Vitamin B6 (pyridoxal 5' phosphate) antagonizes carotid body P2X3 receptors in hypertension

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Short title: Vitamin B6 antagonizes P2X3 receptors

Abstract

Aims: ATP acting on P2X3R within carotid bodies (CBs) underpins chemoreflex-mediated sympathetic overactivity in Spontaneously Hypertensive rats (SHR). Pyridoxal 5'phosphate (PLP), the active form of vitamin B6, is reported as being a non-selective P2X receptor blocker. Hence, we hypothesized that PLP antagonism of P2X3R in the CB would treat hypertension.
Methods: Herein, we employed a multipronged approach to investigate PLP's capability to attenuate CB hyperexcitability in hypertension.
Results: First, PLP inhibited Ca^{2+} responses evoked by α,β-methylene ATP in cells lines expressing human (h) P2X3R with IC50 of 8.7μM. Next, *in-silico* data predicted that PLP binds to the same site of Gefapixant, supporting an allosteric antagonism. Using an isolated perfused carotid artery bifurcation-CB preparation, arterial infusion of PLP (50 μM ;15 min) attenuated CBs sensory firing in SHR (P=0.012). Using the in situ working-heart brainstem preparation, carotid artery injections of PLP (1-5mM) attenuated the chemoreflex-evoked sympathetic (P=0.023) but not phrenic (P=0.62) responses; the CB was stimulated with potassium cyanide (KCN,50 μL; 0.04%). In awake telemetered SHR (n=6), intravenous infusion of PLP (48 mg/Kg/h; 30 min) attenuated KCN-evoked chemoreflex responses and reduced systolic, diastolic, and mean blood pressures (ΔMBP = -15.6 mmHg; P=0.025). Translating our results, we performed a small double-blind randomized clinical trial. In volunteers with hypertension (n=14), oral supplementation with pyridoxine hydrochloride (600 mg) attenuated the hypoxic ventilatory response only in patients with high peripheral chemoreflex sensitivity (P=0.021).
Conclusion: Our findings suggest that PLP binds to and antagonizes P2X3R and is a viable candidate for larger clinical trials to treat CB dysregulation in cardiovascular diseases.

Clinical Perspective

What is new?

- This is the first study to demonstrate that blocking P2X3R in humans attenuates their peripheral chemoreflex sensitivity.
- We show for the first time that PLP antagonizes P2X3R in the CB.
- PLP attenuates high peripheral chemoreflex sensitivity in patients with hypertension at therapeutic doses.

What are the clinical implications?

- The findings indicate that supplementation of vitamin B6 is capable of selectively attenuating CB dysregulation in people with hypertension who have a sensitized peripheral chemoreflex index.

Key words: Chemoreflex, hypertension, P2X3 receptors, carotid body, Vitamin B6, Pyridoxal 5' Phosphate

Non-standard Abbreviations and Acronyms

ALP: alkaline phosphatase
ATP: adenosine 5' triphosphate
BP: blood pressure
CB: carotid body
HVR: hypoxic ventilatory response
KCN: potassium cyanide
P2X3R: P2X3 receptors
PaO_2: arterial partial pressure of oxygen
$PaCO_2$: arterial partial pressure of carbon dioxide
PHC: pyridoxine hydrochloride
PLP: pyridoxal 5’ phosphate
SNA: sympathetic nerve activity
WKY: wistar kyoto rats

Introduction

Hypertension affects over 1 billion people worldwide and is the single most important predisposing risk factor for cardiovascular disease *(1)*. There is emerging evidence to suggest that a major contributor to hypertension and sudden cardiac death is an overactive sympathetic nervous system (i.e., fight or flight system) that is not well controlled by current medications *(2)*. Among the proposed causative mechanisms, aberrant carotid body (CB) discharge is linked to exaggerated sympathetic nerve activity (SNA) generation *(3)*.

The CBs are the main peripheral chemoreceptors in the body and are responsible for monitoring the arterial blood mileu including arterial partial pressures of oxygen (PaO_2) and carbon dioxide ($PaCO_2$), and pH *(4)*. CB activation results in increased ventilation and SNA, leading to increases in heart rate, cardiac contractility, vasoconstriction, and ultimately blood pressure *(5)*. Clinical trials in which one CB was surgically resected in patients with drug resistant hypertension validate it for the first time as an effective/potent therapeutic target *(6, 7)*. However, the nature of this intervention was associated with adverse side effects *(6, 8)*. Therefore, an ideal approach would be to reduce CB sensitivity pharmacologically to physiological levels.

Pre-clinical animal work established a critical role of adenosine 5' triphosphate (ATP) as a transmitter for the CB in the transduction of hypoxia *(9, 10)*. In spontaneously hypertensive rats (SHR), upregulation of P2X3 subunit receptors (P2X3R) underpins CB hypertonicity and hyperreflexia *(11)*. Moreover, targeting P2X3R *in vivo* with a highly potent antagonist, such as MK-7264 (i.e., Gefapixant) demonstrated promising results in both hypertension and heart failure *(11, 12)*. Such an approach awaits clinical trials in human patients as P2X3R antagonists are already employed for other indications *(13, 14)*.

Pyridoxal 5'phosphate (PLP), the active form of vitamin B6 (pyridoxine hydrochloride; PHC), was previously suggested to act as a non-selective P2X receptor blocker *(15)*. Moreover, *low* PLP levels in the blood have been associated with increased risk of cardiovascular disease and all-cause mortality in hypertensive patients *(16, 17)*. Rats placed on a PHC-deficient diet for 8 weeks developed sympathetic-mediated hypertension *(16)*. In humans, oral supplementation with PHC reduced blood pressure and circulating catecholamines in hypertensive patients *(18)*; however, in this study there was no placebo group. Hence, we hypothesized that PLP antagonism of P2X3R in the CB would lower blood pressure in hypertension.

Herein we employed *in silico*, *in vitro*, and *in situ* approaches, along with *in vivo* animal models to investigate the capability of PLP to attenuate CB hyperexcitability in hypertension. We also carried out a small double-blind randomized clinical trial to investigate PLP action on peripheral chemoreflex sensitivity in participants with hypertension. Our results suggest that PLP is a viable candidate for a larger clinical trial to treat CB dysregulation in cardiovascular-respiratory diseases.

Materials & Methods

Detailed description of *Materials & Methods* and *Additional Results,* including *Statistical Output*, are provided in Supplemental Materials.

Ethical approval

A total of eight male Wistar rats and forty-two male spontaneously hypertensive rats (SHRs) were bred by the Vernon Jansen Unit (VJU) of the University of Auckland. All tests were performed in accordance with the biomedical research guidelines for animal welfare and were approved by the University of Auckland committee for the ethical use of animals in scientific research (AEC# 2274, #22280 and #25105). All animal procedures performed were in accordance with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Ethical approval for in human study was provided by the Northen A Health and Disability Ethics Committee, Auckland, New Zealand (20/MTA/29), by the Auckland District Health Board Research Review Committee (A+9025) and was registered with the Australian New Zealand Clinical Trials Registry (URL: https://www.anzctr.org.au/; identifier: ACTRN12620001121954). All participants were provided with a comprehensive written and verbal explanation of the study protocols and provided written informed consent prior to participation. The study was conducted according to the Declaration of Helsinki (2013).

