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

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ABSTRACT

Nontransferrin-bound iron (NTBI) has been detected in iron overload diseases. This form of iron may exert pro-oxidant effects and modulate cellular function and inflammatory response. The present study has aimed to investigate the effects of serum NTBI on monocyte adherence to endothelium. Measured by a recently developed high-throughput fluorescence-based assay, serum NTBI was found to be higher in both homozygotes of HFE C282Y mutation of hereditary hemochromatosis (7.9±0.6 μM, n=9, P<0.001) and heterozygotes (4.0±0.5 μM, n=8, P<0.001), compared with controls (1.6±0.2 μM, n=21). The effects of these sera on monocyte adhesion and endothelial activation were examined. Adhesion of normal human monocytes to C282Y homozygote- and heterozygote-serum-treated human umbilical vein endothelial cells was higher (25.0±0.9 and 22.1±0.7%, respectively) compared with controls (17.6±0.5%, both P<0.001). For the three groups combined, the expression of adhesion molecules, ICAM-1, VCAM-1, and E-selectin, was positively correlated to NTBI levels but not to the inflammatory marker C-reactive protein. Furthermore, accumulation of intracellular labile iron and oxidative radicals within the cells due to NTBI was evidenced. Finally, counteraction of NTBI-induced endothelial activation was observed using iron chelators. These findings therefore identify a physiological function of NTBI in monocyte-endothelial interactions that may also contribute to the development of atherosclerosis and neurodegenerative diseases.

Key words: inflammation • adhesion molecules • hemochromatosis

Nontransferrin-bound iron (NTBI) has been detected in patients with primary and secondary hemochromatosis, particularly in patients with transferrin saturation >45% (1). In subjects with the HFE C282Y mutation of hereditary hemochromatosis, NTBI has been detected not only in the sera of homozygotes but also of carriers, using an HPLC-based method (2). The autosomal recessive mutation of the HFE gene is common among the Caucasian population with prevalence of 10% and is responsible for 80–90% of hemochromatosis cases (3).

In the plasma, NTBI is believed to bind to ligands with substantially less affinity than transferrin, such as citrate, citrate-carbonate (4), albumin, or other serum proteins (5). In contrast to ferritin or transferrin-bound iron, NTBI is hypothesized to be more readily available for catalyzing free radical formation, which are capable of causing cellular damage through various mechanisms (6). Because the chemical nature of NTBI in the plasma is largely unknown, in order to explore the biological function of serum NTBI, it is crucial to use sera with a sufficiently wide range of NTBI in experimental setup.

Oxygen-derived free radicals have been implicated as key mediators of the signaling pathways that underlie inflammation. Immune cells use these radical species to support their functions and therefore need adequate levels of antioxidant defenses to avoid any harmful effects of intracellular reduction-oxidation state imbalance (7). An essential inflammatory process is the binding and transmigration of leukocytes through endothelium to gain access to the inflamed sites. This inflammatory event has been implicated in the development of many diseases, such as atherosclerosis and neurodegenerative diseases. Oxygen-derived free radicals that could be generated by NTBI through the Fenton reaction may augment this particular inflammatory process.

The pro-oxidant iron has been shown to generate free radicals in endothelial cells (8) and induce monocyte adhesion to these cells (9). Moreover, iron has been shown to promote interleukin-6 production by endothelial cells (10) as well as induce cell toxicity (11).

These findings prompt the question whether NTBI present in human sera plays a role in immune function involving monocyte-endothelial interactions. To that purpose, we used the sera from control subjects and HFE C282Y carriers, which provide wide range levels of serum NTBI and investigated the effects of these sera on endothelial activation and subsequent monocyte adherence to the endothelium. NTBI was measured using a recently developed fluorescence-based assay (12). Changes in cellular reduction-oxidation status due to NTBI, which could bring about alteration in cellular function and downstream protein expression, were examined. Furthermore, the potential counteracting effects of iron chelation were also investigated.

MATERIALS AND METHODS

Baseline iron level

The iron content of the EBM-2 medium was measured by Vitros® 950 Chemistry System (Ortho-Clinical Diagnostics, Tilburg, The Netherlands) to monitor the baseline iron level in all of the experiments involving human umbilical cord endothelial cell (HUVEC). In this measurement, iron was first freed from any complexes by lowering the pH of the solution. The released iron was then complexed with a coloring agent for detection at a wavelength of 600 nm.

Serum samples

Serum samples were taken from nine C282Y homozygous hemochromatosis patients, all being treated with phlebotomies (most of them on maintenance treatment), and eight C282Y heterozygous subjects who were from a previous study (2) and methods were approved by the institutional review board of the University Medical Centre Utrecht (Utrecht, The Netherlands). All of these subjects gave informed consent. Twenty-one control sera were obtained from

healthy donors provided and approved by Sanquin blood bank (Utrecht, The Netherlands) to be used for research purposes. These healthy donors were not screened for HFE mutations. None of the subjects studied had inflammatory diseases, liver diseases, or history of alcohol abuse.

Serum samples were isolated by centrifuging blood samples at 2500 *g* for 20 min, followed by heat inactivation (56°C, 60 min), and continued with an additional centrifugation at 2500 *g* for 20 min. The obtained sera were stored at –20°C until experiments were carried out.

Serum iron parameter measurements

Serum ferritin, serum iron, serum transferrin, and transferrin saturation were measured (Central Diagnostic Laboratory, University Medical Center Utrecht) using routine laboratory methods.

Fluorescence-based one-step NTBI measurement

The assay was carried out as described by Breuer and Cabantchik (12). Briefly, the serum sample was mixed with reagent A [HEPES-buffered saline (HBS) containing 10 mM sodium oxalate (BDH Chemicals, Ontario, Canada), 0.1 mM gallium chloride (Sigma-Aldrich, Zwijndrecht, The Netherlands), and 0.6 μM 5-(4,6-dichlorotriazinyl)-aminofluorescein (DCTAF, Molecular Probes, Eugene, OR)-apotransferrin] or reagent B (same as reagent A, but containing 25 μM apotransferrin; Kamada, Haifa, Israel). In reagent A, the accessible iron binds to the fluorescein-apotransferrin and quenches its fluorescence, whereas in reagent B the iron binds to the excess nonfluorescent apotransferrin rather than to fluorescein-apotransferrin, resulting in higher fluorescence. After incubation of 1 h, fluorescence was read using a filter pair for excitation at 485 nm and emission at 530 nm using a CytoFluor II fluorescence microplate reader (PerSeptive Biosystems). The ratio of the fluorescence readings (A/B) was inversely proportional to the concentration of NTBI in the original sample, in which the amount was determined from a Fe(III)nitrilotriacetate (Sigma-Aldrich) calibration curve, in a range of 0.4–25 μM. The arbitrary zero value (12) of the calibration curve was set to the highest value of A/B.

