Pannexin 1 Channels Control the Hemodynamic Response to Hypoxia by Regulating O_2-Sensitive Extracellular ATP in Blood

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Running Title: RBC Panx1 Channels and Hypoxic ATP Export

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Abstract

Pannexin1 (Panx1) channels export ATP and may contribute to increased concentration of the vasodilator ATP in plasma during hypoxia in vivo. We hypothesized that Panx1 channels and associated ATP export contributes to hypoxic vasodilation, a mechanism that facilitates the matching of oxygen delivery to tissue metabolic demand. Male and female mice devoid of Panx1 (Panx1-/-) and wild-type controls (WT) were anesthetized, mechanically ventilated, and instrumented with a carotid artery catheter or femoral artery flow transducer for hemodynamic and plasma ATP monitoring during inhalation of 21% (normoxia) or 10% oxygen (hypoxia). ATP export from WT vs. Panx1-/- erythrocytes (RBC) was determined ex vivo via tonometer experimentation across progressive deoxygenation. Mean arterial pressure (MAP) was similar in Panx1-/- (N=6) and WT (N=6) mice in normoxia, but the decrease in MAP in hypoxia seen in WT was attenuated in Panx1-/- mice (-16±9% vs -2±8%; $P<0.05$). Hindlimb blood flow (HBF) was significantly lower in Panx1-/- (N=6) vs. WT (N=6) basally, and increased in WT but not Panx1-/- mice during hypoxia (8±6% vs -10±13%; $P<0.05$). Estimation of hindlimb vascular conductance using data from the MAP and HBF experiments showed an average response of 28% for WT vs -9% for Panx1-/- mice. Mean venous plasma ATP during hypoxia was 57% lower in Panx1-/- (N=6) vs WT mice (N=6; $P<0.05$). Mean hypoxia-induced ATP export from RBCs from Panx1-/- mice (N=8) was 82% lower than from WT (N=8; $P<0.05$). Panx1 channels participate in hemodynamic responses consistent with hypoxic vasodilation by regulating hypoxia-sensitive extracellular ATP levels in blood.

Keywords: vasodilation, hypoxia, blood flow, erythrocyte

Abbreviation List

Hb, hemoglobin

MAP, mean arterial blood pressure

Panx1, pannexin 1

RBC, red blood cell

New and Noteworthy

- Export of vasodilator ATP from red blood cells requires pannexin 1.
- Blood plasma ATP elevations in response to hypoxia in mice require pannexin 1.
- Hemodynamic responses to hypoxia are accompanied by increased plasma ATP in mice *in vivo* and require pannexin 1.

Introduction

Appropriate matching of oxygen (O_2) delivery to tissue metabolic demand during acute systemic hypoxia is predominantly mediated through augmented tissue blood flow(48). Net local vasodilation of peripheral tissue beds (e.g. skeletal muscle and the integument) during hypoxia is regularly observed in healthy humans(22, 31, 53), is highly phylogenetically conserved(43) and has been shown to be mediated by multiple mechanisms(34, 53, 60). Despite robust sympathetic activation and elevated α-adrenergic vasoconstrictor tone during hypoxia(49, 64), intraluminal stimulation of type 2 purinergic (P2) receptors along the endothelium by extracellular ATP uniquely elicits both pronounced vasodilation and attenuation of sympathetic vasoconstriction in humans(34, 46, 47).

ATP is a primary ligand for P2 receptors and plasma concentrations appear to increase during hemoglobin deoxygenation conditions, presumably via non-lytic, cellular ATP export(16, 23, 56). One well documented conduit for ATP export is pannexin1 (Panx1), a large, non-junctional nonselective membrane channel allowing small molecule passage between the intracellular compartment and the extracellular milieu(8, 13). Panx1 channels are sensitive to hypoxia(35), and importantly, are present in various cell types (i.e. red blood cells (RBCs), vascular endothelium and vascular smooth muscle cells) that are plausible regulators of both vascular tone and the plasma ATP concentration(20, 36, 58). Indeed, vasodilation develops in a concentration dependent manner in congruence with increases in plasma ATP during simultaneous hemoglobin (Hb) deoxygenation in humans(31), and *ex vivo* pharmacological evidence suggests a role for Panx1 in the regulated export of ATP by RBCs in hypoxia(58, 66).

Presently, there is no mechanistic evidence elucidating the contribution of Panx1 channels to ATP-regulated hypoxic vasodilation *in vivo*. While investigations *in vitro* point toward an active role for RBCs in hypoxia-induced ATP export through Panx1 channels(35, 58), the role of Panx1 channels in response to systemic hypoxia *in vivo* has not been defined. Therefore, we tested the hypothesis that Panx1 channel-mediated increases in plasma ATP is

mechanistically linked to hypoxic vasodilation *in vivo*. To do so, we examined systemic (mean arterial blood pressure) and regional hemodynamics (hindlimb blood flow) in wild-type (WT) and Panx1 null(51) (Panx1$^{-/-}$) mice and changes in plasma ATP in response to hypoxia. RBC-mediated ATP export from Panx1-deficient mice was examined to further elucidate the role of Panx1 to plasma ATP and hypoxic vasodilation.

Methods

Transparency and Openness

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Wild-type and Panx1$^{-/-}$ Mice

Animal procedures and protocols were approved by the Duke University and Durham VA Medical Center Animal Care and Use Committees and conformed to APS and Federal guidelines. Wild-type (WT) (C57/BL6, strain control; N = 26) and Panx1$^{-/-}$ (N = 26) mice, 3-6 months of age and of both genders, were used. Panx1 genotyping was confirmed as described in 2011 in Seminario-Vidal et al.(51) At baseline, the mice do not differ from respective WT controls (including littermates) with respect to fertility, viability of offspring, litter sizes, birth weight, or gross organ appearance. No morphological or cell volume differences were observed between WT and Panx1$^{-/-}$ RBCs using microscopy, hematocrit (HCT) and cell counting techniques(14).

Animal Preparation and Induction of Systemic Hypoxia

We used mechanical ventilation (and general anesthesia) to control minute ventilation while exposing mice to hypoxia, thereby preventing compensatory (and potentially confounding) changes in minute ventilation. Mice were initially anesthetized with 4% isoflurane (100% O_2;

0.5ml/minute flow rate) in an induction chamber. Following induction, mice were transferred onto a 37°C temperature-controlled heating pad, anesthetized with 2% isoflurane via nose-cone inhalation, and monitored for breathing rate and also response to toe pinch to assess the plane of anesthesia. Once adequately anesthetized, tracheostomy surgery was performed and mice were mechanically ventilated at a rate of 140 breaths/minute and a tidal volume of 180µL (Harvard Apparatus (Holliston, MA) MiniVent ventilator; Model 845). During tracheostomy placement and for at least 10 minutes thereafter, 100% O_2 was delivered. Gases for inhalation were mixed via a gas blender (Vyaire Bird Blender 3800 Series, Yorba Linda, CA) and tubing was used to connect gas blend to the air inflow port on the ventilator. Upon stabilization, a normoxic period, breathing 21% O_2 (balance N_2) for 10 minutes, was followed by hypoxia at 10% FiO_2 (balance N_2) for 30 minutes. Gas switching in the blender was confirmed using an in-line O_2 analyzer (MiniOx Oxygen Analyzer), and venous blood gases were sampled from the inferior vena cava at experiment termination and measured (Siemens RapidPoint 405). All mice underwent these same procedures regardless of Protocol assignment. Mice were humanely euthanized by rapid exsanguination and bilateral thoracotomy following all hemodynamic experiments.

Protocol 1: Systemic Hemodynamics

To gain broad insight into whether cardiovascular regulation (i.e. vasodilation) during hypoxia is modulated by Panx1 channels, we first determined mean arterial pressure (MAP) and heart rate (HR) during systemic hypoxia (10% O_2) in WT (N = 6; Male = 4, Female = 2) and $Panx1^{-/-}$ mice (N = 6; Male = 6). To do so, a small incision was made midline in the ventral neck and the left carotid artery exposed, isolated, and cannulated with polyethylene tubing for MAP monitoring via a pressure transducer (AD Instruments, MLT844). Systemic blood pressure MAP was measured continuously, recorded with Windaq or LabChart data acquisition software, binned into 30 second averages across the experiment, and partitioned into 5-minute segments

145 throughout the 30-minute exposure to hypoxia. Heart rate was computed from the beat-to-beat
146 blood pressure waveform over 60 seconds for each 5-minute segment.
147
148 ***Protocol 2: Local Hemodynamics (Hindlimb Blood Flow)***
149 Because changes in MAP can result from central (cardiac output) and/or peripheral
150 (vascular resistance) changes, the purpose of this protocol was to determine regional (hindlimb,
151 femoral artery) blood flow (HBF) during systemic hypoxia in a second group of WT (N = 6; Male
152 = 5, Female = 1) and Panx1$^{-/-}$ mice (N = 6; Male = 5, Female = 1). To do so, a small incision
153 was made distal to the inguinal ligament. The left femoral artery was exposed and dissected
154 free of the femoral vein and nerve, and a flow probe (Transonic 0.5PSB) applied to the vessel
155 segment. Mean blood flow was measured continuously, recorded with LabChart acquisition
156 software, binned into 30 second averages across the experiment, and partitioned into 5-minute
157 segments throughout the 30-minute exposure to hypoxia. Technical limitations precluded the
158 blood flow and blood pressure measurements from being made in the same mice.
159
160 ***Venous Plasma ATP and Hb Measurement***
161 Similar to previous reports(17, 25, 31), a venous whole blood sample of 200 μL was
162 drawn into a EDTA-precoated syringe from the inferior vena cava after 30 minutes of hypoxic
163 (10% O_2) exposure and immediately mixed into 270 μL of ATP stop-solution as described by
164 Gorman and colleagues(23, 25) for subsequent measurement of plasma ATP and Hb. In this
165 setting EDTA acts to inhibit ATPases and also serves as the anticoagulant. Samples were
166 centrifuged at 10,000 *g* at room temperature for 2 minutes to thoroughly remove from the
167 plasma any cells that might otherwise artifactually contribute to the measured plasma ATP, and
168 the supernatant was removed and diluted 2-fold with PBS. The sample was again centrifuged
169 to remove any remaining cells. Plasma ATP was assayed by luciferin-luciferase technique with
170 use of a tube luminometer (Promega 20/20) within 10 minutes from blood acquisition. Plasma

