Impaired lymphocyte mitochondrial antioxidant defences in variegate porphyria are accompanied by more inducible reactive oxygen species production and DNA damage

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Summary

This study aimed to analyse lymphocyte reactive oxygen species (ROS) production and detoxification mechanisms and the appearance of oxidative damage in variegate porphyria (VP) patients. Twelve women affected by VP and 12 pair-matched healthy control women participated in the study. VP women presented impaired expression of the mitochondrial proteins protoporphyrinogen oxidase, uncoupling protein-3, Bcl-2 and sirtuin 3. Lymphocytes from VP women presented higher \( \text{H}_2\text{O}_2 \) production than controls after stimulation with phorbol myristate acetate. The inhibition of \( \text{H}_2\text{O}_2 \) production after \textit{in vitro} lymphocyte treatment with myxothiazol pointed towards complex III of the mitochondrial respiratory chain as the main contributor of the higher ROS production in porphyric subjects. No differences were observed between VP and control subjects in the levels of DNA damage, assessed by the comet assay method in un-treated lymphocytes. However, DNA damage, expressed both as a percentage of DNA in tail and as the tail moment, was greater in VP women than controls after lymphocyte treatment with \( \text{H}_2\text{O}_2 \). In conclusion, lymphocytes from VP women showed impaired expression of mitochondrial antioxidant defences but no significant signs of oxidative stress were evidenced in basal, non-stressing conditions; however, lymphocytes of VP women were more susceptible to producing mitochondrial ROS and to suffering oxidative damage when submitted to stressful situations.

Keywords: oxidative damage, protoporphyrinogen oxidase, respiratory chain, UCP-3, white blood cells.

Haem is a prosthetic group present in haem proteins, such as haemoglobin or myoglobin, mitochondrial or microsomal cytochromes, catalase and peroxidases, all of which play very important roles in cell function (Mauzerall, 1998). The porphyrias are a group of metabolic disorders of haem biosynthesis that result in the accumulation of porphyrins or their precursors (Nordmann & Puy, 2002; Badminton & Elder, 2005). Although porphyrias are inherited diseases, there are some agents that can precipitate acute attacks and the appearance of clinical symptoms (Gordon, 1999). Variegate porphyria (VP), an autosomal dominant type of hepatic porphyria, is the result of decreased protoporphyrinogen oxidase (PPOX) activity, the penultimate enzyme of haem biosynthesis, and is characterized by skin lesions and acute attacks (Nordmann & Puy, 2002). The chronic accumulation of haem precursors in erythrocytes, liver or other tissues is responsible for the main clinical and pathological manifestations of this disease (Sassa, 2000; Nordmann & Puy, 2002; Badminton & Elder, 2005). The accumulation of haem precursors in cell types other than erythrocytes or hepatocytes can induce oxidative stress and cellular damage due to their ability to produce free radicals and to activate oxygen. However, other mechanisms could be involved in the generation of greater amounts of reactive oxygen species (ROS) in porphyric patients. The mitochondrial electron transport chain is the main source of ROS in many cell types. Intermediate free radical species from the electron transport chain may interact with dissolved oxygen leading to the formation of superoxide.
mitochondrial ROS and oxidative damage (Takahashi et al., 2004). SIRT3, a mitochondrial member of the sirtuin family of histone deacetylases (Michishita et al., 2005), is known to play an important role over ROS production and scavenging in mitochondria by decreasing mitochondrial membrane potential and increasing cellular respiration (Shi et al., 2005).

Our aim was to evaluate the role of mitochondria in lymphocyte ROS production, the expression of mitochondrial antioxidant defences and the oxidative damage related to VP. We hypothesized that lymphocytes from VP patients would show higher mitochondrial ROS production and oxidative damage markers and impaired mitochondrial antioxidant defences when compared to healthy women.

Materials and methods

Ethical approval

The study protocol was in accordance with the Declaration of Helsinki for research on human subjects and was approved by the Ethical Committee of Clinical Investigation of the CAIB (Palma de Mallorca, Balearic Islands, Spain). All the subjects were informed of the purpose and demands of the study before giving their written consent to participate.

Subjects and study design

The study was performed with 12 women affected by VP and 12 pair-matched healthy control women. All the patients participating in our study had been previously diagnosed to suffer from VP on the basis of different parameters, such as plasma fluorescence peak at 626 nm, levels of excreted urinary and faecal porphyrins, and clinical manifestations, such as abdominal pain during porphyric attacks.

