Endothelial activation and induction of monocyte adhesion by nontransferrin-bound iron present in human sera

Apriliana E. R. Kartikasari,* Niki A. Georgiou,* Frank L. J. Visseren,*,† Henny van Kats-Renaud,* B. Sweder van Asbeck,*,‡ and Joannes J. M. Marx*,[1]

*Eijkman-Winkler Center for Medical Microbiology, Infectious Diseases and Inflammation and Eijkman Graduate School for Immunology and Infectious Diseases; Departments of †Vascular Medicine and ‡Internal Medicine, University Medical Center Utrecht, Utrecht, The Netherlands

To read the full text of this article, go to http://www.fasebj.org/cgi/doi/10.1096/fj.05-4700fje; doi: 10.1096/fj.05-4700fje

SPECIFIC AIMS

Nontransferrin-bound iron (NTBI) has been detected in patients with primary and secondary hemochromatosis, such as in populations with the HFE C282Y mutation of hereditary hemochromatosis. We examined the effects of serum NTBI from control subjects and HFE C282Y carriers on endothelial activation and monocyte adherence to endothelium. Our aim for performing experiments with human sera containing wide range levels of NTBI was to identify biological effects that can be attributed to naturally occurring NTBI.

PRINCIPAL FINDINGS

1. Serum NTBI levels were higher in both homozygotes and heterozygotes of the HFE C282Y mutation of hereditary hemochromatosis, compared with control sera, measured by a recently-developed fluorescence-based one-step assay

A fluorescence-based one-step NTBI assay measured higher levels of NTBI in the homozygotes (7.9±0.6 μM, n=9) compared with heterozygotes (4.0±0.6 μM, n=8, P=0.001) and healthy blood donors as controls (1.6±0.2 μM, n=21, P=0.001) (**Fig. 1*a***). The difference in NTBI levels between heterozygotes and healthy donors was also significant (P=0.001). For the three groups combined, NTBI values were positively correlated to serum iron (R^2=0.872, P=0.001, n=38) and transferrin saturation (R^2=0.909, P=0.001, n=38). Similar correlations within the control group suggest that NTBI was also present in healthy subjects.

Mean values of serum the inflammatory marker hs-CRP (high-sensitivity C-reactive protein) were 1.2 mg/L (HFE C282Y homozygotes, n=9), 1.4 mg/L (C282Y heterozygotes, n=8) and 0.6 mg/L (controls, n=21) (Fig. 1*b*). hs-CRP from all sera ranged from 0.2 to 3.9 mg/L. There was no significant difference in the mean values between the groups, suggesting that the difference in NTBI levels were not due to a difference in inflammation status between the groups.

2. Serum NTBI modifies the levels of adhesion molecule expression and subsequently monocyte adherence to these endothelial cells. In this study, a clear biological function of naturally occurring NTBI in human sera was observed

The adherence of monocytes to endothelial cells was investigated. Human umbilical vein endothelial cells (HUVECs) were incubated with endothelial basal medium-2 (EBM-2) supplemented with 50% individual sample sera for 48 h. The range of adhered monocytes to HUVECs was 13.4% to 21.8% in the control group (n=21) with a mean of 17.6 ± 0.5% (Fig. 1*c*). The adherence of monocytes was significantly higher on HUVECs treated with 50% individual heterozygous sera, ranging from 18.9% to 25.0% (mean=22.1±0.7%, P=0.001, n=8) compared with controls. Moreover, monocyte adhesion ranging from 21.4% to 28.4% (mean=25.0±0.9%, n=9) was observed when homozygote sera were used, and was also significantly higher than both heterozygous sera (P=0.050) and controls (P=0.001).

