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ORIGINAL RESEARCH - CLINICAL

Stimulation of Erythrocyte Soluble Guanylyl Cyclase Induces cGMP Export and Cardioprotection in Type 2 Diabetes

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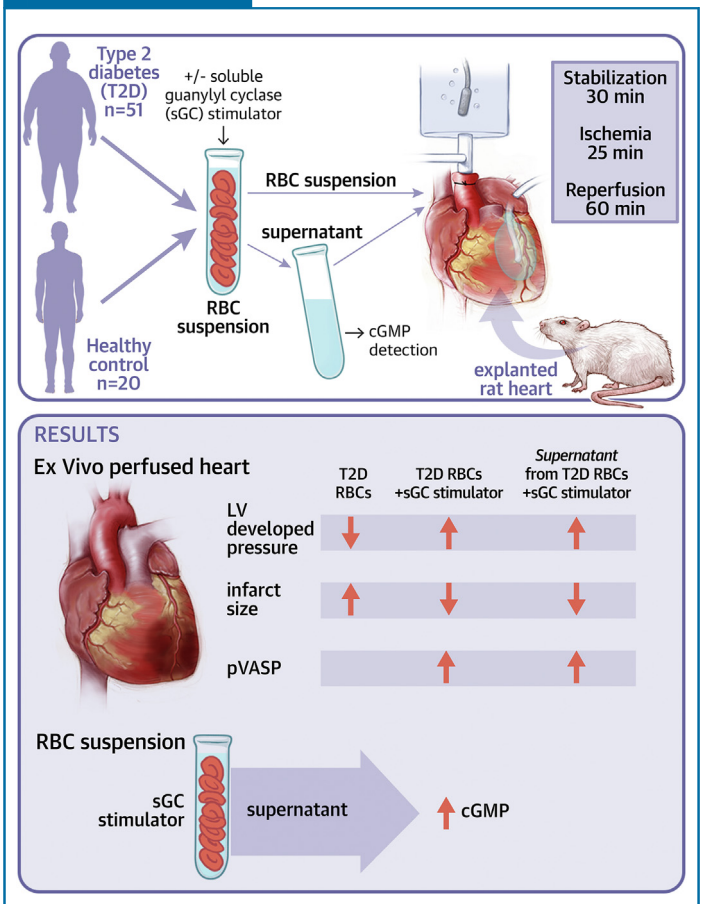
VISUAL ABSTRACT



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HIGHLIGHTS

- **RBCs from patients with T2D impair cardiac function after I/R.**
- **Stimulation of sGC in RBCs induces release of a cardioprotective factor that protects from myocardial I/R injury.**
- **sGC stimulation in RBCs causes release of cGMP and activation of cardiomyocyte PKG.**
- **RBCs sGC provides a novel therapeutic target to prevent cardiac injury in T2D.**

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ABBREVIATIONS AND ACRONYMS

cGMP = cyclic guanosine monophosphate

eNOS = endothelial nitric oxide synthase

KH = Krebs-Henseleit

I/R = ischemia-reperfusion

LVDP = left ventricular developed pressure

LVEDP = left ventricular end-diastolic pressure

MRP = multidrug-resistance protein

NO = nitric oxide

ODQ = 1H-[1,2,4]oxadiazolo-[4, 3-a]quinoxalin-1-one

PKG = protein kinase G

pVASP = phosphorylation of vasodilator-specific phosphoprotein

RBC = red blood cell

sGC = soluble guanylyl cyclase

T2D = type 2 diabetes

SUMMARY

Reduced nitric oxide (NO) bioactivity in red blood cells (RBCs) is critical for augmented myocardial ischemia-reperfusion injury in type 2 diabetes. This study identified the nature of "NO bioactivity" by stimulating the intracellular NO receptor soluble guanylyl cyclase (sGC) in RBCs. sGC stimulation in RBCs from patients with type 2 diabetes increased export of cyclic guanosine monophosphate from RBCs and activated cardiac protein kinase G, thereby attenuating ischemia-reperfusion injury. These results provide novel insight into RBC signaling by identifying cyclic guanosine monophosphate from RBC as a mediator of protection against cardiac ischemia-reperfusion injury induced by sGC stimulation in RBCs. (J Am Coll Cardiol Basic Trans Science 2023;8:907–918)

Multiple observations support the concept that red blood cells (RBCs), in addition to their pivotal role as transporters of oxygen and carbon dioxide, are important for cardiovascular regulation via formation of nitric oxide (NO) and export of NO bioactivity.[1-3] The cardiovascular effects of RBC-derived NO bioactivity include protection of the ischemic heart[4] and vasodilatation, especially under hypoxic conditions.[3,5] However, the mechanism underlying the beneficial cardiovascular effect of NO signaling in RBCs remains unclear.[5-8] It has been shown that RBCs contain key elements of the NO signaling pathway, including endothelial nitric oxide synthase (eNOS) and a functional soluble guanylyl cyclase (sGC).[8,9] Recent studies using mice with specific deletion of RBC eNOS demonstrated the importance of eNOS signaling in RBCs in blood pressure regulation[10] and myocardial ischemia-reperfusion (I/R).[11] Available data suggest that the cardioprotective factor released from RBCs in the setting of experimental myocardial I/R[4] as well as in patients with acute myocardial infarction[12] is derived from eNOS. However, the signaling downstream of eNOS remains unclear and controversial. Because oxygenated hemoglobin reacts very rapidly with NO to form methemoglobin and nitrate, it is considered unlikely that free NO can be released from RBCs.[13] Alternative forms of NO bioactivity released from RBCs have been discussed including more stable NO-derived reaction products such as S-nitrosothiols,[14] but even this remains to be firmly proven. Consequently, there is likely another still unidentified signaling molecule that mediates the NO bioactivity elicited by RBCs in the cardiovascular system. A previous study showed that cyclic guanosine monophosphate (cGMP) may be formed from guanosine triphosphate by sGC in RBCs.[9] However, the functional effects of sGC stimulation in RBCs have previously not been investigated. It is further unknown whether cGMP release occurs from RBCs in response to sGC stimulation and can mediate cardioprotective effects of RBCs. This would be of great interest based on the view that altered cGMP formation and signaling appear to play direct pathophysiological roles in cardiovascular disease.[15]

Besides this protective role of RBCs under physiological situations, alterations in RBC function including impairment of NO signaling have been implicated to be of importance in cardiovascular disease, especially in relation to type 2 diabetes (T2D).[16] We recently demonstrated that RBCs from patients with T2D aggravate postischemic cardiac injury and induce endothelial dysfunction in isolated rodent heart and vascular models ex vivo, effects that are associated with reduced NO signaling and increased oxidative stress.[17,18] Therapeutic strategies that improve NO signaling in RBCs may therefore have the potential to restore RBC function and attenuate cardiovascular injury induced by RBCs in T2D.[16] Stimulation of sGC in RBCs could be an attractive therapeutic approach to prevent the detrimental cardiovascular effects of dysfunctional RBC in T2D. Based on the central role of sGC in mediating NO

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The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the Author Center.

