

## Original Article

# Role of the b93cys, ATP and adenosine in red cell dependent hypoxic vasorelaxation

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**Abstract:** Two of the proposed mechanisms by which red blood cells (RBC) mediate hypoxic vasorelaxation by coupling hemoglobin deoxygenation to the activation of nitric oxide signaling involve ATP-release from RBC and S-nitrosohemoglobin (b93C(SNO)Hb) dependent bioactivity. However, different studies have reached opposite conclusions regarding the aforementioned mechanisms. Using isolated vessels, hypoxic vasorelaxation induced by human, C57BL/6 or mouse RBC which exclusively express either native human hemoglobin (HbC93) or human hemoglobin in which the conserved b93cys was replaced with Ala (HbC93A) were compared. All RBCs stimulated hypoxic vasodilation to similar extents suggesting the b93cys is not required for this RBC-mediated function. Hypoxic vasorelaxation was inhibited by co-incubation of ATP-pathway blockers including L-NAME (eNOS inhibitor) and Apyrase. Moreover, we tested if modulation of adenosine-dependent signaling affected RBC-dependent vasorelaxation using pan- or subtype specific adenosine receptor blockers, or adenosine deaminase (ADA). Interestingly, ADA and adenosine A<sub>2</sub> receptor blockade, but not A<sub>1</sub> receptor blockade, inhibited HbC93, HbC93A dependent hypoxic vasorelaxation. Equivalent results were obtained with human RBC. These data suggest that using isolated vessels, RBC do not require the presence of the b93cys to elicit hypoxic vasorelaxation and mediate this response via ATP- and a novel adenosine-dependent mechanism.

**Keywords:** Hemoglobin, nitric oxide, vasorelaxation, thiol, hypoxia, blood flow

## Introduction

Red blood cells (RBC) are integral players in vascular nitric oxide (NO) homeostasis mechanisms [1-7]. Many studies over the last two decades have demonstrated that as RBC deoxygenate, NO-signaling cascades can be activated [8-11]. This coupling between hemoglobin oxygen sensing and NO-signaling is proposed to underlie hypoxic blood flow, and other NO-dependent processes in the vascular compartment. Moreover, dysfunction in this coupling has been recently proposed to contribute to various disease states [12-20].

The mechanisms by which hemoglobin deoxygenation is linked to activation of NO-signaling remain under investigation with three pathways receiving the most attention involving ATP release, S-nitrosohemoglobin (SNOHb) bioac-

tivity and nitrite-reduction to NO [4, 6, 9, 21-24]. The b93cys residue (b93cys) is conserved on hemoglobin in vertebrates but its function remains unclear. One proposed role is that a small fraction of b93cys residues in RBC, provide the site for S-nitros(yl)ation with the resulting SNOHb mediating hypoxic vasorelaxation [1, 4, 10, 25, 26]. Our previous study described formation of knock-in mice in which RBC were modified to express exclusively human wild type (HbC93) hemoglobin or human hemoglobin in which the b93cys residue was replaced with an Ala (HbC93A) and showed that isolated RBC dependent hypoxic vasorelaxation responses were not dependent on the presence or absence of the b93cys residue, in turn suggesting that SNOHb is not a critical modulator [27]. We also reported that isolated RBC hypoxic vasorelaxation responses are in fact due to ATP release indicated by loss of vasorelaxation by inhibition

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of eNOS, inhibition of P2Y receptors or metabolism of ATP using apyrase (ATP degrading enzyme) [27, 28]. However, subsequent studies showed compelling data that isolated human or C57BL/6 murine hemoglobin containing RBC-dependent hypoxic vasorelaxation responses were similar in endothelial intact or denuded rings as well as in eNOS replete or deficient aortic rings suggesting that ATP-release was not a primary mediator in such ex-vivo vessel bioassay experiments [29]. Interestingly, the observation of a hypoxia and RBC-dependent stimulation of vasorelaxation of isolated vessel segments does not differ. What does however, are conclusions regarding the mechanisms for this effect [3]. We hypothesized that one possible factor confounding comparison of studies is the use of RBC or isolated vessels from different species and different vascular beds. In this study therefore, we took a systematic approach and tested hypoxic vasorelaxation responses of isolated murine, rat or rabbit vessels (from pulmonary artery or aorta) elicited by human or murine (containing murine hemoglobin or human HbC93 or HbC93A hemoglobin) with a focus on understanding mechanisms intrinsic to RBC, by which hypoxic vasorelaxation may occur. We find significant species and vessel source dependence to hypoxic vasorelaxation responses and also present data showing that adenosine can play a previously unrecognized, and significant role in these ex-vivo experiments, which may in turn explain how RBC can stimulate hypoxic vasorelaxation when ATP-dependent mechanisms have been inhibited and provide one mechanism unifying disparate conclusions regarding the role of the b93cys residue.

### Materials and methods

#### *Ethical approval*

All studies involving animals or humans were performed according to Institutional Animal Care and Use Committee or Institutional Review Board approved protocols respectively.

