Red Blood Cell Dysfunction Induced by High-Fat Diet
Potential Implications for Obesity-Related Atherosclerosis

Dusten Unruh, BS*; Ramprasad Srinivasan, PhD*; Tyler Benson, BA*; Stephen Haigh, BA; Danielle Coyle, MS; Neil Batra, BA; Ryan Keil, BS; Robert Sturm, BS; Victor Blanco, PhD; Mary Palascak, BA; Robert S. Franco, PhD; Wilson Tong, MD; Tapan Chatterjee, PhD; David Y. Hui, PhD; W. Sean Davidson, PhD; Bruce J. Aronow, PhD; Theodosia Kalfa, MD, PhD; David Manka, PhD; Abigail Peairs, PhD; Andra Blomkalns, MD; David J. Fulton, PhD; Julia E. Brittain, PhD; Neil L. Weintraub, MD*; Vladimir Y. Bogdanov, PhD*

Background—High-fat diet (HFD) promotes endothelial dysfunction and proinflammatory monocyte activation, which contribute to atherosclerosis in obesity. We investigated whether HFD also induces the dysfunction of red blood cells (RBCs), which serve as a reservoir for chemokines via binding to Duffy antigen receptor for chemokines (DARC).

Methods and Results—A 60% HFD for 12 weeks, which produced only minor changes in lipid profile in C57/BL6 mice, markedly augmented the levels of monocyte chemotactic protein-1 bound to RBCs, which in turn stimulated macrophage migration through an endothelial monolayer. Levels of RBC-bound KC were also increased by HFD. These effects of HFD were abolished in DARC–/– mice. In RBCs from HFD-fed wild-type and DARC–/– mice, levels of membrane cholesterol and phosphatidylserine externalization were increased, fostering RBC-macrophage inflammatory interactions and promoting macrophage phagocytosis in vitro. When labeled ex vivo and injected into wild-type mice, RBCs from HFD-fed mice exhibited ≈3-fold increase in splenic uptake. Finally, RBCs from HFD-fed mice induced increased macrophage adhesion to the endothelium when they were incubated with isolated aortic segments, indicating endothelial activation.

Conclusions—RBC dysfunction, analogous to endothelial dysfunction, occurs early during diet-induced obesity and may serve as a mediator of atherosclerosis. These findings may have implications for the pathogenesis of atherosclerosis in obesity, a worldwide epidemic. (Circulation. 2015;132:1898-1908. DOI: 10.1161/CIRCULATIONAHA.115.017313.)

Key Words: atherosclerosis • erythrocytes • leukocytes • obesity

Obesity caused by chronic consumption of a hypercaloric, high-fat diet (HFD) is a worldwide epidemic, representing one of the greatest threats to global health.1,2 Leukocytes play a key role in fueling adipose tissue inflammation and insulin resistance in obesity, and also promote atherosclerotic lesion formation and myocardial infarction, the leading cause of death in these patients.1,2 Although the proatherogenic effects of HFD and hyperlipidemia on monocytes have been described in mice and humans,3–5 the impact of HFD on other marrow-derived cells that regulate atherosclerosis is ill-defined.

Evidence is emerging that erythrocytes (red blood cells, RBCs) play an important modulatory role in the development of atherosclerosis. RBCs avidly bind proinflammatory chemokines such as monocyte chemotactic protein-1 (MCP-1), KC/interleukin-8, and RANTES via the Duffy antigen receptor for chemokines (DARC).6 Reversible binding to DARC may alter chemokine levels in the atherosclerotic milieu, depending on factors altering the balance of uptake versus release in RBCs traversing through plaque microvessels. Importantly, RBCs can become entrapped within atherosclerotic lesions at sites of intraplaque hemorrhage, where they are taken up by macrophages. RBC membranes are enriched in cholesterol,
and, in human coronary plaques, cholesterol colocalizes with glycoprophorin-A, a membrane marker of RBCs. Intraplaque hemorrhage positively correlates with advanced atherosclerosis, with the extent of RBC extravasation proportional to plaque development. Nevertheless, the impact of chronic HFD on RBC function is largely unexplored.

Here, we examined the effects of HFD on RBC function in mice. We observed that HFD dramatically enhances chemokine release from RBC-DARC, triggering proinflammatory responses in macrophages and increased macrophage binding to the vascular endothelium. Moreover, RBCs from HFD-fed mice exhibit increased levels of reactive oxygen species and membrane phosphatidylserine (PS) externalization, as well as enhanced phagocytosis by macrophages in vitro. In vivo, these RBCs are more avidly taken up by the spleen, the major organ responsible for the clearance of senescent/apoptotic RBCs. These findings have important implications regarding the potential role of RBCs in the pathogenesis of obesity-related atherosclerosis.

Materials and Methods
Animals and Whole Blood Collection
Please see the online-only Data Supplement for details on mice and blood harvesting procedures.

