JACC
JOURNAL OF THE AMERICAN COLLEGE OF CARDIOLOGY
AUGUST 14, 2018
VOLUME 72
NUMBER 7

ORIGINAL INVESTIGATIONS

THE PRESENT AND FUTURE

Audio summaries from Dr. Valentin Fuster, JACC Editor-in-Chief.

■ www.onlinejacc.org

ELSEVIER

ISSN 0735-1097

JOURNAL OF THE AMERICAN COLLEGE OF CARDIOLOGY VOL. 72, NO. 7, 2018

PUBLISHED BY ELSEVIER

Erythrocytes From Patients With Type 2 Diabetes Induce Endothelial Dysfunction Via Arginase I

Zhichao Zhou, MD, PhD,[a,*] Ali Mahdi, BSc,[a,*] Yahor Tratsiakovich, MD, PhD,[a] Szabolcs Zahorán, MSc,[b] Oskar Kövamees, MD, PhD,[a] Filip Nordin, BSc,[a] Arturo Eduardo Uribe Gonzalez, MD,[c] Michael Alvarsson, MD, PhD,[d] Claes-Göran Östenson, MD, PhD,[d] Daniel C Andersson, MD, PhD,[c,e] Ulf Hedin, MD, PhD,[f] Edit Hermesz, PhD,[b] Jon O Lundberg, MD, PhD,[c] Jiangning Yang, MD, PhD,[a] John Pernow, MD, PhD[a,e]

ABSTRACT

BACKGROUND Cardiovascular complications are major clinical problems in type 2 diabetes mellitus (T2DM). The authors previously demonstrated a crucial role of red blood cells (RBCs) in control of cardiac function through arginase-dependent regulation of nitric oxide export from RBCs. There is alteration of RBC function, as well as an increase in arginase activity, in T2DM.

OBJECTIVES The authors hypothesized that RBCs from patients with T2DM induce endothelial dysfunction by up-regulation of arginase.

METHODS RBCs were isolated from patients with T2DM and age-matched healthy subjects and were incubated with rat aortas or human internal mammary arteries from nondiabetic patients for vascular reactivity and biochemical studies.

RESULTS Arginase activity and arginase I protein expression were elevated in RBCs from patients with T2DM (T2DM RBCs) through an effect induced by reactive oxygen species (ROS). Co-incubation of arterial segments with T2DM RBCs, but not RBCs from age-matched healthy subjects, significantly impaired endothelial function but not smooth muscle cell function in both healthy rat aortas and human internal mammary arteries. Endothelial dysfunction induced by T2DM RBCs was prevented by inhibition of arginase and ROS both at the RBC and vascular levels. T2DM RBCs induced increased vascular arginase I expression and activity through an ROS-dependent mechanism.

CONCLUSIONS This study demonstrates a novel mechanism behind endothelial dysfunction in T2DM that is induced by RBC arginase I and ROS. Targeting arginase I in RBCs may serve as a novel therapeutic tool for the treatment of endothelial dysfunction in T2DM. (J Am Coll Cardiol 2018;72:769–80) © 2018 by the American College of Cardiology Foundation.

Type 2 diabetes mellitus (T2DM) is an important risk factor for cardiovascular diseases, including atherosclerosis and ischemic heart disease. Endothelial dysfunction plays a major role in the etiology of diabetes-induced macrovascular and microvascular complications. This encompasses an imbalance between vasodilators and anti-inflammatory molecules including nitric oxide (NO),

Listen to this manuscript's audio summary by *JACC* Editor-in-Chief Dr. Valentin Fuster.

From the [a]Division of Cardiology, Department of Medicine, Karolinska University Hospital, Karolinska Institutet, Stockholm, Sweden; [b]Department of Biochemistry and Molecular Biology, Faculty of Science and Informatics, University of Szeged, Szeged, Hungary; [c]Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden; [d]Division of Endocrinology and Diabetology, Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden; [e]Heart and Vascular Theme, Karolinska University Hospital, Karolinska Institutet, Stockholm, Sweden; and the [f]Division of Vascular Surgery, Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden. *Dr. Zhou and Mr. Mahdi contributed equally to this work. Dr. Pernow has received grants to support this work from the Swedish Research Council, the Swedish Heart and Lung Foundation, the Stockholm County Council (ALF), the Karolinska Institutet/Stockholm County Council Strategic Cardiovascular Program, the Söderberg Foundation, the Novo Nordisk Foundation, and the Diabetes Research and Wellness Foundation. Dr. Zhou has received grants from the Olausson Fund of Thorax, Karolinska University Hospital and the Karolinska Institutet Fund for Young Scientist. All other authors have reported that they have no relationships relevant to the contents of this paper to disclose.

Manuscript received February 5, 2018; revised manuscript received May 5, 2018, accepted May 20, 2018.

ISSN 0735-1097/$36.00 https://doi.org/10.1016/j.jacc.2018.05.052

ABBREVIATIONS AND ACRONYMS

ABH = 2(S)-amino-6-boronohexanoic acid

EC = endothelial cell

EDR = endothelium-dependent relaxation

EIR = endothelium-independent relaxation

eNOS = endothelial nitric oxide synthase

GK = Goto-Kakizaki

H_2O_2 = hydrogen peroxide

Healthy RBC = red blood cell from healthy subjects

KH = Krebs-Henseleit

NAC = N -acetyl-cysteine

NADPH = nicotinamide adenine dinucleotide phosphate

NO = nitric oxide

NOS = nitric oxide synthase

NOX = nicotinamide adenine dinucleotide phosphate oxidase

RBC = red blood cell

ROS = reactive oxygen species

T2DM = type 2 diabetes mellitus

T2DM RBC = red blood cell from patients with type 2 diabetes mellitus

vasoconstrictors, and proinflammatory molecules including reactive oxygen species (ROS) (1). The pathogenesis of endothelial dysfunction in T2DM is complex and multifactorial, and this may explain why glucose-lowering therapy has not convincingly reduced mortality among patients at high risk for cardiovascular events (2). Therefore, there is an unmet need to identify key disease mechanisms behind vascular complications in T2DM to develop novel therapies that specifically target such complications.

SEE PAGE 781

Impaired NO bioavailability occurs early and contributes to progression and prognosis of cardiovascular complications in T2DM. NO is produced from L-arginine by endothelial NO synthase (eNOS), which competes with arginase for their common substrate, L-arginine (3). The expression and activity of the 2 isoforms arginase I and II are increased in cardiovascular diseases, including T2DM triggered by several factors including glucose and ROS (3). The increased arginase activity is an important cause of endothelial dysfunction in T2DM as a result of the competition with eNOS for L-arginine and excessive ROS formation stemming from uncoupled eNOS, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), or mitochondrial complexes (3). Accordingly, arginase inhibition markedly improves endothelial function in patients with T2DM, a finding suggesting that arginase may serve as a potential therapeutic target for improvement of vascular function (4,5).

Red blood cells (RBCs) may play a fundamental role in cardiovascular homeostasis, by contributing to vascular function and integrity independently from their function as oxygen transporters (6). RBCs undergo functional alterations in T2DM, including reduced NO bioavailability (7) or enhanced oxidative stress (8), which subsequently may affect vascular function. We demonstrated a crucial role of RBCs in control of cardiac function through arginase-dependent regulation of export of NO-like bioactivity from RBCs, thus suggesting a direct interaction of RBCs with cardiovascular function (9). However, key mechanisms behind the interaction of RBCs with the vasculature and their importance in cardiovascular diseases are unclear and elusive. Previous studies suggested that arginase is up-regulated in RBCs from patients with T2DM (10,11). The functional implications of this finding are unknown but may imply a possible causative role of RBC arginase for endothelial dysfunction in T2DM.

Therefore, the present study was designed to test the hypothesis that RBCs from patients with T2DM induce endothelial dysfunction and that this effect is mediated by up-regulation of arginase and ROS formation. We demonstrate a detrimental effect of RBCs from patients with T2DM on endothelial function through up-regulation of arginase at both RBC and vascular levels. In addition, we show that RBCs increase endothelial cell (EC) arginase activity and expression through an ROS-dependent mechanism. These results demonstrate a novel role of RBCs in the development of endothelial dysfunction in T2DM.

