ORIGINAL ARTICLE

Antioxidant enzymes in blood of patients with Friedreich's ataxia

G Tozzi, M Nuccetelli, M Lo Bello, S Bernardini, L Bellincampi, S Ballerini, L M Gaeta, C Casali, A Pastore, G Federici, E Bertini, F Piemonte

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See end of article for authors' affiliations

Correspondence to: Dr F Piemonte, Molecular Medicine Laboratory, "Bambino Gesù" Children's Hospital, P.za S. Onofrio, 4-00165 Rome, Italy; Fiorellait@hotmail.com

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Background and Aims: Increased generation of reactive oxygen species and mitochondrial dysfunction may underlie the pathophysiology of Friedreich's ataxia, the most common inherited ataxia, due to GAA expansion in a gene coding for a mitochondrial protein (frataxin), implicated in the regulation of iron metabolism. Because iron overload would cause oxidative stress in Friedreich's ataxia, we investigated the enzyme antioxidant system in the blood of 14 patients by determining superoxide dismutase, glutathione peroxidase, and glutathione trasferase catalytic activities. We also studied the glutathione S-transferase genotype polymorphism in order to evaluate its possible influence on enzyme activity.

Methods: Blood samples were obtained from 14 unrelated patients with Friedreich's ataxia and 21 age matched healthy subjects. Antioxidant enzyme determinations were spectrophotometrically assayed using specific substrates; the glutathione S-transferase genotype polymorphism was analysed by endonuclease restriction mapping of exon 5 and 6 amplification products.

Results: There was a significant elevation of the superoxide dismutase/glutathione peroxidase activity ratio (0.037 (0.01) *v* 0.025 (0.008) of controls) and an 83% rise of glutathione transferase specific activity (0.22 (0.1) *v* 0.12 (0.03) nmol/min/mg protein) in blood of patients with Friedreich's ataxia than in the controls. The genotype polymorphism of glutathione S-transferase enzyme did not show any relevant differences when compared to that of healthy subjects.

Conclusions: Data show an impairment in vivo of antioxidant enzymes in patients with Friedreich's ataxia and provide evidence of an increased sensitivity to oxidative stress, supporting a consistent role of free radical cytotoxicity in the pathophysiology of the disease.

Reactive oxygen species (ROS) have been implicated in the pathophysiology of several neurological diseases.[1–4] Oxidative stress has been proposed as a pathogenic factor for Friedreich's ataxia (FRDA),[5–7] the most common inherited ataxia (prevalence = 1 in 30 000 individuals).

FRDA is an autosomal, recessive, neurodegenerative disease due to GAA expansion in the frataxin gene, which encodes for a ubiquitous mitochondrial protein (frataxin) involved in iron metabolism. The yeast knockout of the homologue gene (YFH1) shows intramitocondrial iron accumulation, a severe defect of mitochondrial respiration, loss of mitochondrial DNA, and increased sensitivity to oxidative stress.[8 9] Reduced activities of respiratory chain complexes I, II, III, and aconitase, and increased iron deposition were found in fibroblast and cardiac muscle samples from FRDA patients.[10–13] The pathogenic hypothesis of the disease is that impaired iron metabolism leads to defective FeS formation with decreased mitochondrial complex I–III activities and iron overload. The consequence might be increased free radical generation followed by cell oxidative damage.

Cells have developed an enzymatic antioxidant pathway against ROS generated during oxidative metabolism: the dismutation of superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2) catalysed by superoxide dismutase (SOD), and the conversion of H_2O_2 to H_2O by glutathione peroxidase (GPX) or catalase.[14–16] The unbalance between these two reactions may be critical, causing an accumulation of O_2^- or H_2O_2, which in the presence of Fe^{2+}, can be converted to $OH^\bullet$ (Fenton reaction). In addition, some reducing agents (reduced glutathione (GSH), vitamins E and C, and β carotene) act as non-enzymatic scavengers of ROS. Collectively, these substances provide a first line of defence against free radicals.

The second line is provided by some detoxification enzymes such as glutathione S-transferase, aldo-keto reductase, and aldehyde dehydrogenase, whose expression has been found to be inducible by several pro-oxidant agents by mean of the antioxidant responsive element.[17]

The glutathione S-transferases (GST) are a family of isozymes which catalyse the conjugation of GSH with several compounds (α,β unsaturated carbonyls, epoxides, and hydroperoxides), produced in vivo during oxidative stress. GSTP1 expression is regulated by the cellular redox status,[18] and represents a sensor able to transmit the redox variation to the apoptosis machinery by modulating the stress kinases pathway.[19]