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signaling, effective pharmacologic therapies that stimulate sGC function have been approved to treat pulmonary arterial hypertension and heart failure.[19-22] The NO-sGC signaling pathway in the cardiovascular system is known to induce cardioprotection via activation of cardiac cGMP-dependent protein kinase G (PKG),[23] but the importance of this pathway in RBCs for cardioprotection has previously not been explored. Knowing the impact of RBCs for cardiac injury in T2D caused by attenuated NO signaling,[17] it is tempting to speculate that stimulation of sGC in RBCs would exert beneficial effects on postischemic cardiac function. However, any cardioprotective effects of targeting RBC sGC as well as the signaling behind such effect of sGC stimulation in RBCs remain unknown.

The objectives of the present study were therefore to determine whether stimulation of sGC in RBCs attenuates the impairment of cardiac tolerance to ischemia in T2D and to identify the underlying molecular signaling mechanism that mediates such effect of RBCs. We hypothesized that stimulation of sGC in RBCs results in formation and release of cGMP that leads to protection against cardiac I/R injury. The results demonstrate that stimulation of sGC in RBCs from patients with T2D markedly improves cardiac postischemic performance and reduces infarct size in isolated rat hearts, and that cGMP released following the stimulation of sGC in RBCs acts as a crucial cardioprotective mediator via activation of cardiac PKG.

TABLE 1 Study Subject Characteristics

	Healthy Subjects (n = 20)	Subjects With T2D (n = 51)
Age, y	59 ± 9	64 ± 10
Male	9	41
BMI, kg/m^2	24.1 ± 3.4	30.7 ± 5.4[a]
Systolic BP, mm Hg	135 ± 18	141 ± 19
Diastolic BP, mm Hg	82 ± 9	83 ± 12
Fasting glucose, mmol/L	5.5 ± 0.4	9.1 ± 2.7[a]
Smokers	1	6
HbA_{1c}, mmol/mol	36 ± 3	67 ± 16[a]
Hemoglobin, g/L	139 ±11	138 ± 14
Creatinine, μmol/l	73 ± 14	87 ± 32
Triglycerides, mmol/L	1.1 ± 0.5	1.9 ± 1.0[a]
Total cholesterol, mmol/L	5.1 ± 0.9	3.9 ± 1.0[a]
HDL, mmol/L	1.5 ± 0.4	1.1 ± 0.3[a]
LDL, mmol/L	3.0 ± 0.7	1.9 ± 0.9[a]
Vascular complications		
Coronary artery disease	0	16
Retinopathy	0	13
Neuropathy	0	11
Nephropathy	0	5
Peripheral vascular disease	0	9
Medication use		
ACE inhibitors/ARBs	0	34
Aspirin	0	18
Lipid-lowering	0	46
β-blocker	0	21
Calcium channel inhibitor	0	8
Insulin	0	34
Metformin	0	39
GLP-1 analog	0	25
DPP-4 inhibitor	0	15
SGLT2 inhibitor	0	16

Values are mean ± SD or n. [a]$P < 0.001$ versus healthy subjects.

ACE = angiotensin-converting enzyme; ARB = angiotensin receptor blocker; BMI = body mass index; BP = blood pressure; DPP = dipeptidyl peptidase; GLP = glucagon like peptide; HbA_{1c} = glycosylated hemoglobin; HDL = high-density lipoprotein; LDL = low-density lipoprotein; SGLT2 = sodium-glucose protein 2 transporter; T2D = type 2 diabetes.

METHODS

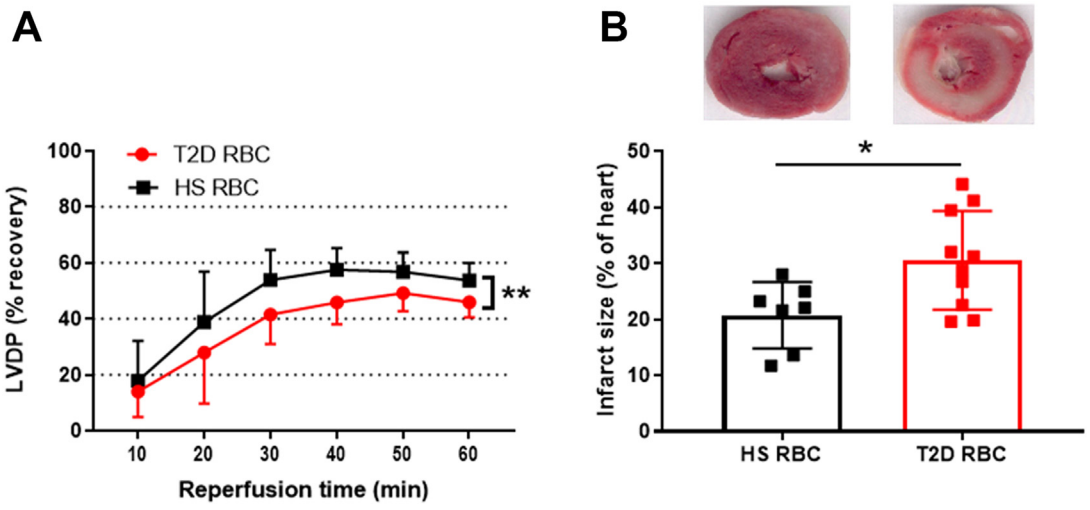
Expanded versions of the Methods are presented in the Supplemental Appendix.

