ORIGINAL ARTICLE

Oxidative modifications of plasma proteins and decreased leukocyte mitochondrial DNA of schizophrenia patients

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Abstract

OBJECTIVES: Schizophrenia is the most frequent and the most important psychiatric disorder. Although the dopaminergic dysfunction is considered as the main pathophysiologic feature of schizophrenia, the exact mechanism leading to dopaminergic dysfunction is still unclear. Different genetic and environmental causative risk factors for schizophrenia have been identified. Despite the strong heterogeneity of the risk factors, oxidative stress could represent the underlying biological mechanism linking these risk factors.
METHODS: We have investigated oxidative modifications of plasma proteins by fluorescent spectroscopy. The conjugates of plasma proteins with 4-hydroxynonenal were determined by Western blot analysis. Relative content of mitochondrial DNA in leukocytes of the patients with confirmed diagnosis of schizophrenia was determined by real time PCR.
RESULTS: We have shown decreased fluorescence of aromatic amino acid residues of plasma proteins and decreased level of mitochondrial DNA in leukocytes of patients suffering from schizophrenia. In addition, we have documented altered pattern of plasma proteins modified with 4-hydroxynonenal.
CONCLUSION: Our results support an idea about important link between oxidative stress, mitochondrial dysfunction and schizophrenia. Although cause consequence relationships are not clear, it seems that mechanism associated with oxidative stress and mitochondrial functions can serve as important targets for development of new strategies to treat schizophrenia.

Introduction

Schizophrenia is the most frequent and the most important psychiatric disorder that is characterized by several neurodevelopmental and behavioural abnormalities, perception deficits, emotion processing and social functioning (Freedman 2003; Picchioni & Murray 2007). It is among the seven most disabling diseases in patients between 20–45 years of age being more common than cardiovascular diseases, HIV, and diabetes (World Health Organisation 2001). Although the dopaminergic hyperfunction in the limbic system and dopaminergic hypofunction in the frontal cortex are considered as the main pathophysi-

ologic features of schizophrenia, the exact mechanism leading to dopaminergic dysfunction is still unclear (Brisch *et al.* 2014). Different genetic (Svrakic *et al.* 2013; Escudero & Johnstone 2014) and environmental causative risk factors for schizophrenia have been identified, including drug and alcohol abuse (Hambrecht & Häfner 1996), stress (Gomes & Grace 2017), prenatal infections (Cheslack-Postava & Brown 2021) and malnutrition (He *et al.* 2018). Despite the strong heterogeneity of the risk factors, oxidative stress could represent the underlying biological mechanism linking these risk factors (Bray & Taylor 1993; Wu & Cederbaum 2003; Lanté *et al.* 2007). Already in 1934, decrease of reduced glutathione and increase of lactate in blood of schizophrenia patients were recognised as molecular alterations associated with schizophrenia (Looney & Childs 1934). These changes indicated important association of oxidative stress and altered energy metabolism attributable to the changes in mitochondrial functions. The further studies have confirmed the important association of oxidative stress with schizophrenia (Ciobica *et al.* 2011; Flatow *et al.* 2013). Oxidative stress is considered as important mechanism associated with development and progress of schizophrenia (Bošković *et al.* 2011; Wu *et al.* 2013) although cause-consequence relationships are not completely clear. Despite the fact that ROS are functioning as pleiotropic physiological signalling agents (Sies & Jones 2020) their overproduction exceeding the intracellular antioxidant capacity is associated with harmful intracellular condition commonly called oxidative stress (Ďuračková 2010; Pizzino *et al.* 2017). Oxidative stress is characterised by oxidative modifications of intracellular lipids, nucleic acids and proteins that can lead to the cell dysfunction often culminating in the cell death (Ďuračková 2010; Pizzino *et al.* 2017). Dysfunction of mitochondrial respiratory chain is considered as the main cause of a higher intracellular production of reactive oxygen species (ROS) and consequent oxidative stress (Sies & Jones 2020). In fact, several alterations of mitochondrial functions and content in brain of schizophrenia patients were described previously (Roberts *et al.* 2015; Bergman & Ben-Shachar 2016; Roberts 2021). Thus, mitochondrial dysfunction was also implicated as an important mechanism of development and progress of schizophrenia (Clay *et al.* 2011; Fizikova & Dragasek 2017; Suárez-Méndez *et al.* 2020). In addition to the dopaminergic dysregulation, oxidative stress and mitochondrial dysfunction, several other cellular mechanisms were considered as a cause associated with development and progress of schizophrenia (Ľupták *et al.* 2021) including neuroinflammation (Müller 2018) or dysfunction of glutaminergic neurotransmission (Moghaddam & Javitt 2012; Poels *et al.* 2014).

