

# Long-lasting blood pressure lowering effects of nitrite are NO-independent and mediated by hydrogen peroxide, persulfides and oxidation of protein kinase G

## 1 $\alpha$ redox signaling

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**Short title:** Nitrite-induced hypotension occurs via H<sub>2</sub>O<sub>2</sub> and cysteine persulfide formation and PKG1 $\alpha$  oxidation

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## Abstract

**Aims:** Under hypoxic conditions, nitrite ( $\text{NO}_2^-$ ) can be reduced to nitric oxide (NO) eliciting vasorelaxation. However, nitrite also exerts vasorelaxant effects of potential therapeutic relevance under normal physiological conditions via undetermined mechanisms. We therefore sought to investigate the mechanism(s) by which nitrite regulates the vascular system in normoxia and, specifically, whether the biological effects are a result of NO generation (as in hypoxia) or mediated via alternative mechanisms involving classical downstream targets of NO (e.g. effects on protein kinase  $\text{G1}\alpha$  ( $\text{PKG1}\alpha$ )).

**Methods and Results:** *Ex vivo* myography revealed that, unlike in thoracic aorta (conduit vessels), the vasorelaxant effects of nitrite in mesenteric resistance vessels from wild-type (WT) mice were NO-independent. Oxidants such as  $\text{H}_2\text{O}_2$  promote disulfide formation of  $\text{PKG1}\alpha$ , resulting in NO-cGMP independent kinase activation. To explore whether the microvascular effects of nitrite were associated with  $\text{PKG1}\alpha$  oxidation, we used a C42Ser  $\text{PKG1}\alpha$  knock-in (KI; “redox-dead”) mouse that cannot transduce oxidant signals. Resistance vessels from these  $\text{PKG1}\alpha$ -KI mice were markedly less responsive to nitrite-induced vasodilation. Intraperitoneal (i.p) bolus application of nitrite in conscious WT mice induced a rapid yet transient increase in plasma nitrite and cGMP concentrations followed by prolonged hypotensive effects, as assessed using *in vivo* telemetry. In the  $\text{PKG1}\alpha$ -KI mice the blood pressure lowering effects were lower compared to WT. Increased  $\text{H}_2\text{O}_2$  concentrations were detected in WT resistance vessel tissue challenged with nitrite. Consistent with this, increased cysteine and glutathione persulfide levels were detected in these vessels

by mass spectrometry, matching the temporal profile of nitrite's effects on  $\text{H}_2\text{O}_2$  and blood pressure.

**Conclusion:** Under physiological conditions, nitrite induces a delayed and long-lasting blood pressure lowering effect, which is NO-independent and occurs via a new redox mechanism involving  $\text{H}_2\text{O}_2$ , persulfides and  $\text{PKG1}\alpha$  oxidation/activation. Targeting this novel pathway may provide new prospects for anti-hypertensive therapy.

### Translational Perspective

Despite current pharmacotherapies and interventional procedures, arterial hypertension remains a global health burden. Thus, novel therapeutic interventions are urgently required. Nitrite exerts vasorelaxant effects of potential therapeutic relevance under normal physiological conditions, yet the mechanism(s) remain unknown. Here, we present evidence of how nitrite lowers blood pressure during normoxia via a novel redox mechanism. This occurs independent of nitric oxide by generating hydrogen peroxide and persulfide formation, which subsequently activates the  $\text{PKG1}\alpha$  by oxidation. Targeting this novel pathway may provide new prospects for anti-hypertensive therapy and other cardiovascular related diseases.

## Introduction

Despite current pharmacotherapies and interventional procedures, arterial hypertension remains a global health burden and a major risk factor for cardiovascular diseases, dementia, renal failure, and premature death.<sup>1</sup> Inorganic nitrite ( $\text{NO}_2^-$ ) elicits vasorelaxation and lowers blood pressure when infused. Due to the potential beneficial effects and the possible therapeutic utility of  $\text{NO}_2^-$  in treating hypertension, it becomes essential to understand the mechanism(s) and efficacy by which  $\text{NO}_2^-$  elicits vasorelaxation under normoxia.<sup>2</sup>

Augmentation of plasma nitrite by oral administration of inorganic nitrate (converted to nitrite by oral/gut bacteria following enterosalivary circulation) has been shown to effectively reduce blood pressure in various animal models and in healthy human subjects.<sup>2-5</sup> It is now clear that at least some of the pharmacological actions of nitrite are mediated via its reduction to NO and increased NO/cyclic guanosine monophosphate (cGMP)-signaling under conditions of low pH and oxygen tension.<sup>6, 7</sup> Whilst this nitrite-NO pathway offers a conceptually attractive explanation for the blood pressure lowering effect during hypoxia, nitrite also has vasorelaxant and blood pressure lowering effects under normoxic conditions. However, the mechanism(s) responsible for these effects remain incompletely understood. We have shown that under normoxic conditions, the rate of conversion of nitrite to NO is extremely slow<sup>8</sup>, suggesting that these effects may be NO-independent. Using single intraperitoneal (i.p.) injections of nitrite in rats we previously demonstrated that nitrite was rapidly absorbed and metabolized to nitrate and nitroso/nitrosyl products in all but two tissues studied (brain and aorta)<sup>9</sup>; this was accompanied by cardioprotection and a sustained (48h) increase in ascorbate oxidation in the heart, reflecting persistent changes in tissue redox status.<sup>10</sup>