HUMAN SUBJECTS. In total, 51 patients with T2D were recruited from the Department of Diabetology and Endocrinology, Karolinska University Hospital and Center for Diabetes, Academic Specialist Center, Health Care Services Stockholm County, Sweden. T2D was defined according to World Health Organization criteria. Twenty age-matched, healthy control subjects without diabetes or history of cardiovascular disease were recruited from a database at the department of Cardiology, Karolinska University Hospital. All procedures involving humans were conducted according to the Declaration of Helsinki. The protocol was approved by the Swedish Ethical Review Authority. All participants were informed of the study's purpose and gave their oral and written informed consent before any study-related procedures were initiated.

ANIMALS. All animal experiment protocols were approved by the Regional Ethical Review Board of Stockholm and conform to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (publication no. 85-23, revised 1996). Male Wistar rats (Charles River) and male *db/db* mice (Janvier Labs) were housed in the Comparative Medicine facility of Karolinska Institutet until 10-15 weeks of age before experimentation.

STATISTICAL ANALYSIS. Left ventricular developed pressure (LVDP) is presented as percentage recovery during reperfusion from baseline level. Left ventricular end-diastolic pressure (LVEDP) is expressed in absolute values. Cardiac performance during the reperfusion period was compared using repeated measures 1- or 2-way analysis of variance with time, treatment, and time-by-treatment interaction as fixed effects. Significant main effects were followed by

FIGURE 1 RBCs From Patients With T2D Aggravate Cardiac I/R Injury



Effect of red blood cells (RBCs) on recovery of left ventricular developed pressure (LVDP) infarct size in isolated rat hearts subjected to global ischemia-reperfusion (I/R). **(A)** Recovery of LVDP and **(B)** infarct size after administration of RBCs from patients with type 2 diabetes (T2D) (n = 17 in **A** and n = 10 in **B**) or from healthy subjects (HS) (n = 13 in **A** and n = 7 in **B**). Postischemic LVDP is presented as percentage recovery from baseline LVDP, and infarct size is expressed as percentage of the total myocardium. Data are presented as mean and SD in **A** and as individual data points and mean ± SD in **B.** Statistical differences were analyzed with 2-way repeated measures analysis of variance in **A** and by unpaired Student's *t*-test in **B;** $*P < 0.05$ and $**P < 0.01$.

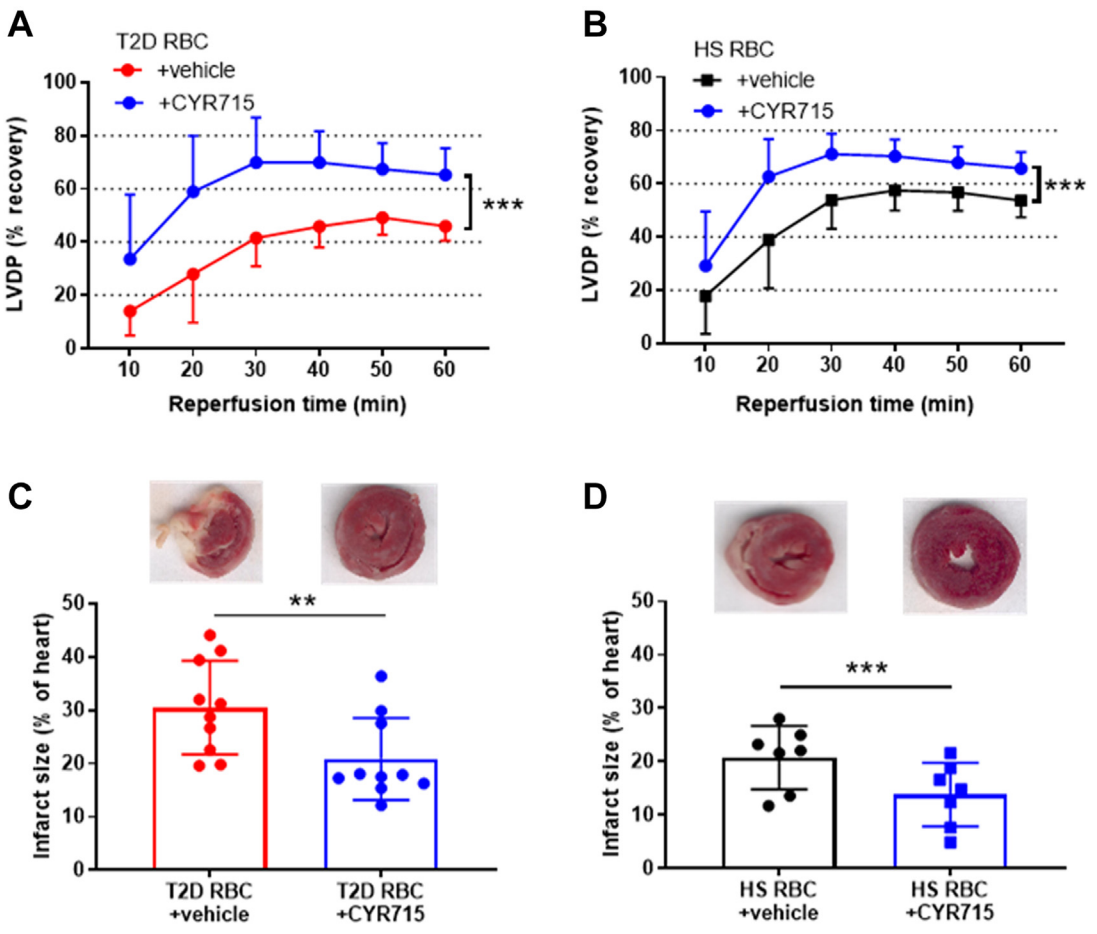
Tukey's post hoc test for multiple pairwise comparisons. Differences between 2 groups were analyzed by unpaired or paired Student's *t*-test or Mann-Whitney *U* test, depending on the distribution. Normality was checked with D'Agostino and Pearson test or Shapiro-Wilk test, depending on the number of observations in each group. All statistical analyses were calculated by using GraphPad Prism (version 7.04, GraphPad Software). Unless otherwise stated, data are presented as mean ± SD, and $P < 0.05$ was considered statistically significant.

