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Red blood cell eNOS is cardioprotective in acute myocardial infarction

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ABSTRACT

Red blood cells (RBCs) were shown to transport and release nitric oxide (NO) bioactivity and carry an endothelial NO synthase (eNOS). However, the pathophysiological significance of RBC eNOS for cardioprotection *in vivo* is unknown. Here we aimed to analyze the role of RBC eNOS in the regulation of coronary blood flow, cardiac performance, and acute myocardial infarction (AMI) *in vivo*. To specifically distinguish the role of RBC eNOS from the endothelial cell (EC) eNOS, we generated RBC- and EC-specific knock-out (KO) and knock-in (KI) mice by Cre-induced inactivation or reactivation of eNOS. We found that RBC eNOS KO mice had fully preserved coronary dilatory responses and LV function. Instead, EC eNOS KO mice had a decreased coronary flow response in isolated perfused hearts and an increased LV developed pressure in response to elevated arterial pressure, while stroke volume was preserved. Interestingly, RBC eNOS KO showed a significantly increased infarct size and aggravated LV dysfunction with decreased stroke volume and cardiac output. This is consistent with reduced NO bioavailability and oxygen delivery capacity in RBC eNOS KOs. Crucially, RBC eNOS KI mice had decreased infarct size and preserved LV function after AMI. In contrast, EC eNOS KO and EC eNOS KI had no differences in infarct size or LV dysfunction after AMI, as compared to the controls. These data demonstrate that EC eNOS controls coronary vasodilator function, but does not directly affect infarct size, while RBC eNOS limits infarct size in AMI. Therefore, RBC eNOS signaling may represent a novel target for interventions in ischemia/reperfusion after myocardial infarction.

1. Introduction

Nitric oxide (NO) generated constitutively by the endothelial NO synthase (eNOS) plays a pleiotropic and fundamental physiological role in the heart [1]. In cardiac tissue, eNOS is mainly expressed in the coronary and endocardial endothelial cells (ECs) and is proposed to regulate coronary blood flow and cardiac performance in an autocrine and paracrine fashion [2,3].

Abbreviations: AMI, acute myocardial infarction; BP, blood pressure; CO, cardiac output; CondKO, conditional global eNOS knock-out mice; DBP, diastolic blood pressure; EF, ejection fraction; EDV, end-diastolic volume; EC(s), endothelial cell(s); eNOS, endothelial nitric oxide synthase; EC eNOS KO, endothelial cell-specific eNOS knock-in mice; ESV, end-systolic volume; FS, fractional shortening; gKO, global eNOS KO mouse; HR, heart rate; IS, infarct size; LV function, left ventricular function; MAP, mean arterial pressure; NO, nitric oxide; PE, phenylephrine; RBC eNOS KO, RBC-specific eNOS knock-out mice; RBC eNOS KI, RBC-specific eNOS knock-in mice; RBCs, red blood cell(s); SV, stroke volume; SBP, systolic blood pressure; WT, wild type.

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Multiple cardioprotective roles have been attributed to NO donors, NO metabolites, and endogenously produced NO [1,4]. However, the role of eNOS-derived NO in cardioprotection from acute myocardial infarction (AMI) is still controversial. Administration of NOS inhibitors or genetic ablation of eNOS in multiple global eNOS knock-out (KO) mice strains have generated conflicting effects on myocardial infarct size and cardiac performance after AMI, as well as different outcomes depending on the species and also the eNOS KO strain under study [5–12]. This may be due to the distinct cell-specific role of eNOS, or to several compensatory mechanisms occurring after constitutive global genetic deletion of eNOS during development or in adulthood.

We have previously reported that eNOS is expressed in RBCs [13,14]. To specifically distinguish the role of RBC eNOS from EC eNOS, we generated RBC- and EC-specific knock-out (KO) and knock-in (KI) mice by Cre-induced inactivation or reactivation of eNOS [15]. We found that RBC eNOS effectively contributes to the regulation of circulating NO metabolites and blood pressure [15]. Available evidence from us and others show that RBC eNOS *ex vivo* plays also a role in protection against myocardial ischemia-reperfusion (I/R) injury [16–18], but its *in vivo* effect is unknown. Moreover, depletion of eNOS from circulating blood, which was achieved by transplanting eNOS KO bone marrow into irradiated wild type (WT) mice, increased infarct size and left ventricular (LV) dysfunction and worsened remodeling following AMI [19,20]. However, these studies using bone marrow chimeras have major limitations, as the phenotype of irradiated chimera mice is confounded by low-grade inflammation and lack of RBC-specificity. Therefore, the pathophysiological significance of RBC eNOS for cardioprotection *in vivo* remains unclear.

Here we aimed to analyze the role of RBC eNOS in the regulation of coronary blood flow, cardiac performance, and acute myocardial infarction (AMI). By comparing RBC eNOS KO and RBC eNOS KI mice we here demonstrate that RBC eNOS does not affect coronary vasodilator function, but limits infarct size in AMI and thus represents a novel target for interventions in ischemia/reperfusion after myocardial infarction.

2. Materials and methods

A detailed description of the methods is available in the Supplemental Information. The data that support the findings of this study are available from the corresponding author on reasonable request.

2.1. Animals

All experiments were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe Treaty Series No. 123). For experiments, 2–6 months old male mice up to 32 g were used. Mice of the same genotype and age were randomly assigned to experimental groups. Experiments were repeated with multiple cohorts of mice to control for variability and assure reproducibility. Evaluation of data obtained by ultrasound was carried out by a blinded researcher.

2.2. Generation of EC eNOS KO/KI mice and RBC eNOS KO/KI mice

We generated two independent founder lines carrying a floxed eNOS ($eNOS^{flox/flox}$) or a gene construct with an inactivated floxed/inverted exon ($eNOS^{inv/inv}$) for a Cre-inducible KI, as described [15]. Phenotypically, $eNOS^{flox/flox}$ are WT mice, and $eNOS^{inv/inv}$ are conditional global eNOS KO mice (CondKO) [15]. These founder lines respectively allow targeted removal or reactivation of eNOS in either EC or RBCs [15] or any other tissue of choice.

To generate global eNOS, KO mice (gKO) $eNOS^{flox/flox}$ mice were crossed with Cre Deleter (C57Bl/6.C-Tg(CMVCre)1Cgn/J) [21]. To generate EC eNOS KO and EC eNOS KI mice and their respective littermate control mice, homozygous $eNOS^{flox/flox}$ mice or $eNOS^{inv/inv}$ mice were crossed with EC-specific tamoxifen-inducible Cdh5-Cre/$ERT2^{pos}$ mice (Tg(Cdh5-Cre/ERT2)1Rha; MGI:3848982) [22]. To induce EC-specific activation of the Cre-recombinase, we treated Cre positive and negative mice of each line with tamoxifen (33 mg/kg/day) for 5 consecutive days and allowed a 21 day waiting period after the last injection. The treatment with tamoxifen generated EC eNOS KO ($eNOS^{flox/flox}$ Cdh5-Cre/$ERT2^{pos}$ + TAM) mice and their WT littermate control ($eNOS^{flox/flox}$ Cdh5-Cre/$ERT2^{neg}$ + TAM), as well as EC eNOS KI ($eNOS^{inv/inv}$ Cdh5-Cre/$ERT2^{pos}$ + TAM) mice and their respective CondKO littermate control mice ($eNOS^{inv/inv}$ Cdh5-Cre/$ERT2^{neg}$ + TAM).

To generate RBC eNOS KO (and their respective WT littermates) and RBC eNOS KI mice (and their respective CondKO littermates), we crossed homozygous $eNOS^{flox/flox}$ mice or $eNOS^{inv/inv}$ mice with erythroid-specific Hbb-Cre^{pos} mice hemoglobin beta chain (C57BL/6-Tg (Hbb-Cre)12Kpe/J; MGI: J:89725) [23], which were obtained from Jackson Laboratory (JAX stock #008314); this breeding strategy generated RBC eNOS KO ($eNOS^{flox/flox}$ Hbb-Cre^{pos}) and WT ($eNOS^{flox/flox}$ Hbb-Cre^{neg}) littermate controls, or RBC eNOS KI ($eNOS^{inv/inv}$ Hbb-Cre^{pos}) and their CondKO ($eNOS^{inv/inv}$ Hbb-Cre^{neg}) littermate controls. For a structured list of the mouse lines, the nomenclature used in the figures and the text, anatomical characteristics of the heart, cardiomyocyte size, and coronary vessel wall area please refer to **Table 1**.

2.3. Analysis of cell-specific loss/reactivation of eNOS expression

The Cre recombinase-dependent recombination of the genetic locus was determined in targeted and non-targeted cells and tissues by real-time polymerase chain reaction (PCR) with specific primers and probes designed to recognize the floxed allele and the allele with targeted deletion (Transnetyx, Cordova, TN). The expression of eNOS and Cre recombinase were analyzed by TaqMan real-time reverse transcription (RT) PCR in ECs ($CD31^+$ $CD45^-$) magnetically isolated from heart homogenates, in erythroid cells ($Ter119^+$ $CD71^+$ $CD45^-$) magnetically isolated from the bone marrow, in cardiomyocytes [24] isolated from the hearts, and in targeted or non-targeted tissues, as explained in detail in the Figure legends and Supplement. The expression of eNOS in RBCs from the mice lines was analyzed here by immunotransmission electronic microscopy [15,25], and by immunoprecipitation and Western blot analysis elsewhere [15]. The expression of eNOS in heart homogenates and other tissues was analyzed by Western blot analysis [13] and quantitative ELISA according to the manufacturer's protocol (Abcam, Cambridge, UK). The expression of iNOS and nNOS in heart or skeletal muscle were analyzed by Western blotting in pre-cast 3–8% Tris Acetate gels (Invitrogen). Mouse anti-iNOS and anti-nNOS antibodies were diluted 1:1000 and membrane staining was carried out as described for eNOS [13].