Hb in the same sample was assessed using a FluoStar (BMG LABTECH, Cary, NC) spectrophotometer and used to estimate the % hemolysis and therefore the extent to which ATP could be due to hemolysis(25, 31). Hemolysis correction formulas were established by lysing RBCs from WT (1.95µmol/ g Hb, r^2 = 0.98) and Panx1$^{-/-}$ (2.39µmol/ g Hb, r^2 = 0.98) mice and performing linear regression relating intracellular ATP and Hb concentrations as outlined in the guidelines by Gorman and Fiegl(23, 25). The hematocrit was measured in three animals from each group, averaged, and used in the computation of total plasma ATP.

Ex Vivo: RBC Deoxygenation, Pharmacological Stimulation, and Measurement of Extracellular ATP

The overall purpose of these procedures was to identify the O_2-sensitive ATP release capacity from WT and Panx1$^{-/-}$ mice through examination of RBCs, a cell type well recognized for its ability to export ATP and suggested to be directly involved in hypoxic vasodilation(15, 31, 57). Fresh whole blood (0.5 mL) was collected into a heparinized vial via submandibular cheek bleeding method from WT (N = 14) and Panx1$^{-/-}$ (N = 14) non-anaesthetized mice. Mice serving as blood donors were not used in other experiments. RBCs were isolated by centrifugation (500*g* at 4ºC for 10 min, in order to separate the majority of plasma and leukocytes) and the plasma and buffy coat were removed. Packed RBCs were re-suspended and washed 3 times in excess PSS (mM: 4.7 KCl, 2.0 $CaCl_2$, 1.2 $MgSO_4$, 140.5 NaCl, 21.0 Tris; 5.5 dextrose; with 0.5% BSA; pH 7.4) to remove remaining plasma and any cells remaining in the supernatant. Since RBCs have little or no surface ATPase activity of their own(66), inhibiting ATPases was not required here, and therefore heparin sufficed as the anticoagulant choice in this series. This method of isolation yields a RBC suspension devoid of platelets and less than one leukocyte per 50 high-power fields(31, 55). All studies on isolated RBCs were performed immediately after processing given the known behavioral changes in non-fresh (3 or more hours post-acquisition)

196 RBCs(3, 32, 66). No attempt was made to preserve the low PO_2 of the venous blood obtained
197 during initial sample processing.
198 A 10% RBC suspension (in Krebs buffer) was placed in a rotating bulb tonometer and
199 warmed to 37ºC (Eschweiler GmbH & Co. KG, Germany). To produce a normoxic isocapnic
200 environment, RBCs were exposed to a blend of 21% O_2 and 5.8% CO_2 (balance N_2) gases.
201 Gases were blended via a custom gas blender (MCQ Gas Blender Series 100, Italy), humidified,
202 and introduced into the closed tonometer system. Progressive RBC deoxygenation was
203 induced by lowering O_2 in step-wise fashion to 10%, 5%, and 2.5% for 10 minutes each. Across
204 all conditions, CO_2 was 5.8% and the balance of gas was N_2. Oxygenation was confirmed by
205 blood gas analysis (Siemens Rapid Point 405 Series Automatic Blood Gas System, Los
206 Angeles, CA)(31). The signal transduction linking hypoxia to Panx1-mediated ATP export from
207 RBCs appears to involve a Gi (inhibitory) protein(42). In order to test whether ATP export from
208 $Panx1^{-/-}$ RBCs was intact downstream of the hypoxic stimulus (but upstream of Panx1), we
209 measured responses to either incremental Hb deoxygenation or mastoparan 7, a highly
210 selective activator of the inhibitory G-protein (G_i) that has been implicated in O_2-sensitive ATP
211 export from RBCs(42).
212 ATP was measured via luciferin-luciferase technique, with light emission during the
213 reaction detected by luminometry. A sample of 10% HCT was diluted 250-fold and a 200µL
214 RBC suspension (0.04% HCT) was injected into a cuvette containing 100µL of 10 mg/mL crude
215 firefly tail extract (Sigma) and 100µL of 0.5 mg/mL D-luciferin (RPI) mixed in PBS. Extracellular
216 ATP was normalized to a cell count of 4×10^8 cells/mL. Intracellular ATP was determined by
217 lysing a 50µL sample of RBC suspension (10% HCT) with water and analyzing for ATP and Hb.
218 A standard curve for ATP (Calbiochem) was obtained in RBC suspension for each individual
219 experiment. To confirm that ATP export was not due to hemolysis, RBC suspension aliquots
220 acquired for ATP analysis were analyzed for cell-free Hb and samples in which cell-free Hb was
221 significantly high was excluded from the study(31, 32, 55). We measured absorbance at

wavelengths of 380, 415, and 450nm in order to calculate cell-free Hb as previously described.(24, 26, 31, 32, 38)

Statistics

All values are reported as means ± S.D. Specific hypothesis testing across groups over time was performed using two-way repeated measures ANOVA. All pairwise multiple comparison procedures were via Student-Newman-Keuls Method. Paired t-tests were used where appropriate and indicated, and inter-group comparisons were made with unpaired t-tests. Significance was set at $P< 0.05$ and SigmaPlot was used for statistical analysis.

Results

The Hypotensive Response to Systemic Hypoxia is Blunted in Panx1$^{-/-}$ Mice

Body weights of Panx1$^{-/-}$ mice did not differ significantly from WT littermates, as previously described (mean $\pm$ SD: 27.75 $\pm$ 3.55 vs. 25.83 $\pm$ 4.32 g, respectively).(51) Baseline MAP did not differ significantly between WT and Panx1$^{-/-}$ mice in normoxia (85±3 vs. 82±2 mmHg, respectively; $P>0.05$), nor at the end of catheterization surgery while mice breathed 100% O_2 (86±3 vs. 82±2 mmHg, respectively; $P>0.05$). Figure 1 displays the values averaged over 30 secs and obtained every 5 mins. Upon exposure to hypoxia (10% O_2 FiO_2), MAP was significantly lower than during the normoxic baseline period at each subsequent time point in WT mice ($P<0.05$) but no decrease was observed in Panx1$^{-/-}$ mice (Figure 1A). Additionally, upon transition from normoxia to hypoxia, the change in MAP differed significantly between WT and Panx1$^{-/-}$ mice at all post-baseline time-points (Figure 1B). The percent changes from baseline MAP in response to hypoxia were (values are at minute 5 and after each 5-minute interval through minute 30): -5.3±4.1%, -6.3±3.0%, -11.0±7.4%, -13.7±6.1%, -14.7±8.0%, and -16.2±9.7% in WT mice; and 1.5±5.2%, 0.4±5.7%, 0.6±4.3%, -0.8±4.0%, -1.7±5.8%, and -1.8±8.6% in Panx1$^{-/-}$ mice, progressing from minute 5 to minute 30, respectively. Basal heart

rate did not differ significantly between WT and Panx1$^{-/-}$ mice (646±28 vs 598±58 bpm; *P*>0.05). Heart rate tended to increase progressively in WT mice from the normoxic baseline to the hypoxia exposure (values are at minute 5 and after each 5-minute interval through minute 30): (%Δ = -0.5±6.0%, 1.1±3.4%, 0.7±4.6%, 2.6±8.1%, 5.2±10.4%, 5.8±12.1%, (*P*>0.05; Figure 3A-B), but the change was not statistically significant, and no change (or trend) was observed in Panx1$^{-/-}$ mice during hypoxia (values are at minute 5 and after each 5-minute interval through minute 30): %Δ = -0.8±6.4%, -0.3±7.1%, -0.1±7.9%, -0.2±8.1%, -0.6±9.3%, -3.8±8.8%, (*P*>0.05; Figure 3A-B.

The Hyperemic Response to Systemic Hypoxia is Blunted in Panx1$^{-/-}$ Mice

At baseline, hindlimb blood flow (HBF) measured in a second group of mice was significantly lower in Panx1$^{-/-}$ vs WT mice during normoxia (0.14±0.04ml/min vs 0.19±0.03ml/min; *P*<0.05), and remained lower throughout hypoxia (Figure 2A). In WT mice, hypoxia evoked increases in HBF from normoxic baseline 0.20±0.04ml/min at minute 5*;* (*P*<0.05), 0.20±0.03ml/min at minute 10 (*P*=0.057), (0.20±0.03ml/min at minute 15 (*P*<0.05), 0.21±0.04ml/min at minute 20 (*P*<0.05), 0.20±0.04ml/min at minute 25 (*P*=0.052), and 0.20±0.04ml/min at minute 30 (*P*<0.05, Figure 2A). In contrast, Panx1$^{-/-}$ mice had a reduction in HBF from baseline to hypoxia, reaching statistical significance (*P*<0.05) at minutes 20, 25, and 30 (0.14±0.04ml/min, 0.14±0.03ml/min, 0.14±0.04ml/min, 0.13±0.04ml/min, 0.13±0.04ml/min, 0.13±0.04ml/min, from minute 5 to minute 30, respectively). Furthermore, the effect of hypoxia (%Δ from normoxia) on HBF was significantly different between WT and Panx1$^{-/-}$ mice across the entire trial (Figure 2B). In a second series of experiments in Panx1$^{-/-}$ and littermate controls (N = 5 Panx1$^{+/+}$ and 6 Panx1$^{-/-}$; all male), the hypotensive response to hypoxia was again blunted in Panx1$^{-/-}$ mice, relative to WT littermates (not shown). In order to further probe the hypoxia-mediated influence on vascular tone, we used the MAP and HBF data from the two mouse groups to estimate hindlimb vascular conductance (HVC; i.e. estimation of vasodilation

or vasoconstriction) in response to hypoxia. Figure 4 depicts increases in HVC reaching over 25% in WT mice, yet a contrasting mild decrease in HVC in Panx1$^{-/-}$ mice.