The mechanism responsible for the vasoactivity of NO (which can be generated from nitrite under hypoxic conditions) is well established and involves activation of the enzyme soluble guanylyl cyclase (sGC), resulting in an increase in the generation of the second messenger cGMP with consecutive activation of the cGMP-binding kinase PKG1 $\alpha$ , which further transduces the signal associated with vasorelaxation<sup>11</sup>. We have previously shown that PKG1 $\alpha$  itself is subject to redox regulation such that the presence of oxidants can result in inter-protein disulfide formation, which translates into an increase in enzyme activity without changes in cGMP<sup>12, 13</sup>. Thus, species such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) that are capable of causing thiol to disulfide oxidation can elicit smooth muscle relaxation and therefore lead to vasodilation via oxidative activation of PKG1 $\alpha$  in an NO-cGMP-independent fashion. Stubbert and coworkers<sup>14</sup> also reported that polysulfur species derived from hydrogen sulfide (H<sub>2</sub>S) oxidation are capable of activating PKG1 $\alpha$  via oxidation. The generation of polysulfur species and/or other sulfur oxidants (e.g. H<sub>2</sub>O<sub>2</sub>) therefore represents a NO-independent mechanism of PKG1 $\alpha$  activation, which could also translate into vasorelaxation. Thus, if nitrite-mediated vasorelaxation under normoxic conditions was found to be NO-independent, it is possible that nitrite is capable of eliciting vasorelaxation via oxidative activation of PKG1 $\alpha$ .

Recently, reactive sulfur species known as persulfides (RSSH) and polysulfides (RS<sub>n</sub>SR, RSS<sub>n</sub>H, n >1) originating from sulfur-containing amino acid metabolism were detected in mammalian tissues and fluids<sup>15</sup>. Specifically, cysteine persulfide (CysSSH), glutathione persulfide (GSSH) and related species may function as potent antioxidants, cytoprotectants and redox signalling intermediates,<sup>16</sup> and CysSSH appears to be involved in the regulation of mitochondrial biogenesis and

bioenergetics<sup>17</sup>. However, the physiological relevance and function of these species is incompletely understood, and whether nitrite has the ability to generate these polysulfur species is unknown.

The significance of nitrite as a redox regulator is not yet widely recognized, and evidence for the possible involvement of these pathways in its blood pressure lowering effects under physiological conditions is lacking. In the present study we therefore sought to explore whether nitrite has the ability to mediate redox signalling and reduce vascular tone under normoxic conditions. We hypothesised that nitrite induces vasodilation and lowers blood pressure by generating endogenous thiol oxidants, such as H<sub>2</sub>O<sub>2</sub> and persulfides, to eventually induce oxidation of PKG1 $\alpha$ . We integrated *in vitro* and *in vivo* approaches to investigate the redox properties of nitrite. Our studies were further supported by the use of “redox-dead” Cys42ser PKG1 $\alpha$  KI mice, which are unable to transduce oxidant signals because the cysteinyl thiol has been replaced by a hydroxyl group, and therefore this mutation prevents the oxidative activation of PKG1 $\alpha$ . We demonstrate that a single bolus application of nitrite induces long-lasting blood pressure lowering effects by generating H<sub>2</sub>O<sub>2</sub> and persulfide species in the resistance vessels to activate PKG1 $\alpha$  in an NO-independent manner.

## METHODS

All experiments were performed under a UK Home Office Licence and conducted according to the Animals Scientific Procedures Act 1986 (United Kingdom) and directive 2010/63/EU of the European Parliament guidelines on the protection of animals used for scientific purposes. All experiments were approved by the

Institutional Animal Welfare and Ethical Review Body. Male wild-type (PKG1 $\alpha$ -WT) or PKG1 $\alpha$  Cys42Ser knock-in (PKG1 $\alpha$ -KI) mice were bred and maintained inhouse<sup>13</sup>. Male C57BL/6 mice (background for the PKG1 $\alpha$  Cys42Ser mice) were purchased from Charles River, and all mice were age- and body weight-matched.

### **Tissue harvest and blood sampling**

C57BL/6 mice were administered a single intraperitoneal (i.p.) injection of sodium nitrite (Sigma-Aldrich) dissolved in sterile saline (Baxter Healthcare Deerfield, IL, USA; 0.9%) at various doses (0 [vehicle control], 0.1, 1.0 and 10 mg/kg body weight<sup>9, 10</sup>). Following nitrite treatment 1 or 24h previously, mice were terminally anaesthetised (intraperitoneal injection of pentobarbital sodium (300mg/kg) mixed 50:50 with anticoagulant heparin (150 units)) and biological specimens were harvested. Blood was collected and supplemented with N-ethylmaleimide (NEM) and EDTA from a 10x stock in phosphate buffered saline to yield final concentrations of 10mM NEM/1 mM EDTA and centrifuged for 10 min at 1000 x g to separate plasma from blood cells. Plasma was then aliquoted, snap frozen in liquid nitrogen and stored at -80°C until further analysis for nitrite/nitrate and cGMP. Thoracic aorta (conduit) and mesenteric resistance vessels were immediately isolated, dissected in ice-cold Krebs Henseleit buffer (KHB)<sup>12, 18</sup>, divided into aliquots, snap-frozen and stored at -80°C until analysis for persulfides and H<sub>2</sub>O<sub>2</sub>. The liver was isolated *in situ*, perfused free of blood, snap frozen, and stored at -80°C for *in vitro* investigations of nitrite's effects on catalase activity. Mice were euthanized by cervical dislocation.