The aim of the study was to investigate oxidative modifications of plasma proteins and relative mtDNA content in leukocytes of the patients with confirmed diagnosis of schizophrenia. By means of fluorometric measurements, we have determined fluorescence of dityrosine, aromatic amino acids and conjugates with 4-hydroxynonenal (HNE) of plasma proteins of schizophrenia patients and persons of control group. We have also performed the Western blot analysis of the plasma proteins focused on proteins modified with HNE. Finally, we have determined the content of the mitochondrial DNA relative to the nuclear DNA performing the real time PCR analysis of total DNA isolated from leukocytes of patients suffering from schizophrenia and persons of control group.

Material and Methods

Study Participants

A total of 60 participants were recruited for the study (39 schizophrenia patients and 21 persons of control group). All schizophrenia patients were diagnosed by psychiatrist at the Psychiatric Hospital of Professor Matulay, Kremnica, Slovakia. All schizophrenia patients during the time of the blood sample collection were on the standard schizophrenia treatment involving haloperidol, olanzapine, risperidone, paloperidone, flupentixol, cariprazine and aripriprazole. The average age of schizophrenia patients was 37.7 ± 10.7 years. The average duration of the disease was 8.1 ± 4.8 years. The female to male (F:M) ratio in group of schizophrenia patients was 1:3.33. Diabetes mellitus was exclusion criteria for the group of schizophrenia patients. The control group consisted of persons examined from professional reasons at the Clinic of Occupational Medicine and Toxicology of University Hospital Martin. The average age of persons of control group was 28.1 ± 14.6 years. The F:M ratio in the control group was 1:2. Neurodegenerative, neuropsychiatric diseases and diabetes mellitus were exclusion criteria for the control group. The study was approved by the Ethics Committee (EC) of Jessenius Faculty of Medicine. The approval was recorded under ID: EK 124/2018 on 13 December 2018 by the EC board. All participants signed written informed consent forms.

Processing of blood samples and isolation of DNA

Blood of patients suffering from schizophrenia and persons of control group was collected in sterile EDTA-containing sampling tubes. Fresh blood samples were subsequently centrifuged at 450× g and 4 °C for 10 min. After plasma separation, total DNA was isolated from blood cellular content by using the Wizard® Genomic DNA Purification Kit (Promega Corporation) according to the manufacturer's protocol. Isolated DNA was stored at −45 °C until analysis. Plasma protein concentrations were determined by a protein DC assay kit (Bio-Rad) with BSA as a standard. Concentration of DNA was determined using NanoPhotometerTM Implen.

Fluorescence Measurements

The steady-state fluorescence measurements were performed in solutions containing 450 μg of plasma