Venous blood samples were obtained from the antecubital vein of control and porphyric women in resting conditions after overnight fasting. The lymphocyte fraction was purified from whole blood following an adaptation of the method described by Boyum (Boyum, 1964; Ferrer et al., 2007) using Ficoll-Paque PLUS reagent (GE Healthcare, Chalfont St Giles, UK). This procedure ensured a lymphocyte purity and viability of 95 ± 5%.

mRNA expression

mRNA expressions were determined by real time reverse transcription polymerase chain reaction (RT-PCR). For this purpose, mRNA was isolated from lymphocytes by extraction with Tripure Isolation Reagent (Roche Diagnostics, Basel, Switzerland). cDNA was synthesized from 1 μg total RNA using reverse transcriptase with oligo-dT primers. Quantitative PCR was performed using the LightCycler instrument (Roche Diagnostics) with DNA-master SYBR Green I. The primers used are shown in Table I. For all PCRs there was one cycle at 95°C for 10 min, followed by 40 cycles at the conditions shown in Table I.
The relative quantification was performed by standard calculations considering $2^{\Delta\Delta C_{t}}$. Basal mRNA levels of control women were arbitrarily referred to as 1. The expression of the target gene was normalized with respect to *RN18S1*.

**Hydrogen peroxide production**

$H_{2}O_{2}$ production in lymphocytes was measured before and after stimulation with phorbol myristate acetate (PMA, Sigma-Aldrich, St Louis, MO, USA) using 2,7-dichlorofluorescin-diacetate (DCFH-DA, Sigma-Aldrich) as an indicator (Soh, 2006). DCFH-DA (30 μg/ml) in phosphate-buffered saline was added to a 96-well microplate containing 50 μl lymphocyte suspension containing about 1 · 10⁵ cells. PMA (3 μmol/l) prepared in Hank’s Balanced Salt Solution (HBSS, Sigma-Aldrich) or HBSS alone was added to the wells and the fluorescence (Ex, 480 nm; Em 530 nm) was recorded at 37°C for 1 h in a FLx800 Microplate Fluorescence Reader (BioTek Instruments, Inc., Winooski, VT, USA). In addition, three treatments with 100 mmol/l allopurinol (Sigma-Aldrich) as a xanthine oxidase inhibitor, 5 mmol/l rotenone (Sigma-Aldrich) as a complex I inhibitor or 2 mmol/l myxothiazol (Sigma-Aldrich) as a complex III inhibitor were performed in PMA-stimulated lymphocytes. All incubations were performed in the presence of digitonin (Sigma-Aldrich) to ensure internalization of inhibitors.

**Comet assay**

Assessment of DNA damage was carried out using the alkaline comet assay method. Briefly, slides were prepared by adding purified lymphocytes, mixed with 0.6% low-melting-point agarose. In order to release the DNA, cells were lysed by immersing slides in lysis solution (2·5 mol/l NaCl, 100 mmol/l EDTA disodium salt, 10 mN Tris, 1% Triton X-100 and 10% DMSO, pH 10) at 4°C for 40 min. After removal from the lysing solution, the slides were placed in an electrophoresis trough containing an alkaline electrophoresis buffer (300 mmol/l NaOH, 1 mmol/l EDTA). A current of 25 V and 300 mA was applied for 30 min. The slides were then removed and Tris buffer 0·4 mol/l adjusted to 7·5 with concentrated HCl was added onto the slides to neutralize excess alkali. DNA was stained by adding ethidium bromide. Comet measurements were made by image analysis using a fluorescence microscope equipped with an excitation filter of 450–490 nm and a barrier filter of 520 nm and the Comet software (TriTek CometScore™). Images of 50 random nuclei concentrated HCl was added onto the slides to neutralize excess alkali. DNA was stained by adding ethidium bromide. Comet measurements were made by image analysis using a fluorescence microscope equipped with an excitation filter of 450–490 nm and a barrier filter of 520 nm and the Comet software (TriTek CometScore™). Images of 50 random nuclei were taken at 200× magnification and were analysed for each sample. The comet measurements that were recorded and subsequently used for analysis were percentage DNA in tail (tail intensity) and tail moment (tail intensity × tail length).

**Statistical analysis**

Statistical analysis was carried out using the Statistical Package for Social Sciences for Windows, version 13.0. Results are expressed as mean ± standard error of the mean (SEM) ($n = 12$) and $P < 0·05$ was considered statistically significant. The statistical significance of the data was assessed by Student’s t-test for unpaired data.