### **Functional studies on isolated blood vessels**

Conduit vessel rings were isolated from the thoracic aorta, and second-order mesenteric arteries (resistance vessels) from PKG1 $\alpha$  WT and KI mice. Blood vessels

were mounted in a tension myograph (Danish Myotechnology) containing KHB (37°C, pH 7.4) and gassed with 95% O<sub>2</sub>/5% CO<sub>2</sub>. After equilibration, vascular reactivity was normalized as previously described<sup>12, 18</sup>. Submaximal vasocontraction in both vessel types was induced by addition of phenylephrine (PE; Sigma-Aldrich) or U46619 (Cambridge Biosciences), respectively, to the organ bath. Relaxation-response curves to sodium nitrite were constructed in the absence or presence of the selective soluble guanylate cyclase inhibitor, 1H-[1,2,4] oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ; Sigma-Aldrich; 10µM<sup>19</sup>) or the NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO; Enzo Life Sciences; 1mM<sup>19</sup>), and results expressed as a percentage of stable precontractile tone achieved.

### **Telemetric blood pressure monitoring *in vivo***

Mean arterial pressure (MAP) in conscious, freely moving PKG1α WT and KI mice was assessed using telemetry, as previously described<sup>13</sup>. Briefly, mice were anesthetised with 2% isoflurane in 0.5L of oxygen per minute, and a TA11PA-C10 probe catheter (Data Science International) was implanted in the aortic arch via the left aortic carotid artery. Pre- and post-operative analgesia (buprenorphine, subcutaneous injection of 0.1mg per kg of body weight; Abbot laboratories) was also provided, and the wellbeing of mice monitored daily. After 1-week recovery, mice were placed on the telemetric receivers, and MAP was recorded every 5 minutes. Blood pressures were measured throughout the day for 72h before the start of the experiment. Mice then received a single i.p. injection of 0.1 mg/kg sodium nitrite dissolved in sterile saline and their blood pressure was monitored for further 24h, followed by the administration of a successive 10-fold higher dose of nitrite at the same time on the subsequent two days while blood pressure monitoring continued.



**Mass spectrometric analyses of tissue persulfide concentrations**

High performance liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) analysis of tissue homogenates following HPE-IAM derivatization was used to determine CysSSH, GSSH, and inorganic persulfide (HSSH; disulfane), as previously described<sup>17</sup>. Briefly, mesenteric and aortic tissues were homogenised with ice-cold 100% methanol solution containing 5 mM HPE-IAM and then incubated at 37 °C for 30 minutes. The homogenates were then centrifuged at 15, 000 rpm for 10 min to remove the protein pellet. After centrifugation, the aliquots of the supernatants were diluted with 0.1% formic acid containing known amounts of isotope-labelled internal standards. The samples were then analysed for CysSSH, GSSH and HSSH by LC-ESI-MS/MS.

To assess the potential of H<sub>2</sub>O<sub>2</sub> to induce the formation of persulfides from thiols and sulfide under various reaction conditions, L-cysteine (100µM) or L-glutathione (100µM), and sodium hydrosulfide (NaHS; 100µM) was mixed with H<sub>2</sub>O<sub>2</sub> (100µM) in Tris/HCl (pH 7.5 or pH 9) or acetate buffer (pH 5.6) containing 2mM HPE-IAM. The reaction solutions were incubated at room temperature for 10, 30, 60, 90 or 120 min and subsequently analysed for cysteine (CysSH), CysSSH, HSH, HSSH, GSH, and GSSH.

**Plasma nitrite/nitrate and cyclic GMP determination**

Nitrite and nitrate were quantified in plasma as previously described<sup>10, 20</sup>. Plasma cGMP was determined using a commercially available assay kit (KGE003 R&D systems).

### **Tissue hydrogen peroxide**

Hydrogen peroxide concentrations were measured in plasma and tissue (mesenteric and thoracic aorta) using a commercially available assay kit (ab102500 Abcam).

### **Tissue catalase activity**

Tissue catalase activity was determined by monitoring the rate of  $\text{H}_2\text{O}_2$  disappearance in the absence and presence of different nitrite concentrations (for order of reagent addition see Supplementary Figure 1D).  $\text{H}_2\text{O}_2$  concentrations were quantified using the horseradish peroxidase (HRP)-mediated oxidation of phenol red and compared to a calibration curve with absorbance readings taken at 610 nm<sup>21</sup>. For these experiments, frozen mouse liver aliquots were homogenized at 1:4 ratio (w/v) in EDTA-containing phosphate buffer pH 7.4 using a glass-glass Potter-Elvehjem tissue homogenizer immersed in an ice/water bath. The homogenization buffer was supplemented with NEM (10 mM, final conc) to minimize potential thiol-mediated metabolic conversion of nitrite to nitroso species. The homogenate was centrifuged (13,000 x g, 15 min, 4°C), and kept on ice in the dark for up to 1h for measurements of catalase activity, in the absence and presence of nitrite.

