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Nitric oxide (NO) regulates blood pressure (BP) by binding the reduced heme iron (Fe$^{2+}$) in soluble guanylyl cyclase (sGC) and relaxing vascular smooth muscle cells (SMC). We previously showed that sGC heme iron reduction (Fe$^{3+} \rightarrow$ Fe$^{2+}$) is modulated by cytochrome b5 reductase 3 (CYB5R3). However, the in vivo role of SMC CYB5R3 in BP regulation remains elusive. Here, we generated conditional smooth muscle cell-specific Cyb5r3 knockout mice (SMC CYB5R3 KO) to test if SMC CYB5R3 loss impacts systemic BP in normotension and hypertension via regulation of sGC redox state. SMC CYB5R3 KO mice exhibited a 5.84 mmHg increase in BP and impaired acetylcholine-induced vasodilation in mesenteric arteries compared to controls. To drive sGC oxidation and elevate BP, we infused mice with angiotensin-II. We found SMC CYB5R3 KO mice exhibited a 14.75 mmHg BP increase and mesenteric arteries had diminished NO-dependent vasodilation, but increased responsiveness to sGC heme-independent activator BAY 58-2667 over controls. Furthermore, acute injection of BAY 58-2667 in angiotensin-II treated SMC CYB5R3 KO mice showed greater BP reduction compared to controls. Together, these data provide the first in vivo evidence that SMC CYB5R3 is a sGC heme reductase in resistance arteries and provides resilience against systemic hypertension development.

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Abstract

Nitric oxide (NO) regulates blood pressure (BP) by binding the reduced heme iron (Fe\textsuperscript{2+}) in soluble guanylyl cyclase (sGC) and relaxing vascular smooth muscle cells (SMC). We previously showed that sGC heme iron reduction (Fe\textsuperscript{3+} → Fe\textsuperscript{2+}) is modulated by cytochrome b5 reductase 3 (CYB5R3). However, the in vivo role of SMC CYB5R3 in BP regulation remains elusive. Here, we generated conditional smooth muscle cell-specific Cyb5r3 knockout mice (SMC CYB5R3 KO) to test if SMC CYB5R3 loss impacts systemic BP in normotension and hypertension via regulation of sGC redox state. SMC CYB5R3 KO mice exhibited a 5.84 mmHg increase in BP and impaired acetylcholine-induced vasodilation in mesenteric arteries compared to controls. To drive sGC oxidation and elevate BP, we infused mice with angiotensin-II. We found SMC CYB5R3 KO mice exhibited a 14.75 mmHg BP increase and mesenteric arteries had diminished NO-dependent vasodilation, but increased responsiveness to sGC heme-independent activator BAY 58-2667 over controls. Furthermore, acute injection of BAY 58-2667 in angiotensin-II treated SMC CYB5R3 KO mice showed greater BP reduction compared to controls. Together, these data provide the first in vivo evidence that SMC CYB5R3 is a sGC heme reductase in resistance arteries and provides resilience against systemic hypertension development.
Introduction

In the vascular wall, nitric oxide (NO) produced by the endothelium binds soluble guanylyl cyclase (sGC) in vascular smooth muscle cells (SMC) and converts guanosine-5' triphosphate (GTP) to the second messenger molecule, cyclic guanosine 3', 5'-monophosphate (cGMP) (1-3). Elevated cGMP, in turn, activates protein kinase G (PKG) leading to SMC relaxation, vasodilation, and a reduction in blood pressure (BP) (4). A prerequisite for activation of NO-sensitive sGC is the presence of reduced heme iron (Fe$^{2+}$) residing in the N-terminus of the heme-NO/O$_2$-binding domain of the sGC-β1 subunit (1-3, 5-9). Binding of NO to reduced sGC heme elicits cleavage of the histidine-Fe$^{2+}$ bond leading to subsequent activation and production of cGMP (10, 11). However, increased production of reactive oxygen species such as hydrogen peroxide and peroxynitrite, as observed in cardiovascular disease, can lead to sGC heme oxidation (Fe$^{2+}$ → Fe$^{3+}$), rendering sGC insensitive or resistant to NO (10, 12, 13). Thus, preserving sGC heme in the reduced state, especially under oxidative stress conditions, is critical for NO-mediated regulation of vasomotor tone and blood pressure control.

NADH cytochrome b5 reductase 3 (CYB5R3), also known as methemoglobin reductase, is a flavoprotein that has numerous enzymatic roles in cellular physiology. In red blood cells, soluble CYB5R3 maintains hemoglobin in the reduced state permitting it to bind oxygen (14, 15). Membrane-bound CYB5R3 has been shown to control elongation and desaturation of fatty acids (16, 17), cholesterol biosynthesis (18), drug metabolism (19, 20) and nutrient and oxidative stress responses (21). In endothelial cells, CYB5R3 has been found to reduce the heme of hemoglobin alpha to mediate NO-scavenging and impair NO-diffusion from endothelial cells to smooth muscle cells at myoendothelial
junctions (22, 23). Recently, we demonstrated for the first time that CYB5R3 is expressed in cultured SMC and reduces the oxidized sGC heme (Fe$^{3+} \rightarrow$ Fe$^{2+}$) (22, 24). Specifically, we showed that transient knockdown and pharmacological inhibition of CYB5R3 in SMCs impairs NO-mediated cGMP production by SMC in vitro and aortic relaxation via ex vivo myography (24). However, the in vivo role of CYB5R3 in SMC remains unknown.

Herein, we investigated whether SMC derived CYB5R3 acts as a sGC heme reductase in vivo in the context of normotension and systemic hypertension. To test this, we generated tamoxifen-inducible, SMC-specific CYB5R3 knockout mice (Cyb5r3$^{Δ/Δ}$Myh11-CreER$^{T2}$, hereafter referred to as SMC CYB5R3 KO). We found that SMC CYB5R3 KO mice had significantly higher mean arterial pressures as compared to WT controls (Cyb5r3$^{wt/wt}$Myh11-CreER$^{T2}$) that was further exacerbated with angiotensin-II (Ang-II) induced hypertension. In addition, Ang-II treated SMC CYB5R3 KO mice show an enhanced vasodilation response to the sGC activator BAY 58-2667 compared to Ang-II treated WT mice suggesting loss of CYB5R3 in SMC results in more oxidized or heme-deficient sGC. Combined, these data provide the first in vivo evidence that SMC CYB5R3 is a sGC heme reductase in resistance arteries and confers resilience to systemic hypertension.
Results