**Results**

No significant differences were observed in anthropometric data between the porphyric patients and controls participating in the study. Controls presented the same age and similar lifestyle habits as porphyric patients (data not shown) (Romaguerera et al., 2006).

Lymphocyte $H_{2}O_{2}$ production is shown in Fig 1. In basal conditions, $H_{2}O_{2}$ production was similar in control and VP women. However, when lymphocytes were stimulated with

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**Table I. Primers and conditions used in real time PCRs.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>RN18S1</td>
<td>Fw: 5’-ATG TGA AGT CAC TGT GCC AG-3’  95°C, 10 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rv: 5’-GTG TAA TCC GTC TCC ACA GA-3’  60°C, 7 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>72°C, 12 s</td>
</tr>
<tr>
<td>PPOX</td>
<td>Fw: 5’-TTT GCC TAG TTT GAG GTC AC-3’  95°C, 10 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rv: 5’-TCC CCA TTT GCA TTA ACC AG-3’  60°C, 10 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>72°C, 15 s</td>
</tr>
<tr>
<td>UCP3</td>
<td>Fw: 5’-GGT GAT GTT CAT AAC CTA TG-3’  95°C, 5 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rv: 5’-CGG TGA TTC CCG TAA CAT CTG-3’  60°C, 10 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>72°C, 10 s</td>
</tr>
<tr>
<td>BCL2</td>
<td>Fw: 5’-CTG GTG GGA GCT ATC AC-3’  95°C, 5 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rv: 5’-ACA GGC TGC AGC TTT GTT TC-3’  65°C, 5 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>72°C, 5 s</td>
</tr>
<tr>
<td>SIRT3</td>
<td>Fw: 5’-GAG CTT CTG GGC TGG ACA GA-3’  95°C, 10 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rv: 5’-TGG GAT GTG GAT GTC TCC TAT G-3’  65°C, 5 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>72°C, 7 s</td>
</tr>
</tbody>
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PMA, H$_2$O$_2$ production rose and this was significantly higher in the VP group when compared to controls. The possible source of the production of H$_2$O$_2$ by lymphocytes was studied using different inhibitors of target candidates to produce ROS. Three different inhibitors were used to determine the possible source of the increased ROS production after lymphocyte stimulation with PMA. Myxothiazol, as an inhibitor of respiratory chain complex III, returned ROS production back to basal levels in both groups and the difference between porphyrins and controls found in the absence of myxothiazol disappeared. Rotenone, as an inhibitor of respiratory chain complex I, induced a rise in H$_2$O$_2$ production in the control group but not in the porphyric group, leading to the disappearance of the difference in ROS production found between controls and VP patients in the absence of rotenone treatment. Allopurinol, as an inhibitor of xanthine oxidase, did not modify H$_2$O$_2$ production, but no differences between VP and control women were detected in the presence of this inhibitor. Taken together, these results suggest that the PMA activation of lymphocytes induced an increase in H$_2$O$_2$ production mainly due to mitochondrial production, and lymphocytes from porphyric women produced greater mitochondrial H$_2$O$_2$ amounts than controls after PMA activation. This mitochondrial H$_2$O$_2$ overproduction in porphyric women could be related with lower antioxidant defences, in addition to other causes. The gene expression of some key antioxidant proteins in mitochondrial H$_2$O$_2$ production was analysed. Figure 2 shows the gene expression of PPOX, UCP3, BCL2 and SIRT3 genes in lymphocytes of control and porphyric women. PPOX (Fig 2A) and UCP3 (Fig 2B) gene expressions were decreased in porphyric patients to 80% of the expression in controls. BCL2 (Fig 2C) and SIRT3 (Fig 2D) expressions were also reduced in the porphyric group when compared to controls (70% and 65% respectively). These results indicate that lymphocytes from women affected by VP had lower expression of the enzyme that define their pathology (PPOX), but also in other key antioxidant proteins such as UCP-3, Bcl-2 and SIRT3, even to a greater extent than PPOX.