## **RESULTS**

### **Nitrite regulates mesenteric resistance vasorelaxation NO-independently during normoxia**

To determine whether in the presence of oxygen nitrite mediates vasorelaxation in an NO-independent or independent fashion, we first constructed cumulative

concentration-response curves to nitrite in isolated pre-constricted mesenteric resistance vessels mounted in wire-myographs (Figure 1). Pre-incubation of resistance vessels from the WT mice with either the NO-scavenger, carboxy-PTIO or the sGC inhibitor, ODQ failed to abrogate vasorelaxation in the resistance vessels from WT mice (Figure 2A-B), indicating that the effects of nitrite in this tissue were largely NO/sGC-independent. By contrast, when wire-myography was performed in conduit vessels (thoracic aorta) nitrite's vasorelaxant effects were clearly NO/sGC-dependent (Figure 2C-D).

In light of the above, we hypothesized that nitrite-mediated vasorelaxation may perhaps occur via PKG1 $\alpha$  oxidation, independent of the classical NO-cGMP pathway. To explore this possibility we used a "redox-dead" genetic variant of PKG1 $\alpha$  whereby the important regulatory SH moiety has been mutated to a redox inactive OH group (by replacing a single cysteine, Cys42, against serine)<sup>13</sup>. Using this PKG1 $\alpha$  Cys42S knock-in (KI) mouse model and WT littermates as a comparator, we next investigated the molecular target involved in nitrite-mediated vasorelaxation (Figure 1). Nitrite relaxed WT vessels in a concentration-dependent fashion, but did not relax resistance vessels from PKG1 $\alpha$ -KI mice (Figure 2E); whereas in the conduit vessels, nitrite was not mediated through PKG1 $\alpha$  oxidation (Figure 2F).

To translate our findings into the vivo setting, we administered i.p. injections of sodium nitrite (0.1, 1 and 10 mg/kg) in WT mice on three consecutive days following 72h continuous telemetric arterial blood pressure monitoring. Compared to the hourly averages for the 3 days baseline blood pressure measurements shown, reductions in blood pressure were not apparent until 4-5h after the nitrite application (Figures 1, 3A; WT- black trace), but blood pressure remained reduced for almost 24h,

especially during the nocturnal period when the animals were most active (Figure 3B, WT-black bars). Unexpectedly, the magnitude of blood pressure lowering by the three doses was similar.

We next assessed the *in vivo* blood pressure lowering effects of nitrite in redox-dead PKG1 $\alpha$ -KI using the same treatment regime as in their PKG-WT littermates (see above). Of note, the PKG-KI had modestly, but consistently higher mean blood pressure than the WT.<sup>13</sup> In contrast to the long-lasting hypotensive effects seen with nitrite in WT mice, PKG-KI animals did not show a blood pressure lowering effect. Figure 3A compares the 24hr MAP data in both genotypes following i.p administration of sodium nitrite (0.1, 1 and 10 mg/kg). Figure 3B is further simplified to illustrate the delta difference between baseline and response to nitrite treatment at night. There was a significant difference between the two genotypes (\*P<0.05 determined by repeated measures two-way ANOVA). These results are consistent with the notion that the ability of nitrite to elicit persistent hypotension depends on its capacity to oxidatively activate PKG1 $\alpha$ .

In a separate group of C57BL/6 mice, the same nitrite injection regime was used and blood samples were taken 1h and 24h post-treatment for measurement of plasma nitrite, nitrate and cGMP (Figures 1, 3C-E). Plasma nitrite and nitrate acutely increased in a dose-dependent manner, and this was statistically significant at the highest dose (i.e 3-4 hours before a BP-lowering effect was observed), as determined by two-way ANOVA. Following Tukey's multiple comparisons test 10mg/kg sodium nitrite, plasma nitrite was significantly higher at one hour when compared to control vehicle, 0.1 and 1mg/kg; \*P<0.05). However 24h later plasma nitrite, nitrate and cGMP were similar to baseline concentrations even at the highest

dose (despite persistent BP reduction persisting for almost 24h); these changes were consistent with our previous results in rats subjected to i.p nitrite administration, demonstrating that plasma nitrite levels rapidly increased, but returned close to baseline values in approximately 1h.<sup>9, 10</sup>

### **Nitrite increases hydrogen peroxide levels**

We next focused on the mechanism of nitrite-mediated vasorelaxation of resistance arteries and blood pressure lowering. Nitrite has the potential to inhibit catalase,<sup>22, 23</sup> which may conceivably lead to an increased oxidative tone by reducing the breakdown of endogenously produced H<sub>2</sub>O<sub>2</sub>. This action was confirmed in complementary studies in which nitrite was found to be capable of inhibiting catalase activity in hepatic tissue *in vitro* (Supplementary Figure 1). In order to determine whether this can also occur in vascular tissue, we measured *ex vivo* H<sub>2</sub>O<sub>2</sub> concentrations in blood vessels harvested 1 and 24h after nitrite pre-treatment *in vivo* (Figure 1). As depicted in Figure 4A, nitrite caused a significant and similar increase in steady-state concentrations of H<sub>2</sub>O<sub>2</sub> in mesenteric resistance vessels with both 1 and 10 mg/kg nitrite at 1h, which persisted at 24h. Whilst nitrite also triggered an increase of this oxidant in the aorta at 1h, H<sub>2</sub>O<sub>2</sub> concentrations had returned to normal at 24h (Figure 4B), revealing kinetic differences in H<sub>2</sub>O<sub>2</sub> handling between these two types of vessels.