To investigate how sera of C282Y carriers promote monocyte adherence, the expression of the adhesion molecules, ICAM-1, VCAM-1, and E-selectin, on treated HUVECs was analyzed. Incubation of endothelial cells for 48 h with 50% C282Y homozygote and heterozygote sera resulted in significantly higher adhesion molecule expression compared with controls (Fig. 1*d–f*, circles). The expression levels were tested for association with the

[1] Correspondence: Eijkman-Winkler Center for Medical Microbiology, Infectious Diseases and Inflammation, University Medical Center Utrecht, 100 Heidelberglaan, G04.614, Utrecht 3584CX, The Netherlands. E-mail: jmarx@azu.nl

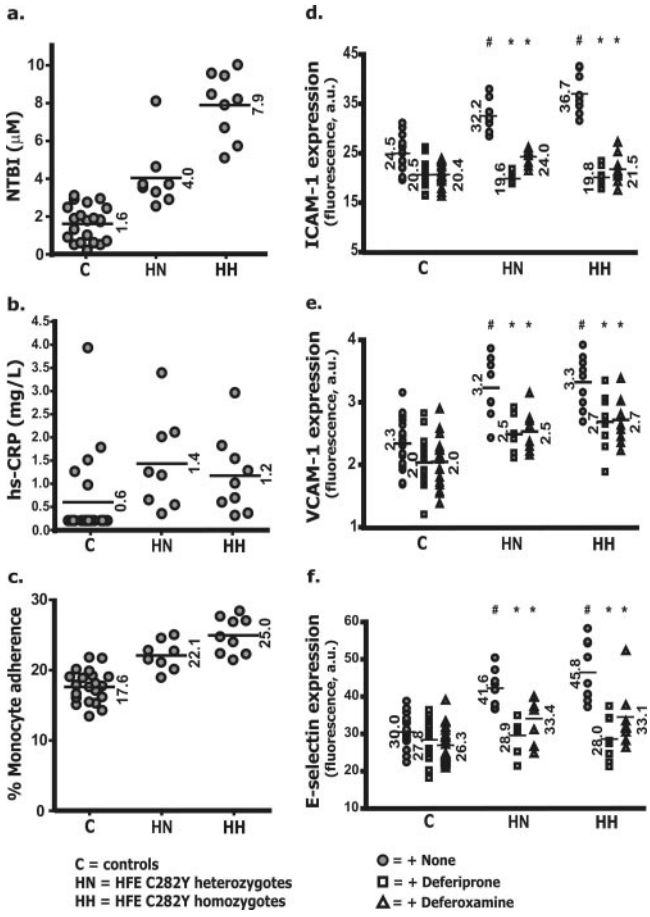

Figure 1. Serum NTBI levels and hs-CRP of HFE C282Y homozygotes, heterozygotes and controls, and their effects on endothelial activation and the adhesiveness of monocytes to endothelium. Scatter plots with the means of the three measured groups, including controls (C, n=21), HFE C282Y heterozygotes (HN, n=8), and homozygotes (HH, n=9), of (*a*) NTBI, (*b*) hs-CRP, and (*c*) percentage of monocyte adherence to HUVECs treated for 48 h with 50% individual sera (each value is the mean of 4 independent experiments in triplicate). *d)* ICAM-1, (*e*) VCAM-1, and (*f*) E-selectin expression of respective HUVECs from groups as indicated, with lines showing the means of each group. HUVECs were treated with 50% individual sera of controls, C282Y heterozygotes, or C282Y homozygotes for 48 h, ±10 μM deferoxamine or 30 μM deferiprone for the last 24 h of incubation with sera. Each value of point is the mean of 4 to 9 independent experiments. #Higher than the control group (P=0.001); *P = 0.010, lower than the respective heterozygote or homozygote group with no chelation; a.u., arbitrary unit.

values of NTBI or hs-CRP using Pearson regression analysis. Positive correlations to NTBI levels in sera (n=38) were found with ICAM-1 (R^2=0.574, P=0.001), VCAM-1 (R^2=0.399, P=0.001), and E-selectin (R^2=0.453, P=0.001). Hs-CRP, on the other hand, did not positively correlate to the adhesion molecule expression (ICAM-1, $R2$=0.054,P=0.161; VCAM-1, R^2=0.028,P=0.313; E-selectin, $R2$=0.120,P=0.123, n=38). This finding suggests that serum NTBI, under the current experimental conditions, modulated the levels of adhesion molecule expression, and consequently the extent of monocyte adhesion to the endothelial cells.