![Fig 1. Effects of variegate porphyria on lymphocyte H$_2$O$_2$ production after addition of different inhibitors. All incubations were performed in the presence of digitonin (DIG). Phorbol meristate acetate (PMA) was used to activate lymphocytes. Inhibitors tested were 2 mmol/l myxothiazol (MYX), 5 mmol/l rotenone (ROT) and 100 mmol/l allopurinol (ALL). Results are expressed as mean ± SEM. Statistical analysis: Student’s t-test for unpaired data. (#) Indicates significant differences between porphyria and control groups, (§) indicates significant differences versus PMA/DIG alone treatment (P < 0.05).](image1)

![Fig 2. Effects of variegate porphyria on lymphocyte PPOX (A), UCP3 (B), BCL2 (C) and SIRT3 (D) gene expression. The relative quantification was performed by standard calculations considering $2^{-\Delta\Delta Ct}$. mRNA levels of control women were arbitrarily referred to as 1. Results are expressed as mean ± SEM. Statistical analysis: Student’s t-test for unpaired data. (#) Indicates significant differences between porphyria and control groups (P < 0.05).](image2)
with H2O2, the degree of DNA damage, expressed both as a percentage of DNA in tail (A) and tail moment (B) in lymphocytes measured in basal conditions and after H2O2 treatment. Results are expressed as mean ± SEM. Statistical analysis: Student’s t-test for unpaired data. (#) Indicates significant differences between controls and porphyric patients, (*) indicates significant differences between basal and H2O2 treatment ($P < 0.05$).

The imbalance between lymphocyte H2O2 production and antioxidant gene expression could increase oxidative damage. The levels of DNA damage in basal conditions were similar in controls and porphyric women with no significant differences between groups (Fig 3). However, after treating lymphocytes from porphyric and control women, the degree of DNA damage, expressed both as a percentage of DNA in tail and as the tail moment, was significantly increased in women affected by VP compared to controls.

**Discussion**

VP is the result of decreased PPOX activity, the penultimate enzyme of haem biosynthesis. This disease is characterized by the accumulation of some haem precursors in erythrocytes and by a low rate of haem biosynthesis as a result of the genetic error in PPOX. All the patients participating in our study had been previously diagnosed on the basis of different parameters, such as plasma fluorescence peak at 626 nm, levels of excreted urinary and faecal porphyrins and clinical manifestations. We first investigated whether the PPOX expression is impaired in lymphocytes, in which the demand and utilization of haem is not as high as in the erythrocytes. We found a downregulation (80% of control expression) of PPOX gene expression in the lymphocytes of VP women when compared to controls, which makes it evident that the mutation in the PPOX gene has phenotypic consequences, like decreased PPOX expression in white blood cells, such as lymphocytes. This lower PPOX expression in lymphocytes is in accordance with previous studies reporting decreased PPOX activity in lymphocytes from variegate porphyria patients (Siepker & Kramer, 1985; Meissner et al, 1986) and could compromise the haem synthesis in these patients and hence alter their oxidative status by impairing the antioxidant systems and/or increasing ROS production.