### **Nitrite increases cysteine persulfides and glutathione persulfide in resistance vessels during normoxia**

The results presented thus far indicate that nitrite produces H<sub>2</sub>O<sub>2</sub> and induces vasorelaxation by oxidation of PKG1 $\alpha$ . We next examined whether there was a possible intermediate that links these effects. We surmised that nitrite may have the

ability to oxidatively activate PKG1 $\alpha$  via the generation of polysulfur species. To establish whether nitrite generates polysulfur species *in vivo*, mesenteric resistance and thoracic aorta blood vessels were harvested from WT mice (C57BL/6) treated with bolus i.p administration of vehicle control or sodium nitrite (0.1, 1 and 10 mg/kg) for 1 or 24h. The levels of persulfides (CysSSH, GSSH and HSSH) were analysed by liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) following derivation with  $\beta$ -(4-hydroxyphenyl)ethyl iodactamide (HPE-IAM). We observed dose-dependent increases in CysSSH and GSSH in the resistance vessels (Figure 5A-B) with progressively lower concentrations of HSSH (Figure 5C). In contrast, we observed no changes in CysSSH, GSSH, or HSSH formation in the conduit vessels (Figure 5D-F, respectively). In further *in vitro* studies, we sought to confirm experimentally that H<sub>2</sub>O<sub>2</sub> is capable of inducing polysulfidation when inorganic sulfide and cysteine are present. This was indeed found to be the case under a variety of pH and H<sub>2</sub>O<sub>2</sub> conditions (Figures 6A-C), and similar results were observed with glutathione/sulfide/H<sub>2</sub>O<sub>2</sub> (Figure 6D).

## DISCUSSION

In the present study, we demonstrate that a single bolus application of nitrite elicits a long-lasting blood pressure lowering effect that is unrelated to the generation of NO. We furthermore show that a marked temporal dissociation exists between plasma nitrite and cGMP concentrations, and the blood pressure lowering effects of i.p. injections of nitrite, with the hypotensive effects being delayed in onset vs circulating nitrite/cGMP concentrations and persisting despite the return of the latter to baseline values. These observations differ considerably from the short-lived BP-lowering effects of a single intravenous injection of nitrite, thus supporting the role of a non-canonical vasodilator effect via a mechanism that is independent of the NO-cGMP

pathway. Our results are consistent with the notion that nitrite has signaling properties in its own right<sup>9</sup> and provides evidence for the notion that nitrite can act as an indirect oxidant. Using a combination of biochemical analytical tools and a genetic KI mouse model, we finally demonstrate that the blood pressure lowering effects of nitrite are mediated by PKG1 $\alpha$  oxidation following H<sub>2</sub>O<sub>2</sub> and cysteine/glutathione persulfide formation in the resistance vasculature.

We previously demonstrated that oxidants such as H<sub>2</sub>O<sub>2</sub> can activate PKG1 $\alpha$  independently of the classical NO-sGC-cGMP pathway by forming a disulfide bond at Cys42 to dilate resistance vessels and lower blood pressure<sup>13</sup>. Consistent with this finding, PKG1 $\alpha$ -KI mice in which WT kinase has been replaced by a “redox-dead” serine residue (C42S mutant) cannot transduce oxidant signals because their PKG does not harbour the redox-sensing thiol, rendering these mice unable to lower blood pressure via an oxidative mechanism.<sup>13</sup> Independent of this, we demonstrated that nitrite chemically/functionally interacts with hydrogen sulfide (H<sub>2</sub>S)<sup>24</sup> and that a single bolus application of nitrite triggers long-lasting alterations in myocardial redox status with dramatic alterations in the cardiac mitochondrial proteome<sup>10</sup>. We here sought to integrate these different strands of information and assessed whether nitrite could produce a metabolic signature consistent with a process involving thiol/sulfide oxidation and whether this might be associated with PKG1 $\alpha$  dimerization to mediate vasorelaxation and blood pressure reduction.

Whilst the resistance vessels are the primary determinant of diastolic and mean blood pressure, most vascular studies investigated the effects of nitrite in the aorta and have shown NO-dependent effects. Here, we assessed both the aorta and the mesenteric resistance vessels from WT and PKG1 $\alpha$  KI mice. Unlike the aorta, it was

evident in the isolated resistance vessels from WT mice that nitrite mediated vasodilatory effects were NO-sGC independent. Moreover, the mesenteries from mice expressing the “redox dead” C42S form of PKG1 $\alpha$  were relatively insensitive in their vasodilatory response to nitrite compared to WT. Based on these findings, we anticipated that the long-lasting *in vivo* blood pressure lowering effect of nitrite would also be diminished in the KI mice. Indeed, this was found to be the case because nitrite decreased blood pressure via non-canonical oxidative activation of PKG1 $\alpha$ , whereas the KI mice were resistant to this hypotensive response.