2.4. Assessment of coronary hemodynamics and cardiac function in isolated perfused hearts

Coronary hemodynamics and cardiac function were determined *ex vivo* in isolated perfused hearts according to Langendorff as previously described [26] with some modifications. To assess coronary endothelial function, we induced 20 s (s) or 1 min of global ischemia, followed by 5 min of reperfusion. The effects of NOS inhibition on reactive hyperemia were studied by injecting the potent non-selective inhibitor S-ethyl-isothiourea (ETU [27], 5 μmol) 15 min before performing the second set of brief ischemia/reperfusion. Bradykinin (5 μmol), adenosine (1 μmol), and isoproterenol (12 pmol) were infused into the aortic cannula one after the other with 10–15 min washout intervals in between. The reactive hyperemic response to brief ischemia (20 s or 1 min) and the flow response to bradykinin were quantified as area under the curve during 1 min after stimulation after subtraction of the baseline resting coronary flow measured before intervention.

Table 1
Basal characteristics of the mouse lines analyzed in this study. Data are reported as mean ± SD; n = number of analyzed mice. Differences between KO/WT or KI/CondKO were calculated by unpaired *t*-test with Welch correction. None of the comparisons shows any statistical significance.

Line No	Experimental Groups	Genotype	Litter size	Body weight (n)	Heart weight (n)	Heart weight index (n)	Cardiomyocyte area (n)	Coronary vessel perimeter ranging from 50 to 100 μm (n)	Coronary vessel wall area (n)	Cardiomyocyte area/Vessel area ratio (n)
1	**EC eNOS KO**	**eNOS$^{flox/flox}$ Cdh5-Cre/ ERT2^{pos} + TAM**	6 ± 2	30.4 ± 3.1 (14)	168.7 ± 32.2(14)	5.5 ± 0.7 (14)	179.3 ± 27.0(5)	61.4 ± 2.2(5)	106.7 ± 20.7 (5)	1.7 ± 0.4 (5)
	WT control	**eNOS$^{flox/flox}$ Cdh5-Cre/ ERT2^{neg} + TAM**		30.9 ± 1.7 (12)	173.4 ± 26.0(12)	5.6 ± 0.8 (12)	169.2 ± 13.1(5)	61.5 ± 2.2(5)	106.5 ± 12.9 (5)	1.7 ± 0.4 (5)
2	**EC eNOS KI**	**eNOS$^{inv/inv}$ Cdh5-Cre/ ERT2^{pos}+ TAM**	4 ± 2	29.9. ± 12.2 (7)	172.5 ± 61.2(7)	5.1 ± 1.9 (7)	188.7 ± 23.7(3)	58.8 ± 1.8(3)	118.1 ± 17.8 (3)	1.6 ± 0.5 (3)
	CondKO control	**eNOS$^{inv/inv}$ Cdh5-Cre/ ERT2^{neg}+ TAM**		29.1 ± 1.6 (8)	186.1 ± 26.8(8)	6.4 ± 0.6 (8)	213.6 ± 17.3(3)	62.3 ± 3.4(3)	137.9 ± 15.4 (3)	1.6 ± 0.2 (3)
3	**RBC eNOS KO**	**eNOS$^{flox/flox}$ HbbCre^{pos}**	6 ± 2	32.4 ± 1.3(13)	186.1 ± 26.9(13)	5.7 ± 0.8 (13)	192.6 ± 17.5(3)	69.4 ± 7.1(3)	142.4 ± 22.4 (3)	1.3 ± 0.2 (3)
	WT control	**eNOS$^{flox/flox}$ HbbCre^{neg}**		31.1 ± 1.6(11)	181.2 ± 21.2(11)	5.8 ± 0.7 (11)	165.1 ± 5.2(3)	73.1 ± 5.1(3)	155.7 ± 44.5 (3)	1.2 ± 0.3 (3)
4	**RBC eNOS KI**	**eNOS$^{inv/inv}$ HbbCre^{pos}**	4 ± 2	29.5 ± 3.4(8)	160.0 ± 19.0(8)	5.5 ± 0.7 (8)	n.d	n.d	n.d.	
	CondKO control	**eNOS$^{inv/inv}$ HbbCre^{neg}**		30.7 ± 2.0(8)	189.6 ± 28.1(8)	6.2. ± 0.7 (8)	n.d.	n.d.	n.d.	

2.5. Invasive assessment of hemodynamic parameters by Millar catheterization

Invasive assessment of hemodynamic parameters was carried out in intubated anesthetized (2.5% isofluorane) mice by using a 1.4 F Millar pressure-conductance catheter (SPR-839, Millar Instrument, Houston, TX, USA) placed into the left ventricle through the right carotid artery according to the closed chest method as described [26]. The pressure was recorded by a Millar Box and analyzed with LabChart 7 (AD Instruments, Oxford, UK) to assess LV developed pressure, rate of pressure development (dP/dt_{max}), and rate of pressure decrease (dP/dt_{min}).

2.6. Induction of AMI and measurement of infarct size and LV function

Myocardial ischemia was induced in intubated anesthetized (2% isofluorane) mice by occlusion of the left anterior descending coronary artery for 45 min followed by 24 h of reperfusion, as previously described with some modifications [28]. Infarct size was evaluated by staining with 2,3,5-triphenyl tetrazolium chloride (TTC), as described [28]. The area at risk (AAR) and nonischemic areas were evaluated by computer-assisted planimetry. The size of the myocardial infarct is expressed as a percentage of the infarcted tissue area compared to the total AAR. Left ventricular (LV) function was analyzed before and after AMI in mice anesthetized with 2.5% isoflurane by transthoracic echocardiography, as described previously [28]. Left ventricular (LV) end-systolic (ESV) and end-diastolic volumes (EDV), LV ejection fraction (EF), fractional shortening (FS), cardiac output (CO), and stroke volume (SV) were evaluated by using the manufacturer's software (18–38 MHz; Vevo 2100, Visual Sonics Inc., Toronto, Canada).

2.7. Statistical analysis

Statistical analysis was carried out with GraphPad Prism 9 for macOS (Version 9.3.0(345)). Unless stated otherwise, the results are reported as means ± standard deviation (SD). Normal distribution was tested by the D'Agostino-Pearson test. Comparisons among multiple groups were performed using 1-way and 2-way analysis of variance (ANOVA) or 2-way repeated measures (RM)-ANOVA, as appropriate, followed by Tukey's or Sidak's post-hoc analysis, as indicated. Where indicated, an unpaired Student's t-test with Welch correction was used to determine if the two groups of data were significantly different. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Characterization of eNOS expression in the heart of EC eNOS KO and EC eNOS KI mice

Due to the role of eNOS in EC differentiation, angiogenesis, and vasculogenesis the temporal deletion of eNOS in endothelial cells is currently the best approach we have to minimize off-target effects and limit long-term adaptation mechanisms. Therefore, EC eNOS KO and EC eNOS KI mice were generated by breeding eNOS$^{flox/flox}$ (phenotypically "WT" mice) or eNOS$^{inv/inv}$ (phenotypically conditional global eNOS KO mice, CondKO) with mice expressing a tamoxifen-inducible Cre recombinase in ECs (Cdh5-Cre/ERT2^{pos} mice) (Fig. 1A). Treatment of eNOS$^{flox/flox}$ Cdh5-Cre/ERT2$^{pos/neg}$ with tamoxifen induces an EC-specific Cre-mediated DNA recombination leading to excision of exon 2 in the Nos3 genomic locus to obtain EC eNOS KO (eNOS$^{flox/flox}$ Cdh5-Cre/ERT2^{pos}) or their littermate WT (eNOS$^{flox/flox}$ Cdh5-Cre/ERT2^{neg}) controls (**Table 1**). Tamoxifen treatment of eNOS$^{inv/inv}$ Cdh5-Cre/ERT2$^{pos/neg}$ mice leads to the genetic re-inversion of exon 2 of Nos3; this reactivates eNOS expression only in ECs and generates EC eNOS KI (eNOS$^{inv/inv}$ Cdh5-Cre/ERT2^{pos} + TAM) mice and their CondKO (eNOS$^{inv/inv}$ Cdh5-Cre/ERT2^{neg} + TAM) littermate control (**Table 1**). In EC eNOS KO mice we found deletion of exon 2 (Fig. S1, blue) and loss of eNOS expression in cardiac ECs ($CD31^+$ $CD45^-$ by real-time RT-PCR (Fig. 1B). In cardiomyocytes preparations from hearts of WT or EC eNOS KO mice, we found that eNOS expression was very low or undetectable (Fig. S2A). In heart tissue, eNOS expression was not detectable by immunoblot (Fig. 1 D, blue) and showed very low levels by ELISA (Fig. S2B). We did not find any DNA recombination in WT littermate control mice (Fig. S1). Vice versa in EC eNOS KI mice we found reinversion of exon 2 (see also [15]) (Fig. S1, green) and reactivation of