METHODS

All experimental protocols regarding human materials were conducted according to the Declaration of Helsinki and were approved by the regional ethical review board in Stockholm. All subjects were informed of the purpose and gave their oral and written consent. All protocols regarding animal studies were approved by the regional ethical committee and conformed to the Guide for Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication no. 85-23, revised 1996). An expanded methods section is available in the Online Appendix.

RBC-TISSUE CO-INCUBATION AND FUNCTIONAL EXPERIMENTS. RBCs were isolated from patients and rats with T2DM. Subsequently, RBCs were diluted with Krebs-Henseleit (KH) or serum-free culture medium to a hematocrit of 45% or 10%, and were incubated with aortic rings isolated from rats or internal mammary arteries (IMAs) from nondiabetic patients in cell culture incubator at 37°C with 5% carbon dioxide for 18 h or 1 h. Control arteries were incubated with isolated RBCs from healthy controls, with KH buffer, or with supernatant from RBCs that underwent 18 h incubation. Following incubation, the vessels were thoroughly washed and mounted in wire myograph for determination of endothelium-dependent relaxation (EDR) and endothelium-independent relaxations (EIR) by cumulatively increasing concentrations (10^{-9} to 10^{-5} M) of acetylcholine and sodium nitroprusside, respectively. Additional RBCs and RBC-incubated vessels were subjected to molecular analysis. The same protocols were repeated in the presence of various inhibitors

against arginase, NOX, ROS, and hydrogen peroxide (H_2O_2).

STATISTICS. Concentration-response curves were analyzed with 2-way analysis of variance with repeated measurement. Differences between 2 groups were determined with the *t*-test or nonparametric equivalent, depending on distribution. Analyses of 3 or more groups were determined with 1-way analysis of variance. Data are presented as mean ± SEM unless otherwise indicated; $p < 0.05$ was considered statistically significant.

RESULTS

BASAL CHARACTERISTICS. Basal characteristics are shown in Table 1. The patients with T2DM had higher body mass index, fasting glucose, glycated hemoglobin, and triglycerides, whereas they had lower total cholesterol, high-density lipoprotein, and low-density lipoprotein cholesterol in comparison with the healthy subjects. Seven of the patients with T2DM and 1 of the healthy subjects were smokers at the time of inclusion. None of the healthy subjects took medication.

To evaluate endothelial function in vivo in patients with T2DM and healthy subjects, forearm endothelium-dependent vasodilation and endothelium-independent vasodilation were determined (4). Baseline forearm flow did not differ significantly between patients with T2DM (37.4 ± 5.0 ml/min/1,000 ml) and healthy subjects (29.1 ± 2.6 ml/min/1,000 ml). Endothelium-dependent vasodilation was significantly lower in patients with T2DM than in healthy subjects (Online Figure 1A), whereas endothelium-independent vasodilation did not differ (Online Figure 1B).

TABLE 1 Basal Characteristics of Subjects

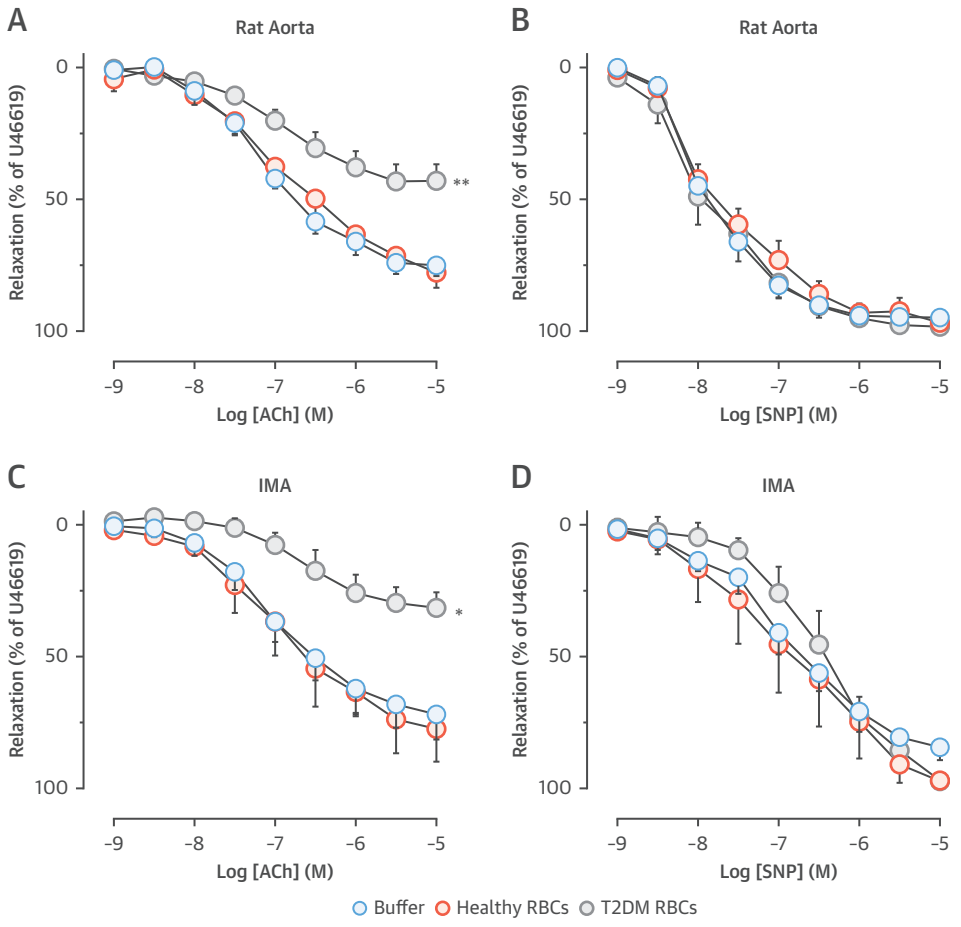
	Healthy Subjects (n = 34)	Type 2 Diabetes (n = 46)
Age, yrs	61 ± 7	60 ± 12
Male	31	36
BMI, kg/m^2	25.0 ± 2.7	30.6 ± 5.1*
Systolic BP, mm Hg	134 ± 16	137 ± 16
Diastolic BP, mm Hg	81 ± 8	79 ± 9
Fasting glucose, mM	5.7 ± 0.5	11.4 ± 3.2*
Smokers	1	7
HbA_{1c}, mmol/mol	36 ± 3	70 ± 20*
Hemoglobin, g/l	144 ± 8	139 ± 17
Creatinine, µmol/l	83 ± 14	88 ± 29
Triglycerides, mmol/l	1.1 ± 0.4	1.9 ± 1.0*
Total cholesterol, mmol/l	5.2 ± 0.9	4.3 ± 1.3*
HDL, mmol/l	1.5 ± 0.4	1.1 ± 0.3*
LDL, mmol/l	3.2 ± 0.8	2.2 ± 1.0*
Vascular complications		
Coronary artery disease	0	7
Retinopathy	0	6
Neuropathy	0	5
Nephropathy	0	5
Peripheral vascular disease	0	5
Medications		
ACE inhibitor/ARB	0	27
Aspirin	0	18
Lipid lowering	0	37
β-blocker	0	12
Calcium-channel blocker	0	12
Insulin	0	31
Metformin	0	31
GLP-1 analogue	0	14
DPP-4i	0	9
SU	0	2
SGLT2i	0	3

Values are mean ± SD or n. *$p < 0.001$ versus healthy subjects.

ACE = angiotensin-converting enzyme; ARB = angiotensin receptor blocker; BMI = body mass index; BP = blood pressure; DPP-4i = dipeptidyl peptidase-4 inhibitor; GLP-1 = glucagon like peptide-1; HbA_{1c} = glycosylated hemoglobin; HDL = high-density lipoprotein; LDL = low-density lipoprotein; SGLT2i = sodium-glucose co-transporter inhibitor; SU = sulfonylurea.