RESULTS

SUBJECT CHARACTERISTICS. Subject characteristics are shown in Table 1. In the patients with T2D, body mass index, glucose, glycated hemoglobin, and triglyceride levels were higher, and total cholesterol, high-density lipoprotein, and low-density lipoprotein levels were lower compared with those in healthy subjects. None of the healthy subjects took any medication.

sGC STIMULATION IN RBCs IMPROVES CARDIAC POSTISCHEMIC FUNCTIONAL RECOVERY. First, we investigated the functional impact of human RBCs on the cardiac postischemic recovery and infarct size in isolated perfused hearts from rats subjected to I/R. Postischemic recovery of LVDP was impaired and infarct size was larger in hearts given RBCs from patients with T2D compared with hearts given RBCs from healthy subjects (Figures 1A and 1B). Incubation of RBCs either from patients with T2D or from healthy controls with the ferrous-dependent sGC stimulator CYR715[24] significantly enhanced recovery of LVDP (Figures 2A and 2B). The percentage improvement in postischemic recovery of LVDP induced by sGC stimulation in RBCs was significantly greater in hearts given RBCs from patients with T2D than in those given RBCs from healthy controls ($P = 0.002$), resulting in comparable postischemic cardiac function in hearts exposed to RBCs from healthy subjects and patients with T2D after incubation with the sGC stimulator. Consequently, CYR715 prevented the negative effect of RBCs from patients with T2D on postischemic cardiac function. In addition, compared with vehicle, myocardial infarct size was significantly reduced by CYR715 (Figures 2C and 2D). Preincubation of the RBCs with CYR715—regardless of whether the RBCs were from patients with T2D or from healthy subjects—also reduced LVEDP compared with vehicle (Supplemental Figure 1).

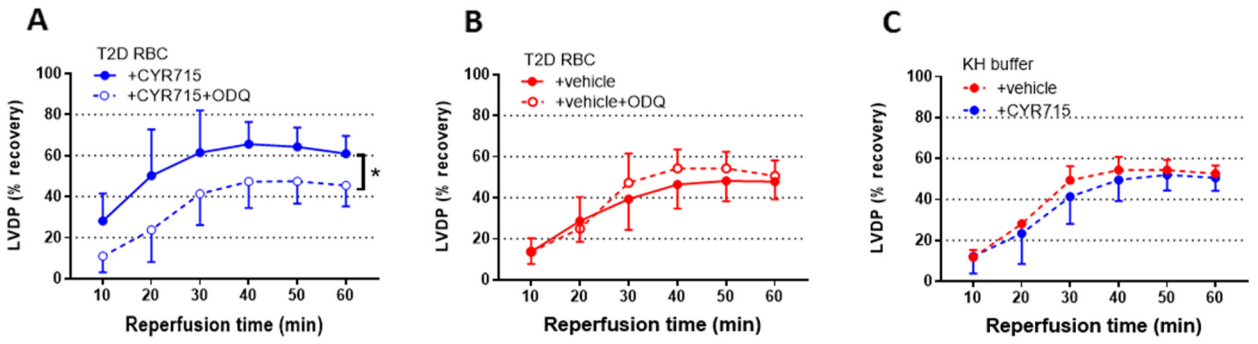
FIGURE 2 **sGC Stimulation in RBCs Improves Cardiac Postischemic Function and Reduces Infarct Size**



Recovery of LVDP after administration of RBCs from **(A)** patients with T2D (n = 17) or **(B)** HSs (n = 13) incubated with vehicle or the soluble guanylyl cyclase (sGC) stimulator CYR715. Myocardial infarct size in hearts given RBCs from **(C)** patients with T2D (n = 10) or **(D)** HSs (n = 7) incubated with vehicle or CYR715. Postischemic LVDP is presented as percentage recovery from baseline LVDP. Data are presented as mean and SD in **A** and **B** and as individual data points and mean ± SD in **C** and **D**. Statistical differences in **A** and **B** were analyzed with 2-way repeated measures analysis of variance and with paired Student's *t*-test in **C** and **D**; $^{**}P < 0.01$ and $^{***}P < 0.001$. Abbreviations as in Figure 1.

CARDIOPROTECTION IS MEDIATED VIA STIMULATION OF sGC IN RBCs AND RELEASE OF A CARDIOPROTECTIVE FACTOR. Next, we investigated the mechanisms behind the cardioprotection induced by sGC stimulation in RBCs. Because the therapeutic effect was more pronounced when studying RBCs from patients with T2D than from healthy subjects, these investigations were focused on RBCs from patients with T2D. The improvement in postischemic recovery of LVDP by CYR715 was attenuated by coincubation with the sGC inhibitor 1H-[1,2,4]oxadiazolo-[4, 3-a]quinoxalin-1-one (ODQ) (Cayman Chemical) (Figure 3A), supporting the hypothesis that the cardioprotection induced by CYR715 in RBCs was sGC-dependent. There was no effect of ODQ on recovery in the absence of CYR715 (Figure 3B). Interestingly, CYR715 administered with Krebs-Henseleit (KH) buffer only (without RBCs) to the isolated heart failed to improve postischemic recovery of LVDP compared with vehicle (Figure 3C). These findings demonstrate that the cardioprotective effect is dependent on sGC in the RBCs but independent of cardiac sGC. To explore whether the beneficial effects could be enhanced by additional exogenous NO, the RBCs were incubated with CYR715 and the NO donor diethylamine NONOate diethylammonium (DEA-NO) (Cayman

FIGURE 3 sGC Stimulation Protects From I/R Injury via sGC in RBCs



Recovery of LVDP following administration of RBCs incubated with **(A)** the sGC stimulator CYR715 (n = 5), or CYR715 + the sGC inhibitor ODQ (n = 5) and **(B)** vehicle (n = 4) or vehicle + ODQ (n = 5). **(C)** Recovery of LVDP following administration of Krebs-Henseleit (KH) buffer incubated with vehicle (n = 4) or CYR715 (n = 6). Postischemia LVDP is presented as percentage recovery from baseline LVDP. Data are presented as mean and SD. Statistical differences between groups were analyzed with 2-way repeated measures analysis of variance. *$P < 0.05$. Abbreviations as in Figures 1 and 2.