To determine the function of NADH cytochrome b5 reductase 3 (CYB5R3) in smooth muscle cells (SMC) in vivo, we generated Cyb5r3 floxed animals and crossed them with the previously characterized Myh11-CreERT2 mice (25) to generate tamoxifen-inducible SMC CYB5R3 knockout animals (Cyb5r3Δ/Δ Myh11-CreERT2 or SMC CYB5R3 KO) (Supplemental Figure 1, Table 1). No gross morphological changes were observed as a consequence of SMC CYB5R3 KO though they had significantly fewer circulating monocytes compared to WT mice (Table 2). We confirmed SMC-specific knockout of Cyb5r3 via staining post-tamoxifen treated Cyb5r3wt/wt Myh11-CreERT2 (WT) and SMC CYB5R3 KO mice aorta and mesenteric arteries with CYB5R3. CYB5R3 levels were significantly reduced within the media areas of both the aorta and mesenteric arteries in SMC CYB5R3 KO (n=5) compared to WT (n=5) mice (Supplemental Figure 2A-C). In order to determine if levels of sGC in SMC were impacted by loss of SMC CYB5R3, the aorta and mesenteric arteries of WT and SMC CYB5R3 KO mice were stained for the beta subunit of sGC (sGC-β) (Supplemental Figure 3A). We observed no significant difference in sGC-β levels in the media of the aorta or mesenteric arteries between WT (n=5) and SMC CYB5R3 KO (n=4) animals (Supplemental Figure 3B).

We hypothesized, given our previous work (24), that loss of SMC CYB5R3 would impair reduction of oxidized sGC (Fe2+ → Fe3+) consequently increasing vasoconstriction and blood pressure. To test this, WT (n=10) and SMC CYB5R3 KO (n=10) mice were surgically implanted with telemetry units and blood pressure and heart rate continuously recorded for 24-hours (Figure 1A). We found that SMC CYB5R3 KO mice averaged a significant 5.84 mmHg higher mean arterial pressure over the total 24-hour period (Figure
This baseline blood pressure difference was driven by the fact that SMC CYB5R3 KO versus WT mice averaged significantly higher systolic and diastolic pressures over the total 24-hour period (Supplemental Figure 4A-B). Consistent with this, SMC CYB5R3 KO mice averaged significantly lower heart rates over the total 24-hour period compared to WT mice (Figure 1C, Table 3).

To determine the cause of the increased blood pressure in SMC CYB5R3 KO mice, WT (n=5-7) and SMC CYB5R3 KO (n=7-8) mesenteric arteries were subjected to ex vivo wire myography to assess vessel reactivity (Figure 2A). Vasoconstriction with $10^{-7}$ M U46619, a thromboxane mimetic, showed no significant difference in vasoconstriction between WT and SMC CYB5R3 KO mice (Figure 2B). Next, we assessed WT versus SMC CYB5R3 KO mesenteric artery response to cumulative doses of three different vasodilators: acetylcholine, sodium nitroprusside (SNP), and BAY 58-2667 (sGC activator). Acetylcholine was used to assess whether loss of SMC CYB5R3 impacts endothelial cell-mediated vasodilation (26). We found that SMC CYB5R3 KO mice had a significantly impaired response to acetylcholine as compared to WT mice particularly at the $10^{-5}$ M dose (Figure 2C). To test whether NO-mediated vasodilation was disrupted in SMC as a consequence of loss of CYB5R3, mesenteric arteries from WT and SMC CYB5R3 KO mice were given cumulative doses of SNP, a NO-donor molecule that can deliver NO to reduced sGC in SMC to induce vasodilation. We found no differences in SNP response between WT and SMC CYB5R3 KO mice (Figure 2D). In order to determine whether SMC CYB5R3 regulates sGC state in these animals, we treated mesenteric arteries with sGC-activator BAY 58-2667. This compound activates oxidized sGC and heme-deficient sGC (apo-sGC) independent of NO in SMC to induce cGMP.
production and vasodilation (4, 27). We found SMC CYB5R3 KO had a slight but significant impairment in vasodilation response to BAY 58-2667 compared to WT mice (Figure 2E). Combined, these data suggest that SMC CYB5R3 may lead to dysfunctional SMC and endothelial cross-talk under physiological conditions.

Next, we wanted to assess whether SMC CYB5R3 was important for blood pressure regulation in the context of systemic hypertension. Hypertension and increased Ang-II signaling has been shown to result in oxidation or loss of the sGC heme (28). WT (n=8) and SMC CYB5R3 KO mice (n=8) were implanted with osmotic mini-pumps delivering 750ng kg\(^{-1}\) min\(^{-1}\) Ang-II and blood pressure and heart rate changes were measured continuously for 14 days (Figure 3A). Ang-II induced hypertension resulted in a significant increase in mean arterial, systolic, and diastolic pressures and decreased heart rate in SMC CYB5R3 KO animals compared to WT animals during the first 7 days of Ang-II treatment (Figure 3B-C, Supplemental Figure 5A-B). Days 6-7 of Ang-II treatment appeared to represent the culmination of the increased hypertensive state of SMC CYB5R3 KO mice compared to WT (Figure 3D-E, Supplemental Figure 5C-D). Indeed, Days 6-7 of Ang-II treatment further exacerbated the difference in average mean arterial pressure between WT and SMC CYB5R3 KO mice from 5.84 mmHg difference in untreated animals to a peak 14.75 mmHg difference (Table 3). Notably, WT heart rate declined between Days 8-9 of Ang-II while SMC CYB5R3 KO mice maintained a relatively consistent heart rate throughout the study (Figure 3C). Combined, the data indicate that SMC CYB5R3 KO mice, as compared to WT mice, are more susceptible to hypertensive agonists and likely have an impaired ability to mitigate Ang-II effects.
After the 14 days of Ang-II treatment, mesenteric arteries from WT (n=9-12) and SMC CYB5R3 KO (n=11-12) mice were subjected to ex vivo wire myography and treated with vasoconstrictor U46619 and cumulative doses of vasodilators (acetylcholine, SNP, and BAY 58-2667) to assess the impact of systemic hypertension on resistance artery function (Figure 4A). U46619-mediated vasoconstriction was significantly increased in Ang-II treated SMC CYB5R3 KO mice compared to WT (Figure 4B). Additionally, SMC CYB5R3 KO mesenteric arteries showed an impaired acetylcholine mediated vasodilation compared to WT controls (Figure 4C). Cumulative treatment with NO-donor SNP resulted in a significant impairment of SMC CYB5R3 KO vasodilation compared to WT (Figure 4D). WT and SMC CYB5R3 KO mesenteric arteries were also treated with increasing concentrations of oxidized and apo-sGC targeted vasodilator BAY 58-2667 where it was found that SMC CYB5R3 KO mice were significantly more responsive to BAY 58-2667 induced vasodilation than WT controls (Figure 4E). This indicates that loss of SMC CYB5R3 in Ang-II treated mesenteric arteries results in a greater shift in the sGC pool to a more oxidized or heme-deficient state as compared to WT mesenteric arteries.