#### *Materials*

All reagents were purchased from Sigma (St. Louis, MO, USA). Male Sprague-Dawley rats (250 - 300g) and male C57BL/6 mice were purchased from Harlan (Indianapolis, IN). Rabbits were purchased from Myrtles Rabbitry Inc (TN,

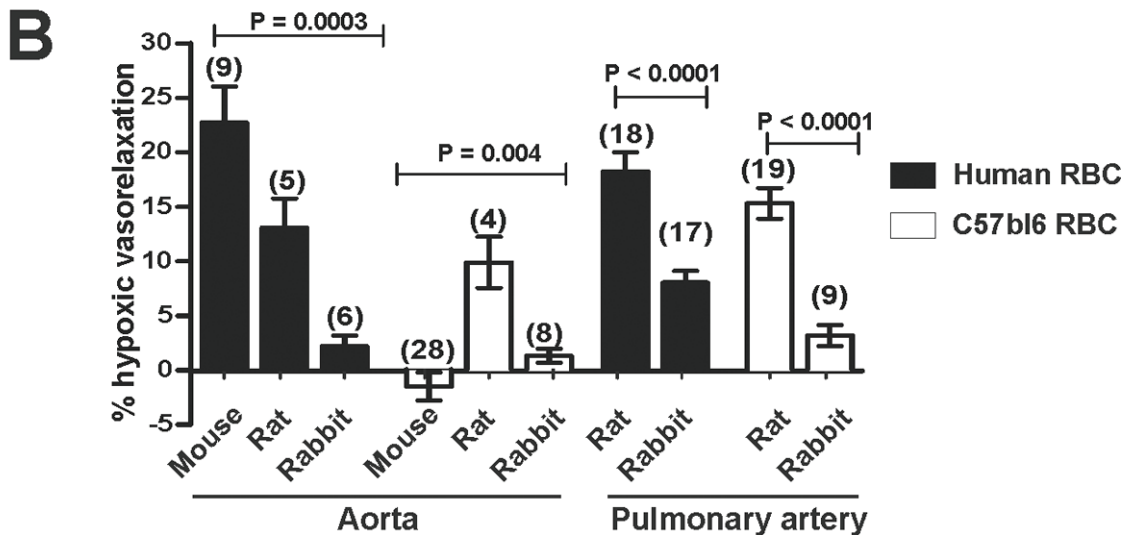
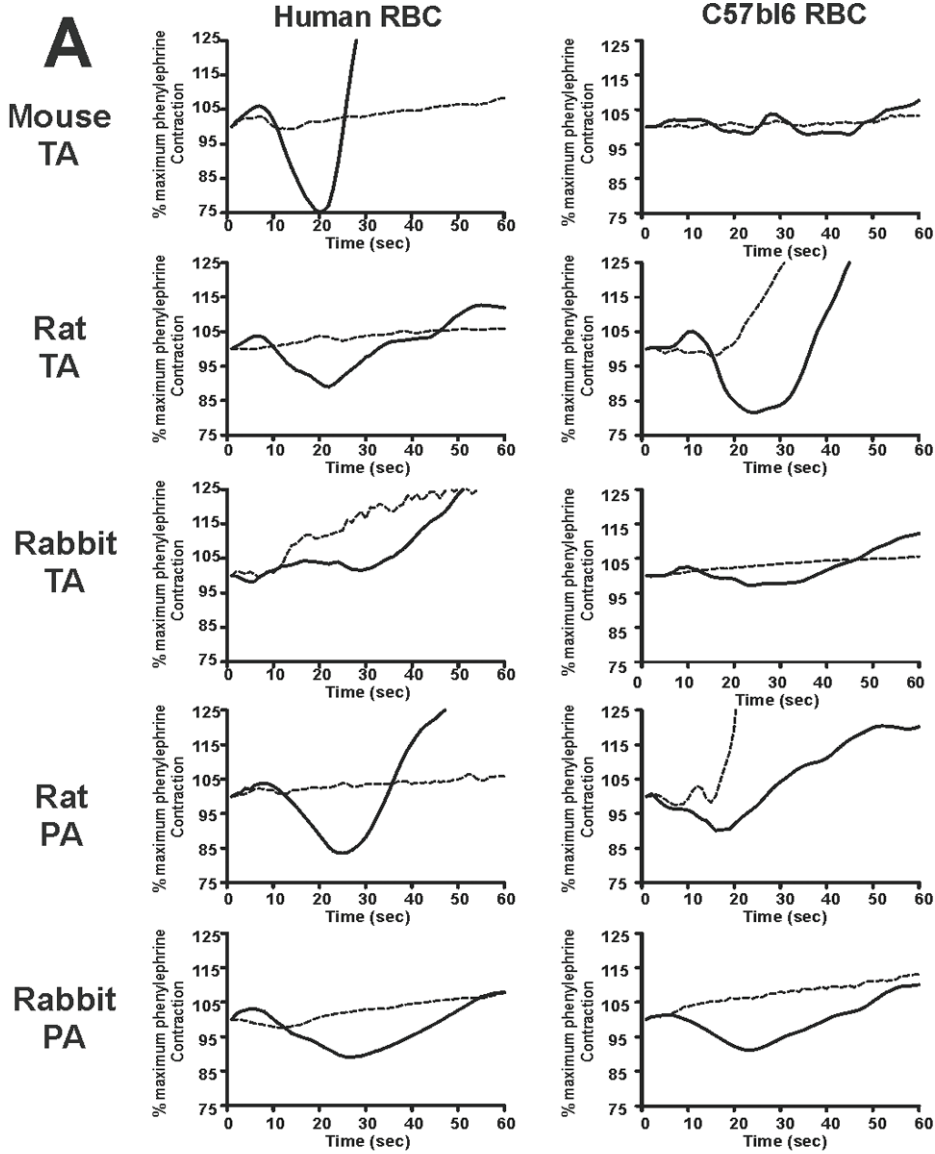
USA). All animal studies were performed following Institutional Animal Care and Use approved procedures. HbC93 and HbC93A male mice were used for all studies and generated as described previously [27].