Study design:

Six experiments were performed to investigate whether PLP (Fig. 1e, i) is a suitable candidate for treating CB-mediated hypersensitivity in cardiovascular disease. First, in 1321N1 cells stably expressing hP2X2/3R, we investigated whether PLP could inhibit Ca2+ responses evoked by α,β-methylene ATP in vitro. Next, using a crystal structure of the hP2X3 receptor in complex with the negative allosteric modulator MK-7264 (PDB ID 5YVE19), we investigated how PLP may interact on the same negative allosteric binding site as MK-7264 (Fig. 1d, i) via a molecular docking analysis and molecular dynamic simulations. Third, we tested PLP's ability to attenuate CB excitability as measured via carotid sinus nerve (CSN) recordings *in vitro*. Fourth, using an *in situ* preparation, we investigated the effect of PLP on the peripheral chemoreflex motor responses. Fifth, in adult SHRs telemetered for arterial pressure measurement, an intravenous infusion of PLP was carried out to test its ability to both attenuate the peripheral chemoreflex response and lower blood pressure in vivo. We also quantified the level of gene expression in the CB of SHR relative to Wistar rats for the enzymes responsible for the breakdown of PLP. Finally, we carried out a small double-blind, randomized clinical trial to test whether PLP would attenuate peripheral chemoreflex sensitivity in patients with hypertension.

Results

PLP allosterically inhibits recombinant hP2X3R activity in vitro

PLP inhibited α,β-methylene ATP-evoked Ca^{2+} responses in 1321N1 cells stably expressing human P2X2/3R (Fig. 1a) with an IC_{50} of 8.7 ± 0.7 μM (N=5; Fig. 1a and b). Furthermore, PLP significantly increased the α,β-methylene EC_{50} from 433 ± 92 nM to 1706 ± 189 nM (N=5; P<0.01) (i.e., right-shift reducing its potency) and reduced the maximal response to α,β-methylene by 63% (N=5; P<0.01) (i.e. reducing its efficacy), suggesting an allosteric mode of antagonism (Fig. 1c). We have previously characterised the action of the allosteric antagonist MK-7264 at human P2X3 and P2X2/3 receptors *(19)*, which is proposed to exert action through binding an allosteric site created by contacts in the left flipper, lower body and dorsal fin receptor domains *(20)*.

In silico analysis predicts less strong PLP binding to hP2X3's negative allosteric site

The predicted binding of PLP to the same pocket as MK-7264 in the crystal structure of its complex with hP2X3 (PDB ID 5YVE) was evaluated via molecular docking and supports its antagonistic activity. In the crystal structure of its complex with hP2X3R (Fig. 1d), MK-7264 engages with residues defining the site through a network of interactions: a hydrogen bond/electrostatic interaction between the sulphonamide oxygen atoms and the sidechain of Lys176; a hydrogen bond between the pyrimidine ring N-2 position and the backbone NH group of Leu191, and two hydrophobic interactions between the phenyl ring and the carbon atoms of Asp266 (backbone C-α) and Lys176 (sidechain C-β). In addition, the amine group at position 4 of the pyrimidine ring is at H-bond distance from the carbonyl oxygen of the sidechain of Asn190.

Following a molecular docking analysis performed with GlideXP*(21)*, PLP is predicted to bind to the upper portion of the pocket defined by MK-7264 (Fig. 1e), with its phosphate group overlapping the sulphonamide function of MK-7264 (Fig. 1f, i) and its pyridine ring mainly occupying the space defined by the phenyl ring of MK-7264 (Fig. 1f, i). PLP is predicted to make an ionic bond and a hydrogen bond, through its phosphate group, with the sidechain of Lys176. One oxygen atom in the phosphate group is also predicted to form a weaker interaction with the C-α of Pro276. The pyridine ring appears to retain the ability to form a hydrophobic interaction with the sidechain C-β of Lys176, while the methyl group is near the sidechain of Val238, with the potential for an additional hydrophobic interaction. Next, we performed triplicate, 100 ns molecular dynamic simulations of MK-7264 and PLP in complex with hP2X3R, to compare their predicted binding energies to the negative allosteric site. MK-7264 optimised its occupation of the site, maintaining a stable position during the entire simulation. The compound adjusted its orientation in the binding pocket, establishing a network of polar interactions with the residues defining the binding site (i.e., Lys176, Asn190, Leu191, Asp266, Ser267 and Gly277), as summarized in Fig. S5. In the opposite direction, PLP did not assume a stable conformation in the binding pocket, showing variable results in terms of occupation of the binding site across the three simulations performed, consistently moving towards, and

protruding from, the upper part of the allosteric pocket. Although the protein-ligand complex reached stability after about 20 ns, as observed for MK-7264, highly variable binding modes were obtained for this compound, suggesting weaker binding to this site compared to MK-7264. This was supported by the predicted binding energies ($\Delta G_{binding}$), evaluated using the Prime/MM-GBSA calculation method*(22)*, of -52.6 ±4.9 KJ/mol (N=3) and -22.7 ± 9.7 KJ/mol (N=3) for MK-7264 and PLP, respectively.

