Interactions of nitric oxide and superoxide dismutase in Behcet's disease

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Abstract

Objective

Nitric oxide (NO) is produced in increased amounts in inflammatory conditions and may cause tissue injury by reacting with superoxide to yield peroxynitrite, a powerful toxin. Superoxide dismutase (SOD) scavenges superoxide and inhibits the formation of peroxynitrite, thereby suppressing the resulting injury and regulating the bioavailability of NO. We conducted a study to assess serum NO and SOD in patients with Behçet's disease (BD) and correlate their levels with disease activity.

Methods

Serum NO concentrations and SOD activities were determined in 25 BD patients (mean age: 36 years; male/female: 13/12) and in 15 healthy controls. BD patients were allocated into two groups according to disease activity (active/inactive: 11/14).

Results

In patients with active disease, NO levels were found to be significantly elevated, while SOD activities were comparable to the control group. Conversely, patients with inactive disease exhibited markedly high SOD activities and normal NO levels. Moreover, there was a positive correlation between SOD activity and NO levels in patients with inactive BD ($r = 0.562$, $p < 0.05$).

Conclusion

We propose that NO-associated injury of tissues, particularly the endothelium, may be important in the etiopathogenesis of vasculitis in BD, and SOD may play a protective role against inflammation.

Key words

Behçet's disease, nitric oxide, superoxide dismutase.

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Introduction

Behçet's disease (BD) was first described by Hulusi Behçet, a Turkish dermatologist, in 1937 as a triad of relapsing iridocyclitis with oral and genital ulcers (1). It is now recognized as a polysymptomatic systemic vasculitis which may present with skin lesions, arthritis, central nervous system manifestations and vascular involvement in addition to the original triad (2).

The etiology and pathogenesis of BD have not yet been clarified. Histopathologically, lesions of BD are typically seen in small blood vessels,and there is marked exudative inflammation and leukocyte infiltration in the surrounding tissue (3). Therefore, extensive research efforts have been focused on inflammatory reactions and the role of neutrophil functions in the etiopathogenesis of BD. Pathologically enhanced neutrophil functions including chemotaxis, phagocytosis, active oxygen radical production and lysosomal secretions have been demonstrated in BD patients (4). Excessive generation of the active oxygen species by activated neutrophils has been suggested to lead to tissue injury (4).

NO is a highly reactive molecule that possesses pro- and anti-inflammatory effects depending on the concentration and the origin of release (5). Neutrophils and macrophages generate NO via an NO synthase that is inducible by endotoxin and various cytokines. Accordingly, NO levels increase in inflammatory conditions (5). NO is potentially cytotoxic to the host, because in the presence of large amounts of superoxide and NO, peroxynitrite, a powerful toxic oxidant, is spontaneously formed (6). Superoxide dismutase (SOD) scavenges superoxide and inhibits the formation of peroxynitrite, thereby suppressing the resulting injury and regulating the bioavailability of NO (6). Therefore, it acts as a protective mechanism against tissue injury in inflammatory reactions.

NO has been proposed to be involved in the pathogenesis of autoimmune disorders. Excessive NO production has been demonstrated in patients with rheumatoid arthritis, systemic lupus erythematosus, Kawasaki disease, and tissue injury caused by deposition of immune complexes (7-10). In an experimental model of vasculitis, cytokine-mediated neutrophil-dependent endothelial injury was shown to be associated with NO (11). As another pathologic condition with inflammation and endothelial dysfunction (12), BD might comprise alterations in NO production.

In view of the finding that SOD plays a protective role against tissue injury, SOD preparations have been administered to BD patients and have resulted in remission of active disease (13). However, studies on the role of SOD in the pathogenesis of BD are very scarce and have produced conflicting results (14-17). We conducted a study to assess serum NO and SOD in patients with Behçet's disease (BD) and correlate their levels with disease activity.

Materials and methods

Study group

Twenty five patients with BD and 15 healthy controls were included in this study. All patients fulfilled the criteria of the International Study Group for BD (18). The demographic characteristics of the patients and controls are given in Table I. None of the patients or the controls was receiving any medication which might affect neutrophil functions. All subjects were given a standard diet poor in exogenous sources of NO (green vegetables, preserved food, etc.) (19) for at least 48 hours before blood sampling.

Disease manifestations of the patients are summarized in Table II. Patients with one or more of these features at

Table I. Demographic characteristics of patients and controls.