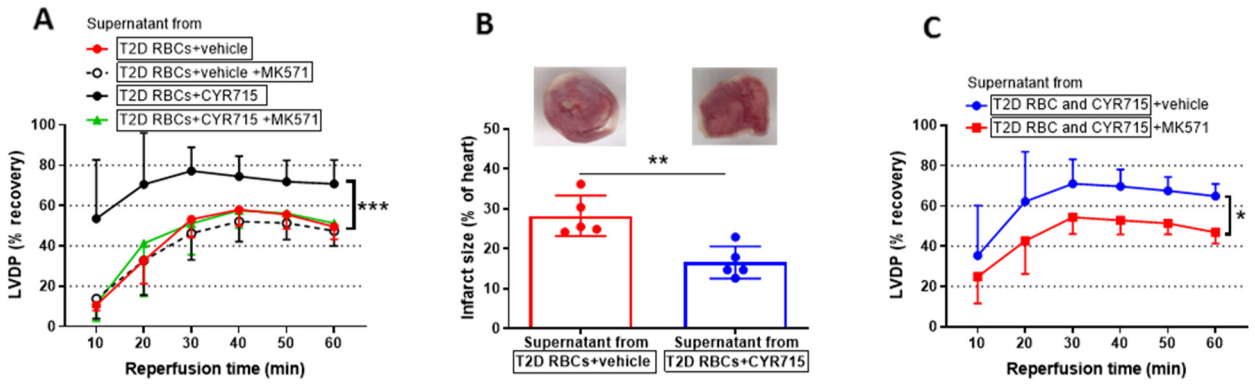
Chemical). The postischemic recovery of LVDP induced by CYR715 in the presence of RBCs from patients with T2D was not further improved by the combined treatment (Supplemental Figure 2).

To determine whether the cardioprotection is mediated by release of a cardioprotective signal, the supernatant from the coincubation of RBCs with either CYR715 or vehicle was administered to the isolated heart. Notably, administration of the supernatant from the T2D RBCs incubated with CYR715 improved postischemic recovery of LVDP (Figure 4A). This cardioprotective effect of the supernatant was abolished by the addition of the cGMP transport inhibitor MK-571 to the coincubation of RBCs and CYR715 (Figure 4A). Moreover, the infarct size was smaller in hearts that received supernatant from T2D RBCs incubated with CYR715 compared with that in hearts that received supernatant from vehicle-incubated RBCs (Figure 4B), further supporting a cardioprotective effect. Postischemic recovery of cardiac function was not affected by the administration of CYR715 to the supernatant collected from RBCs (Supplemental Figure 3A) or the administration of ODQ to the supernatant collected from RBCs incubated with CYR715 (Supplemental Figure 3B), supporting the notion that the cardioprotective effect was mediated by a signal downstream of sGC in the RBCs and released following sGC stimulation in RBCs but independent of sGC in the heart. Interestingly, the cardioprotective effect of the supernatant from RBCs incubated with CYR715 was attenuated when the cGMP transport inhibitor MK-571 was added to the supernatant after the separation of the supernatant and the RBCs (Figure 4C), suggesting transport of a cardioprotective mediator to the heart. MK-571 also blocked the cardioprotection induced by CYR715 when RBCs were administered to the heart, but it did not affect cardiac recovery in the absence of CYR715 (Supplemental Figure 4).

To determine whether stimulation of sGC in RBCs from patients with T2D also provides protection to the diabetic heart, supernatant from RBCs incubated with CYR715 was administered to hearts from *db/db* mice. Postischemic recovery of LVDP in hearts from *db/db* mice was significantly improved following administration of supernatant from RBCs of T2D mice incubated with CYR715 in comparison with vehicle (Supplemental Figure 5).

STIMULATION OF RBC sGC INDUCES RELEASE OF cGMP. Incubation of RBCs from patients with T2D with the sGC stimulator induced a significant increase in cGMP levels in the supernatant (Figure 5A). The cGMP level detected in the supernatant was further enhanced in the presence of sildenafil, an inhibitor of the cGMP-hydrolyzing enzyme phosphodiesterase-5 (Figure 5A), but it was not affected by the cGMP transport inhibitor MK-571 (Supplemental Figure 6). To verify that exogenous cGMP exerts cardioprotection in the present model, we show that administration of either 8-bromo cGMP or cGMP in buffer resulted in enhanced recovery of LVDP (Figures 5B and 5C). Finally, the cardioprotective effect of cGMP was attenuated by the cGMP transport inhibitor MK-571 (Figure 5C), suggesting the transport of cardioprotective cGMP to the heart was blocked by MK-571. The concentration of nitrate in the

FIGURE 4 Cardioprotection by sGC Stimulation in RBCs Is Mediated via a Releasable Factor



(A) Recovery of LVDP following administration of supernatant from RBCs from patients with T2D following incubation with vehicle (n = 5), CYR715 (n = 5), vehicle + the cyclic guanosine monophosphate transport inhibitor MK-571 (n = 4), or CYR715 + MK-571 (n = 4). **(B)** Myocardial infarct size in hearts that received supernatant from T2D RBCs incubated with vehicle (n = 5) or CYR715 (n = 5). **(C)** Recovery of LVDP induced by supernatant collected from RBCs stimulated by CYR715 or vehicle and subsequent addition of vehicle (n = 6) or MK-571 (n = 6) to the supernatant. Text within frames denotes incubation together with the RBCs. LVDP is presented as percentage recovery from baseline LVPD. Data are presented as mean and SD in **A** and **C** and as individual data points and mean ± SD in **B**. Statistical differences in **A** and **C** were analyzed with 2-way repeated measures analysis of variance and with paired Student's *t*-test in **B**. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. Abbreviations as in Figures 1 and 2.

supernatant of RBCs was not changed following incubation with CYR715 (Supplemental Figure 7), suggesting that the sGC stimulator did not alter NO formation or export of nitrate from the RBC. Collectively, these observations suggest that the cardioprotection induced by sGC stimulation is mediated by cGMP released from the RBCs.

THE CARDIOPROTECTIVE SIGNAL FROM RBCs ACTIVATES CARDIAC PKG. To identify the downstream target in the heart, cardiac PKG-dependent phosphorylation of vasodilator-specific phosphoprotein (pVASP) was measured. The expression of pVASP at Ser239, which is the major phosphorylation site of PKG, was increased in hearts after administration of RBCs incubated with CYR715 (**Figure 6**).