Figure 1

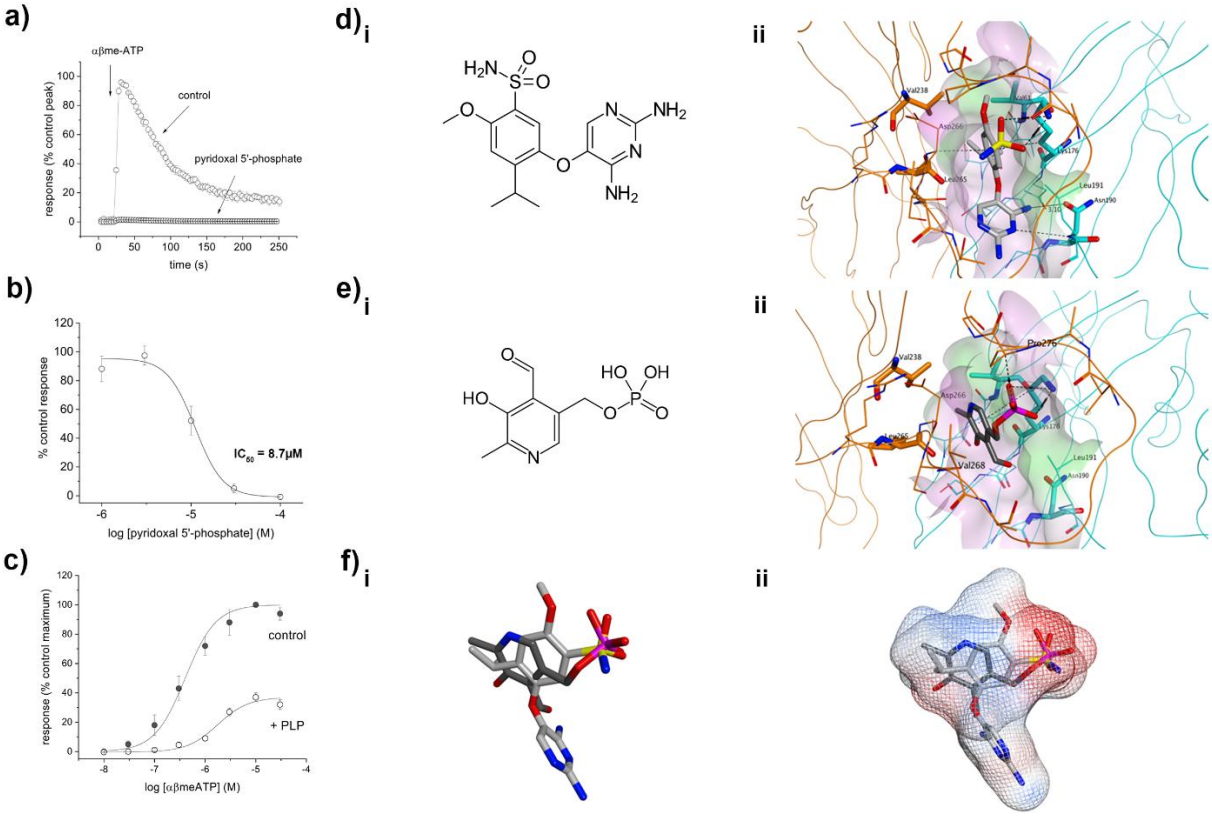

Fig. 1: Pyridoxal 5' phosphate (PLP) vs MK-7264 binding to human P2X2/3 receptor (hP2X3R). a) In cell lines expressing hP2X3R, PLP inhibits Ca^{2+} responses evoked by α,β-methylene ATP (αβme-ATP, 10 μM). **b)** The dose-response curve indicate an IC_{50} of 8.7 μM for PLP. **c**) In pharmacodynamic studies, PLP reduced both the potency and efficacy of αβme-ATP on hP2X3R, revealing an allosteric mode of antagonism. **d) i:** Molecular structure of MK-7264. **ii:** Binding of MK-7264 (carbon atoms in light grey) to the allosteric antagonist site of hP2X3R (PDB ID 5YVE), between the extracellular domains of two subunits (LB from one subunit, LF and LB from the adjacent subunit) (ribbon and carbon atoms in orange and light blue, respectively; third subunit hidden for clarity). The receptor molecular surface defining the site is represented according to its lipophilic/hydrophilic nature (green: lipophilic, pink: hydrophilic, white: neutral). **e) i:** Molecular structure of PLP. **ii:** Predicted binding pose of PLP (carbon atoms in dark grey) to the allosteric antagonist site in the hP2X3R crystal structure (PDB ID 5YVE). The receptor molecular surface defining the site is represented according to its lipophilic/hydrophilic nature (green: lipophilic, pink: hydrophilic, white: neutral). **f)** Superposition between the structure of MK-7264 co-crystallised with hP2X3 (PDB ID 5YVE, carbon atoms in light grey) and the predicted docking pose of PLP to the same site of the 5YVE crystal structure (carbon atoms in dark grey). **i**: ligands represented as simple sticks. **ii:** the ligand electrostatic surface shown for both ligands (blue: positive (partial) charge, red: negative (partial) charge, white: neutral).

PLP infusion suppresses carotid sinus nerve (CSN) firing in SHR

To test functionally whether PLP was capable of antagonizing P2X3R in the peripheral chemoreceptors of SHR, we tested its ability to attenuate CB hypertonicity and hyperreflexia *(11)* via CSN recordings. First, as proof-of-principle, PLP was bolus perfused at a high dose (5 mM; 5 mL) to test whether it would block potassium cyanide-evoked CSN responses (Fig. 2a; KCN, 100 µL, 0.08%); the perfusate was gas-equilibrated with carbogen (5% CO_2 in 95% O_2, PO_2>400 mmHg). PLP strongly suppressed CSN firing evoked by KCN injection (Fig. 2a; ΔCSN= -0.86 µV (95%CI, -1.07 to -0.66); P<0.001, Table S5). In a few instances, the evoked sensory discharge was completely abolished (Fig. 2a). Interestingly, 5 min after the infusion was finished, the effect began to wane (ΔCSN= -0.74 µV (95%CI, -0.95 to -0.53); P<0.001, Table S5). Next, in normoxia (i.e., perfusate PO_2= 90-100 mmHg), continuously intra-arterial infusion of PLP (50 µM; 15 min) significantly attenuated ongoing CBs sensory firing (Fig. 2b; ΔCSN= -11.44 impulse/s (95%CI, -20.8 to -2.11); P=0.022, Table S6, Fig. 2b), indicating its ability to also reduce tonicity, which has been previously demonstrated in SHR *(11)*.

Intra-arterial injections of PLP attenuates the CB-evoked sympathetic reflex in situ

To determine the effect of PLP on the peripheral chemoreflex motor responses, we challenged the CB with KCN whilst recording thoracic sympathetic chain activity (tSNA), phrenic nerve (PN), and ECG (i.e., heart rate - HR) in the *in situ* working heart-brainstem preparation - WHBP*(23, 24)*. Focal injections of PLP (1-5 mM) into the internal carotid artery significantly attenuated the CB-evoked sympathetic reflex ($\Delta tSNA_{PLP}$= -0.12 µV versus control KCN (95%CI, -0.23 to -0.019); P=0.023, Table S7, Fig. 2c). We also observed an attenuating trend in the CB-evoked bradycardia (i.e., P=0.056, Table S8, Fig. $S6_a$), whereas no effect was observed on either chemoreflex evoked PN rate or PN amplitude (i.e., P>0.05, Table S10 and S11). However, PLP significantly attenuated the KCN-evoked increase in neural inspiratory drive (i.e., PN amplitude/ inspiratory time -Δ PN amp/Ti = -0.67 µV/s versus control KCN (95%CI, -1.05 to -0.304); P=0.023, Table S9, Fig. $S6_d$).