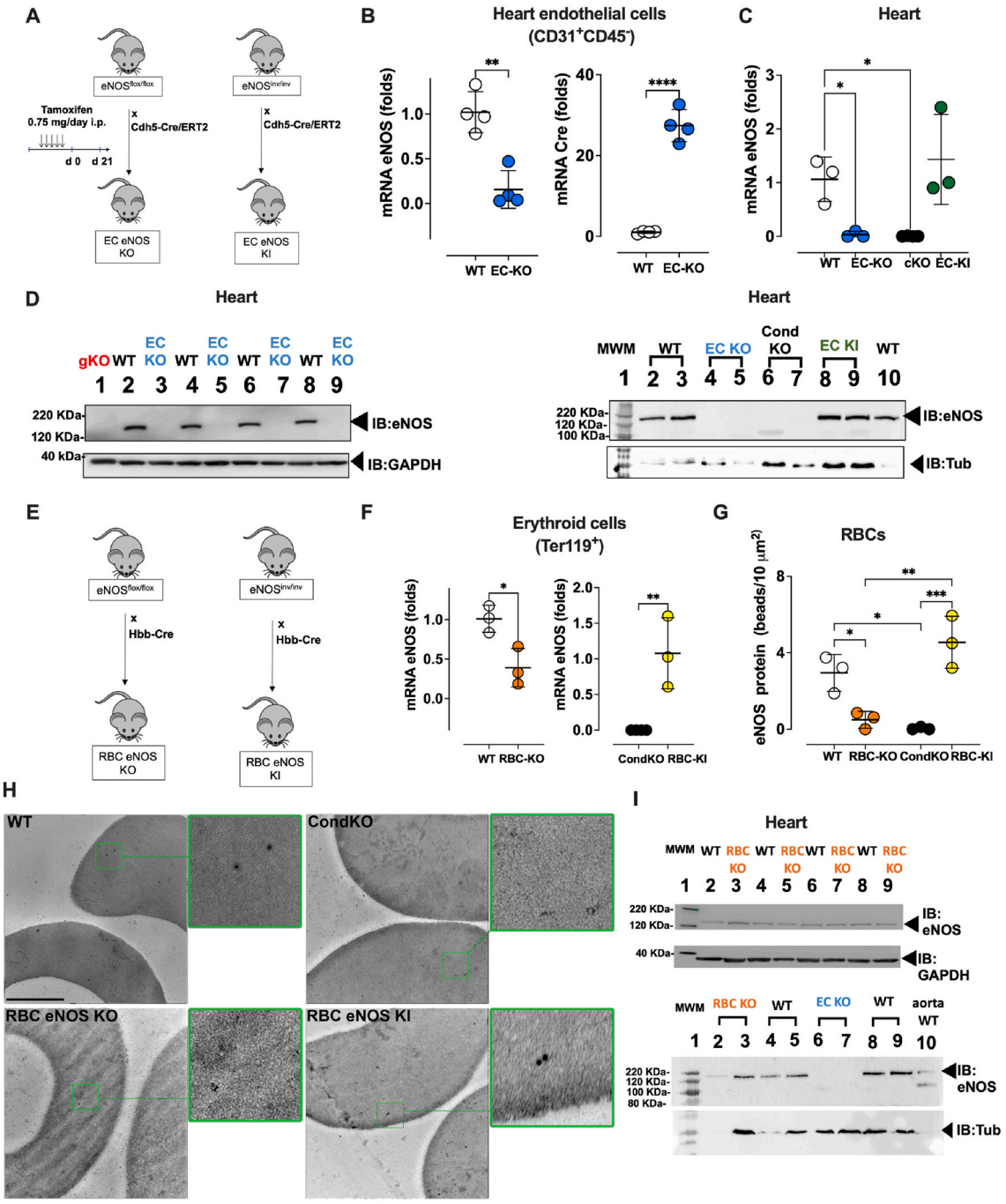

Fig. 1. Characterization of eNOS expression in the heart of EC-specific and RBC-specific eNOS KO and KI mice. **(A)** To generate EC eNOS KO and EC eNOS KI mice and their respective littermate controls, the founders $eNOS^{flox/flox}$ (= WT) and $eNOS^{inv/inv}$ (= CondKO) mice were crossed with endothelial-specific tamoxifen (TAM)-inducible ($Cdh5\text{-}CreERT2^{pos}$) mice. Activation of Cre recombinase was induced by treatment with tamoxifen (TAM) for 5 days, and analyzed after 21 days. **(B)** Real-time RT-PCR analysis of expression of eNOS (left) and Cre recombinase (right) in ECs ($CD31^+$ $CD45^-$) magnetically sorted from the heart of EC eNOS KO (blue) (T-test $^{**}p < 0.01$; $^{****}p < 0.0001$) **(C)** Real-time RT-PCR analysis showed lack/presence of expression of eNOS in heart lysates of EC eNOS KO/WT and EC eNOS KI mice/CondKO mice (1-way ANOVA $p = 0.0050$; Dunnett's vs. WT $^{*}p < 0.05$). **(D)** Western blot analysis of eNOS protein expression in the heart of EC eNOS KO/WT and EC eNOS KI/CondKO mice show a lack of eNOS in EC eNOS KO (left panel), and CondKO (right panel) and reactivation in EC eNOS KI (right panel). For data on DNA recombination and eNOS expression in other cells and tissues of EC eNOS KO and EC eNOS KI mice please refer to Figs. S1–2 in the Supplemental information. **(E)** To generate RBC eNOS KO and WT mice, or RBC eNOS KI and CondKO mice, the founders $eNOS^{flox/flox}$ (WT) and $eNOS^{inv/inv}$ (CondKO) mice were crossed with erythroid cell-specific Cre ($Hbb\text{-}Cre^{pos}$) mice. **(F)** Real-time RT-PCR analysis of expression of eNOS in erythroid cells ($Ter119^+$ $CD45^-$) magnetically sorted from the bone marrow of the mice shows a lack of eNOS in erythroid cells from RBC eNOS KO (orange) and CondKO mice (black) and presence of eNOS in erythroid cells from WT mice (white) and RBC eNOS KI mice (yellow) (T-test $^{*}p < 0.05$; $^{**}p < 0.01$) **(G, H)** Quantification **(G)** of immunotransmission electronic microscopy **(H)** showing lack of eNOS expression in RBCs from RBC eNOS KO and CondKO mice and its presence in WT mice and RBC eNOS KI mice (1-way ANOVA $p = 0.0006$; Turkey's $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). Each data point is average of 5 fields of view from one mouse. **(I)** Western blot analysis shows no changes in eNOS protein expression in the heart of RBC eNOS KO mice and their WT littermate and its lack in the heart of EC eNOS KO mice. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

eNOS expression in heart tissue (Fig. 1C, green). As expected, in CondKO littermate mice, we did not find any DNA recombination (Fig. S1, black) and eNOS expression was non-detectable in the heart or any other cell or tissue (Fig. 1D; Fig. S3), demonstrating that CondKO mice are phenotypically global eNOS KO mice. Transient loss and gain of eNOS function in EC did not affect body weight, heart weight index, or coronary vessel perimeter as compared to respective controls (**Table 1**).

3.2. Characterization of eNOS expression in the heart of RBC eNOS KO/KI mice

Due to the continuous turnover of RBCs and the plasticity of the bone marrow, the most efficient gene targeting approach for erythroid cells is the use of a constitutive promoter highly specific for erythroid cells for controlling the expression of the Cre recombinase. Therefore, RBC eNOS KO and RBC eNOS KI mice were generated by breeding $eNOS^{flox/flox}$ or $eNOS^{inv/inv}$ with Hbb-Cre mice (Fig. 1E). By using allele-specific primers, we found Cre-induce DNA recombination and deletion of exon 2 in the bone marrow of RBC eNOS KO, and not in WT littermates (Fig. S4A, **orange vs. white**); moreover, we found Cre-induced inversion of exon 2 in RBC eNOS KI, but not in its CondKO counterpart (Fig. S4A black vs. yellow). We did not detect any DNA recombination events in the heart of RBC eNOS KO or RBC eNOS KI or their littermate controls (Fig. S4B). Accordingly, in RBC eNOS KO we found a lack of eNOS expression in erythroid cells ($Ter119^{+}$ $CD45^{-}$) (Fig. 1F, orange) and RBCs as detected by transmission electron microscopy (Fig. 1G, orange; Fig. 1H). Loss of eNOS function in RBC did not affect body weight, heart weight index, coronary vessel perimeter, or cardiomyocyte area to coronary vessel wall area in RBC eNOS KO as compared to respective controls (**Table 1**). In RBC eNOS KI we found eNOS expression in erythroid cells ($Ter119^{+}$ $CD45^{-}$) (Fig. 1F, yellow) and RBCs (Fig. 1G, yellow; Fig. 1 H). Instead, eNOS mRNA and protein expression were fully preserved in the heart of RBC eNOS KO mice (Fig. 1C, I; Fi. S5) and other tissues including the aorta, lung, kidney, and liver (Fig. S6). Accordingly, eNOS expression was absent in the heart and other tissues of RBC eNOS KI mice and their CondKO littermates (Fig. S5, yellow; Fig. S6).

