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Hemodialysis-Induced Release of Hemoglobin Limits Nitric Oxide Bioavailability and Impairs Vascular Function

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Objectives This study sought to characterize the impact of hemodialysis (HD)-induced release of hemoglobin on the bioavailability of nitric oxide (NO) and endothelial function.

Background Patients on chronic HD suffer from endothelial dysfunction and a massively increased risk for cardiovascular events. Although dialysis-dependent and -independent factors are discussed, the exact mechanisms are not fully understood.

Methods In 14 HD patients (56 ± 15 years of age), endothelial function was determined by measuring flow-mediated dilation (FMD) of the brachial artery using high-resolution ultrasound before and after treatment. The NO consumption activity of plasma isolated from patients before and after hemodialysis was studied with an NO-sensitive electrode.

Results HD impaired FMD (3.5 ± 2.6% to 1.7 ± 1.4%, $p = 0.04$) without affecting brachial artery diameter (4.7 ± 0.6 mm vs. 4.4 ± 0.9 mm, $p = 0.27$). This was accompanied by an increase in cell-free plasma hemoglobin (196 ± 43 mg/l to 285 ± 109 mg/l, $p = 0.01$), which led to a decrease in the bioavailability of free NO by more than 70%. Oxidation of the released plasma ferrous hemoglobin prevented the consumption of NO. The amount of decompartmentalized hemoglobin after HD correlated inversely with the change in FMD ($r = -0.65$, $p = 0.041$).

Conclusions Our data support a role of HD-induced release of hemoglobin in the pathogenesis of endothelial dysfunction in patients with end-stage renal disease. Approaches that oxidize free plasma hemoglobin may restore NO bioavailability and may have potential beneficial effects on vascular function. (Influence of Hemodialysis on Endothel-Depending Dilatation of Peripheral Arteries; NCT00764192) (J Am Coll Cardiol 2010;55:454–9)

Cardiovascular complications are the major cause of death in patients with end-stage renal disease (ESRD) undergoing hemodialysis (HD) (1). Endothelial dysfunction is an early key step in the development of atherosclerosis (2) and has been attributed to impaired nitric oxide (NO) bioactivity as well as enhanced formation of oxygen-derived free radicals (3). Previous reports showed a decline in NO bioactivity during HD (4). The underlying mechanisms of altered NO bioavailability in these patients are not fully understood. Although activation of cytokines during HD may increase the production of NO (5), NO might be decreased because of increased degradation, diminished NO synthase activity (6), altered serum levels of asymmetric dimethylarginine (7), decreased bioavailability of L-arginine (8), and/or a removal of NO metabolites by dialysis itself (4).

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Gladwin et al. (9) have recently reported a novel mechanism by which the bioavailability of NO is dramatically reduced during decompartmentalization of hemoglobin. Central to this investigation is the understanding that free NO is scavenged at least 1,000 times more rapidly by cell-free hemoglobin than by red blood cells. The rates of

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NO consumption by cell-free and intraerythrocytic hemoglobin suggest that only when hemoglobin is physically compartmentalized within red blood cells will endothelium-derived NO reach concentrations within smooth muscle necessary to activate guanylyl cyclase and cause vasodilation (10). This mechanism has been associated with the vasculopathy of hereditary, acquired, and iatrogenic hemolytic states (11). Importantly, intravascular hemolysis has also been described during HD (12,13). Whether decompartmentalization of hemoglobin contributes to impaired endothelial function in patients undergoing HD is unknown. We therefore hypothesized that HD increases cell-free plasma hemoglobin, which then blunts endothelial function by scavenging NO.

Methods

Study design. In this proof-of-concept study, vascular function and blood parameters were studied immediately before and within 30 min after a single HD session in patients with ESRD. The NO scavenging activity of plasma from patients before and after HD was determined by an NO-sensitive electrode. The study protocol was approved by the institutional review board, and all patients gave written informed consent.

Patients. Patients with ESRD (21 to 80 years of age) who had been on chronic HD for at least 6 months were investigated. Other cardiovascular risk factors and pre-existing cardiovascular disease did not preclude patients from participation in the study. Exclusion criteria were congestive heart failure with a cardiac ejection fraction of <30%, HD-associated hypotension, severe cardiac arrhythmias, acute inflammation (C-reactive protein >5 mg/l), and heart rhythm other than sinus.