The accumulation of the haem precursors has been shown to constitute an endogenous source of ROS, triggering oxidative damage to cellular components (Monteiro et al, 1986; Pereira et al, 1992). Therefore, we studied H2O2 production in lymphocytes as an indicator of intracellular ROS formation. In basal conditions, lymphocytes from porphyric and control women produced approximately the same amount of H2O2. However, when lymphocytes were activated with PMA, greater H2O2 production was detected in the porphyric group when compared to controls, indicating that after activation lymphocytes from VP women are more susceptible to producing ROS and hence to suffering oxidative damage. Lymphocyte ROS sources are the mitochondrial respiratory chain, nitric oxide synthase (NOS) activity in the lack of arginine, xanthine oxidase (XOD) and glucose oxidase (GOD) activities. The rate of increase in the H2O2 production when lymphocytes are activated with PMA, in a medium lacking xanthine, and with no changes in arginine availability, as well as iNOS, XOD or GOD ‘de novo’ synthesis seems unlikely to occur, suggests that this increase could be mitochondrial. In order to demonstrate that the increased H2O2 production in activated lymphocytes from porphyric women was due to mitochondrial respiratory chain impairment, we tested the effects of three different inhibitors of ROS production such as myxothiazol (complex III inhibitor), rotenone (complex I inhibitor) and allopurinol (xanthine oxidase inhibitor). The allopurinol treatment did not modify H2O2 production in lymphocytes, thus ruling out the possibility of a XOD activity derived increase in ROS production in VP women. The inhibition of the mitochondrial respiratory chain with different inhibitors induced different changes. The inhibition of complex I with rotenone has been shown to induce both an increase (Gao & Wolin, 2008) and a decrease (Chen et al, 2008) in ROS production. It would seem that the effects of rotenone depend on whether the primary site of superoxide generation in the mitochondrial electron transport chain is complex I (Turrens & Boveris, 1980) or complex III (Chen et al, 2003), thus increasing or decreasing ROS production, respectively. No effect of complex I inhibition with rotenone was found in the porphyric patients. In the control group, however, an increase in ROS production was detected after inhibition with rotenone while no differences in H2O2 production were found between porphyric and control groups. This might indicate that, in porphyric patients, complex III could play an important role in producing more ROS.
ROS than in healthy people. To further corroborate this hypothesis, the effects of the complex III inhibitor, myxothiazol, were tested. Myxothiazol inhibits the binding of ubiquinol at complex III, thereby preventing the oxidation of ubiquinol and the formation of ubisemiquinone, and reducing ROS production (Crofts et al., 1999; Chen et al., 2003). In our study, when complex III was inhibited with myxothiazol, decreased \( \text{H}_2\text{O}_2 \) production was detected and no differences were found between porphyric and control women. This reaffirms that the increase in \( \text{H}_2\text{O}_2 \) production in lymphocytes after stimulation with PMA and the increased production in porphyric women when compared to controls might be due to the mitochondrial respiratory chain function, and probably due to respiratory complex III, rather than to other sources, such as XOD activity. As haem is an essential factor present in several cytochromes belonging to complex III, the dysfunction in haem biosynthesis as a result of the limited PPOX content seems to lead to a disturbed function of the respiratory chain, thus leading to increased ROS production. NADPH oxidases have been described in lymphocytes (Jackson et al., 2004). However, NADPH oxidase is localized in the plasmatic membrane and releases ROS to the extracellular compartment, while we determined intracellular \( \text{H}_2\text{O}_2 \) production – as the DCFH-DA probe only reacts with ROS after intracellular hydrolysis – so it is not probable that the observed increase in \( \text{H}_2\text{O}_2 \) production was due to this enzyme. The fact that the inhibition of the mitochondrial respiratory chain highly decreases PMA-induced ROS production also reaffirms the exclusion of NADPH oxidases as a major source of ROS in activated lymphocytes.

Having shown the increased susceptibility of porphyric patients to produce mitochondrial ROS, we studied the expression of some proteins that have been related to the regulation of ROS by the electron transport chain. UCP-3 is a member of the mitochondrial uncoupling protein family mainly detected in skeletal muscle but also present in human spleen and thymus (Carroll & Porter, 2004), and it has been recently detected in human lymphocytes (Ferrer et al., 2009b). This uncoupling protein is not involved in thermogenesis (Rousset et al., 2004) but has been shown to act as an antioxidant in skeletal muscle by reducing ROS production (Schrauwen & Hesselink, 2004). It has been recently proposed that UCP-3 may partially alleviate the proton gradient across the inner membrane, thereby reducing further ROS production by the electron transport chain (Jiang et al., 2009). In our study, women affected by VP presented downregulated UCP-3 expression in lymphocytes when compared to controls. We have previously shown that UCP-3 expression was downregulated after a single bout of exercise and was directly correlated to Bcl-2 expression, thus supporting the idea that both proteins could have related functions in protecting mitochondrial stability by acting as antioxidants (Ferrer et al., 2009b). Several lines of evidence support the idea that Bcl-2 might as an antioxidant (Fleury et al., 2002). Bcl-2 is localized in the outer mitochondrial membrane and could be important in the regulation of proton flux in mitochondria, mitochondrial membrane potential (\( \Delta \psi_M \)) and the appearance of mitochondrial ROS and oxidative damage (Takahashi et al., 2004). Furthermore, it has been previously described that Bcl-2 prevented ROS accumulation during tumour necrosis factor-\( \alpha \)-induced apoptosis (Sidoti-de Fraisse et al., 1998) and cells over-expressing Bcl-2 have increased total glutathione levels and reduced ratios of oxidized glutathione to total glutathione, thus suggesting that Bcl-2 could protect cells by shifting the cellular redox potential to a more reduced state (Ellerby et al., 1996). The present study found that lymphocytes from VP women presented lower Bcl-2 expression than those from controls. The decreased gene expression of both antioxidant proteins UCP-3 and Bcl-2 indicates that the antioxidant capabilities of the lymphocytes from porphyric patients are impaired, and thus they could be more susceptible to suffering oxidative stress.