3.3. Coronary flow is impaired in isolated perfused hearts of EC eNOS KO mice

In the heart, eNOS was proposed to modulate coronary hemodynamics [2] and cardiac contractility in an endocrine as well as paracrine fashion, involving the coronary endothelium [29], the cardiomyocytes [30], or RBC-mediated release of vasoactive compounds [16] (defined as "NO bioactivity") [16,31]. We first carried out measurements on

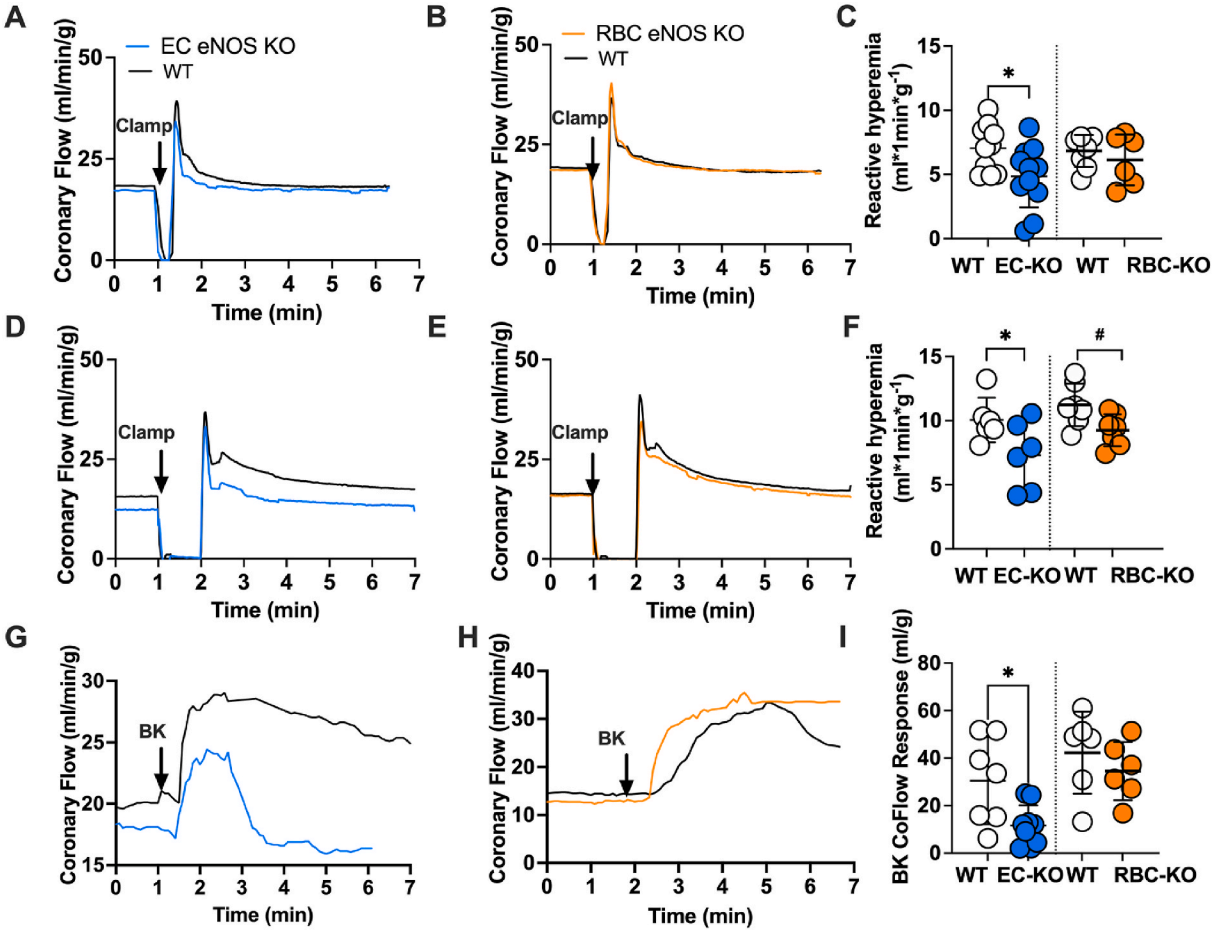

Fig. 2. Coronary endothelial function is impaired in EC eNOS KO hearts but preserved in RBC eNOS KO. Hyperemic flow responses were measured after 20 s (A–C) or 1 min (D–F) of coronary flow occlusion in isolated perfused mice hearts. (**A**). Flow curves are averaged from n = 11 EC eNOS KO ($eNOS^{flox/flox}$ Cdh5-Cre/ERT^{pos}) and n = 10 WT ($eNOS^{flox/flox}$ Cdh5-Cre/ERT^{neg}). (**B**) n = 8 RBC eNOS KO ($eNOS^{flox/flox}$ $HbbCre^{neg}$) and n = 7 WT ($eNOS^{flox/flox}$ $HbbCre^{neg}$). (**C**) The area under the curve (AUC) of the reactive hyperemic response after 20-sec ischemia. T-test *p < 0.05. See also **Table S1**. (**D**) Flow curves are averaged from n = 6 EC eNOS KO and n = 6 WT; (**E**) Flow curves are averaged from n = 7 RBC eNOS KO and n = 7 WT. (**F**) The area under the curve (AUC) of the reactive hyperemic response after 1 min ischemia. T-test *p < 0.05; **p < 0.01. See also **Table S3**. (**G**) Representative coronary flow response to BK of one EC eNOS KO and one WT mouse. (**H**) Representative coronary flow response to BK of one RBC eNOS KO and one WT mouse. (**I**) Bradykinin flow response from EC eNOS KO/WT and RBC eNOS KO/WT mice as assessed as AUC; T-test **p < 0.01. See also **Table S5**.

coronary flow in isolated perfused hearts *ex vivo* using the Langendorff technique (Fig. 2; Tables S1–S5). Resting coronary flow was decreased in the hearts of EC eNOS KO mice as compared to WT mice (Fig. 2A, D), but the differences did not reach statistical significance (**Tables S1 and S3**). After a global brief (20 s or 1 min) ischemia, EC eNOS KO hearts, and their respective controls responded with a uniform increase in coronary flow (Fig. 2A and D), but the peak flow (assessed as max flow) and repayment flow (measured as the area under the curve during 1 min of reperfusion) were significantly decreased in EC eNOS KO (Fig. 2C; Table S1; Table S4). Moreover, acute administration with the NOS-inhibitor ETU decreased resting coronary flow and the repayment flow in WT hearts, but not in EC eNOS KO mice (**Table S2 and Table S4**). Moreover, EC eNOS KO hearts showed an impaired flow response to bradykinin (Fig. 2G, **I**), while the effects of adenosine were not different as compared to WT littermate controls (**Table S5**). Diastolic and systolic function in response to adrenergic stimulation was fully preserved in hearts from EC eNOS KO as determined by no differences in isoproterenol-induced changes in LVDP and $dPdT_{min}$ (**Table S5**).

3.4. Coronary flow regulation is preserved in RBC eNOS KO

Contrary to what was observed in EC eNOS KO mice, RBC eNOS KO mice showed a fully preserved coronary flow response to brief 20-sec global ischemia (Fig. 2B and C; Table S1) and NOS inhibition by ETU (**Table S2**). After a longer ischemia time of 1 min, coronary flow response was partially decreased (Fig. 2E and F; Tables S3 and S4). The coronary flow response to bradykinin (determined as the area under the curve) was also preserved in RBC eNOS KO (Fig. 2H and I; Table S5). The coronary flow response to adenosine was not significantly different from WT control littermates (**Table S5**). The expression of iNOS was non-detectable in the heart or skeletal muscle of RBC eNOS KO or WT mice, while in RBC eNOS KO hearts the expression of nNOS was comparable to WT mice (Fig. S7). Systolic and diastolic function in response to adrenergic stimulation was fully preserved in the heart of RBC eNOS KO mice, as determined by no differences in isoproterenol-induced changes in LVDP and $dPdT_{min}$ (**Table S5**).

3.5. LV function is altered in EC eNOS KO mice and preserved in RBC eNOS KO mice

Next, we aimed to verify whether a lack of eNOS in the ECs affects cardiac contractility, relaxation, or heart rate (HR) *in vivo* as determined by measuring these parameters invasively. We found that EC eNOS KO mice had significantly increased systolic, diastolic, and mean arterial pressure (MAP) (**Table 2**) as shown before [15]. In addition, EC eNOS KO showed a significantly altered LV function characterized by increased LVDP and $dPdt_{max}$ and improved relaxation velocity ($dPdt_{min}$) with no significant changes in HR (**Table 2**). Contrary to EC eNOS KO, systolic and diastolic function *in vivo* was not altered in RBC eNOS KO mice, although blood pressure was increased [15] (**Table 2**).

3.6. Infarct size and LV dysfunction after AMI are unchanged in EC eNOS KO and EC eNOS KI mice

To test whether removal or reactivation of eNOS expressed in the vascular endothelium may affect the outcome of AMI, EC eNOS KO mice and their WT littermates or EC eNOS KI mice and their CondKO littermate controls underwent 45 min open-chest coronary occlusion followed by 24 h of reperfusion (Fig. 3). AAR did not differ between the groups (Fig. 3A). In EC eNOS KO mice, infarct size was not significantly different from that in respective littermate WT controls (Fig. 3B blue vs. white). Accordingly, in EC eNOS KI reactivation of eNOS in ECs did not affect infarct size or LV dysfunction after AMI as compared to the respective CondKO littermate control (Fig. 3B green vs. black). Interestingly, Fig. 3B shows that infarct size in CondKO mice (which are phenotypically global eNOS KO mice) (black) did not differ from infarct size in WT controls (white), which confirms data obtained before in two (of the three) independent strains of global eNOS KO mice [11,12,32].

The effects of AMI and EC-specific loss/gain of eNOS expression on LV function *in vivo* were determined by echocardiography measurements carried out in all mice before and 24 h after induction of AMI (Fig. 4, Table 3, Fig. S8). As shown in Fig. 4, after AMI LV function in EC eNOS KO (blue) and EC eNOS KI mice (green) did not differ as compared to the respective WT (white) or CondKO (black) littermates; specifically, we did not find any significant differences in cardiac output (CO), stroke volume (SV), HR, ejection fraction (EF) or fractional shortening (FS). At baseline under homeostatic conditions, genetic depletion or reactivation of eNOS in ECs did not modify LV function as determined by no significant differences in CO, EF, or FS compared with their respective WT (white) or CondKO (black) littermate control mice. These data provide compelling evidence that eNOS expressed in ECs per se does not modulate infarct size or LV function after AMI in mice.