High-sensitivity C-reactive protein test

The measurement of high-sensitivity C-reactive protein (hs-CRP) in each serum was performed by a commercial high-sensitivity assay on a Behring Nephelometer Analyzer II (BNII, Dade-Behring, Germany).

HUVEC isolation and culture

HUVEC were isolated and cultured as described by Jaffe et al. (13) with minor modifications. Briefly, fresh umbilical cords, after informed consent from the parents, were collected in PBS and stored at 4°C for no more than 3 days until isolation. The cord was canulated at each end and washed gently with phosphate-buffered saline (PBS, BioWhittaker, Verviers, Belgium); 0.02% trypsin containing 0.21 mM ethylenediamine tetraaceticacid (EDTA, GIBCO, Ontario, Canada) was injected into the vein, and left at 37°C for 20 min. The cells were collected by flushing the vein with PBS, continued with centrifuging the suspension at 250 g for 10 min. The pellet was resuspended in endothelial cell basal medium (EBM-2, Clonetics®) supplemented with 2% fetal bovine serum. Cells were cultured in tissue culture flasks (Costar®, New York, NY), precoated with fibronectin (GIBCO). Culturing was carried on in a humidified 37°C incubator with 5%

CO_2, and confluent cells from passages 2–3 were used for all experiments. HUVECs were always used during and maintained at a cobblestone confluent density for all conducted experiments.

Monocyte isolation

Peripheral blood mononuclear cells (PBMC) were isolated from donor blood (Sanquin Blood Bank, Utrecht) by Ficollpaque density gradient centrifugation. Monocytes were isolated using the negative immunoselection monocyte isolation kit (MiltenyiBiotec, CLB Sanquin, Amsterdam, The Netherlands) according to supplier's instructions. This method resulted in purity of >90% as analyzed by flow cytometry. Purified monocytes were suspended at a concentration of 5×10^6 cells/ml in RPMI1640 (BioWhittaker) supplemented with 2 mM L-glutamine and 0.2% human serum albumin (CLB Sanquin) before use.

Preparation of iron and chelators

A 10 mM Fe(III)citrate (Sigma, 1:6 iron-to-citrate molar ratio) solution was made by dissolving the iron crystals in distilled water at 56°C for 30 min. Iron solutions were always freshly prepared and filter-sterilized prior to use. The iron chelators were prepared as stocks in PBS of 10 mM deferoxamine (Novartis, Arnhem, The Netherlands) and 30 mM deferiprone (Duchefa Biochemie, Haarlem, the Netherlands) and stored at –20°C before use. Final pH was maintained 7.8 in incubation medium.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide viability assay for HUVECs and trypan blue exclusion test for monocytes

Cellular viability of HUVECs was monitored by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT; Sigma-Aldrich) method (14). Briefly, cells were seeded into a 96-well microtiter plate and grown to confluence. Individual sera with iron chelator over a range of concentrations (0−1000 μM) were added to the plate. Incubation was for a period of 48 h before the MTT assay was performed. Compound cytotoxicity was expressed as a TC_{50} denoting the concentration resulting in 50% loss of cell viability, as calculated by Calcusyn (15). After 48 h of serum treatment, HUVEC viability was >95%.

The amount of viable freshly isolated monocytes was monitored by trypan blue (Sigma-Aldrich) exclusion. Briefly, 10 μl cell suspension was mixed with 90 μl trypan blue isotonic solution (0.2% w/v). The amount of viable cells was >95%, determined microscopically using a hemocytometer.

In vitro cytoadherence assay

Confluent HUVECs were pretreated with 50% individual sera in EBM-2 medium for 48 h prior to the assay. Monocytes were labeled with 2 μM 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethylester (BCECF-AM, Molecular Probes). Cytoadherence of monocytes (25×10^4 cells/well) to HUVEC monolayer (5×10^4 cells/well) was performed in a 96-well plate for 30 min at 37°C with gentle agitation. After addition of monocytes to HUVECs at monocytes-to-HUVECs ratio of 5:1, fluorescence was read using a filter pair for excitation at 485 nm and emission at 530 nm using the Cytofluor II microplate reader. This value represented

total fluorescing monocytes added to each well. After being thoroughly washed with RPMI1640, fluorescence was again measured and the value represented the remaining monocytes firmly attached to the HUVEC monolayer. The percentage of adhesion was defined as the value of remaining fluorescence divided by the value of total fluorescence multiplied by 100.

Measurement of cell adhesion molecules by fluorescence-activated cell sorting (FACS)

After treatment with 50% individual sera for 48 h with or without iron chelator for the last 24 h of treatment, HUVECs were harvested by incubating with 0.2% trypsin-EDTA at 37°C for 3 min. The cells were then incubated with fluorescence-labeled monoclonal antibodies against the surface proteins: FITC-conjugated ICAM-1 antibody (R&Dsystem, Minneapolis, MN), PE-conjugated VCAM-1 antibody (BDBiosciences, San Diego, CA), or Cychrome-conjugated E-selectin antibody (BD Biosciences), for 30 min at 4°C. Each flow cytometric measurement was performed using a Becton-Dickinson (San Jose, CA) FACScan and 10,000 events were analyzed.

2,7-Dichlorofluorescein assay

Carboxydichlorofluorescein diacetate (DCFH-DA, Molecular Probes) is a nonpolar compound that is converted into a membrane-impermeable nonfluorescent polar derivative (DCFH) by cellular esterase after incorporation into cells. The trapped DCFH is rapidly oxidized to fluorescent 2,7-dichlorofluorescein (DCF) by intracellular hydrogen peroxide and hydroxyl radicals (16). After incubation with 50% serum in EBM-2 medium for 48 h or otherwise indicated, HUVEC were harvested by incubating with 0.2% trypsin-EDTA at 37°C for 3 min. Cells were then resuspended in DCFH-DA at a final concentration of 5 μM, incubated for 30 min at room temperature, and washed. The emission of the trapped, oxidized DCF in 10,000 cells was analyzed on a FACScan.

Calcein assay

The possible accumulation of labile iron inside HUVEC due to incubation with serum NTBI was tested by the calcein-acetoxymethylester (AM) assay (17). Calcein-AM (Molecular Probes) is a fluorescent probe with a lipophilic AM moiety that makes it permeable through cell membranes. Once inside the cell, the AM group will be cleaved by nonspecific esterases, resulting in a charged form that hardly leaks out of cells. As a weak iron chelator, once inside the cells, the fluorescence signal of calcein can be quenched by iron. This property is useful to detect any intracellular labile iron.

