57**	**2.17**	**29.73**	**2.78**	9.46	−0.13	17.57	1.70
Mismatch repair	**33.33**	**2.32**	**44.44**	**2.12**	11.11	0.12	22.22	1.03
Nucleotide metabolism	**27.78**	**2.51**	**38.89**	**2.39**	22.22	1.75	22.22	1.45
Changes due to disease that interact with culture condition								
Oxidative stress	**22.22**	**2.11**	**48.15**	**4.20**	14.81	0.85	**37.04**	**4.22**
Selenium metabolism/selenoproteins	**28.13**	**3.41**	**40.63**	**3.45**	15.63	1.08	**28.13**	**3.00**
TGF beta signaling pathway	**19.23**	**2.22**	**34.62**	**3.26**	11.54	0.39	**26.92**	**3.56**
Translation factors	**21.43**	**2.46**	26.19	1.48	**19.05**	**1.99**	**21.43**	**2.07**
Changes due to interaction disease-culture condition only								
Cell cycle	14.12	1.26	**32.94**	**3.78**	10.59	0.21	**24.71**	**3.92**
G1 to S cell cycle control	12.31	0.61	**27.69**	**2.17**	9.23	−0.19	**21.54**	**2.61**
Androgen receptor signaling pathway	8.41	−0.57	**25.23**	**2.12**	6.54	−1.19	**21.50**	**3.35**
Glutathione metabolism	6.25	−0.51	**37.50**	**2.10**	0.00	−1.33	**37.50**	**3.30**
Keap1–Nrf2	7.69	−0.28	**46.15**	**2.72**	15.38	0.66	**38.46**	**3.08**
DNA damage response	11.94	0.52	**31.34**	**3.00**	11.94	0.56	**23.88**	**3.26**
Glycogen metabolism	11.43	0.27	**31.43**	**2.17**	14.29	0.87	**25.71**	**2.69**
TGF-beta receptor signaling pathway	10.27	0.09	22.60	1.64	10.96	0.43	**17.12**	**2.24**
Id signaling pathway	6.12	−0.92	22.45	0.91	10.20	0.07	**20.41**	**2.01**
TNF-alpha/NF-κB signaling pathway	8.00	−0.93	20.00	0.88	8.57	−0.61	**16.00**	**1.98**

Every pathway counts at least 7 genes measured by the microarray.
A Z score of more than 1.96 indicates that a pathway is significantly changed (shown in bold).