3.7. Infarct size and LV dysfunction after AMI are increased in RBC eNOS KO mice

To test whether removal or reactivation of eNOS expressed in RBCs may affect the outcome of AMI, we compared the effects of AMI on infarct size and LV dysfunction in RBC eNOS KO vs. littermate WT mice and RBC eNOS KI mice vs. littermate CondKO mice (Fig. 5). Again, AAR did not differ between the groups (Fig. 5A). However, in RBC eNOS KO we measured an Δ%-increase in infarct size of +31 ± 17% as compared to infarct size determined in WT control (calculated as Δ% vs. WT) (Fig. 5B). Accordingly, in RBC eNOS KO mice we found a significantly

Table 2

Systemic hemodynamics and cardiac function as assessed by the Millar catheter. Data are reported as mean ± SD. n = number of analyzed mice. HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; PED, end-diastolic pressure; PES end-systolic pressure; LVDP, left ventricular developed pressure. Differences between WT and respective eNOS KO were calculated by unpaired *t*-test with Welch correction *p < 0.05; **p < 0.01; ***p < 0.001).

Parameter	Unit	WT	EC eNOS KO		WT	RBC eNOS KO	
		$eNOS^{flox/flox}$ Cdh5-Cre/$ERT2^{neg}$ + TAM	$eNOS^{flox/flox}$ Cdh5-Cre/$ERT2^{pos}$ + TAM	p	$eNOS^{flox/flox}$ $HbbCre^{neg}$	$eNOS^{flox/flox}$ $HbbCre^{pos}$	p
n		6	10		6	7	
HR	bpm	502 ± 88	578 ± 58	0.1017	508 ± 55	436 ± 81	0.0854
SBP	mmHg	87 ± 16	119 ± 13**	0.0026	92 ± 10	108 ± 5	0.0073
DBP	mmHg	56 ± 15	85 ± 12**	0.0036	59 ± 11	74 ± 7	0.0245
MAP	mmHg	66 ± 15	99 ± 13**	0.0019	70 ± 11	85 ± 6	0.0159
PED	mmHg	1 ± 8	0 ± 8	0.8154	3 ± 7	3 ± 7	0.9329
PES	mmHg	89 ± 14	119 ± 12**	0.0020	95 ± 10	101 ± 5	0.2507
DP	mmHg	92 ± 11	122 ± 11***	0.0003	96 ± 11	106 ± 6	0.0874
$dPdt_{max}$	mmHg/s	9144 ± 3045	13350 ± 2635*	0.0195	9202 ± 2895	7546 ± 1071	0.2318
$dPdt_{min}$	mmHg/s	−7150 ± 1267	−11408 ± 1672***	<0.0001	−8162 ± 2265	−7928 ± 1516	0.8348

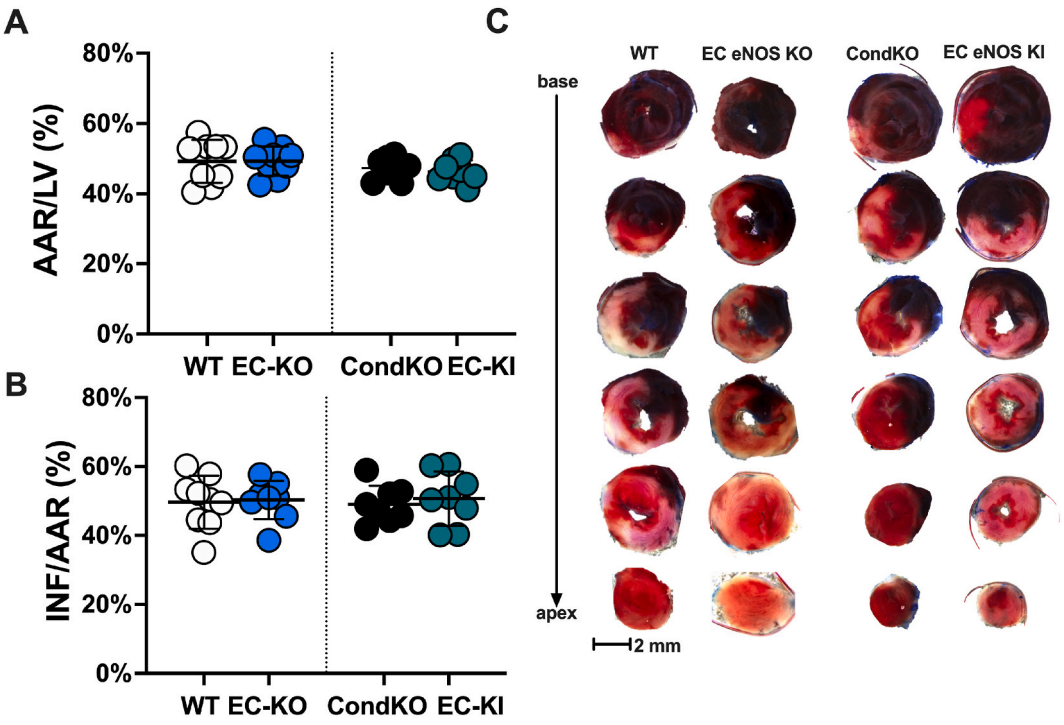

Fig. 3. Myocardial damage after acute myocardial infarction is not affected by the inactivation/activation of eNOS in endothelial cells. Acute myocardial infarction (AMI) was induced in EC eNOS KO (blue) and respective WT littermate controls (white), and in EC eNOS KI (green) and respective CondKO (black) mice. The experimental protocol consisted of 45 min ischemia and 24 h of reperfusion. (**A**) Area-at-risk (AAR)/left ventricle (LV) is not significantly different in EC eNOS KO vs. WT or EC eNOS KI vs. CondKO. (**B**) IS/AAR is not significantly different in EC eNOS KO vs. WT or EC eNOS KI vs. CondKO as compared by *t*-test. (**C**) Representative TTC staining showing no differences in myocardial damage after AMI. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

increased AMI-induced LV dysfunction as compared to WT littermate mice (Fig. 6, Table 4, and Fig. S9). In fact, in RBC eNOS KO mice CO decreased by $\Delta\% = 39 \pm 14\%$ after AMI (calculated as Δ% vs. baseline) and instead in WT mice CO decreased by $\Delta\% = -6 \pm 15\%$ after AMI (Fig. 6B, Table 4, and Fig. S9B). The AMI-induced decrease in CO in RBC eNOS KO was due to a significant reduction in stroke volume (SV) after AMI (vs. baseline; Fig. S9C) related to an increase in end-systolic volume (ESV), while end-diastolic volume (EDV) was preserved (Table 4, Figs. S9D and E). EF decreased by 37 ± 8% in RBC eNOS KO after AMI (Fig. 6C), while in WT mice EF decreased by only 15 ± 11% (Fig. 6C).

3.8. *Infarct size and LV dysfunction after AMI are decreased in RBC eNOS KI mice*

An important finding was that reactivation of eNOS specifically in erythroid cells in RBC eNOS KI rescued the conditional global eNOS KO mouse from myocardial damage with a decrease in infarct size of a $\Delta\% = -21 \pm 12\%$ in the RBC eNOS KI as compared to their CondKO littermate controls (Fig. 5). Accordingly, AMI-induced LV dysfunction in RBC eNOS KI was not different from the littermate CondKO littermates (Fig. 6, black) or WT mice (Fig. 6, white). See also in **Table 4**. Genetic depletion/reactivation of eNOS in RBC did not modify LV function at baseline with no significant differences in CO, EF, or FS compared with their respective WT control mice. These data provide compelling evidence that RBC eNOS limits infarct size and left ventricular dysfunction after AMI.

4. Discussion

In this work, we analyzed the role of RBC eNOS and EC eNOS in the regulation of coronary blood flow and left ventricular performance in acute myocardial infarction (AMI).

The major findings of this study are: (1) RBC eNOS KO mice presented with a preserved coronary flow regulation, while EC eNOS KO had significantly impaired coronary flow response to reactive hyperemia and bradykinin (2) RBC eNOS KO mice show preserved cardiac performance, while EC eNOS KO showed a significant compensatory increase in developed LV pressure, the velocity of contraction and relaxation at comparable stroke volume and heart rate. (3) After AMI, EC eNOS KO/KI mice did not show any differences in infarct size or AMI-induced LV dysfunction, as compared to the respective littermate controls. Instead (4) RBC eNOS KO mice showed greater infarct size and reduced LV function as compared to littermate WT control mice; on the contrary RBC, eNOS KI showed reduced infarct size and preserved LV function after AMI as compared to the littermate CondKO control mice.

RBC eNOS KO mice showed preserved coronary hemodynamics, characterized by a preserved reactive coronary dilatory response to brief ischemia and bradykinin as also found in conduit vessels of these mice [15]. We also observed some impairment of reactive hyperemic response to longer ischemia time (1 min) and of peak response to bradykinin, which is likely related to hypertension-induced resistive vessel dysfunction [33] in these mice limiting maximal vasodilation.