In this assay, confluent HUVECs were pretreated with 50% serum in EBM-2 medium for 48 h or otherwise indicated before the assay. HUVECs were then washed one time and followed by incubation with 0.125 μM calcein-AM (30 min at 37°C). The cells were washed twice to remove the remaining extracellular calcein-AM before fluorescence signal of calcein (excitation=485 nm; emission=530 nm) was read in the Cytofluor II microplate reader. After a stable basal fluorescence signal was observed, deferiprone (100 μM) or Fe(III)citrate (50 μM) was added to the incubation medium. Addition of this permeant membrane iron chelator led to its competitive binding of intracellular labile iron, subsequent release of calcein-bound iron, and an increase in fluorescence intensity by dequenching of calcein signal. This fractional increase in fluorescence intensity after addition of a chelator correlates with the amount of labile iron within HUVEC.

Calcein-fluorescing cells were fixed in PBS containing 3% paraformaldehyde (Polysciences) and 0.02% glutaraldehyde (Merck, Darmstadt, Germany) and visualized using Leica TCS SP2 confocal scanning laser microscope and Leica confocal software (Leica Microsystems, GmbH, Heidelberg, Germany).

Data analysis

Results are expressed as means ± SE of the means. Differences in quantitative measures were tested for significance using one-way ANOVA with Bonferroni posttest for further analysis of the data. Between NTBI and other variables, two-tailed Pearson regression coeficient (r^2) was computed. Significance was established when $P < 0.050$. All statistics were performed using the GraphPad Prism 4 statistical program (San Diego, CA).

RESULTS

The baseline iron level

With no external iron addition, the baseline iron level in the basal cell culture growth medium EBM-2 was 0.36 μM. To avoid any external iron contaminant, in all experiments, plastic materials with low affinity for iron were used.

Serum ferritin, serum transferrin, serum iron, transferrin saturation, and NTBI levels

Mean values of serum ferritin, serum iron, transferrin saturation, and NTBI were higher in HFE C282Y homozygotes compared with the HFE unscreened controls (all $P<0.050$). Serum iron and transferrin saturation were higher in the sera of HFE C282Y homozygotes compared with heterozygotes and heterozygotes compared with controls (all $P<0.050$; Table 1). In this study, the HFE unscreened control subjects included some blood donors with serum ferritin levels indicating iron depletion. The fluorescence-based one-step NTBI assay measured higher levels of NTBI in HFE C282Y homozygotes (7.9±0.6 μM, $n=9$) compared with heterozygotes (4.0±0.6 μM, $n=8$, $P<0.001$) and controls (1.6±0.2 μM, $n=21$, $P<0.00$; Fig. 1*A*). The difference in NTBI levels between heterozygotes and controls was also significant ($P<0.001$). These NTBI values were calculated after adjusting for the arbitrary zero value (see Materials and methods), with within sample variation of 0.24 μM as the average of the errors of the means. The result is in agreement with the previous study (2), using an HPLC-based method, which unlike the assay used in this present study measures NTBI with a separation step from other serum proteins. Furthermore, NTBI values were positively correlated to the standardized measurement of iron parameters, including transferrin saturation ($r^2=0.909$, $P<0.001$, $n=38$) and serum iron ($r^2=0.872$, $P<0.001$, $n=38$) in all the three groups combined (Fig. 2*A–B*), which is in agreement with another study (18). Furthermore, serum ferritin was correlated to NTBI with a positive exponential relationship (log-linear $r^2=0.635$, $P<0.001$, $n=38$) (Fig. 2*C*). Considering the value of arbitrary zero that falls between 0 to 1.5 μM of iron concentrations (19), the plot of serum ferritin vs. NTBI indicates that NTBI was detectable from the sera with serum ferritin of as low as 10 μg/l. Additionally, similar significant correlations found within the control group (NTBI vs. transferrin saturation, $r^2=0.605$, $P<0.001$, $n=21$; NTBI vs. serum iron $r^2=0.555$, $P<0.001$, $n=21$; and NTBI vs. serum ferritin, log-linear $r^2=0.321$, $P=0.008$, $n=21$) also identify the presence of NTBI in these healthy subjects.

Serum hs-CRP levels of HFE C282Y homozygotes and heterozygotes

Mean values of serum inflammatory marker, hs-CRP, 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, indicating that the difference in NTBI levels were not due to a difference in inflammation status between the groups.

HFE C282Y homozygote and heterozygote sera modulate human monocyte adhesion to human endothelial cells

The adherence of monocytes to endothelial cells was investigated, where HUVECs were conditioned with EBM-2 containing 50% individual sample sera for 48 h. The range of adhered monocytes to HUVECs was 13.4–21.8% 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 ($P<0.001$). Collectively, the results suggest that constituents present in both of the sera of HFE C282Y homozygotes and heterozygotes are capable of modifying the level of monocyte adherence to endothelial cells.

Serum NTBI, hs-CRP, and the expression of VCAM-1, ICAM-1, and E-selectin on HUVECs

To investigate how sera of C282Y carriers promote monocyte adherence, the expression of the adhesion molecules on treated HUVECs was analyzed. The expression of VCAM-1, ICAM-1, and E-selectin, the adhesion molecules involved in monocyte recruitment to endothelium (20), was measured by FACS analysis. The sera were heat inactivated before use to avoid the influence of complement systems as well as other heat-labile proteins. The complement systems have been shown to promote leukocyte-endothelial interactions (21) and endothelial adhesion molecule expression (22). The conventional method of heat-inactivation largely abrogates the activity of heat-labile enzymes, such as superoxide dismutase (23), and cytokines (24), as well as lipopolysaccharide-potentiating activity of the serum (25), without affecting the iron-bound proteins, such as transferrin (26) and albumin (27). The heat-inactivation procedure did not affect our NTBI, serum ferritin, serum iron, serum transferrin, transferrin saturation (data not shown), or hs-CRP (28) measurements.