Enzyme-Linked Immunosorbent Assay and Chemokine Array Assays
Specimens of EDTA anticoagulated blood were divided in half, treated with either 50 U/mL of heparin in plasma-buffered saline (PBS) or vehicle control (PBS), and incubated at room temperature for 30 minutes on a rocking platform. Platelet-poor plasma was harvested by centrifugation at 1500g for 10 minutes at 25°C and used to measure MCP-1 and KC protein levels with and without heparin treatment by commercial enzyme-linked immunosorbent assay kits according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN). Mouse chemokine array (R&D Systems, ARY020) was performed according to the manufacturer’s protocol. In brief, array membranes were blocked and incubated with 100 µg of RBC membranes and array panel detection antibody cocktail overnight at 4°C. After washing, membranes were incubated with streptavidin-horseradish peroxidase, washed, and visualized by using chemiluminescence; mean pixel density was measured using the Bio-Rad Gel Doc XR+ imaging system.

Monocyte Transmigration Assay
bEnd.3 cells (murine endothelial cells, ATCC) were grown to confluence on 3.0-µm pore size 24-well inserts (BD Biosciences); 1×10⁶ packed RBCs from chow diet (CD)-fed (CD-RBC) or HFD-fed (HFD-RBC) mice in defined medium were added to the bottom of the wells. Then, J744.1 cells (murine monocyte/macrophage cell line, ATCC) were added to the inserts and allowed to adhere for 1 hour at 37°C, 5% CO₂. Nonadherent cells were removed by aspiration, and the inserts were filled with fresh defined medium. Migration proceeded for 16 hours at 37°C, 5% CO₂. Inserts were excised and fixed in ice-cold methanol, after which nonmigrated macrophages on the abluminal side of the inserts were removed by using a cotton swab, whereas transmigrated macrophages on the abluminal side were preserved by placing the inserts on slides and mounted by using Vectashield/DAPI (Vector Labs). Images were captured using fluorescence (Nikon Microphot-FXA, 10x, n=5 representative fields per insert), and results were analyzed using Image J (National Institutes of Health).

Flow Cytometry
PS externalization, reactive oxygen species (ROS), and DARC protein levels were measured by flow cytometry after labeling RBCs with Annexin V (Molecular Probes), 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA, Invitrogen), and anti-DARC antibody (R&D Systems, catalog no. AF6695), respectively, using standard techniques as described previously.

Assessment of RBC Deformability
RBCs were subjected to uniform shear stress ranging from 0.3 to 100.0 Pa and the elongation index was determined by using an automated rotational analyzer (LoRRAc Maxxis, Mechatronics, The Netherlands) and the manufacturer’s protocol. The elongation index is calculated by dividing the difference between the major and minor RBC axes by the sum of the axes.

Determination of RBC Membrane Cholesterol Content
For all experiments, RBC membranes (pink ghosts) were prepared according to Hanahan et al; in brief, blood was centrifuged at 1000g for 30 minutes at 4°C, plasma and buffy coat were removed, and RBCs were suspended in 1 mL of 310 mOsm/L (0.172 mol/L) Tris-HCl buffer (pH 7.6). Samples were washed twice with 310 mOsm/L Tris-HCl buffer and resuspended to a final hematocrit of 50%. One mL of RBC suspension was pelleted, RBCs were resuspended in 1 mL of hypotonic (20 mOsm/L) Tris-HCl buffer (pH 7.6) and lysed on ice for 5 minutes. RBC membranes were centrifuged at 20000g for 40 minutes at 4°C, washed 4 times, and resuspended in 20 mOsm/L Tris-HCl buffer to a final volume of 1 mL. Colorimetric assay was performed in 96-well plates; samples were prepared by combining 36 µL of RBC membrane suspensions with 324 µL of Infinity cholesterol reagent (Thermo Scientific catalog no. TR13521), and incubating the mixtures for 1 hour at 37°C. Plates were read at 500 nm; commercial cholesterol preparation (Pointe Scientific, Core Laboratory Supplies catalog no. C7510) was used to derive a standard curve.

Electron Paramagnetic Resonance Analysis of SNO-Hb
Six-week-old C57BL/6 mice were either continued on CD or switched to HFD for 12 weeks. Mice were anesthetized by using isoflurane and blood collected via retro-orbital bleed using EDTA capillary tubes and heparin-coated microfuge tubes. Whole blood was then drawn into a 1-mL syringe, snap-frozen to make a blood core, transferred to a 15-mL conical tube, and stored in liquid nitrogen. S-nitrosohemoglobin (SNO-Hb) measurements were performed by using a Bruker E-scanner (Bruker) and an electron paramagnetic resonance (EPR) finger dewar filled with liquid nitrogen; blood cores were transferred to the finger dewar to keep them frozen during measurements and placed in e-scan sample cavity. All e-scan spectra were collected as an average of 30 scans with the following settings: sweep time of 5.243 seconds, modulation amplitude of 3.0906 Gauss, modulation frequency of 86.000 kHz, microwave power of 0.00219 mW, and microwave frequency of 9.742222 GHz. To compare SNO-Hb content in RBCs collected from mice on CD and HFD, spectra for each group were constructed and the area under the curve was calculated from the double integral by using WinEPR processing software.