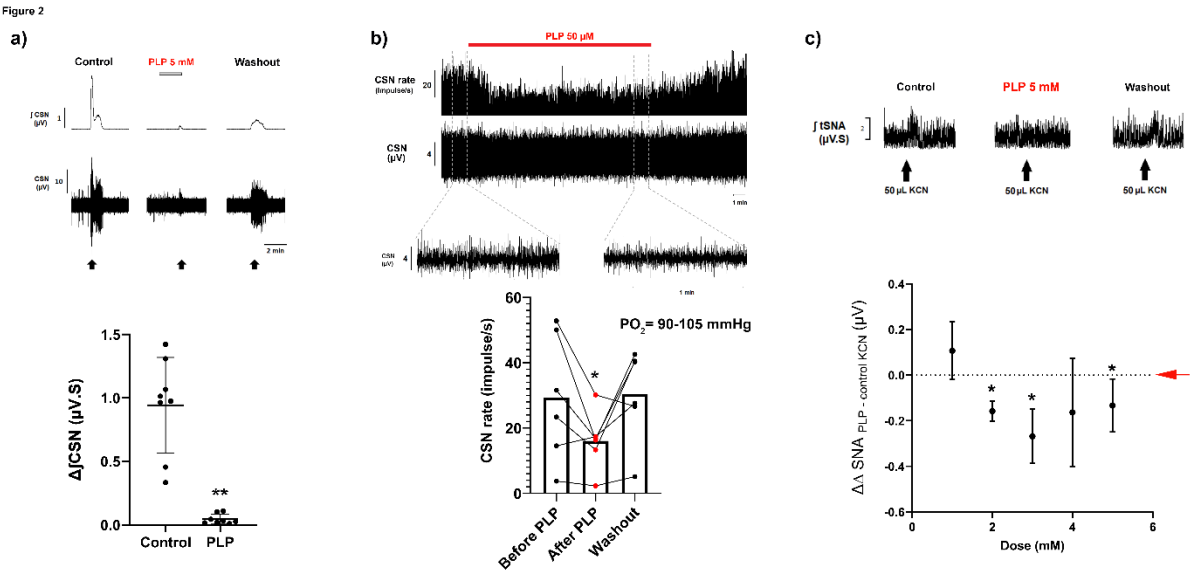

Fig. 2: Effect of Pyridoxal 5'Phosphate (PLP) on carotid body (CB) activity *in vitro* and *in situ*. a) In the isolated perfused carotid artery bifurcation-CB preparation of pre-hypertensive SHR (4-6 weeks, n=8), we used potassium cyanide (KCN; black arrow - 100 µL, 0.08%) to evoke carotid sinus nerve (CSN) responses either in the presence or absence of PLP (5 mL, 5 mM) (i.e., assessment of hyperreflexia). At the top is a typical tracing of raw (CSN) and integrated sinus nerve discharge (∫CSN). **b)** Under normoxia (PO_2 = 90-105 mmHg), PLP (50 µM, 15 min) was continuously infused through the bifurcation to record its effect on resting CSN firing (SHR, 4-6 weeks, n= 6). At the top is a typical tracing of raw CSN and its discharge rate . **c)** In the *in situ* working heart-brainstem preparation, focal injections of PLP (1-5 mM) into the internal carotid artery attenuated the CB-evoked sympathetic chemoreflex (SHR, 4-6 weeks, n= 15). At the top is a typical tracing of integrated thoracic sympathetic chain activity (∫tSNA); data are shown as ΔΔ, which means the difference between the Δ∫tSNA responses from PLP versus the first KCN (i.e., control response). The further the data departs from the dashed line at zero (red arrow), the more attenuated the response. Data were analyzed using a mixed regression model with either linear (i.e., normal) or gamma distribution. Mean ± SD, * P<0.05, **P<0.01.

PLP infusion attenuated the KCN-evoked pressor response in telemetered conscious SHR in vivo.

To test whether PLP would reproduce the above-mentioned results *in vivo*, we evoked chemoreflex responses with bolus injections of KCN -1 µg/µL; 15µg *(25)* in adult telemetered SHR (n=5) and 30µg in Wistar rats (n=5). Ventilation was recorded via the barometric whole-body Plethysmography *(26)*. In SHRs, KCN-evoked pressor response was attenuated during PLP infusion (24 mg/Kg) when compared to control KCN injection (ΔMBP= -48.3 mmHg versus control KCN (95%CI, -73.3 to -23.34), P=0.002, Table S14, Fig. 3_{A3}); whilst neither bradycardia (Δ*HR*= 46 bpm versus control KCN (95%CI, -97 to 188), P=0.48, Table S16, Fig. 3_{A4}) nor tachypnea were significantly affected (Δf_R= -86 breaths/min versus control KCN (95%CI, -180 to 9), P=0.07, Table S15, Fig. 3_{A5}). Likewise, no effect was observed on tidal volume (V_T) (ΔV_T= -0.1 mL/Kg versus control KCN (95%CI, -0.9 to 0.7), P=0.82, Table S17, Fig. 3A5), minute ventilation (V_E) (ΔV_E= -150 mL/min/Kg versus control KCN (95%CI, -360 to 59), P=0.137, Table S18, Fig. 3A5), or respiratory efficiency (V_E/VCO_2) (ΔV_E/VCO_2= -3.27 versus control KCN (95%CI, -8.2 to 1.7), P=0.168, Table S19, Fig. 3A5).

Likewise, in Wistar rats, KCN-evoked cardiovascular responses were attenuated during PLP infusion - both pressor (ΔMBP= -24.1 mmHg versus control KCN (95%CI, -40.3 to -7.84), P=0.011, Table S20, Fig. S7a) and bradycardic responses (Δ*HR*= 96.4 bpm versus control KCN (95%CI, 26 to 166), P=0.015, Table S21, Fig. S7b). On the other hand, neither tachypnea (ΔfR= 28.9 breaths/min versus control KCN (95%CI, -52 to 109), P=0.442, Table S22, Fig. S7c) nor ventilation (ΔVE= -160 mL/min/Kg versus control KCN (95%CI, -353 to 32), P=0.101, Table S23, Fig. S7e) were significantly affected.

