Acute and Sustained Effects of Dihydropyridine-Type Calcium Channel Antagonists on Oxidative Stress in Systemic Sclerosis

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PURPOSE: To evaluate the potential antioxidant properties of dihydropyridine calcium channel antagonists in systemic sclerosis.

METHODS: Forty-two patients with systemic sclerosis were included (mean ± SD age, 54 ± 12 years; mean disease duration, 8 ± 7 years). Plasma markers of oxidative stress (carbonyl residues, advanced oxidation protein products, malondialdehyde, nitrosothiols, and total thiol groups) were determined 72 hours after the discontinuation of usual dihydropyridine treatment (with either nifedipine or nicardipine), shortly after reinitiation of treatment, and 9 to 12 months later (long-term treatment) in 19 of the patients. Baseline values were compared with those in 23 healthy volunteers.

RESULTS: Mean levels of plasma markers of oxidative stress were much higher in patients with systemic sclerosis than in controls (carbonyls, 0.4 ± 0.1 nmol/mg protein vs. 0.3 ± 0.1 nmol/mg protein, P = 0.0001; advanced oxidation protein products, 111 ± 13 μmol/L vs. 47 ± 7 μmol/L, p = 0.003; malondialdehyde, 11.3 ± 3.3 μmol/L vs. 5.5 ± 1.3 μmol/L, P < 0.0001; nitrosothiols, 1.6 ± 0.2 μmol/L vs. 0.6 ± 0.2 μmol/L, P < 0.0001). In contrast, thiol levels were lower in systemic sclerosis patients (264 ± 80 μmol/L vs. 435 ± 50 μmol/L, P < 0.0001). Short-term treatment led to a significant decrease in oxidative stress markers (carbonyls, 0.3 ± 0.1 nmol/mg protein, P < 0.0001), advanced oxidation protein products (60 ± 3 μmol/L, P < 0.0001), malondialdehyde (8.8 ± 5.6 μmol/L, p = 0.0002), and nitrosothiols (1.4 ± 0.2 μmol/L, p = 0.0001), but an increase in thiol levels (340 ± 84 μmol/L, P < 0.0001). These decreases persisted with long-term treatment.


Systemic sclerosis is a connective tissue disease characterized by early generalized microangiopathy, including vasospastic tendencies, which culminates in systemic fibrosis.

Several lines of evidence suggest that generation of free radicals may be important in systemic sclerosis. Free radicals, which are generated by reperfusion injury (Raynaud’s phenomenon) and the inflammatory process (1), damage proteins, lipids, DNA, collagens, and the immune system. The damage can be assessed indirectly; for example, protein carbonyl groups and advanced oxidation protein products reflect protein oxidation. Markers of lipid peroxidation, including plasma malondialdehyde (2), thiobarbituric acid–reactive substances (3), antibodies against oxidized low-density lipoproteins (4), and bioactive F2-isoprostane concentrations, are elevated in patients with systemic sclerosis (5).

These phenomena do not seem to depend on only the ischemia or reperfusion injuries (6). Monocytes from patients with systemic sclerosis can produce large amounts of the superoxide anion (7), and fibroblasts produce reactive oxygen species (8). Moreover, some tissue antigens are susceptible to fragmentation in an oxidative microenvironment (9). An increase in susceptibility to oxidation may be related to an increase in oxidative stress or to a decrease in antioxidants (10).

Two clinical trials of antioxidants had conflicting results in these patients. A combination of micronutrient antioxidants with allopurinol failed to demonstrate benefit (11), whereas probucol reduced lipoprotein oxidation (12). Calcium channel blockers, mostly of the dihydropyridine type, are often used to treat Raynaud’s phenomenon in patients with systemic sclerosis (13) and may have beneficial effects on cardiac involvement (14,15). Some data suggest that their antiatherogenic effects may be related to their antioxidant activity (16,17). We evaluated the potential antioxidant properties of dihydropyridines in patients with systemic sclerosis.

METHODS

Sample
We included consecutive patients with systemic sclerosis who had been hospitalized for systematic follow-up. Patients were classified as having limited or diffuse cutaneous...
ous disease (18). We excluded patients who could not stop vasodilator therapy, as well as those who were pregnant or current smokers, or who had diabetes, hypercholesterolemia, or severe disease (e.g., cardiac or hepatic failure, cancer, or gangrene). We also excluded patients who had not been stable on their current treatment for at least 3 months. Onset of disease was defined as the time when skin involvement occurred.