Biotin Switch Assay
Six-week-old C57BL/6 mice were either continued on CD or switched to HFD for 12 weeks. Mice were anesthetized by using isoflurane, and blood was collected via retro-orbital bleed using EDTA capillary tubes and heparin-coated microfuge tubes. Whole blood was separated by centrifugation and washed twice with PBS. Packed RBCs (300 µL) were transferred to a new microfuge tube and lysed with 800 µL of 100 µM HEPEs, 1 mM EDTA, and 1 mM Neocuproine; pH 7.7 buffer containing 1% NP40 and protease inhibitors. Samples were spun at 12000g for 10 minutes at 4°C. Fifty µL of RBC lysate were then transferred to a new tube and treated with 5 mL of 100% acetone for 20 minutes at –20°C to precipitate proteins. Lysates were...
pelleted by spinning at 4000g for 5 minutes at 4°C and then washed 3 times with 70% acetone. Protein pellets were then dried and resuspended in HEN buffer+1% sodium dodecyl sulfate (SDS). A positive control was generated by treating one of the samples with 100 µmol/L of Cys-nitrile oxide (NO) and incubating in the dark for 30 minutes. After another round of acetone precipitation and wash, proteins were resuspended in HEN buffer+2.5% SDS with 20 mmol/L s-methyl methanethiosulfonate and incubated at 50°C with vortexing every 4 minutes. Proteins were then precipitated in acetic acid and washed, and pellets were resuspended in 1 mL of PBS containing 1% SDS, 10 mmol/L copper, 10 mmol/L ascorbate, and 200 µmol/L HDPP-biotin, and incubated in the dark for 1 hour. Proteins were precipitated and washed, and pellets were resuspended in 250 µL of HEN+1% SDS and 200 µmol/L dl-dithiothreitol, and aliquots of total protein were stored at −20°C. Neutralization buffer (750 µL) was added to the remaining protein suspension, samples were transferred to a microfuge tube containing 80 µL of washed streptavidin beads, and the samples were incubated overnight at 4°C with gentle rotation. The following day, streptavidin complexes were washed 3 times with neutralization buffer and eluted at 95°C with 100 µL of HEN+2.5% SDS and 3 mMol/L biotin. Eluted samples and total protein aliquots were normalized to total protein content and the degree of S-nitrosylation determined by Western blotting and quantified by densitometry.

In Vitro Erythrophagocytosis Assay

RBCs from EDTA-anticoagulated whole blood were washed 3 times (PBS, 1000g, 5 minutes, room temperature) and buffy coat was removed. Packed washed RBCs were fluorescently labeled by using 1 µmol/L calcein-AM (BD Biosciences) for 30 minutes at 37°C, washed 3 times with PBS, and resuspended in defined medium. Either CD-RBC or HFD-RBC at 2% hematocrit were added to 1×10⁶ thiglycolate-elicited peritoneal macrophages obtained from chow or HFD mice in a 24 well plate. After 4 hours of incubation, the medium was aspirated, nonphagocyotc RBCs were lysed by using RBC lysis buffer, and the macrophages were washed 3 times with PBS. Macrophages were then lysed using 0.1% Triton-X in PBS, and fluorescence was measured using Omega Polaris plate reader (excitation 485 nm, emission 525 nm).

In Vivo Splenic Uptake Assay

One hundred µL of packed CD-RBC or HFD-RBC were labeled with 15 µmol/L Cell-Tracker Red CMTPX fluorescent dye (Invitrogen) for 30 minutes at 37°C, washed with PBS, and suspended in defined medium at 50% hematocrit. Two hundred µL of RBCs were infused via retro-orbital route into CD-fed mice, and after 24 hours the animals were anesthetized and euthanized by cardiac puncture/perfusion with PBS. Spleens were then isolated, rinsed in PBS, embedded in optimum cutting temperature medium cross-sectioned using a cryotome, fixed in formalin, and analyzed under fluorescence. Images were captured, and macrophages that adhered to the luminal endothelium were counted.

Microarray Analysis of Gene Expression

Peritoneal macrophages were obtained by thioglycolact induction of C57BL/6 mice fed either CD or HFD. Erythrophagocytosis was performed as described above by using CD-RBC or HFD-RBC. A third group (no RBCs) served as control. At the end of incubation, macrophages were washed with PBS to remove free RBCs, and total RNA was extracted by using RNeasy kit (Qiagen, Valencia, CA). RNA was then converted to cDNA, amplified, fragmented, and labeled for microarray analysis by using WTA-Ovation FFPE V2 kit and Encore biotin module (Nugen, San Carlos, CA) according to the manufacturer’s instructions. Affymetrix Human Gene 1.0 ST microarray chips were used to assess the gene expression profile (Microarray Core Facility, Cincinnati Children’s Hospital and Medical Center). Transcripts that were differentially expressed as a result of either CD-RBC or HFD-RBC phagocytosis were identified based on filtering for probe sets with Robust Multichip Average–normalized raw expression of >6.0 in either of the 3 pairs of replicates, that differed between treated and untreated macrophages by at least 1.5-fold with P<0.05 using a Welch t test. A total of 642 genes were identified that exhibited significant expression responses as a function of exposure to CD-RBC/HFD-RBC, HFD, or both; 41 genes of the 642 were >1.6-fold upregulated or downregulated in macrophages isolated from HFD versus CD-fed animals. Primary .cel files have been uploaded into GEO database (accession no. GSE50240). The relative levels of expression of mRNA encoding CCL3, interleukin-1β, and chemokine C-X-C motif ligand (CXC) were determined by using the corresponding Taqman probe/primer sets (all from Roche Diagnostics, Indianapolis, IN).