#### *Aorta or pulmonary artery vessel studies*

All animals were anesthetized using ketamine / xylazine. Aorta from rabbit, rat or mice and pulmonary artery from rabbit or rats were isolated, cut into ~3-5mm sections then suspended between two hooks connected to a force transducer and placed within a water jacketed vessel bath chamber (Radnoti, Monrovia, CA) as described previously [30]. Vessels were bathed with 10 ml Krebs Henseleit buffer perfused with 21% O<sub>2</sub>, 5% CO<sub>2</sub> balanced with N<sub>2</sub> and maintained at 37°C and allowed to equilibrate for ~30min. A basal tension of 1.5g (rat pulmonary artery, mouse thoracic aorta), 2g (rat thoracic aorta) and 3g (rabbit thoracic aorta and pulmonary artery) was applied and the rings equilibrated for 20 min. A hyperpolarizing concentration of KCl (70mM) was added to the baths to check for viability and assess maximum contraction. The vessel rings were washed 3-times, allowed to rest for 15 min before the KCl dose was repeated. Any vessel segment that was unresponsive to KCl was excluded from experiments.

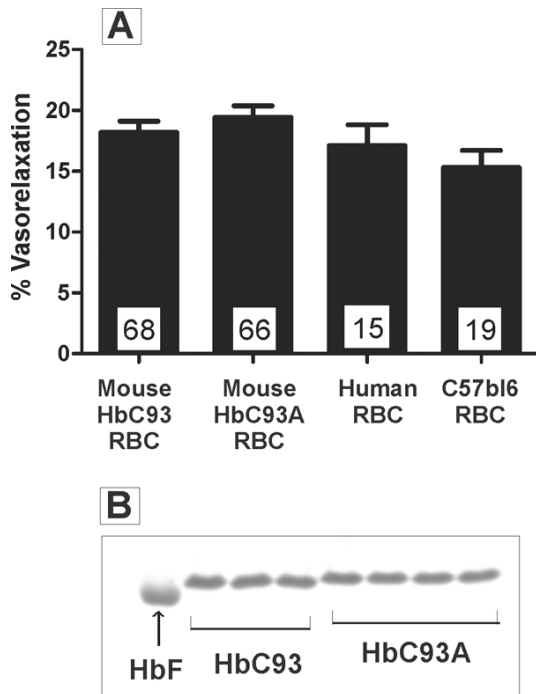
After KCl contractions, vessels were washed and either oxygen perfusing the buffer maintained at 21% O<sub>2</sub> or decreased to 1% O<sub>2</sub>, 5% O<sub>2</sub> balanced with N<sub>2</sub>. Vessel rings were allowed to acclimate to hypoxic conditions for 15 minutes treated with indomethacin (50nM) and then pre-contracted with phenylephrine (PE) to achieve 50-75% maximal contraction (300nM at 21% O<sub>2</sub> and 1μM at 1% O<sub>2</sub>). Both 21% O<sub>2</sub> and 1% O<sub>2</sub> were only used for data shown in **Figure 1**. All subsequent studies were performed at 1% O<sub>2</sub> only. For these latter studies, after equilibration and precontraction with PE, ATP or adenosine dose dependent vasorelaxation was assessed. Vessels were subsequently washed 3-times and rested at 21% O<sub>2</sub> for 15-30min. Vessels were re-exposed to 1% O<sub>2</sub> for 15 min and treated with indomethacin and PE as described above and upon reaching a stable tone (5-6 min for rat pulmonary artery). RBC (60μl of 50% Hct stock) was added (0.3% Hct final) to assess hypoxic vasorelaxation. Compounds used to inhibit ATP or adenosine-

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**Figure 1.** Effects of vessel and RBC source on hypoxic vasorelaxation responses. A. Shown are representative vessel tension vs. time traces for indicated vessel segments exposed to RBC (0.3% Hct final concentration) from human or C57BL/6 mice at 21% O<sub>2</sub> (dashed (---) line) or 1% O<sub>2</sub> (solid (-) line). RBC suspended in room air equilibrated PBS were added at time 0 to vessels that were pre-contracted and pre-equilibrated to either 21% or 1% O<sub>2</sub>, as described in methods. B. Average percent hypoxic (1% O<sub>2</sub>) vasorelaxation responses (measured 20sec after RBC addition) in response to human or mouse C57BL/6 RBC. Source of vessels are indicated on x-axis. Data are mean ± SEM. n-values are denoted in parentheses and correspond to individual vessel segments (with segments collected from 2-7 animals). Indicated P-values are from 1-way ANOVA or unpaired t-test.