Figure 3

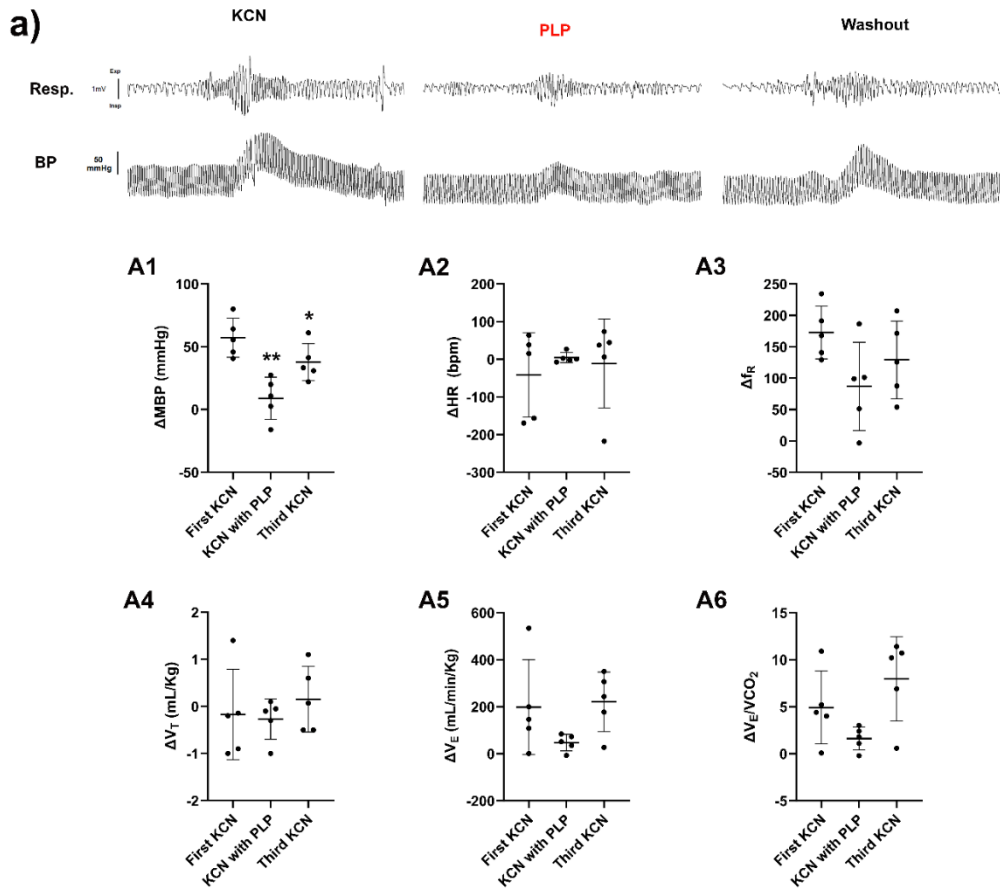

Fig. 3: Effect of Pyridoxal 5' Phosphate (PLP) on cardiorespiratory chemoreflexes. a) In adult telemetered SHR (n=5), we used potassium cyanide (KCN, 15µg/rat; bolus injection i.v.) to stimulate the peripheral chemoreceptors either in the presence or absence of PLP (intravenous infusion - 48 mg/kg/h, 30 min i.v.). At the top is a typical tracing of breathing in the whole-body plethysmography and blood pressure (BP) before, during, and after PLP infusion. PLP attenuated but did not abolish the KCN-evoked increase in mean blood pressure (MBP, A1). Other KCN-evoked responses quantified are bradycardia (HR, A2), tachypnea (f_R, A3), changes in tidal volume (V_T, A4), minute ventilation (V_E, A5), and respiratory efficiency (V_E/VCO_2, A6). Data are shown as Δ response relative to the immediate baseline. Data were analyzed using mixed regression model with either linear (i.e., normal) or gamma distribution. Mean ± SD, * P<0.05, **P<0.01.

Intravenous infusion of PLP lowers blood pressure and CO_2 production (VCO_2) of SHR

We evaluated PLP infusion on resting blood pressure, breathing, and metabolism of both Wistar rats (n=5) and SHRs (n=5) (Table S1 and S2). In SHRs, PLP prompted significant falls in systolic (ΔSBP = -17 mmHg (95%CI, -29 to -5); P= 0.019, Table S25, Fig. 4_{A1}), diastolic (ΔDBP = -16 mmHg (95%CI, -22.4 to -9.3); P= 0.003, Table S26, Fig. 4_{A2}), mean blood pressures (ΔMBP = -16.2 mmHg (95%CI, -24.3 to -8.0); P= 0.005, Table S27, Fig. 4_{A3}), and heart rate (ΔHR = -35 bpm (95%CI, -57.6 to -12.7); P= 0.012, Table S28, Fig. 4_{A4}). PLP did not produce significant changes in breathing (P>0.05; Table S29-33), (Table S1). Regarding metabolism, PLP reduced CO_2 production (ΔVCO_2 = -8.53 mL/min/Kg (95%CI, -14.1 to -2.98); P= 0.020, Table S35, Table S1) without a change in oxygen consumption (ΔVO_2 = -2 mL/min/Kg (95%CI, -9.1 to 4.78); P= 0.490, Table S34), which reduced the respiratory exchange ratio (ΔRER = -0.22 (95%CI, -0.42 to -0.02); P= 0.054, Table S36), (Table S1).

In Wistar rats, PLP also prompted significant falls in blood pressure (Table S2); however, the latter was only a fraction of what we have seen in SHRs (ΔSBP = -4.6 mmHg (95%CI, -8.5 to -0.8); P= 0.042, Table S38; ΔDBP = -3.5 mmHg (95%CI, -6.3 to -0.8); P= 0.033, Table S39; ΔMBP = -4.0 mmHg (95%CI, -7.2 to -0.9); P= 0.035, Table S40). PLP did not produce significant changes in HR and breathing (P>0.05; Table S41-49) (Table S2).

The CBs of SHR display elevated gene expression for alkaline phosphatase (ALP) subtype Alpl

Given that the effects of PLP were short lived, we analyzed the gene expression of alkaline phosphatases (ALP), which is one of its main degrading enzymes. Based on published RNA-seq data *(27)*, the CB of SHR displays increased transcript abundance of 3 out 4 ALP subtypes relative to Wistar-Kyoto (WKY) rats; these are, Germ line *(Alpg),* Biomineralization associated *(Alpl),* and Placental *(Alpp)*. Using RT-qPCR*(28)* to validate these results, we observed a 2-fold increase in mRNA expression for *Alpl* ($t_{(14)}$=14.0, P<0.001, Cohen'D= 6.98; Fig. 4_{B2}) in the CB of SHR relative to Wistar rats, whereas both *Alpg* ($t_{(14)}$=-2.64, P= 0.019, Cohen'D= -1.32; Fig. 4_{B1}) and pan-ALP - a primer pair recognizing multiple ALP isoforms (*Alpg*, *Alpp*, *Alpi*) - ($t_{(14)}$= -3.17, P= 0.007, Cohen'D= -1.59; Fig. 4_{B3}) were slightly downregulated.