To further test this, we looked at cGMP pathway activation by quantifying phosphorylation of vasodilator - stimulator phosphoprotein (pVASP) which is specifically phosphorylated at Serine 239 by PKG. Mesenteric arteries isolated from 14 day Ang-II treated SMC CYB5R3 KO mice and given NO-donor diethylamine nonoate (DEA-NONOate) showed decreased levels of pVASP239 compared to WT mice (Supplemental Figure 6). To assess whether vasodilation in Ang-II treated conduit arteries was similarly impacted by SMC CYB5R3 loss, we performed these same ex vivo myography experiments in aortas. We found no differences in phenylephrine mediated vasoconstriction, or vasodilation via
SNP or BAY 58-2667 between WT and SMC CYB5R3 KO aortas (Supplemental Figure 7A-B, D-E) However, WT hypertensive aorta and mesenteric arteries were found to be significantly more responsive to acetylcholine as compared to SMC CYB5R3 KO (Figure 4C, Supplemental Figure 7C). Taken together, these data provide evidence that SMC derived CYB5R3 acts as a sGC reductase and impacts NO-mediated vasodilation in hypertensive resistance arteries.

To determine whether loss of SMC CYB5R3 had secondary effects on vessel and cardiac fibrosis or remodeling, aorta, mesenteric arteries, and heart tissue treated with Ang-II for 7, 14, and 28 days were stained with Masson’s trichrome stain. We observed no gross differences in fibrosis of any of the tissues at any point of Ang-II treatment (Supplemental Figures 8A, 9A, and 10A). We also found no significant differences in aorta or mesenteric artery medial area between WT and SMC CYB5R3 KO at any point of Ang-II treatment (Supplemental Figures 8B and 9B). Similarly, no differences in cardiac hypertrophy or intracardiac fibrosis were observed between groups at any point of Ang-II treatment (Supplemental Figure 10B).

To test whether loss of CYB5R3 in SMC can affect sGC heme redox state and consequently blood pressure in vivo, hypertensive WT and SMC CYB5R3 KO mice were intraperitoneally (I.P.) injected at 1 dose per day with increasing concentrations of 10^-7 - 10^-3 M BAY 58-2667 (Figure 5A). We reasoned these studies would extend our ex vivo myography vasodilation experiments in vivo for assessment of BAY 58-2667 influence on blood pressure between WT and SMC CYB5R3 KO mice. Mice were anesthetized briefly via isoflurane when injected I.P. to minimize stress-induced blood pressure changes. The first dose at 10^-7 M was given to both WT and SMC CYB5R3 KO mice on Day 20 of Ang-
II treatment. At the $10^{-4}$ M dose of BAY 58-2667 on Day 23 of Ang-II treatment, the equivalent of 0.04 mg/kg BAY 58-2667, we observed a decrease in mean arterial pressure in both WT and SMC CYB5R3 KO mice between 15-30 minutes post-injection though no differences in response to BAY 58-2667 were observed between groups for up to two hours post-injection at this dose (Figure 5B). Similarly, no significant differences in systolic or diastolic pressures were observed between WT and SMC CYB5R3 KO at this dose of BAY 58-2667 (Supplemental Figure 11A-B). Lastly, WT mice had an increased heart rate at 15 minutes post injection of 0.04 mg/kg BAY58-2667 (Figure 5C).

Importantly, the subsequent higher $10^{-3}$ M dose which is 0.4 mg/kg BAY 58-2667 given the following day resulted in a significant and drastic decrease in change in mean arterial blood pressure in SMC CYB5R3 KO (n=4) compared to WT (n=4) (Figure 5D). SMC CYB5R3 KO mice also showed increased vasodilatory response to 0.4 mg/kg BAY 58-2667 dose as the change in systolic and diastolic pressures were significantly decreased post injection compared to WT controls (Supplemental Figure 11C-D). While the 0.4 mg/kg BAY 58-2667 dose increased the change in heart rate in both groups, no significant differences in change in heart rate were observed between WT and SMC CYB5R3 KO mice (Figure 5E). This suggests that the enhanced BAY 58-2667 blood-pressure lowering effects in Ang-II treated SMC CYB5R3 KO mice are the result of increased peripheral vascular resistance in these mice as compared to WT mice. Taken together, these data provide evidence that in Ang-II induced hypertensive conditions, CYB5R3 is the sGC reductase in SMC modulating sGC heme state thereby influencing blood pressure and vessel function (Figure 6).
Discussion

The NO-sGC-cGMP pathway is well-established in regulating systemic blood pressure. We previously discovered in cultured SMC that CYB5R3 restores NO-sGC-cGMP vasodilation signaling by reducing the oxidized sGC heme (Fe$^{3+}$→Fe$^{2+}$) (24, 29). However, the function of CYB5R3 in SMC in vivo and its potential impact in blood pressure regulation had yet to be explored. Using conditional, SMC-specific CYB5R3 KO mice, we provide the first evidence that SMC CYB5R3 is a sGC reductase and that SMC CYB5R3 confers protection against Ang-II induced systemic hypertension via regulation of sGC redox state.

Under normal physiological conditions, we observed SMC CYB5R3 KO resulted in a significant elevation in physiological blood pressure as compared to WT mice. Moreover, ex vivo myography studies on mesenteric arteries provide evidence that the elevation in physiological blood pressure in SMC CYB5R3 KO mice involves dysfunction in endothelial cell acetylcholine-mediated signaling. Acetylcholine can vasodilate resistance arteries, in part, through the activation of endothelial nitric oxide synthase (eNOS) leading to NO synthesis, NO diffusion to SMC, and then activation of reduced sGC mediated signaling (26, 30). Independent of NO-sGC signaling, acetylcholine can induce vasodilation via activation of other endothelium-derived hyperpolarizing factor (EDHF) pathways involving lipooxygenases, cyclooxygenases, and cytochrome p450 enzymes (26, 31). In particular, cytochrome p450 enzymes can and may be regulated by CYB5R3 activity adding additional complexity to the system (19, 32, 33). It is thus possible that SMC CYB5R3 may have an as yet unidentified role in one of these other mechanisms of acetylcholine action for vasodilation. Therefore, a further in-depth analysis of the
complex and multifaceted acetylcholine-dependent pathway that is pertinent for SMC CYB5R3 regulation of physiological blood pressure is warranted.