RBCs FROM PATIENTS WITH T2DM INDUCE ENDOTHELIAL DYSFUNCTION. EDR and EIR of rat aortic rings were determined following 18 h co-incubation with RBCs. EDR was significantly impaired following incubation with RBCs (hematocrit ~45%) from patients with T2DM (T2DM RBCs), whereas it was unaffected by RBCs from healthy subjects (Healthy RBCs) in comparison with incubation in KH buffer (**Figure 1A**). EIR was unaffected by T2DM RBCs (**Figure 1B**), a finding suggesting that the RBCs selectively impair endothelial function. Incubation with T2DM RBCs at ~10% hematocrit for 18 h or at ~45% hematocrit for 1 h did not induce endothelial dysfunction (Online Figures 2A and 2B). Incubation with the supernatant collected from isolated RBCs diluted in buffer to hematocrit 45% stored for 18 h or from the final washing step of RBCs did not affect endothelial function, a finding demonstrating that the endothelial dysfunction was mediated by the RBCs (Online Figures 2C and 2D). T2DM RBCs also induced endothelial dysfunction in human internal mammary arteries (**Figure 1C**), whereas internal mammary artery smooth muscle cell function was not affected (**Figure 1D**). T2DM RBCs induced endothelial dysfunction irrespective of whether the patients were receiving statin treatment or not (Online Figure 2E). EDR was also impaired in healthy rat aortas incubated with RBCs from Goto-Kakizaki (GK) rats (12) to an extent similar to that observed in GK rat aortas incubated with KH buffer (Online Figure 2F). EIR was not affected in aortas from nondiabetic rats incubated with RBCs from GK rats or aortas from GK rats incubated with KH buffer (Online Figure 2G). T2DM RBCs

FIGURE 1 RBCs From Patients With T2DM Impair Endothelial Function

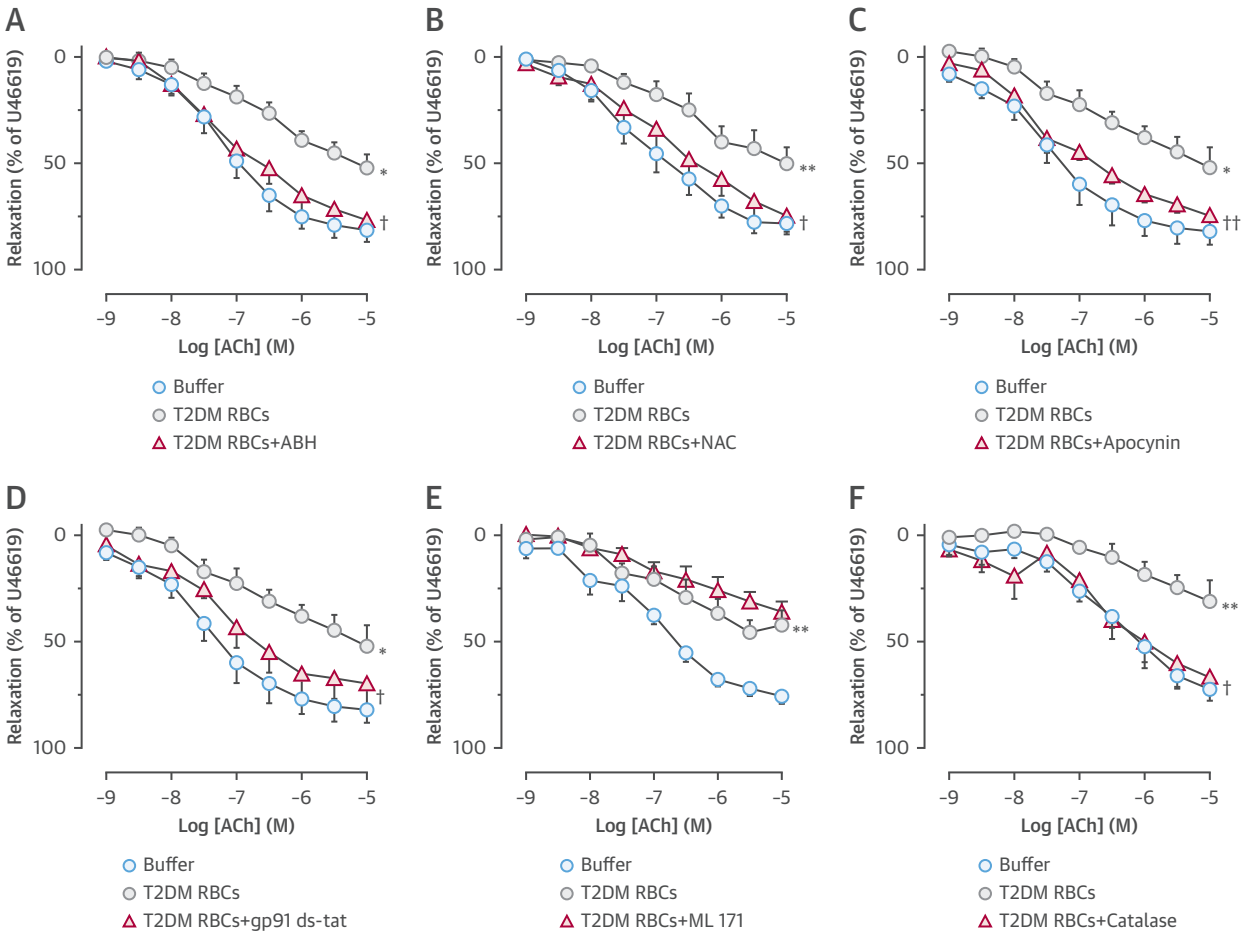


(A and C) Endothelium-dependent relaxation to acetylcholine (ACh) and **(B and D)** endothelium-independent relaxation to sodium nitroprusside (SNP) in isolated rat aortas (**A and B**, n = 5 to 9) and human internal mammary arteries (IMAs) (**C and D**, n = 3 to 5) incubated with buffer, red blood cells from healthy subjects (Healthy RBCs), and red blood cells from patients with type 2 diabetes mellitus (T2DM RBCs) for 18 h. Values are mean ± SEM. *p < 0.05, **p < 0.01 versus buffer or healthy red blood cells representing the concentration-response relation by 2-way analysis of variance.

or RBCs from GK rats incubated with aortas from GK rats did not induce additional endothelial dysfunction beyond that observed after incubation with Healthy RBCs or buffer (Online Figures 2H and 2I).

RBC ARGINASE AND ROS ACCOUNT FOR ENDOTHELIAL DYSFUNCTION IN T2DM. Next, we investigated whether RBC arginase and ROS account for the development of endothelial dysfunction. Co-incubation with the arginase inhibitor 2(S)-amino-6-boronohexanoic acid (ABH) for 18 h prevented the impairment of EDR induced by T2DM RBCs in rat aortas (**Figure 2A**). Moreover, ROS scavenging by N-acetyl-cysteine (NAC) prevented endothelial dysfunction induced by T2DM RBCs (**Figure 2B**). The involvement of ROS is likely derived from NOX isoform 2 (NOX2) because the nonselective NOX inhibitor apocynin (**Figure 2C**) and the selective NOX2 inhibitor gp91 ds-tat (**Figure 2D**) markedly attenuated the impairment in EDR. By contrast, the NOX1 inhibitors ML171 (**Figure 2E**) and NoxA1ds (data not shown) did not block the effect. The H_2O_2 decomposition catalyst catalase prevented the development of impaired EDR (**Figure 2F**). None of these inhibitors affected EDR following incubation with Healthy RBCs (Online Figures 3A to 3E) or KH buffer (Online Figures 4A to 4F), with the exception that EDR was reduced by catalase in aortas incubated with Healthy RBCs (Online Figure 3F).