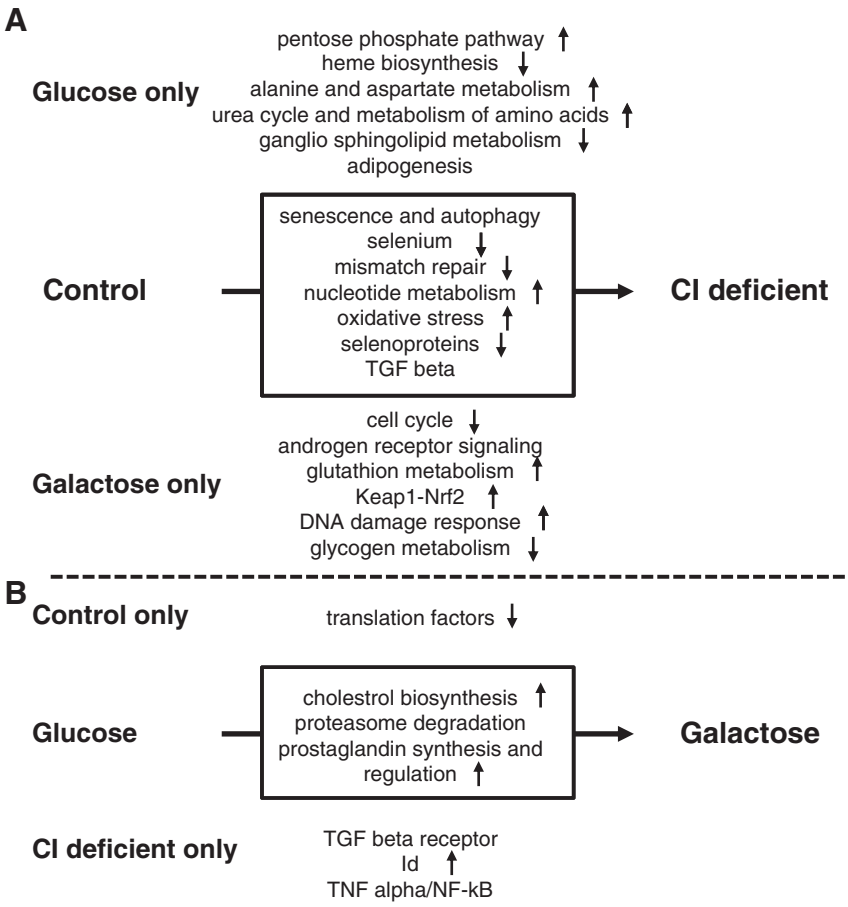

Fig. 1. Pathways significantly different (A) in controls and patients and (B) in glucose and galactose conditions. Pathways in the box are changed in both culture conditions (A) or groups (B). Pathways above and below the box are only changed in the indicated condition (A) or group (B). Arrows indicate increased or decreased expression; for pathways without arrow, the direction of the change is not clear.

pathway (increased in patients), heme biosynthesis (decreased in patients), alanine and aspartate metabolism (increased in patients), urea cycle and metabolism of amino groups (increased in patients) and ganglio spingolipid metabolism (decreased in patients). Furthermore, some pathways were significantly altered in both normal glucose and galactose condition: senescence and autophagy (miscellaneous effect), selenium (decreased in patients), mismatch repair (decreased in patients) and nucleotide metabolism (increased in patients) (Fig. 1A).

3.1.3. Changes due to disease and interacting with the culture condition

There were 1584 genes that differed between patients and controls in the glucose and/or galactose culture condition, but for which patients showed a different response to the galactose condition compared with the controls. A different response could be a response to galactose in only the patients or the controls, a quantitative difference or an opposite effect. When considering these genes, part of them were altered as a result of the CI deficiency, but additionally showed an interaction between this deficiency and the culture condition. Altered pathways included oxidative stress (increased in patients and further increased in galactose medium), selenium metabolism and selenoproteins (decreased in patients and further decreased in galactose medium), the TGF beta signaling pathway (miscellaneous effect) and translation factors (decreased in patients). The remainder of the genes was only altered in patient cells relative to control cells on galactose medium but not in glucose medium. This last group of genes was mapped to pathways involved in cell cycle progression (decreased in patients), oxidative stress (increased in patients; Keap1–NRF2, glutathione metabolism, DNA damage response) and signaling (miscellaneous effect; androgen receptor, TGF beta receptor, Id, TNF alpha/NF-κB). A general overview of all significantly changed pathways is shown in Fig. 1.

3.2. QPCR and western blot validation of microarray data

From the pathway analysis, representative genes with fold changes of more than 50% were selected and validated by QPCR (Table 3). For all except one gene (*C-MYC*), the QPCR method detected a difference between patients and controls comparable with the microarray analysis. Furthermore, to correlate gene expression and protein levels, western blot analysis was performed for proteins selected from the genes with relatively high gene expression fold changes in significantly altered pathways for patients versus controls in galactose medium. Significantly increased expression was confirmed for HMOX and GCLC, a similar trend in protein expression was observed for TXNRD1 but no change was detected for GSR (Fig. 2). Additionally, western blot analysis also revealed that complex I deficiency in the cell lines was accompanied by reduced protein levels of complex I (Fig. 2).

4. Discussion

During the last decade, studies have generated extensive knowledge on the pathogenesis of CI deficiency [4, 28, 29, 32]. In spite of these advances in physiological, biochemical and genetic insights, the total picture is far from complete. In the present study, gene expression analysis was performed for a more general overview of the processes involved in CI deficiency in fibroblasts. According to

Table 3
Selected genes for the QPCR signature.

