Evidence of Oxidative Stress in Familial Amyloidotic Polyneuropathy Type 1

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Objective: To evaluate the oxidative state in patients with familial amyloidotic polyneuropathy type 1 (FAP1).

Design: From 3 unrelated families, patients with FAP1 carrying a transthyretin Met-30 mutation were studied. The diagnosis was confirmed by genetic analysis. Eleven of 21 patients carried the mutation; all were symptomatic and were clinically assessed using a clinical score. All of the patients were evaluated for copper-zinc superoxide dismutase type 1 activity in red blood cells using spectrophotometry. Plasma total reactive antioxidant potential was studied using a chemiluminescent method. The results were compared with those obtained from an age-matched control group.

Setting: A public and academic multidisciplinary research clinic.

Results: Six of the 11 FAP1-positive patients disclosed superoxide dismutase type 1 activity values greater than 55 U/mg of protein (upper control limit), whereas 9 of 10 patients in whom total reactive antioxidant potential was measured had values below the lower limit of the control group. No relationship was found between the levels of superoxide dismutase type 1 activity and the severity of the clinical involvement.

Conclusions: Oxidative stress may be part of the mechanisms leading to tissue damage in patients with FAP1. The lack of correlation between the laboratory findings and the severity of clinical involvement may signal that oxidative processes are at work throughout the natural history of the disease.

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FAMIAL AMYLOIDOTIC polyneuropathies (FAPs) are diseases that affect the nervous system, mainly expressed as peripheral neuropathy.1,2 Many patients have been found to have a mutation in position 30 at exon 2 of the transthyretin (TTR) gene (chromosome band 18q11.2-q12.1), which results in a single methionine and/or valine substitution.3,4 This condition has been named FAP type 1 (FAP1); the mutated protein has been proposed to be the origin of the deposit of amyloid fibrils that characterizes the illness.5,6

Familial amyloidotic polyneuropathy type 1, also known as type 1 amyloidosis, is the most frequent type of FAP, and it has been described in many countries around the world, including Portugal,4 Greece,7 Japan,8 Sweden,9 Brazil,10 Turkey,11 the United States,12 and Argentina.13 Clinically, FAP1 is expressed by peripheral neuropathy, usually starting at the lower limbs and progressively spreading to the upper limbs and cranial nerves, associated with varying degrees of bowel, cardiac, or urinary disturbances.1,14

Some evidence15-17 suggests that oxidative stress may play a role in the development of FAP1. However, the activity of the circulating antioxidant enzyme copper-zinc superoxide dismutase type 1 (SOD1) and the total reactive antioxidant potential (TRAP) in plasma, considered an index of the level of antioxidants,18 have not yet been assessed.

Reactive oxygen species might be important pathogenic factors in different conditions, ranging from cardiovascular, inflammatory, and degenerative diseases to cancer.19 Reactive oxygen species are generated in living systems, and they oxidize various cellular constituents such as lipids, proteins, and DNA. Consequently, antioxidants come into play and act as free radical scavengers, inhibiting lipid peroxidation and other free radical-mediated processes, thus protecting the body from several diseases attributed to the reaction of free radicals.20 These biological effects, induced by free radicals, are...
neutralized in vivo by antioxidative defense mechanisms, which include vitamins C and E, carotenoids, glutathione, and antioxidant enzymes. However, the extensive generation of reactive oxygen species in some pathologic conditions seems to overwhelm natural defense mechanisms, thereby dramatically reducing the levels of endogenous antioxidants. The TRAP is an index used to evaluate the total endogenous antioxidant compounds present in plasma.\(^1\)

The present article describes a series of patients with FAP1 from 3 different families in whom SOD1 activity in red blood cells and TRAP in plasma were evaluated. Increased activity of the antioxidant cytosolic enzyme copper-zinc SOD1 measured in red blood cells and decreased TRAP in plasma are the most remarkable findings in this work.

## METHODS

### PATIENTS

This protocol was approved by the Ethics Committee of FITEN (Fundación para la Investigación y Tratamiento de las Enfermedades Neurolo[ gicas, Buenos Aires) and the Board for Human Research of the Hospital Ramos Mejía. Eleven patients (7 women and 4 men aged 19-49 years) from 3 unrelated families were studied. None of the patients were receiving any medication, and all proved to have clinically evident FAP1, which was confirmed by the genotyping procedure described herein.

The intensity of the clinical manifestations varied among individuals. Sensory symptoms were hands and feet paresthesias in the form of pins and needles, and motor symptoms included weakness of the upper and lower limbs, hands, legs, and feet. Autonomic symptoms included diarrhea, constipation, urinary incontinence, delayed voiding, dry mouth, and syncope.