Increase of Venous Plasma ATP during Systemic Hypoxia is Suppressed in Panx1$^{-/-}$ Mice

Plasma levels of ATP were similar in WT (N=4; 95.1±34.2 nM) and Panx1$^{-/-}$ mice (N=4; 88.4±30.8 nM; P>0.05) under normoxic conditions. Blood was sampled after 30 minutes of hypoxia in groups of mice separate from those sampled for normoxia exposure only (N=6 WT and N=6 Panx1$^{-/-}$). After 30 minutes of systemic hypoxia (10% FiO_2; PaO_2 = 47±6 mmHg in WT vs. 44±6 mmHg in Panx1$^{-/-}$), venous plasma ATP was higher in hypoxic WT mice than at baseline, while in Panx1$^{-/-}$ mice plasma ATP was not higher during hypoxia (Figure 5). Plasma ATP in hypoxia was greater in WT (N=6) than Panx1$^{-/-}$ mice (N=6) (166±23 nM vs. 71±6 nM; P<0.05; Figure 5). The difference in the mean plasma ATP between normoxia and hypoxia was +70.4 nM in WT and -17.1 nM in Panx1$^{-/-}$ mice (note: data are unpaired). To determine whether this observation could be simply the result of differences in levels of circulating ATP during the 35-minute experiment, plasma ATP was measured during 30 minutes of normoxia in a separate group of mice from those exposed to hypoxic gases, and plasma ATP did not differ significantly between WT vs. Panx1$^{-/-}$ mice (values noted above for normoxic conditions).

Regulated Export of ATP from RBCs of Panx1$^{-/-}$ Mice is Impaired; P_{50} is Unaltered

Exported ATP from isolated RBCs of WT mice during graded hypoxia was significantly greater than in normoxia (P<0.05). In contrast, no hypoxia-induced increase in ATP was observed from RBCs from Panx1$^{-/-}$ mice (Figure 6A). Moreover, despite a non-significantly lower basal ATP from Panx1$^{-/-}$ RBCs, the ATP export response to hypoxia in Panx1$^{-/-}$ mice was impaired at each level of deoxygenation (Figure 6B). RBC intracellular ATP was similar between WT (N=8) and Panx1 null mice (N=8) (2.03±0.51 vs. 2.31±0.56 μmoles/g Hb; P>0.05). Congruent with responses to the hypoxic stimulus, ATP export from WT RBCs in response to

300 the Gi-protein activator Mastoparan 7 increased by 31%, whereas ATP release from Panx1$^{-/-}$
301 RBCs did not significantly increase in response to Mastoparan 7 (P<0.05) (Figure 7).
302 In the present experiments, RBC lysis rarely exceeded 0.4%, and the predicted [ATP]
303 that would result from this amount of RBC lysis was smaller by an order of magnitude than the
304 differences in supernatant ATP we measured when comparing WT vs. Panx1$^{-/-}$ RBCs in
305 hypoxia. Furthermore, the extent of RBC lysis, as calculated based on the measured cell-free
306 [Hb] detected in the supernatant in response to hypoxia did not differ between WT RBCs (0.250
307 $\pm$ 0.067 % RBC lysis at 5% O_2) vs Panx1$^{-/-}$ RBCs (which displayed weaker ATP export, 0.213 $\pm$
308 0.046 % RBC lysis at 5% O_2). Additionally, cell-free [ATP] and cell-free [Hb] in supernatant in
309 response to deliberate RBC lysis were strongly correlated (r^2 = 0.98), as expected, but cell-free
310 [ATP] correlated very weakly (r^2 = 0.04) with cell-free [Hb] in supernatants from RBC
311 suspensions exposed to hypoxia. Collectively, these observations show a dissociation between
312 cell-free ATP and cell-free Hb during hypoxia and indicate that hemolysis is not the driving
313 factor in the hypoxia-induced appearance of extra-RBC ATP.
314 Hb-O_2 affinity of RBCs from WT and Panx1$^{-/-}$ was equivalent (P_{50} = 45.1 vs. 45.3 mmHg;
315 Figure 8).
316
317 **Discussion**
318 The present investigation suggests that Panx1-deficient mice have an impaired
319 vasodilatory response to hypoxia *in vivo*, a finding associated with lower plasma ATP
320 concentrations *in vivo* and depressed ATP export from RBCs during hypoxia *ex vivo*. The
321 blunted hemodynamic response to hypoxia in Panx1$^{-/-}$ mice was evidenced via the inability to
322 neither effectively lower systemic MAP nor augment regional HBF. Although examined
323 independently, coupling MAP and HBF observations (and computing an estimated hindlimb
324 vascular conductance) supports the notion for poor modulation of O_2 sensitive regulation of
325 vascular tone in Panx1 null mice. Furthermore, attenuated hemodynamic changes in Panx1-

deficient mice during hypoxia could not be explained by differences in heart rate, which was essentially unchanged in both groups of mice. In response to hypoxia, the change in MAP was accompanied by concordant changes in plasma ATP concentrations across WT and Panx1$^{-/-}$ mice, with Panx1 deficient mice demonstrating less change in plasma ATP in hypoxia as compared to WT mice. Reflecting one potential explanation and cellular source for plasma ATP, increases in extracellular ATP generated by Panx1$^{-/-}$ RBCs in hypoxia were significantly lower than those by WT RBCs. The decreased ability of Panx1$^{-/-}$ RBCs to export ATP in hypoxia could not be explained by differences in Hb O_2 affinity, intracellular ATP content, RBC susceptibility to hemolysis in hypoxia (which was low in both WT and Panx1$^{-/-}$ RBCs these experiments), as these did not differ from those of WT RBCs. Indeed, RBC lysis is an important potential confounder when interpreting experiments addressing the determinants of regulated ATP export from RBCs, as we and others have discussed.(28, 33, 37, 52) Furthermore, the Panx1-dependent differences (Panx1$^{-/-}$ vs. WT) in the accumulation of extracellular ATP in response to hypoxia were greater by an order of magnitude than the predicted [ATP] that would result from the small degree of RBC lysis we observed. Taken together, these data point to an important role for intravascular ATP in the control of vascular tone during hypoxemia and for Panx1 channels in physiologically relevant, hemolysis-independent extracellular ATP export.

Hypoxia Elicits Panx1-Sensitive Vasodilation and Plasma ATP Elevation In Vivo

In humans and certain animal species (e.g. canines and rats) hypoxemia elicits regional vasodilation in multiple vascular beds, with associated increases in plasma ATP concentration.(31) Intravascular ATP administration evokes dose sensitive and robust vasodilation in humans(22, 46). Accordingly, we sought to test the hypothesis that Panx1 channel-mediated increases in plasma ATP is mechanistically linked to hypoxic vasodilation *in vivo.* In support of this hypothesis, we observe that Panx1-deficient mice exposed to hypoxia fail to: (1) increase plasma ATP concentration, (2) exhibit the anticipated hypotensive and

352 regional hyperemic responses suggestive of vasodilation, and (3) increase ATP export from
353 RBCs.

354 In the present study, WT mice exhibited a drop in blood pressure during systemic
355 hypoxia, whereas blood pressure in Panx1 null mice did not change significantly. Unlike
356 humans, blood pressure reduction to hypoxia is the expected response in mice.(59) In support
357 of the notion that the hypotension during hypoxia in WT mice was in fact due to peripheral
358 vasodilation, we also observed increases in hindlimb hyperemia in response to hypoxia. Given
359 that reductions in perfusion pressure in the face of increased blood flow equate to an elevated
360 vascular conductance, peripheral vasodilation as indicated by the estimated increases in
361 hindlimb vascular conductance is apparent (Figures 1A, 2A, & 4). The absolute values of
362 femoral blood flow were similar to values previously reported in anesthetized, resting mice(54,
363 62). Interestingly, and in striking contrast to WT mice, a ~10% reduction in baseline hindlimb
364 flow was observed in Panx1$^{-/-}$ mice, raising the possibility that basal (non-hypoxic) Panx1-
365 mediated ATP export may contribute to basal control of peripheral vascular resistance.
366 Whether the differences in baseline HBF might complicate the interpretation of the subsequent
367 hypoxia-induced HBF changes is not known definitively, but we would not expect the baseline
368 differences to weaken or qualify the conclusion that the differences in hypoxia are significant.
369 Notably, the differences in BP and HBF remained significant when expressed as percent
370 change. The time courses of the changes in blood flow and pressure were roughly similar.
371 Isolated RBC microfluidic chamber studies suggest that the release dynamics are quite fast (on
372 the order of milliseconds), therefore ATP export is unlikely to be rate-limiting in the
373 hemodynamic response to hypoxia(19, 61). Differences in the flow and pressure responses
374 between Panx1$^{-/-}$ vs. WT mice were apparent by approximately 5 minutes after the onset of
375 hypoxia. Additionally, there was a nonsignificant trend toward a compensatory increase in heart
376 rate in the WT mice that was not seen in the Panx1$^{-/-}$ mice (Figure 3A-B). Therefore, differences
377 in the (direct, reflexive or mediator-driven) chronotropic response to systemic hypoxia could not

account for the differences in blood pressure to hypoxia in Panx1$^{-/-}$ vs. WT mice. Taken together, these data indicate that the hemodynamic responses during hypoxia consistent with hypoxic vasodilation is in part mediated by Panx1 channels. Whether Panx1 deletion may alter other determinants of blood flow, such as microvascular density, has not been investigated to our knowledge and was not examined in our studies. Panx1 may contribute to hemodynamic responses to other metabolic and pathologic stimuli (metabolic change, hemorrhage), but we focused exclusively on one stimulus, hypoxia, and future work is needed to determine the role of Panx1 more broadly in adaptive vasoregulation.