Nitrite has the ability to reduce the rate of H<sub>2</sub>O<sub>2</sub> decomposition by binding to the heme moiety of catalase thereby inhibiting enzyme activity<sup>23, 25</sup>; thus we reasoned that nitrite may inhibit catalase activity and possibly increase H<sub>2</sub>O<sub>2</sub> levels, especially in resistance vessels, and this may be responsible for the vasoactivity observed. Exploratory studies on the inhibition of catalase activity by nitrite in mouse liver tissue *in vitro* confirmed that this may be a viable mechanism. In the vasculature, H<sub>2</sub>O<sub>2</sub> concentrations were found to remain elevated for prolonged periods of time in the mesenteric resistance vessels when compared to conduit vessels. Although these observations would seem to provide a mechanism that could explain the NO-independent actions of nitrite as a vasorelaxant, previous studies reporting H<sub>2</sub>O<sub>2</sub>-mediated PKG1 $\alpha$  activation required high concentrations of this oxidant<sup>12</sup>, suggesting the effect may not be entirely due to H<sub>2</sub>O<sub>2</sub> directly.

Reactive sulfur containing species, such as CysSSH have been known to exist endogenously, but the physiological relevance of these species remain incompletely understood. CysSSH evidently generates GSSH, and previous work from our group has shown that these reactive species act as potent scavengers of oxidants such as



H<sub>2</sub>O<sub>2</sub>, which contributes to cellular redox signalling.<sup>16</sup> Furthermore, Stubbert and colleagues<sup>14</sup> reported that polysulfur species derived from hydrogen sulfide (H<sub>2</sub>S) oxidation are capable of activating PKG1 $\alpha$  via oxidation. Therefore, the generation of polysulfur species may represent an NO-independent mechanism of PKG1 $\alpha$  activation, which could mediate vasorelaxation. Thus, the ability for nitrite to oxidatively activate PKG1 $\alpha$  may be due to additional generation of polysulfur species. Indeed, there is precedence for the interaction of nitrite with sulfide in forming another reactive sulfur species, nitrosopersulfide, in the literature<sup>24</sup>. Previous reports indicate that analysis of CysSSH, GSSH and HSSH (disulfane) can serve as detectable surrogates for overall levels of cellular polysulfur species, since they are all in dynamic equilibrium<sup>26, 27</sup>. Thus, to test the hypothesis that nitrite generates polysulfur species *in vivo*, the concentrations of all three compounds were determined by mass spectrometry in conduit and mesenteric resistance vessels of WT mice 1h and 24h after nitrite administration. We observed dose-dependent increases in CysSSH and GSSH in the resistance vessels with progressively lower concentrations of HSSH. Since disulfane is highly reactive, it may immediately react as intracellular disulfide concentrations rise to produce CysSSH and GSSH. In contrast to our findings in resistance vessels, no increases in CysSSH or GSSH were apparent in conduit vessels. These observations demonstrate that bolus applications of nitrite trigger polysulfur species formation through an NO-independent mechanism already at low-to-moderate pharmacological doses with marked differences between vascular beds. Interestingly, persulfide formation from the resistance vessels matched the temporal profile of nitrite effects on H<sub>2</sub>O<sub>2</sub> and blood pressure.

Thus far, we demonstrated that nitrite is capable of eliciting increases in  $\text{H}_2\text{O}_2$  and polysulfur species, with greater propensity for resistance vessels than conduit vessels. It is intriguing to speculate that both events are related and that the source of the polysulfur species is via  $\text{H}_2\text{O}_2$ -mediated oxidation of reduced thiol species. In further *in vitro* studies, we thus sought to confirm experimentally that  $\text{H}_2\text{O}_2$  is capable of inducing polysulfidation when inorganic sulfide and cysteine are present. This was indeed found to be the case under a variety of conditions (pH dependence and  $\text{H}_2\text{O}_2$  concentrations) and similar results were observed with glutathione/sulfide/ $\text{H}_2\text{O}_2$ . Taken together, these reactions strongly suggest that nitrite can indirectly contribute to polysulfide formation by acting as an amplifier of endogenous  $\text{H}_2\text{O}_2$  concentrations.

Although it is intriguing to speculate that nitrite may elicit vasorelaxation via its capacity to raise  $\text{H}_2\text{O}_2$  levels which subsequently give rise to organic persulfides which, in turn, activate  $\text{PKG1}\alpha$ , it is important to reiterate that detection of CysSSH/GSSH is merely indicative of the general presence of polysulfur species (including protein per- and polysulfides)<sup>26</sup> and that these particular species are not necessarily solely responsible for the biological effects observed herein. What detection of these species does indicate, however, is the presence of an oxidizing environment consistent with the notion of oxidative  $\text{PKG1}\alpha$  activation. Due to the analytical challenges and rapid interconversion of reactive sulfur species it is currently not possible to disentangle these reaction channels further. Nevertheless, the key findings of this work are that nitrite – when applied i.p. - is capable of creating an oxidizing environment *in vivo*, as evidenced by increases in  $\text{H}_2\text{O}_2$  and polysulfide concentrations in vascular tissue, favouring  $\text{PKG1}\alpha$  dimerization, and that

these effects occur primarily in resistance vessels. While it has been reported that nitrite is capable of inhibiting catalase (which we confirm to also occur in tissues), and this is a possible pathway through which nitrite creates an oxidizing environment, the exact mechanism(s) by which this occurs are not established. We cannot exclude, for example, that nitrite also increases vascular NADPH oxidase activity<sup>28</sup> and/or upregulates SOD<sup>29</sup> and/or increases superoxide/H<sub>2</sub>O<sub>2</sub> formation by interfering with electron transfer processes in the mitochondria; these possibilities warrant further investigation. Furthermore, increases in resistance vessel H<sub>2</sub>O<sub>2</sub> concentration were similar at 1 and 10mg nitrite, which may explain the absence of dose-response for blood pressure lowering over the range of doses explored.