**Figure 2.** Effect of the b93cys on hypoxic vasorelaxation of rat pulmonary arteries: Panel A: RBC were collected from either wild type C57BL/6 mice, humanized HbC93 or HbC93A containing mice or from healthy humans and maximal hypoxic vasorelaxation (1%) response (20-30sec after addition) of isolated rat pulmonary arterial segments determined as described in methods. Data show mean ± SEM (n values are indicated in figure and correspond to individual vessel segments). P = 0.17 by one-way ANOVA. Panel B shows a representative IEF gel of blood collected from HbC93 (n=3) or HbC93A (n=4) mice. Also shown is a positive control for fetal Hb expression (HbF) detected by IEF in blood from a transgenic mouse model of sickle cell disease.

dependent vasorelaxation were added to vessels 5min prior to addition of RBC or initiation of ATP or adenosine dose responses at the following final concentrations L-NAME (L-NG-Nitroarginine methyl ester, 200μM), CSC (8-(3-Chlorostyryl)caffeine, 1μM), DPCPX (Dipropylcyclopentylxanthine, 200nM), theophylline (100μM), Apyrase (2U/ml), MRS 1754 (8-[4-[(4-Cyanophenyl)carbamoylmethyl]oxy]

phenyl]-1,3-di(n-propyl)xanthine hydrate, 20nM).

### RBC preparation

RBC were collected from healthy humans by venipuncture using UAB Institutional Review Board approved protocols, or from C57BL/6, HbC93 or HbC93A mice by cardiac puncture after isoflurane induced anesthesia. Blood was collected in ACD coated tubes and 1ml immediately centrifuged (in a mini centrifuge for 45 sec). Plasma was discarded and RBC washed 3-times in 500μl ice-cold, room air-equilibrated PBS. After the final wash, RBC were resuspended in an equal volume of ice-cold, room air-equilibrated PBS to achieve 50% Hct. 60μl of this suspension was then added to vessels bath to get a final concentration of 0.3% RBC. The time taken from blood collection to addition of RBC to vessels was <5min.

### Isoelectric gel focusing

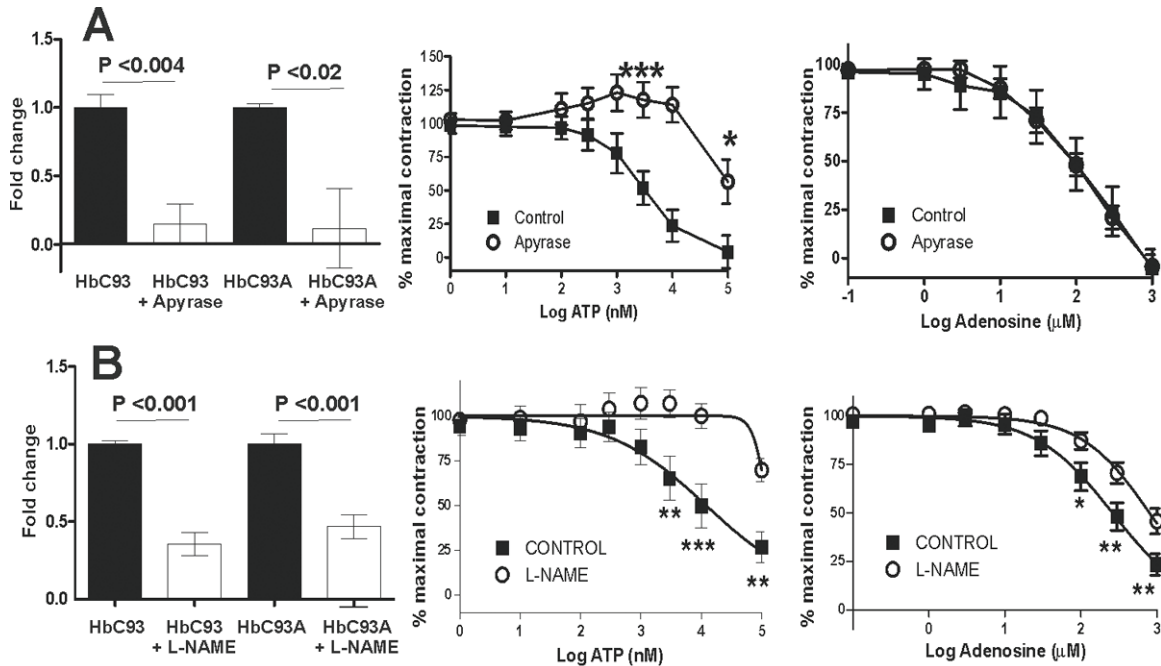
50μl of whole blood from HbC93, HbC93A or HbS expressing mouse [31, 32] was washed in 500 μl PBS. Cell pellets were resuspended in 100μl lysis solution (5 mM sodium phosphate, 0.5 mM EDTA, pH 7.4) and incubated on ice for 15 minutes. One tenth volume of 10% NaCl was added and the sample was centrifuged at maximum speed in an Eppendorf microfuge (Hamburg, Germany). 1μl of the supernatant was analyzed on an isoelectric focusing (IEF) gel. IEF was performed using the Isothermal Controlled Electrophoresis system (Fisher Scientific, Pittsburgh, PA) with precast agarose IEF gels (RESOLVE from PerkinElmer, Helsinki, Finland) as previously described [31].