In response to hypoxia, the change in MAP correlated significantly with changes in plasma ATP concentrations across WT and Panx1$^{-/-}$ mice (r^2 = 0.39, P<0.05), with Panx1-deficient mice demonstrating lower hypoxia-induced changes in plasma ATP as compared to WT littermate mice. These data collectively show for the first time that Panx1 channels mediate an increase in plasma ATP during hypoxia and are mechanistically linked to hypoxic vasodilation *in vivo*. Panx1 channels do conduct ATP (2), in contrast to the cystic fibrosis transmembrane conductance regulator (CFTR) channel, which was once thought to be a candidate as an “ATP channel”.(63) While our animal preparation included mechanical ventilation as a means to regulate respiration during hypoxia, future studies are needed to determine whether Panx1 may contribute to such hemodynamic responses to hypoxia in awake mice, as in our anesthetized mice. In addition, future investigations examining whether the role of Panx1 and ATP on vascular tone might vary by anatomic region (e.g., cerebral blood flow) is warranted. Mouse models and receptor-pharmacologic approaches could inform whether ATP itself or a derivative such as adenosine is ultimately responsible for stimulating vasodilation in response to hypoxia. Nevertheless, we observe clear impairments in systemic and regional hemodynamics and attenuated plasma ATP during hypoxia in whole body Pan1 deficient mice.

RBC Export of ATP is Mediated via Panx1 Channels

Cellular export of ATP can variably increase or decrease vascular tone depending on the cell type, stimulus, and purinergic receptor involvement. For example, phenylephrine-induced vasoconstriction via the alpha-$_{1D}$ (α_{1D}) adrenergic receptor on vascular smooth muscle cells (VSMCs) appears to involve the export of ATP via Panx1(5). Alternatively, intravascular ATP can act as a sympatholytic agent, blunting vasoconstrictor (pressor) responses to adrenergic agonists(30). Luminal ATP can act as an endothelium-dependent vasodilator by stimulating the activity of endothelial nitric oxide synthase and endothelium-dependent hyperpolarization(6, 41). The cellular source(s) contributing to increased plasma ATP during hypoxia have been extensively debated[46]. However, evidence has suggested against roles for sympathetic nerves, or skeletal muscle act as the primary sources for the release of vasodilator plasma ATP during hypoxemia(10, 11, 40), in part due to the physical barrier of the vascular smooth muscle coupled to a high density of membrane-bound ectonucleotidases.(27, 41, 67) Nevertheless, tissue perfusion and the resupply of O_2-carrying blood to the region of low oxygen tension is sufficient to increase intravascular (luminal) ATP (17, 21, 31, 41). Thus, the cellular source of the increased plasma ATP presumably lies within the blood itself or vascular lumen, with red blood cells and endothelial cells being candidates.

Hypoxia appears to promote ATP release from isolated endothelial cells or vascular smooth muscle cells only weakly(29). Therefore, given that RBCs can directly regulate vascular tone and export ATP in response to hypoxia, coupled to the fact that ATP elicits vasodilation capable of overriding sympathetic vasoconstrictor tone, RBCs are a strong candidate contributor to plasma ATP accumulation during hypoxia. Moreover, RBC export of ATP basally and in response to Hb deoxygenation has been previously linked to Panx1, which may serve as a conduit for hypoxic ATP export from RBCs(58). ATP export from RBCs can occur in response to various stimuli, and the underlying mechanisms and responsible signaling elements may vary(33). However, a line of evidence compliments a strong role for Panx1 as one mediator in

the release of ATP from RBCs. For example, we have shown previously that pharmacological inhibitors of Panx1 inhibit *hypoxic* ATP export from human RBCs(66). During non-hypoxic conditions, Panx1 inhibitors also decrease the release of ATP from malaria-infected RBCs basally and in response to a combination of stimuli promoting cAMP formation such as the beta-adrenergic agonist isoproterenol, the adenylate cyclase activator forskolin, and the phosphodiesterase inhibitor papaverine(1). Shear-stress-induced RBC ATP export is also sensitive to Panx1 inhibitors(19). Leal-Denis reported that cAMP agonists promoted a minor but significant release of ATP from WT (but not $Panx1^{-/-}$) murine RBCs attached to poly-D-lysine-coated coverslips(14). Notably, pharmacological inhibitors of Panx1 can have off-target (Panx1-independent) actions, limiting the ability to link their effects with Panx1 function. In this regard, $Panx1^{-/-}$ mice become an important tool to test the role of Panx1 in hypoxia-induced ATP export *in vivo,* as in our study. Consistent with this notion, the presented data indicate that RBCs from Panx1 deficient mice do not strongly export ATP in response to the physiological stimulus of hemoglobin deoxygenation. To this end, lack of export cannot be explained by hemolysis (see Results) nor depletion of intracellular ATP pools, which is several orders of magnitude more concentrated than is plasma ATP(32) and is regenerated via glycolysis during hypoxia(4, 32, 45). Collectively, these findings support the assertion that hypoxia-induced ATP export via Panx1 channels from RBCs may be sufficient to trigger the *in vivo* vasodilatory response to hypoxia.

In hypoxia, the docking of deoxygenated Hb with RBC membrane-resident Band 3 protein (anion exchanger 1 (AE1) is favored, and cytoskeletal ankyrin-dependent RBC deformability increases in concert with increases in RBC glucose uptake and ATP export(9, 18). However, the specific mechanism linking hypoxia to Panx1-mediated ATP export is unknown. The increase in ATP export does not appear to drive the increase in RBC deformability, as we showed previously that the Panx1 inhibitors (and ATP-release inhibitors) carbenoxolone and glibenclamide did not attenuate human RBC deformability(19, 66). Finally, Leal Denis et al.(14)

showed that the ATP release in response to mastoparan 7-induced cell swelling of RBCs was Panx1-dependent. We are not aware of published data on RBCs from Panx1$^{-/-}$ mice examining whether Band 3 binding and downstream effects such as glycolytic flux and glucose transport are modulated.

Plasma ATP in the Context of Other Vasodilatory Mediators

Hypoxic vasodilation is understood to be largely endothelium-dependent as are pharmacologically-elicited ATP-mediated responses(10, 12, 44). That is, both systemic hypoxia *and* direct agonism by intraluminal ATP are mediated in part via endothelium-dependent hyperpolarization, NO, and vasodilating prostaglandins. When acting as an *endothelium-dependent* vasodilator, ATP exported by RBCs is postulated to elicit vasodilation principally via luminal P2Y (likely subtypes 1, 2, and/or 4) purinergic receptors(7). Moreover, ATP-induced vasodilation evokes an ascending vasodilatory response upstream from the longitudinal site of mediator release within the vasculature(50, 65). Because Panx1-mediated export of ATP occurs upstream of endothelial cell stimulation and consequential vasodilation, inhibition of Panx1 would limit ATP movement into the plasma and thus could presumably precede and account for contributions observed by specific downstream mediation from adenosine, nitric oxide, vasodilator prostaglandins, and endothelium-dependent hyperpolarization. RBCs are capable of exporting not only the vasodilator ATP, but also vasodilator S-nitrosothiols (SNOs; nitric oxide derivatives) during hypoxia, and both mediators have been implicated in hypoxic vasodilation and the regulation of blood flow distribution in various model systems. The relative roles of these two mediators remain to be fully parsed experimentally, including their differential roles in micro- vs macrohemodynamic responses to hypoxia or other metabolic signals of tissue demand. RBC-derived SNO, alternatively, acts locally as an *endothelium-independent* vasodilator. ATP-dependent conducted vasodilatory activity may function in tandem with SNO-induced vasodilatory activity, and both mediators are exported by the RBC and exquisitely O_2-

sensitive via direct coupling to Hb saturation/desaturation(39), may fine-tune regional blood flow (re)distribution in accordance with the local O_2 demand.

Experimental Considerations

While we aimed to first examine the hypoxic vasodilatory response to hypoxia in whole body Panx1 null mice, we recognize that in order to unequivocally link RBCs and the (lysis-independent) release of ATP via Panx1 channel to hypoxic vasodilation *in vivo*, cell-specific deletion is desired. Future studies using cell-specific Panx1$^{-/-}$ mice and/or cross-transfusion will aid in deeper understanding for the singular contribution of RBC ATP export to hypoxic vasodilation *in vivo.* Along these lines, once exported into the plasma, ATP may undergo hydrolysis leading to formation of ADP or adenosine, which also act as vasodilators. Whether ATP itself or such a derivative/precursor is the responsible vasodilator agent to hypoxia in the present study cannot be determined from these studies and is worthy of future investigation. Nonetheless, the present study demonstrates that Panx1 coupled with high plasma ATP are at minimum required to observe hypoxic vasodilation.