Most importantly, we provide evidence that CYB5R3 in SMC acts as a sGC reductase in resistance arteries in the context of Ang-II induced systemic hypertension. Indeed, we show Ang-II treated SMC CYB5R3 KO mice given sGC activator BAY 58-2667, which enhances cGMP production through acting on heme-deficient and oxidized sGC, resulted in a significant decrease in mean arterial pressure in vivo and increased resistance artery vasodilation in ex vivo myography experiments compared to WT mice. In addition, NO-dependent cGMP production and vasodilation were blunted in Ang-II SMC CYB5R3 KO mice compared to WT mice. Of interest, Ang-II treated WT and SMC CYB5R3 KO aortas didn’t differ in response to SNP or BAY 58-2667 but did show a blunted acetylcholine response in SMC CYB5R3 KO mice compared to WT controls. This again highlights the importance of CYB5R3 in peripheral vascular resistance and a potential role for SMC CYB5R3 in acetylcholine-dependent vasodilation. Ang-II treated SMC CYB5R3 KO mice were also more sensitive to Ang-II treatment with further enhanced elevation in mean arterial pressure detected compared to WT mice for the first week of Ang-II treatment. Combined, this suggests that SMC CYB5R3, particularly in resistance arteries, confers protection from systemic hypertension. This is consistent with work published by Martin-Montalvo et al. showing that CYB5R3 overexpression confers protection from oxidative stress induced lipid peroxidation and enhanced survival (17). During the second week of Ang-II treatment, however, no differences in mean arterial pressure or heart rate were detected between groups. Interestingly at Day 8 of Ang-II treatment, the WT mice heart rate decreased to nearly the level of the SMC CYB5R3 KO.
We hypothesize these observations indicate a pressure natriuresis event and/or change in sympathetic drive that occurred in Ang-II treated WT mice that is absent in the SMC CYB5R3 KO mice.

We had previously published that spontaneously hypertensive rats treated with a CYB5R3 inhibitor acutely resulted in a decrease in mean arterial pressure compared to vehicle treated controls (29). It is worth considering these differences in observations are likely due to the net effect of pharmacological inhibition of CYB5R3 on both endothelial cells and SMCs. Indeed, we previously found that pharmacological inhibition of CYB5R3 aortas resulted in reduced acetylcholine and SNP induced vasodilation providing evidence the CYB5R3 inhibitor likely impacts NO-signaling in both SMCs and endothelial cells (24). In endothelial cells, CYB5R3 reduces ferric hemoglobin (methemoglobin) alpha to its ferrous hemoglobin alpha state (22, 23, 29). However, the oxidation of ferrous hemoglobin to ferric hemoglobin occurs very rapidly in the presence of oxygen due to a NO dioxygenation reaction that occurs at a rate of 6-8 x 10^{-7} M^{-1} s^{-1} (22, 23, 29, 34). In comparison, the oxidation of ferrous sGC to ferric sGC, which does not involve oxygen or a NO dioxygenation reaction, likely occurs much more slowly. Taken together, it is plausible that the short-term, acute Ang-II and CYB5R3 inhibitor treatment used in our previous studies in hypertensive rats was not given long enough to impact sGC and therefore CYB5R3 action on hemoglobin alpha in endothelial cells took precedence and caused the previously observed increased vasodilation response (9, 10).

CYB5R3 has been shown in vitro to be the reductase for several hemoproteins including hemoglobin alpha, cytoglobin, and as evidenced herein sGC (22-24, 29, 35, 36). Recently, Liu et al showed that loss of CYB5R3 in cultured SMC resulted in impaired NO
scavenging (35, 36). This was attributed to CYB5R3 action on cytoglobin which is found in SMC and via NO dioxygenase reaction transitions from a ferrous to ferric heme state (35, 36). In addition, global genetic knockout of cytoglobin was shown to result in systemic hypotension in untreated and Ang-II treated mice, the opposite of our observations in our SMC CYB5R3 KO mice (36). Combined with the fact that we observed no differences in SNP vasoreactivity between WT and SMC CYB5R3 KO mesenteric arteries under physiological conditions, our data suggests CYB5R3 likely does not act on cytoglobin in SMC under physiological conditions in vivo.

Ang-II treatment has been shown to increase NADPH oxidase dependent production of superoxide at the plasma membrane and contribute to mitochondrial reactive oxygen species generation (37). In SMC specifically, numerous studies have provided evidence that Ang-II superoxide production is NADPH oxidase 1 (NOX1) dependent and that NOX1 is localized to caveolae at the plasma membrane (38-45). Interestingly, work in cardiac hypertrophy models have shown that cytosolic sGC-β1 can be oxidized while sGC-β1 sequestered in caveolae microdomains at the plasma membrane is protected from oxidation (46, 47). Consistent with this, Zabel et al has shown that sGC-β1 localized to the membrane of the rat heart is more sensitive to NO-signaling (48). We previously published that in sGC oxidant 1h-[1,2,4]oxadiazolo-[4,3-a]quinoxaline-1-one (ODQ) treated SMC, there are CYB5R3-sGC interactions occurring mostly within the cytoplasm (24). Given that CYB5R3 is present in the plasma, mitochondrial, and endoplasmic reticulum membranes, an intriguing future study would be to determine if SMC CYB5R3 subcellular localization is important for regulation of NO-sGC signaling during oxidative stress. Moreover, the fact that Ang-II SMC CYB5R3 KO
mice still responded to SNP suggests it may be possible that an unidentified reductase may also contribute to activation of NO-sGC vasodilation in SMC in the context of systemic hypertension.