HD. All patients underwent HD 3 times per week with a session time of 4 h. All patients were dialyzed with a synthetic low-flux hollow-fiber filter (polysulfone, F-series, Fresenius Medical Care, Bad Homburg, Germany) with a mean blood pump speed of 296 ± 97 ml/min. Bicarbonate-buffered dialysate was used in all sessions. The ultrafiltration rate during the HD procedure was set to reach individual dry weight.

Ultrasound measurement of flow-mediated vasodilation. Endothelial function was measured as flow-mediated dilation (FMD) of the brachial artery as recently described (14). Briefly, the diameter of the brachial artery was measured 1 to 2 cm above the cubital fossa before and after ischemia of the forearm using a 15-MHz transducer (Vivid 7, GE Healthcare, Princeton, New Jersey) (15). Endothelium-independent dilation was measured 4 min after sublingual application of 400-μg glycerol trinitrate (GTN) after HD because GTN may influence hemodynamics and NO bioavailability (16).

Measurements of blood pressure, standard clinical blood parameters, arginase 1, and cell-free plasma hemoglobin. Blood pressure was measured by a sphygmomanometric cuff. Blood was drawn through large-bore angiocatheters to prevent artifactual hemolysis into pre-chilled tubes. Standard clinical blood parameters, including parameters of hemolysis and anemia, were immediately analyzed in a central laboratory using standard techniques. Cell-free hemoglobin was measured in plasma via the QuantiChrom Hemoglobin Assay Kit (BioAssaySystems, Hayward, California). Plasma levels of arginase 1, a cytosolic protein found predominantly in liver and red blood cells (17), were measured via enzyme-linked immunosorbent assay (Human Arginase 1 ELISA Test-kit, Hycult Biotechnology, Uden, the Netherlands; n = 5).

Abbreviations and Acronyms

ESRD = end-stage renal disease
FeCN = ferricyanide
FMD = flow-mediated dilation
GTN = glycerol trinitrate
HD = hemodialysis
NO = nitric oxide

NO consumption assay. The NO consumption was measured using an NO-sensitive electrode as described (9,18). The NO scavenging activity of test substances was measured as a decrease in electrode current, indicating lower NO concentration in the solution. The NO was generated in situ by the decay of the NO donor PROLI NONOate (Cayman Chemical, Axxora, Loerrach, Germany), in argon-purged, essentially anaerobic, phosphate-buffered saline at pH 7.4 (9,19). The NO was continuously monitored with an ISO-NO Mark-II NO meter and an amperometric, NO-specific electrode (WPI Europe, Berlin, Germany) (9,20). Plasma samples (50 μl) were added into the reaction chamber by means of a gas-tight syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland) after NO production by the NO donor reached a stable plateau. The NO consumption was quantitated by dividing the instantaneous decrease in electrode current produced on the addition of samples by the slope of the standard curve, which was generated by additions of oxyhemoglobin standards. Ferricyanide (FeCN) oxidizes Fe^{2+} in the heme group to Fe^{3+}, thereby abolishing the NO-binding capacity of hemoglobin (9). To show that the NO-scavenging activity of plasma is heme dependent, potassium FeCN was added to post-HD patient plasma (2 mmol/l) and incubated for 15 min.

Statistical analysis. Continuous variables are presented as mean ± SD. Pre- and post-HD values were compared using paired *t* tests. Univariate correlations were Pearson correlations. A multivariate regression analysis was performed to determine independent predictors of the change in FMD. Values of $p < 0.05$ were considered to be statistically significant. Statistical analyses were performed using SPSS version 14.0 (SPSS Inc., Chicago, Illinois).

