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Significance of NOx concentration in red blood cells

Takaharu Ishibashi[CA]

Department of Pharmacology, Kanazawa Medical University, Uchinada, Ishikawa, 920-0293, Japan

[CA]Corresponding author
Tel: +81 76 286 2211, ext. 3722
Fax: +81 76 286 8191
E-mail: ishitaka@kanazawa-med.ac.jp

Dear Sir,

I would like to address the earlier Letter to the Editor 'The pathophysiological significance of red blood cell nitric oxide concentrations in inflammatory Behçet's disease' by Everklioglu *et al*. in *Mediators of Inflammation*.[1] It is not hard to accept that NO production is enhanced in active inflammation in Behçet's disease, and this may be reflected in the elevation of end-products (NO_2^- and NO_3^-: NO_x) in circulating blood as is supported by some other groups. However, in the article, elevation of NO_x in red blood cells (RBCs) in patients with Behçet's disease is reported. I would like to ask the authors about the following points:

1. How NO_x contamination was eliminated from every experimental procedure.
2. How hemoglobin was removed from hemolysate before the Griess reaction.

The first was very important to quantify NO_x, because NO_x contamination in laboratory ware[2] sometimes makes accurate evaluation of NO_x difficult. In addition, NOx contamination in water[3] used for buffer preparation could be a cause of fatal error.

In our experience, NO_3^- easily passes the membrane of RBCs bi-directionally through an anion exchanger. The rate of the influx or efflux of NO_3^- is very rapid and equilibrium (ratio of NO_x concentration of intra-RBCs to extra-RBCs around 0.8) is established within 2 min.[4] This means that when RBCs were washed with a buffer containing NO_x, the NO_x level in RBCs would become similar to that of the buffer. When the NO_x level in the buffer is near zero, only a scar level of NO_x is recognized in RBCs.[4] However, the NO_x content reported in the Letter to the Editor is rather high (when hemoglobin and hematocrit are set at 15 g/dl and 45%, respectively, the RBC NO_x level in the control subjects is calculated to be about 43 μM). This may indicate NO_x contamination of the buffer used to wash RBCs, or a new finding that the anion exchanger in RBCs in Behçet's disease is impaired, in that NO_x was retained within RBCs. If the latter is the case, it would be worthwhile to examine the rate of influx and efflux of NO_x through an anion exchanger.

The second question also concerns the NOx contamination. Because hemoglobin interferes with absorption at 540 nm, it is inevitable to remove the substance before colorimetric measurement. For this sake, ultrafiltration is commonly used. However, the membrane of the unit is heavily contaminated with NO_x without exception.[3] This may also be a cause of high NO_x contents in RBCs in both patients and control subjects.

I hope the authors took particular care of these points, and the difference in RBC

NO_x level between the patients and the control subjects did not rest on a large number of chance events.

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Authors' reply

Cem Evereklioglu[1,CA] and Mustafa Cekmen[2]

[1]Department of Ophthalmology, Erciyes University, Turkey, and [2]Department of Biochemistry, Kocaeli University, Turkey

[CA]Corresponding author
E-mail: everekliogl u@hotmail.com

Dear Sir,

We thank Dr Ishibashi for his important contributions on our previous paper.[1] Because our published paper was a Letter to the Editor, the study protocol was not stated in detail. All stock and working solutions were prepared with double-distilled and deionized water ($NO_x < 1$ μmol). After obtaining fasting whole-blood samples from all subjects, hematocrit and hemoglobin analyses were performed. After the centrifugation at $3000 \times g$ for 10 min, the plasma and buffy coat were carefully discarded and the RBC pellet was subsequently washed three times with isotonic (295 mOsM) NaCl phosphate buffer containing 150 mM NaCl, 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4 (pH 7.4).

All laboratory ware (polypropylene disposable tips, vacuum blood sampling tubes, centrifuge tubes, conical tubes and other glassware) were washed three times (for 5 min) in large containers with pure water before use to minimize NO_x contamination. Therefore, we do not expect any contamination during the procedure of our experimental process. In addition, NOx concentrations were also measured in two samples of pure water as it was for the patients and control subjects. If an excess contamination occurred ($NO_x > 2$ μmol/l), we repeated the experiment.

Because we deproteinized the hemolysate and studied with a transparent supernatant, there will not be hemoglobin (Hb) interference at 540 nm. In addition, we would like to correct the calculated values of Ishibashi (when Hb and hematocrit are set at 15 g/dl and 45%, respectively, the RBC NO_x level in the control subjects is calculated to be about 43 μmol/l) as follows; control Hb = 14.1 g/dl, $HbNO_x$ = 18.29 μmol/l and hematocrit = 43.1%. Control NO_x:Hb = 18.29 μmol/l:14.1 g/dl = 0.12971 μmol/g Hb (129.7 nmol/g Hb). This means that whole blood RBC HbNO levels in control subjects were 18.29 μM. This value is about one-third of the plasma levels found in our previous paper.[2]

NO can be found in RBC in various forms including ($HbFe^{2+}NO$, $NO^{\bullet}$, $HbFe^{3+}NO_2$, $HbFe^{III}OONO$, NO_2^-, NO_3^- SNO-Hb, etc.). Therefore, although there may be equilibrium after washing with the buffer, NO that is bound to Hb will still be in the RBCs.

One reason for the increased RBC NO levels found in our study may be the drugs that the patients used (not stated in the paper). All patients in our study were under the treatment of steroids, colchicines and immunosuppressives. It is therefore possible that these agents may prevent NO_3^- transport through the anion exchanger. As a result, it is open to further study whether these agents could modify NO_3^- transport across the RBC membrane via the anion exchanger. Another hypothesis may be that the anion exchanger may be impaired in patients with Behçet's disease, which also awaits further investigation.

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