Lastly, the present investigation did not measure blood pressure and blood flow in the same mice, and as a result vasodilation can only be inferred. Direct measures of vasodilation *in vivo* in mice are challenging outside of nude mouse window model experiments and rely on vascular tone computations. The present experiment sought to prioritize mechanical ventilation and functional organ stability over the potentially confounding responses associated with ventilation, carotid cannulation, vessel excision, and blood sampling. To this end, the data presented minimize reflex changes in hemodynamics due to trauma and provide more stable profile by which to observe singular unperturbed endpoints. Nonetheless, a computation of vascular tone by estimating hindlimb vascular conductance across the two groups of mice suggests modulation of peripheral vasodilation to hypoxia in Panx1 null mice (Figure 4).

Conclusions

In this study, we show a key role for the Panx1 channel in O_2-sensitive plasma ATP accumulation and concomitant hypoxic vasodilation in mice *in vivo*. RBCs from WT, but not Panx1$^{-/-}$ mice exported ATP in proportion to the degree of RBC deoxygenation *ex vivo*. Collectively, these findings reveal the distinct contribution of Panx1 to hypoxic vasodilation, and highlight its role as an O_2-sensitive ATP conduit and controller of vascular tone. These new findings suggest future work examining whether Panx1-mediated ATP export from RBCs may contribute to O_2-sensitive hemodynamic regulation and disturbance, as in anemia or sepsis.

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Conflicts of Interest/Disclosures

The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. Mean arterial blood pressure (MAP) for WT or Panx1$^{-/-}$ mice during a 30-minute hypoxic challenge. Individual values and mean ± SD are shown. **A**, absolute values and **B,** percent change from baseline. * indicates $P < 0.05$ vs. respective baseline. † indicates $P < 0.05$ compared to WT as determined by two-way repeated measures ANOVA with Student-Newman-Keuls post hoc analysis. N = 6 for WT mice (4 males, 2 females) and N = 6 for Panx1$^{-/-}$ mice (6 males). Red shading denotes normoxia (21% FiO_2) and blue shading denotes hypoxia (10% FiO_2).

Figure 2. Hindlimb blood flow (HBF) for WT or Panx1$^{-/-}$ mice during a 30-minute hypoxic challenge. Individual values and mean ± SD are shown. **A,** absolute values and **B,** percent change from baseline. * indicates $P < 0.05$ vs. respective baseline. † indicates $P < 0.05$ compared to WT as determined by two-way repeated measures ANOVA with Student-Newman-Keuls post hoc analysis. N = 6 for WT mice (5 males, 1 females) and N = 6 for Panx1$^{-/-}$ mice (5 males, 1 females). Red shading denotes normoxia (21% FiO_2) and blue shading denotes hypoxia (10% FiO_2).

Figure 3. Heart rate (HR) for WT or Panx1$^{-/-}$ mice during a 30-minute hypoxic challenge. Individual values and mean ± SD are shown. **A,** absolute values and **B,** percent change from baseline. N = 6 for WT mice (4 males, 2 females) and N = 6 for Panx1$^{-/-}$ mice (6 males). Red shading denotes normoxia (21% FiO_2) and blue shading denotes hypoxia (10% FiO_2).

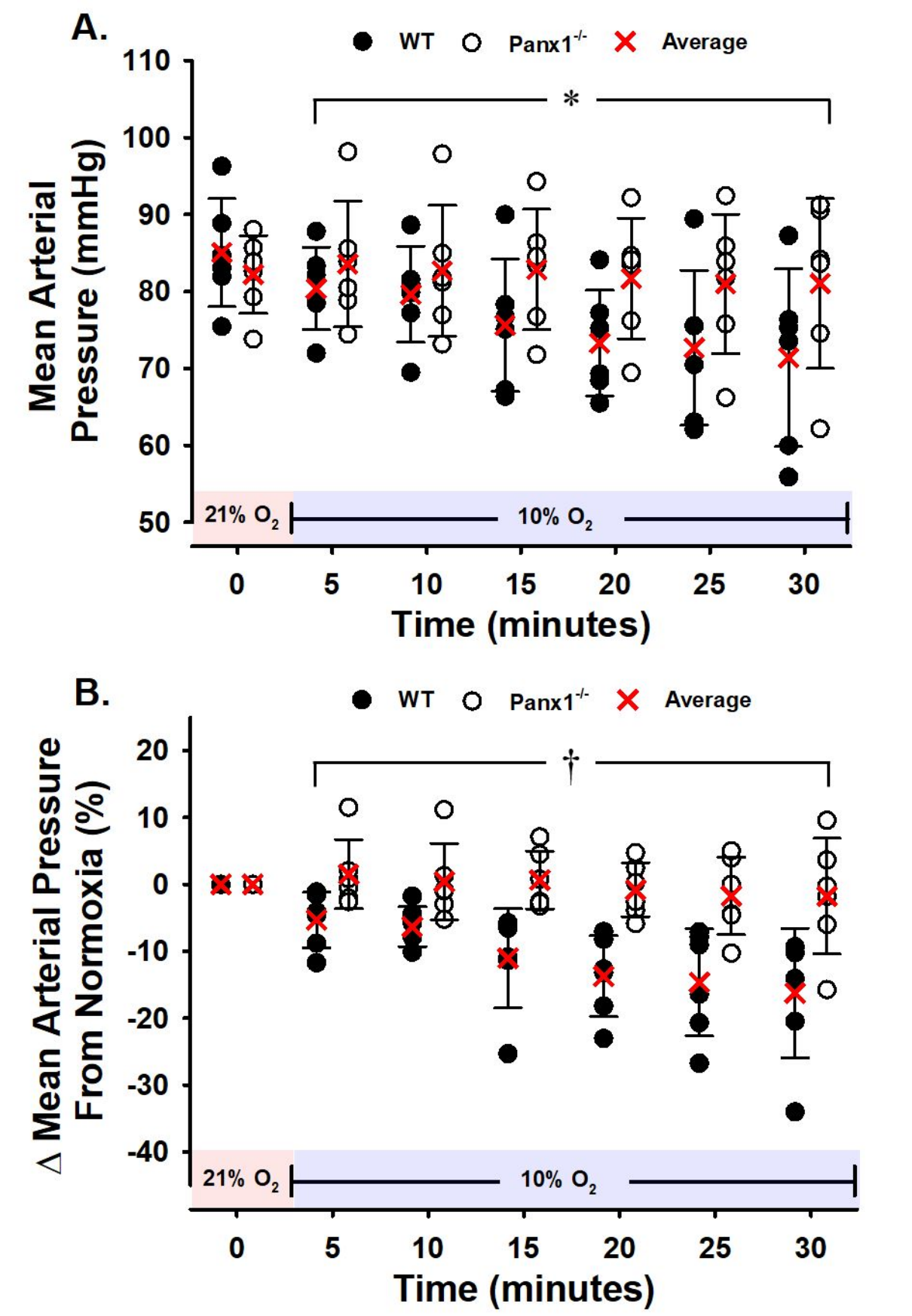
Figure 4. Calculated, estimated hindlimb vascular conductance during a 30-minute hypoxic challenge using MAP from mouse group 1 and HBF from mouse group 2. Hindlimb vascular conductance = (group average HBF / group average MAP) * 100. Values are % change from baseline. N = 12 for WT mice (9 males, 3 females) and N = 12 for Panx1$^{-/-}$ mice (11 males, 1 females). Red shading denotes normoxia (21% FiO_2) and blue shading denotes hypoxia (10% FiO_2).