FIGURE 2 Functional Involvement of RBC Arginase and ROS in Endothelial Dysfunction in T2DM

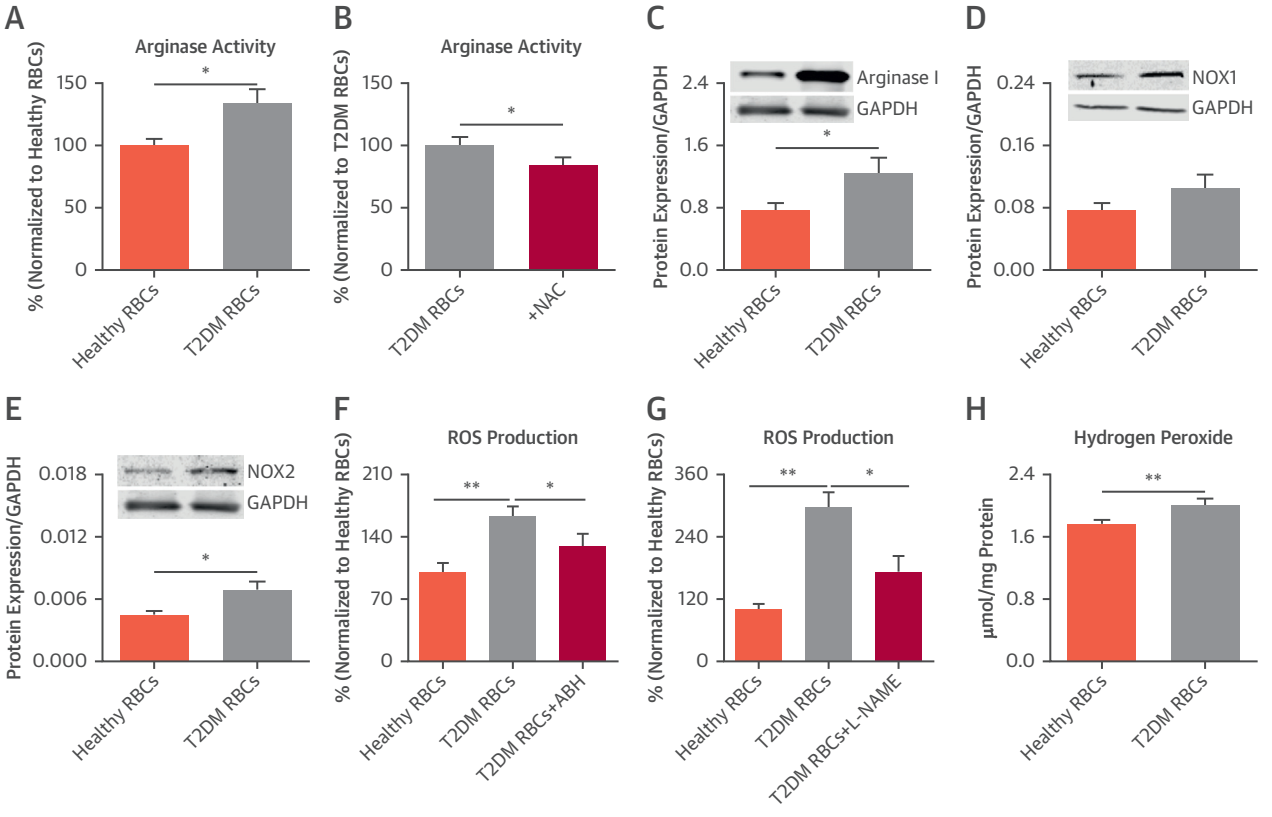


Endothelium-dependent relaxation to ACh in isolated rat aortas following incubation with buffer, Healthy RBCs, and T2DM RBCs for 18 h. RBCs from patients with T2DM were co-incubated with **(A)** the arginase inhibitor 2(S)-amino-6-boronohexanoic acid (ABH) (n = 8), **(B)** the reactive oxygen species (ROS) scavenger N-acetyl-cysteine (NAC) (n = 9), **(C)** the reduced nicotinamide adenine dinucleotide phosphate oxidase (NOX) inhibitor apocynin (n = 6), **(D)** the selective NOX2 inhibitor gp 91 ds-tat (n = 6), **(E)** the selective NOX1 inhibitor ML171 (n = 6), and **(F)** the hydrogen peroxide decomposition catalyst catalase (n = 5). Values are mean ± SEM. *p <0.05, **p <0.01 versus buffer; †p <0.05, ††p <0.01 effect of RBCs with drug versus RBCs without drug representing the concentration-response relation by 2-way analysis of variance. Abbreviations as in Figure 1.

To test whether there were carryover effects by the pharmacological inhibitors from the co-incubation with RBCs to the functional studies, aortic rings isolated from wild-type (WT) and GK rats were pre-incubated with KH buffer for 18 h in the absence and presence of ABH, NAC, and catalase. The impaired EDR in GK rat aortas was not affected by those inhibitors (Online Figures 5A to 5C). By contrast, EDR of the GK rat aortas was improved when these aortas were pre-incubated with and exposed to the inhibitors in the organ baths (Online Figures 5A to 5C). In addition, impairment of EDR induced by T2DM RBCs was prevented when the RBCs were pre-incubated for 1 h with gp91 ds-tat before the 18-h incubation (Online Figure 6A), whereas the inhibitor incubated with Healthy RBCs had no effect on EDR (Online Figure 6B). These observations suggest that there was no carryover effect to the functional studies and that the improvement in EDR induced by the inhibitors during pre-incubation with RBCs results from inhibitory effects in RBCs acting on the vascular wall.

ARGINASE IS UP-REGULATED IN RBCs FROM PATIENTS WITH T2DM BY A ROS-DEPENDENT MECHANISM. Arginase activity and arginase I protein expression were significantly elevated in T2DM RBCs as compared with Healthy RBCs (**Figures 3A and 3C**). Arginase I protein expression was elevated in