Aortic Ring Assay

Abdominal aortas were isolated from CD- or HFD-fed mice (n=1 mm) were prepared, and 2 rings were used per each experimental condition. Citrated blood from either CD- or HFD-fed mice was centrifuged. RBCs were washed 2 times with PBS, and 100 µL of packed RBC (hematocrit-normalized) were added and incubated with aortic rings in defined media for 4 hours at 37°C (90 rpm). A third group (no RBCs) served as control. The aortic rings were then washed to remove RBCs, and calcein-AM–labeled mouse peritoneal macrophages from mice on CD were added and incubated for 2 hours at 37°C (90 rpm). Subsequently, the aortic rings were washed with PBS and embedded in optimum cutting temperature medium crosswise, snap-frozen, and stored at −80°C. Multiple 10-μm sections were cut using a cryotome, fixed in formalin, and analyzed under fluorescence. Images were captured, and macrophages that adhered to the luminal endothelium were counted.

Statistical Analyses

Statistical analyses were performed and dot plots were generated using SigmaPlot v12.5. The following tests were used: for data sets with ≥2 groups, Kruskal-Wallis; for 2-group data sets, Wilcoxon rank sum; and for paired data sets (heparin versus no heparin), Wilcoxon signed-rank. The appropriate 2-group comparisons were performed and the Bonferroni-Dunn multiple comparisons procedure was used. In the dot plots, thick bars depict mean values and thin bars show standard deviation. P≤0.05 was deemed significant.

Results

RBCs Exhibit a Proinflammatory Phenotype in Response to HFD

To determine the effects of HFD on RBC, mice were fed an obesogenic, 60% fat diet for 12 weeks. Wild-type (WT) and DARC−/− mice similarly gained weight on this diet (Figure IA in the online-only Data Supplement); the fasting lipid profile showed increased cholesterol and triglyceride levels (not shown). Overt diabetes mellitus did not develop in either HFD group, as evidenced by nonelevated hemoglobin A1C levels, although fasting glucose levels were elevated in HFD-fed DARC−/− mice (Figure IB and IC in the online-only Data Supplement). To test whether RBCs carry increased levels of MCP-1 as a consequence of high-fat feeding, we measured plasma MCP-1 before and after RBCs were treated with heparin to release DARC-bound MCP-1.6 Plasma MCP-1 levels in
HFD-fed WT mice were significantly increased in comparison with those in CD-fed WT mice; heparin treatment caused HFD-RBCs to release MCP-1, whereas there was no release from CD-RBC (Figure 1A). Significantly elevated levels of MCP-1 were also detected on RBC membranes (pink ghosts) of HFD-fed WT mice (Figure 1C). As expected, DARC−/− mice exhibited little or no membrane-associated MCP-1. Plasma MCP-1 levels in CD-fed DARC−/− mice were much lower than in CD-fed WT mice, which is consistent with previous reports,11 and HFD produced only a slight elevation of MCP-1 levels in the plasma of DARC−/− mice (Figure 1A and 1C). To examine whether the decreased circulating MCP-1 in DARC−/− mice may be caused by a decrease in its biosynthesis, we also evaluated MCP-1 expression in adipose tissues, a major source of MCP-1 production in obesity, via real-time PCR and enzyme-linked immunosorbent assay. In subcutaneous adipose tissue, no significant differences were noted in MCP-1 mRNA expression levels (not shown) and secreted protein levels between WT and DARC−/− mice (Figure II in the online-only Data Supplement), whereas, in the epididymal adipose tissues, MCP-1 expression tended to be higher in DARC−/− mice (not shown). These data suggest that alterations in MCP-1 biosynthesis do not account for the reduced RBC-bound MCP-1 in DARC−/− mice.

Evidence is emerging that neutrophils play a major role in atherogenesis12; thus, we examined the levels of KC, a critical neutrophil chemoattractant that binds to DARC,13 in plasma and on RBC membranes. Overall, the results were analogous to those for MCP-1 (Figure 1B and 1D); interestingly, treatment with heparin could not dislodge additional KC protein from RBC membranes, indicating that, in comparison with MCP-1, KC likely binds DARC with a higher affinity (Figure 1B). To examine whether HFD affects the levels of other RBC-bound chemokines, we evaluated pink ghost preparations by using a murine chemokine array. HFD significantly decreased the levels of DARC-bound eosinophil-specific chemokine CCL11 (eotaxin, linked to atherosclerotic progression), and increased the levels of DARC-bound chemokine CXCL5, a chemokine that is protective in the context of atherosclerosis13–15 (Figure III in the online-only Data Supplement). Interestingly, the levels of CXCL12, a chemokine that plays a complex role in atherosclerosis and does not bind to DARC,13,16 were significantly decreased on RBC membranes of HFD-fed WT and DARC−/− mice, suggesting reduced production or binding to