	Number of subjects	Sex (M/ F)	Age (years)	Age of disease onset (years)
Behçet's disease	25	13/12	36 ± 11	23 ± 9
Healthy controls	15	7/8	33 ± 9	

Table II. Clinical features depending on the history and at the time of blood sampling of the patient group (n = 25). Serum NO concentrations and SOD activities are also given. Note that the first 14 patients were clinically inactive and the remaining 11 were active at the time of blood sampling.

No	Age	Sex	OU	Art I	GU	Pathergy*	Skin I	EN	Ocu I	Vasc I	NI	Epidymitis	SOD	NO
1	42	M	+/-	+/-	-/-	+	+	+/-	-/-	-/-	-/-	-/-	22.2	25
2	18	M	+/-	-/-	+/-	-	-	-/-	+/-	-/-	-/-	-/-	6.5	19
3	35	F	+/-	-/-	+/-	+	+	-/-	-/-	-/-	-/-	-/-	18.6	33
4	29	M	+/-	+/-	-/-	-	+	+/-	+/-	-/-	-/-	-/-	3.5	48
5	51	M	+/-	-/-	-/-	+	+	+/-	-/-	-/-	-/-	-/-	24.5	34
6	38	F	+/-	+/-	+/-	-	+	-/-	-/-	-/-	-/-	-/-	14	60
7	29	F	+/-	+/-	+/-	-	+	-/-	-/-	-/-	-/-	-/-	3.5	12
8	17	F	+/-	+/-	-/-	+	-	-/-	+/-	+/-	-/-	-/-	6.9	22
9	20	F	+/-	-/-	-/-	+	+	-/-	-/-	-/-	-/-	-/-	9.8	30
10	39	M	+/-	-/-	-/-	+	+	+/-	-/-	-/-	-/-	-/-	10.6	19
11	42	F	+/-	+/-	+/-	-	+	-/-	-/-	-/-	-/-	-/-	31.5	21
12	41	M	+/-	-/-	-/-	-	+	+/-	+/-	-/-	-/-	-/-	42.1	30
13	32	M	+/-	+/-	+/-	-	+	+/-	-/-	-/-	-/-	-/-	20	37
14	22	F	+/-	+/-	+/-	+	-	-/-	-/-	-/-	-/-	-/-	6.6	22
15	19	F	+/+	+/+	+/+	+	-	-/-	-/-	-/-	-/-	-/-	0.2	95
16	47	M	+/+	-/-	-/-	-	+	+/-	+/-	-/-	+/-	-/-	0.6	57
17	30	F	+/+	-/-	-/-	+	+	+/+	-/-	-/-	-/-	-/-	0	45
18	21	F	+/+	+/-	-/-	-	+	-/-	+/-	-/-	-/-	-/-	0.5	38
19	26	M	+/-	-/-	+/-	-	+	-/+	-/-	-/-	-/-	-/-	1.1	68
20	27	F	+/+	+/-	+/-	-	-	-/-	+/+	-/-	-/-	-/-	0.2	67
21	36	M	+/+	-/-	-/-	+	+	-/-	-/-	+/+	-/-	+/-	0.2	65
22	41	M	+/+	+/-	+/-	-	+	-/-	-/-	-/-	-/-	-/-	0.4	90
23	25	M	+/+	+/-	-/-	+	+	+/-	-/-	+/+	-/-	-/-	0.5	81
24	20	F	+/+	-/-	-/-	-	+	-/-	+/-	-/-	-/-	-/-	0.7	40
25	19	M	+/+	-/-	+/-	-	+	-/-	-/-	-/-	-/-	-/-	0.4	38

OU:oral ulcer; Art I: articular involvement; GU: genital ulcer; Skin I:skin involvement:EN:erythema nodosum; Ocu I: ocular involvement; Vasc I: vascular involvement; NI: neurological involvement; SOD: superoxide dismutase; NO: nitric oxide; M: male; F: female; +: present; -: absent.

the time of blood sampling were considered to have active disease. Accordingly, the study population was allocated as Group I (patients with inactive BD), Group II (patients with active BD), and Group III (healthy controls).

Measurement of superoxide dismutase activity and levels of NO

SOD activity determinations were performed on plasma supernatants after centrifugation of the chloroform-/ethanol treated plasma sample. Plasma was assayed by a recently defined spectrophotometric method (20) via using Bioxytech SOD-525 commercial research kit (Oxis Int., Portland®, USA). Briefly, this assay is based on the SOD-mediated increase in the rate of autooxidation of 5,6,6a,11b-tetrahydroxybenzo(c)fluorene in aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm. SOD activity is determined from the ratio of autooxidation rates measured in the presence (Vs) and in the absence (Vc) of SOD. The Vs/Vc ratio as a function of SOD concentration is independent of the isozyme type of SOD being measured. One SOD activity is defined as the activity that doubles the autooxidation background (Vc/Vs=2).