We measured Westergren erythrocyte sedimentation rate; C-reactive protein levels; serum creatinine concentration; and antinuclear, anticientromere (indirect immunofluorescence on HEp2 cells), and antitopoisoenrase I (counterimmunoelctrophoresis) antibody levels in all patients. Pulmonary involvement was assessed by computed tomographic scan, forced vital capacity, and carbon monoxide diffusing capacity. Systolic pulmonary artery pressure was determined by Doppler echocardiography. Control subjects were healthy nonsmokers from the laboratory staff.

**Study Design**

Patients were asked to stop taking their dihydropyridine medication 3 days before admission (the half-life of nifedipine and nicardipine is between 6 and 11 hours). The baseline evaluation of plasma markers of oxidative stress was performed the morning of admission, after a 1-hour rest. The second evaluation (short-term treatment) was performed during the patient’s stay in hospital after vasodilator treatment had been restarted (25 were treated with nifedipine [20 mg] and 17 were treated with nicardipine [50 mg], based on previous treatment, given orally twice daily). This second evaluation was carried out in the morning, 1 hour after the third dose of medication. Blood samples (10 mL) were collected in tubes containing ethylenediaminetetraacetic acid (EDTA), centrifuged at 3000 g for 10 minutes within an hour of their collection, and immediately stored in aliquots at −80°C until use.

Patients living close to Paris and attending regular appointments in our department were asked to undergo a third evaluation on stable treatment (including dihydropyridine) 9 to 12 months after hospitalization, in the morning after a 1-hour rest (long-term treatment).

As the symptoms of Raynaud’s phenomenon may be influenced by temperature, most of the patients were enrolled in the fall and winter of 2001, and all patients who underwent the third evaluation were investigated in the fall of 2002. The study was approved by the local ethics committee (Cochin Hospital, Paris, France), and all patients gave written informed consent.

**Laboratory Assay Techniques**

Carbonyl groups were measured in plasma samples that had been normalized to a concentration of 1 mg protein/mL (19). We then treated 0.5 mL of plasma with 0.5 mL of 10 mM dinitrophenylhydrazine in 2 M hypochlorous acid or with 0.5 mL of 2 M hypochlorous acid alone for the blank. Samples were then treated with 10% trichloroacetic acid and centrifuged. The pellet was washed in ethanol/ethyelacetate and solubilized in 1 mL of 6 M guanidine in 20 mM potassium phosphate, adjusted to pH 2.3 with trifluoroacetic acid; the resulting solution was incubated for 15 minutes. Carbonyl concentration (nmols/mg protein) was determined by spectrophotometry (370 nm), with $e_{370} = 22 \text{ M}^{-1} \text{cm}^{-1}$.

To measure advanced oxidation protein products levels (20), we dispensed 200 μL of plasma diluted 1:5 in phosphate-buffered saline into a 96-well microtiter plate and added 20 μL of acetic acid to each well. For the standards, we added 10 μL of 1.16 M potassium iodide (Sigma, St. Louis, Missouri) to 200 μL of chloramine-T solution (0 to 100 μmol/L) (Sigma) in a well and then added 20 μL of acetic acid. The absorbance of the reaction mixture was immediately read at 340 nm against a blank consisting of 200 μL of phosphate-buffered saline, 10 μL of 1.16 M potassium iodide, and 20 μL of acetic acid. Advanced oxidation protein product concentrations were expressed as μmol/L of chloramine-T equivalents.

Malondialdehyde was determined by spectrophotometry at 586 nm, using a lipid peroxidation assay kit (Calbiochem-Novabiochem Corporation, San Diego, California) according to the recommendations of the manufacturer. The reaction is based on the principle that the condensation of one molecule of malondialdehyde with two molecules of N-methyl-2-phenylindole at 45°C yields a stable chromophore with maximal absorbance at 586 nm.

S-nitrosothiol levels were determined by fluorimetry (21). Briefly, ammonium sulfamate (50 μL of a 0.1-mM solution) was added to 100 μL of a 1:2 dilution of plasma. Thereafter, 50 μL of reaction mixture (one part of 1.1 mM mercuric chloride to four parts of 0.05 g/L diaminoonaphthalene in 0.62 M hypochlorous acid) was incubated for 10 minutes, and the reaction was stopped by adding 20 μL of 2.8 M sodium hydroxide. Fluorescence intensity was measured in a microtiter plate (Pharmacia Biotech, Saint-Quentin en Yvelines, France) at excitation wavelengths of 360 nm and emission of 450 nm. The sample results were compared with an S-nitrosothiol standard curve (Sigma).