GST P1-1 is the prevalent form in mammalian cells and its expression is raised in tumours from a wide range of human tissues.[20] However, among individuals with similar levels of GSTP1-1 expression, enzyme catalytic activity would be expected to vary according to the presence of variant *GSTP1* genotype. It is well known that at least four allelic variants exist (A, B, C, and D), which differ from each other by a single conservative aminoacid substitution.[21] The substitutions residing in the xenobiotic substrate binding site (H site), result in steric modification of this region; consequently the three isoforms show different cosubstrate specificity and different termostability.[22–24]

To our knowledge, no study has so far reported evidence of an impairment of antioxidant enzymes in blood of FRDA

Abbreviations: dNTP, deoxynucleotide triphosphate; FRDA, Friedreich's ataxia; GPX, glutathione peroxidase; GSH, reduced glutathione; GST, glutathione S-transferase; ROS, reactive oxygen species; SOD, superoxide dismutase

Table 1 Clinical data for FRDA patients

Case		Age (y)	Sex	GAA repeats	Cardiomyopathy	Diabetes
1	S	14	M	848/848	Yes	No
2	C	8	M	448/848	Yes	No
3	J	10	M	750/750	No	No
4	L	10	M	699/699	Yes	No
5	L	14	F	1000/1000	No	No
6	B	14	M	848/1300	Yes	No
7	MA	22	F	300/810	No	No
8	SA	14	M	1160/833	Yes	No
9	SE	12	F	1160/1160	No	No
10	TC	19	F	680/350	No	No
11	BM	17	M	614/848	Yes	No
12	B	8	F	681/914	Yes	No
13	RF	18	M	888/888	Yes	Yes
14	BR	15	M	848/848	Yes	No

patients, and we therefore analysed the enzymatic activity of SOD, GPX, and GST in 14 patients and 21 healthy subjects. We also studied the GSTP1-1 genotype polymorphism in order to investigate its possible influence on GST activity.

METHODS

Chemicals

All substrates and reagents were purchased from Sigma Chemical Co. (St Louis, Missouri, USA). "Ransod" and "Ransel" kits were obtained from Randox Laboratories Ltd, UK. Electrophoresis reagents were from BioRad (Hercules, California, USA). The Taq DNA polymerase, dNTPs (deoxynucleotide triphosphates), and the hydrophobic interaction column (XK 16/20) were from Amersham Pharmacia-Biotech, Inc. (Piscataway, New Jersey, USA).

Sample collection and preparation

Blood samples were obtained from 14 unrelated FRDA patients (nine males, five females) and 21 age matched healthy controls (13 males, eight females). Clinical diagnosis used accepted criteria.[25] Table 1 summarises clinical and genetic information for patients.

Samples were collected into Vacutainer tubes (Becton Dickinson) containing heparin. For SOD determinations, erythrocytes were obtained from 1 ml of fasting venous blood by centrifugation at 500 *g* for 10 minutes, at room temperature, immediately after the blood was drawn; they were washed three times in a 9 g/l NaCl solution and stored at −70°C until analysis. For GPX activity assays, fresh whole blood was collected and stored at −70°C. Samples were haemolysed by the addition of ice cold distilled water (1/10), cell membranes were removed by centrifugation, and the supernatant used for the analysis. For GST activity determination, 200 µl haemolysed erythrocytes were centrifuged for five minutes at 3000 *g* and supernatant loaded on a hydrophobic interaction column, to eliminate haemoglobin interference. GST elution was performed by washing column with 10 mmol/l phosphate buffer (pH 6.8); 1 ml fractions were collected and assayed for the enzymatic activity.

Antioxidant enzyme determinations

SOD (EC 1.15.1.1) and GPX (EC 1.11.1.9) activities were spectrophotometrically assayed in erythrocytes with "Ransod" and "Ransel" kits, respectively, using a DU-640 spectrophotometer (Beckman Instruments, Inc., California, USA). SOD activity was expressed as the amount of protein causing a 50% inhibition of formazan dye (505 nm), employing xanthine and xanthine oxidase to generate superoxide radicals. Units of GPX activity were calculated following NADPH oxidation at 340 nm using cumene hydroperoxide as the substrate.

GST activity was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as cosubstrate, as described previously.[26] In a typical experiment, 100 µl partially purified GST was added to 1 ml (final volume) of 0.1 mol/l potassium phosphate buffer (pH 6.5), containing 2 mmol/l CDNB and 1 mmol/l GSH. The reaction was monitored at 340 nm (ϵ = 9600/M/cm), using a DU-640 spectrophotometer (Beckman Instruments, Inc., California, USA) equipped with a thermostated cuvette holder at 25°C.