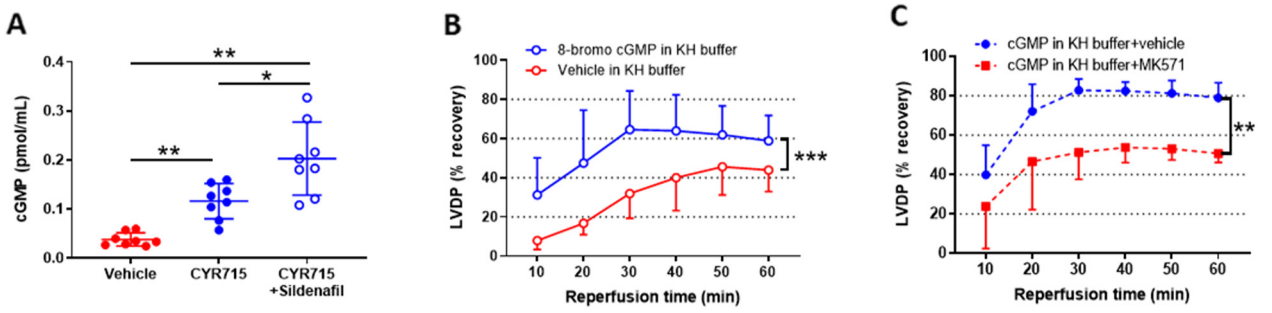
To further specify the cardiac effect of the protective mediator released from RBCs of patients with T2D, the supernatant from RBCs incubated with CYR715 was administered to the hearts followed by determination of pVASP expression using immunofluorescence. Administration of supernatant from RBCs incubated with the sGC stimulator induced a marked increase in pVASP immunofluorescence compared to vehicle (**Figure 7**). The increased expression was colocalized with the cardiomyocyte marker sarcomeric alpha-actinin (**Figure 7**). Lack of unspecific staining was confirmed using mouse and rabbit immunoglobulin G (Supplemental Figure 8). Collectively, these observations suggest that sGC stimulation in RBCs induces release of a cardioprotective factor activating cGMP-dependent PKG in cardiomyocytes.

DISCUSSION

The present study provides novel and important insights into the molecular signaling of RBCs in their role in cardiovascular regulation as well as the potential therapeutic effect of interventions targeting RBC dysfunction to treat cardiovascular injury. To the best of our knowledge, these results for the first time demonstrate that stimulation of sGC in RBCs from patients with T2D protect against cardiac I/R injury, and that this protective effect is mediated by a cardioprotective factor released from the RBCs and subsequent activation of cardiac PKG. Furthermore, stimulation of sGC in the RBCs resulted in increased export of cGMP from the RBCs, which led to cardioprotection. These observations demonstrate a novel mechanism by which RBCs regulate cardiovascular function via a signaling pathway involving activation of erythrocytic sGC that leads to extracellular release of cGMP that in turn activates cardiac PKG to protect the heart during I/R.

The mediator(s) of the biological effects of RBCs on cardiovascular function has been a matter of great discussion.[16,25-28] Although NO can be produced by eNOS in RBCs, it is assumed to be rapidly scavenged

FIGURE 5 Stimulation of sGC in RBCs Induces Release of Cardioprotective cGMP

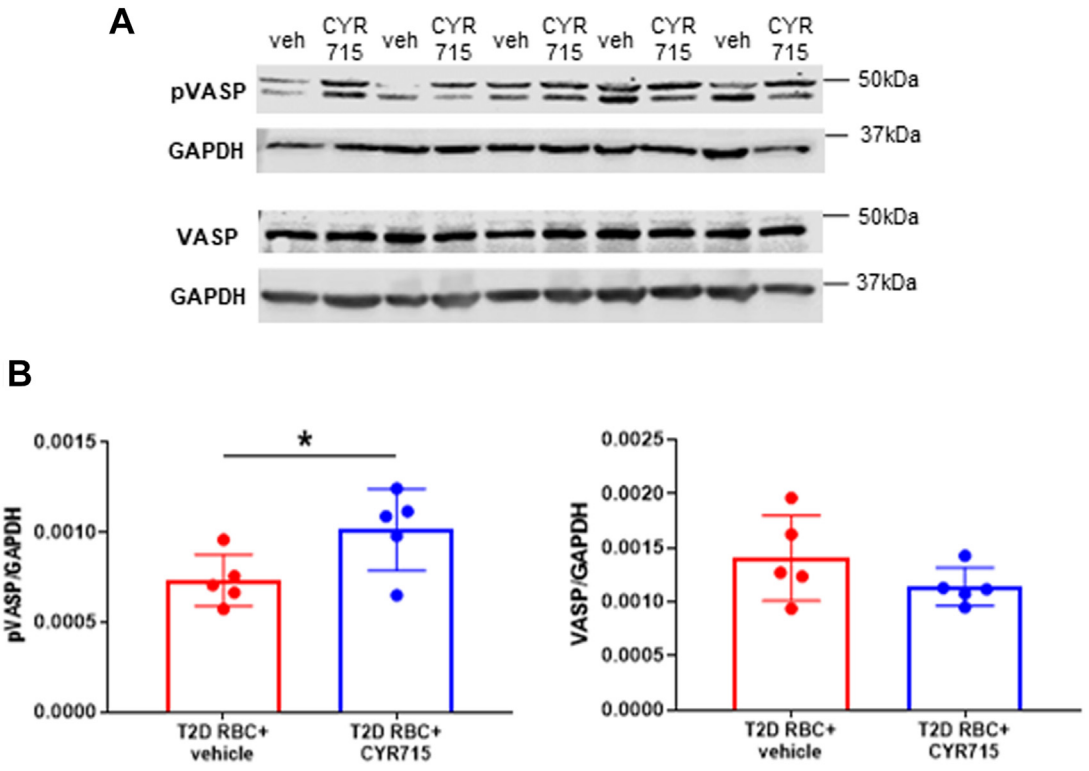


(A) Cyclic guanosine monophosphate (cGMP) levels in supernatant from RBCs of patients with T2D following incubation with vehicle (n = 8), CYR715 (n = 8) or CYR715 + the inhibitor of phosphodiestrase-5 sildenafil (n = 8). **(B)** Recovery of LVDP following administration of the cGMP analogue 8-bromo cGMP (n = 4) or vehicle (n = 4) in KH buffer and **(C)** cGMP in KH buffer in combination with vehicle (n = 5) or the cGMP transporter inhibitor MK-571 (n = 5). cGMP expression is expressed in absolute values. Postischemia LVDP is presented as percentage recovery from baseline LVPD. Data are presented as individual data points and mean ± SD in **A** and mean ± SD in **B** and **C**. Statistical differences in **A** were analyzed with 1-way analysis of variance followed by Tukey post hoc test and in **B** and **C** with 2-way repeated measures analysis of variance including all time points. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. Abbreviations as in Figures 1 to 3.