Sensory signs included touch and pain hypoesthesia and diminished vibratory and joint position senses in the distal segments of the 4 limbs; motor signs were muscle weakness and wasting, diminished tendon jerks, and fasciculations. It was accepted that the patient was affected by postural blood pressure hypotension when changing from lying to standing: a decrease of 10 mm Hg in diastolic blood pressure or 30 mm Hg in systolic blood pressure was achieved.\(^2\) The degree of clinical involvement was correlated with oxidative stress by using the score described by Dyck et al.\(^3\)

### BLOOD SAMPLING AND MEASUREMENTS

#### Copper-Zinc SOD1 Activity

A 3-mL volume of blood was sampled in 4% citrate and centrifuged at 2500 rpm for 10 minutes. Serum and white blood cells were discarded, and the red blood cells were washed twice with 8% sodium chloride, lyzed in bidistilled water for 5 minutes, and extracted with chloroform and/or ethanol. The samples were then centrifuged at 5000 rpm for 10 minutes. The supernatants were stored at −20°C until assayed. Superoxide dismutase type 1 activity was evaluated using the epinephrine system described by Misra and Fridovich.\(^4\) One unit of SOD1 activity was defined as the amount of enzyme that induced a 50% decrease in the rate of epinephrine auto-oxidation. Data are expressed as units per milligram of protein. The results were compared with those from a group of 125 healthy subjects of different ages\(^5\) and 10 healthy relatives (FAP1[−]).

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**Plasma TRAP**

Plasma samples were obtained from 15 age-matched controls, 5 relatives, and 10 patients. The reaction medium consisted of 20mM 2,2'-azo-bis-2-amidinopropane and 40µM luminol. The former is a source of free radicals that reacts with the latter, yielding chemiluminescence, which is then measured in a Packard tri-Carb liquid scintillation counter (Hewlett-Packard Company, Palo Alto, Calif) with the circuit coincidence out of mode. The addition of 10 µL of plasma decreases chemiluminescence to basal levels for a period proportional to the amount of charge in antioxidants present in plasma until luminol radicals are regenerated (induction time [TI]). The system is calibrated with a vitamin E hydrosoluble analogue (Trolox; F. Hoffman-La Roche Ltd, Basel, Switzerland). The relationship between the induction time before and after the addition of known concentrations of Trolox and plasma defines the TRAP, which is the equivalent of the Trolox concentration necessary to suppress the emitted luminescence:

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\text{TRAP (µM Trolox)} = D \times (8s/8T),
\]

where D is a dilution factor, 8s is the induction time of the sample, and 8T is the time elicited by the addition of 1µM Trolox. Data are expressed as micromoles of Trolox, as previously described.\(^6\)

**GENOTYPING FOR TTR Valine 30 Methionine**

Genotyping was carried out in blood samples by polymerase chain reaction–restriction fragment length polymorphism and immunoblotting of cyanogen bromide–cleaved TTR.

**Polymerase Chain Reaction–Restriction Fragment Length Polymorphism**

Genomic DNA was isolated from 200 µL of total blood or from buffy coat using DNA blood adsorption columns (Qiagen Inc, Valencia, Calif) following the manufacturer’s instructions. Exon 2 of TTR was amplified using flanking primers (5'-CTT gTT TCG ATT TC-3' and 5'-ggg CAA ACg gAA AgA TAA-3') and approximately 1 µg of the isolated DNA as substrate with a hot-start Taq DNA polymerase (Platinum Taq; Invitrogen Corp, Carlsbad, Calif). The 278-base pair polymerase chain reaction product was digested with NsiI re-

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**Cyanogen Bromide Cleavage and Immunoblotting**

Serum samples (400 µL) were incubated with 400 µL of a 50% suspension of a murine anti-TTR monoclonal immuno-

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Superoxide dismutase type 1 (SOD1) and total reactive antioxidant potential (TRAP) values in patients with familial amyloid polyneuropathy type 1 (FAP1(+)) and controls. A, Copper-zinc SOD1 activity in erythrocytes of 11 FAP1(+) individuals and 10 FAP1(−) relatives. The upper and lower limits (19 and 55 U/mg of protein, respectively) correspond to control data reported previously24 in 125 controls (mean±2 SD, 37±18 U/mg of protein). B, Plasma TRAP in 10 FAP1(+) patients, 5 FAP1(−) relatives, and 15 controls. The means±SD values for these groups are 129.1±29.0, 237.8±56.8, and 246.3±74.1µM of Trolox (F. Hoffman–La Roche Ltd, Basel, Switzerland), respectively. P<.001, FAP1(+) individuals vs controls and FAP1(−) relatives.

Evaluation of the clinical damage in FAP1(+) patients yielded the following results: 2 patients scored 6, 3 scored 10, and the remaining 6 scored 12, 16, 17, 79, 88, and 97.