NO synthesis was determined via nitrate on microtiter plates by Nitric Oxide Colorimetric Assay® (Boehringer, Mannheim). NO is detected in biological fluids via nitrite. The nitrate present in the sample which is originated from NO itself and from peroxynitrite, is reduced to nitrite by reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of the enzyme nitrate reductase. The nitrite formed reacts with sulfanilamide and N-(1-naphthyl)-ethyl-enediamine dihydrochloride to give a red-violet diazo dye. The diazo dye is measured on the basis of its absorbance in the visible range at 550 nm. According to this principle, solutions were prepared and 300 ml serum and 300 l of potassium phosphate buffer were placed in an ultrafilter (Centrisart cut-off 10000, from Sartorius) and centrifuged for 45 min at 2000 x g (4000 rpm, r: 7 cm). Ultrafiltrate was collected and used in the test. Separate membrane filters were used for each sample in order to avoid nitrate. The results were calculated by the calibration curves derived from standard solutions. The change in absorbance obtained for the potassium nitrate standard solutions were piloted on the Y-axis against the corresponding nitrate concentrations (mM) on the X-axis.

Statistical analysis

Results of the 3 groups were analyzed by Kruskal-Wallis analysis. Post-hoc additional comparisons between individual groups were performed by Mann-Whitney U test. Significant p results were assigned to values lower than 0.05. The results were expressed as median (min-max). The correlation between NO levels and SOD activity was evaluated by Spearman correlation test.

Table III. NO levels and SOD activity in the study groups. Values are expressed as median (min-max).

	Inactive BD (Group I, n = 14)	Active BD (Group II, n = 11)	Control (Group III,n = 15)
NO (mol/ ml)	27.5 (12-60)	65 (38-95)*	32 (19-66)
SOD (U/ ml)	16.3 (3.5-42) †	0.4 (0.0-1.1)	0.2 (0.0-1.1)

$p < 0.05$, * Group II vs. Group I and Group III, † Group I vs. Group II and Group III.

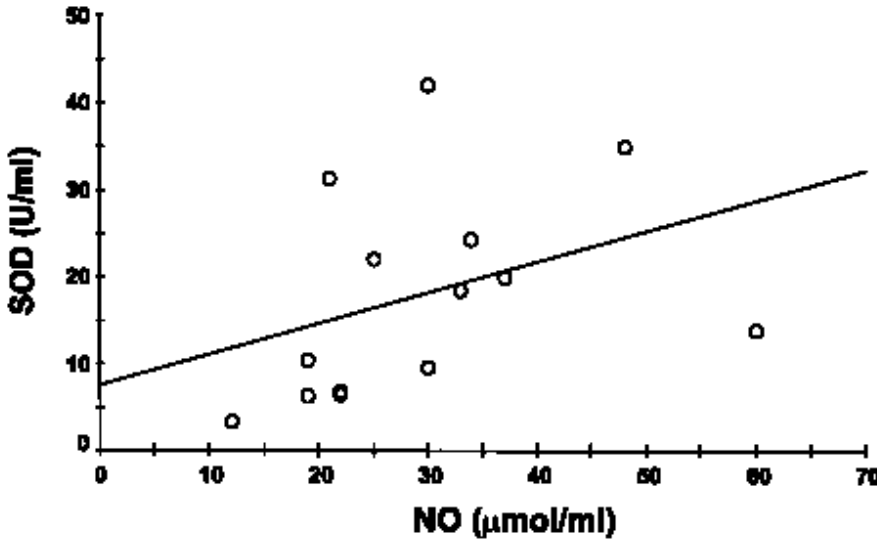

Fig. 1. Scatterplot graphic demonstrating the positive correlation between serum NO levels and SOD activity in patients with inactive Behçet's disease ($r = 0.562$, $p < 0.05$).

Results

In active BD patients, plasma SOD activities (0.4 U/ml) were comparable to the control group (0.2 U/ml), whereas NO levels were significantly elevated (65 mmol/ml vs 32 mmol/ml, $p < 0.05$). In patients with inactive disease, plasma SOD activities were elevated (16.3 U/ml vs 0.2 U/ml, $p < 0.05$) while NO levels were not different from those of the controls (27.5 mol/ml vs 32 mol/ml, $p < 0.05$) (Table III).