Endonuclease restriction mapping *GSTP1*

Genomic DNA was amplified by polymerase chain reaction (PCR) performed with a DNA thermal cycler (Perkin Elmer 480). PCR for GSTP1 exons 5 and 6 was carried out after a preheating step at 94°C for five minutes through 30 cycles (denaturation at 94°C for one minute, annealing at 64°C for one minute, extension at 72°C for one minute) and a final extension at 72°C for eight minutes.

The mixture for each single reaction in a final volume of 50 µl was as follows: 100 ng genomic DNA, 10 mM Tris (pH 8.3) PCR buffer, 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 5% (v/v) DMSO, 1 unit Taq polymerase, and 0.2 µM of the listed primers:

Exon 5 sense: 5′ CTC TAT GGG AAG GAC CAG CAG G 3′

Exon 5 antisense: 5′ CAA GCC ACC TGA GGG GTA AGG 3′

Exon 6 sense: 5′ AAT CTG GGA CTC TGG TGT CTG G 3′

Exon 6 antisense: 5′ TCT TGC CTC CCT GGT TCT GGG A 3′

The amplification product was a 192 bp fragment for exon 5 and a 182 bp product for exon 6. The 192 amplification product was digested overnight at 37°C with Alw26I, which in the presence of the variants *B* and *C* generates two fragments of 108 and 84 bp. The 182 bp amplification product was digested overnight at 37°C with Aci I, which in the presence of the variants *A* and *B* generates two fragments of 98 and 84 bp. The restriction fragments were evaluated by 12% polyacrilamide non-denaturating gel and electrophoresed for three hours under constant current (45 mA). After electrophoresis, the gel was stained with 0.01% (w/v) ethidium bromide for 10 minutes and DNA fragments were visualised with Fluor-S Max.

Statistical analysis

Data are expressed as mean (SD). The comparison between values obtained in patients and controls was performed by Student's *t* test for unpaired data.

RESULTS

As fig 1 shows, SOD activity showed a significant increase in FRDA erythrocytes (1.56 (0.45) *v* 1.04 (0.28) U/mg,

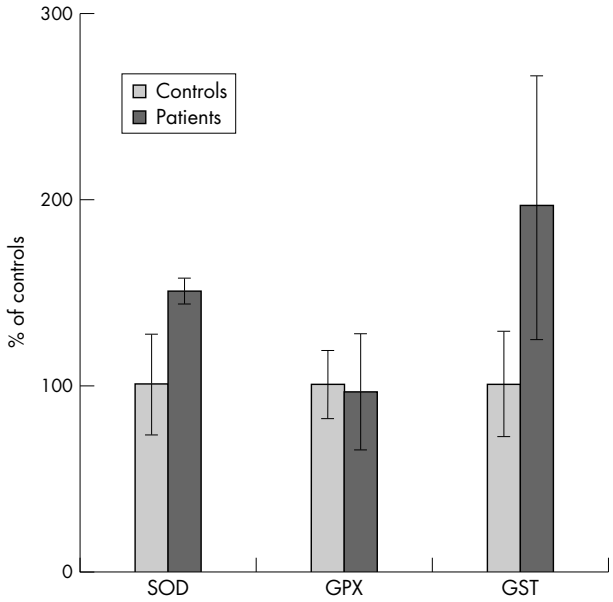

Figure 1 SOD, GPX, and GSTP1-1 enzyme activities in erythrocytes of 14 FRDA patients. Values are expressed as % of age matched controls (n = 21).

Table 2 GSTP1-1 genotype

Genotype	Frequency (%)
FRDA patients	
A/A	64.3
A/B	28.6
B/B	7.1
Controls	
A/A	68.8
A/B	18.7
A/C	12.5

$p < 0.0001$), whereas GPX activity was comparable to the controls (41.2 (13) *v* 43.6 (8) nmol/min/mg protein), thus leading to a consistent rise of SOD:GPX activity ratio (0.037 (0.01) *v* 0.025 (0.008), $p < 0.05$) in patients. Furthermore, the determination of GST specific activity in FRDA erythrocytes showed an 83% rise (0.22 (0.1) *v* 0.12 (0.03) nmol/min/mg protein, $p = 0.0061$), related to healthy subjects.

As at least four GSTP1-1 allelic variants exist which encode functionally different GSTP proteins, we also analysed GSTP1-1 genotype polymorphism in patients with FRDA. However, endonuclease restriction mapping of exon 5 and 6 amplification fragments did not find any significant differences in the relative frequencies of the allelic variants with respect to healthy subjects (table 2).