by hemoglobin and is therefore unlikely to be the mediator. The term "NO bioactivity" is often used to describe the biological effect mediated by the NO-sGC signaling pathway in RBCs,[27] which reflects the uncertainty of its molecular identity. In the present study we provide several lines of evidence suggesting that RBCs mediate protection against I/R injury following sGC stimulation by exporting cGMP, which transfers the cardioprotective effect by activating cardiac PKG. First, RBCs preincubated with the sGC stimulator CYR715 and administered to the buffer-perfused isolated heart protected the heart from postischemic dysfunction and reduced infarct size. By contrast, administration of CYR715 in buffer only or addition of CYR715 to the supernatant collected from the RBCs failed to protect the heart, suggesting that stimulation of sGC in the RBCs, rather than in the cardiac tissue, is essential for the cardioprotection. Second, the supernatant from the coincubation of RBCs and the sGC stimulator protected the ischemic heart, suggesting transfer of a cardioprotective factor via a mechanism that was sensitive to inhibition of multidrug-resistance protein (MRP) types 4 and 5, which are known to be responsible for cGMP transport.[29,30] Third, the cardioprotective signal released from the RBCs into the supernatant induced cardiomyocyte phosphorylation of VASP, a marker of cGMP-dependent PKG activation.[31] Finally, cGMP levels were increased in the cardioprotective supernatant following incubation with the sGC simulator, and exogenous cGMP exerted cardioprotection that was blocked by the cGMP transport inhibitor. Collectively, these observations demonstrate that stimulation of sGC in RBCs from patients with T2D induces export of cGMP from RBCs followed by transport to the cardiomyocytes to exert cardioprotection.

The demonstration that cGMP levels were increased in the supernatant following incubation of the RBCs with the sGC stimulator indicates that the RBCs export cGMP. The cGMP levels detected in the supernatant were further enhanced by the inhibitor of the cGMP-degrading enzyme phosphodiesterase-5, lending support to the conclusion that the detected signaling molecule is cGMP. Somewhat surprisingly, the inhibitor of cGMP transport, MK-571, did not affect the cGMP level in the supernatant, suggesting that MK-571 did not affect the export of cGMP from RBCs, but MK-571 did attenuate the cardioprotection induced by the supernatant. Interestingly, we found that MK-571 also attenuated the cardioprotective effect by the RBC supernatant when administered after separation of the supernatant from the RBCs, as well as the cardioprotective effect induced by exogenously administered cGMP. These observations suggest that MK-571 also interferes with the use of cGMP by the myocardium. Although MRP5 is expressed in the vasculature and myocardium,[32] the MRPs are considered to induce efflux but not influx of cGMP.[33] Interestingly, MK-571 also inhibits organic anion transporters,[34] which are known to transport cGMP into cells,[35] and explains the inhibitory effect of MK-571 on the cardioprotection induced by exogenous cGMP in our study. Moreover, we demonstrate that administration of the supernatant from RBCs incubated with CYR715 resulted in increased

FIGURE 6 **Stimulation of sGC in RBCs Increases Cardiac pVASP Expression**

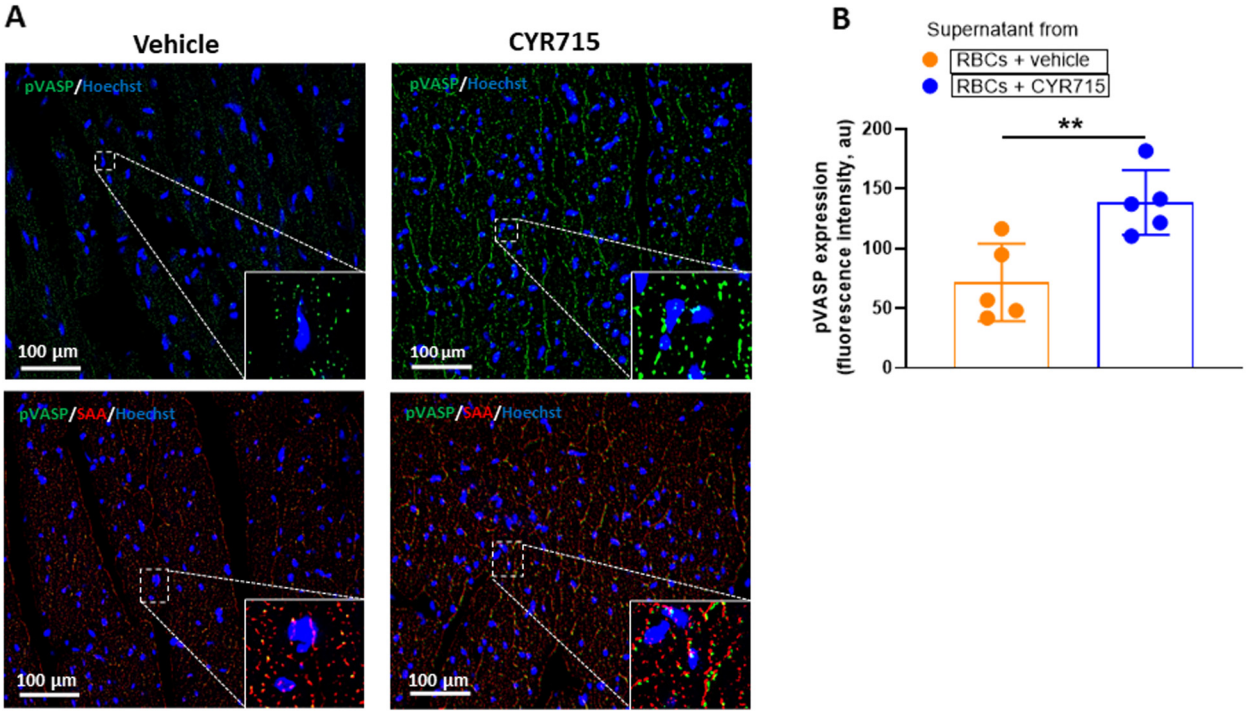


(A) Expression of phosphorylated vasodilator-specific phosphoprotein (pVASP) and VASP in hearts that received RBCs incubated with vehicle (veh) or CYR715. **(B)** Quantification of pVASP and VASP expression in hearts that received RBCs incubated with vehicle (n = 5) or CYR715 (n = 5) normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. Data are presented as individual data points and mean ± SD. Statistical differences between groups were analyzed with paired Student's *t*-test. **P* < 0.05. Abbreviations as in Figure 1.