Gene symbol	Disease effect				Medium effect			
	Glucose condition		Galactose condition		Controls		Patients	
	Array	QPCR	Array	QPCR	Array	QPCR	Array	QPCR
Keap1–Nrf2								
NRF2		0.77	1.30	1.42	0.82	0.77	1.25	1.42
KEAP1		0.77	1.28	1.27			1.68	1.58
Oxidative stress								
CYBA		*0.56*	*0.63*	*0.56*		*0.62*	*0.63*	*0.62*
FOS	0.44	0.48	1.55	2.10	0.62		2.19	2.85
HMOX1			7.23	17.58			9.76	28.95
Glutathione metabolism								
GSR			1.96	2.46			2.31	3.23
IDH1			1.50	1.79		1.27	1.88	2.61
GCLC			1.90	1.82			2.38	2.66
GCLM			1.94	2.67			1.91	2.81
Selenium metabolism/selenoproteins								
SELK			2.14	2.30			2.01	2.36
SEPP1	*0.38*	*0.33*	*0.21*	*0.33*	*0.59*	*0.39*	*0.32*	*0.39*
Pentose phosphate pathway								
PGD			1.75	1.55			2.10	2.36
DNA damage response								
GADD45A		1.86	2.25	3.20			1.89	2.08
GADD45B	*1.71*	*3.56*	*3.40*	*3.56*		*1.81*	*1.84*	*1.81*
TNFRSF10B			2.05	1.69			2.12	2.11
C-MYC			1.90			1.78	1.75	1.78
Cell cycle–anaphase regulation								
ESPL1			0.60	0.35	1.69	2.04		
CDC20		*0.48*	*0.38*	*0.48*	*2.55*			
Nucleotide metabolism								
PRPS1	1.77	1.79		0.59			0.50	0.29
Glycogen metabolism								
GBE1	0.87		1.43	1.74			1.63	1.70
Senescence and autophagy								
ING1		0.71	1.60				1.91	1.69
TGF beta signaling pathway								
LTBP1	1.70		0.60	0.23		1.97	0.44	0.54
TGIF			1.46	1.65			1.71	2.60
Adipogenesis								
RORA		1.49	1.86	2.59			1.84	2.41
DDIT3			2.61	2.50		1.65	2.24	3.46
LMNA			0.45	0.44			0.45	0.46
ADFP		0.66	1.41				1.95	1.84

Genes in italics did not show an interaction between disease effect and medium effect with QPCR analysis (in contrast to microarray analysis) but are still able to distinguish patients from controls in both culture media.

recent models of complex I assembly, the selected patients all had mutations in structural CI subunits of the dehydrogenase and hydrogenase modules of the hydrophilic peripheral arm of complex I protruding in the matrix. These modules are involved in oxidation of NADH to NAD^+ and electron transfer to ubiquinone [41] and mutations in their proteins disturb these fluxes, leading to CI deficiency. Therefore, we consider these patients as a group with a common pathophysiological basis, making them suitable fro genome-wide gene expression approaches.

Differential expression of a number of genes and proteins in relevant pathways was validated and related to available physiological and biochemical data.

4.1. Experimental set-up

To be able to examine the consequences of CI deficiency in fibroblasts (which are mainly glycolytic), cell lines were challenged to use their OXPHOS system for energy production by culturing in galactose medium in the absence of glucose [33, 34]. Our analytical model corrected for a number of potential 'noise' factors (biological *e.g.* age, sex and passage; technical *e.g.* chip effect), in contrast to a previous study [34]. The approach differed further from this study [34] by type and scope of the array (Affymetrix GeneCHIPS versus home-made two-color cDNA microarrays with selected mitochondria-related genes). The current study provides a well-controlled and more complete picture of the molecular processes in fibroblasts of CI patients. These differences can explain why only eleven genes showing differential expression in the previous study could be confirmed [34] (Supplementary Table 4). Unfortunately, nine additional genes could not be analyzed due to the use of the more accurate updated probe set definitions [36] and thirteen genes had low signal intensities, which made comparison impossible. However, the eleven confirmed results included multiple metallothionein transcripts, which were key elements in the previous study [34].