Results

Patient characteristics. The clinical baseline characteristics are shown in Table 1. The ESRD resulted from diabetic nephropathy (n = 2), nephrosclerosis (n = 2), polycystic kidney disease (n = 3), suspected glomerulonephritis (n = 3), and hypertensive/vascular renal damage (n = 4). The average dialysis vintage was 30 ± 23 months. Ultrafiltration (1,385 ± 598 ml) varied according to the patient's actual weight. The latter decreased during HD from 76 ± 14 kg to

Table 1 Clinical Characteristics of Hemodialysis Patients

n (male/female)	14 (12/2)
Age, yrs	56 ± 15
BMI, kg/m^2	24 ± 4
Current smoker	3
Past smoker	2
Diabetes mellitus	3
Hypertension	9
Dyslipidemia	6
CAD	6
1-vessel	1
2-vessel	1
3-vessel	4
CVD	3
Time on dialysis, months	30 ± 23
Medication	
Beta-blockers	12
ACE inhibitors/AT-II antagonists	7
Calcium antagonists	5
Central sympatholytics	4
Diuretic agents	8
Statins	4
Oral antidiabetes agents	1
Insulin	2
Blood parameters	
Serum protein, g/l	67 ± 9
Serum albumin, g/l	40 ± 7
Total cholesterol, mg/dl	154 ± 43
HDL cholesterol, mg/dl	51 ± 13
LDL cholesterol, mg/dl	95 ± 37
Triglycerides, mg/dl	156 ± 55
Plasma glucose, mg/dl	97 ± 24

Values are n or mean ± SD.
ACE = angiotensin-converting enzyme; AT-II = angiotensin II type 1 receptor; BMI = body mass index; CAD = coronary artery disease; CVD = cerebrovascular disease; HDL = high-density lipoprotein; LDL = low-density lipoprotein.

74 ± 14 kg after HD ($p < 0.001$). None of the patients had received blood transfusions during the preceding 6 months.

HD leads to decompartmentalization of hemoglobin. The total concentration of hemoglobin was unaffected by HD (114 ± 15 g/l vs. 115 ± 15 g/l, p = 0.8). Cell-free hemoglobin (196 ± 43 mg/l; equivalent to 19.6 ± 4.3 mg/dl [12.2 ± 9.1 μM] heme) was elevated in patients at baseline before HD as compared with values reported for control subjects who are in the nanomolar range (9). Hemodialysis led to a significant further increase in cell-free plasma hemoglobin to 285 ± 109 mg/l (equivalent to 28.5 ± 10.9 mg/dl [16.8 ± 4.8 μM] heme) (p = 0.01) (Fig. 1), indicating decompartmentalization of hemoglobin. This was accompanied by an increase in plasma arginase 1 concentrations (1.8 ± 0.4 ng/ml to 2.7 ± 0.2 ng/ml, p = 0.03), which is characteristic for red blood cell damage. No signs of apparent hemolysis were observed as evidenced by unchanged levels of lactate dehydrogenase, bilirubin, haptoglobin, and red blood cells (Table 2).

HD leads to a decrease in FMD. Endothelial vasodilator function as measured by FMD decreased after HD as compared with baseline (3.5 ± 2.6% vs. 1.7 ± 1.4%, p = 0.04) (Fig. 1). The GTN response as measured after HD was 7.6 ± 3.7%, showing that the smooth muscle compartment was still responsive to NO.

No differences were seen in baseline diameters of the brachial artery (4.7 ± 0.6 mm vs. 4.4 ± 0.9 mm, before vs. after HD, p = 0.27) or in blood flow at baseline (106 ± 32 ml/min vs. 111 ± 32 ml/min, p = 0.81) and during hyperemia (652 ± 209 ml/min vs. 655 ± 138 ml/min, p = 0.94). This confirmed that the degree of shear stress representing the driving force of FMD was unaffected (Table 2).

Cell-free hemoglobin inversely correlates with the change in FMD. The change in FMD univariately correlated inversely with the baseline diameter of the brachial artery (r = −0.65, p = 0.029), with erythrocytes (r = 0.56, p = 0.046), as well as inversely with cell-free plasma hemoglobin (r = −0.65, p = 0.041) (Table 3) after HD.