Thiol groups were determined with Ellman’s reagent (22). We mixed 50 μL of plasma with 1.0 mL of 0.1 M Tris, 10 mM EDTA, pH 8.2. We determined the absorbance at 412 nm, and then added 40 μL of 10 mM Ellman’s reagent (Sigma) in methanol to the sample. The absorbance obtained before the addition of Ellman’s reagent was subtracted from that obtained after incubation with Ellman’s reagent. A control containing Ellman’s reagent only was included, and the concentration of thiol groups was calculated using a molar extinction coefficient of 13,600 M$^{-1}$ cm$^{-1}$ at 412 nm.
Statistical Analysis
Data were analyzed with the Mann-Whitney (unpaired data) and Wilcoxon (paired data) tests. Spearman’s rank correlation test was used to assess the relation between quantitative variables. P values less than 0.05 were considered significant.

RESULTS
We included 42 successive systemic sclerosis patients (38 [90%] women; mean [± SD] age, 54 ± 12 years). All patients had Raynaud’s phenomenon (Table 1). The control group of 23 healthy subjects included 20 (87%) women, with a mean age of 49 ± 10 years. Of the 19 patients included in the long-term evaluation, 16 (84%) were women; they had a mean age of 58 ± 12 years and a mean disease duration of 8 ± 8 years, and 12 (63%) had limited cutaneous disease.

Oxidative Stress Markers at Baseline and after Treatment
Levels of plasma markers of oxidative stress were significantly higher at baseline in systemic sclerosis patients (n = 42) than in healthy volunteers (n = 23), whereas thiol levels were lower (Table 2).

Short-term treatment significantly improved all oxidative stress parameters in patients with systemic sclerosis (Table 2). There was a significant correlation between

Table 1. Clinical and Biological Characteristics of 42 Patients with Systemic Sclerosis

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number (%) or Mean ± SD (Range)</th>
</tr>
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<tbody>
<tr>
<td>Disease duration (years)</td>
<td>8 ± 7 (1–32)</td>
</tr>
<tr>
<td>Cutaneous form</td>
<td></td>
</tr>
<tr>
<td>Limited</td>
<td>28 (67)</td>
</tr>
<tr>
<td>Diffuse</td>
<td>14 (33)</td>
</tr>
<tr>
<td>Raynaud’s syndrome</td>
<td>42 (100)</td>
</tr>
<tr>
<td>Current digital vascular ulceration</td>
<td>8 (19)</td>
</tr>
<tr>
<td>Lung fibrosis on computed tomographic scan</td>
<td>22 (52)</td>
</tr>
<tr>
<td>Forced vital capacity &lt;75% predicted value</td>
<td>8 (19)</td>
</tr>
<tr>
<td>Carbon monoxide diffusion capacity &lt;80% predicted value</td>
<td>28 (57)</td>
</tr>
<tr>
<td>Pulmonary hypertension (systolic pulmonary arterial pressure &gt;35 mm Hg)</td>
<td>15 (35)</td>
</tr>
<tr>
<td>Positive antitopoisomerase I antibodies</td>
<td>15 (35)</td>
</tr>
<tr>
<td>Positive anticentromere antibodies</td>
<td>7 (17)</td>
</tr>
<tr>
<td>Creatinine (μmol/L)</td>
<td>76.4 ± 13.5 (56–102)</td>
</tr>
<tr>
<td>Erythrocyte sedimentation rate (mm/h)</td>
<td>17.6 ± 15</td>
</tr>
<tr>
<td>C-reactive protein (mg/L)</td>
<td>8.6 ± 7.1</td>
</tr>
<tr>
<td>Prednisone dose</td>
<td>14 ± 7</td>
</tr>
<tr>
<td>Angiotensin-converting enzyme inhibitor use</td>
<td>9 (21)</td>
</tr>
<tr>
<td>D-penicillamine use</td>
<td>8 (19)</td>
</tr>
<tr>
<td>Omeprazole use</td>
<td>42 (100)</td>
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</tbody>
</table>

Table 2. Plasma Concentrations of Oxidative Stress Markers in Patients with Systemic Sclerosis and Controls