FIGURE 3 Mutual Regulation Between Arginase and ROS in RBCs

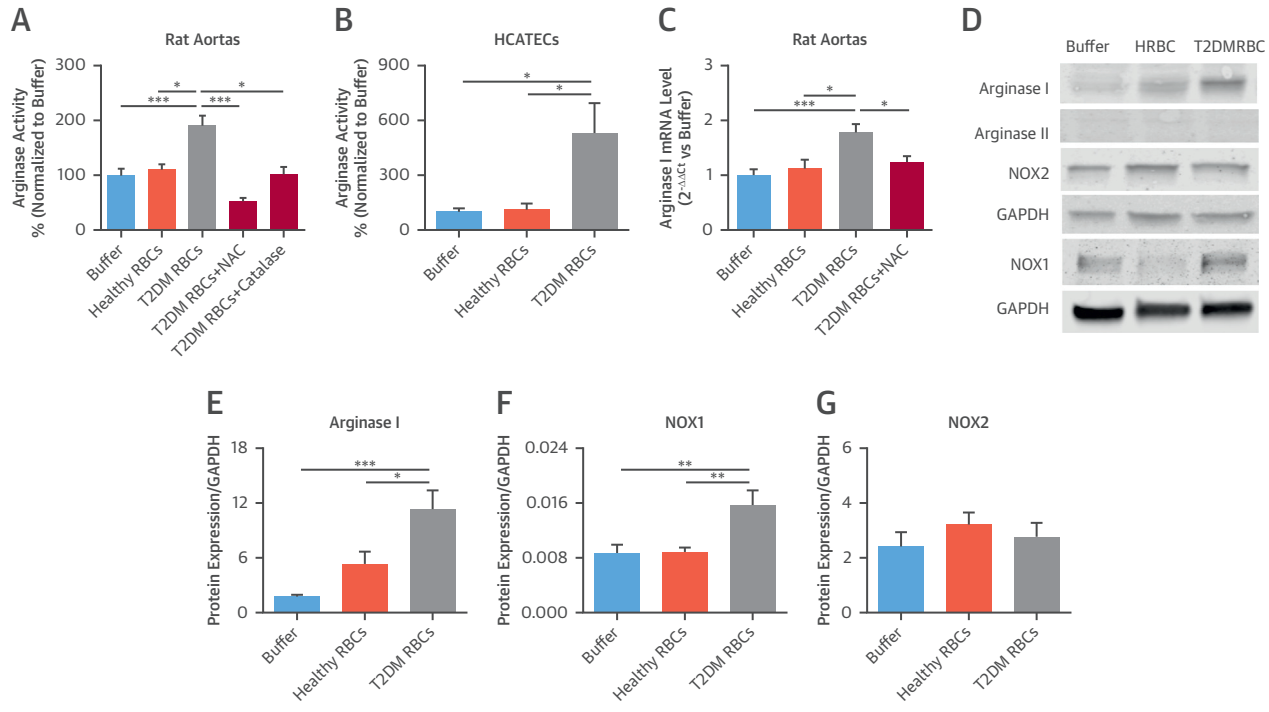


(A) Arginase activity in Healthy RBCs (n = 15) and T2DM RBCs (n = 20). **(B)** Arginase activity in T2DM RBCs following 24-h incubation with the ROS scavenger NAC (n = 8). Protein expression of **(C)** arginase I, **(D)** NOX1, and **(E)** NOX2 normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in T2DM RBCs (n = 8 to 9) and Healthy RBCs (n = 8). Reactive oxygen species formation in Healthy RBCs and T2DM RBCs in the absence and presence of **(F)** the arginase inhibitor 2(S)-amino-6-boronohexanoic acid (ABH) (n = 9) and **(G)** the nitric oxide synthase inhibitor L-NAME (n = 8). **(H)** Hydrogen peroxide formation in Healthy RBCs and T2DM RBCs (n = 12). Values are mean ± SEM. *p <0.05, **p <0.01. Abbreviations as in Figures 1 and 2.

T2DM RBCs (Figure 3C), whereas no arginase II was detected. Interestingly, NAC decreased arginase activity in T2DM RBCs (Figure 3B) but not Healthy RBCs (Online Figure 7C). Moreover, ROS formation was increased in T2DM RBCs in comparison with Healthy RBCs (Figures 3F and 3G). The ROS formation was attenuated by ABH (Figure 3F) and the NOS inhibitor N^{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME) (Figure 3G) in T2DM RBCs but not in Healthy RBCs (Online Figures 7E and 7F). H_2O_2 formation was increased in T2DM RBCs (Figure 3H). In addition, NOX2 expression but not NOX1 expression (p = 0.19) was significantly increased in T2DM RBCs (Figures 3D and 3E). These observations indicate a vicious cycle in which ROS, likely derived from NOX2 and uncoupled NOS, increases RBC arginase activity in T2DM that, in turn, stimulates ROS production. High glucose slightly but significantly increased arginase activity in Healthy RBCs (Online Figure 7A). This increase was not affected by NAC (Online Figure 7A). Further, high glucose did not affect ROS formation in Healthy RBCs (Online Figure 7D).

RBC-INDUCED UP-REGULATION OF VASCULAR ARGINASE ACCOUNTS FOR ENDOTHELIAL DYSFUNCTION IN T2DM. We next investigated the molecular mechanisms at the vascular level for the development of endothelial dysfunction induced by T2DM RBCs. Arginase activity in aortic rings and human carotid arterial ECs (HCATECs) was increased following incubation with T2DM RBCs as compared with Healthy RBCs or KH buffer (Figures 4A and 4B). Co-incubation with NAC or catalase abolished the increase in aortic arginase activity induced by T2DM RBCs (Figure 4A), but not in vessels incubated with Healthy RBCs or KH buffer (Online Figures 7G and 7H).

FIGURE 4 Vascular Arginase Activity and Expression Profile Following RBC Incubation



(A) Arginase activity in isolated rat aortas following incubation with buffer, Healthy RBCs, and T2DM RBCs for 18 h in the absence and presence of the ROS scavenger NAC (n = 5) and hydrogen peroxide decomposition catalyst catalase (n = 7). **(B)** Arginase activity in cultured human carotid arterial endothelial cells (HCATECs) incubated with buffer, Healthy RBCs, and T2DM RBCs for 24 h (n = 5 to 7). **(C)** mRNA level of arginase I in aortas incubated with buffer, Healthy RBCs, and T2DM RBCs for 8 h in the absence and presence of NAC (n = 5 to 13). **(D)** Western blot images of arginase I, arginase II, NOX1, and NOX2 in isolated rat aortas incubated with buffer, Healthy RBCs, and T2DM RBCs for 18 h. Quantification of protein expression of **(E)** arginase I, **(F)** NOX1, and **(G)** NOX2 normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in isolated rat aortas incubated with buffer, Healthy RBCs, and T2DM RBCs for 18 h (n = 5 to 6). Values are mean ± SEM. *p <0.05, **p <0.01, ***p <0.001. Abbreviations as in Figures 1 and 2.

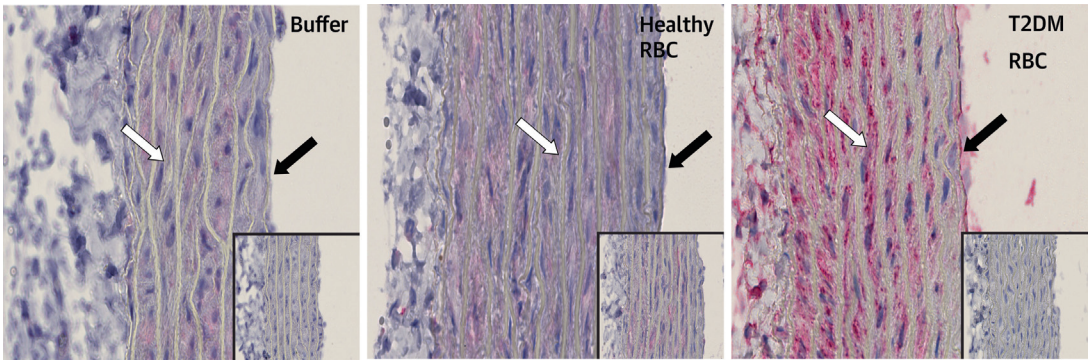
These observations indicate that H_2O_2 likely serves as a mediator initiating signaling between RBCs and the vascular wall. Vascular arginase I protein level was also elevated by T2DM RBCs (Figures 4D and 4E). Immunohistochemical analysis showed that arginase I was abundantly expressed in ECs and smooth muscle cells (SMCs) following incubation with T2DM RBCs (Figure 5). No arginase II protein was detected using either Western blot or immunohistochemistry (Figure 4D, Online Figure 8). Incubation with T2DM RBCs induced an increase in rat aortic messenger RNA (mRNA) level of arginase I that was evident at 8 h (Figure 4C, Online Figure 7J). The increase in vascular arginase I mRNA induced by T2DM RBCs was attenuated by NAC (Figure 4C), whereas NAC had no effect following incubation with Healthy RBCs (Online Figure 7I). NOX1 but not NOX2 protein expression in rat aortas was increased by T2DM RBCs (Figures 4D, 4F, and 4G).

The impairment of aortic EDR induced by T2DM RBCs was fully recovered by inhibition of vascular arginase with ABH (Figure 6A), scavenging of ROS with NAC (Figure 6B), apocynin (Figure 6C), ML171 (Figure 6D), and catalase (Figure 6E). By contrast, the NOX2 inhibitors gp91 ds-tat (Figure 6F) and GSK2795039 (data not shown) failed to improve EDR in rat aortas incubated with T2DM RBCs, a finding consistent with the lack of changes in NOX2 protein expression. None of the inhibitors applied in the organ baths mentioned previously had any effects on EDR in vessels incubated with Healthy RBCs (Online Figures 9A to 9F) or KH buffer (Online Figures 10A to 10F).

DISCUSSION

The main findings of the present study are as follows: 1) T2DM RBCs induced endothelial dysfunction in rat

FIGURE 5 Immunohistological Expression of Arginase I in Rat Aortas Incubated With Buffer, Healthy RBCs, and T2DM RBCs for 18 h (n = 3)



Rabbit immunoglobulin G was used as an isotype control for rabbit primary antibody (shown in **insets**). The **black arrows** indicate endothelial cells, and the **white arrows** indicate smooth muscle cells. Abbreviations as in Figure 1.