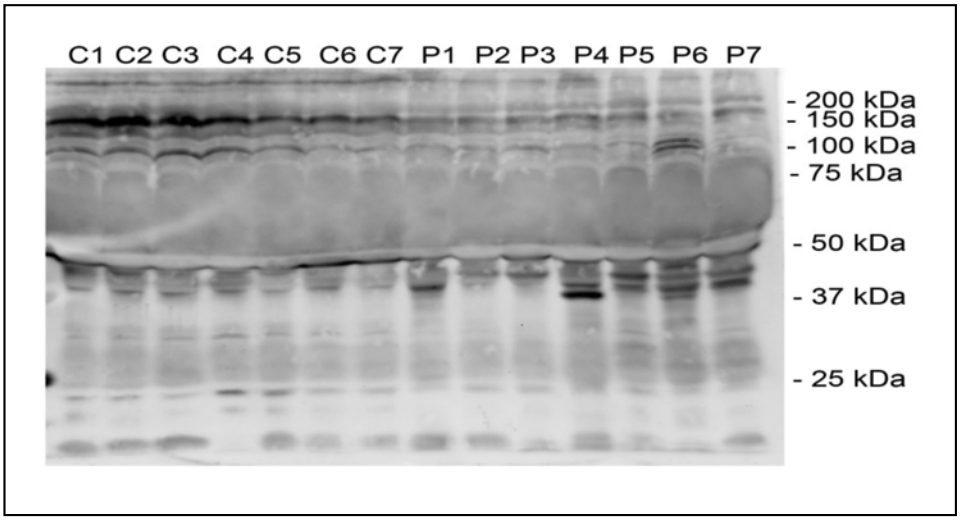

Fig. 1. Representative Western blot detection of HNE conjugates of plasma proteins of control volunteers and schizophrenia patients.
Plasma proteins of control volunteers (C) and schizophrenia patients (P) were analysed by Western blot as described in Material and Methods using anti-HNE antibodies. The position of molecular mass standards is indicated.

protein/ml, in 10 mM phosphate buffer (pH 7.4), 150 mM NaCl at 25°C on a Perkin Elmer LS-55 spectrofluorometer. Fluorescence of HNE conjugates with plasma proteins (Shimasaki 1994) were recorded at an emission wavelength 405 nm (slit width 5 nm) at an excitation wavelength of 365 nm (slit width 5 nm). Fluorescence of dityrosine (Giulivi & Davies 1994) were recorded at an emission wavelength 440 nm (slit width 5 nm) at an excitation wavelength 325 nm (slit width 5 nm). Fluorescence of aromatic amino acids (Dousset *et al.* 1994) were measured at an emission wavelength 330 nm with excitation at 295 nm (5 nm slit width).

Western blot analysis for detection of HNE-modified plasma protein

Plasma proteins (20 μg proteins loaded per lane) diluted in 4xsample loading buffer (250 mM Tris–HCl (pH 6.8), 8% (w/v) sodium dodecyl sulphate, 0.2% (w/v) bromophenol blue, 400 mM dithiothreitol, 40% (v/v) glycerol) and denaturated by incubation at 95 °C for 5 min were separated using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) using 10% gels under denaturing conditions. Separated proteins were transferred to nitrocellulose membranes using a semidry transfer protocol. Non-specific binding was blocked by overnight incubation of membranes with blocking buffer (2 % BSA in TBS-T, Tris base saline and 0.05 % Tween 20) at 4 °C. After blocking, the membranes were incubated with the monoclonal mouse anti-HNE antibodies diluted in blocking buffer (1:2000, Oxis International Inc.) at 4 °C overnight. The, the membranes were washed 3 times with TBS-T and further incubated with the rabbit anti-mouse secondary antibodies (1:10,000; Santa Cruz Biotechnology) for 1 h at room temperature. After washing 4 times with TBS-T, the membranes were incubated in SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) solution for 5 min in dark. After exposition of membranes on Chemidoc XRS system (Bio-Rad), the immunopositive bands were visualised by Quantity One software (Bio-Rad).