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Controls (n = 23)</th>
<th>Patients with Systemic Sclerosis (n = 42)</th>
<th>P Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Short-term Treatment</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td></td>
</tr>
<tr>
<td>Carbonyls (nmol/mg protein)</td>
<td>0.3 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.0001</td>
</tr>
<tr>
<td>Advanced oxidation protein products</td>
<td>47 ± 7</td>
<td>111 ± 13</td>
<td>0.003</td>
</tr>
<tr>
<td>(μmol/L of chloramine-T equivalents)</td>
<td></td>
<td>60 ± 3</td>
<td></td>
</tr>
<tr>
<td>Malondialdehyde (μmol/L)</td>
<td>5.5 ± 1.3</td>
<td>11.3 ± 3.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Nitrosothiols (μmol/L)</td>
<td>0.6 ± 0.2</td>
<td>1.6 ± 0.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Thiols (μmol/L)</td>
<td>435 ± 50</td>
<td>264 ± 80</td>
<td>&lt;0.0001</td>
</tr>
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</table>
Evidence of a sustained effect was provided by comparison of the results at short and long term, which demonstrated a statistical difference only for advanced oxidation protein products (60 ± 43 μmol/L vs. 85 ± 54 μmol/L, P = 0.03).

**DISCUSSION**

Our data demonstrate excessive oxidative stress in patients with systemic sclerosis after cessation of dihydropyridine treatment (at baseline). In addition, we found a
substantial acute and sustained decrease in oxidative stress after treatment with a dihydropyridine.

We chose different markers to explore different aspects of oxidative stress. Carbonyl residues are well-accepted markers of protein oxidation, whereas advanced oxidation protein products have been validated more recently. In our study, their concentrations were not correlated with carbonyl concentrations; this was expected because these two markers react with different oxidative agents (23). Advanced oxidation protein products have been associated with monocyte activation in renal failure, which may be important in systemic sclerosis, a disease in which mononuclear cells are implicated (24,25). Although statistically significant, correlations between oxidative stress parameters and inflammation were weak and require confirmation; however, this observation is consistent with data suggesting that free radicals are not strictly dependent on Raynaud’s phenomenon in systemic sclerosis (6).

We investigated lipid peroxidation by determining malondialdehyde concentrations. These levels decreased more with short-term treatment in patients with pulmonary hypertension, consistent with previous studies that have suggested an association between pulmonary hypertension and lipid peroxidation (26).

Nitrosothiols are bioactive agents that may generate nitric oxide, which is thought to form peroxynitrite in systemic sclerosis; a decrease in nitrosothiol concentration is therefore beneficial. Total thiol groups protect against oxidative stress. Previous studies have reported low plasma thiol concentrations in patients with systemic sclerosis (27).

The hypothesis that rest during hospitalization might contribute to changes in plasma markers of oxidative stress after short-term treatment cannot be excluded. However, the sustained decreases after long-term treatment—albeit in a limited sample of only 19 patients—do not support this hypothesis.

In a previous study, probucol (an antioxidant) was compared with nifedipine for 12 weeks (12). Probucol improved the clinical parameters of Raynaud’s phenomenon and lag times for low-density lipoprotein oxidation, effects that were not observed with nifedipine. However, the dose of nifedipine (10 mg twice daily) was lower than that used in our study, and the investigators used a different method to measure lipid peroxidation.

In hypertensive patients, nifedipine (30 to 60 mg/d) decreases circulating plasma lipoperoxide and isoprostane levels and increases antioxidant capacity (28,29). The molecular mechanism is thought to be an electron or hydrogen transfer reaction (17). However, the antioxidant effect of dihydropyridine in systemic sclerosis may also be a secondary effect due to improvement of the vasospastic disease. Whatever the mechanism, our results are consistent with dihydropyridine having beneficial effects on oxidative stress in systemic sclerosis.

The results of this study confirm that systemic sclerosis is associated with excessive oxidative stress, as reflected by plasma markers. We also observed an acute and sustained decrease in oxidative stress in response to treatment with
dihydropyridine. These findings suggest that a trial of dihydropyridines, with careful assessment of biological and clinical outcomes, may be of value in patients with systemic sclerosis.

REFERENCES

17. Mak IT, Zhang J, Weglicki WB. Protective effects of dihydropyridine Ca-blockers against endothelial cell oxidative injury due to combined nitric oxide and superoxide. Pharmacol Res. 2002;45:27–33.