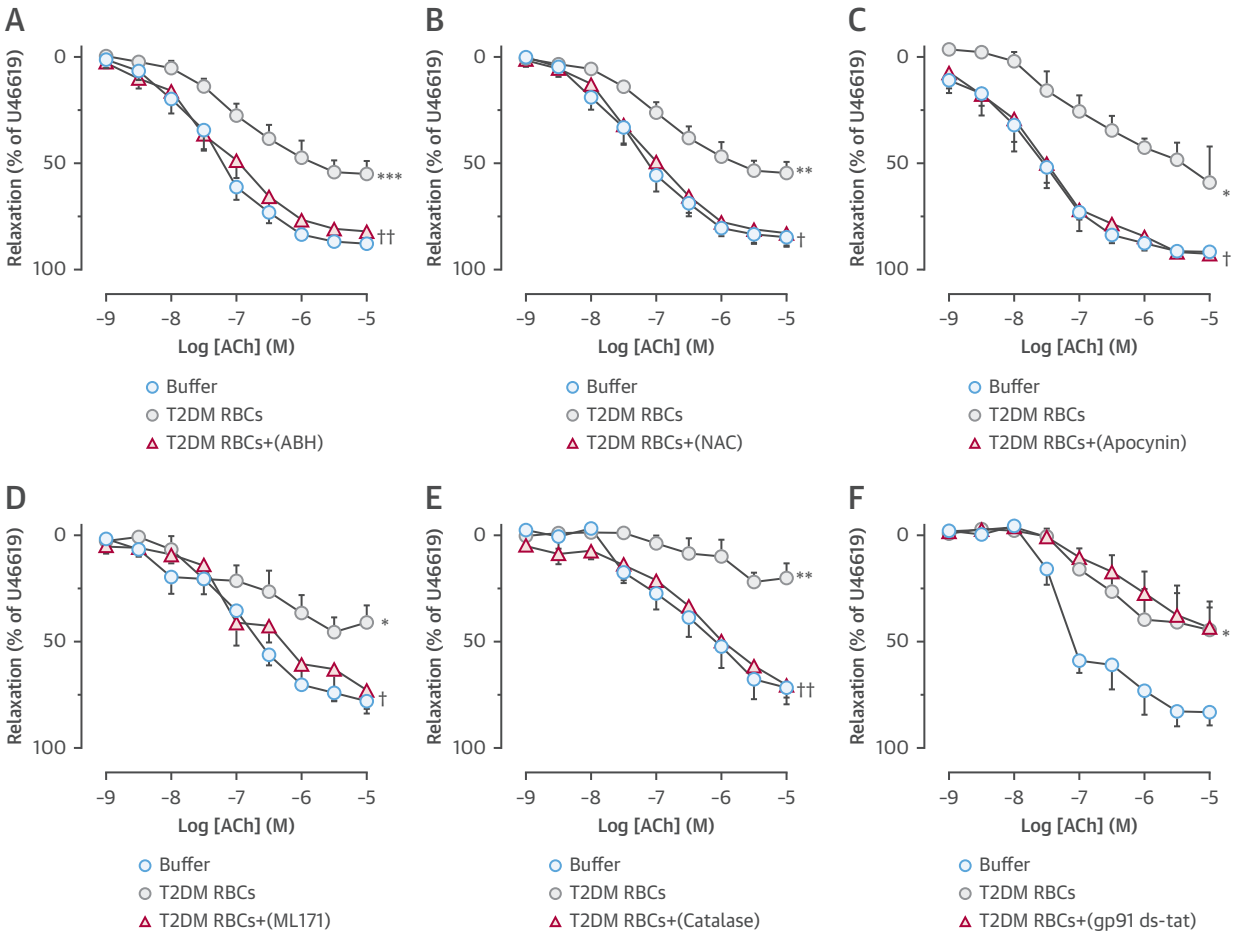
and human arteries; 2) the endothelial dysfunction induced by T2DM RBCs was attenuated by arginase inhibition, ROS scavenging, and H_2O_2 decomposition at both the RBC and vascular levels; 3) arginase activity, arginase I expression, NOX2 expression, ROS formation, and H_2O_2 production were increased in T2DM RBCs; 4) the increased ROS production in T2DM RBCs was driven by arginase; and 5) T2DM RBCs increased vascular arginase activity and arginase I expression through a ROS-dependent mechanism. Our findings demonstrate a novel disease mechanism in T2DM by which RBCs induce vascular endothelial dysfunction through up-regulation of arginase I and ROS signaling.

Studies have revealed that RBCs contribute to vascular function and integrity, in addition to their function as oxygen transporters (6). Although several studies have implied a role of RBCs in regulation of vascular function in diabetes (7,8), key mechanisms are unclear, and direct evidence behind the interaction of RBCs with the endothelium in T2DM is lacking. We therefore aimed to investigate the role of RBCs as novel mediators of endothelial dysfunction in patients with T2DM. Indeed, T2DM RBCs were demonstrated to induce endothelial dysfunction in both healthy rat aortas and internal mammary arteries from nondiabetic patients. In accordance with our previous findings (13), vascular endothelial dysfunction was demonstrated in vivo in the cohort of patients with T2DM who donated RBCs for the in vitro experiments, thus suggesting that RBCs play an important role and may serve as a key mechanism for the development of vascular endothelial dysfunction observed in vivo in T2DM. In addition to human RBCs, RBCs from GK rats induced endothelial dysfunction to an extent similar to that observed in GK rat aortas. This finding suggests that it is the T2DM per se that contributes to the detrimental effect of RBCs rather than co-morbidities, co-medication, or other confounding factors associated with the group of patients with T2DM.

We and other investigators previously demonstrated that arginase is up-regulated in the vasculature in both animal models and patients with T2DM, and it contributes to resistance artery and microvascular endothelial dysfunction (4,5,13,14). Of further interest, we demonstrated that arginase expressed in RBCs regulated cardiac function in an ex vivo model of ischemia and reperfusion (9). This finding indicates an important interaction between RBC arginase and cardiovascular function. We therefore hypothesized a potential involvement of arginase in RBC-induced endothelial dysfunction in T2DM. Accordingly, arginase activity is significantly elevated in T2DM RBCs as compared with Healthy RBCs. The status of arginase in RBCs from patients with T2DM remains debatable. The majority of arginase is bound to the membrane of RBCs, with small amounts present in the cytoplasm (15). Arginase activity has been found to be elevated in membrane fractions of RBCs in T2DM (10), but it has been shown to be decreased in RBCs from patients with T2DM at first clinical onset (16). This variation may indicate different levels of arginase activity at different stages of T2DM. We also demonstrated increased expression of arginase I protein.

These observations suggest that up-regulation of arginase I already exists in the early phase of erythropoiesis (e.g., in reticulocytes). Indeed,