Figure 4

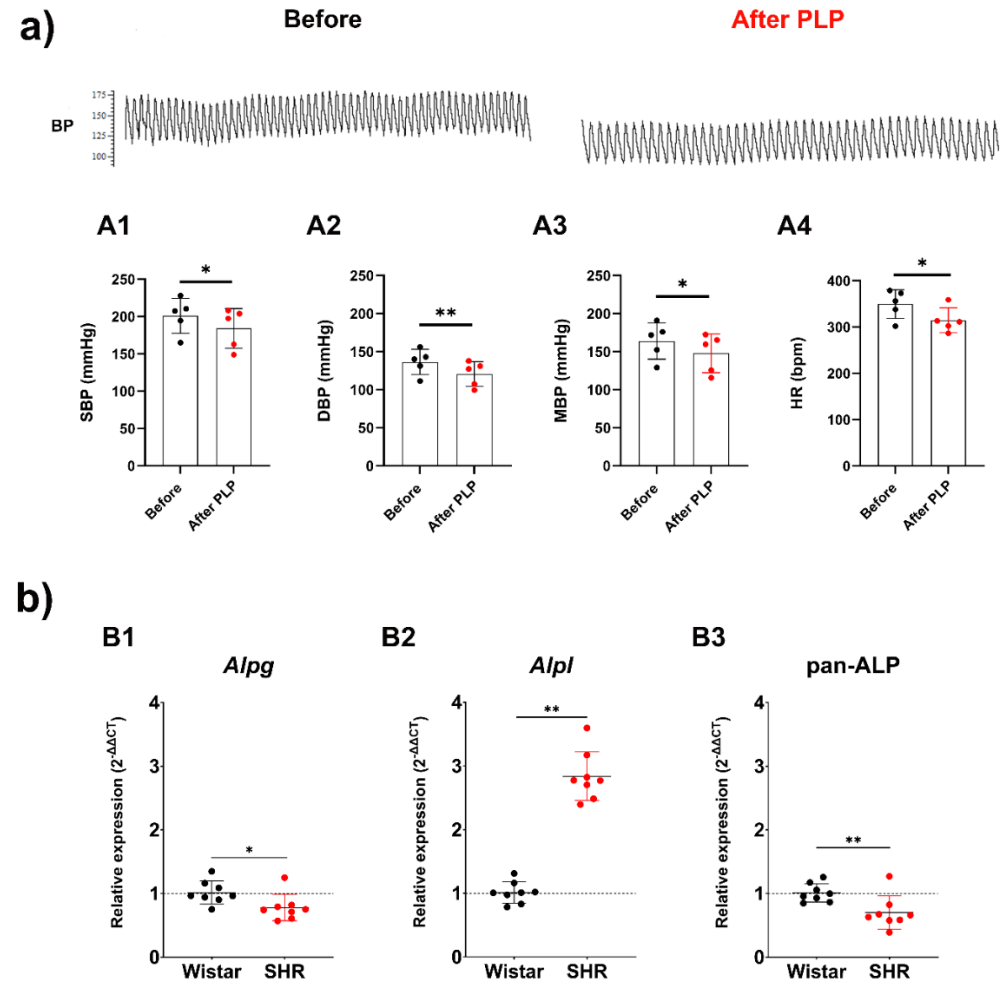

Fig. 4: Effect of Pyridoxal 5'Phosphate (PLP) on resting blood pressure *in vivo*. a) In adult SHRs (n=5), PLP was intravenously infused (48 mg/kg/h, 30 min) while recording the animal's BP. At the top is a typical tracing of BP before and immediately after the infusion. At the bottom, the effect of PLP infusion on resting SBP (A1), DBP (A2), MBP (A3) and HR (A4) **b)** RT-qPCR validation of *Alpl*, *Alpg*, and pan-ALP expression in the CB of male Wistar (4-6 weeks, n=8) and prehypertensive male (4-6 weeks, n=8) SHR. *Alpg* (B1), *Alpl* (B2), and pan-ALP (B3) expression were normalized to *Eif4b* – a housekeeping gene – and presented as relative change to Wistar. Data were analyzed either independent t-test or mixed regression model with either linear (i.e., normal) or gamma distribution. Mean ± SD, * P<0.05, **P<0.01.

PHC attenuates the hypoxic ventilatory response (HVR) of human hypertensive patients with sensitized peripheral chemoreflex index

In a double-blind randomized placebo-controlled cross-over study, 18 participants with hypertension stage 2 or above (i.e., averaged SBP = 151 ± 22 mmHg, DBP = 83 ± 8 mmHg) who met the inclusion criteria were recruited and attended two visits to our laboratory (for more information see supplementary material *Section III-Additional Results*). The sample size was calculated *a priori* based on the work of Bock et al. *(29)*. Participant demographics and current medications are reported in Table 1; baseline hemodynamic and blood biochemistry *(30)* are reported in supplementary materials (Table S3). Using the isocapnic hypoxic rebreathing method *(31)*, an index of peripheral chemoreflex sensitivity was quantified via the change in

patient's $\dot{V}_E$ relative to their estimated arterial oxygen saturation *(32)* (S_aO_2) (i.e., $\Delta\dot{V}_E/\Delta S_aO_2$). 4 participants did not complete the isocapnic hypoxic rebreathing due to frequent ectopics (n=1) and technical issues (n=3). Of the 14 participants completing the isocapnic hypoxic rebreathing test, half (n=7) were deemed to have a "sensitized" peripheral chemoreflex index (i.e., the response was greater than -0.5 L/min/% during the placebo visit). This stratification was based on the work of Narkiewicz et al. *(7)*. Oral supplementation with 600 mg PHC, which is a dose sold over the counter in supplement formulations, significantly attenuated the HVR in patients with a sensitized peripheral chemoreflex index ($\Delta\dot{V}_E/\Delta S_aO_{2\ \text{treatment*level of chemosensitivity}}$ = 0.496 L/min/% (95%CI, 0.09 to 0.90); P=0.021, Table S50*,* Fig. 5). In contrast, PHC had no effect on either resting blood pressure *(33)* or the hypoxic evoked pressor response (P>0.05; Tables S51 and S52, Table 2). Blood analysis of B6 vitamers indicate therapeutic concentrations in the micromolar range for pyridoxine, pyridoxal, and pyridoxic acid (Table S4) about 2 hours after the treatment, which allows us to infer that PLP also reached therapeutic concentrations.

393 **Table 1:** Hypertensive participant characteristics and medications

Demographics	
n	18
Age (yr)	70 ± 8
Female, n (%)	12 (67)
Height (cm)	168 ± 10
Weight (kg)	76 ± 20
BMI ($kg.m^{-2}$)	27 ± 5
Medication used	
ACE inhibitor, n (%)	5 (28)
Aldosterone receptor agonist, n (%)	1 (6)
Alpha blocker, n (%)	1 (6)
Anti-platelet, n (%)	2 (11)
Antipsychotic, n (%)	1 (6)
ARBs, n (%)	5 (28)
β blocker, n (%)	4 (22)
Ca^{2+} channel blocker, n (%)	8 (33)
Cholesterol lowering, n (%)	1 (6)
Islet enhancer / biguanide, n (%)	1 (6)
Statin, n (%)	7 (39)
Thiazide diuretic, n (%)	1 (6)
Thiazide-type diuretic, n (%)	4 (22)
Xanthine oxidase inhibitor, n (%)	1 (6)

394 Values are expressed as mean ± SD for continuous variables and frequency (%) for discrete variables. BMI: body
395 mass index, ACE: angiotensin converting enzyme, ARB: angiotensin receptor blocker
396