Combined, our data provide substantive evidence that CYB5R3 in SMC regulates the NO-sGC-cGMP pathway by acting as an sGC reductase during systemic hypertension. However, it is important to recognize that CYB5R3 has been shown to have varied roles in cellular processes including in cellular respiration and metabolism (17, 21, 32, 33, 49-51), oxygen-delivery (14, 15), and NO-sGC-cGMP signaling (22-24, 29, 35, 36). Additionally, data from our lab and others have shown that CYB5R3 reductase activity in hepatocytes (17, 21, 49), red blood cells (14, 15), endothelial cells (22, 23) and smooth muscle cells (24) have the capacity to target different hemoproteins and redox molecules (e.g. hemoglobin alpha, sGC, and coenzyme Q) that then differentially modulate cell-signaling and functional output. A crucial future direction will be to determine how the differential use of CYB5R3 reductase activity in various cells and tissues translates to that of human vascular physiology and cardiovascular diseases. This will be of utmost importance when considering personalized treatment of patients with and without genetic variants for CYB5R3 and the possibility that they might have different responsiveness to NO but also sGC stimulators and sGC activators.
Methods

Generation of Cyb5r3\textsuperscript{fl/fl} and SMC-specific CYB5R3 knockout mice (Cyb5r3 Myh11-Cre\textsubscript{ERT2}). A Cyb5r3 gene targeting plasmid was generated by the University of California Davis - Knockout Mouse Project Repository (Cyb5r3\textsuperscript{tm1a(KOMP)Wtsi}, CSD29891, reproduced and simplified in Supplemental Figure 1A) (52, 53). Exon 3 of Cyb5r3 was identified via computational model to be a “critical exon” where excision of exon 3 would result in a frame-shift mutation and non-sense mediated decay of the Cyb5r3 transcript conferring loss of CYB5R3 (52). This gene targeting vector was transduced into JM8.N4 embryonic stem cells (54) which are an agouti C57BL/6N line and selected for via neomycin resistance before being implanted into pseudo-pregnant C57BL/6J mice at the University of Alabama. C57BL/6NJ chimeras were then crossed to C57BL/6J FLPase mice to target FRT sites and remove the embryonic stem cell selection cassette. The resulting Cyb5r3 floxed female mice were then crossed to male Myh11-Cre\textsubscript{ERT2} mice (019079, C57BL/6, Jackson Laboratory) (25) to generate tamoxifen-inducible SMC-specific Cyb5r3 floxed mice: Cyb5r3\textsuperscript{fl/fl}Myh11-Cre\textsubscript{ERT2} (Supplemental Figure 1B-C). Mice were backcrossed for 10 generations. Given that the Myh11-Cre\textsubscript{ERT2} is present on the Y chromosome, only male mice were used in our studies. Intraperitoneal injections of 1mg per day per mouse of tamoxifen (Sigma, T-5648) for 10 days into Cyb5r3\textsuperscript{fl/fl}Myh11-Cre\textsubscript{ERT2} results in activation of Cre-loxp mediated excision of exon 3 and loss of CYB5R3 expression solely in Myh11 expressing cells (Supplemental Figure 1C). Tamoxifen treatment therefore generates SMC-specific Cyb5r3 knockout mice (Cyb5r3\textsuperscript{3A/3A}Myh11-Cre\textsubscript{ERT2} or SMC CYB5R3 KO - Supplemental Figure 1C, Table 1). Cyb5r3\textsuperscript{wt/wt}Myh11-Cre\textsubscript{ERT2} or WT mice from this breeding scheme were used as control animals. Both WT and SMC
CYB5R3 KO mice were subjected to the same tamoxifen dose regimen prior to any analysis described herein. Mice were between 12-24 weeks at the completion of experiments. The exception to this is 1 SMC CYB5R3 KO mouse in the 7 day Ang-II trichrome analysis and 2 SMC CYB5R3 KO mice in the 28 day Ang-II trichrome analysis which includes mice at 40 weeks of age at the completions of the experiment.

**Animal harvesting for immunohistochemical analysis.** Mice were euthanized by CO$_2$ asphyxiation and aorta, heart, and mesenteric arteries removed. Tissues were post-fixed overnight in 4% paraformaldehyde (sc-281692, Santa Cruz), paraffin-embedded, and sectioned at 7 µM thickness. Artery sections were deparaffinized with xylene and rehydrated by decreasing 100%-70% ethanol followed by distilled H$_2$O. Sections underwent heat-mediated citric acid-based antigen retrieval (H-3300, Vector Laboratories) for 20 minutes then cooled for 30 minutes at 4°C. Subsequently, sections were blocked with phosphate buffered saline (PBS) containing 10% horse serum (Sigma H1270) for 1 hour at room temperature. Primary antibodies for CYB5R3 (10894-1-AP, Proteintech, 1:100) or sGC-β (160897, Cayman Chemicals, 1:100) in conjunction with platelet and endothelial cell adhesion molecule 1 (PECAM1, sc-1506, Santa Cruz, 1:250) were diluted in PBS with 10% horse serum and incubated on sections overnight at 4°C in a humidity chamber. One section per slide was stained with both rabbit (I-1000, Vector Laboratories) and goat IgG controls (I-5000, Vector Laboratories) matching the concentration of primary antibodies. Sections were then washed in PBS and incubated with the pre-conjugated smooth muscle alpha-actin (ACTA2)-FITC (F3777, Sigma (clone 1A4), 1:500), DAPI (D3571, ThermoFisher Scientific, 1:100), and secondary
antibodies donkey anti-rabbit AlexaFluor 596 (A21207, Invitrogen, 1:250) and donkey anti-goat AlexaFluor 647 (A21447, Invitrogen, 1:250) for 1 hour at room temperature in a humidity chamber. Sections were then washed with PBS and cover slipped with Prolong Gold Antifade with DAPI reagent (P36931, Invitrogen). Arteries were imaged using a Nikon A1 Confocal Laser microscope at 40x magnification with 1096 x 1096 resolution at the Center for Biological Imaging at the University of Pittsburgh. Z-stacks in 1 µM increments were taken for both stained and IgG control sections. Representative images are the maximum intensity projections of the Z-stack images (Supplemental Figures 2A and 3A). In ImageJ, regions of interest were drawn for the maximum intensity projection of ACTA2 (SMC) which represents the vessel media and superimposed onto the maximum intensity projection for CYB5R3 and sGC. The raw integrated intensity per area in ImageJ was then quantified to represent the amount of CYB5R3 or sGC staining in the media (Supplemental Figures 2B-C and 3B-C).