Instead, EC eNOS KO mice displayed an impaired coronary flow response to brief ischemia and a significantly decreased endothelium-dependent flow response to bradykinin, demonstrating reactive hyperemia and bradykinin flow responses (AUC and Δ% of max flow) are mainly dependent on eNOS expressed in the coronary endothelium. This is consistent with previous findings obtained by pharmacological NOS inhibition [34] or in global eNOS KO mice [2,34]. Interestingly, EC eNOS KO mice show, a partially decreased basal coronary flow, but differences were not statistically significant; Accordingly, previous studies show that all global eNOS KO mice (except in the Huang eNOS KO model [9]), have a fully preserved basal coronary flow as compared to WT controls, which was attributed to a compensatory increase of

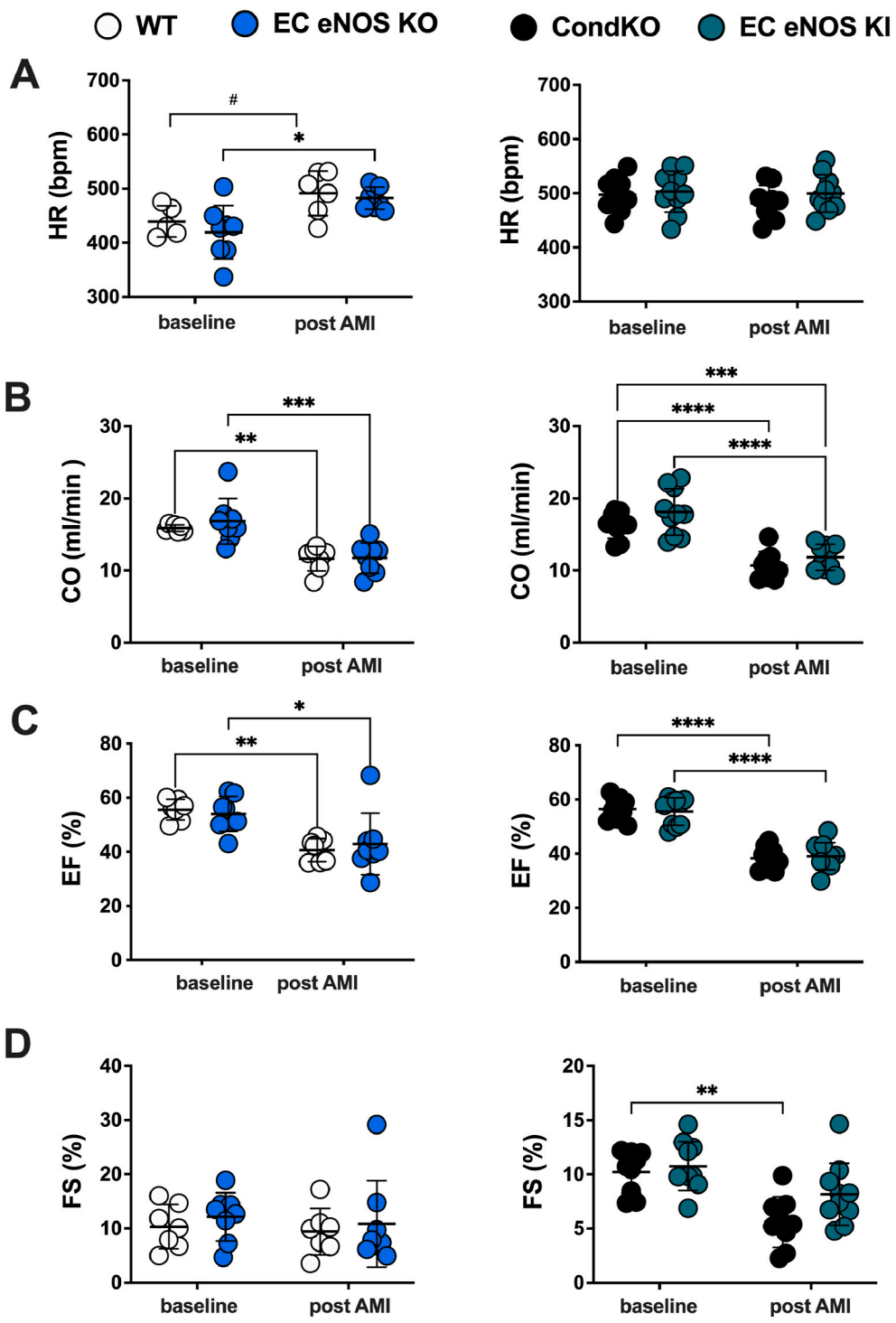

Fig. 4. LV dysfunction after acute myocardial infarction is not affected by inactivation/activation of eNOS in ECs. Echocardiographic evaluation of left ventricular (LV) function before and 24 h after acute myocardial infarction (AMI). LV function was evaluated by comparing cardiac output (CO), ejection fraction (EF), and fractional shortening (FS) before (baseline) and after AMI. LV function after AMI is not different in EC eNOS KO (Blue) vs. littermate WT controls (white), or EC eNOS KI vs. littermate CondKO mice. Please note that CondKO mice are genetically global eNOS KO mice. Comparisons were tested 2-way ANOVA $p < 0.001$; Sidak's * $p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; T-Test $^{\#}p < 0.05$. See also Table 4 and Fig. S7. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

other dilatory metabolites like adenosine, COX-derived prostaglandins [2], or upregulation of other NOS isoforms [5]. Importantly, acute administration of the NOS inhibitor ETU decreased basal coronary flow in WT mice (as observed before [2,34]), and in RBC eNOS KO mice but not in EC eNOS KO mice, confirming the role of EC eNOS in the regulation of the basal tone of coronary vessels.

RBC eNOS KO mice showed increased BP but fully preserved cardiac contractility and LV function under homeostatic conditions both *in vivo* and *ex vivo*. These data indicate that RBC eNOS KO hearts can cope with the moderate increase in blood pressure found in these mice, as also evidenced by maintained SV and CO. Notably, increased BP in RBC eNOS KO mice did not affect baseline coronary flow and maximal vasodilatory capacity of coronary circulation *ex vivo*. Differently from the peripheral circulation (e.g. in the skeletal muscle and other organs), coronary circulation is characterized by a high baseline flow and oxygen extraction capacity that is governed by several metabolic pathways. This may contribute to limiting the role of RBC eNOS in the regulation of coronary hemodynamics under unstressed/homeostatic conditions.

Interestingly, EC eNOS KO mice showed a more prominent BP increase, which was accompanied by compensatory increases in LVDP and $dPdt_{max}$ and improved relaxation velocity ($dPdt_{min}$). As reported by some studies in mice and humans, NO produced and released by

Table 3
Echocardiographic parameters were assessed in EC eNOS KO vs. WT littermates, and EC eNOS KI vs. CondKO littermates by high-resolution ultrasound before and after AMI. Data are reported as mean ± SD; n = number of mice. Differences between KO/WT pre/post-AMI or KI/CondKO pre/post-AMI were calculated by 2-way-ANOVA followed by Sidak's multiple comparisons test. The p values of the comparison of KO/WT or KI/CondKO are reported in the tables. Please refer also to Fig. 4 for further comparisons and statistical analysis. HR, heart rate; CO, cardiac output; SV, stroke volume; EF, ejection fraction; FS, fractional shortening; ESV, end-systolic volume; EDV, end-diastolic volume.

Parameter		Baseline			Post-AMI			Baseline			Post-AMI		
		WT	EC eNOS KO		WT	EC eNOS KO		CondKO	EC eNOS KI		CondKO	EC eNOS KI	
	Unit	$eNOS^{flox/flox}$ Cdh5-Cre/ $ERT2^{neg}$ + TAM	$eNOS^{flox/flox}$ Cdh5-Cre/ $ERT2^{pos}$ + TAM	p	$eNOS^{flox/flox}$ Cdh5-Cre/ $ERT2^{neg}$ + TAM	$eNOS^{flox/flox}$ Cdh5-Cre/ $ERT2^{pos}$ + TAM	p	$eNOS^{inv/inv}$ Cdh5-Cre/ $ERT2^{neg}$ + TAM	$eNOS^{inv/inv}$ Cdh5-Cre/ $ERT2^{pos}$ + TAM	p	$eNOS^{inv/inv}$ Cdh5-Cre/ $ERT2^{neg}$ + TAM	$eNOS^{inv/inv}$ Cdh5-Cre/ $ERT2^{pos}$ + TAM	p
n		7	8		7	8		9	10		9	10	
HR	bpm	444 ± 30	419 ± 50	0.4016	482 ± 45	483 ± 21	0.9994	497 ± 33	503 ± 38	0.9272	482 ± 33	500 ± 35	0.4713
CO	ml/min	16 ± 1	17 ± 3	0.6225	12 ± 2	12 ± 2	0.9949	16 ± 2	18 ± 3	0.1858	11 ± 2	12 ± 2	0.5024
SV	µl	36 ± 3	40 ± 7	0.1607	24 ± 4	24 ± 4	0.9999	33 ± 5	36 ± 6	0.3134	22 ± 3	24 ± 4	0.7070
EF	%	56 ± 4	54 ± 6	0.8974	41 ± 4	43 ± 11	0.7994	57 ± 4	56 ± 5	0.8906	38 ± 4	39 ± 5	0.9261
FS	%	10 ± 4	12 ± 4	0.7775	9 ± 4	11 ± 8	0.8578	10 ± 2	11 ± 2	0.8660	6 ± 2	8 ± 3	0.0492
ESV	µl	29 ± 3	35 ± 8	0.2969	36 ± 9	34 ± 10	0.8573	25 ± 5	29 ± 7	0.5783	37 ± 9	38 ± 12	0.9127
EDV	µl	65 ± 3	75 ± 11	0.0954	60 ± 12	58 ± 10	0.9039	58 ± 9	65 ± 12	0.3938	59 ± 11	62 ± 14	0.8135