NO consumption by cell-free hemoglobin. To provide a mechanistic link between decreased NO-dependent vasodilation and HD-associated decompartmentalization of hemoglobin, the NO consumption of plasma isolated from patients before and after HD was tested ex vivo and compared with oxyhemoglobin standards (Fig. 2). Post-HD plasma containing 28.3 ± 5.7 μM heme consumed significantly more NO (14.0 ± 4.1 μM vs. 8.0 ± 5.3 μM, p = 0.02) as compared with pre-HD plasma containing 12.4 ± 4.3 μM heme (Fig. 3). Consistent with rapid dioxygenation or nitrosylation by plasma hemoglobin, or other ferrous heme species, the quantity of NO consumed by plasma correlated with plasma hemoglobin-related heme levels (r = 0.7, $p < 0.01$). The slope of the linear least-square fit (0.66 μM NO/μM

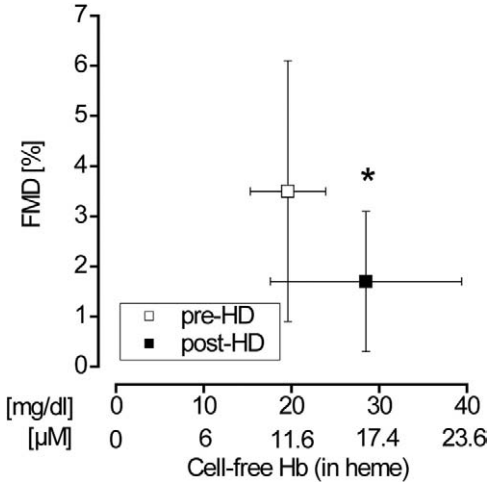

Figure 1 Decompartmentalization of Hb Is Associated With Endothelial Dysfunction During HD

Cell-free plasma hemoglobin (cell-free Hb) increases from 196 ± 43 mg/l (equivalent to 19.6 ± 4.3 mg/dl [12.2 ± 9.1 μM] heme) to 285 ± 109 mg/l (equivalent to 28.5 ± 10.9 mg/dl [16.8 ± 4.8 μM] heme), and flow-mediated dilation (FMD) significantly decreases during a single hemodialysis (HD) session. Data given as mean ± SD (n = 14). *Each $p < 0.05$.

Table 2 Hemodynamics, Vascular Function, and Blood Parameters Before and After HD

	Before HD	After HD	p Value
Hemodynamics			
MAP, mm Hg	103 ± 18	99 ± 22	0.6
Heart rate, beats/min	70 ± 11	71 ± 11	0.9
Vascular function			
Diameter BA, mm	4.7 ± 0.6	4.4 ± 0.9	0.3
FMD, %	3.5 ± 2.6	1.7 ± 1.4	0.04
GTN, %	ND	7.6 ± 3.7	
Blood parameters			
Serum creatinine, mg/dl	8.5 ± 3.3	3.9 ± 1.8	<0.01
Blood urea nitrogen, mg/dl	116 ± 31.0	42 ± 13.5	<0.01
Potassium, mM/l	4.8 ± 0.7	3.9 ± 0.4	<0.01
Calcium, mM/l	2.2 ± 0.4	2.2 ± 0.4	0.5
Phosphate, mM/l	1.7 ± 0.7	0.9 ± 0.4	<0.01
Erythrocytes, T/l	3.6 ± 0.4	3.5 ± 0.4	0.7
Reticulocytes, G/l	76 ± 34	79 ± 34	0.9
Hemoglobin, g/l	114 ± 15	115 ± 15	0.8
Cell-free Hb, mg/l	196 ± 43	285 ± 109	0.01
Cell-free Hb (in heme), mg/dl (μM)	19.6 ± 4.3 (12.2 ± 9.1)	28.5 ± 10.9 (16.8 ± 4.8)	0.01
Arginase 1, ng/ml	1.8 ± 0.4	2.7 ± 0.2	0.03
Hematocrit, %	34 ± 4	36 ± 4	0.08
Haptoglobin, g/l	1.6 ± 1.1	1.4 ± 0.7	0.6
Total bilirubin, mg/dl	0.7 ± 0.4	0.8 ± 0.4	0.4
Lactate dehydrogenase, mg/dl	215 ± 52	217 ± 52	0.4
Iron, μM/l	14 ± 7	15 ± 7	0.7
Ferritin, μg/l	566 ± 325	566 ± 325	0.9
Transferrin, g/l	1.9 ± 0.4	1.9 ± 0.4	0.7
Transferrin saturation, %	30 ± 19	37 ± 19	0.2