Tissues were also stained with a Masson’s Trichrome Kit (ThermoFisher 87019) according to manufacturer’s protocol to assess tissue fibrosis. For aorta and mesenteric arteries, images were taken on the Olympus Provis 1 microscope at 10x and 40x objectives respectively and medial area quantified using ImagePro Plus V9 software (Supplemental Figures 7-8). For heart tissues, stained sections were imaged using Tissuegnostics Microscope at 20x objective and stitched using TissueQuest Analysis Software (Snake Stitch, 8% overlap). Total tissue area was determined by converting individual images into binary images and measuring area using Fiji software (Supplemental Figure 10).
**Telemetry.** Mice were anesthetized with isoflurane and telemetry units (HDX-10, Data Sciences International (DSI) surgically implanted in the right common carotid artery. Mice were allowed to recover for two weeks from surgery before telemetry units were turned on. Blood pressure was then measured continuously for between 24-48 hours to assess baseline physiological blood pressure (Figure 1, Supplemental Figure 4). Measurements of mean arterial blood pressure, heart rate, systolic pressure, and diastolic pressure were recorded by Ponemah Software (DSI) and exported into Microsoft Excel version 16.16.6 for analysis. For Ang-II studies (Figures 3 and 5, Supplemental Figure 5-6), mice were then anesthetized with isoflurane and osmotic 28-day mini-pumps (Model 1004, Alzet) were inserted subcutaneously between mice shoulders. Ang-II was delivered from the pumps at a rate of 750 ng kg⁻¹ min⁻¹. Blood pressure was monitored continuously immediately after osmotic mini-pump insertion and for the duration of the experiments.

**Mesenteric artery and aorta ex vivo wire myography.** Myography experiments were done similarly to those previous published by our lab (24). In summary, mice were euthanized by CO₂ asphyxiation and mesenteric arteries cleaned, removed, and placed in a physiological salt solution (PSS) containing 0.026 mM EDTA, 119 mM NaCl, 5.5 mM d-glucose, 25 mM NaHCO₃, 4.7 mM KCl, 1.17 mM MgSO₄, 1.18 mM KH₂PO₄, and 2.5 mM CaCl₂. PSS is then set to a pH 7.4, and bubbled with 95% O₂ 5% CO₂ at 37°C. Vessels were placed on the wire myograph (Multiple Myograph Model 620 M, Danish Myotechnology) using a 25 µm wire and allowed to rest for 30 minutes in PSS. After rest, fresh PSS was added for an additional 10 minutes then mesenteric arteries were stretched to a tension equivalent to physiological 80 mmHg of pressure. For aortic rings,
vessels were gradually stretched to a tension of 500 mg. 60mM potassium (K) in PSS (KPSS) is then added to vessels for 5 minutes to constrict the vessels and determine vessel viability. Vessels are then washed thrice with PSS and allowed to rest in PSS for 30 minutes. To test for vessel viability, untreated mice mesenteric arteries and Ang-II treated aortas were treated for 5 min with 60mM potassium (K) in PSS (KPSS) while Ang-II treated mesenteric arteries were given increasing doses of constrictor U46619 \((10^{-7} - 10^{-6} \text{ M})\), 16450, Cayman Chemicals) at 5-minute intervals until vessels reached maximal constriction. The vessels were then washed thrice with PSS and allowed to rest in PSS for an additional 30 minutes. Untreated and Ang-II treated mesenteric arteries were then constricted with \(10^{-7} - 10^{-6} \text{ M}\) U46619 and Ang-II treated aortas given \(10^{-6} - 10^{-5} \text{ M}\) phenylephrine (PE) in 5 minute intervals to induce maximal constriction prior to vasodilator treatment. After vessels reached maximal constriction, aorta and mesenteric arteries were given cumulative increasing doses of the following vasodilator drugs in 5-minute increments: acetylcholine \((10^{-8} - 10^{-4} \text{ M}, \text{A6625, Sigma})\), sodium nitroprusside (SNP, \(10^{-9} - 10^{-4} \text{ M}, \text{71778, Sigma})\), or BAY 58-2667 \((10^{-12} - 10^{-6} \text{ M})\). Vessels were then treated with \(\text{Ca}^{2+}\) free PSS containing 100 \(\mu\text{M}\) SNP to determine maximal dilation.

Myography data was recorded on Lab Chart Software (AD Instruments). The data were normalized to the change in maximal constriction by U46619 or PE to maximal dilation in \(\text{Ca}^{2+}\) free PSS to determine the reported percent relaxation of the vessels (Figures 2 and 4, Supplemental Figure 7).

**cGMP pathway activation quantification.** Intracellular cGMP production activates protein kinase G (PKG) which phosphorylates Serine 239 of vasodilator-stimulated
phosphoprotein (pVASP\textsuperscript{239}); this phosphorylation can serve as a surrogate indicator of cGMP-activation\textsuperscript{(55)}. Mesenteric artery cascades from two separate mice of the same genotype were isolated, pooled, and then pooled material split equally into two separate tubes each containing in 1 mL of a 1-part Lonza Smooth Muscle Growth Medium-2 with SmGM-2 SingleQuot kit containing growth factors and 5\% FBS (Lonza CC-3182) to 9-parts solely Lonza Smooth Muscle Growth Medium-2 and were put at 5\% CO\textsubscript{2} at 37°C. For \textbf{Supplemental Figure 6}, the n=3 reported therefore represents the equivalent of 6 WT and 6 SMC CYB5R3 KO mice total used for the experiment. Vessels from both tubes were then treated with 100 µM 3-isobutyl-1 methylxanthine (IBMX) for 15 minutes to inhibit phosphodiesterase activity. Vessels are subsequently either left untreated or treated with 100 µM NO-donor diethylamine NONOate (DEA-NONOate) for 30 minutes. Vessels are then washed thrice with phosphate buffered saline (PBS) then placed in 1 X Cell Lysis buffer (Cell Signaling, 9803) containing protease (Sigma, P8340) and phosphatase (Sigma, P5726) inhibitors and lysed. Tissue protein levels were then quantified. For analysis of protein phosphorylation levels by Western blot, lysates were boiled and run on a 4-12\% NuPAGE Bis-Tris gels (Invitrogen, NP0335BOX). Proteins were then transferred to a nitrocellulose membranes (LiCOR, 926-31092) and blocked for 1 hour in 1 part LiCOR Odyssey blocking buffer (LiCOR Biosciences, 927-40000) to 1 part PBS at room temperature. Membranes were then probed for pVASP\textsuperscript{239} (Cell Signaling 3114S) and beta-actin (Santa Cruz sc-47778) overnight in 1 part LiCOR Odyssey blocking buffer to 1 part PBS with 0.0625\% tween 20 at 4°C. Membranes were washed with PBS and 0.05\% tween 20 and then incubated with secondary antibodies (1:15,000 LiCOR IRDye 680RD 926-68072; LiCOR IRDye 800CW: 926-32213) for 1 hour.
at room temperature. Membranes were washed and imaged using a LiCOR Odyssey with bands quantified using Image Studio Lite software.