4.2. General gene expression analysis results

Linear regression analysis showed significantly altered expression of in total 3279 genes due to disease, culture medium or a combination of both. As expected, gene expression differences were the highest when comparing patient and control cells cultured in galactose medium and when comparing the glucose and galactose condition in patient fibroblasts. Both the number of differentially expressed genes and the expression difference were higher. In control cells, less than 10% of the differentially expressed genes showed fold changes of more than 50% in galactose compared with glucose indicating that control cells were better capable of handling the galactose challenge. The altered expression of a number of genes was validated by QPCR and all of these, except for one gene (*C-MYC*), showed a response similar to the microarray thus confirming the overall reliability of the microarray data. Furthermore, for two genes, increased gene expression was accompanied by increased protein expression, validating the biological relevance of the detected differences in gene and pathway expression.

4.3. Differentially expressed processes

To identify processes that were altered due to the cumulative effect of differentially expressed genes in such a process, pathway analysis was performed. Pathways that were only changed in patient versus control fibroblasts both cultured in the presence of glucose were mainly involved in metabolism and included in most cases only a small number of genes with limited fold changes. This suggests that under glycolytic conditions, fibroblasts can adapt relatively easily to alternative energy sources. Therefore, we focused on processes that were changed due to the combination of disease and galactose culturing when the cells are forced to use their oxidative phosphorylation system.

4.3.1. Oxidative stress

Although the oxidative stress pathway was significantly changed when comparing patient and control cells on glucose, the difference was more significant (higher Z score) and more genes were involved on galactose medium. This is consistent with the observation of increased ROS levels in the CI deficient fibroblasts (Table 1; [4, 28]). Furthermore, in an independent study with fibroblasts from OXPHOS (including CI deficient) patients, we observed higher ROS levels when fibroblasts (including controls) were cultured with galactose compared with glucose (manuscript in preparation). Transcripts of genes involved in glutathione homeostasis were increased, but transcription of the other antioxidant enzymes, catalase and superoxide dismutase (mitochondrial and extracellular), was decreased. Although enzyme activity measurements should confirm these results, this suggests that the glutathione antioxidant system is the main defense mechanism counterbalancing increased ROS production in CI fibroblasts. Additionally, the induction of the DNA damage response through increased expression of *GADD45A* and *GADD45B* could further emphasize cellular stress. Gadd45-mediated growth arrest [42]