Figure 5

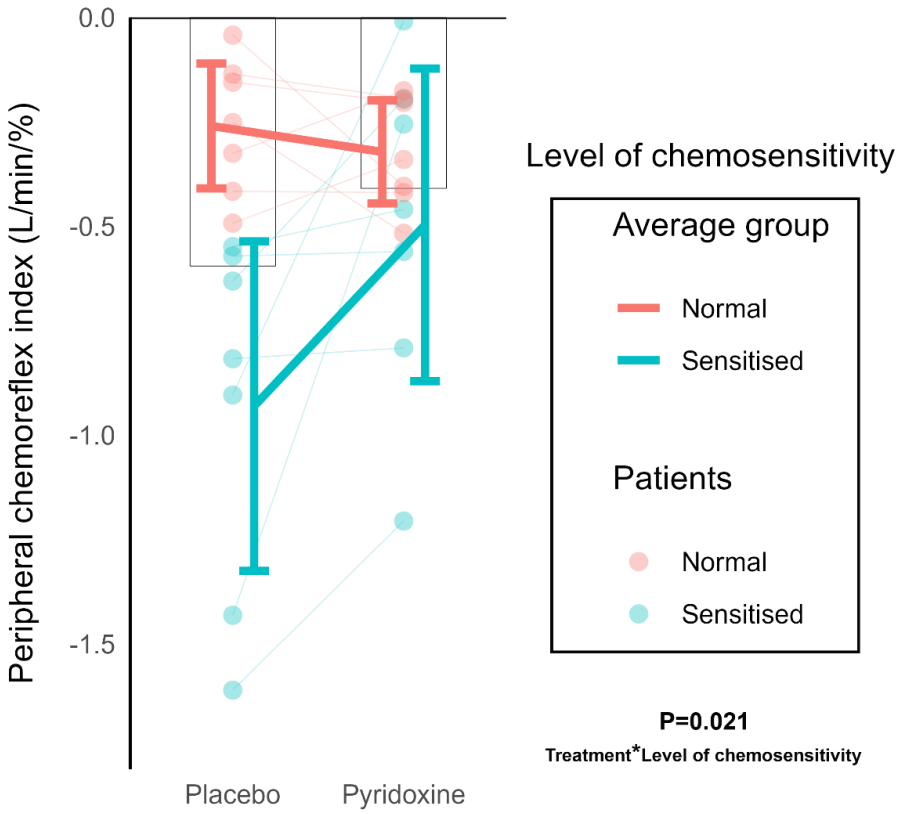

Fig. 5: Effect of Pyridoxine Hydrochloride (PHC) on the index of peripheral chemoreflex sensitivity in patients with hypertension (n=14). Using the isocapnic hypoxic rebreathing method,*(31)* the index of peripheral chemoreflex sensitivity was quantified as the patients' peak $\dot{V}E$ response relative to their nadir arterial oxygen saturation (S_aO_2) (i.e., $\Delta\dot{V}E/\Delta SaO_2$). Half of the patients (n=7) were deemed to have a "sensitized" index (i.e., the slope was steeper than -0.5 L/min/% during the placebo visit – this threshold was based on the work of Narkiewicz et al.*(7)*). Our analysis shows a significant interaction (P=0.021) between the "treatment" and "level of chemosensitivity". Data were analyzed using a linear mixed regression model (i.e., normal distribution). Mean ± 95%CI (confidence interval).

Table 2: Peripheral and central blood pressures with PHC supplementation in hypertensive participants

	Placebo	PHC	P-value
SBP (mmHg)	151 ± 22	157 ± 20	0.279
DBP (mmHg)	83 ± 8	86 ± 9	0.203
Mean pressure (mmHg)	115 ± 15	118 ± 12	0.456
Pulse pressure (mmHg)	69 ± 19	71 ± 18	0.437
Pulse rate (beats/min)	61 ± 8	62 ± 12	0.608
Central SBP (mmHg)	145 ± 20	148 ± 18	0.439
Central DBP (mmHg)	83 ± 9	86 ± 8	0.142
Central mean pressure (mmHg)	111 ± 12	114 ± 12	0.324
Central pulse pressure (mmHg)	62 ± 18	62 ± 15	0.981

Values are expressed as mean ± SD. SBP: systolic blood pressure, DBP: diastolic blood pressure. The main effect of treatment was assessed using a paired Student's t-test.

Discussion

Our findings provide the first evidence that PLP, the active form of vitamin B6, binds to and antagonizes the P2X3R of both SHR and humans with hypertension, thus attenuating CB hyperexcitability. Our *in vitro* data showed that PLP allosterically blocked $[Ca^{2+}]_i$ responses evoked by α,β-methylene-ATP in cell lines expressing recombinant hP2X3R. Its antagonist effect is supported by our *in silico* data, which predicts that PLP binds less strongly to the same negative allosteric pocket as MK-7264 (the highly potent antagonist Gefapixant). *In situ*, PLP attenuates both CB hypertonicity and hyperreflexia in SHR and attenuated preferentially the chemoreceptor sympathetic reflex. Likewise, *in vivo*, PLP preferentially attenuated the peripheral chemoreflex pressure response and lowered resting blood pressure in conscious SHR. In a small double-blind randomized clinical trial, oral supplementation with PHC, vitamin B6, attenuated peripheral chemoreflex respiratory sensitivity in participants with a sensitized chemoreflex index based on their HVR. This is the first study to demonstrate that blocking P2X3R attenuates the peripheral chemoreflex in humans.

In the field of drug discovery, computer-aided drug design (CADD) is an important method to identify molecular scaffolds with biological activity. Molecular docking is an *in silico* method to predict the three-dimensional orientation/conformation (i.e., binding pose) of a molecule within the binding site of a target of interest *(34)*. This technique, alongside molecular dynamic simulations, another *in silico* method for CADD, was recently used to investigate the interaction of MK-7264 and its analogues with the negative allosteric site of hP2X3R *(20)*. In this study, the authors reported that direct contacts of MK-7264 with Asn190 and Leu191 in hP2X3R play an important role in compound binding to the pocket. In addition, Gly189 seems to be critical for allosteric inhibition, even though MK-7264 did not display direct contacts with it in the 300 ns simulations we ran. Our molecular dynamic simulations predict a network of polar interactions with the residues defining the binding site, which include hydrogen bonds between oxygen atoms from MK-7264's sulphonamide group and Lys176. Comparatively, according to our molecular docking analyses, PLP is predicted to bind the upper portion of the pocket defined by MK-7264 with its phosphate group overlapping the sulphonamide function of MK-7264 (Fig. 1i) and its pyridine ring occupying the space defined by the phenyl ring of MK-7264 (Fig. 1h, i). Our molecular dynamics and binding energy calculations suggest PLP binds to the same binding pocket of MK-7264 albeit less strongly, supporting the relatively low potency of PLP in comparison to MK-7264 (IC_{50} = 8.7 μM versus 42.6 nM *(35)*, respectively).

In the WHBP, intra-carotid bolus injections of PLP (1-5 mM) preferentially attenuated the CB-evoked sympathetic reflex over the respiratory and bradycardia responses. Similar results were observed with AF-353 in the same *in situ* preparation *(11)*. In addition, PLP lowered blood pressure in the SHR *in vivo*. This preferential response on the chemoreflex-evoked sympathoexciation is not unique and was observed after denervating the sympathetic innervation to the CB *(25)* and following CB exposure to exedin-4, *(27)* a glucagon-like peptide-1 analogue. This sympathetic selectivity is consistent with the ribbon cable hypothesis that proposes separate lines of afferent transmission with different transmitter/receptors regulating distinct chemoreflex circuits governing separate target organs *(36)*. Similarly,

intravenous infusions of PLP in telemetered conscious SHR preferentially attenuated the cardiovascular component of the peripheral chemoreflex response evoked by KCN (Fig. 3a). It is important to acknowledge that we can observe attenuation trends in the respiratory component of the chemoreflex, e.g., the KCN-evoked tachypnea was marginally attenuated (P=0.07). In addition, in the present study, we quantified the neural inspiratory drive differently (i.e., PN amp/Ti), *in situ*, during KCN evoked responses (Fig. $S6_d$), which was not measured in the above-mentioned previous studies *(11, 25, 27)* but may be a more sensitive measure and explain the difference.

The effect of PLP on blood pressure of SHR was pronounced. While its ability to reduce blood pressure in hypertension is well-known *(18, 37–39)*, its underlying mechanism is not. The fact that PLP could reduce blood pressure within 30 minutes of infusion supports its mechanism via P2X3R antagonism, as previously demonstrated with MK-7264 *(11)*. However, this might not be the only mechanism by which PLP can reduce blood pressure if given chronically. Lellig et al. *(40)* investigated the ability of PLP to convert angiotensin II (Ang II) into pyruvamide–Ang II, which has less affinity to Ang II type 1 receptors (AT1R). Using an osmotic pump, the authors delivered PLP at a dose of 4 µg/Kg/day in SHR; after 3 days of treatment, the authors observed significant falls in both SBP and DBP. In addition, the authors treated WKY rats with Ang II to induce hypertension, which was blocked in PLP-treated rats. These results indicate that PLP could lead to falls in blood pressure via synergic mechanisms (i.e., P2X3R antagonism and scavenging of Ang II) depending on the dose. Despite that, the authors did not demonstrate increased levels of pyruvamide–Ang II in the blood or urine of PLP-treated rats, therefore, not confirming their theory that such a reaction happens, at significant levels, *in vivo*.