BA = brachial artery; FMD = flow-mediated dilation; GTN = glycerol trinitrate; Hb = hemoglobin; HD = hemodialysis; MAP = mean arterial pressure; ND = not defined.

heme) indicates that nearly 70% of the measurable hemoglobin-related heme is competent to consume NO. A hemoglobin-based mechanism for NO consumption was further substantiated by the elimination of plasma NO-consuming activity by potassium FeCN, which oxidizes ferrous hemoglobin to methemoglobin. The FeCN reduced the post-HD plasma consumption of NO to levels observed with pre-HD plasma (Fig. 3). These data indicate that an HD-related increase in NO-consuming activity of patient plasma is iron dependent and is related to the heme concentrations of this plasma, which nearly stoichiometrically consumes micromolar quantities of NO.

Discussion

The key findings of the present study are: 1) HD leads to a decompartmentalization of hemoglobin with an increase in cell-free plasma hemoglobin after a single HD session; 2) FMD is impaired during HD, and the degree of impairment is determined by cell-free hemoglobin levels; and 3) the NO scavenging activity of post-HD plasma is linked to the decompartmentalized hemoglobin and can be reversed by oxidation of the ferrous hemoglobin.

Corroborating previous studies, we observed an acutely blunted endothelial function as measured by FMD after a single HD session (21–23). The FMD of the brachial artery is almost entirely NO synthase–dependent, corre-

Table 3 Univariate Analysis for Predictors of the Change in FMD During a Single HD Session

	Δ FMD	
	r	p Value
Risk factors		
BMI	−0.35	0.238
MAP	−0.11	0.704
Total cholesterol	0.44	0.132
Age	−0.21	0.485
Glucose	−0.18	0.59
Vascular function		
Diameter BA	−0.65	0.029
GTN-induced dilation	−0.22	0.561
Hemodialysis parameters		
Serum creatinine	−0.43	0.181
Blood urea nitrogen	0.08	0.808
Ultrafiltration	−0.12	0.745
Blood pump speed	−0.14	0.659
Hemolysis parameters		
Erythrocytes	0.56	0.046
Reticulocytes	−0.09	0.846
Cell-free hemoglobin	−0.65	0.041
Arginase 1	0.75	0.245
Haptoglobin	−0.07	0.861
Lactate dehydrogenase	−0.33	0.518
Total bilirubin	−0.43	0.158

Abbreviations as in Tables 1 and 2.

lates with endothelial function of most conduit arteries, and can therefore be used as a surrogate for systemic NO bioactivity (24). Here we show the concept that HD leads to the release of hemoglobin, which limits free NO bioavailability. The NO reacts with oxyhemoglobin in a rapid and irreversible reaction that produces nitrate and methemoglobin. The speed and irreversibility of this reaction is such that small amounts of cell-free hemoglobin are sufficient to completely offset endothelial NO production and result in endothelial dysfunction (20). This study is the first to test this theory in an iatrogenic setting. Alternative explanations of the impaired NO-dependent vasodilation include arginase-dependent depletion of the NO synthase substrate L-arginine in vivo. However, our in vitro data show that the majority of NO scavenging activity of post-HD patient plasma is explained by the plasma cell-free hemoglobin/heme content. Furthermore, a predominant hemoglobin-based mechanism responsible for NO consumption in the HD patients presently studied is supported by the elimination of plasma NO consumption after treatment of plasma with FeCN, leading to transition of Fe(II) hemoglobin to methemoglobin. Future studies are necessary to define the time dependence of these effects and to show whether clearance or therapeutic removal of cell-free hemoglobin leads to restoration of NO bioavailability and hence endothelial function.