The ability of nitrite to elicit its vasoactive effects to a greater extent in resistance versus conduit vessels is intriguing and requires independent confirmation. This mechanism is at odds with nitrite's currently accepted mode of action (Figure 7) and represents an unusual case of non-canonical pharmacology whereby a short-lived pro-oxidant elicits long-lasting hemodynamic effects *in vivo*. To our knowledge, this is the first report for such a mechanism in resistance vessels *in vivo*.

These findings may potentially be relevant not only to pharmacological, but also to physiological concentrations of nitrite. A previous study reported that antibacterial mouthwash reduced plasma nitrite and increased blood pressure in treated hypertensive patients.<sup>30</sup> Herein, we demonstrated that the delayed but sustained blood pressure lowering effect of nitrite was markedly attenuated in the C42S PKG1 $\alpha$  KI versus the WT mice. Furthermore, we also confirm a previous report that the baseline blood pressure was higher in the C42S PKG1 $\alpha$  KI mice.<sup>13</sup> This could potentially be related to effects of physiological concentrations of nitrite on PKG oxidation state or may be consistent with a previous report of impaired acetylcholine

mediator effects observed in the C42S PKG1 $\alpha$  KI.<sup>13</sup> Mesenteries from KI mice were deficient in their responses to H<sub>2</sub>O<sub>2</sub> or acetylcholine, as well as the latter in the presence of L-NAME and indomethacin to remove vasodilation by classical nitric oxide- or prostacyclin-dependent mechanisms.<sup>6</sup> This deficiency in acetylcholine-dependent relaxation in the C42S PKG1 $\alpha$  KI is consistent with the endothelium-derived hyperpolarizing factor-dependent component of vasodilation induced by this neurotransmitter being significantly mediated by H<sub>2</sub>O<sub>2</sub> generation.<sup>31</sup> Indeed, evidence was presented that C42S PKG1 $\alpha$  is deficient in activation by cGMP, although whether this mutation impacts the affinity with cGMP was not determined, and this was suggested to underlie the loss of function observed in C42S PKG1 $\alpha$  KI mice.<sup>32</sup> However, the studies comparing the response of isolated resistance vessels of these mutant mice to WT controls, showed their vasodilation to NO donors or a cell-permeable cGMP analogue were the same.<sup>13</sup>

Although oxidation of C42 has been implicated in the targeting and the oxidation of PKG1 $\alpha$ , there is additional complexity because of the potential roles of other redox-active cysteines and the interrelationship between cGMP binding and kinase oxidation. It may be that C42 is more important for targeting the kinase to its substrates, and that cGMP or oxidation of another cysteines is required for kinase competency.<sup>33</sup> For example, nitroxyl donors were shown to induce a disulfide at C42 as well as an intradisulfide between C117 and 195 in the high-affinity cGMP binding site of PKG1 $\alpha$  – with the latter oxidation mediating most of the activation induced by this nitro-oxidative intervention.<sup>34</sup> C195 was also shown to adduct the electrophile 8-nitro-cGMP, with this S-guanylation modification leading to persistent activation of the kinase.<sup>35</sup> Hyperoxidation of C117 has been implicated as a mechanism of PKG1 $\alpha$  activation by H<sub>2</sub>O<sub>2</sub><sup>36</sup>, whilst activation by this oxidant was not observed by

others.<sup>32</sup> In summary, the redox control of PKG1 $\alpha$  is complex, with our understanding continuing to evolve as contemporary studies on the subject emerge. Nevertheless, a casual role for PKG1 $\alpha$  C42 oxidation in oxidant or endothelium-derived hyperpolarizing factor-dependent vasodilation is supported by a number of studies.

The generation of polysulfur species is dependent on the presence of cysteine and/or H<sub>2</sub>S<sup>17, 37</sup>. Thus, if PKG1 $\alpha$  activation is polysulfide-mediated (as our *in vivo* experiments suggest) the generation of H<sub>2</sub>S in the target tissues becomes an important factor for the action of nitrite and may contribute to the tissue specificity of the observed effects. The overall outcome of these reactions on the effective concentrations of cysteine persulfide and related species (and thus the net effect on physiological function) appears to be complex and tissue/organ-specific, as illustrated by the differences in regulation between resistance and conduit vessels in the present study. The factors contributing to these differences (e.g. oxygen tension, oxidative stress) warrant further investigation.

In summary, this study reports a novel finding, whereby under physiological conditions nitrite induces long-lasting blood pressure lowering effects independent of NO-induced cGMP elevations. We show that nitrite lowers blood pressure by mediating oxidation of PKG1 $\alpha$ . This mechanism has been shown by using “redox-dead” Cys42ser PKG1 $\alpha$  KI mice, which cannot activate oxidant signals. We observed dose-dependent vasorelaxant responses to nitrite in the PKG1 $\alpha$  WT isolated resistance vessels, but the vasodilatory response to nitrite were blunted in the PKG1 $\alpha$  KI mice. These findings were further substantiated by monitoring blood pressure *in vivo*, since nitrite was unable to lower blood pressure in the redox-dead PKG1 $\alpha$  KI mice compared with WT littermates. We also identified that the effect of

nitrite was mediated via the generation of  $H_2O_2$ , with consecutive production of persulfide species (CysSSH and GSSH) in the resistance vessels, which oxidatively activate  $PKG1\alpha$ . Our study concludes that a single bolus application of nitrite has the ability to act as a redox regulator during physiological conditions to induce long-lasting blood pressure lowering effects, providing a new prospect for therapeutic strategy in treating the global healthcare burden of hypertension. Future investigations are warranted to address the significance of this redox pathway in hypertensive and other cardiovascular disease models. Furthermore, our findings raise the possibility of identifying other molecules capable of modulating this novel pathway, that may have therapeutic potential as hypertensive agents.