3. Iron added as serum NTBI accumulates inside the endothelial cells and increased levels of oxygen-derived free radicals were formed as a result

NTBI-induced adhesion molecule expression on endothelial cells may possibly be mediated by alteration in cellular reduction-oxidation status. The levels of cellular oxidative stress in HUVECs were therefore examined, using the oxygen radical sensitive DCF fluorescence probe, where the fluorescence intensifies with increased level of oxidative stress. HUVECs incubated for 48 h with 50% serum containing 9.4 μM NTBI produced 2-fold higher fluorescence compared with those incubated with 50% serum containing 0.6 μM NTBI. When HUVECs were incubated with 50% serum containing 0.6 μM NTBI that had been premixed with 30 μM Fe(III)citrate, a 3-fold increase in fluorescence was observed. Here, Fe(III)citrate was added to supplement NTBI to the incubating serum, since the majority of NTBI is found in complex forms to citrate. This finding indicates that NTBI increased cellular oxidative stress and that NTBI-induced adhesion molecule expression on HUVECs was mediated by oxygen-derived free radicals.

The pro-oxidant condition in cells could have been promoted by available intracellular labile iron. Influx of iron from the various iron species in the serum may take place during the 48 h incubation of HUVECs. Some may accumulate in the cytoplasmic labile iron pool. HUVECs incubated for 48 h with 50% serum containing 9.4 μM NTBI has lower basal calcein fluorescence compared with those incubated with 50% serum containing 0.6 μM NTBI (**Fig. 2*a–b***), due to quenching of calcein signal. This result demonstrates increased level of intracellular labile iron due to NTBI. Further quenching of calcein signal was observed when HUVECs were incubated for 48 h with 50% serum containing 0.6 μM NTBI, premixed with 50 μM Fe(III) citrate. When Fe(III)citrate at a final concentration of 15 μM was added to the incubation medium, after calcein loading, quenching of the basal calcein signal was also observed (Fig. 2*a*). On the other hand, dequenching of basal calcein signal was observed when 45 μM deferiprone, an iron chelator, was added to the incubation medium. Collectively, these results indicate that serum NTBI augmented the level of cytoplasmic labile iron, leading to oxidative radical formation and endothelial activation.

4. Iron chelation counteracted NTBI-induced endothelial activation

Since there is a positive correlation between adhesion molecule expression and NTBI, addition of a chelator with high specificity for iron would be expected to lower adhesion molecule expression. To test this, deferiprone and deferoxamine, iron chelators used to treat secondary iron overload, were included in the experimental set-up. HUVECs were treated in total for 48 h with 50% individual sera. After 24 h of serum

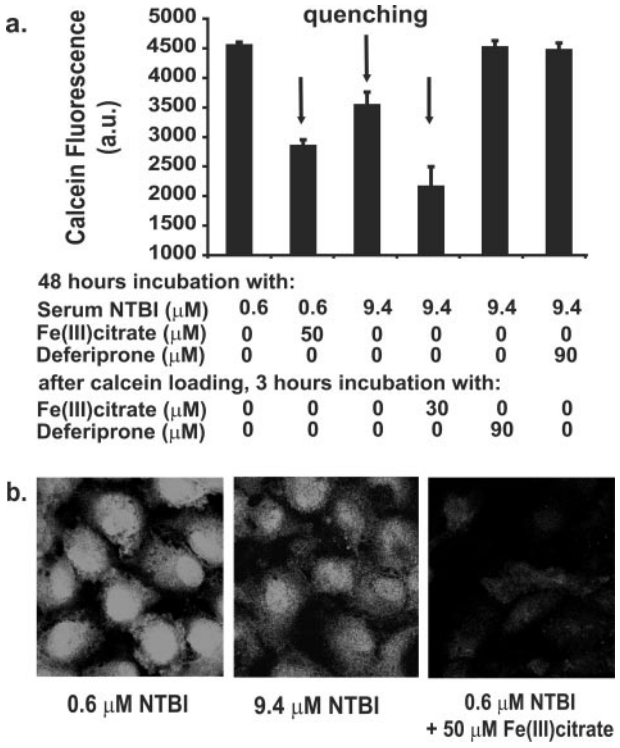

Figure 2. Accumulation of labile iron intracellularly. *a)* Fluorescence of intracellular calcein signal of HUVECs (n=3), which is quenched by iron, after treatments as indicated. *b)* Depiction of the calcein signal from HUVECs after treatments for 48 h, using a confocal laser microscope. All concentrations are half of the final concentrations in the incubation medium. a.u., arbitrary unit.

incubation, 30 μM deferiprone or 10 μM deferoxamine was added to the incubation solution.