Real time polymerase chain reaction (qPCR)

Aliquots of the isolated DNA (1 ng) were used for qPCR. Sequences of primers used for amplification of DNA (shown in Table 1) were designed and verified by using the nucleotide database of the National Center for Biotechnology Information (NCBI). Amplification of the DNAs was initiated by denaturation at 95 °C for 3 min, followed by 40 PCR cycles (initial denaturation at 95 °C for 3 min followed by cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s) in iQ™5 Multicolor Real-Time PRC Detection System (Bio-Rad). The amplification was followed by final analysis of PCR product melting curve. The threshold cycle (C_T) was automatically assigned by the iQTM5 Multicolor Real-Time PRC Detection System Software, version 2.0 (Bio-Rad). The relative ratio in mtDNA versus nDNA content between schizophrenia patients and persons of control group was determined using the $2^{-\Delta\Delta C_T}$ method (Schmittgen & Livak 2008) where $\Delta\Delta C_T = (C_{TtmtDNAgene} - C_{TnDNA-gene})_{schizophrenia\ patients} - (C_{TtmtDNAgene} - C_{TnDNAgene})_{control\ group.}$ Relative ratio (RR) was determined using the equation $RR = 2^{-\Delta\Delta C_T}$.

Statistical analysis

Tukey's test (GraphPad InStat V2.04a, GraphPad Software) was used to determine the differences between the experimental groups. Data are presented as mean ± S.D. A $P<0.05$ was considered as being significant.

Tab. 1. Sequences of primers used for amplification of mitochondrial and nuclear genes.
ND3 NADH dehydrogenase subunit 3, ATP6 ATP synthase F0 subunit 6, RPL7 ribosomal protein L7, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Gene	Front primer	Reverse primer
ND3	ACA CCC TCC TAG CCT TAC TAC	GAT ATA GGG TCG AAG CCG CAC
ATP6	GTT CGC TTC ATT CAT TGC CCC	GGG TGG TGA TTA GTC GGT TGT
RPL7	GGT CGA GTC TGT GTG AAA GAC	CCA TTT CGA ATC ACT CAG CAC
GAPDH	GCT ACA GCA ACA GGG TGG TGG	GTC TCT CTC TTC CTC TTG TGC

Results

In order to study whether possible oxidative stress leads also to the modifications of plasma proteins we have measured fluorescence corresponding to modification of plasma proteins with HNE and formations of dityrosines as well as fluorescence of aromatic amino acids of plasma proteins. As shown in Table 2, we have documented significant decrease of fluorescence of aromatic amino acids in plasma proteins of patients suffering from schizophrenia ($p<0.0001$). Other investigated parameters were not significantly changed.

Western blot analysis showed HNE modified plasma proteins with discrete molecular masses in the range 100 – 200 kDa in control group while HNE modified plasma proteins with discrete molecular masses 37 – 50 kDa were detected in plasma of schizophrenia patients (Fig. 1).

Despite some critical view (Chinopoulos, 2020), content of mtDNA in leukocytes could reflect the relative content of mitochondria in affected peripheral tissue. In order to determine relative content of mtDNA in leukocytes of schizophrenia patients and persons of control group we have selected two mitochondrial and two nuclear genes serving as markers of mtDNA and nDNA, respectively. The analysis was performed by qPCR. We have observed significantly higher values of C_T of mitochondrial genes related to DNA isolated from schizophrenia patients (Table 3A). With respect to the nuclear genes, C_T values of RPL7 were not significantly different (Table 3A). We have documented that C_T value of GAPDH related to DNA isolated from schizophrenia patients was higher at the border of significance (Table 3A). In order to avoid bias related to differences in both amount and quality of isolated DNA, we have compared all possible differences among C_T values of mitochondrial and nuclear genes. All possible values were significantly decreased for DNA isolated from schizophrenia patients (Table 3B). Obtained values were used for calculation of the mtDNA content relative to the content of nDNA. All obtained values were less than 1 (Table 3B), indicating decreased amount of mtDNA in leukocytes of schizophrenia patients.

Discussion

In this study, we have shown decreased fluorescence of aromatic amino acid residues of plasma proteins and decreased level of mtDNA in leukocytes of patients suffering from schizophrenia. In addition, we have documented altered pattern of plasma proteins modified with HNE. These findings are in accord with a view about importance of oxidative stress and mitochondrial dysfunction associated with schizophrenia although cause consequence relationships are not completely clear.