Incubation of endothelial cells for 48 h with 50% individual sera resulted in various levels of adhesion molecule expression. The mean values of those three adhesion molecules were found to be higher in both C282Y-homozygote- and heterozygote-serum-incubated HUVECs compared with controls (both $P<0.001$; Fig. 1*D–F*, circles). The levels of expression were then tested for association with the values of NTBI or hs-CRP of the respective sera, using Pearson regression analysis. All of the above-mentioned molecules were positively correlated to the NTBI levels of the sera (ICAM-1, $r^2=0.574$, $P<0.001$; VCAM-1, $r^2=0.399$, $P<0.001$; E-selectin, $r^2=0.453$, $P<0.001$, n=38) depicted as linear regression on Fig. 3). Hs-CRP, on the other hand, did not correlate to the adhesion molecule expression (ICAM-1, $r^2=0.054$, $P=0.161$; VCAM-1, $r^2=0.028$,

P=0.313; E-selectin, r^2=0.120, P=0.123, n=38). This finding suggests that NTBI, under the current experimental conditions, modulated the levels of adhesion molecule expression and consequently the extent of monocyte adhesion to the endothelial cells.

Intracellular radical formation and accumulation of labile iron within HUVECs

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 a twofold higher fluorescence compared with those incubated with serum containing 0.6 μM NTBI (Fig. 4*A*). When HUVECs were incubated with serum containing 0.6 μM NTBI, which had been premixed with 30 μM Fe(III)citrate, a threefold increase in free radicals formation was observed (Fig. 4*A*). 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 can be promoted by an increase of intracellular labile iron (29). 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 have lower basal calcein fluorescence compared with those incubated with serum containing 0.6 μM NTBI (Fig. 5*A–B*), due to quenching of the 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 (Fig. 5*A–B*). Additionally, when Fe(III)citrate at a final concentration of 15 μM was added to the incubation medium, after calcein was loaded to the cells, quenching of the basal calcein signal was also observed (Fig. 5*A*). On the other hand, dequenching of basal calcein signal was observed when 45 μM deferiprone 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.

HUVECs incubated for 48 h with 50% serum containing 9.4 μM NTBI premixed with 90 μM deferiprone or 50% serum containing 0.6 μM NTBI premixed with 30 μM Fe(III)citrate and 90 μM deferiprone were subjected to DCF and calcein assays. The amount of oxygen-derived free radicals (Fig. 4*B*) and the basal calcein fluorescence (Fig. 5*A* and not shown) in these HUVECs were at the same level as HUVECs treated with 50% serum containing 0.6 μM NTBI. This result confirms that iron chelation prevented while NTBI induced the formation of oxygen-derived free radical formation and labile iron accumulation.

Iron chelation significantly reduced the induction of endothelial adhesion molecule expression

Since there is a positive correlation between adhesion molecule expression and NTBI, addition of a chelator with high specificity for iron would then be expected to lower adhesion molecule

expression. To test this, deferiprone and deferoxamine were included in this experimental set-up. HUVECs were treated in total for 48 h with 50% individual sera. After 24 h of serum incubation, 30 μM deferiprone or 10 μM deferoxamine were added to the incubation solution. The concentration of 10 μM for deferoxamine was chosen to cover the highest NTBI level in this experiment. This concentration is below the TC_{50} (15±2.3 μM) of the compound on HUVECs (9). Deferoxamine is a hexadentate chelator, in which one molecule can bind all six ligands of one iron atom. Three molecules of a bidentate iron chelator, like deferiprone, are required to fully chelate one iron atom. Therefore, in this experiment 30 μM concentration was chosen for deferiprone, which is also below its TC_{50} value (100±11.3 μM) (9).

Treated HUVECs were grouped based on C282Y genetic variation of the incubating sera and the chelator added. The expression of adhesion molecules was then analyzed. The mean value of each group and significance of difference between groups are shown in Fig. 1*D–F*. NTBI-induced VCAM-1, ICAM-1, and E-selectin expression was significantly lowered by the addition of either deferiprone or deferoxamine. Both chelators most effectively reduced ICAM-1 expression. This finding confirms the involvement of NTBI in the induction of adhesion molecule expression. Deferiprone lowered the expression of ICAM-1 and E-selectin more effectively than deferoxamine. The different effectiveness of chelation, shown in this study, might be due to the nature of the two chelators, as deferoxamine is slowly entering the cells and therefore, needs longer time to chelate intracellular labile iron (9). Chelation of intracellular labile iron is necessary to reduce the upregulation of adhesion molecule expression (9, 30).

DISCUSSION

We investigated the relation between serum NTBI and adhesion of monocytes to endothelium. This process plays a significant role in host-defense mechanism against microbial infections and homing of monocytes to the tissues. However, in many age-associated diseases, like atherosclerosis and neurodegenerative diseases, this inflammatory event has been implicated as an initiating process toward the development of the disease.

The involvement of iron in atherosclerosis and coronary heart disease has recently been extensively investigated. A review of epidemiological studies and experimental data suggested evidence for iron involvement in atherosclerosis (31). However, an early meta-analysis of prospective studies could not yet confirm this evidence (32). In this study, we focused on NTBI, the nonsequestered form of iron, which unlike ferritin and transferrin-bound iron, is potentially capable of catalyzing free radical formation. Recently, it has been shown that the redox active component of NTBI, so-called labile plasma iron (LPI), is measurable in the sera of HFE C282Y hemochromatosis patients (33). In this study, sera of HFE C282Y homozygotes, heterozygotes, and normal subjects with wide-range levels of serum NTBI were used. Our results showed for the first time that NTBI present in human sera were involved in the process of monocyte adherence to endothelium. This finding suggests the involvement of iron in the process of inflammation and furthermore in the development of diseases like atherosclerosis. In experimental animal models of atherosclerosis, the pro-oxidant iron has been shown to promote plaque formation (34–36).

Iron chelation markedly improved vascular-endothelial function in patients of coronary artery disease (37), HFE C282Y hemochromatosis (38, 39), and in subjects with homocysteine-induced

endothelial dysfunction (40). Blood donation with substantial depletion of storage iron proves to acutely improve vascular function without pharmacological interventions. (41, 42). Serum ferritin is known to be a measure for body iron storage. This parameter, however, can be highly influenced by inflammation. Considering the lack of relationship to hsCRP in our study, serum ferritin could therefore describe the level of iron storage in our population. For those blood donors with ferritin levels indicating iron depletion, NTBI was found to be very low or lacking. The very low NTBI level was associated and may have lead to the measured least state of endothelial activation. Our findings may therefore explain the results from the aforementioned studies on how low iron status in vivo could benefit vascular endothelial function.

