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The pathophysiological significance of red blood cell nitric oxide concentrations in inflammatory Behçet's disease

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Nitric oxide ($NO^{\bullet}$) is a free oxygen radical with powerful vasodilator properties studied in many tissues. It is produced by $NO^{\bullet}$ synthases in endothelial cells upon stimulation by various agents like cytokines and tumor necrosis factor. The end-products of $NO^{\bullet}$ are nitrite and nitrate. Their levels are used biochemically to determine $NO^{\bullet}$ synthase activity. Evidence is accumulating for the role of $NO^{\bullet}$ in the pathophysiology of inflammatory Behçet's disease (BD). Indeed, we first demonstrated that serum $NO^{\bullet}$ production is increased in active BD, suggesting a possible new activity marker.[1] More recently, we have reported that pro-inflammatory cytokines, inducers of $NO^{\bullet}$, and lipid peroxidation are increased and associated with decreased antioxidant enzyme activities in BD.[2,3] Our findings were then supported by four independent investigations, one of which was from the present group.[4–7] Moreover, aqueous humor $NO^{\bullet}$ levels were found to be increased in uveitic BD patients.[8] Furthermore, Salvarani *et al*.[9] have recently shown that the Glu-Asp298 polymorphism of endothelial $NO^{\bullet}$ synthase gene is associated with BD susceptibility. Therefore, to support our previous studies, this report further investigated for the first time $NO^{\bullet}$ levels in red blood cells of BD patients compared with age-matched and sex-matched healthy control volunteers.

A total of 20 patients with BD (16 men, four women), with a mean age of 33.4 years, and 15 healthy control subjects (12 men, three women), with a mean age of 32.1 years, were enrolled in this study. Patients with BD had to fulfil the International Study Group criteria for the diagnosis of BD.[10]

Fasting whole-blood samples were taken from BD patients and control subjects by venipuncture from an antecubital peripheral vein into heparinised plain tubes to prepare the erythrocyte sediment. The buffy coat on the erythrocyte sediment was carefully separated. The erythrocyte sediment was subsequently washed three times with 10-fold volumes of 0.9% sodium chloride solution to remove the plasma remnants. Following this, the erythrocyte sediment was treated with four-fold volumes of ice-cold deionised water to obtain hemolysate. $NO^{\bullet}$ is a labile compound and has a brief half-life, and therefore its detection as the native $NO^{\bullet}$ molecule is difficult. It is rapidly converted to the stable end-products nitrate (NO_3^-) and nitrite (NO_2^-) in typical oxygenated aqueous solutions and tissues. Thus, erythrocyte total nitrite levels were measured as an index of $NO^{\bullet}$ production. For total nitrite detection, lysate was treated with copperised cadmium in glycine buffer at pH 9.7 (2.5–3.0 g of cadmium granules for a 4 ml reaction mixture) to reduce NO_3^- to NO_2^-.[11] Measurement of total nitrite was based on the Griess reaction, in which a chromofore with a strong absorbance at 540 nm is formed by the reaction of nitrite with a mixture of sulphanilamide and *N*-(1-

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naphthyl)-ethylenediamine. A standard curve was established with a set of serial dilutions (10^{-8} to 10^{-3} mol/l) of sodium nitrite.

The analysis was performed by the same examiner (M.Ç.), who was blinded to diagnosis, and was assayed in duplicate. Data were presented as nanomoles per gram of hemoglobin (Hb) (mean $\pm$ standard deviation). The Mann–Whitney U-test was used for statistical analysis and $p < 0.05$ was considered significant. BD patients had significantly ($p < 0.001$) higher erythrocyte $NO^{\bullet}$ concentrations (179.7 ± 33.6 nmol/g Hb) when compared with control subjects (129.7 ± 23.4 nmol/g Hb). The significantly increased $NO^{\bullet}$ levels in erythrocytes from BD patients indicated a role of $NO^{\bullet}$ in the pathogenesis of this unique disorder.

$NO^{\bullet}$ is an important intercellular physiological messenger of immunity and inflammation for the vascular system, with the inhibition of platelet adhesion.[12] It is produced by various cell types including the endothelium to facilitate communication. The formation of $NO^{\bullet}$ is catalysed by $NO^{\bullet}$ synthase, an enzyme present in a variety of cell types including red blood cells, which can be induced by cytokines during inflammatory and infectious processes, resulting in large amounts of $NO^{\bullet}$ production.[11,13] In addition, $NO^{\bullet}$ is accepted as an oxygen radical and also recognised as a major messenger molecule involved in the regulation of vasodilatation.

$NO^{\bullet}$ is present in the blood at 10^{-7} M under physiological conditions, but at concentrations higher than 10^{-6} M during inflammatory disease states.[14] Because the erythrocyte membrane is highly permeable to $NO^{\bullet}$, it is quickly scavenged by Hb in red blood cells when $NO^{\bullet}$ is released into the bloodstream or oxidised to nitrite.[15] Nitrite can also enter erythrocytes rapidly and reacts with oxyhemoglobin but does not exert a strong oxidant stress on these cells. Peroxynitrite anion, the reactive species formed *in vivo* by the reaction of $NO^{\bullet}$ with the superoxide anion, is also capable of diffusing across erythrocyte membranes via anion channels and passive diffusion.[16] Moreover, recent studies have revealed that human erythrocytes possess a $NO^{\bullet}$ synthase. Therefore, we think that excess $NO^{\bullet}$ levels found in this study may be liberated from the endothelium of conductance and vessels in inflammatory BD, which is then taken up by red blood cells, and/or $NO^{\bullet}$ may be produced primarily in the erythrocytes by the enzyme $NO^{\bullet}$ synthase.

Excess $NO^{\bullet}$, in turn, may cause lipid peroxidation, cellular dysfunction and death. Indeed, we demonstrated the presence of strong pro-oxidants (radicals) during the course of BD, suggesting the presence of an imbalance in the oxidant–antioxidant system in the pathogenesis of BD.[1–3] In other words, because $NO^{\bullet}$ participates in the compensatory response to chronic vascular injury,[14] its increased levels seem to be pathophysiologically significant during the course of this unique vasculitic disorder. Moreover, these results are consistent with the hypothesis that $NO^{\bullet}$ may have a crucial role in vasoregulatory mechanisms in BD patients.[1,5] Likewise, cytokine-induced overproduction of $NO^{\bullet}$ by immunocompetent cells may be the other possible pathophysiological consequences with subsequent development of oxidative stress in BD. Therefore, the present finding indicates $NO^{\bullet}$-dependent alterations of oxidative metabolic burst in such patients, and also questions the possible participation of erythrocytes during the course of inflammation in BD. In other words, $NO^{\bullet}$-Hb may serve as a store of $NO^{\bullet}$ in red blood cells, and oxidative stress status in the blood seems to be deteriorated as a whole in BD, both in plasma and erythrocytes, confirming an impaired oxidant–antioxidant equilibrium.

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