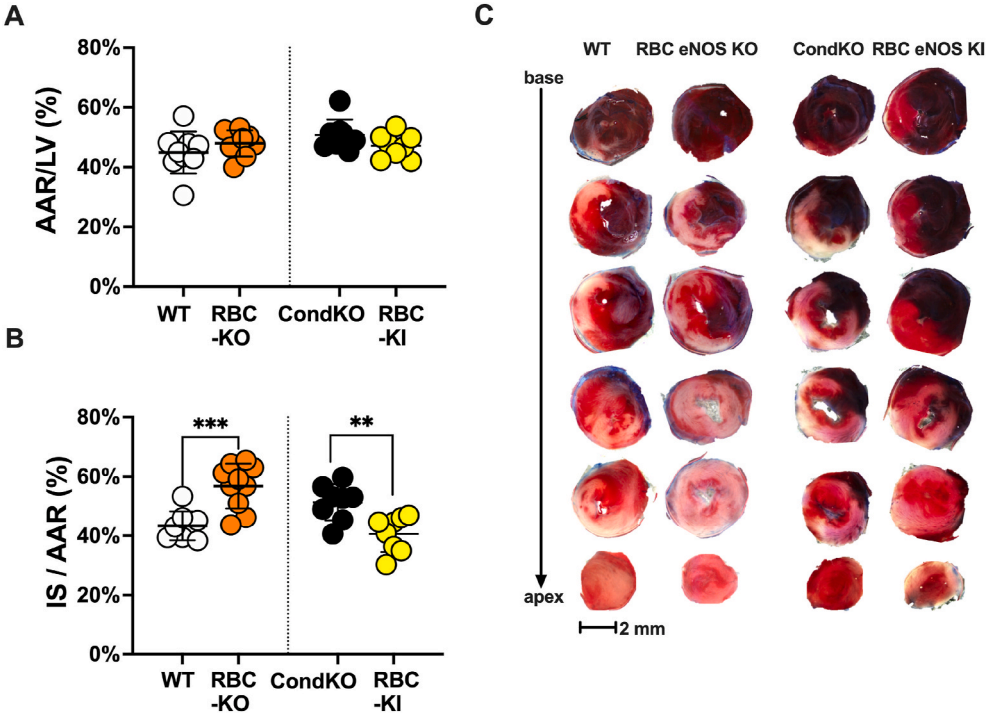

Fig. 5. RBC eNOS limits infarct size after acute myocardial infarction. Acute myocardial infarction (AMI) was induced in RBC eNOS KO (orange) and their WT littermate controls (white), and in RBC eNOS KI mice (yellow) and their littermate CondKO controls (black). The experimental protocol consisted of 45 min ischemia and 24 h of reperfusion. (**A**) AAR/LV is not significantly different in RBC eNOS KO vs. WT or RBC eNOS KI vs. CondKO. (**B**) IS/AAR is increased in RBC eNOS KO (orange) vs. WT mice; while RBC eNOS KI (yellow) show decreased IS as compared to global CondKO mice (black).T-test $^{**}p < 0.01$; $^{***}p < 0.001$. (**C**) Representative TTC staining showing increased myocardial damage after AMI. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

coronary endothelial cells affects cardiomyocyte lusitropy and increases inotropy [3,35]. However, in EC eNOS KO mice increase in cardiac inotropy was not observed *ex vivo* in perfused Langendorff hearts, indicating that those effects are not intrinsic but may be linked to the increase of systemic blood pressure *in vivo* rather than structural changes in the myocardium. In fact, in isolated perfused Langendorff hearts, the contractile response to isoproterenol was not altered. Therefore, the results obtained in EC eNOS KO hearts indicate that lack of EC eNOS significantly affects coronary blood flow but its effects on myocardial performance are rather modest.

We induced AMI to verify the role of EC eNOS and RBC eNOS in myocardial injury. We found that loss or reactivation of EC eNOS did not affect myocardial damage nor reversed LV dysfunction after AMI. This was somehow unexpected as NO inhalation and NO donors such as nitroglycerine are well known to exert cardioprotective effects via cGMP-dependent signaling in the heart; cardioprotection was also observed with overexpression of eNOS in cardiomyocytes [36] (for a review see Refs. [1,4]). However, previous studies carried out in global eNOS KO strains showed different outcomes after AMI depending on the particular eNOS KO strain under study, and the ischemia time and conditions [5–12]. Specifically, the Huang's global eNOS KO model showed increased infarct size and myocardial necrosis [7,37], which was attributable to a permanent decrease in coronary flow [9]. The eNOS KO strain created by Shesely et al. [38] showed no differences in infarct size [11], and in a later study a decreased infarct size after AMI, which was attributed to iNOS upregulation [7]. The eNOS KO strain created by Gödecke et al. [2] showed no differences in infarct size and cardiac performance after AMI *ex vivo* [10] or *in vivo* [28], and acute

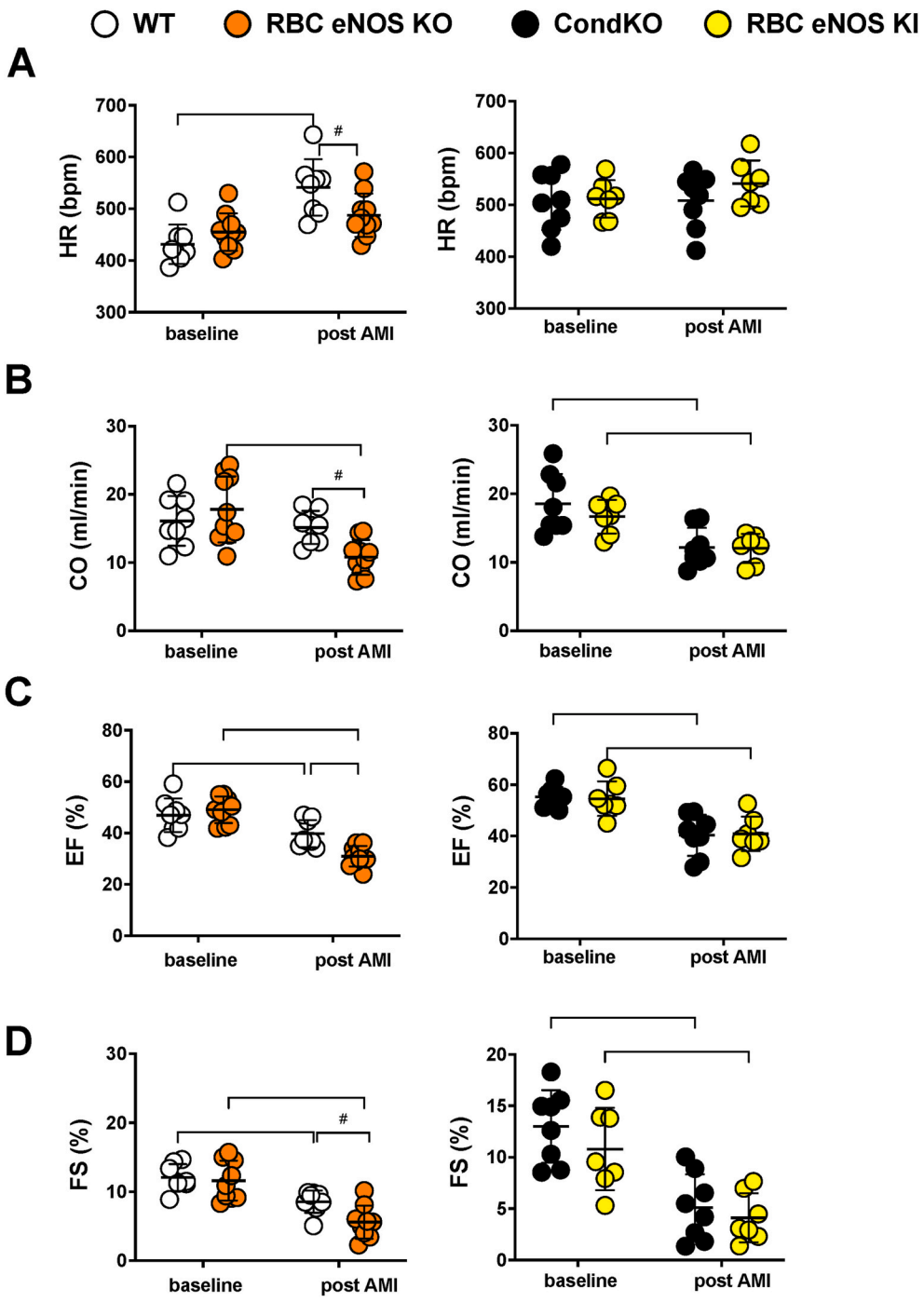

Fig. 6. RBC eNOS is critical to limit left ventricular dysfunction after acute myocardial infarction. Echocardiographic evaluation of left ventricular (LV) function before and 24 h after acute myocardial infarction (AMI). In RBC eNOS KO (orange) a significant decrease in cardiac output (CO), ejection fraction (EF), and fractional shortening (FS) are observed as compared to their WT control group (white). In RBC eNOS KI mice (yellow) LV function after AMI is not different from CondKO mice (black). Comparisons were tested 2-way ANOVA $p < 0.001$; Sidak's * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; T-Test $^{\#}p < 0.05$. See also Table 4 and Fig. S8. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

NOS inhibition by ETU neither affected infarct size nor LV function in global eNOS KO or WT mice [28]. Here we show that EC eNOS KO, EC eNOS KI, or global CondKO mice showed a similar AMI-induced infarct size and changes in CO and EF as found in WT mice. Taken together these data demonstrate that the extent of acute myocardial damage seems to be primarily independent of endogenous NO production derived from EC eNOS in the heart and may be affected instead by other NOS isoforms and redox enzymes (e.g. COX), which may be differently regulated under homeostatic and pathophysiological conditions. Further studies should address the role of circulating nitrite/nitrate levels, other mediators like adenosine/adenine, as well as redox signaling/inflammation (including iNOS/nNOS expression) after AMI and cardiac remodeling in EC eNOS KO/KI and RBC eNOS KO/KI mice.