Although the evidence from epidemiological studies linking HFE C282Y mutation to the development of cardiovascular disease is inconclusive, a large-scale study involving 12.239 postmonopausal women revealed an 18.85-fold increased risk of cardiovascular death in subjects who were heterozygous for HFE C282Y and additionally were smokers and hypertensive, while 2.26-fold increased for noncarriers who were smokers and hypertensive, both compared with nonsmoking and nonhypertensive controls (43). NTBI in these C282Y carriers may have modulated the reduction-oxidation status, while interaction with environmental stressors like smoking and hypertension leads to overexposure of radicals and triggers their sensitivity toward cardiovascular disease. Furthermore, in our study, a correlation analysis between endothelial adhesion molecule expression and serum NTBI levels of the three groups combined revealed a strong positive association. This suggests that the NTBI levels could influence the levels of adhesion molecule expression, regardless of the HFE genotypes. This also implies that populations with increased NTBI levels may have an inclination toward enhanced monocyte adhesion to endothelium.

In neurodegenerative diseases including Alzheimer's, Parkinson's, and Huntington's diseases, accumulation of iron has been characterized, which catalyzes metal-induced oxidative stress in the disease process (44). Chronic inflammation has been associated with a broad spectrum of neurodegenerative diseases of aging (45). Neuroinflammation, characterized by the accumulation of reactive microglia, is present in the degenerating areas. The intensity of the activation of these microglial cells, as the brain representatives of the monocyte phagocytic system, is related to a spectrum of inflammatory mediators like adhesion molecules and free radicals generated by a variety of local cells, including the vascular endothelium of the blood-brain barrier (45, 46). Evidence for the role of inflammation and infiltration of inflammatory cells through the brain endothelium in neurodegenerative diseases has recently emerged (47–49). Moreover, leukocyte-mediated breakdown of blood–brain barrier followed by recruitment into the central nervous system is indeed a process characteristic of several neurodegenerative diseases (47, 49). Our findings on the role of NTBI in endothelial activation as well as monocyte-endothelial interactions identify a potential pathophysiological nature of NTBI in the development of neurodegenerative diseases.

Monitoring the levels of oxygen-derived free radical formation and labile iron inside the cells unraveled the mechanism underlying NTBI-induced endothelial activation. Influx of iron initially joins the labile iron pool in cytoplasm, which is soon stored safely in ferritin or being used for the synthesis of iron-containing proteins. Iron in this transit cytoplasmic pool is metabolically and catalytically reactive (17, 50); therefore, the level is tightly regulated to minimize any potentially toxic reactions. Prolonged exposure to high serum NTBI on HUVECs

in this study had augmented the level of intracellular labile iron. This coincided with induction of cellular reduction-oxidation state imbalance, which led to the phenotype of primed endothelial cells.

Both deferiprone and deferoxamine were capable of reducing the induction of endothelial adhesion molecule expression by C282Y sera. In vivo, the two iron chelators have been shown to prevent hydroxyl radical damage (51). In this study, deferiprone and deferoxamine may have chelated NTBI in those sera, reduced the formation of free radicals, and thereby inhibited endothelial activation. These results not only confirm the involvement of naturally occurring NTBI present in the sera in enhancing the process of monocyte adhesion to endothelium but also demonstrate the possible beneficial effects of iron chelation or induced iron depletion to lessen monocyte-endothelial interactions in individuals with increased serum NTBI levels.

Recently, the adhesion molecule ICAM-1 has been shown to have a predictive value of carotid atherosclerosis progression independently of traditional risk factors and hs-CRP (52). Concordantly, in our experimental conditions, NTBI induced the expression of endothelial adhesion molecules regardless the levels of hs-CRP. Additionally, many factors other than NTBI in the sera could also be involved in the process of endothelial activation.

In conclusion, NTBI from human sera promotes monocyte adhesion to endothelium, by upregulating the expression of endothelial adhesion molecules. The findings support the involvement of NTBI in physiological process of monocyte homing to the tissues, as well as in host-defense mechanism against microbial infections. This study also suggests for a role of NTBI in diseases involving monocyte-endothelial interactions, such as artherosclerosis or neurodegenerative diseases.

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Table 1

Iron parameters and NTBI levels in sera of HFE C282Y homozygotes, heterozygotes, and normal controls

	Normal controls (*n*=21)	HFE C282Y heterozygotes (*n*=8)	HFE C282Y homozygotes (*n*=9)
Serum ferritin (μg/l)	25.5 (3.0–88.0)	343.0 (35.0–1045.0)	928.3 (43.0–4229.0)*
Serum iron (μM)	15.8 (6.0–28.0)	24.9 (16.0–49.0)*	40.0 (31.0–45.0)*†
Transferrin (g/l)	2.5 (1.5–3.6)	2.2 (1.3–2.5)	1.9 (1.5–2.5)*
Transferrin saturation (%)	24.1 (9.1–40.7)	44.5 (27.7–92.8)*	84.7 (56.2–99.6)*†
NTBI (μM)	1.6 (0.2–3.1)	4.0 (2.5–8.1)*	7.9 (5.1–10.0)*†

Values represent the mean; ranges of values are shown in parentheses. NTBI: nontransferrin-bound iron. *$P < 0.050$, for differences between heterozygotes or homozygotes and normal controls; †$P < 0.050$, for differences between homozygotes and heterozygotes.