SIRT3 is a member of the sirtuin family of histone deacetylases. Of the seven known sirtuins in mammals, three of them – including SIRT3 – are located in the mitochondria (Michishita et al., 2005). It has been postulated that SIRT3 activity can upregulate the expression of mitochondrial biogenesis genes such as \( \text{PPARGC1A} \) (Shi et al., 2005), but the pathways involved in these upregulations are unclear (Shi et al., 2005). SIRT3 has been shown to decrease mitochondrial membrane potential and ROS production, while increasing cellular respiration (Shi et al., 2005). The mechanism by which SIRT3 increases respiration is not completely understood as yet, but recent evidence points towards deacetylation and activation of the mitochondrial enzymes involved in the respiratory metabolism and the control of the citric acid cycle, such as isocitrate dehydrogenase – which produces NADPH, important for the NADPH-dependent regeneration of antioxidants – (Schlicker et al., 2008), glutamate dehydrogenase (Schlicker et al., 2008) and acetyl coenzyme A synthetase 2 (North & Sinclair, 2007). Therefore SIRT3 clearly plays an important role in ROS production and scavenging in mitochondria. In our study, lymphocytes of porphyric women showed 65% of the SIRT3 expression found in the control group, which could result in a decreased respiratory capacity when compared to control healthy subjects, and then could be related to the higher ROS production after lymphocyte stimulation observed in porphyric patients. The combined downregulation of UCP3, BCL2 and SIRT3 – all of them genes encoding proteins directly or indirectly related to mitochondrial ROS formation and detoxification – in VP women is indicative of impaired antioxidant defences in these patients.

Taking into account these results, lymphocytes from VP women have been shown to present impaired mitochondrial antioxidant potential, but in basal conditions this impairment is not related to increased ROS production. However, when lymphocytes are activated and then exposed to a stress condition, lymphocytes from porphyric women produce greater amounts of mitochondrial ROS than control women. The combination of both impaired antioxidant defences and
increased ROS production could lead to a situation of oxidative stress, thus we measured DNA damage as an indicator of cellular damage. The comet assay is a sensitive method for detecting DNA damage at the level of individual cells, including detection of single and double strand breaks, incomplete excision repair sites and cross links. It has been proved to be a good marker of oxidative damage in the DNA of lymphocytes (Sinha et al, 2007; Devaraj et al, 2008; Sliwinska et al, 2008). Tail moment represents both the amount of DNA migrated into the tail and the distance migrated, and it has been reported as a valid marker of single-strand DNA breakage (Mastaloudis et al, 2004) while at low levels of damage the tail length seems to be a more sensitive marker than percentage DNA in tail (Collins, 1998). Once again we found no evidence of DNA damage in the lymphocytes of porphyric women when compared to controls in basal conditions. However, after a short treatment of isolated lymphocytes with 10 µmol/l H2O2, greater DNA damage was evidenced in porphyric women, evidenced both as % DNA in tail and tail moment. The increased oxidative damage after oxidative treatment suggests that VP patients have difficulties in countering the excess of ROS produced as a consequence of a stressful situation and are hence more susceptible to suffering oxidative stress.

This study showed that VP – a disease that primarily affects the liver and erythrocytes – induces oxidative stress in lymphocytes, a cell type in which the demand for haem is not as high as in erythrocytes or hepatocytes. The factor triggering lymphocyte oxidative stress could be the altered haem synthesis in the lymphocyte, but indirect effects of erythrocyte metabolites on lymphocytes cannot be discarded, as has been observed in other blood diseases. For instance, thalassaemia – a heterogeneous group of inherited anemias resulting from reduced or absent synthesis of α- or β-globin chains of haemoglobin A – generates iron overload and premature haemolysis in the plasma and in major organs such as heart and liver (Kushner et al, 2001). Iron released by the erythrocyte can then induce oxidative alterations and DNA damage in nearby cells (Halliwell & Aruoma, 1991; Comporti et al, 2002; Fibach & Rachmilewitz, 2008). The porphyria-induced oxidative stress observed in lymphocytes could also be related to an impairment of the immune function in these patients, as happens in some haemolytic anemias. In thalassaemia, chronically stressed polymorphonuclear cells have reduced capacity to elicit a respiratory burst, which compromises their antibacterial capacity and results in recurrent infections (Amer & Fibach, 2005). However, no studies concerning the effects of VP on immunity have been performed until date.

In conclusion, in basal conditions, lymphocytes from VP patients showed impaired mitochondrial antioxidant defences but no significant signs of oxidative stress were found; however, lymphocytes of VP patients were more susceptible to producing mitochondrial ROS and to suffering oxidative damage when submitted to stressful situations, such as lymphocyte activation and H2O2 treatment, respectively.

Acknowledgements

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