Treated HUVECs were grouped based on C282Y genetic variation of the incubating sera and the chelator added. Means of adhesion molecule expression are shown in Fig. 1*d–f*. NTBI-induced VCAM-1, ICAM-1, and E-selectin expression were significantly lowered by the addition of deferiprone or deferoxamine; both reduced ICAM-1 expression. Deferiprone lowered the expression of ICAM-1 and E-selectin more so than deferoxamine. The different effectiveness of chelation might be due to the nature of the two chelators, as deferoxamine is slowly entering the cells. Chelation of intracellular labile iron is necessary to reduce the up-regulation of adhesion molecule expression.

CONCLUSIONS AND SIGNIFICANCE

The inflammatory process has been recently associated with alterations in iron metabolism. Lack of iron nutrient has been shown to weaken immune response. Our findings relate to the importance of iron in inflammatory processes by revealing the role of nontransferrin-bound iron (NTBI) in the process of monocyte adherence to endothelium.

We showed the presence of NTBI in human sera, and therefore NTBI may play a physiological role in cellular metabolism. Prolonged exposure to serum NTBI on HUVECs had augmented the level of intracellular iron pool. Induction of cellular reduction-oxidation state imbalance occurred as a result, which had led to the phenotype of primed endothelial cells (**Fig. 3**). Deferiprone and deferoxamine were capable of chelating intracellular labile iron, and therefore reducing the formation of oxygen-derived free radicals and NTBI-induced endothelial adhesion molecule expression. Collectively, the findings suggest a role for NTBI in endothelial activation that leads to recruitment of monocytes to the endothelium. This study supports the involvement of NTBI in the physiological process of monocyte homing to the tissues, as well as in host-defense mechanism against microbial infections.

Accumulation of iron has been found in atherosclerosis as well as neurodegenerative diseases, including Alzheimer's, Parkinson's, and Huntington's. The present study suggests a role for NTBI in atherogenesis, as monocyte adherence to endothelium is a crucial initial event in atherosclerotic plaque formation. FJ

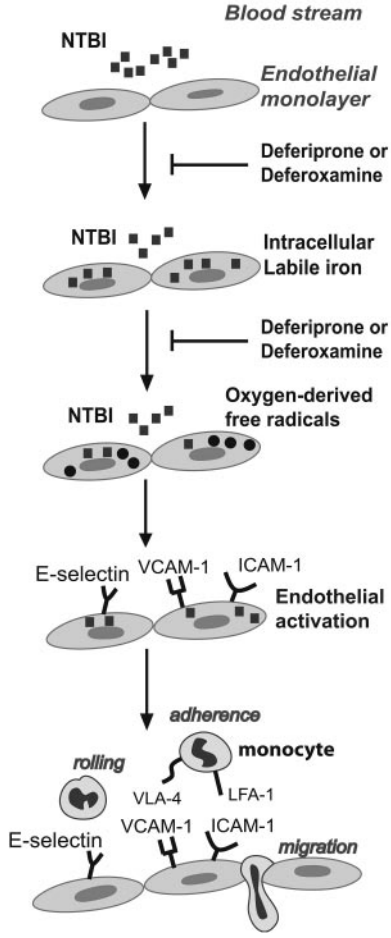

Figure 3. Illustration of the reported findings with current knowledge showing a hypothetical sequence of events initiated by NTBI leading to inflammation. NTBI increases the level of intracellular labile iron, and oxygen-derived free radicals. The radicals activate the cellular machinery of endothelial cells leading to the expression of endothelial surface adhesion molecules. These adhesion molecules promote monocyte adherence to the endothelium.