Fig. 1

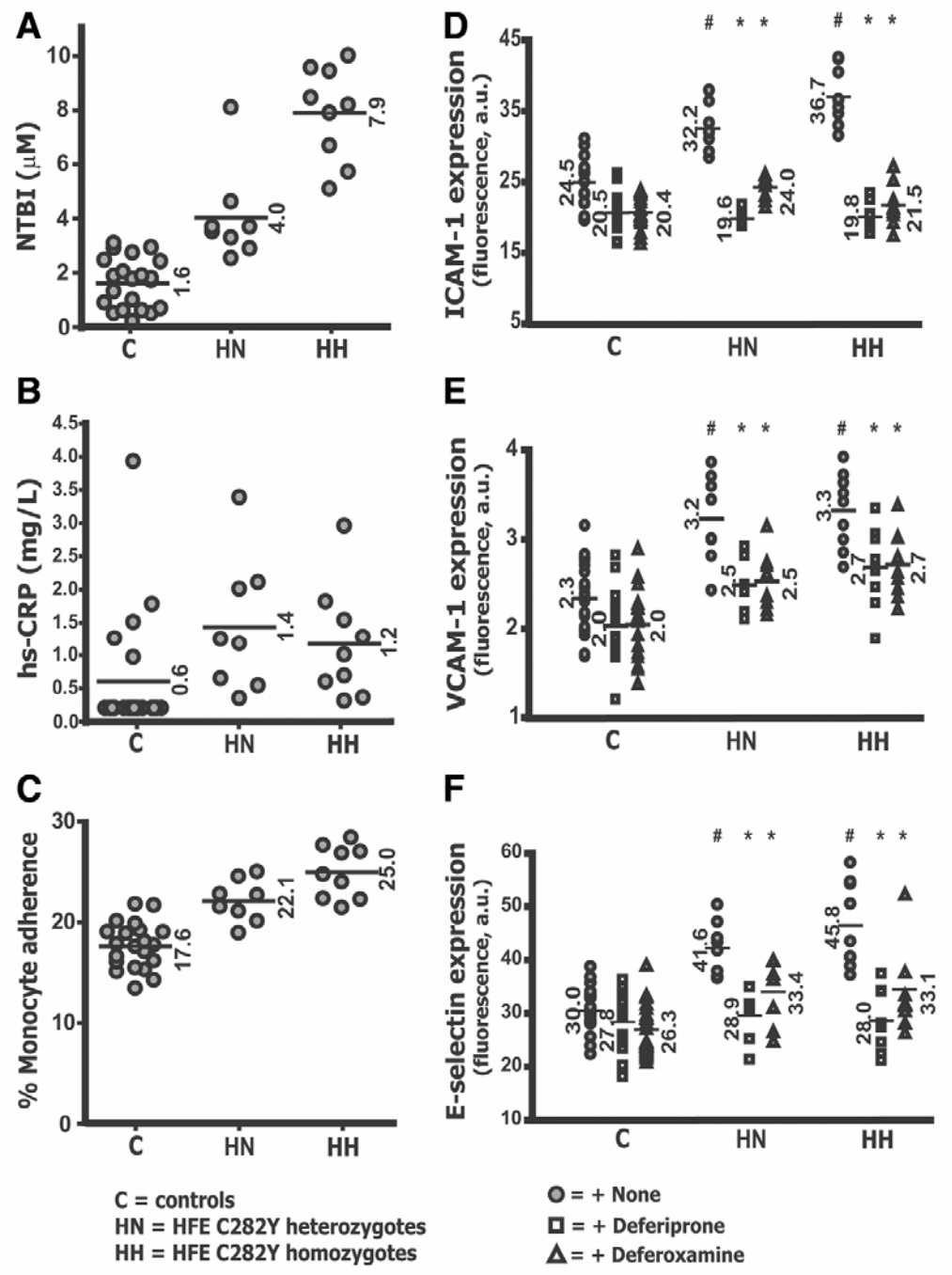

Figure 1. Serum NTBI levels and hs-CRP of HFE C282Y homozygotes, heterozygotes, and controls and their effects on endothelial activation and adhesiveness of monocytes to endothelium. Scatter plots with means of the 3 measured groups, including controls (C, *n*=21), HFE C282Y heterozygotes (HN, *n*=8), and homozygotes (HH, *n*=9), of NTBI (***A***), hs-CRP (***B***), and percentage of monocyte adherence to HUVECs (**C**) treated for 48 h with 50% individual sera (each value is mean of 4 independent experiments in triplicate). ICAM-1 (***D***), VCAM-1 (***E***), and E-selectin (***F***) expression of respective HUVECs from indicated groups is shown, with lines indicating means of each group. HUVECs were treated with 50% individual sera either 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, as indicated. Each value of point is mean of 4–9 independent experiments. #$P < 0.001$, higher than control group. *$P < 0.010$, lower than respective heterozygote or homozygote group with no chelation; a.u.: arbitrary unit.

Fig. 2

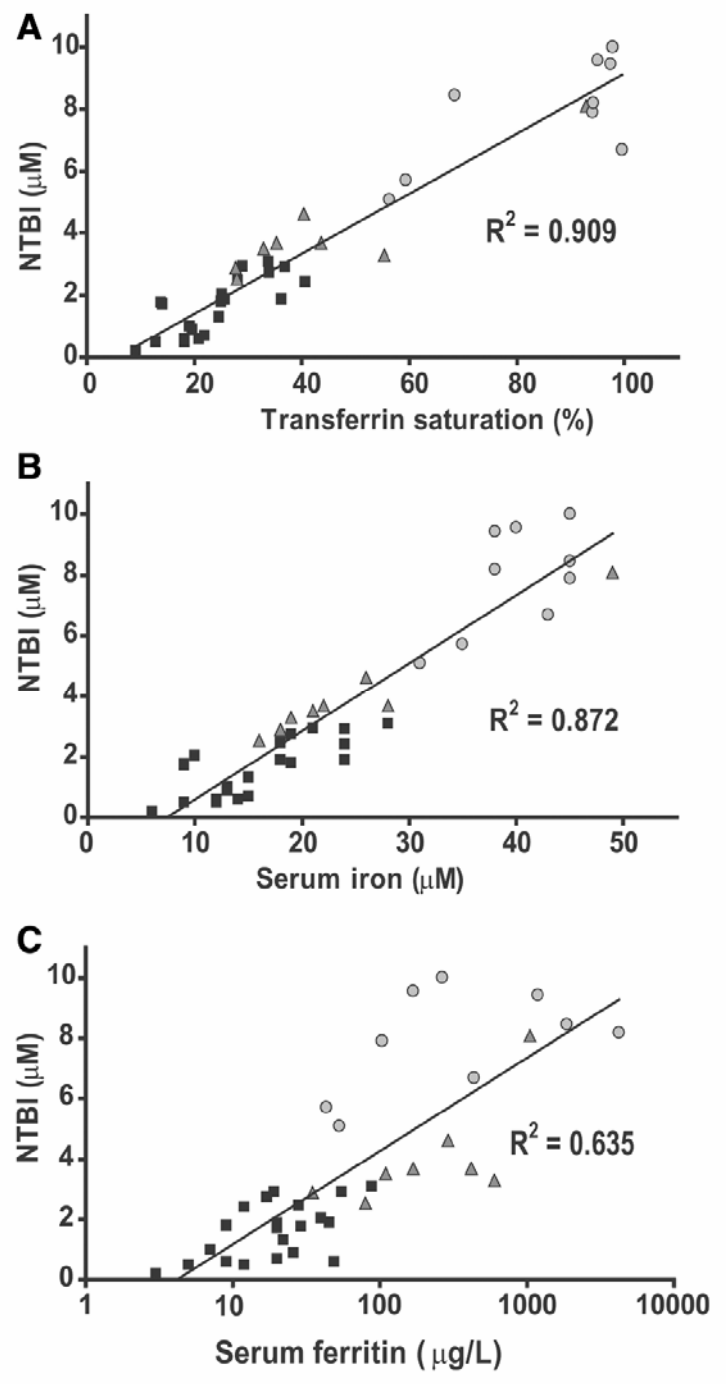

Figure 2. Positive correlation between NTBI and transferrin saturation or serum iron. Linear regression plots of NTBI (*y*-axis) and transferrin saturation (***A***), serum iron (***B***), or serum ferritin (***C***) (*x*-axis). Black squares: controls (*n*=21); dark gray triangles: HFE C282Y heterozygotes (*n*=8); light gray circles: HFE C282Y homozygotes (*n*=9).