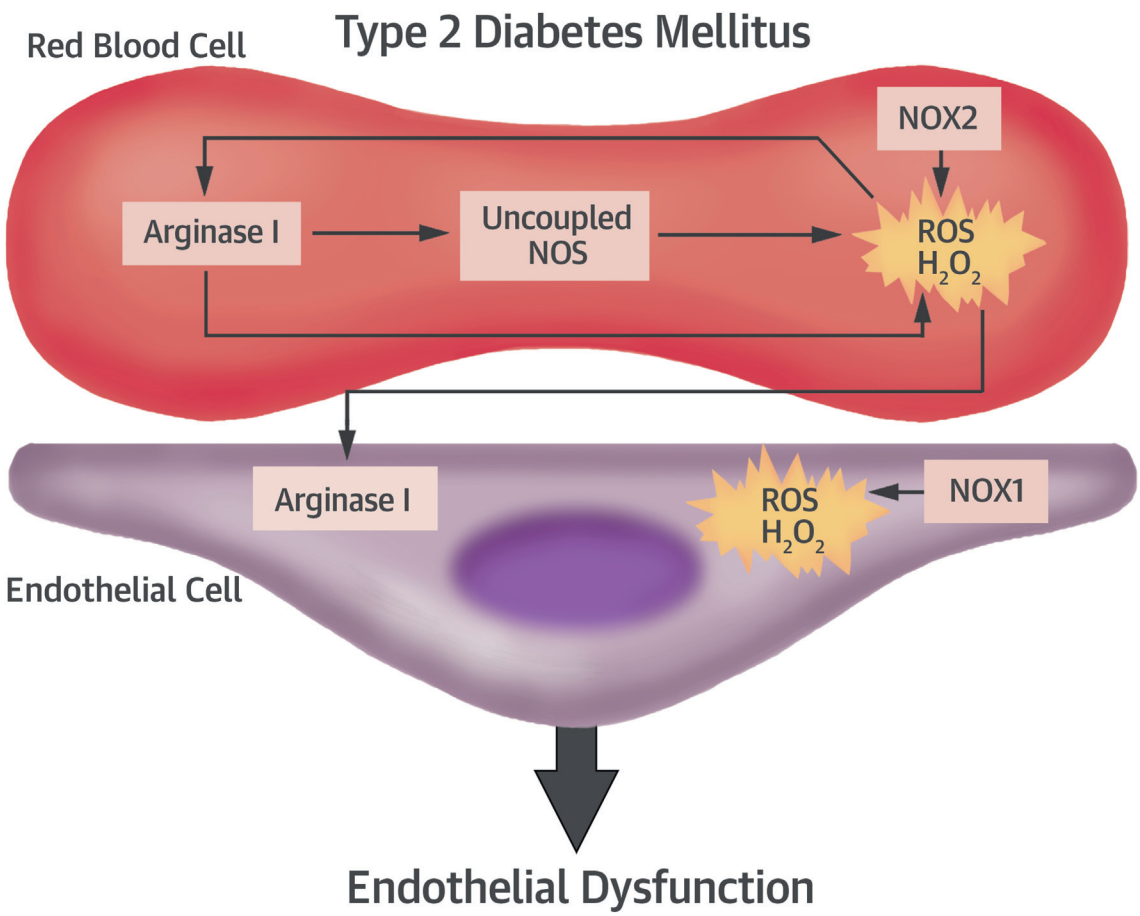
FIGURE 6 Functional Involvement of Vascular Arginase and ROS in Endothelial Dysfunction in T2DM



Endothelium-dependent relaxation to ACh in isolated rat aortas following incubation with buffer, Healthy RBCs, and T2DM RBCs for 18 h. Following the 18-h incubation with red blood cells from patients with T2DM and rinsing, the rat aortas were treated with **(A)** the arginase inhibitor 2(S)-amino-6-boronohexanoic acid (ABH) (n = 6), **(B)** the ROS scavenger NAC (n = 7), **(C)** the NOX inhibitor apocynin (n = 3), **(D)** the selective NOX1 inhibitor ML171 (n = 5), **(E)** the hydrogen peroxide decomposition catalyst catalase (n = 3 to 4), and **(F)** the selective NOX2 inhibitor gp 91 ds-tat (n = 3). Values are mean ± SEM. *p <0.05, **p <0.01, ***p <0.001 vs. buffer; †p <0.05, ††p <0.01 effect of drug representing the concentration-response relation by 2-way analysis of variance. **Parentheses** indicate that the inhibitors were added in the organ baths following the 18 h RBC incubation. Abbreviations as in Figures 1 and 2.

reticulocytes isolated from an animal model of diabetes had increased arginase activity compared with reticulocytes from nondiabetic controls (17). ROS scavenging decreased arginase activity in T2DM RBCs, and the increased ROS formation in T2DM RBCs was attenuated by arginase inhibition. The observed up-regulation of arginase and ROS is of critical importance for the detrimental effect of RBCs on endothelial function. Thus, inhibition of RBC arginase prevented the impairment of EDR induced by T2DM RBCs. Moreover, ROS scavenging, H_2O_2 decomposition, NOX inhibition, and NOX2 inhibition also prevented development of endothelial dysfunction, a finding supported by elevated levels of H_2O_2 and NOX2 protein in T2DM RBCs. Catalase reversed endothelial dysfunction induced by T2DM RBCs, thus suggesting an important role of RBC-derived H_2O_2 in RBC-induced endothelial dysfunction, but it attenuated endothelial function in the presence of Healthy RBCs. In line with previous observations indicating that H_2O_2 may mediate EDR (18), this finding may indicate a role of RBC-derived H_2O_2 in the regulation of endothelial function under healthy conditions. Together with the evidence that increased ROS formation was attenuated by NOS inhibition, the present data suggest a mutual regulation between NOX2-derived and uncoupled NOS-derived ROS and arginase behind RBC dysfunction in T2DM.

CENTRAL ILLUSTRATION **Interaction Between Red Blood Cells and Endothelium**



Zhou, Z. et al. J Am Coll Cardiol. 2018;72(7):769-80.

Red blood cells (RBCs) from patients with type 2 diabetes mellitus induce endothelial dysfunction. At the red blood cell level, up-regulated arginase I causes nitric oxide synthase (NOS) uncoupling, leading to reactive oxygen species production. The reactive oxygen species are also derived from activation of reduced nicotinamide adenine dinucleotide phosphate oxidase (NOX) 2 in RBCs in type 2 diabetes mellitus, which mutually interact with arginase I, thus resulting in endothelial dysfunction. The reactive oxygen species, specifically hydrogen peroxide (H_2O_2), may play a crucial role in the interplay between red blood cells and endothelium. H_2O_2 derived from RBCs in type 2 diabetes mellitus induces increased vascular arginase activity. Together with vascular NOX1-derived H_2O_2, up-regulation of vascular arginase accounts for endothelial dysfunction in type 2 diabetes mellitus.

High glucose slightly but significantly increases arginase activity in Healthy RBCs. The biological significance of the effect of glucose incubation on arginase activity in relation to the elevated RBC arginase in T2DM is unclear, however. The elevated arginase activity in T2DM RBCs was ROS dependent, whereas the increase in RBC arginase by glucose in Healthy RBCs was not significantly changed by ROS scavenging. Similarly, there is increased ROS production in T2DM RBCs, but incubation of Healthy RBCs with high glucose did not increase ROS production. Thus, several differences exist between incubation with high glucose ex vivo and T2DM, and it therefore seems less likely that the slight increase in arginase activity induced by high glucose is a single trigger for the larger difference observed between T2DM RBCs and Healthy RBCs and also for the endothelial dysfunction induced by T2DM RBCs. Hyperglycemia is only 1 of multiple factors contributing to RBC and endothelial dysfunction in T2DM. Future studies are warranted to clarify the role of long-term hyperglycemia in RBC function in T2DM.

We also demonstrated that alterations of the T2DM RBCs induce specific changes in vascular gene and protein expression and enzyme activity that translate into functional changes. RBCs induced

increased arginase activity in aortas and cultured human carotid arterial ECs, as well as increased arginase I mRNA and protein expression in aortas. The functional importance of endothelial arginase was demonstrated by the ability of the arginase inhibitor to fully reverse the endothelial dysfunction in rat aortas induced by T2DM RBCs. Furthermore, ROS scavenging and H_2O_2 decomposition resulted in decreased vascular arginase activity in T2DM RBCs, a finding suggesting cross-talk between RBC ROS and vascular arginase I. Collectively, our data demonstrate a crucial role of both RBC and vascular arginase in the development of endothelial dysfunction by an ROS-dependent mechanism in T2DM.

T2DM RBCs also affect vascular oxidative stress, as revealed by the observation that ROS scavenging and H_2O_2 decomposition following RBC incubation restored the impaired EDR. ECs express 4 NOX isoforms (NOX1, 2, 4, and 5), of which NOX1 and NOX2 are the most important sources of ROS formation and endothelial dysfunction in experimental animals with diabetes (19). In the present study, nonselective NADPH oxidase inhibition and selective NOX1 but not selective NOX2 inhibition improved EDR following incubation with T2DM RBCs. This is consistent with the increased protein expression of vascular NOX1 induced by T2DM RBCs. These observations suggest that vascular NOX1-derived ROS is involved in the impaired EDR induced by T2DM RBCs.