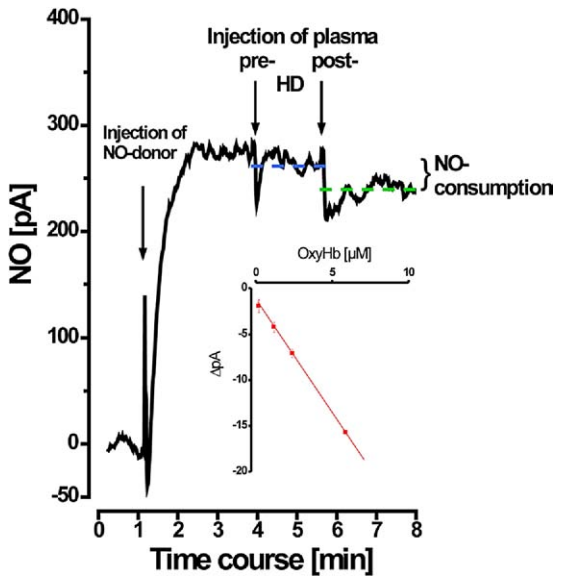

Figure 2 HD Increases NO Consumption in Plasma

Original nitric oxide (NO)-sensitive electrode registration showing that plasma from a patient post-hemodialysis (HD) **(dotted green line)** consumes more NO than plasma from the same patient pre-HD **(dotted blue line)**. The NO was generated in situ, which we measured using amperometrics (current in pA). The NO donor and plasma samples additions are indicated by **arrows**. **(Inset)** Representative plot of the change in current (ΔpA) in response to cell-free oxyhemoglobin (OxyHb) standards in phosphate-buffered solution at pH 7.4. The **solid red line** represents the linear best fit to the data. Data given as mean ± SD (n = 3).

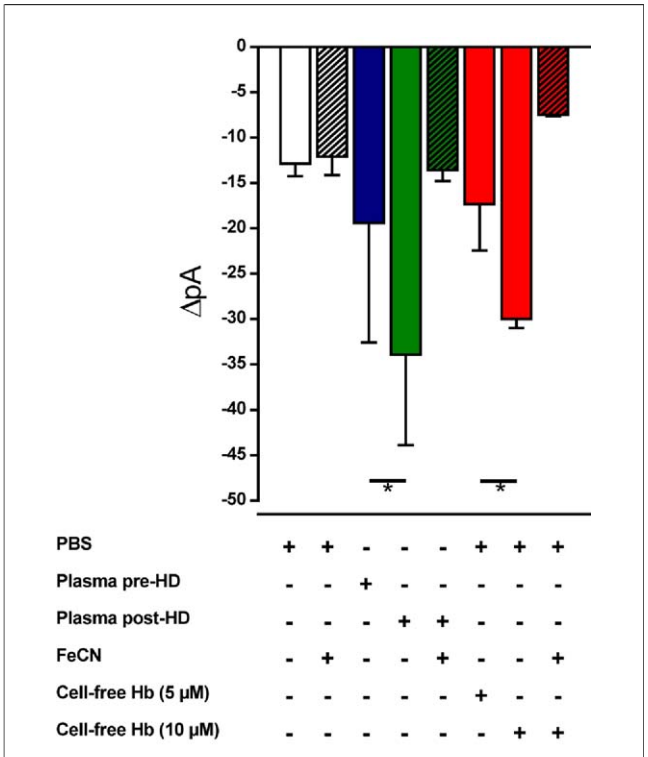

Figure 3 Cell-Free Hb Accounts for NO Consumption

The NO consumption by post-HD plasma **(green bar)** was significantly greater than that exerted by pre-HD plasma ($p < 0.05$) **(blue bar)** and was similar to the effects exerted by 5 and 10 μM cell-free Hb standards (17 ± 5 pA and 30 ± 2 pA, $p < 0.05$, n = 3). Confirming Hb dependence of these effects, the treatment of samples with ferricyanide (FeCN) reduced NO consumption to control levels (phosphate-buffered saline). Data given as mean ± SD. *$p < 0.05$. Abbreviations as in Figures 1 and 2.

Conclusions

Our data suggest that HD-induced release of hemoglobin plays an important role in the pathogenesis of endothelial dysfunction in patients with ESRD. This mechanism is likely to be relevant for other medical interventions that entail red blood cell damage, including coronary artery bypass grafting, cell-saver interventions, extracorporeal membrane oxygenation, and transfusion of aged blood. Therapies that inactivate cell-free plasma hemoglobin by oxidation, such as inhaled NO gas, NO donor infusions, and L-arginine supplementation, restore NO bioavailability and may have potential beneficial effects on vascular function.

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Key Words: nitric oxide ■ endothelial function ■ hemodialysis ■ hemolysis.