![Figure 1](http://circ.ahajournals.org/). HFD-RBCs promote vascular inflammation: increased MCP-1 and KC levels on the surface of the HFD-RBCs in wild-type (WT) mice, but not Duffy antigen receptor for chemokine-deficient (DARC−/−) mice. A and B, ELISA was performed on platelet-poor plasma prepared from whole blood of WT and DARC−/− mice fed CD vs HFD for 12 weeks, with or without heparin treatment. C and D, ELISA was performed on RBC membrane preparations. n=3 to 5 (1 dot = 1 mouse); *P<0.05. CD indicates chow diet; ELISA, enzyme-linked immunosorbent assay; HFD, high-fat diet; MCP-1, monocyte chemoattractant protein-1; n.s., not significant; and RBC, red blood cell.
an alternative RBC receptor in the setting of HFD (Figure III in the online-only Data Supplement). Importantly, we note that the level of DARC expressed on RBC surfaces was not affected by HFD (Figure IV in the online-only Data Supplement).

Because RBC-bound MCP-1 protein levels are significantly higher in HFD, we reasoned that HFD-RBCs would elicit enhanced monocyte recruitment in comparison with CD-RBCs. To test this hypothesis, we performed monocyte transendothelial migration assays by using WT CD-RBCs and HFD-RBCs as the source of chemoattractant. An increase in monocyte transmigration of 1.6-fold (Figure 2) was observed with HFD-RBCs in comparison with CD-RBCs. No significant monocyte transmigration was observed in control experiments in which RBCs were omitted (data not shown).

Production of ROS in WT RBC was studied by flow cytometry by using 2,7'-dichlorodihydrofluorescein fluorescence, which detects peroxides and other oxidant species. 2,7'-Dichlorodihydrofluorescein fluorescence was increased ≈1.2-fold in HFD-RBCs in comparison with CD-RBCs (Figure 3A). Damaged and senescent RBCs externalize PS, which is recognized by macrophages via lactadherin-mediated mechanisms, resulting in RBC clearance from the circulation through phagocytosis. Staining for Annexin V demonstrated greater externalization of PS in HFD-RBCs than in CD-RBCs (Figure 3B). This difference was not attributable to a higher percentage of reticulocytes (immature RBCs with increased levels of externalized PS) in the HFD mice, suggesting that HFD did not result in a large change in RBC lifespan (data not shown).

Phagocytosis and Splenic Uptake of RBCs Are Potentiated by HFD

We next sought to determine whether the alterations in RBCs induced by HFD lead to an increase in RBC phagocytosis. In vitro phagocytosis assays performed by using peritoneal macrophages revealed a ≈1.4-fold increase in phagocytosis of HFD-RBCs in comparison with CD-RBCs (Figure 4A). The spleen is a major organ for clearance of senescent RBCs, and splenic monocytes are involved in systemic inflammation. In the mouse, the spleen acts as a depot from which monocytes efflux to sites of inflammation in a triggered manner. Hence, we tested whether HFD-RBCs are preferentially taken up by the spleen in comparison with CD-RBCs. We injected labeled packed RBCs retro-orbitally into CD-fed mice, because this is an effective and reliable way to introduce cells into the circulation. After euthanization, all mice were perfused with PBS to remove circulating RBCs, and spleens were harvested for analysis. We observed a ≈3-fold increase in splenic uptake of HFD-RBCs in comparison with CD-RBCs (Figure 4B). Judging from the dye pattern, the labeled RBCs were predominately phagocytosed in the marginal zone, which is enriched in monocytes/splenic macrophages. It is noteworthy that reduced RBC deformability (Figure 3D and 3G) has been reported to directly promote increased splenic sequestration.

**Phagocytosis of HFD-RBCs Induces a Proinflammatory Phenotype in Macrophages**

The increased phagocytosis and splenic uptake of proinflammatory HFD-RBCs prompted us to examine whether HFD-RBCs elicit a proinflammatory shift in the monocytes/macrophages with which they interact. To study the effects on global gene expression in macrophages during RBC phagocytosis, we performed a microarray-based gene expression analysis of the macrophages following in vitro phagocytosis of CD-RBCs and HFD-RBCs. Macrophages elicited from mice on both CD and HFD were used in our...
assay. As shown in Figure 5A, macrophages from mice on HFD were basally activated toward a proinflammatory phenotype, consistent with previous reports. In macrophages isolated from mice on CD, RBC phagocytosis per se yielded a change in the global gene expression pattern, rendering it similar to that in macrophages from mice on HFD: genes involved in leukocyte metabolism/tissue repair were upregulated, whereas the expression of chemoattractants was decreased (Figure 5A, and Figure VI in the online-only Data Supplement). In comparison with phagocytosis of CD-RBCs, phagocytosis of HFD-RBCs by the macrophages from mice on HFD caused a significantly more pronounced upregulation of proinflammatory chemokines known to be involved in atherosclerosis: \( \text{Ccl3, Il1b, and Cxcl2} \) by \( \approx 2.5 \), \( \approx 3 \)-fold, and \( \approx 12 \)-fold, respectively (Figure 5A and 5B; Figures VI and VII in the online-only Data Supplement).