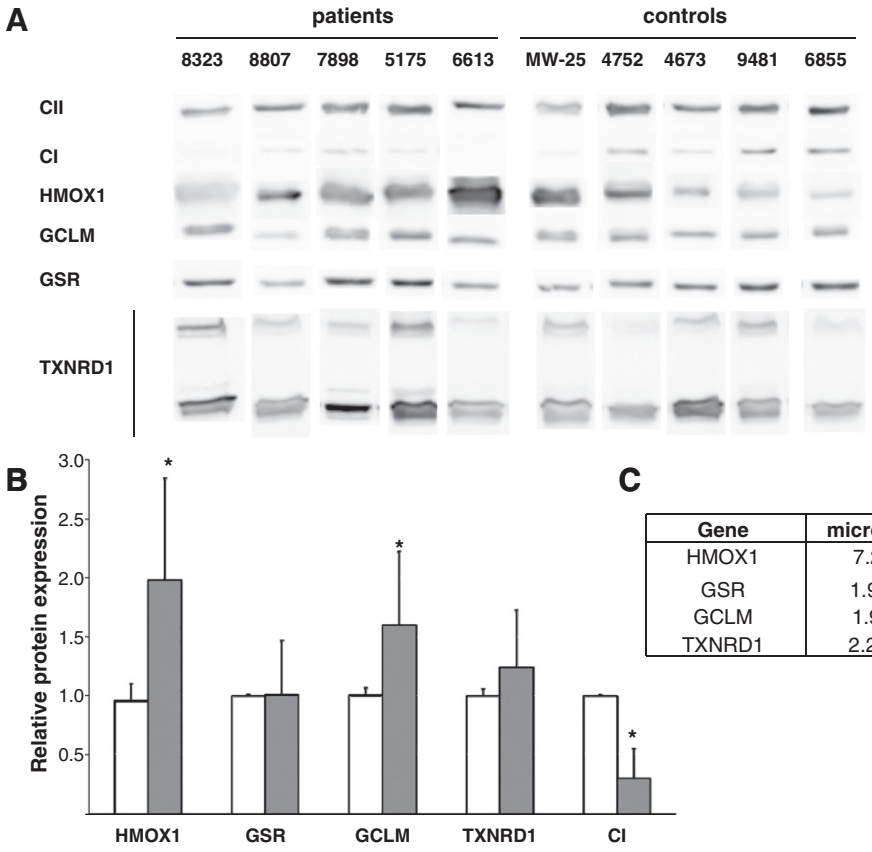

Fig. 2. Western blot validation of mRNA results. (A) Original western blot results and (B) a graphical representation of differences in protein levels for complex I (CI), HMOX, GSR, GCLC and TXNRD1 between control (white bars) and patient (grey bars) fibroblasts cultured with galactose medium, normalized for the expression in controls (* $p<0.05$ patients versus controls galactose). Complex II (CII) was used as a reference. In panel C, microarray, QPCR and western blot results for these genes in patients versus controls in galactose medium were compared.

might explain why fibroblasts of CI deficient patients proliferate at a slower rate than healthy control fibroblasts.

A number of redox-sensitive genes that were significantly changed in this study (*HMOX1*, *CAT* and *SOD3*) were not reported in a previous study [34], most likely due to a different analytical approach. However, the induction of a number of metallothionein genes was detected in both studies (Supplementary Table 4). Metallothioneins are considered potent antioxidants of which the expression is induced by ROS, heavy metals and other forms of cellular stress [34]. Another study with different genetically characterized CI deficient patient fibroblast cell lines (*NDUFV1* p.W51X + p.T423M and *NDUFA1* p.R37S + G8R) did not detect differences in catalase and glutathione reductase protein expression and only one of the three patient cell lines showed the induction of mitochondrial superoxide dismutase protein [33]. This could be explained by the fact that, in contrast to our cell lines (Table 1; [4, 28]), their cell lines did not show increased ROS production, explaining the lack of increased antioxidant defense. It also suggests that the CI mutations of these patients might have different consequences than the ones studied here.

4.3.2. KEAP1–NRF2–glutathione

One of the most obvious differentially expressed pathways was the *KEAP1–NRF2* pathway, leading to the activation of a large number of genes involved in glutathione homeostasis and the thioredoxin and peroxiredoxin antioxidant defense (Fig. 3). ROS-mediated modification of cysteine (Cys) residues in Keap1 is known to result in diminished binding of Nrf2, leading to decreased Nrf2 degradation, its translocation to the nucleus and transcription of genes containing the antioxidant response element (ARE) in their promoter region [43, 44]. Activation of *NRF2* signaling was described previously in fibroblasts of patients with a mutation in the mitochondrial *ATP6* gene [45]. Consistent with a lack of *NRF2* signaling, glutathione levels in patient fibroblasts were not significantly different from controls in de glucose situation (Table 1; [29]). However, reduced glutathione (GSH) levels of cell lines 7898 and 5175 were at the lower limit of control values and oxidized glutathione (GSSG) levels were at the higher limit of control values for cell line 6613 [29]. It has been suggested previously that antioxidants present in the culture medium may quench ROS [46], which might make up-regulation of the antioxidants unnecessary. With the induction of glutathione homeostasis genes, it can be expected that glutathione levels are increased in the galactose situation to buffer the additional free radicals and prevent oxidative damage. Up-regulation of GCLC was confirmed at the protein level.