## Results

### Effects of vessel type and RBC source on hypoxic vasorelaxation

We suggest that differences in conclusions about the mechanisms involved in RBC induced

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**Figure 3.** Role of ATP in RBC-mediated hypoxic vasorelaxation: Rat pulmonary arterial segments were exposed to HbC93 or HbC93A RBC in the presence or absence of A) Apyrase or B) LNAME. Also shown are effects of each inhibitor on ATP and adenosine dose-dependent vasorelaxation. Hypoxic vasorelaxation was measured 20-30sec after RBC addition. Data are shown as fold change relative to RBC alone and are mean  $\pm$  SEM, n= 6-10 vessel segments from 2-7 rats. Indicated p-values were calculated by unpaired t-test. For ATP and adenosine dose dependent vasorelaxation lines shown are best fit using sigmoidal dose response (variable slope) fitting algorithm, \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, #P<0.0001 by 2-way RM-ANOVA with Bonferonni post test.

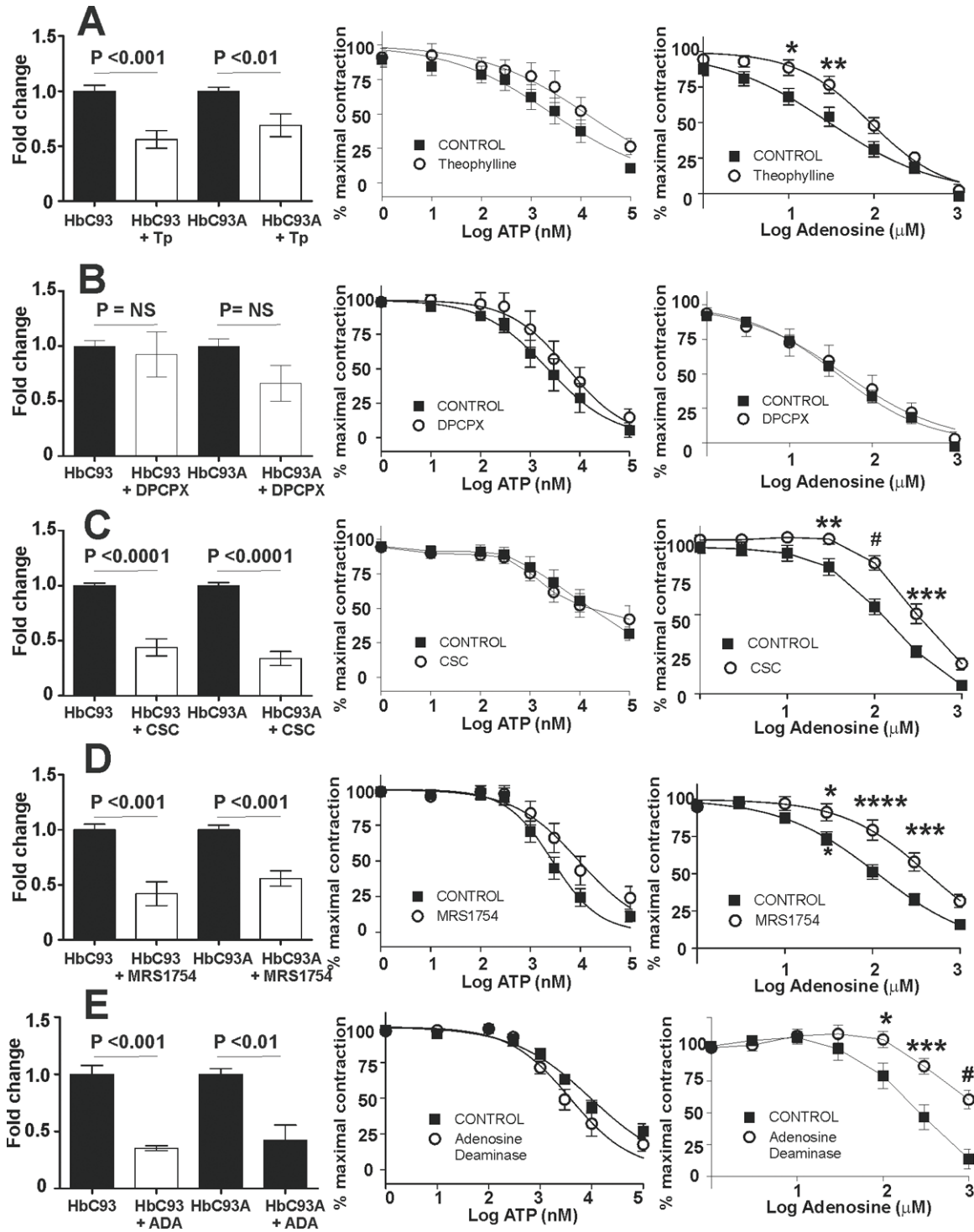
hypoxic vasorelaxation of isolated vessels may result from differences in responses of vessels from various vascular beds (e.g. aorta vs. pulmonary artery) or species (rat, mice, rabbit). Potential differences in responses elicited by RBC from different species (human, mice) may also contribute to conflicting conclusions. To examine these issues, we measured hypoxic vasorelaxation responses using various combinations of vessels and RBC. **Figure 1A** shows representative tension vs. time traces after addition of RBC to PE-constricted vessels at 21% or 1% O<sub>2</sub> and shows that vasorelaxation was not observed with all vascular preparations, and in instances when it was, the response only occurred at 1% O<sub>2</sub> and was transient (over ~10-30sec). Moreover, no vasodilation occurred in the absence of RBC addition (not shown). These results are consistent with previous studies and with the requirement for hemoglobin deoxygenation [17, 26-28, 33]. **Figure 1B** presents mean hypoxic vasorelaxation responses and interestingly, demonstrates a marked dependence on the source of either the vessel and / or RBC. Notable obser-