Figure 3. HFD alters the biochemical properties of RBCs. Mice were maintained on CD or HFD for \( \geq 12 \) weeks. A, Intracellular ROS levels (DCFH fluorescence). B, PS externalization as measured by Annexin V staining. C, Cholesterol content of RBC membranes. D, RBC deformability index as a function of various shear rates (black line, CD RBCs; red line, HFD RBCs), WT mice. E through G, Annexin V staining, cholesterol content, and elongation index, respectively, in DARC–/– mice fed CD vs HFD. A through C, E, F, n=3 to 8 (1 dot = 1 mouse); *P<0.05. H, RBC nitrosohemoglobin levels (EPR assay), n=6; *P<0.05. AUC indicates area under the curve; CD, chow diet; DARC–/–, Duffy antigen receptor for chemokine-deficient; DCFH, \( 2',7' \)-dichlorodihydrofluorescein; EPR, electron paramagnetic resonance; HFD, high-fat diet; PS, phosphatidylserine; RBC, red blood cell; ROS, reactive oxygen species; and WT, wild type.
HFD-RBCs Promote Endothelial–Macrophage Interactions

Activation of the vascular endothelium is one of the earliest events observed in experimental models of atherosclerosis. In humans, endothelial dysfunction is associated with traditional cardiovascular risk factors, including hyperlipidemia, and is a prominent feature of obesity. Accordingly, we tested whether exposure of HFD-RBCs to blood vessels leads to
endothelial cell activation. We performed an ex vivo experiment wherein we incubated freshly isolated aortic rings with CD-RBCs or HFD-RBCs and then exposed them to fluorescently labeled macrophages. The aortic rings were ≈1 mm thick, and, to ensure the appropriate representation of macrophage adhesion, sections were cut that covered the entire aorta (n=50 per ring). Macrophages that bound to the lumen were counted. Exposure of blood vessels to CD-RBCs had no appreciable effect in comparison with control incubations (in the absence of RBCs); in contrast, HFD-RBCs promoted a ≈3-fold increase in macrophage adhesion to the aortic endothelium (Figure 6).

Discussion

In this report, we demonstrate that HFD induces dramatic alterations in RBCs which promote inflammatory interactions with macrophages and endothelial cells. These RBC alterations are in part dependent on DARC, because HFD-induced proinflammatory responses in RBCs are abrogated in mice lacking this chemokine receptor. Furthermore, the effects of HFD on RBCs lead to enhanced uptake by the spleen in vivo and by macrophages in vitro. Notably, RBCs become entrapped at sites of intraplaque hemorrhage in advanced atherosclerotic lesions, and uptake of cholesterol from RBC membranes contributes to foam cell formation. This process is likely further accelerated by alterations in RBCs induced by HFD. Thus, our findings may be pertinent to mechanisms of atherosclerosis in the setting of obesity, an emerging worldwide epidemic.

Diets high in saturated fat have long been associated with endothelial dysfunction,28 the precursor to atherosclerosis. To our knowledge, however, the effects of HFD on RBCs have not been previously examined. RBCs can functionally bind to lipoproteins, resulting in lipid transfer and perturbation of membrane fluidity and cell morphology.29 In humans, severe hypercholesterolemia is associated with alterations in RBC cholesterol and membrane fluidity,30 which are improved by treatment with statins.31 However, the majority of obese humans are not severely hypercholesterolemic, as was also the case in the present murine study. The diet we used in this study is rich in saturated fat and sucrose, leading to obesity and glucose intolerance, but the duration was not long enough to induce frank diabetes mellitus. Thus, RBC dysfunction, analogous to endothelial dysfunction, may occur early in the course of diet-induced obesity and function as a marker and, possibly, mediator of atherosclerosis.

HFD caused an increase in MCP-1 and KC levels on the surface of murine RBCs, likely via the increase in binding to DARC, as evidenced by our data with DARC−/− mice (Figure 1). RBC-DARC has been previously shown to bind MCP-1 protein and proposed to regulate its circulating concentration in mice and humans.11,32 The levels of plasma MCP-1 were higher in CD-fed WT mice than in CD-fed DARC−/− mice, suggesting that DARC serves as a buffer-sink for MCP-1 under CD conditions; however, we did not detect increased release of MCP-1 in response to heparin in CD-fed WT mice (Figure 1A). The most likely explanation for this observation is that, at low concentrations, MCP-1 preferentially binds to DARC with high affinity and thus cannot be effectively dislodged by heparin. It is possible that RBC-DARC–bound MCP-1 can elicit vascular inflammation at sites of low flow rates, including atherosclerotic lesions, which contain an extensive microvascular network because of proliferation of vasa vasorum.33 Disruption of the delicate vasa vasorum network is a major cause of intraplaque hemorrhage, which likely contributes to atherosclerosis progression and the development of myocardial infarction.7 MCP-1 released from trapped RBCs may also augment inflammation and thus contribute to this process.