STUDY LIMITATIONS. Patients with T2DM had other comorbidities and ongoing medication, which may influence the effect of the RBCs. However, RBCs from GK rats, a pure T2DM model, also impaired EDR to an extent comparable to that induced by RBCs from patients with T2DM and to that observed in aortas isolated from GK rats, thereby suggesting the T2DM per se is capable of inducing endothelial dysfunction. Moreover, several pharmacological compounds of our diabetic patients are known to improve endothelial function by various mechanisms (20). It may therefore be expected that the medication may even counteract the observed negative effect, thus leading to an underestimation of the difference between the diabetic patients and the healthy subjects. Interestingly, RBCs collected from a subgroup of patients without statin treatment induced endothelial dysfunction that was comparable to that induced by the group with statin treatment. The study demonstrates that RBCs induce endothelial dysfunction in isolated arteries ex vivo. Despite the observations that the patients who donated the RBCs had impaired endothelial function in vivo, and that arginase inhibition improves resistance and microvascular endothelial function in patients with T2DM (4,5), it cannot be determined to what degree the RBCs contribute to endothelial dysfunction in T2DM patients in vivo. However, only an isolated vascular model permits the identification of the specific pathophysiological mechanism mediated by RBCs because it is not feasible to distinguish effects mediated by RBCs from those of other cell types in the in vivo setting.

CONCLUSIONS

We demonstrate a novel disease mechanism by which RBCs induce endothelial dysfunction in T2DM (**Central Illustration**). This effect is triggered by increased NOX2- and NOS-derived ROS formation driven by arginase in the RBCs. H_2O_2 seems to play a crucial role in the interplay between RBCs and vasculature that leads to endothelial dysfunction in T2DM. This involves an RBC-derived H_2O_2-induced increase in vascular arginase I and vascular H_2O_2 formation derived from vascular NOX1, which ultimately induce endothelial dysfunction. Our findings suggest that RBCs, specifically RBC arginase I, are potential therapeutic targets for the treatment of endothelial dysfunction in patients with T2DM.

ACKNOWLEDGMENTS The technical assistance of Marita Wallin and Tong Jiao and the patient coordination of David Ersgård, Anette Härström, and Kajsa Sundqvist are gratefully acknowledged. The authors thank Dr. Matthias Corbascio of the Division of Thoracic Surgery, Karolinska Institutet, for providing human internal mammary arteries.

ADDRESS FOR CORRESPONDENCE: Dr. Zhichao Zhou, CMM L8:03, Division of Cardiology, Department of Medicine, Karolinska University Hospital, Karolinska Institutet, Stockholm 17176, Sweden. E-mail: zhichao.zhou@ki.se OR zhzhou2015@gmail.com. Twitter: @karolinskainst.

PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Changes in the function of erythrocytes induced by ROS stimulating arginase I cause endothelial dysfunction in patients with T2DM.

TRANSLATIONAL OUTLOOK: Additional studies are needed to develop therapeutic interventions that address this mechanism of endothelial injury and assess their impact on vascular outcomes.

REFERENCES

1. Vanhoutte PM, Shimokawa H, Tang EH, Feletou M. Endothelial dysfunction and vascular disease. Acta Physiol (Oxf) 2009;196:193–222.

2. Palmer SC, Mavridis D, Nicolucci A, et al. Comparison of clinical outcomes and adverse events associated with glucose-lowering drugs in patients with type 2 diabetes: a meta-analysis. JAMA 2016;316:313–24.

3. Pernow J, Jung C. The emerging role of arginase in endothelial dysfunction in diabetes. Curr Vasc Pharmacol 2016;14:155–62.

4. Shemyakin A, Kovamees O, Rafnsson A, et al. Arginase inhibition improves endothelial function in patients with coronary artery disease and type 2 diabetes mellitus. Circulation 2012;126:2943–50.

5. Kovamees O, Shemyakin A, Checa A, et al. Arginase inhibition improves microvascular endothelial function in patients with type 2 diabetes mellitus. J Clin Endocrinol Metab 2016;101:3952–8.

6. Cortese-Krott MM, Rodriguez-Mateos A, Sansone R, et al. Human red blood cells at work: identification and visualization of erythrocytic eNOS activity in health and disease. Blood 2012;120:4229–37.

7. Sprague RS, Bowles EA, Achilleus D, Stephenson AH, Ellis CG, Ellsworth ML. A selective phosphodiesterase 3 inhibitor rescues low PO2-induced ATP release from erythrocytes of humans with type 2 diabetes: implication for vascular control. Am J Physiol Heart Circ Physiol 2011;301:H2466–72.

8. Wautier JL, Wautier MP, Schmidt AM, et al. Advanced glycation end products (AGEs) on the surface of diabetic erythrocytes bind to the vessel wall via a specific receptor inducing oxidant stress in the vasculature: a link between surface-associated AGEs and diabetic complications. Proc Natl Acad Sci U S A 1994;91:7742–6.

9. Yang J, Gonon AT, Sjoquist PO, Lundberg JO, Pernow J. Arginase regulates red blood cell nitric oxide synthase and export of cardioprotective nitric oxide bioactivity. Proc Natl Acad Sci U S A 2013;110:15049–54.

10. Bizjak DA, Brinkmann C, Bloch W, Grau M. Increase in red blood cell-nitric oxide synthase dependent nitric oxide production during red blood cell aging in health and disease: a study on age dependent changes of rheologic and enzymatic properties in red blood cells. PLoS One 2015;10:e0125206.

11. Jiang M, Jia L, Jiang W, et al. Protein disregulation in red blood cell membranes of type 2 diabetic patients. Biochem Biophys Res Commun 2003;309:196–200.

12. Ostenson CG, Khan A, Abdel-Halim SM, et al. Abnormal insulin secretion and glucose metabolism in pancreatic islets from the spontaneously diabetic GK rat. Diabetologia 1993;36:3–8.

13. Kovamees O, Shemyakin A, Pernow J. Amino acid metabolism reflecting arginase activity is increased in patients with type 2 diabetes and associated with endothelial dysfunction. Diab Vasc Dis Res 2016;13:354–60.

14. Beleznai T, Feher A, Spielvogel D, Lansman SL, Bagi Z. Arginase 1 contributes to diminished coronary arteriolar dilation in patients with diabetes. Am J Physiol Heart Circ Physiol 2011;300:H777–83.

15. Jiang M, Ding Y, Su Y, Hu X, Li J, Zhang Z. Arginase-flotillin interaction brings arginase to red blood cell membrane. FEBS Lett 2006;580:6561–4.

16. Savu O, Iosif L, Bradescu OM, Serafinceanu C, Papacocea R, Stoian I. L-arginine catabolism is driven mainly towards nitric oxide synthesis in the erythrocytes of patients with type 2 diabetes at first clinical onset. Ann Clin Biochem 2015;52:135–43.

17. Gupta BL, Preet A, Baquer NZ. Protective effects of sodium orthovanadate in diabetic reticulocytes and ageing red blood cells of Wistar rats. J Biosci 2004;29:73–9.

18. Chuaiphichai S, McNeill E, Douglas G, et al. Cell-autonomous role of endothelial GTP cyclohydrolase 1 and tetrahydrobiopterin in blood pressure regulation. Hypertension 2014;64:530–40.

19. Drummond GR, Sobey CG. Endothelial NADPH oxidases: which NOX to target in vascular disease? Trends Endocrinol Metab 2014;25:452–63.

20. Chaudhury A, Duvoor C, Reddy Dendi VS, et al. Clinical review of antidiabetic drugs: implications for type 2 diabetes mellitus management. Front Endocrinol (Lausanne) 2017;8:6.

KEY WORDS arginase, endothelial dysfunction, reactive oxygen species, red blood cell, type 2 diabetes, vascular complication

APPENDIX For a supplemental Methods section, supplemental references, and supplemental figures, please see the online version of this paper.