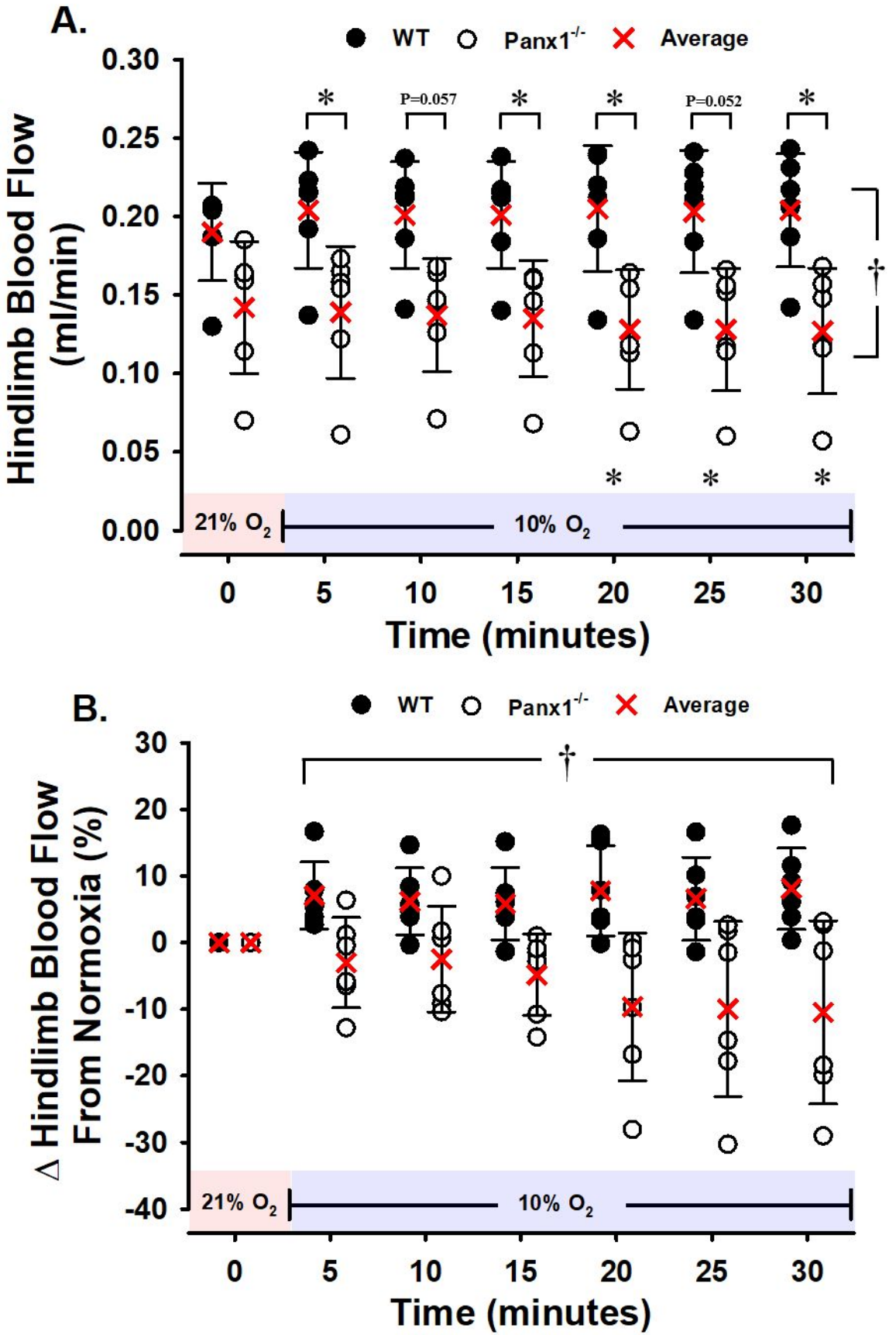
Figure 5. Plasma ATP concentration (nM) for WT or Panx1$^{-/-}$ mice after 30 minutes of breathing either 21% or 10% O_2. Individual values and mean ± SD are shown. † $P < 0.05$ compared to WT as determined by unpaired t-test. For normoxia time control, N = 4 for WT mice (4 males) and N = 4 for Panx1$^{-/-}$ mice (4 males). For hypoxia, N = 6 for WT mice (4 males, 2 females) and N = 6 for Panx1$^{-/-}$ mice (6 males).

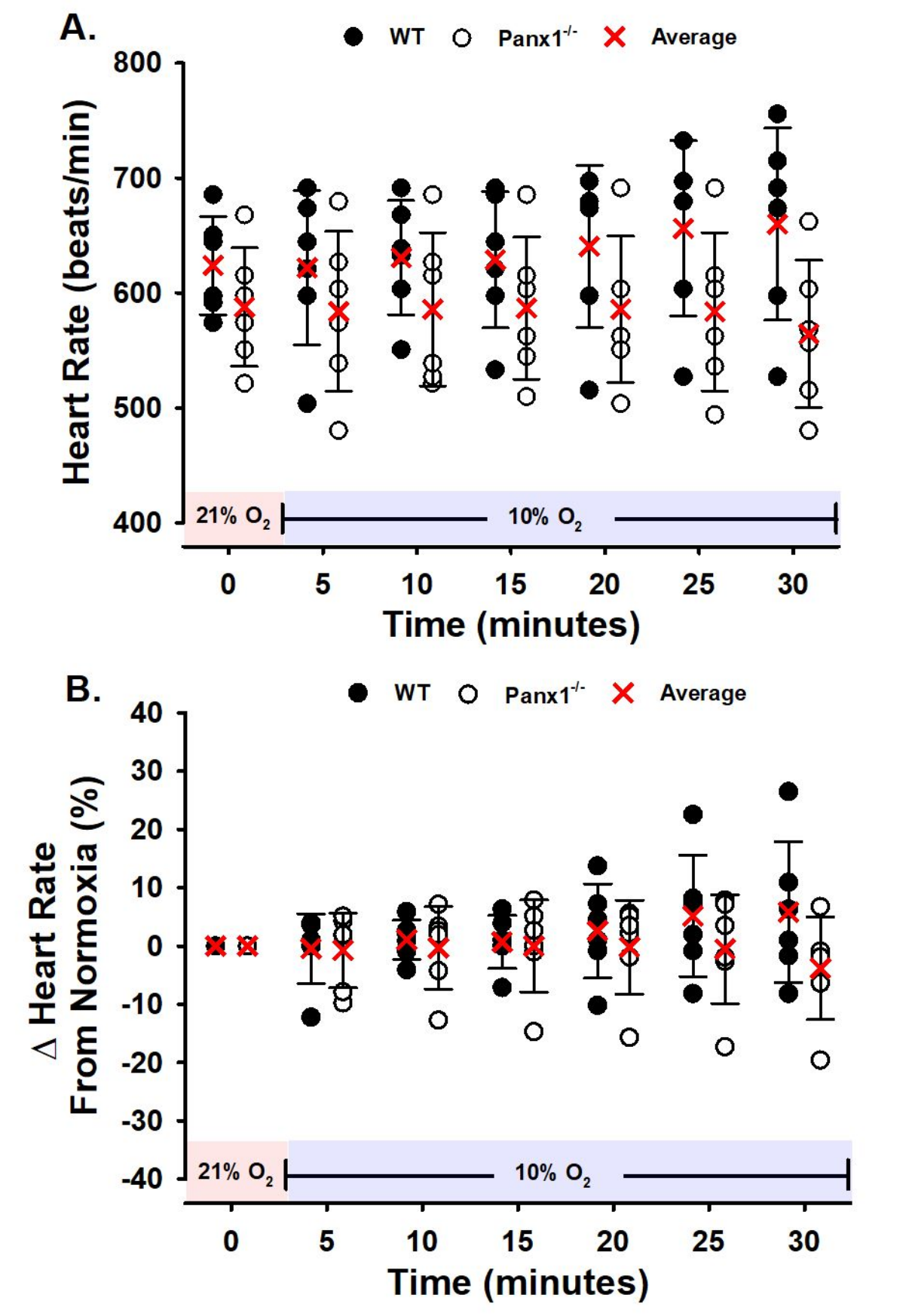
Figure 6. Extracellular ATP increased progressively with the deoxygenation of WT and, to a lesser extent, Panx1$^{-/-}$ RBCs. Individual values and mean ± SD are shown. Absolute values are summarized in **A**. and the percent change from baseline in panel **B**. * indicates $P < 0.05$ vs. respective baseline and † indicates $P < 0.05$ compared to WT as determined by two-way repeated measures ANOVA with Student-Newman-Keuls post hoc analysis. N = 8 for WT mice (6 males, 2 females) and N = 8 for Panx1$^{-/-}$ mice (6 males, 2 females).

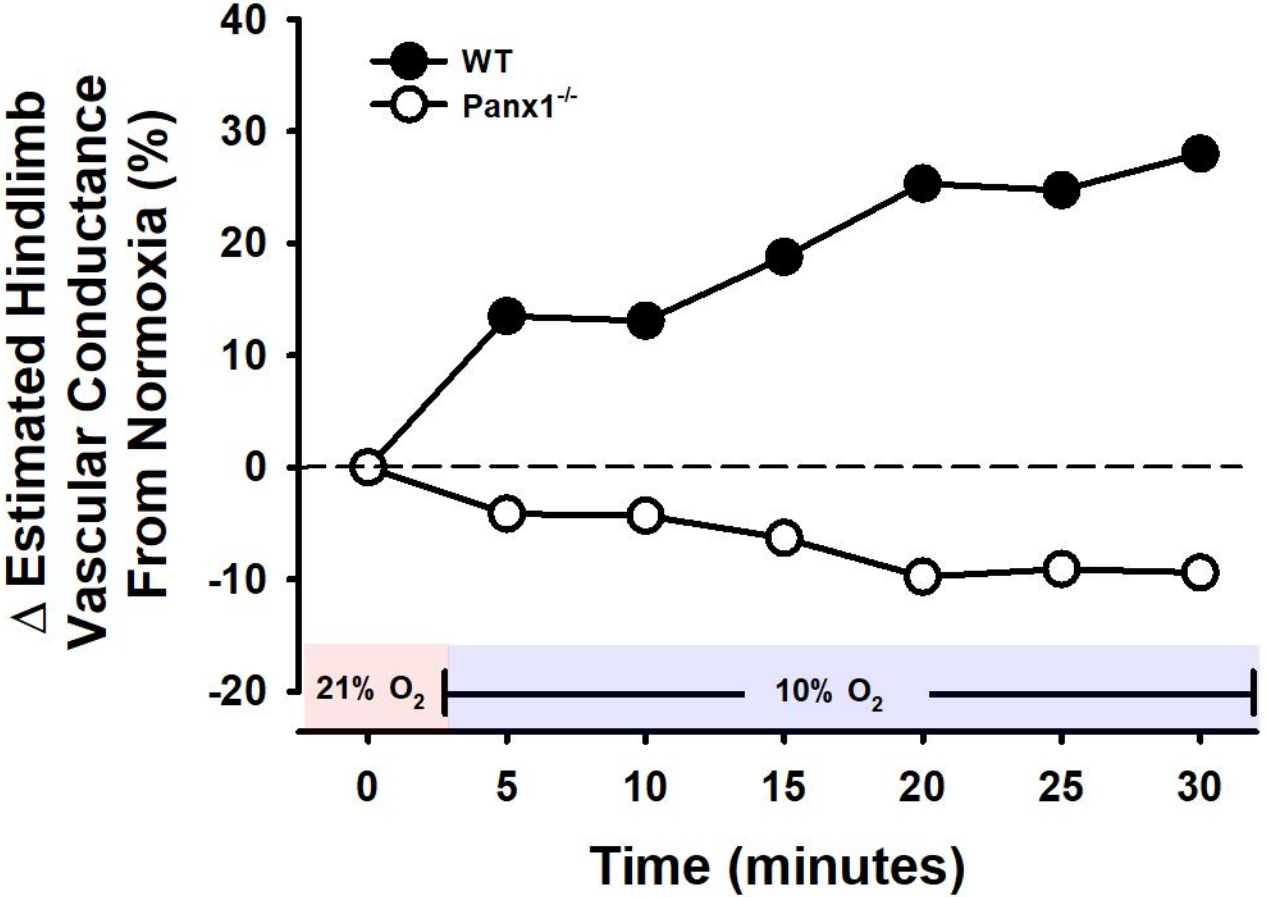
Figure 7. Percent change in extracellular ATP following RBC incubation of RBCs from WT or Panx1$^{-/-}$ mice with mastoparan 7 (10 μM). Individual values and mean ± SD are shown. † indicates $P < 0.05$ compared to WT as determined by unpaired t-test. N = 4 for WT mice (4 males) and N = 4 for Panx1$^{-/-}$ mice (4 males).