phosphorylation of VASP, suggesting activation of PKG in the cardiomyocytes. As cGMP-dependent PKG is suggested to mediate protection against cardiac I/R injury,[23] it is reasonable to assume that the functional improvement in cardiac postischemic recovery induced by the sGC stimulation in RBCs was mediated by cardiomyocyte PKG activated by cGMP released from the RBCs. Thus, our results suggest that after sGC stimulation, RBCs export cGMP that is transferred to the heart to protect from I/R injury. The suggestion that cGMP may exert paracrine action is supported by the observations that fibroblast-derived cGMP affects cardiomyocytes,[36] and cGMP released from mucosal epithelial cells acts on and inhibits nociceptors.[37]

The present results have potential therapeutic implications. Available treatments for T2D focus on regulation of glucose metabolism, whereas treatments that specifically target and prevent cardiovascular complications in the T2D patient population are limited or nonexistent.[38] This is partly because the mechanisms behind cardiovascular complications in T2D are incompletely understood. There is therefore a clinical need for identification of specific disease mechanisms that drive cardiovascular complications in T2D in order to develop novel therapies targeting such complications. In this study, we confirmed previous results demonstrating that RBCs from patients with T2D exert detrimental effects on cardiac recovery after I/R because of reduced NO-signaling.[17] We now provide ex vivo evidence that the function of RBCs from patients with T2D can be improved by sGC stimulation and cGMP release, and that this effect is achieved by actions in RBCs. Interestingly, after incubation of the RBCs with the sGC stimulator, the postischemic recovery of cardiac function was similar in hearts exposed to RBCs from healthy subjects and in hearts exposed to RBCs from patients with T2D. This finding indicates that the function of RBCs from patients with T2D was normalized after sGC

FIGURE 7 Supernatant of RBCs Following sGC Stimulation Increases pVASP Expression in Cardiomyocytes



(A) Representative immunofluorescence images and **(B)** quantification in an arbitrary unit (au) of pVASP in cardiomyocytes following administration of supernatant from RBCs incubated with vehicle or CYR 715. Text within frames in **B** denotes incubation together with the RBCs. Immunoreactivity of pVASP was visualized using Alexa Fluor 488 antibody (Life Technologies) **(green)** and the cardiomyocyte specific marker sarcomeric alpha-actinin (SAA) was visualized using an Alexa Fluor 594 secondary antibody (Life Technologies) **(red)**. Nuclei were stained with Hoechst (Sigma-Aldrich) **(blue)**. Statistical differences were analyzed with paired Student's *t*-test; $^{**}P < 0.01$. Abbreviations as in Figures 1, 2, and 6.

stimulation. Based on these results, stimulation of sGC in RBCs may provide a new therapeutic strategy for attenuating cardiovascular complications in T2D. It is also of interest that incubation of RBCs from healthy subjects with the sGC stimulator also improved the recovery of postischemia cardiac function and reduced infarct size. This indicates that sGC in RBCs from healthy individuals who are nondiabetic can be further stimulated and, even in RBCs with physiological NO bioactivity, sGC stimulation may exert beneficial therapeutic effects in the setting of myocardial I/R. Interestingly, coronary artery disease, myocardial infarction, and other diabetes-associated cardiovascular complications are described to be related to dysfunction of cGMP signaling based on reduced cGMP synthesis and/or increased cGMP breakdown.[39] sGC stimulators can increase cGMP generation and restore physiological cGMP response when tissue levels of NO are low.[15] The results of the present study indicate that these beneficial effects may be mediated via effects in RBCs. Based on the present results, it is tempting to speculate that part of the cardioprotective effect demonstrated with sGC stimulators in vivo[40,41] and the beneficial effect of sGC stimulation in the clinical setting[20-22,42] may be related to effects mediated via RBCs. However, this potential effect needs further investigations.

STUDY LIMITATIONS. The effect of the RBCs was evaluated in an isolated rat heart model ex vivo, and therefore it cannot be determined from these data the extent to which sGC stimulation in RBCs may contribute to cardioprotection in vivo in humans. The clear advantage of this model is that it permits investigation of the specific actions of human RBCs. Furthermore, much of the functional data are based on experiments using pharmacologic approaches. Thus, off-target effects of the drugs used in the current study cannot be excluded, although the sGC stimulator is known to be highly selective.[24] The use of genetic deletion or gene silencing is unfortunately

not possible when using human RBCs. Moreover, it remains to be established how cGMP is transmitted from the RBCs to the target cell to exert the protective effects. Previous findings suggested that membrane vesicles may be involved in the transport of cGMP from human and mouse RBCs.[30] However, it remains to be investigated whether such a mechanism is involved under the conditions investigated in the current study. Finally, it cannot be excluded that additional signaling molecules besides cGMP or closely related derivatives of cGMP are involved in the communication between the RBCs and target cells.

CONCLUSIONS

Our study demonstrates a novel mechanism whereby RBCs provide protection against cardiac injury by exporting cardioprotective cGMP after stimulation of sGC in the RBCs. The study identifies cGMP being exported from RBCs as a cardioprotective signal mediating protection to the heart during I/R. We further extend the well-established NO-sGC-cGMP signaling pathway to the RBC, a previously overlooked cell type in cardiovascular disease, leading to activation of cardiac PKG. These findings advance our understanding of the significant role of RBCs in cardiovascular regulation by identifying the underlying signaling pathway providing cardiovascular protection and illustrating the therapeutic potential of sGC stimulation to protect from cardiovascular injury via an effect in RBCs.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Erythrocytes regulate cardiovascular function and contribute to cardiovascular injury in T2D. Stimulation of sGC in human erythrocytes protects the heart from I/R injury, with a particularly pronounced effect in erythrocytes from patients with T2D. Erythrocytic sGC stimulation induces release of cGMP, which can be transported to cardiomyocytes and lead to activation of cardiac PKG and cardioprotection.

TRANSLATIONAL OUTLOOK 1: Novel understanding of how NO bioactivity is transmitted from the erythrocyte and protects the target organ.

TRANSLATIONAL OUTLOOK 2: Therapeutic strategies that target NO-sGC signaling in erythrocytes have the potential to protect against cardiovascular injury in T2D.

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