Low plasma protein nitrotyrosine levels distinguish primary Raynaud’s from scleroderma

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ABSTRACT

Increased formation of reactive nitrogen species is thought to contribute to the vascular pathology that develops in patients with connective tissue disease such as scleroderma. To investigate this hypothesis, we measured the level of protein-bound nitrotyrosine in plasma in patients with primary Raynaud's, scleroderma, chronic renal impairment, and in healthy controls.

There was a marked decrease in plasma protein-bound nitrotyrosine in patients with primary Raynaud's phenomenon (0.60 ± 0.06 ng/mg dry protein) compared to patients with scleroderma (1.78 ± 0.21 ng/mg protein), chronic renal impairment (1.42 ± 0.17 ng/mg) or healthy controls (1.63 ± 0.15 ng/mg protein, ANOVA p < 0.001). These data suggest that there is decreased nitration of plasma proteins, or increased degradation of nitrated proteins from the circulation of patients with primary but not secondary Raynaud's phenomenon.

INTRODUCTION

Raynaud's phenomenon is commonly associated with connective tissue disorders, notably scleroderma (systemic sclerosis) but also systemic lupus erythematosus, polymyositis and others. It also occurs frequently in otherwise healthy individuals (primary Raynaud's phenomenon) when the symptoms are generally milder and more responsive to vasodilator therapy. Although almost all scleroderma patients manifest secondary Raynaud's phenomenon, only a minority of Raynaud's sufferers develop systemic features of scleroderma, suggesting that the pathogenic mechanisms and structural vascular consequences of primary or secondary Raynaud's are distinct.

Patients with Raynaud's phenomenon have abnormal digital vasoconstriction, which may be secondary to impaired synthesis of nitric oxide, and recent studies have suggested that topical application of an NO donor may improve the microcirculation of Raynaud's (1). Scleroderma is a heterogeneous multi-system disorder that involves inflammatory, vascular and fibrotic pathology. Cutaneous fibrosis and Raynaud's phenomenon are almost always present. Recent studies have shown increased lipid peroxidation products in both diffuse and limited cutaneous subsets of scleroderma (2), and it is now recognised that reactive nitrogen species, including peroxynitrite, contribute to oxidative and nitrative stress (nitroxidation) in a variety of clinical disorders. The formation of reactive nitrogen species such as peroxynitrite can be inferred by quantification of the levels of nitrated tyrosine residues (nitrotyrosine) in proteins.

The aim of this study was to measure the nitrotyrosine content of circulating plasma proteins in patients with Primary or secondary Raynaud's phenomenon and to compare it with a group of patients with impaired renal function, and healthy controls.
Patients and Methods

Samples were obtained from randomly selected patients with primary Raynaud’s phenomenon, scleroderma, chronic renal impairment, and healthy controls, after informed consent. The diagnosis of primary Raynaud’s phenomenon was based on a detailed history and/or observation of bilateral, cold-induced blanching and/or cyanosis of the fingers, or fingers and toe without having any clinical features of connective tissue disease, antinuclear antibodies or antibodies to extractable nuclear antigens or abnormal digital nail-fold capillaroscopy (3). All scleroderma patients satisfied the American College of Rheumatology preliminary classification criteria and were categorised into two subgroups of diffuse or limited cutaneous scleroderma (4). Since many patients with scleroderma have impaired renal function, patients with stable impaired renal function were selected from outpatients to serve as an additional control group. GFR was calculated using equation developed in the Modification of Diet in Renal Disease (MDRD) study (5).

Protein-bound tyrosine (TYR) and nitrotyrosine (NT) were measured by stable isotope dilution gas chromatography negative ion chemical ionisation mass spectrometry as previously described (6). This method avoids the introduction of artefactual nitration of proteins during work-up that is inherent in some methods. Results were compared using ANOVA.

Results

Nitrotyrosine levels are expressed as either the absolute amount per mg of dry protein, or as a ratio to tyrosine. Expression as a ratio reflects the percentage of tyrosine residues in protein that are nitrated. Patient characteristics and the plasma protein nitrotyrosine/tyrosine ratios for the patients and control groups are shown in Table 1. Plasma protein nitrotyrosine levels for each of the groups are shown in Figure 1.

