

Assessment of Plasma S-Nitrosothiol Concentration by Electron Paramagnetic Resonance Spectrometry and Plasma Nitrotyrosine Levels by ELISA in Behçet's Disease

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Introduction

Behçet's Disease (BD) is a chronic inflammatory, multisystemic disease characterized by oral, genital ulcerations, uveitis, vasculitis, and arthritis. BD is endemically higher in the Republic of Turkey, the Middle East, and Japan compared with other countries (1, 2, 3).

Although the exact underlying mechanism of BD is still unknown, recent findings have focused on the role of reactive oxygen species (ROS) produced by activated neutrophils and other inflammatory cells during inflammation (4,5). Recently, it has been also shown that nitrite and nitrate concentrations, used as indirect markers of nitric oxide (NO) production are increased in BD patients and associated with disease activity (6,7,8). NO produced by endothelial cells may be an important mediator in the development of BD.

However, human blood nitrite/nitrate measurements may be confounded by dietary intake of nitrate and nitrite (9). Some studies have also shown that S-nitrosothiols, which are bioactive forms of NO, are increased in certain inflammatory diseases such as RA (rheumatoid arthritis), and are associated with the disease activity (11). S-nitrosothiols are derived from NO by S-nitrosation of thiol-containing proteins. S-nitrosothiol formation may prevent the formation of peroxynitrite that results from the reaction between NO and superoxide anion (O₂⁻) through sequestration of available NO (10,11). Although S-nitrosothiols have been measured by various techniques, electron paramagnetic resonance spectrometry, in conjunction with spin trapping, is considered to be a more sensitive and specific method (11).

Recently, nitrotyrosine (3-NT) has been identified as another marker of NO production. Nitrotyrosine is a relatively stable product and forms from the reaction of peroxynitrite with free and protein bound tyrosine. It has been found to be elevated in various inflammatory processes such as atherosclerosis, celiac disease, rheumatoid arthritis, chronic renal failure and septic shock (12)

Aim of study

The aim of the project was to investigate S-nitrosothiols and 3-NT in plasma from patients with BD, using a sensitive and specific electron paramagnetic resonance spectrometry method for S-nitrosothiols and an ELISA assay for 3-nitrotyrosine(3-NT). It was anticipated that the results of the study would help to identify if S-nitrosothiols or peroxynitrite are involved in BD pathogenesis and if they can be an indicator for disease activity.

Methods

We selected 75 patients (men and women) among those admitted to the Behçet's Clinic at Ege University Hospital in Izmir, Turkey, during the period from May 2006 - September 2006. As a control group, 25 healthy persons (men and women), matched for age and sex, volunteered to participate in this study.

All plasma samples were kept at -80°C until they were shipped to the Institute of Biomedical & Clinical Science of the Peninsula Medical School, Exeter, UK. S-nitrosothiols levels were measured using a previously described EPR method in 15 plasma samples (10). In this technique the RSNOs were degraded using an alkaline pH (pH 10.5), and the NO^{\bullet} released was measured in the presence of the spin trap complex $(\text{MGD})_2\text{-Fe}^{2+}$, in which MGD is *N*-methyl-D-glucamine dithiocarbamate. In order to further improve the detection limit for S-nitrosothiols, the EPR method was modified using different techniques. The modifications of this method included the following:

1. The samples were ultrafiltered using Whatman (30 kDa) devices.
2. The samples were incubated with the spin trap at both room temperature and at 50°C at different time intervals, such as 1 min, 2 min, and 5 min.
3. Since the low molecular weight S-nitrosothiols produced by proteolytic digestion are less stable than large protein S-nitrosothiols, the samples were subjected to enzymatic proteolysis using proteinase K and pronase.

Each procedure was tried several times.

We measured 3-NT levels (in triplicate) in 53 plasma samples using a commercial kit Cambridge BioScience Ltd, Cambridge, England). The principle of this 3-NT ELISA test kit is based on the sandwich method. All samples were diluted at least 1:10 prior to conducting the assay.

Results

S-nitrosothiols could not be detected in any of the BD plasma samples (n=15), despite the detection of S-nitrosothiols in RA synovial fluid and plasma samples (9). The limit of detection of the assay is about 50 nM (10).

We were also unable to detect 3-NT in BD samples (Table 1). All results were below the limit of detection (2.1 nM) of the assay. In contrast, 3-NT was detected in some, but not all, samples of synovial fluid and plasma from RA patients (data not shown). 3-NT was not detected in normal plasma, BD patients in remission or in BD patients with active disease.

Table 1. Characteristics of patients and healthy subjects used in 3-NT analysis (n=53).

<i>Sample groups</i>	<i>n</i>	<i>Age (years) (mean ± SD)</i>	<i>Sex (M/F)</i>
Healthy Subjects	17	33.2 ± 8.7	11/6
BD-R	17	39.5 ± 6.7	11/6
BD-A	19	33.2 ± 8.7	12/7

BD: Behcet's disease, R: Remission period, A: Active period.

Discussion

Autoimmune diseases such as rheumatoid arthritis (RA) and Behcet's disease (BD) feature chronic inflammation, in which excessive levels of reactive oxygen (ROS) and nitrogen (RNS) species are produced by activated inflammatory cells. These may cause tissue damage and contribute to the development or progression of the disease, and may also modify cellular proteins. Such oxidative/nitrosative modifications could potentially be utilised as biomarkers of the stage or severity of the disease. The aim of the present study was to ascertain if such correlations could be reliably observed in patient samples. For this reason, we wanted to measure NO derived oxidative stress markers, S-nitrosothiols and 3-NT.

We employed different techniques to concentrate the S-nitrosothiols (which are mostly present within the albumin fraction of plasma), such as ultrafiltration (Whatman devices). We also incubated samples with the spin trap at different temperatures (room temperature, 50 °C) and different times (1 min, 2 min, 5 min) and tried enzymatic proteolysis using proteinase K and pronase. Even though the EPR method was modified to increase either the S-nitrosothiol levels in the samples, or to improve the detection limit of the assay using different techniques (ultrafiltration, enzymatic proteolysis etc) S-nitrosothiols could not be detected in plasma

samples of patients with Behçet's disease, indicating that S-nitrosothiol concentrations were low in Behçet's disease. We concluded that the detection limit of EPR for S-nitrosothiols was insufficient to measure S-nitrosothiols in plasma from Behçet's disease patients.

Having established that S-nitrosothiols were undetectable in BD plasma, we carried out measurements of 3-NT, which is another inflammation and nitric oxide production marker, using a commercial kit. We measured 3-NT levels (in triplicate) in 53 BD plasma samples. Again, we were unable to detect 3-NT in BD samples, suggesting that, whilst there is significant evidence that this disease is associated with oxidative stress, the role of nitrosative stress is much less clear. Interestingly, we were able to detect 3-NT in some knee-joint synovial fluid samples from patients with rheumatoid arthritis, confirming that 3-NT was detectable in inflammatory extracellular fluids from patients with a disease known to involve nitrosative stress.

Conclusion

We conclude that the levels of S-nitrosothiols and 3-NT are low in BD plasma compared to RA synovial fluid and plasma.

Implications for Further Research

The previous literature suggested that BD may be associated with increased NO production. We have been unable to obtain supporting data for this idea, despite our use of sophisticated and selective techniques for the detection of NO metabolites. Further studies, using more sensitive methods for the detection of NO metabolites, may yet be able to detect a difference in NO production between BD patients and healthy subjects.

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