**Acute intraperitoneal (I.P.) injections of BAY 58-2667 in vivo.** Following telemetry implantation and Ang-II treatment (750ng kg⁻¹ min⁻¹) via osmotic minipump, WT (n=4) and SMC CYB5R3 KO mice (n=4) were briefly anesthetized (< 2 minutes per mouse) with isoflurane and intraperitoneally (I.P.) injected at 1 dose per day of increasing concentrations of 10⁻⁷ - 10⁻³ M BAY 58-2667. With this design, we rationalized we could mimic our ex vivo myography experiments in vivo. Mice were briefly anesthetized with isoflurane for each I.P. injection to minimize stress induced variation in blood pressure in the animals. Injections began at Day 20 of Ang-II treatment with the 10⁻⁷ M BAY 58-2667 dose given. As blood pressure was measured continuously, we were able to assess the impact of BAY 58-2667 injection at each dose per day. We found that the 10⁻⁴ M and subsequent 10⁻³ M doses of BAY 58-2667 at Days 23 and 24 of Ang-II treatment respectively led to a sufficient reduction in mean arterial pressure and increase in heart rate at 30 minutes post-injection in WT mice suggesting the drug was efficacious at these doses (**Figure 5, Supplemental Figure 11**). We then extrapolated that these 10⁻⁴ M and 10⁻³ M doses of BAY 58-2667 given I.P. were equivalent to 0.04 and 0.4 mg/kg BAY 58-2667 in our mice. The time to briefly anesthetize and I.P. inject the 8 animals (4 WT and 4 SMC CYB5R3 KO) used in this study averaged 15 minutes. To analyze if blood pressure and heart rate changed as a consequence of acute doses of BAY 58-2667, blood pressure and heart rate parameters were averaged across 15-minute intervals and
normalized to the readings taken 15 minutes prior to the start of injection (Figure 5 and Supplemental Figures 11).

**Statistics.** Statistical analyses were done using Graphpad Prism Software version 7.0d. For telemetry experiments, data normality was assessed and analyzed by two-way repeated measures ANOVA with post-hoc Sidak’s multiple comparisons test (Figures 1B-C, 3B-E, and 5B-E, and Supplemental Figures 4A-B, 5A-D, and 6A-D). For ex vivo myography, two-way ANOVA analyses were conducted for vasodilator dose-response treatments with P values representing significance across genotype and *P<0.05 representing significance by post-hoc Sidak multiple comparison test (Figures 2C-E and 4C-E, Supplemental Figure 7C-E). For the vasoconstrictor responses, immunohistochemical staining, and Western blot analyses, data normality was assessed by Shapiro-Wilk test and analyzed by unpaired two-tailed t-test, $unpaired$ two-tailed t-test with Welch’s correction, or $^\text{Mann Whitney U}$ test based on normality results (Figures 2B and 4B, Supplemental Figures 2B-C, 3B-C, 6B, 8B, 9B, and 10B).

**Study Approval.** All animal studies were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (Protocol #17019505).

**Author Contributions**

B.G.D, S.A.H., and A.C.S. conceived and designed experiments. B.G.D prepared tissues for histology and performed immunofluorescence staining and analysis. S.A.H performed ex vivo myography and telemetry experiments with both B.G.D and S.A.H analyzing data.