Oxidative stress induced modifications of plasma proteins associated with schizophrenia were investigated in several previous studies by means of several different methods. For example, statistically increased levels of markers of oxidative/nitrative stress such as carbonyl groups or 3-nitrotyrosine, determined by ELISA, were observed in the plasma proteins from schizophrenia patients (Dietrich-Muszalska *et al.* 2009, 2012). Further study has also documented that the level of the plasma protein oxidation assed spectrofotometrically was significantly higher in the schizophrenia acute psychotic attack patients compared to control group (Tunçel *et al.* 2015). The same study has showed that protein oxidation level was not significantly changed in schizophrenia remission patients compared to the

Tab. 2. Fluorescence intensities of modified amino acid residues of plasma proteins

	Fluorescence intensity (a. u./mg of protein)		*P* value
	Controls	Patients	
Aromatic amino acids	20.41 ± 2.73	11.74 ± 2.64	0.0001
Dityrosine	2.95 ± 0.44	2.76 ± 0.81	0.34
HNE conjugates	6 ± 1.04	5.7 ± 1.75	0.47

Tab. 3A. C_T values of nuclear and mitochondrial genes

	Controls	Patients	*P* value
ND3	15,06 ± 1,14	16,78 ± 1,95	0,0034
ATP6	13,65 ± 1,97	15,83 ± 1,96	0,0025
RPL7	24,86 ± 1,85	24,96 ± 1,96	0,44
GAPDH	19,13 ± 1,02	20,33 ± 1,95	0,043

control group while the level of total oxidized guanine species was statistically higher in both schizophrenia acute psychotic attack patients and schizophrenia remission patients compared to the control group. Our simple and fast fluorescence analysis revealed significantly decreased fluorescence of aromatic amino acids of plasma proteins of patients suffering from schizophrenia regardless their disease status. We did not detect significant changes of dityrosines that seems to be apparently controversial to the results of aromatic amino acid fluorescence. The setup of aromatic amino acid fluorescence measurements used in this study corresponds mainly to the fluorescence of tryptophane residues while the contribution of tyrosine residues is significantly lower whereas contribution of phenylalanine residues is almost equal to zero (Kang *et al.* 2014). Tryptophan is one of the amino acids most susceptible to oxidation, reacting with different types of ROS at significant rates under physiological conditions (Ehrenshaft *et al.* 2015). In most cases, decrease of tryptophan fluorescence can provide useful, if not definitive, evidence of alterations in tryptophan residue structure caused by ROS attack and can be considered as important marker of oxidative stress (Ehrenshaft *et al.* 2015). For example, profound decrease of fluorescence of tryptophan residues of membrane proteins was documented after incubation of membranes of endoplasmic reticulum with Fe^{2+} as a source of super oxide anion radical (Kaplan *et al.* 2000, 2003). In addition to the oxidation of plasma proteins, significantly increased concentration of oxidized guanine as a marker of oxidative damage to the DNA was documented in the blood of schizophrenia patients (Tunçel *et al.* 2015; Goh *et al.* 2021).

HNE as the major product of lipid peroxidation produces conjugates with proteins mainly via the ε-amino group of lysine residues (Barrera *et al.* 2015). Although we did not detect significant changes in fluorescence of plasma proteins modified with HNE we have shown altered pattern of plasma proteins modified with HNE using Western blot analysis. While in the control group HNE modified plasma proteins with discrete molecular masses in the range 100 – 200 kDa were detected, HNE modified proteins with discrete molecular masses in the range 37 – 50 kDa were detected in the plasma of schizophrenia patients. Changed pattern of plasma proteins modified with HNE might explain the reason of non-significant results obtained by fluorescence measurements. Similarly to our study, increased levels of HNE modified proteins with molecular mass around 50 kDa were observed in the plasma of patients suffering with Rett syndrome (Pecorelli *et al.* 2011). However, in contrast to our study, the same study has also documented increased HNE modified proteins with molecular mass between 150 – 200 kDa in plasma of patients suffering with Rett syndrome (Pecorelli *et al.* 2011). Massive modification of plasma proteins with HNE was also documented in plasma of patients with classic autistic disorder (Pecorelli *et al.* 2013). Elevation of HNE modification of discrete proteins observed mainly in the mitochondria isolated from rat brains was age-dependent (Tatarkova *et al.* 2016). In addition, previous study documented cytochrome c oxidase, terminal complex of mitochondrial respiratory chain, as one of specific targets of HNE (Kaplan *et al.* 2007). HNE modification of cytochrome c oxidase was associated with inhibition of its activity possibly leading to mitochondrial dysfunction. With respect to schizophrenia, results of the recent study showed elevated levels of HNE-protein adducts in brains of schizophrenia patients when compared to their content in the brains of control group (Manzoor *et al.* 2022). Interestingly, increased HNE-protein adducts were documented in hippo-