Oxidative stress induces proinflammatory gene expression, antagonizes the beneficial actions of nitric oxide, and precedes the onset of HFD-induced insulin resistance in obesity.34 In this light, we found that HFD induced an increase in the generation of intracellular ROS in murine RBCs, although the mechanism through which this occurs is poorly understood. Increased RBC ROS could potentially diminish NO bound to RBC; however, we were not able to detect a significant reduction in RBC NO/SNO-Hb content in mice fed HFD for 12 weeks, although we cannot exclude the possibility that longer-term HFD feeding would have a greater impact on this parameter. Also, an increase in externalized PS, a marker of senescence in normal RBCs, and a marker for apoptosis in deranged RBCs, as well,35 was noted in HFD-RBCs. Furthermore, as a consequence of increased PS externalization, erythropagocytosis was increased in HFD-RBCs in an in vitro setting (Figure 4A). These findings may be pertinent with regard to the uptake of extravasated RBCs by macrophages following intraplaque hemorrhage in atherosclerotic lesions.

Swirsky et al21 demonstrated that the spleen acts as a reservoir for undifferentiated monocytes, which are sequestered...
and subsequently deployed to sites of injury/inflammation. In this context we found that HFD-RBCs are also avidly taken up by the spleen (Figure 4B), where they are phagocytosed by macrophages, especially those surrounding the splenic sinuses. The enhanced phagocytosis of HFD-RBCs by macrophages could potentially act as a trigger for the splenic recruitment and deployment of monocytes, thus influencing atherosclerosis. Considering that splenectomized mice exhibit accelerated atherosclerosis in comparison with sham-operated controls,36 it is tempting to speculate that sequestration of monocytes and RBCs in the spleen in the setting of HFD plays a protective role in atherosclerosis. However, it is important to recognize that the spleen also plays a broad role in regulating immune function, particularly antibody production, and responses to infections. Thus, the role of the spleen in regulating atherosclerosis is likely very complex and merits further investigation.

In macrophages derived from HFD mice, phagocytosis of HFD-RBCs caused a more pronounced upregulation of several proinflammatory genes than phagocytosis of CD-RBC; most notably, \( \text{Il1b} \), \( \text{Ccl3} \), and \( \text{Cxcl2} \) expression was markedly upregulated (Figure 5). Bone marrow from \( \text{Ccl3}(-/-) \) mice, when transplanted to \( \text{LDLr}(-/-) \) mice maintained on Western diet, yielded milder atherosclerotic lesions and lower blood cholesterol and triglycerides, implying a role for bone marrow–derived \( \text{Ccl3} \) in atherosclerosis. However, it is important to recognize that the spleen also plays a broad role in regulating immune function, particularly antibody production, and responses to infections. Thus, the role of the spleen in regulating atherosclerosis is likely very complex and merits further investigation.

In summary, our findings suggest that RBCs play an important role in linking HFD to endothelial dysfunction and activation of macrophages in the pathogenesis of obesity-related atherosclerosis (Figure 7). We propose that erythrocyte dysfunction induced by HFD may be a novel and significant contributor to and mediator of atherogenesis.
B.J. Aronow, A. Peairs, A. Blomkalns, D. Fulton, and J. Brittain participated in designing experiments, analyzed data, and wrote the article. N.L. Weintraub and V.Y. Bogdanov designed research, analyzed data, and wrote the article.

Disclosures

None.

References


**CLINICAL PERSPECTIVE**

High-fat diet (HFD) promotes endothelial dysfunction and proinflammatory monocyte activation, which contribute to atherosclerosis in obesity. Here, we examined the impact of HFD on red blood cells (RBCs), which may play an important modulatory role in atherosclerosis by binding inflammatory chemokines and interacting with macrophages and endothelium within atherosclerotic plaques. We detected a marked increase in the level of chemokines bound to the RBCs of mice fed a 60% HFD for 12 weeks in comparison with mice fed a normal chow diet. Further investigations demonstrated that these chemokines were bound to RBCs via the Duffy antigen receptor for chemokines. Exposure of RBCs from HFD-fed mice to an endothelial monolayer in vitro significantly enhanced macrophage transendothelial migration, confirming the functional importance of RBC-bound chemokines in the setting of HFD. In addition to increasing the level of chemokines bound to RBCs, HFD increased RBC membrane cholesterol content and phosphatidylserine externalization (a marker of RBC damage or senescence), fostering RBC-macrophage inflammatory interactions and promoting the uptake of RBC by macrophages in vitro and by the spleen in vivo. Finally, RBCs from HFD-fed mice augmented macrophage adhesion to the endothelium when incubated with isolated aortic segments, indicating endothelial activation. We propose that RBC dysfunction, analogous to endothelial dysfunction, occurs early during diet-induced obesity and may serve as a mediator of atherosclerosis. These findings may have implications for the pathogenesis of atherosclerosis in obesity, a worldwide epidemic.
Red Blood Cell Dysfunction Induced by High-Fat Diet: Potential Implications for Obesity-Related Atherosclerosis


_Circulation_. 2015;132:1898-1908; originally published online October 14, 2015; doi: 10.1161/CIRCULATIONAHA.115.017313

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/132/20/1898

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2015/10/14/CIRCULATIONAHA.115.017313.DC1
http://circ.ahajournals.org/content/suppl/2016/12/26/CIRCULATIONAHA.115.017313.DC2