Acknowledgments

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References


Figure 1: SMC CYB5R3 KO mice have a significantly elevated mean arterial pressure and reduced heart rate compared to WT mice under physiological conditions. A) Schematic of the experimental design where WT (n=10, gray) and SMC CYB5R3 KO (n=10, red) mice post-tamoxifen treatment were implanted with telemetry units and blood pressure monitored continuously for 24 hours. B) SMC CYB5R3 KO mice have a significantly increased mean arterial pressure compared to WT mice. C) SMC CYB5R3 KO mice averaged a significantly decreased heart rate compared to WT mice. B-C) P values represent significant differences between WT and SMC CYB5R3 KO by two-way repeated measures ANOVA with *P<0.05 by Sidak multiple comparison tests. Error bars represent SEM.
Figure 2: SMC CYB5R3 KO results in an acetylcholine-dependent impairment of vasodilation under physiological conditions. A) Experimental design showing that mesenteric arteries from WT (n=5-7) and SMC CYB5R3 KO (n=7-8) mice were subjected to ex vivo wire myography to assess vasoreactivity. B) There are no differences in WT and SMC CYB5R3 KO response to U46619-mediated vasoconstriction. P values determined by unpaired two-tailed t-test. Error bars are SEM. C) WT mesenteric arteries had a significantly increased responsiveness to acetylcholine compared to acetycholine compared to SMC CYB5R3 KO mesenteric arteries. D) No significant differences were seen between WT and SMC CYB5R3 KO responsiveness to the vasodilator SNP. E) SMC CYB5R3 KO mice are less responsive to BAY 58-2667 compared to WT. C-E) P values represent statistical differences between WT and SMC CYB5R3 KO by two-way ANOVA with * representing P<0.05 by post-hoc Sidak multiple comparison tests. Error bars are SEM.
Figure 3: SMC CYB5R3 KO mice have an exacerbated hypertensive response to Angiotensin-II (Ang-II) compared to WT mice. A) Experimental design schematic showing WT (n=8, gray) and SMC CYB5R3 KO (n=8, red) mice were implanted with telemetry units then subsequently with osmotic pumps delivering 750ng kg⁻¹ min⁻¹ Ang-II. Blood pressure (BP) was measured continuously 24-hours prior to and for the duration of Ang-II treatment. B) Mean arterial pressure (mmHg) and C) heart rate (beats per minute - bpm) averaged and plotted per day for the course of 14 days shows SMC CYB5R3 KO mice have an increased mean arterial pressure and decreased heart rate as compared to WT particularly during the first 7 days of Ang-II treatment. Shaded regions highlight Days 6-7 of Ang-II where peak differences were observed between WT and SMC CYB5R3 KO. D) The hourly mean arterial pressure (mmHg) recording and E) heart rate for the shaded regions in B-C for the 24-hours between Days 6-7 of Ang-II treatment. A-E) The P value represents a difference across genotype by two-way repeated measures ANOVA with * representing P values < 0.05 as determined by post-hoc Sidak multiple comparison tests. B-E) Error bars are SEM.
Figure 4: SMC CYB5R3 acts as a sGC heme reductase in the context of Ang-II induced systemic hypertension.
A) Schematic of ex vivo wire myography experiments on mesenteric arteries from WT (n=9-12, gray) and SMC CYB5R3 KO mice (n=10-12, red) after 14 days Ang-II (750ng kg⁻¹min⁻¹) treatment. B) SMC CYB5R3 KO mice have increased U46619-mediated vasoconstriction than WT mice. P values determined by two-tailed, unpaired t-test. C) SMC CYB5R3 KO mice are significantly less responsive to acetylcholine than WT mice. D) WT mice are more responsive to vasodilator sodium nitroprusside (SNP) than SMC CYB5R3 KO mice. E) SMC CYB5R3 KO mice are more responsive than WT mice to BAY 58-2667. C-E) P values represent statistical differences between WT and SMC CYB5R3 KO by two-way ANOVA with *P<0.05 representing significance by post-hoc Sidak multiple comparison tests. Error bars are SEM.
Figure 5: Ang-II treated SMC CYB5R3 KO mice are more responsive to acute BAY 58-2667 induced vasodilation than WT controls. A) Schematic of experimental design with Ang-II treated WT (n=4, gray) and SMC CYB5R3 KO (n=4, red) mice receiving intraperitoneal (I.P.) injections of BAY 58-2667 (1 dose/day) and changes in blood pressure (BP) measured. B) Acute injection (time 0 minutes, purple arrow) with 0.04 mg/kg BAY 58-2667 resulted in no significant differences in change in mean arterial pressure between WT and SMC CYB5R3 KO mice. C) 0.04 mg/kg BAY 58-2667 injection (time 0 minutes, purple arrow) results in a significant increase in change in heart rate in WT mice over SMC CYB5R3 KO at 15 minutes post injection. D) The higher 0.4 mg/kg BAY 58-2667 dose (time 0 minutes, purple arrow) resulted in a significant reduction in the change in mean arterial pressure in SMC CYB5R3 KO compared to WT mice post injection indicating SMC CYB5R3 KO mice are more sensitive to BAY 58-2667 blood pressure lowering effects. E) Both WT and SMC CYB5R3 KO mice had an elevated change in heart rate after 0.4 mg/kg BAY 58-2667 injection (time 0 min, purple arrow) though no differences in heart rate occurred between groups. B-E) P value represents significant difference between WT and SMC CYB5R3 KO by two-way repeated measures ANOVA with * representing P value < 0.05 by post-hoc Sidak multiple comparison tests. Error bars are SEM.
Figure 6: Schematic of CYB5R3 acting as a sGC reductase in systemic hypertension. In normotension, NO binds to reduced heme sGC in SMC to induce vasodilation. Ang-II causes systemic hypertension as well as oxidation and loss of the sGC heme rendering it insensitive to NO. CYB5R3 confers resilience to systemic hypertension by reducing the oxidized sGC heme back to its reduced state.
Table 1: Primers for genotyping *Cyb5r3* mice

<table>
<thead>
<tr>
<th></th>
<th>Forward (5’ – 3’)</th>
<th>Reverse (5’ – 3’)</th>
<th>*Cyb5r3^fl/fl^</th>
<th>*Cyb5r3^wt/wt^</th>
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<tr>
<td></td>
<td>GCAGCCTCAGGACTGTTTCT</td>
<td>TTGACCCTCTGCTGAACTG</td>
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<td>360 bp</td>
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bp indicates base pairs
Table 2: Baseline hematology of WT and SMC CYB5R3 KO mice.

<table>
<thead>
<tr>
<th></th>
<th>WT (n=7)</th>
<th>SMC CYB5R3 KO (n=8)</th>
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<tbody>
<tr>
<td>White Blood Cells</td>
<td>6.37 ± 0.58</td>
<td>5.95 ± 0.58</td>
<td>0.56</td>
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<td>(x 10^3/µL)</td>
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<tr>
<td>Lymphocytes</td>
<td>4.96 ± 0.47</td>
<td>4.96 ± 0.40</td>
<td>0.99</td>
</tr>
<tr>
<td>(x 10^3/µL)</td>
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<tr>
<td>Monocytes</td>
<td>0.69 ± 0.06</td>
<td>0.46 ± 0.05</td>
<td>^0.03</td>
</tr>
<tr>
<td>(x 10^3/µL)</td>
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</tr>
<tr>
<td>Granulocytes</td>
<td>0.73 ± 0.11</td>
<td>0.51 ± 0.05</td>
<td>0.1</td>
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<td>(x 10^3/µL)</td>
<td></td>
<td></td>
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<tr>
<td>Hematocrit</td>
<td>36.71 ± 0.95</td>
<td>36.28 ± 0.52</td>
<td>0.68</td>
</tr>
<tr>
<td>(%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>13.43 ± 0.33</td>
<td>13.49 ± 0.25</td>
<td>0.92</td>
</tr>
<tr>
<td>(g/dL)</td>
<td></td>
<td></td>
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<tr>
<td>Red Blood Cells</td>
<td>8.39 ± 0.18</td>
<td>8.28 ± 0.13</td>
<td>0.64</td>
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<tr>
<td>(x 10^6/µL)</td>
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<td></td>
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<tr>
<td>Platelets</td>
<td>470.90 ± 21.52</td>
<td>476.90 ± 28.80</td>
<td>0.87</td>
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<tr>
<td>(x 10^3/µL)</td>
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g/dL is grams per deciliter

P values are unpaired two-tailed t-test; ^P value is Mann Whitney U test
Table 3: Mean change between WT and SMC CYB5R3 KO telemetry measurements.

<table>
<thead>
<tr>
<th>SMC CYB5R3 KO&lt;sub&gt;mean&lt;/sub&gt; - WT&lt;sub&gt;mean&lt;/sub&gt;</th>
<th>Untreated</th>
<th>Ang-II (Day 6-7)</th>
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<tbody>
<tr>
<td></td>
<td>Day</td>
<td>Night</td>
</tr>
<tr>
<td>Mean arterial pressure (mmHg)</td>
<td>5.22</td>
<td>6.49</td>
</tr>
<tr>
<td>Heart Rate (bpm)</td>
<td>-31.6</td>
<td>-27.3</td>
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<tr>
<td>Systole (mmHg)</td>
<td>4.9</td>
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<tr>
<td>Diastole (mmHg)</td>
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<td>5.8</td>
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