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

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 transferase 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.
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 “Ranse” 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. A Table 1 summarises clinical and genetic information for patients.

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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 MgCl₂, 0.2 mM dNTPs, 5% (v/v) DMSO, 1 unit Taq polymerase, and 0.2 mM of the listed primers:

- **Exon 5 sense:** 5′ CCA GCC ACC TGA GGG GTA AGG 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 Aci I, 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-denaturing 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) U/mg, GST activity was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as cosubstrate, as described previously. 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 MgCl₂, 0.2 mM dNTPs, 5% (v/v) DMSO, 1 unit Taq polymerase, and 0.2 mM of the listed primers:

- **Exon 5 sense:** 5′ CTC TAT TAT GGG AAG GAC CAG CAC 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′

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 “Ranse” 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.

**Table 1 Clinical data for FRDA patients**

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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, 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. 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.

Our findings show evidence of an increased sensitivity to oxidative stress in the disease and are in agreement with two recent papers, 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.

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.

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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. Our findings show evidence of an increased sensitivity to oxidative stress in the disease and are in agreement with two recent papers, 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.

Table 2  GSTP1-1 genotype

<table>
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<th>Genotype</th>
<th>Frequency (%)</th>
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<td>A/B</td>
<td>28.6</td>
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<td>Controls</td>
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<td>A/A</td>
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<td>A/B</td>
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<td>A/C</td>
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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. Our findings show evidence of an increased sensitivity to oxidative stress in the disease and are in agreement with two recent papers, 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.

DISCUSSION

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REFERENCES

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ARCHIVIST

A touch of the ‘flu

W

hen 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 1997; 350:1021–2) 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.
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doi: 10.1136/adc.86.5.376

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