To explore an eventual environmental factor that might affect oxidative stress, we included 10 relatives of these patients as another control group. Their ages ranged from 21 to 55 years. These 10 FAP(−) relatives showed SOD1 activity ranging from 27 to 54 U/mg of protein. In a previous study,24 SOD1 activity varied between 19 and 55 U/mg of protein in a general control population comprising 125 individuals of both sexes whose ages ranged from 16 to 89 years (Figure, A). In the 11 FAP1(+) patients examined, SOD1 activity values for 6 (5 women and 1 man) were greater than the upper control limit reported previously (55 U/mg of protein) (Figure, A). The mean±SD SOD1 activity of the 11 FAP1(+) patients was significantly different than that of the 10 FAP1(−) patients (52±13.8 vs 40.3±9.5 U/mg of protein; P<.001). Comparing SOD1 activity and the level of clinical involvement, the Pearson correlation coefficient was not significant (NS) (r=0.149; P=NS), and there were no correlations between the length of the history and SOD1 values (r=0.108; P=NS) or between the age of the patients and SOD1 values (r=−0.409; P=NS).

The TRAP was measured in the plasma of FAP1(+) patients and compared with that of a control population and 5 FAP1(−) individuals who agreed to be sampled for this procedure (Figure, B). The mean±SD values obtained from 15 healthy individuals do not differ significantly from those obtained from 5 FAP1(−) relatives (246.3±74.1 vs 237.8±56.8±µM of Trolox). A substantial decrease in TRAP was observed in 10 FAP1(+) patients (129.1±29.0±µM of Trolox) vs controls and FAP1(−) relatives (P<.001) (Figure, B). Only 1 patient was within the control range.

Familial amyloidotic polyneuropathy type 1 due to the TTR Met-30 mutation is an autosomal dominant disease2 with a relentless course that starts, in most patients, in the fourth decade or later.2,6

Only a few studies suggest the participation of oxidative stress in the development of the disease. Sakashita et al15 found in tissue samples from patients with different clinical forms of amyloidosis, including FAP1, increased levels of hydroxynonenal and thiobarbituric acid reactive substances, which are considered products of lipid peroxidation.

The results reported herein show that SOD1 activity is increased in the red blood cells of 54.5% of affected patients, whereas the antioxidant potential of the plasma is decreased in 9 of 10 patients tested. These changes in the biochemical variables cannot be attributed to the effect of environmental factors because relatives living in the same place as the patients had values similar to those of the healthy nonselected controls.

It is known that SOD1 is the main intracellular scavenger of superoxide anions, which are converted to hydrogen peroxide. An increase in SOD1 may be interpreted as an obvious response against an increased amount of superoxide anions, as reported in other conditions, such as chronic renal failure or experimental exposure to paraquat or kainic acid used as free radical inducers.27-29

Superoxide dismutase type 1 converts superoxide to hydrogen peroxide, a pro-oxidant molecule. A sustained increased in SOD1 activity may induce tissue dam-
age by increasing the concentration of hydrogen peroxide, leading to an excess of hydroxyl radicals, which are powerful oxidant elements. In this regard, increased SOD1 activity has been postulated as the main cause of tissue degeneration observed in Down syndrome.30 Experiments31,32 carried out in a transgenic mouse model that overexpresses SOD1 and resembles Down syndrome in humans revealed damage in many tissues, which is correlated with increased SOD1 and hydrogen peroxide concentrations.

Usually, and in the context of putative oxidative stress, living organisms react by increasing the amount of antioxidant molecules, which act as free radical scavengers, blocking free radical–mediated processes and preventing any eventual damage that reactive oxygen species may impose on different tissues. Although SOD1 activity increases in individuals with FAP1, TRAP measurements demonstrate that the system is responding to an oxidative situation, with the consequent consumption of endogenous antioxidants. Similar data about TRAP were reported33 in patients undergoing high-dose chemotherapy and radiochemotherapy; a decrease in TRAP was associated with a decrease in the concentrations of antioxidant molecules.

The source of free radicals in FAP1 remains unknown and deserves further investigation. Recent studies34 carried out in human tissues and cell cultures described the activation of an apoptotic pathway by TTR fibrils, due to the triggering of the receptor of advanced glycation end-product pathway that involves cytokines, iNOS, and other mediators of inflammation and oxidation. According to data reported by other researchers,35 this mechanism can be extended to other pathologic conditions in which amyloid deposits play a major role in their pathogenesis, such as Alzheimer disease.

In summary, these results support the hypothesis that oxidative stress plays a role in the pathogenesis of FAP1. We conclude that increased activity of the enzyme SOD1 reflects the response of the protective defense system against oxygen free radicals, whereas the decreased value of TRAP signals that antioxidants are consumed as a consequence of scavenging prooxidative species. The lack of correlation between clinical damage and SOD1 activity suggests that oxidative stress is present throughout the development of the disease, triggering the onset and maintaining tissue damage.

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