Comparison of Group I and II revealed that plasma SOD activities were significantly higher and NO levels lower in the inactive group (Table III).

Correlation analysis revealed a significant positive correlation between plasma SOD activities and NO levels in patients with inactive disease (Fig. 1). There was no such correlation in BD patients with active disease or in the control group.

Discussion

In this study, plasma NO and SOD concentrations were investigated in the active and inactive clinical phases of BD in comparison to healthy controls. Active disease was characterized by elevated NO and normal SOD, while inactive patients harbored elevated SOD and normal NO, as compared to controls.

NO, a multifunctional intracellular messenger molecule, modulates endothelial functions, blood flow, homeostasis, thrombosis, immune reactions, and inflammation via its wide spectrum of biological activities (21). In inflammatory reactions, many pro-inflammatory cytokines lead to expression of inducible NO synthase in monocytes/macrophages, neutrophils, and in many other cells (5). Under physiological conditions, NO does not cause tissue damage as it is rapidly cleared by reaction with oxyhemoglobin (6). However, in pathological conditions, expression of the inducible NO synthase leads to increased generation of NO. Excessive amounts of NO can damage endothelium either as a direct toxicity of the molecule itself, or more likely via peroxynitrite which is formed by its reaction with a superoxide radical (6,21). Excessive NO production has been demonstrated in several autoimmune disorders and vasculitic processes (7-10). NO was also studied in BD, and its serum levels were found to be decreased in the active phase of the disease (22). Authors argued their findings as the assays of NO measure both nitrite and nitrate, not the NO itself, and the decreased levels of NO in BD might be due to its interaction with superoxide anion yielding peroxynitrite molecule and concluded that measurement of peroxynitrite level would be the best way to evaluate the production of NO. However, it has been clearly demonstrated that peroxynitrite is an unstable molecule and measurement of *in vivo* is difficult as it promptly isomerases to nitrate anions or leads to nitration of tyrosine residues on proteins (23, 24). Therefore, from the technical point of view, the nitrite and nitrate measured originate not only from NO but also from peroxynitrite as well (24). In our study, NO levels were found to be significantly elevated only during the active phase of BD. As enhancement of cytokine production in active BD leading to induction of NO synthase has been recently reported (25), increased plasma NO levels is most probably related to the inflammation due to active disease. On the other hand, NO synthase activity has been reported to be increased in experimental models of endothelial injury (26). In Behçet's disease, endothelial injury itself might also alter NO production in addition to the inflammatory reaction.

Increase in superoxide concentrations was previously demonstrated in BD (27). Yoshida and coworkers (28) demonstrated that sera of BD patients have a priming effect on normal neutrophils to induce significantly increased amounts of superoxide. SOD levels were previously studied in BD, but produced discrepant results (14-17). Wang *et al.* (14) found an increased serum SOD activity in the active phase of BD, while other authors reported decreased activity (15-17). The levels of NO in BD patients were not determined in these studies, so to our knowledge this study is the first in which SOD and NO levels are concomitantly evaluated. Plasma SOD levels were markedly increased during the inactive period while NO levels were elevated during active period in BD patients. Moreover, we found a positive correlation between serum SOD activity and NO levels in the inactive phase.

Inflammatory reactions include very complex interactions and levels of each participating molecule are determined by many factors. Pronai *et al*. (15) demonstrated that when generation of free oxygen radicals were stimulated, SOD activity of neutrophils decreased by 50%. Peroxynitrite was demonstrated to have direct stimulatory effects on

MnSOD gene expression (29). Since the levels of superoxide and NO are both elevated in BD, the levels of peroxynitrite, and therefore SOD activity could also increase in BD patients.

These findings suggest that SOD might act as a compensatory mechanism against proinflammatory trigger(s) of BD in the inactive period. However, in patients with active disease, plasma SOD activities were not different from those of normal controls. Two mechanisms may be envisaged for the active period: either SOD levels were normalized due to further increase in superoxide levels due to disease activity, or vice versa, decreased production of SOD causes decompensation of protective mechanisms leading to development of endothelial damage and vasculitis.

In conclusion, we propose that, endothelial damage mediated via NO-associated processes and SOD-mediated inhibition of tissue injury should be considered in the etiopathogenesis of vasculitis in BD. Due to the limitations of a cross-sectional study to highlight the overall dynamic process, further experimental and clinical investigations are strictly needed to determine the exact pathogenetic roles of these molecules in BD.

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