vations include i) the magnitude of human RBC dependent hypoxic vasorelaxation of aorta decreases from mouse > rat > rabbit, ii) C57BL/6 RBC eliciting significant hypoxic vasorelaxation of aortic segments from rat, but not from mouse or rabbit, iii) similar hypoxic vasorelaxation responses of rat pulmonary artery were observed to both human RBC and C57BL/6 RBC; and iv) a decreased sensitivity of rabbit pulmonary artery to both human RBC and C57BL/6 RBC relative to rat pulmonary artery.

### *Effects of the b93cys on RBC dependent hypoxic vasorelaxation*

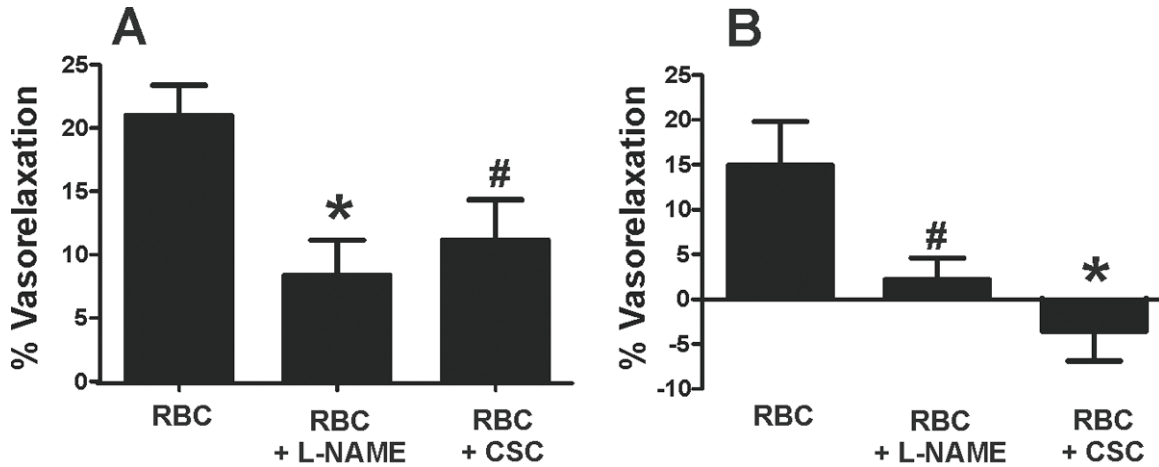
To determine whether b93cys is essential for hypoxic vasorelaxation in isolated vessels, we utilized isolated rat pulmonary artery segments since the degree of hypoxic vasorelaxation stimulated by mouse or human RBC was similar (**Figure 1B**); this precluded potential effects of mouse vs. human RBC. **Figure 2A** shows that murine RBC containing either human HbC93 or HbC93A elicited hypoxic vasorelaxation to the

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**Figure 4.** Role of adenosine in RBC-mediated hypoxic vasorelaxation: Rat pulmonary arterial segments were exposed to HbC93 or HbC93A RBC in the presence or absence of A) theophylline, B) DPCPX, C) CSC, D) MRS1754, or E) adenosine deaminase (ADA). Also shown are effects of each receptor antagonist / inhibitor on ATP and adenosine dose-dependent vasorelaxation. Hypoxic vasorelaxation was measured 20-30sec after RBC addition. Data for RBC hypoxic vasorelaxation are shown as fold change relative to RBC alone and are mean  $\pm$  SEM, n= 4-14 vessel segments from 2-7 rats. Indicated p-values were calculated by unpaired t-test. For ATP and adenosine dose dependent vasorelaxation lines shown are best fit using sigmoidal dose response (variable slope) fitting algorithm, \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001, #P<0.0001 by 2-way RM-ANOVA with Bonferonni post test.