Plasma protein nitrotyrosine levels were significantly decreased in patients with primary Raynaud’s phenomenon compared with patients with scleroderma, chronic renal impairment or healthy volunteers (mean ± SEM: 0.60 ± 0.06 ng/mg, vs 1.78 ± 0.21, 1.42 ± 0.17 and 1.63 ± 0.15 respectively) (Figure 1, ANOVA p<0.001). The plasma protein nitrotyrosine/tyrosine ratio was also significantly reduced in patients with primary Raynaud’s phenomenon compared with all non-Raynaud’s subjects (table 1, ANOVA p<0.001). The levels of protein-bound tyrosine were similar in each of the 5 groups (data not shown). There were no statistically significant differences between plasma protein nitrotyrosine or plasma protein nitrotyrosine/tyrosine ratios between patients with scleroderma, chronic renal impairment or controls. Intra group comparisons of plasma protein nitrotyrosine or plasma protein nitrotyrosine/tyrosine ratios between subsets of the scleroderma group did not identify significant differences (table 1). There was no correlation between plasma protein nitrotyrosine or plasma protein...
nitrotyrosine/tyrosine ratio and the duration of scleroderma, maximum skin score, calculated glomerular filtration rate or history of smoking.

**Table 1.** Patient characteristics, mean glomerular filtration rate (GFR) and the plasma protein nitrotyrosine/tyrosine ratio.

<table>
<thead>
<tr>
<th></th>
<th>No.</th>
<th>Age Mean (Range)</th>
<th>Sex M:F</th>
<th>Smokers</th>
<th>Mean GFR (ml/min) ± SEM</th>
<th>Mean protein nitrotyrosine/tyrosine ratio (pg/µg) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Healthy Controls</strong></td>
<td>23</td>
<td>31 (21-50)</td>
<td>10:13</td>
<td>5</td>
<td>90 ± 3</td>
<td>24.8 ± 2.2</td>
</tr>
<tr>
<td><strong>Chronic renal impairment</strong></td>
<td>13</td>
<td>44 (22-88)</td>
<td>6:7</td>
<td>4</td>
<td>66 ± 8</td>
<td>21.9 ± 2.4</td>
</tr>
<tr>
<td><strong>Diffuse cutaneous Scleroderma</strong></td>
<td>12</td>
<td>56 (31-77)</td>
<td>1:11</td>
<td>0</td>
<td>70 ± 6</td>
<td>31.7 ± 7.2</td>
</tr>
<tr>
<td><strong>Limited cutaneous Scleroderma</strong></td>
<td>25</td>
<td>53 (33-74)</td>
<td>5:20</td>
<td>5</td>
<td>78 ± 6</td>
<td>29.1 ± 5.9</td>
</tr>
<tr>
<td><strong>Primary Raynaud’s</strong></td>
<td>11</td>
<td>54 (38-70)</td>
<td>2:9</td>
<td>1</td>
<td>86 ± 5</td>
<td>10.6 ± 1.0</td>
</tr>
</tbody>
</table>

**CONCLUSIONS**

This study demonstrates a striking reduction in plasma protein nitrotyrosine in patients with primary Raynaud’s phenomenon when compared with scleroderma patients, and control groups. This finding was unexpected, particularly since virtually all patients with scleroderma also have Raynaud’s phenomenon. Others studies have identified nitrotyrosine by immunohistochemistry in scleroderma skin (7). The mean plasma protein nitrotyrosine in our sample of scleroderma patients, who tend to have more severe and more frequent episodes of Raynaud’s, was not significantly different from the control groups. However, the highest plasma nitrotyrosine values seen in this study were identified in the
scleroderma group, suggesting that some patients have increased formation of reactive nitrogen species.

A number of mechanisms might account for our observations. Firstly, there may be decreased formation of nitric oxide and reactive nitrogen species in patients with primary Raynaud's. However, previous studies have suggested that plasma nitrite/nitrate concentrations are normal in patients with primary Raynaud's (8). Secondly, since protein nitration is a dynamic process and nitrated proteins are degraded more rapidly than normal proteins (9,10), there may be up-regulated degradation or de-nitration of nitrated proteins in patients with primary Raynaud's phenomenon. It is possible that reduced levels of nitrotyrosine are a significant factor in determining the vascular consequences of Raynaud's phenomenon and that the low levels observed in primary Raynaud's are protective. If so, then strategies that aim to reduce plasma nitrotyrosine levels in cases of severe secondary Raynaud's may abrogate the effects of longstanding Raynaud's in the context of connective tissue disease.

These data suggest that decreased formation of reactive nitrogen species or increased degradation of nitrated proteins may be important in the pathogenesis of primary Raynaud's, and further suggest that there is a distinct difference between this group of patients and patients with scleroderma who also exhibit Raynaud's phenomenon. Further studies are needed to elucidate some of the mechanisms involved.

References


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Figure 1. Protein bound nitrotyrosine is decreased in patients with primary Raynaud’s phenomenon.
Plasma protein-bound nitrotyrosine (ng/mg) was measured in healthy controls (n=23), chronic renal impairment (CRI, n=13), scleroderma (SSc, n=37), and primary Raynaud’s patients (n=11).
Low plasma protein nitrotyrosine levels are a biochemical hallmark of primary Raynaud's phenomenon

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