Fig. 3

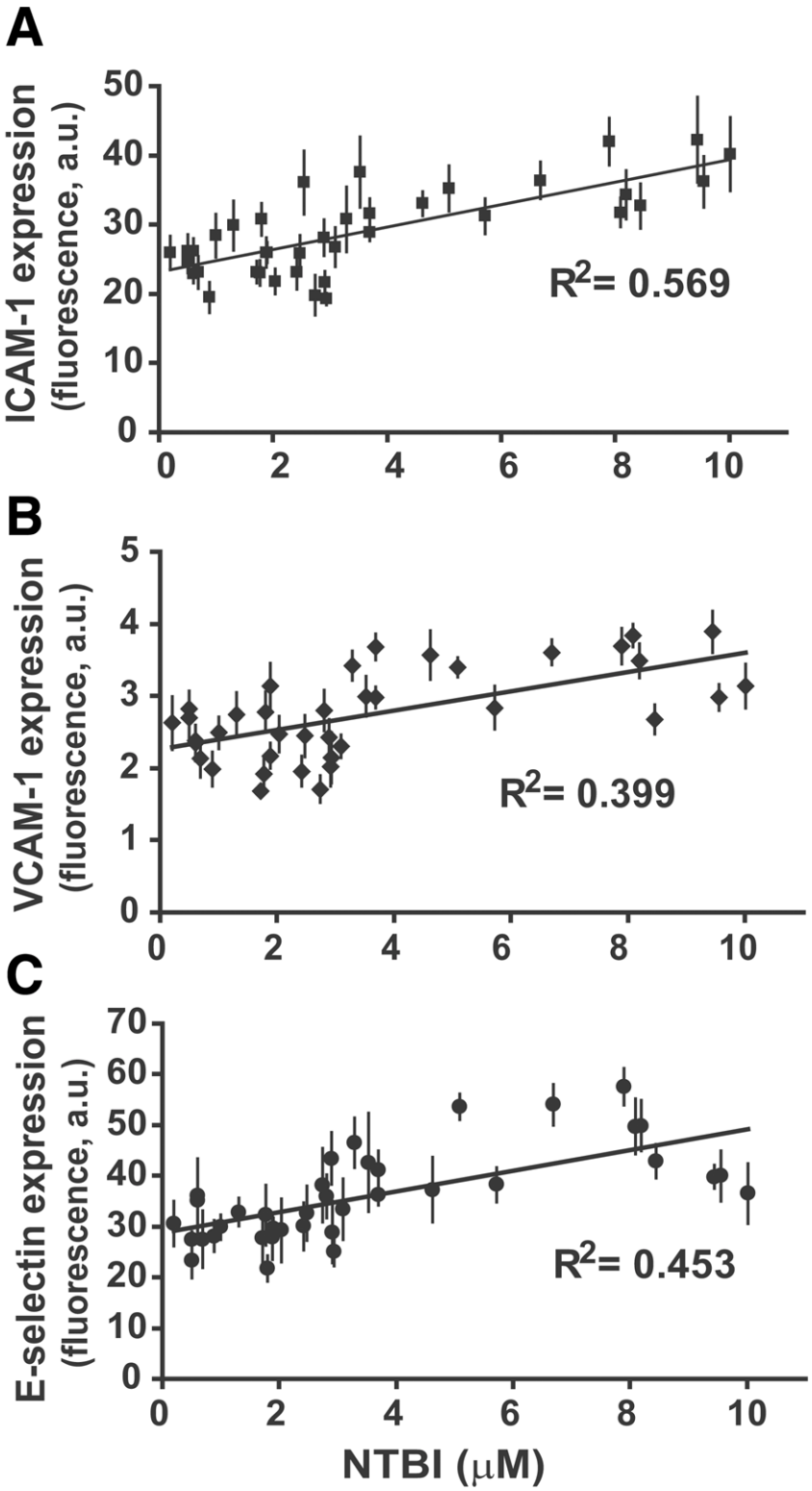

Figure 3. Positive correlation between NTBI and expression of the endothelial adhesion molecules ICAM-1 (***A***), VCAM-1(***B***), and E-selectin (***C***). Linear regression plots of NTBI (*x*-axis) and endothelial adhesion molecule expression (*y*-axis) on HUVECs treated for 48 h with 50% individual sera containing various levels of NTBI. Each value of adhesion molecule expression is mean ± SE of 7–9 independent experiments; a.u.: arbitrary unit.