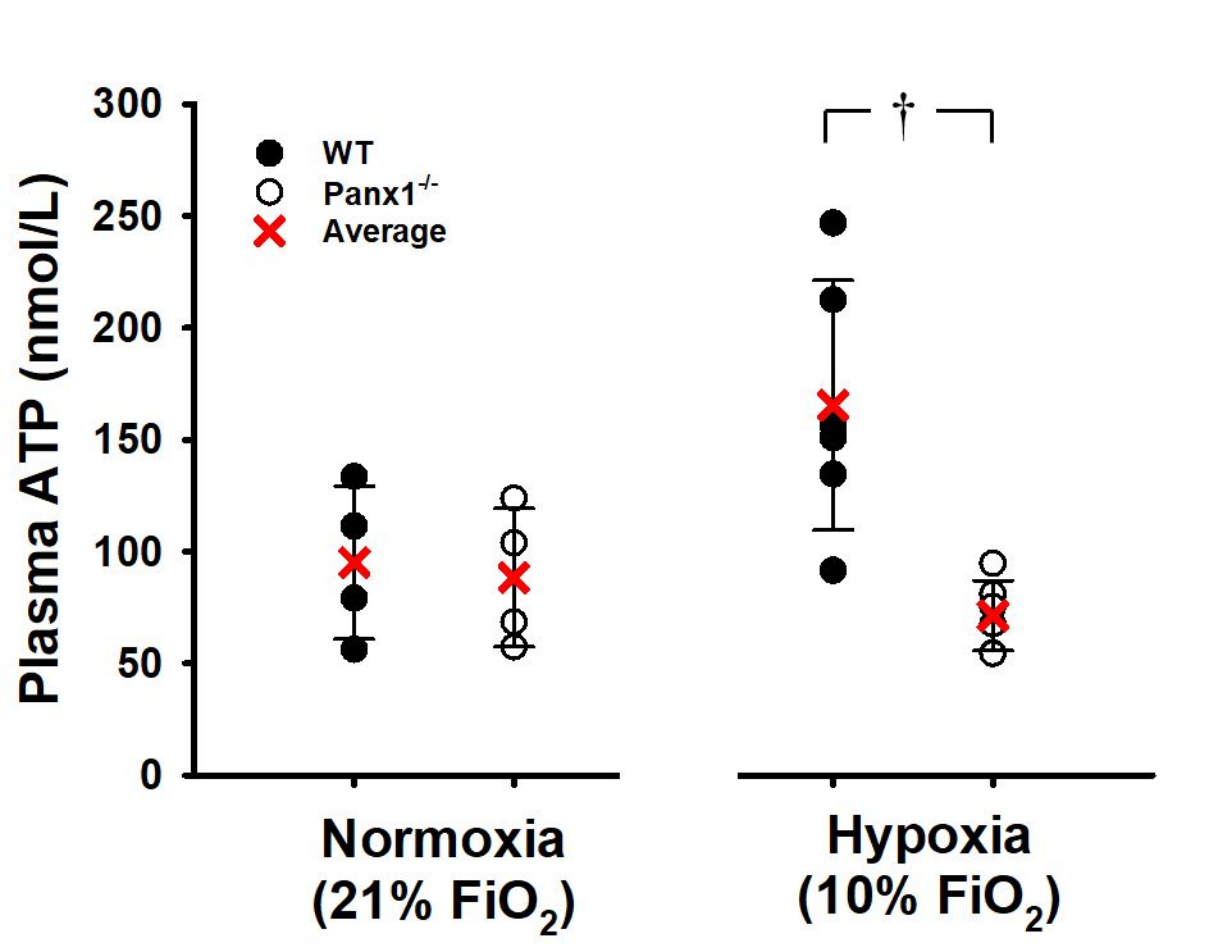
Figure 8. Oxygen binding curves of RBCs from WT (N=6 males) or Panx1$^{-/-}$ mice (N=7 males). The insert shows mean and standard error of P_{50} values. Mean P_{50} values (inset) did not differ significantly as determined by unpaired t-test.

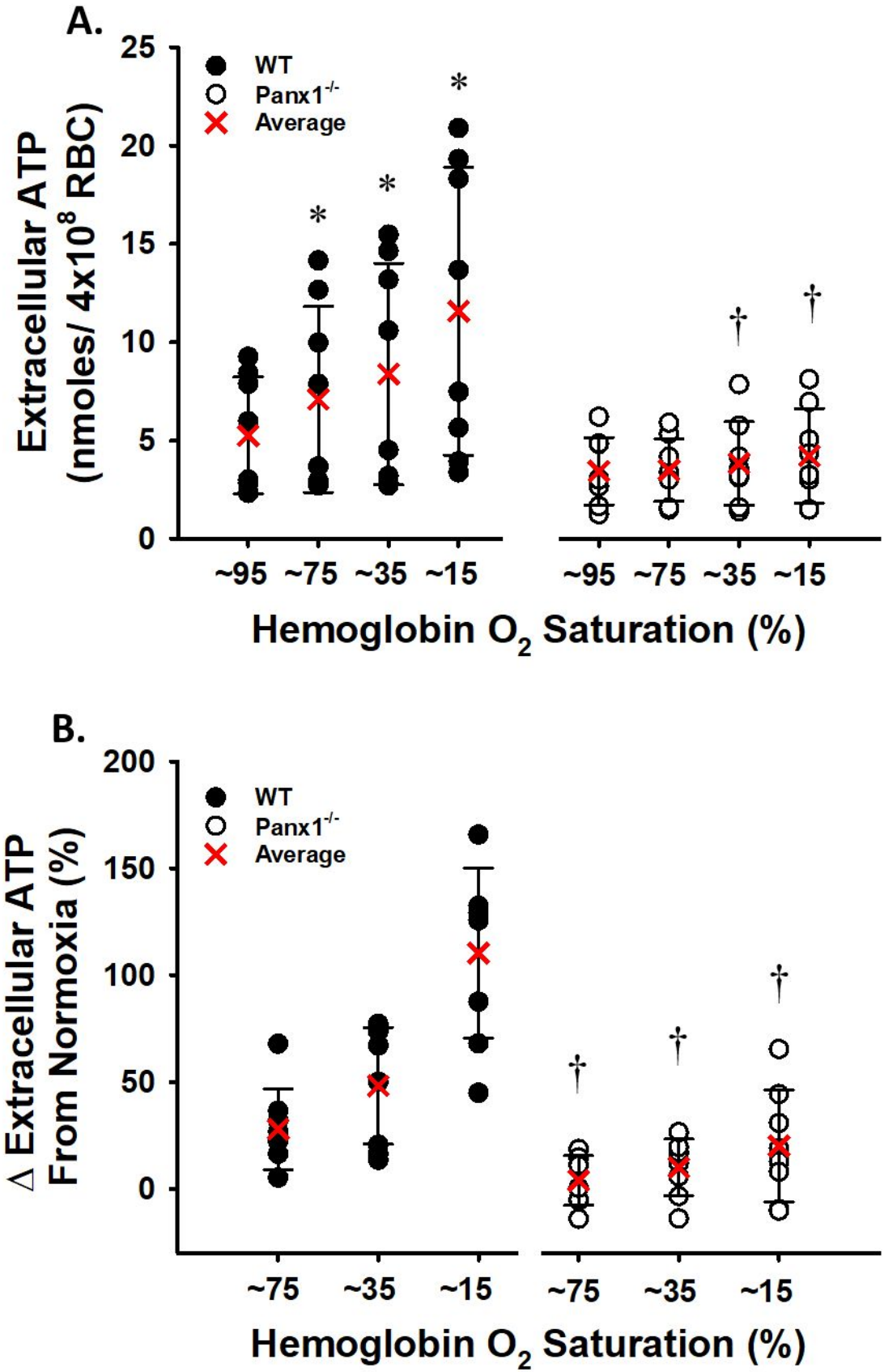

A.
WT
Panx1-/-
Average
*
Mean Arterial Pressure (mmHg)
110
100
90
80
70
60
50
21% O2
10% O2
0
5
10
15
20
25
30
Time (minutes)
B.
WT
Panx1-/-
Average
†
Δ Mean Arterial Pressure From Normoxia (%)
20
10
0
-10
-20
-30
-40
21% O2
10% O2
0
5
10
15
20
25
30
Time (minutes)