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**Figure 5.** LNAME and CSC inhibit human RBC dependent hypoxic vasorelaxation. Rat pulmonary arterial segments (Panel A) or rat thoracic aorta (Panel B) were exposed to human RBC (0.3% Hct) at 1% O<sub>2</sub>, in the presence or absence L-NAME or CSC. Hypoxic vasorelaxation was measured 20-30sec after RBC addition. Data are mean ± SEM (n = 4-11 vessel segments from 4-5 rats). \*P < 0.05 and #P < 0.02 relative to RBC alone.

same extent, recapitulating our previous results [27]. Moreover, human RBC and murine RBC containing normal mouse hemoglobin stimulated a similar degree of hypoxic vasorelaxation suggesting that human Hb in murine RBC does not compromise basal hypoxic vasorelaxation responses. Use of the knock-in mice have been questioned on the basis of potential compensatory increases in fetal hemoglobin (hence potential hemoglobin thiol substrate for S-nitrosothiol formation) [34, 35] However, no fetal Hb was detected in blood collected from HbC93 nor HbC93A mice RBC (Figure 2B) by IEF; a positive control for HbF is shown by comparing to HbS blood. These data are consistent with our previous biochemical measurements characterizing hemoglobin chain and thiol content in this experimental system [27].

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Figure 3A and 3B demonstrates that apyrase (an ATP degrading enzyme), or L-NAME (inhibitor of eNOS), respectively, both inhibited HbC93 and HbC93A RBC dependent hypoxic vasorelaxation, consistent with previous results using human RBC [28] and suggesting a role for ATP release in RBC-dependent hypoxic vasorelaxation. As a positive control to ensure that the Apyrase and L-NAME selectively inhibit the targeted pathways, Figure 3 also shows the effects of each inhibitor on reagent ATP and adenosine-dose dependent vasorelaxation. As

expected Apyrase inhibited ATP, but not adenosine-dependent vasorelaxation. However, L-NAME inhibited both ATP and adenosine dependent vasorelaxation, although the potency of inhibition was much lower for the latter. To test if adenosine-dependent vasorelaxation could be playing a role in hypoxic RBC responses, the effects of theophylline (a pan adenosine receptor antagonist, Figure 4A); DPCPX (A<sub>1</sub> adenosine receptor antagonist, Figure 4B), CSC (A<sub>2a</sub> adenosine receptor antagonist, Figure 4C) and MRS1754 (A<sub>2b</sub> adenosine receptor antagonist, Figure 4D), were determined. Theophylline, CSC, MRS1754 all inhibited HbC93 and HbC93A dependent hypoxic vasorelaxation. These inhibitors also attenuated adenosine-dependent, but not ATP-dependent vasorelaxation, demonstrating specificity for adenosine-dependent signaling. DPCPX had no effect on HbC93, HbC93A nor on adenosine dependent vasorelaxation suggesting the lack of expression/function of the adenosine A<sub>1</sub> receptor. Collectively these data suggest a role for adenosine A<sub>2a</sub> and A<sub>2b</sub> receptors in mediating RBC-dependent hypoxic vasorelaxation of isolated rat pulmonary arteries. A role for adenosine was confirmed by ~60% inhibition of RBC-dependent vasorelaxation by addition of adenosine deaminase (ADA, Figure 4E), which metabolizes adenosine to inosine. Finally, Figure 5A and 5B show that both L-NAME and CSC inhibit human RBC dependent hypoxic vasorelaxation of rat pulmonary arteries and rat thoracic aorta, respectively, suggesting that

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a role for adenosine is not an artifact associated with humanized mouse RBC.

### Discussion

How red cells control vascular nitric oxide (NO)-signaling is the topic of much interest, with implications ranging from understanding of physiologic mechanisms of blood flow control to how inflammatory diseases characterized aberrant NO-signaling develops. The hemoglobin b93cys residue has been proposed to be a key modulator of RBC dependent hypoxic vasorelaxation. However, data presented herein show no differences between hypoxic vasorelaxation responses stimulated by HbC93 vs. HbC93A RBC suggesting no significant role for the b93cys. Our previous data suggest that ATP release from deoxygenated RBC mediated vasorelaxation of isolated aortic or pulmonary arterial ring preparations [27, 28]. However, other studies using aorta from eNOS deficient mice showed no loss of hypoxic RBC-dependent vasorelaxation, suggesting little role for ATP release [29].