DISCUSSION

An increased oxidative stress, caused by accumulation of hydroxyl radicals produced by the iron catalysed Fenton reaction and/or a deficiency of iron–sulphur containing proteins with mitochondrial dysfunction are the proposed pathogenic mechanisms in FRDA.[5–7]

Our findings show evidence of an increased sensitivity to oxidative stress in the disease and are in agreement with two recent papers,[27 28] showing increased concentrations of plasma malondialdehyde and urine 8-hydroxy-2-deoxyguanosine in patients. We assayed antioxidant enzymes in FRDA erythrocytes and obtained a disequilibrium between SOD and GPX activities, with a significant increase of SOD:GPX activity ratio.

SOD plays a fundamental role in modulating ROS toxicity and its induction seems related to the extent of the redox abnormality in the cell. An increased SOD activity represents an adaptive response to a higher superoxide ion production,[29 30] and a critical ratio of the enzymes metabolising ROS is needed to protect the cells from free radicals toxicity. Transgenic mice overexpressing SOD activity develop morphological and biochemical abnormalities of neuromuscular tissue similar to those seen in aged animals and exhibit an increased neuronal susceptibility to apoptotic death.[31] Furthermore, changes in the physiological ratios of SOD to GPX have a profound effect on the cellular resistance to oxidant induced damage and on cell killing. An elevation in SOD/GPX ratio induces cellular senescence in transfected cell lines, suggesting a role for ROS in aging.[32–34] Moreover, patients with Down's syndrome, that carry three copies of SOD and only two copies of GPX gene, display increased SOD activity resulting in higher concentrations of H_2O_2.[35]

Furthermore, as human GSTP 1-1 gene expression is regulated by the redox status of the cell and H_2O_2 treatment has been shown to induce protein transcription in human cell cultures,[18] we analysed GSTP1-1 catalytic activity and gene polymorphism in patients with FRDA. We obtained a significant increase of blood GSTP1-1 specific activity, but not relevant differences in frequencies of expression variants.

Our suggestion is that the unbalanced SOD:GPX ratio in FRDA may contribute to an overload of H_2O_2 and, speculatively, lead to a modulation of the second defence line against ROS provided by an increase of GSTP1-1 specific activity. This enzyme activation, however, seems to not reside on a different pattern of protein expression, but it could be caused by a favourable conformational change following the oxidative insult.

In conclusion, our findings give evidence of an impairment in vivo of enzymatic antioxidants in FRDA disease, supporting a relevant role of free radical damage in the pathophysiology of neurodegeneration. This study may also assist in finding a biochemical marker of oxidative stress in FRDA, and in future therapeutic trials.

Authors' affiliations

G Tozzi, L M Gaeta, E Bertini, F Piemonte, Molecular Medicine Unit, Children's Hospital "Bambino Gesù", P.za S. Onofrio, 4, 00165 Rome, Italy
A Pastore, G Federici, Biochemistry Laboratory, Children's Hospital "Bambino Gesù"
M Nuccetelli, M Lo Bello, Department of Biology, Tor Vergata University, Rome, Italy
S Bernardini, L Bellincampi, S Ballerini, Department of Internal Medicine, Tor Vergata University
C Casali, II Neurological Institute, "La Sapienza" University, Viale dell'Università, 30, Rome, Italy

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ARCHIVIST

A touch of the 'flu

When I sat my final M.B. written exams one of the questions was, "Discuss the uses and abuses of the word influenza". I didn't know then what the examiners were getting at and I still don't now although I know considerably more about influenza than I did then.

Clinical 'influenza' (or influenza-like illness) may be caused by influenza viruses A and B, respiratory syncytial viruses (RSVs) A and B and probably other viruses including the newly identified human metapneumovirus (see Lucina page 386). Although paediatricians are familiar with RSV because of bronchiolitis it is, in fact, responsible for many influenza-like illnesses in children and adults.

During the winters of 1995–96, '96–97, and '97–98 a virological study (MC Zambon and colleagues. *Lancet* 2001;358:1410–6, see also editorial, ibid: 1382–3) was carried out in 10–15 general practices across England and Wales. Nasopharyngeal swabs were collected from people with an influenza-like illness (loosely defined) and examined for influenza virus and RSV by tissue culture and multiplex reverse transcription PCR. Seven hundred and sixty-two of the 2226 patients tested were children under the age of 14. In the three successive winters 32%, 33%, and 21% of the children under 5 years and 49%, 38%, and 41% of the children aged 5–14 years tested positive for influenza viruses. During the same winters 20%, 37%, and 41% of the under 5s and 7%, 22%, and 17% of the 5–14 year olds tested positive for RSV. In all three winters RSV was the predominant virus in infants.

Influenza and RSV outbreaks occur at about the same time in probably half of winters and they may be clinically indistinguishable. New virus-specific drug treatments and the development of an effective RSV vaccine may make it important to develop rapid tests to distinguish between them.