Tab. 3B. Differences of C_T values of nuclear and mitochondrial genes and relative ratio (RR) of mtDNA/nDNA in patients compared to controls

	Controls	Patients	*P* value	RR mtDNA/nDNA patients/controls
RPL7 – ND3	9,8 ± 1,49	8,19 ± 0,6	0,0004	0,33
RPL7 – ATP6	11,21 ± 1,98	9,13 ± 0,87	0,0004	0,24
GAPDH – ND3	4,08 ± 0,90	3,57 ± 0,52	0,035	0,7
GAPDH – ATP6	5,48 ± 1,41	4,51 ± 0,82	0,014	0,51

campus compared to the cortex of schizophrenia brains (Manzoor *et al.* 2022). HNE is important molecule with significant impact on cellular functioning and survival (Dalleau *et al.* 2013). Thus, it seems that HNE might play some role in pathophysiology of schizophrenia but the exact role remains elusive.

Oxidative stress could be a result of increased ROS production and/or decreased antioxidant capacity. Decreased antioxidant systems associated with schizophrenia were documented previously (Raffa *et al.* 2012). Mitochondria are considered as the main intracellular source of ROS (Brand 2016; Tauffenberger & Magistretti 2021) that can play important role in normal physiology as well as in different pathologic conditions including schizophrenia (Ben-Shachar 2017). In this study, we have documented decreased relative mtDNA amount in leukocytes of schizophrenia patents compared with relative mtDNA amount in leukocytes of control group persons. Our results agree with recently published results documenting decreased mtDNA copy number in the leukocytes of schizophrenia patients (Li *et al.* 2015; Kumar *et al.* 2018; Shivakumar *et al.* 2020). While Li *et al.* (2015) showed that decreased mtDNA copy number in leukocytes of schizophrenia patients is not dependent on the antipsychotic treatment, Kumar *et al.* (2018) showed that mtDNA copy number in leukocytes of schizophrenia patients is associated with psychosis severity and anti-psychotic treatment. Decreased mtDNA copy number in manic and depressed patients, which may reflect disturbances of energy metabolism was also documented in leukocytes of bipolar disorder patients (Wang *et al.* 2018). On contrary, higher mtDNA copy number in leukocytes was documented in association with ADHD (Kim *et al.* 2019). Despite some critical view (Chinopoulos 2020), content of mtDNA in leukocytes could reflect the relative content of mitochondria in the affected peripheral tissue. For example, in the largest investigation of mtDNA copy number associated with Parkinson's disease, it has been shown that reduced mtDNA copy number is restricted to the substantia nigra pars compacta, but is also reflected by decreased mtDNA copy number in the peripheral blood (Pyle *et al.* 2016). Finally, mtDNA copy number, measured by the number of mitochondrial genomes per nuclear genome, could be a useful biomarker of mitochondrial dysfunction associated with a number of different pathological conditions (Castellani *et al.* 2020).

In conclusion, the results presented in this study and in the several previous studies support an idea about important link between oxidative stress, mitochondrial dysfunction and schizophrenia. Although cause consequence relationships are not completely clear, it seems that mechanisms associated with oxidative stress and mitochondrial functions can serve as important targets for development of new strategies to treat schizophrenia.

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Competing interests

The authors have declared that no competing interests exist.

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