Therefore we undertook a systematic approach to assess mechanisms of RBC-mediated hypoxic vasorelaxation using isolated aorta and pulmonary artery segments. These vessels were chosen as they offer convenient experimental models and importantly have been used extensively to elucidate hypoxic vasorelaxation mechanisms intrinsic to RBC [17, 19, 26-29, 36], thereby allowing direct comparison with previous data. Significant differences in the hypoxic vasorelaxation response were observed based on the source of the RBC and the vessel segment used (**Figure 1**). Our primary goal was to determine mechanisms intrinsic to the RBC and not on differences between aorta and pulmonary artery per se; although we note that this could reflect differences between the magnitude of hypoxic vasorelaxation responses observed in previous studies [26, 27, 33]. With this in mind, we opted to utilize the rat pulmonary artery system further since there were no differences in hypoxic vasorelaxation of these vascular rings stimulated by human or mouse RBC (in contrast to mouse aorta for example) thus allowing direct comparison of human, mouse and humanized mouse RBC. We recognize that pulmonary arteries constrict during hypoxia, and therefore verified key findings with aortic vessels (**Figure 5**) and note that RBC

were only added after stable PE-induced contractile tone was achieved. Using pulmonary arterial segments also allowed direct comparison of our results with previous studies that suggest a role for b93 S-nitrosylation in RBC-dependent hypoxic vasodilation [26, 33]. Using these systems, no difference was observed between HbC93 and HbC93A RBC confirming the lack of requirement for the b93cys residue. Moreover the hypoxic vasorelaxation response was the same as observed with human and murine (mouse Hb containing) RBC demonstrating the expression of human hemoglobin in mouse RBC does not result in dysfunctional RBC-dependent hypoxic vasorelaxation responses and validates the use of these knockin RBC.

Having established no role for b93cys, we then asked how RBC mediate hypoxic vasorelaxation. Previous studies suggested a role for ATP [5, 8, 18, 27, 28, 37] and consistent with this hypothesis, Apyrase or L-NAME inhibited both HbC93 and HbC93A dependent effects. However, L-NAME also prevented adenosine dependent dilation consistent with studies showing adenosine dependent vasorelaxation is eNOS and NO-dependent [38, 39]. Moreover, we have noted previously that theophylline can also attenuate HbC93 and HbC93A RBC dependent hypoxic vasorelaxation of rabbit pulmonary artery segments [27] suggesting a potential role for adenosine. The effect of theophylline was confirmed in the current study and extended upon by demonstration that adenosine  $A_{2A}$  and  $A_{2B}$  receptors are involved in RBC hypoxic vasorelaxation. Furthermore, the inhibitory effect of adenosine deaminase verified that hypoxic RBC utilized adenosine to stimulate vasorelaxation. Importantly, human RBC dependent hypoxic vasorelaxation was also attenuated by inhibition of adenosine signaling, and this was observed with both rat pulmonary arterial and thoracic aorta segments indicating that a role for adenosine was not exclusive to humanized mouse RBC, nor to pulmonary arterial segments only. Since adenosine can dilate smooth muscle directly, we hypothesize that experimental protocols employing vessel denudation or eNOS inhibition are not sufficient to exclude ATP-dependent effects. Coupled with the fact that L-NAME can also attenuate adenosine-dependent vasorelaxation, and that there was no difference in responses between



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HbC93A vs. HbC93 RBC, we posit that adenosine dependent vasorelaxation offers an alternative mechanism to SNO-Hb in explaining how RBC dependent vasorelaxation may occur when ATP-signaling has been ablated.

Many cells can release adenosine in response to hypoxia, we note that in the studies performed here however, hypoxic vasorelaxation was absolutely dependent on addition of RBC. This raises the question of how RBC elicit adenosine-dependent effects. Adenosine influx into RBC has been studied extensively [40] but little is known about whether adenosine may also be released from RBC. Hypoxemic RBC may stimulate adenosine release by endothelial or smooth muscle cells and a final alternative is that adenosine is derived from ATP metabolism; the latter is also indicated since Apyrase almost completely ablated the hypoxic vasorelaxation response. These possibilities remain to be tested. We note that in terms of hypoxic blood flow in vivo, convincing evidence that adenosine does not play a role in coupling RBC deoxygenation with NO-signaling in human skeletal muscle has been presented [41]. Therefore, the biological function for adenosine signaling induced by RBC-deoxygenation remains to be determined. Nevertheless this provides a novel mechanism through which RBC may affect various vascular processes and underscores that adenosine effects must be considered when evaluating RBC-dependent effects ex-vivo. Moreover, we note that further studies are needed to test these mechanisms using resistance vessels, where hypoxic vasodilatation occurs, as opposed to large conduit vessels used in this study. In summary, we confirm that the b93cys residue is not required for RBC dependent hypoxic vasorelaxation and demonstrate that both ATP and adenosine may play significant roles in coupling RBC deoxygenation to stimulation of vasorelaxation.

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