**Permissions:** Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

**Reprints:** Information about reprints can be found online at:
http://www.lww.com/reprints

**Subscriptions:** Information about subscribing to *Circulation* is online at:
http://circ.ahajournals.org//subscriptions/
Supplementary Materials and Methods

Animals and collection of whole blood

Six week old C57BL/6 WT or DARC-/- mice (strain #29873, MMRRC-UNC, Chapel Hill, NC) were fed either 10% fat chow diet (CD) or 60% HFD (Research Diets, Inc., D12492) for ≥ 12 weeks. The animals were anesthetized using isoflurane, and whole blood collected in EDTA tubes by cardiac puncture unless indicated otherwise. All animal experiments were approved by the University of Cincinnati and Georgia Regents University Institutional Animal Care and Use Committees.
Suppl. Fig 1: Weight gain (A), fasting glucose (B), and Hb1AC levels (C) in WT and DARC-/- mice maintained on chow and HFD. Animals were fed an obesogenic, 60% fat diet for 12 weeks. n=3-4; *, P<0.05.
Suppl. Fig 2: Levels of MCP-1 protein secreted by subcutaneous adipose tissues are similar in WT and DARC-/- mice maintained on chow and HFD. n=3, *, P<0.05.
Suppl. Fig 3: Levels of CCL11, CXCL5, and CXCL12 on RBC membranes of WT and DARC/-/- mice maintained on chow and HFD. n=5; *, P<0.05.
Suppl. Fig 4: Levels of RBC-DARC are unaffected by HFD. A, representative flow cytometry experiment; B, calculated mean fluorescence in graph form; n=3, P<0.05.
Suppl. Fig 5: Levels of SNO-Hb as assessed by biotin switch assay are unaffected by HFD. 

A, representative western blot; B, calculated mean levels by analysis of relative densitometry in graph form; n=4-5, n.s. chow vs HFD.
Suppl. Fig. 6: Complete unsupervised clustering analysis, changes in gene expression (n=642) observed in macrophages isolated from WT mice maintained on CD or HFD, and exposed to either CD-RBC or HFD-RBC. Note that the bulk of the RBC response signature is recapitulated by HFD-exposed macrophages not exposed to RBC.
Suppl. Fig. 7: Pathway map depicting the major physiological and pathophysiological pathways (group color coded squares: green and brown, respectively) and target genes (red hexagons) affected by the changes in gene expression depicted in Suppl. Fig. 6. The map was compiled using ToppGene Suite (http://toppgene.cchmc.org/). In light green: pathways highly specific for the cells of myeloid lineage as per the compendium maintained by Immunological Genome Project (www.immgen.org).
고지방 식이는 적혈구 세포막의 기능이상을 유발하여 죽상경화를 촉진한다: 비만에서 죽상경화 발생이 촉진되는 기전

김 상 현 교수 서울대학교 보라매병원 순환기내과

초록

배경
고지방 식이는 비만 환자에서 내피세포 기능장애와 염증반응 유발 단핵구(proinflammatory monocyte)를 활성화시켜, 죽상경화를 촉진한다. 본 연구는 고지방 식이가 적혈구 세포막의 기능이상을 유발하는지, 그리고 그에 따라 duffy antigen receptor for chemokine(DARC)에 염증반응 유발인자들이 부착-유리되는 비율이 변화되며, 결과적으로 염증반응을 촉진하는지를 확인하기 하였다.

방법 및 결과
C57/BL6 생쥐에게 12주 동안 60% 고지방 식이를 먹인 결과, 클레ست롤과 중성지방 수치는 약간 증가하고, 적혈구 세포막에 부착된 monocyte chemotactic protein-1(MCP-1)은 매우 증가하는데, 이것은 대식세포가 내피세포층을 통과하여 이동하는 것을 촉진한다. 또한, 고지방 식이는 적혈구 세포막에 부착된 KC 단백질 수치를 증가시켰다. 이러한 고지방 식이의 대식세포 이동 촉진효과는 DARC⁻⁺ 생쥐에서는 관찰되지 않았다. 고지방 식이를 섭취한 정상 생쥐와 DARC⁻⁺ 생쥐의 적혈구 in vitro 실험에서, 세포막 클레스트롤과 phosphatidylserine의 세포 박 이동(externalization)이 증가하였고, 그에 따른 적혈구-대 식세포 상호작용을 향상시켜 대식세포 이동과 대식작용을 증가시켰다. 고지방 식이를 섭취한 생쥐의 적혈구에 표지자를 부착하여 정상 생쥐에 주입하면 표지된 적혈구들은 정상 적혈구보다 3배 이상 비장에 부착되었다. 그리고 고지방 식이를 섭취한 생쥐의 적혈구를 대동맥 조직과 박테리아, 대동맥 내피세포가 활성화되어 대식세포의 내피세포 부착이 증가하였다.

결론
비만을 유발하는 식이 섭취는 내피세포의 기능이상 외에도 적혈구의 기능이상을 유발하여 죽상경화를 촉진한다. 전세계적으로 증가하고 있는 비만 환자들에서 죽상경화의 병태생리 진행 과정에 적혈구가 관여한다는 것을 알 수 있다.