PLP infusion produced a small reduction in VCO_2 without changes in breathing (Table S2). Since VO_2 was unchanged, this led to a reduction in the respiratory exchange ratio (RER) favoring lipid oxidation. This is possible since PLP is a co-factor in many metabolic reactions, including carbohydrate and lipid catabolism *(41–44)*. For instance, Zemel & Bruckbauer*(43)* demonstrated that PHC increased fat oxidation and reduced R in overweight/obese patients.

In our experiments, the effect of PLP on CB activity was relatively short-lived with recovery around 30 min after the end of the infusion. The latter reflects the known PLP pharmacokinetics with a half-life ($T_{1/2}$) of 2.3h in humans *(45)*, 49 min in goats, and 16 min in pigs *(46)*. PLP is metabolised by two enzymes, PLP Phosphatase and ALP *(47)*. Interestingly, increased serum ALP activity is associated with both low levels of PLP *(48)* and increased risk for cardiovascular disease *(49)*, which includes hypertension *(50)*. Therefore, adequate ALP activity seems pivotal for maintaining physiological blood levels of PLP. Herein, we report higher levels of mRNA expression for *Alpl* in the CB of SHR. Whether the increased *Alpl* gene expression is translated into increased enzymatic activity that reduces PLP translation in the CB of SHR is unknown but might explain limitations on $T_{1/2}$.

Translating these studies, we carried out a small double-blind randomized clinical trial to investigate the capability of PLP to attenuate CB hyperexcitability in patients with hypertension. Using the index of peripheral chemosensitivity from the placebo visit, we

categorized patients having either sensitized or normal CB activity. This categorization was based on collective work from the literature, which defines a normal response as approximately -0.35 L/min/% - for review see Felippe et al. *(3)*. In addition, Narkiewicz et al. *(7)* were able to sort patients with hypertension who underwent unilateral CB resection into responders and non-responders based on their baseline HVR (-0.50 ± 0.05 and -0.32 ± 0.06 L/min/%, respectively). Interestingly, PHC's ability to attenuate the HVR occurred preferentially in patients with sensitized peripheral chemoreflex index (i.e., steeper than -0.5 L/min/%). In contrast to our rodent SHR studies, we did not see any effect on either resting blood pressure or the hypoxic pressor response. The reason for that might be that the hypertension was mild or reflected the acute, single dose, regimen of our protocol; however, when given chronically, oral supplementation with PHC reduced blood pressure and circulating catecholamines in hypertensive patients *(18, 39)*. It is worth mentioning that this is the first study to show that blocking P2X3R can attenuate the peripheral chemoreflex sensitivity in humans, thus reproducing previous animal work with MK-7264 *(11, 12)*.

Our initial study protocol, as per trial registration in 2020, involved a longitudinal study with different endpoints and plans to record the peroneal muscle sympathetic nerve activity using microneurography; however, the study protocol was negatively impacted by the COVID-19 pandemics. Thus, after amending the study with the ethics committee, we changed our protocol to the one presented herein. Therefore, not been able to carry out the protocol as initially registered is a clear limitation of our study. As another limitation, we were not successful to recruit a diverse cohort of participants; this compromised our ability investigate whether there are any sex and race/ethnicity specific responses to PHC supplementation.

It is important to recognize inter-species differences between humans and rodents in the responses to vitamin B6 treatment. Even though P2X3R is highly homologous between rat and humans (i.e., 97%)*(51)*, there are differences between the receptor conductance and kinetics of these two species *(51, 52)*. Additionally, when treated with vitamin B6, rats (SHR) showed an acute fall in blood pressure; humans did not, and rats seemed to need a higher concentration of plasma PLP and to have a shorter $T_{1/2}$. Finally, the respiratory component of the chemoreflex in rats seems to be less sensitive to vitamin B6 treatment than humans.

In addition to these inter-species differences, we need to acknowledge that the aetiology of hypertension in humans is not necessarily the same as in SHR, where it remains unknown if the increase in sympathetic outflow mediated by CB hyperexcitability contributes to a greater proportion to the high blood pressure phenotype. However, an increase in sympathetic activity is reported in approximately 60% of the patients with essential hypertension *(53)*. In addition, previous clinical trials of CB resection proved that the CB is a viable target to treat autonomic imbalance in patients with resistant hypertensive *(7)*.The latter reproduced findings in SHR*(54)*, which demonstrates that SHRs is a good animal model for translating CB interventions.

To further comprehend why the treatment did not lower blood pressure in humans with hypertension versus SHR may relate to the mild hypertension in these patients. Although

patients were asked to abstain from any cardioactive medications on the morning of the study, it is unlikely their medication were completely cleared from the blood, which could act as cofound variable. Finally, in humans, we used oral PHC instead of intravenous PLP as used in the rats, which means that PHC needed to be absorbed and converted into PLP and reach blood concentration at micromolar levels. Although blood analysis of B6 vitamers indicate therapeutic concentrations in the micromolar range for pyridoxamine, pyridoxal, and pyridoxic acid, the blood concentration of PLP in SHR were probably higher, since animals received direct intravenous infusions of PLP (24 mg/Kg). This higher concentration might be the reason why the blood pressure falls acutely in SHRs rather than in humans. Nonetheless, as previously stated, it is important to note that clinical trials with B6 supplementation in patients with hypertension showed that the latter can decrease blood pressure in humans when given chronically *(18, 39)*.

Altogether, our experiments support that PLP can attenuate CB hyperexcitability in hypertension of both rats and humans. Most importantly, the attenuating effect does not fully block the chemoreflex and showed selectivity for patients with a sensitized peripheral chemoreflex index. Given that an effective dose is sold over the counter as a supplement relatively cheaply, this could have meaningful therapeutic implications especially in developing economies (see Lellig et al. *(40)* for further discussion on the topic). It is important to acknowledge possible adverse side effects of long-term supplementation with vitamin B6, such as peripheral neuropathy, which could limit its therapeutic use and should be taken into consideration by healthcare providers. Nevertheless, our findings suggest that PLP is a viable candidate for larger clinical trials to treat CB dysregulation in cardiovascular disease, such as hypertension, heart failure, sleep apnoea, and possibly non-CB related disease, for instance refractory chronic cough.

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Conflict of interest:

Dr. Bates is Founder and CEO of LSF Medical Solutions. Work at LSF does not overlap topically with the content of this manuscript.

Dr. Paton is a founder member and Chief Scientific Officer of Ceryx Medical Ltd. Work at Ceryx does not overlap topically with the content of this manuscript.

Data Availability

The authors declare that all supporting data have been made publicly available at the Figshare and can be accessed using DOI (http://dx.doi.org/10.17608/k6.auckland.28191725). Raw data is available from the corresponding author upon reasonable request.

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