Perhaps the most surprising finding of this study is that eNOS expressed in RBCs is cardioprotective after AMI. RBC eNOS KO mice showed an increased infarct size and AMI-induced LV dysfunction as compared to WT littermates. Vice versa, regain of eNOS in RBCs limited infarct size after AMI. These results significantly expand previous findings on the role of RBCs in cardioprotection. By using a model of I/R injury in the Langendorff system, Pernow and co-workers demonstrated that eNOS in RBCs is required for RBC-mediated release of NO metabolites and cardioprotection *ex vivo* [16]. Interestingly, later studies showed that RBC-mediated cardioprotection is lost in RBCs from patients affected by diseases like type 2 diabetes [17,18] and in patients with anemia and acute coronary syndrome [39]. Moreover, we have found that chimeric mice lacking eNOS in blood cells (obtained by

Table 4
Echocardiographic parameters were assessed in RBC eNOS KO and WT littermate mice, and RBC eNOS KI and CondKO littermate mice by high-resolution ultrasound before and after AMI. Data are reported as mean ± SD; n = number of mice. Differences between KO/WT pre/post-AMI or KI/CondKO pre/post-AMI were calculated by 2-way-ANOVA followed by Šídák's multiple comparisons test. The p values of the comparison of KO/WT or KI/CondKO are reported in the tables. Please refer also to Fig. 6 for further comparisons and statistical analysis. HR, heart rate; CO, cardiac output; SV, stroke volume; EF, ejection fraction; FS, fractional shortening; ESV, end-systolic volume; EDV, end-diastolic volume.

Parameter		Baseline			Post-AMI			Baseline			Post-AMI		
		WT	RBC eNOS KO		WT	RBC eNOS KO		CondKO	RBC eNOS KI		CondKO	RBC eNOS KI	
	Unit	$eNOS^{flox/flox}$ $HbbCre^{neg}$	$eNOS^{flox/flox}$ $HbbCre^{pos}$	p	$eNOS^{flox/flox}$ $HbbCre^{neg}$	$eNOS^{flox/flox}$ $HbbCre^{pos}$	p	$eNOS^{inv/inv}$ $HbbCre^{neg}$	$eNOS^{inv/inv}$ $HbbCre^{pos}$	p	$eNOS^{inv/inv}$ $HbbCre^{neg}$	$eNOS^{inv/inv}$ $HbbCre^{pos}$	p
n		8	10		8	10		8	8		8	8	
HR	bpm	431 ± 38	455 ± 36	0.4425	541 ± 55	488 ± 42	0.0249	507 ± 55	512 ± 36	0.9773	509 ± 53	542 ± 44	0.3579
CO	ml/min	16 ± 4	18 ± 5	0.5382	15 ± 2	11 ± 3	0.0278	19 ± 4	17 ± 2	0.4490	12 ± 3	12 ± 2	0.9949
SV	µl	38 ± 9	39 ± 9	0.9090	25 ± 5	22 ± 5	0.1534	36 ± 7	33 ± 5	0.4452	25 ± 8	22 ± 4	0.7436
EF	%	47 ± 7	49 ± 5	0.6300	40 ± 5	31 ± 4	0.0026	55 ± 4	55 ± 7	0.9734	40 ± 8	41 ± 7	0.9822
FS	%	12 ± 2	12 ± 3	0.8960	9 ± 2	6 ± 2	0.0238	13 ± 4	11 ± 4	0.3845	5 ± 3	4 ± 2	0.8098
ESV	µl	42 ± 5	40 ± 8	0.9232	43 ± 9	50 ± 14	0.2473	29 ± 4	28 ± 10	0.9781	36 ± 9	33 ± 7	0.6163
EDV	µl	79 ± 10	79 ± 14	0.9997	72 ± 13	73 ± 13	0.9885	66 ± 9	61 ± 15	0.9164	61 ± 14	55 ± 9	0.5729

transplanting bone marrow from eNOS KO mice in irradiated WT mice) showed decreased circulating nitrite/nitrate, increased blood pressure [40], increased infarct size, and left ventricular remodeling after AMI [19,20]. To the best of our knowledge, the present data are the first showing a specific role of RBC eNOS in cardioprotection after AMI *in vivo*. Since infarct size and impaired LV function are important predictors of outcome after AMI [41], RBC-mediated protection against myocardial damage may have important clinical implications.

Of note, genetic background, Cre recombinase expression, and tamoxifen treatment may have effects on their own and therefore potentially confound the interpretation of the results. To control for differences in genetic background, founder lines were backcrossed for 10 generations with C57Bl/6J mice, and the Cre-positive controls were compared with the Cre-negative littermate from the same line. To control for tamoxifen effects in EC-specific models Cre-positive and Cre-negative littermates were treated with tamoxifen. Of note, we found that treatment with tamoxifen increased the eNOS expression in the heart by 1.7 fold, as determined by eNOS quantification by ELISA, but it did not significantly affect the pathophysiological parameters analyzed.

Moreover, to assure optimal gene targeting efficiency we tailored the gene targeting strategy to the cell type we were targeting: EC-specific lines were generated by tamoxifen-inducible temporal deletion of EC eNOS, while RBC-specific lines in mice were generated by using a constitutively expressed Cre-recombinase under the control of a strong, erythroid-specific promoter like the Hbb promoter. For EC eNOS we chose a transient approach to minimize off-target effects and limit long-term adaptation mechanisms linked to its role in embryogenesis, vasculogenesis, and angiogenesis; in fact, constitutive genetic deletion of eNOS strongly affects litter size (as also observed here in global CondKO mice) and the "survivors" are characterized by multiple adaptation mechanisms, including upregulation of other genes (NOS, COX) or morphological changes in vessel network characteristics, cardiac hypertrophy, and changes in life span. Instead, for targeting bone marrow erythroid cells a constitutive approach is more reliable and efficient than a transient approach, as erythroid cells are characterized by a rapid turnover and RBCs persist for only 40 days in the mouse circulation. The use of both loss-of-function and gain-of-function models was therefore necessary to drive conclusions about the role of EC eNOS and RBC eNOS in the targeted cells.

An essential question from this work is how eNOS-signaling in RBCs may contribute to modulating infarct size and LV function after AMI? Reductions in blood flow in the penumbra of the infarct area are likely to have profound detrimental effects on the infarct size and LV function after AMI. Although we did not observe differences in coronary blood flow hearts of RBC eNOS KO *ex vivo* in the absence of RBCs, RBCs may contribute to regulating regional coronary flow in the infarct border zone *in vivo* via several independent mechanisms, including the export of NO bioactivity [16,31,42–44], nitrite bioactivation [45,46], the release of ATP [47] or sulfide [48].

The circulating levels of NO metabolites before and after AMI may play a major role in cardioprotection after AMI and in modulating remodeling. Of note, we have shown that RBCs from RBC eNOS KO mice show decreased levels of heme-NO and circulating NO metabolites, while reactivation of eNOS in RBCs fully restored NO levels in RBCs at baseline [15]. Moreover, a lack of eNOS-dependent release of NO metabolites [15] or increased release of pro-oxidative molecules [17,18,39] (including reactive oxygen species and free Hb) increased myocardial damage *ex vivo*.

In a previous study from our laboratory carried out in bone marrow chimera mice [19], we observed that after I/R injury the levels of nitrite and nitrate in plasma were decreased by 50%, as compared to baseline conditions in both mice lacking eNOS in bone marrow/blood and WT chimera control mice, but eNOS KO chimeras had lower levels of NO metabolites than controls before and after AMI. It is important to point out that bone marrow eNOS KO chimera mice lack RBC specificity and show low-grade systemic inflammation, which may strongly affect NO metabolism. In fact, the nitrite levels at baseline are around 2–3 µM in the chimeras, while in WT mice are around 0.5–1 µM; this difference may affect the outcome after AMI [49]. The role of circulating nitrite/nitrate levels in EC eNOS and RBC eNOS KO in cardiac remodeling after AMI should be investigated in future studies.

In vivo, RBC extravasation and intramyocardial hemorrhage [50,51] may further aggravate local oxidative stress, scavenging of NO, and vasoconstriction. In addition, a limited oxygen delivery capacity of RBCs may also contribute to the enlargement of infarct sizes in RBC eNOS KO mice. Interestingly, we found that RBCs from RBC eNOS KO show decreased ability to release oxygen at a certain oxygen pressure as demonstrated by increased oxygen affinity and lower oxygen-binding cooperativity [15]. Importantly, these effects were enhanced at lower pH [15], a condition typically occurring during ischemia. To which extent do RBC dysfunction, modulation of circulating NO metabolites, oxygen consumption/delivery, or mitochondrial function link RBC eNOS activity to infarct size and LV function after AMI warrants further investigation.

To summarize, we found that EC eNOS affects coronary hemodynamics but has no detrimental effects on AMI. Conversely, lack of RBC eNOS does not affect coronary or myocardial function under homeostatic conditions but limits AMI-induced cardiac damage and

deterioration of LV function. Importantly, reactivation of eNOS in RBCs is cardioprotective in AMI. Taken together, we show that EC eNOS and RBC eNOS have independent and complementary roles in the regulation of coronary vascular tone and myocardial performance under basal conditions and after AMI.

Diabetes mellitus, chronic kidney disease, and anemia exert deleterious effects on the course and outcome of AMI. Once these risk modulators occur at the same time their negative impact becomes additive and the prognosis in and after AMI is detrimental [52]. Multiple studies show that each of these comorbidities is associated with a larger infarct size [41]. We and others have shown that RBC dysfunction with disturbed redox balance and eNOS activity is a characteristic of these disease conditions [17,18,39]. In RBC-specific loss and gain of function models, we show here that RBC eNOS represent a novel target to limit infarct size. This may have important implications for the development of future strategies in cardioprotection, for improving the quality of packed RBCs in blood banking, and for the treatment of hematological disorders such as hemoglobinopathies.

Author contributions

M.M.C.-K. and M.K. conceived the study and designed the research; M.M.C.-K., T. Suv., F. L., S.K.H, A.L.B., J.L., S.B., R.S., T. Sri, R.E., G. W., J.P.S., M.G., J.O.L., J.P., B.E.I., E.W., M.K. planned/performed research and contributed to data analysis and statistics; M.M.C.-K., T. Suv., J.P.S., M.G., J.O.L., J.P., B.E.I., E.W, M.K. provided essential intellectual input; M.M.C.-K. and M.K. wrote the paper, and all authors edited the manuscript.

Declaration of competing interest

No competing interests to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2022.102370.

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