Fig. 4

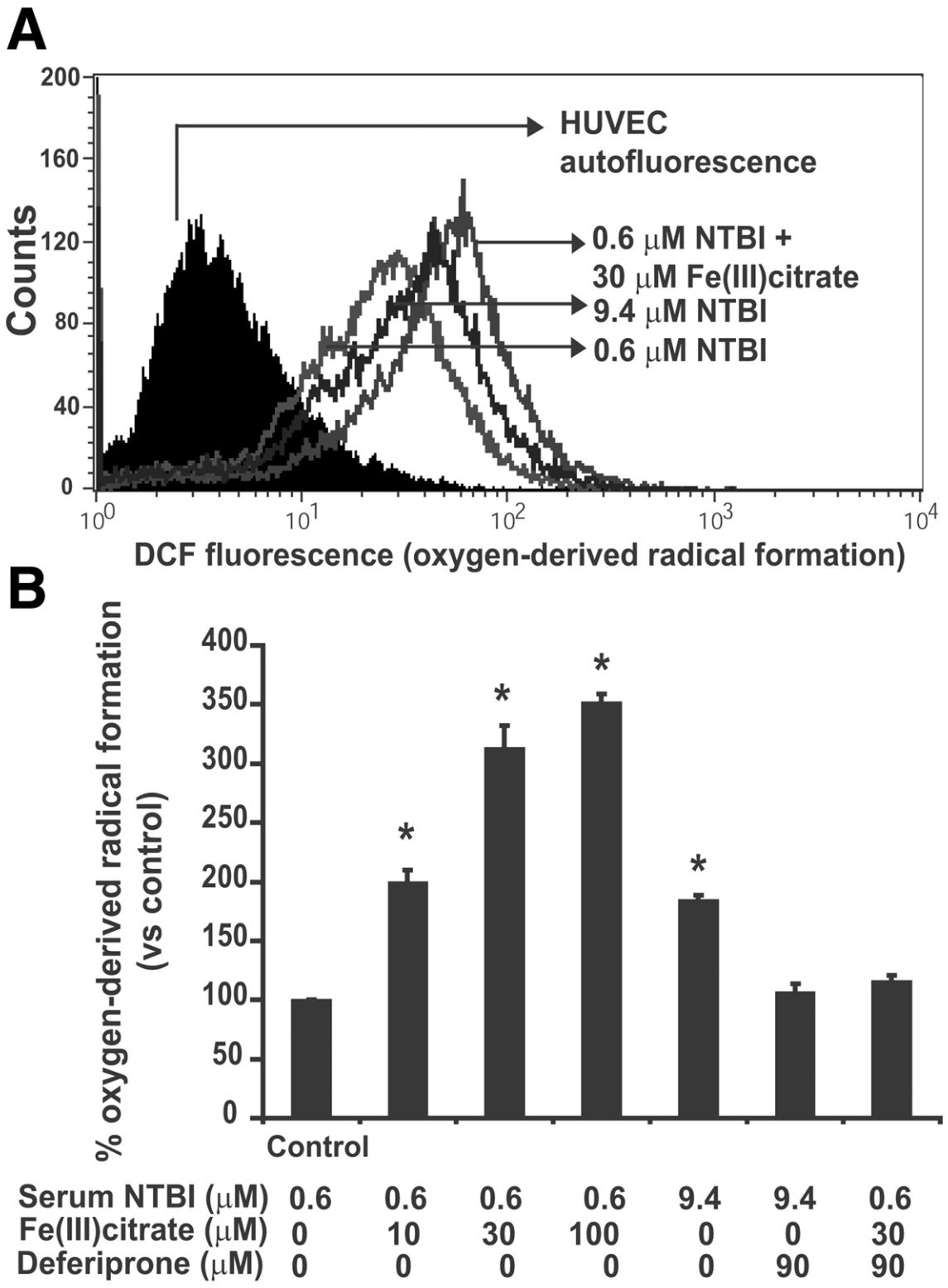

Figure 4. Oxygen-derived radical formation within HUVECs. ***A*)** FACS analysis on DCF fluorescence in HUVECs after indicated treatments for 48 h. ***B*)** Percentage of oxygen-derived radicals formed in HUVECs after indicated treatments for 48 h compared with control. **P* < 0.001, higher than control (*n*=3). All indicated concentrations are half of final concentrations in incubation medium (dilution 1:1).

Fig. 5

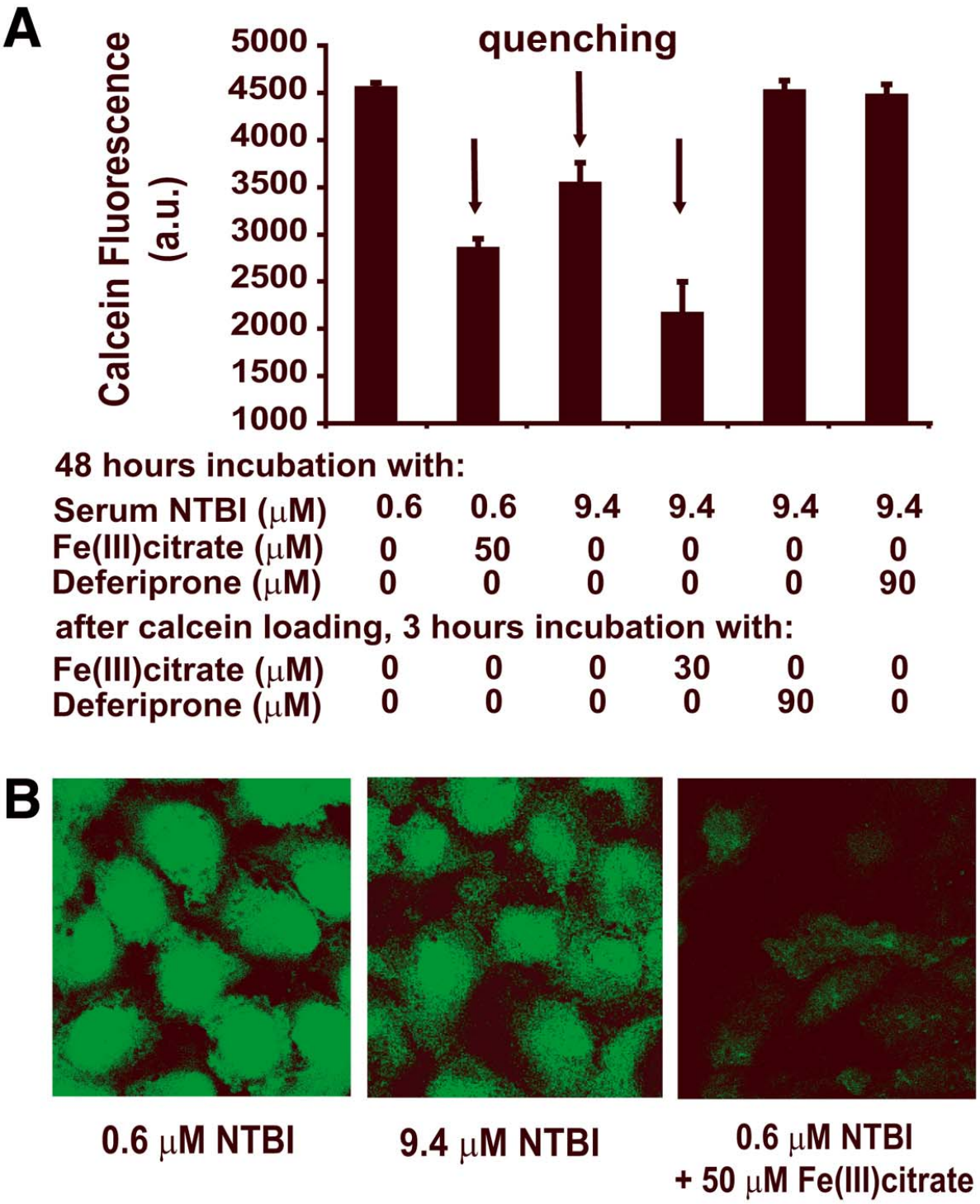

Figure 5. Accumulation of labile iron intracellularly. ***A***) Fluorescence of intracellular calcein signal of HUVECs (*n*=3), which is quenched by iron, after indicated treatments. ***B***) Calcein signal from HUVECs after indicated treatments for 48 h, using a confocal laser microscope. All indicated concentrations are half of final concentrations in incubation medium (dilution 1:1); a.u.: arbitrary unit.