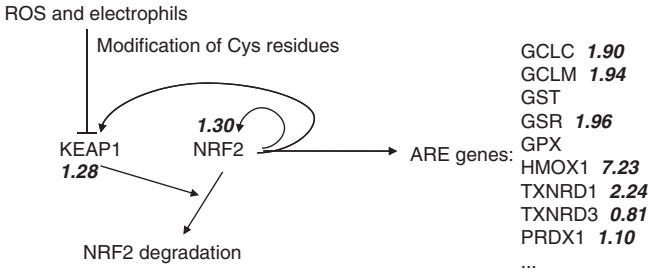

Fig. 3. Activation of the KEAP1–NRF2 pathway in patient fibroblasts cultured with galactose. Fold changes of the significantly changed genes (patients versus control cultured with galactose) are shown in bold italics.

4.3.3. Selenium and selenoproteins

Pathway analysis revealed significant differences in the expression of the selenium (metabolism) and selenoprotein genes in control and patient fibroblasts. Although some overlap exists between these pathways and the oxidative stress pathway, the functions of many of the selenoproteins are unknown [47]. Selenoproteins are a group of proteins that contain selenocystein as an integral part of their polypeptide chain and include different protein families such as the thioredoxin reductases (TXNRDs) and glutathione peroxidases (GPXs), and a number of alphabetically annotated selenoproteins (SelK, SelM, SelN…) [47, 48]. While some classes are definitely involved in antioxidant pathways (GPXs) and redox pathways (TXNRDs), others have been implicated to play a role in calcium homeostasis (SelN) [47]. Recently, it was shown that association of SelN with the ryanodine receptor was necessary to control release of calcium from intracellular endoplasmic reticulum stores [49]. Thus, a decrease of *SEPN1* transcripts, might correlate with the decreased calcium fluxes measured in the studied patient cell lines (Table 1; [31, 32]). If confirmed this might provide a new lead in the pathogenesis of CI deficiency. In HepG2 cells, SelK was induced by endoplasmic reticulum stress and protected the cells from stress induced apoptosis [50]. The increased mRNA expression of *TXNRD1* and *SelK*, opposed to the decreased expression of multiple other selenoproteins, might suggest the preferential expression of selenoproteins involved in stress reduction in oxidative CI deficient patient fibroblasts. The expression of selenoproteins is depending on the cellular selenium status [51]. Selenium deficiency has been shown to induce the expression of Nrf2 target genes [51, 52], providing a link between the observed induction of the Keap1–Nrf2 pathway and the decreased expression of selenoproteins. Therefore, monitoring and adjusting selenium levels might be beneficial for patients with complex I deficiency. Dietary selenium has been supplemented for the treatment or prevention of cancer [53] and Alzheimer's disease [54]. This study emphasizes the relevance of testing the applicability and efficiency of selenium supplementation also for CI deficiency, which should be tested first *in vitro* and in animal models and later in clinical trials.

5. Conclusion

The gene expression analysis supports the changes in oxidative stress levels previously measured in fibroblasts of CI deficient patients. It was shown that the cells adapt to and protect themselves against a higher oxidative state through transcriptional regulation of the *NRF2* pathway. Additionally, altered expression of selenoproteins provided a new way to explain diminished calcium fluxes in the CI deficient fibroblasts. Altogether, the glutathione and selenium pathways might be considered as (new) targets for future therapeutic interventions. Extending our approach to other CI deficient patient groups with functionally different defects (assembly factor or mtDNA defects affecting the CI proton translocation) in different genes will elucidate if the mechanisms identified are common to all complex I patients or if heterogeneity of pathophysiological processes exist.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbadis.2011.10.009.

Acknowledgements

This work was supported by the Dutch IOP Genomics grant IGE05003. We would like to thank S. Vanherle for technical expertise and help.

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