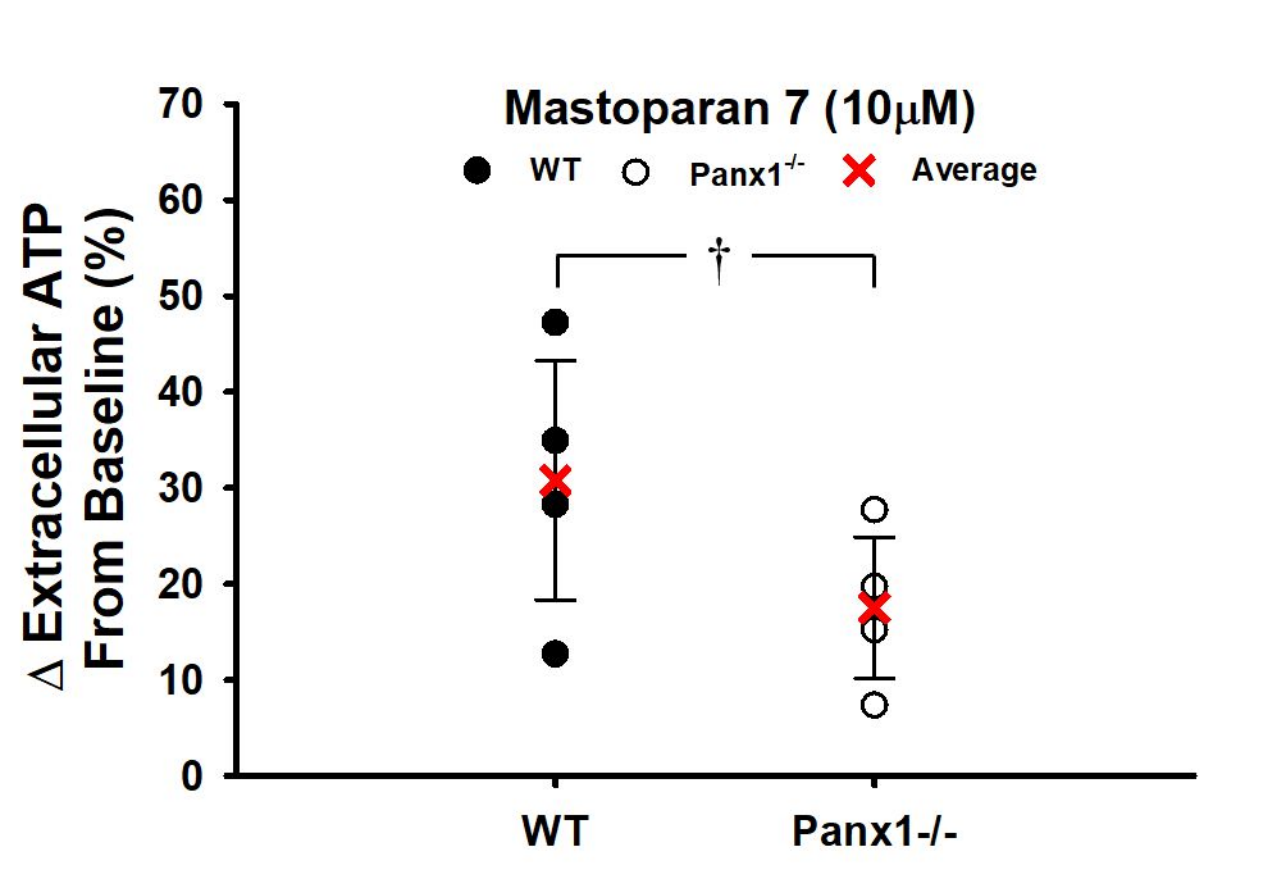

A.
WT
Panx1-/-
Average
Hindlimb Blood Flow (ml/min)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
P=0.057
*
*
P=0.052
*
†
21% O2
10% O2
0
5
10
15
20
25
30
Time (minutes)
B.
WT
Panx1-/-
Average
Δ Hindlimb Blood Flow From Normoxia (%)
30
20
10
0
-10
-20
-30
-40
†
21% O2
10% O2
0
5
10
15
20
25
30
Time (minutes)

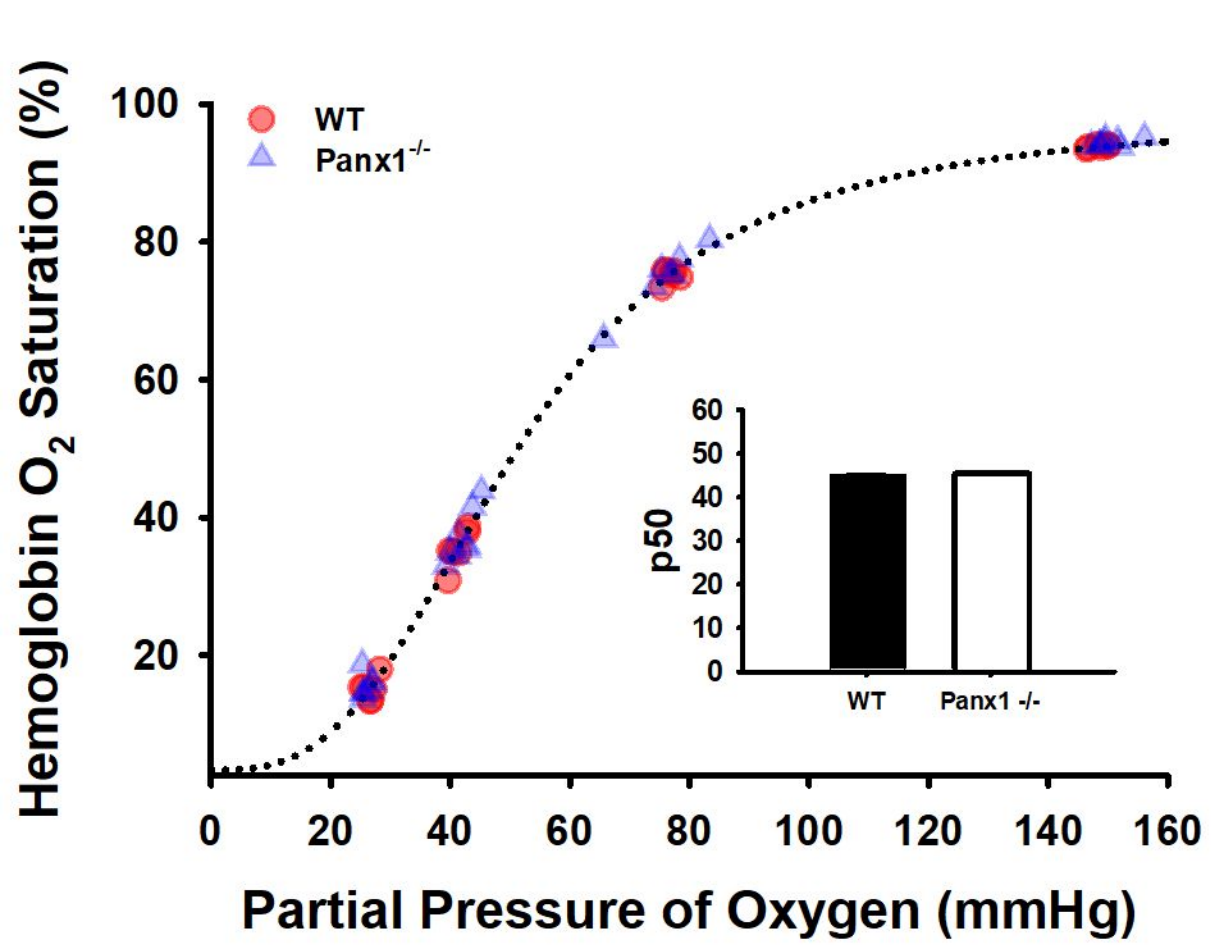

A.
WT
Panx1-/-
Average
Heart Rate (beats/min)
800
700
600
500
400
21% O2
10% O2
0
5
10
15
20
25
30
Time (minutes)
B.
WT
Panx1-/-
Average
Δ Heart Rate From Normoxia (%)
40
30
20
10
0
-10
-20
-30
-40
21% O2
10% O2
0
5
10
15
20
25
30
Time (minutes)


Δ Estimated Hindlimb Vascular Conductance From Normoxia (%)
40
30
20
10
0
-10
-20
WT
Panx1-/-
21% O2
10% O2
0
5
10
15
20
25
30
Time (minutes)


300
250
200
150
100
50
0
Plasma ATP (nmol/L)
WT
Panx1-/-
Average
†
Normoxia
(21% FiO2)
Hypoxia
(10% FiO2)


A.
Extracellular ATP
(nmoles/ 4x10^8 RBC)
25
20
15
10
5
0
WT
Panx1-/-
Average
*
*
*
†
†
~95
~75
~35
~15
~95
~75
~35
~15
Hemoglobin O2 Saturation (%)
B.
Δ Extracellular ATP
From Normoxia (%)
200
150
100
50
0
WT
Panx1-/-
Average
†
†
†
~75
~35
~15
~75
~35
~15
Hemoglobin O2 Saturation (%)


Mastoparan 7 (10μM)
WT
Panx1-/-
Average
†
Δ Extracellular ATP From Baseline (%)
70
60
50
40
30
20
10
0
WT
Panx1-/-


Hemoglobin O_2 Saturation (%)
100
80
60
40
20
WT
Panx1$^{-/-}$
p50
60
50
40
30
20
10
0
WT
Panx1 -/-
0
20
40
60
80
100
120
140
160
Partial Pressure of Oxygen (mmHg)