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## Figure Legends

**Figure 1.** Schematic experimental design to assess nitrite-induced vasodilatory and blood pressure lowering effects as well as biochemical changes during normoxia.

**Figure 2.** Mesenteric and thoracic blood vessels from PKG-1 $\alpha$  WT and PKG-1 $\alpha$  Cys42 KI were mounted in myograph and submaximally constricted with U46619 and phenylephrine, respectively. Pre-incubation of isolated mesenteric resistance vessels from WT to **A.** NO scavenger, carboxy-PTIO (CPTIO; 1mM; n=15) or **B.** soluble guanylate cyclase inhibitor (sGC), ODQ (10 $\mu$ M; n=14) did not attenuate the vasodilatory response to nitrite in WT mice. Concentration response curves to nitrite in isolated thoracic aorta. Vasorelaxant effects of nitrite was **C.** NO-dependent (n=7) and **D.** sGC-dependent in WT mice (n=11). Concentration response curves to sodium nitrite in **E.** isolated mesenteric resistance vessels from both genotypes (n=19). Nitrite was significantly less effective as a vasodilator in PKG-1 $\alpha$  KI mice, but not in the **F.** thoracic aorta (conduit vessels; n=13-14). Data are mean  $\pm$  s.e.m. \*\*\*P $\leq$ 0.001 as determined by two-way ANOVA with Bonferroni's test (**A-F**).

**Figure 3.** PKG-1 $\alpha$  Cys42 KI mice are resistant to nitrite-induced blood pressure lowering compared with age-matched WT littermates, as measured by in vivo telemetry (n=6 WT and 7 KI). **A.** Comparison of the 24h averaged changes in MAP in both genotypes following bolus administration of sodium nitrite (0.1, 1 and 10 mg/kg). WT mice, but not for KI mice, there was a significant difference in blood pressure lowering effect with each nitrite dose when compared to baseline **B.** Delta indicates the difference between baseline and response to treatment at night. In separate group of WT mice (C57BL/6), following bolus i.p administration with sodium nitrite

(vehicle control, 0.1, 1 and 10 mg/kg) for 1 or 24h treatment, plasma samples were taken **C.** nitrite, **D.** nitrate and **E.** cGMP (n=4-7 mice/group). Data are representative or mean  $\pm$  s.e.m. \* $P \leq 0.05$  when WT mice compared to each nitrite dose with baseline (repeated measures one-way ANOVA, **A**). \* $P \leq 0.05$  between two different genotypes was determined by repeated measures two-way ANOVA (**B**). Significant time and concentration interaction for plasma nitrite and nitrate, as determined by two-way ANOVA. Following Tukey's multiple comparisons test 10mg/kg sodium nitrite was significant when compared to control vehicle, 0.1 and 1mg/kg (\* $P \leq 0.05$ ; **C**, **D**). There was no significant difference between nitrite concentration for cGMP levels at either time ( $P > 0.05$ ; two-way ANOVA followed by Tukey's multiple comparisons test; **E**).

**Figure 4.** In separate group of mice (C57BL/6), mesenteric resistance and thoracic aorta blood vessels were harvested following bolus i.p administration of vehicle control or sodium nitrite (0.1, 1 or 10 mg/kg) in WT mice (C57BL/6) for 1 or 24h. Hydrogen peroxide ( $H_2O_2$ ) levels were measured in **A.** mesenteric resistance vessels (n=5-8) and **B.** thoracic aorta (n=7-9). Data are mean  $\pm$  s.e.m. \* $P \leq 0.05$  as determined by one-way ANOVA with Dunnett's test.

**Figure 5.** Mesenteric resistance and thoracic aorta blood vessels were harvested from WT mice (C57BL/6) treated with bolus i.p administration of vehicle control or sodium nitrite (0.1, 1 and 10 mg/kg) for 1 or 24h. The levels of persulfides (CysSSH, GSSH and HSSH) were analysed by liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) following derivation with  $\beta$ -(4-hydroxyphenyl)ethyl iodactamide (HPE-IAM). Data are represented as mean  $\pm$  s.e.m (n=5-8). \* $P \leq 0.05$  as determined by two-way ANOVA with Tukey's test (**A-F**).

**Figure 6.** Cysteine persulfide (CysSSH) and polysulfides formation during various reactions. **A.** pH conditions (5,6, 7.5 & 9) in the presence of cysteine (100µM), sulfide (NaHS; 100µM), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ; 100µM) in 50mM Acetate buffer (pH 5 & 6), or Tris/HCL (pH 7.5 & 9) or HPE-IAM (2mM) **B.**  $\text{H}_2\text{O}_2$  concentrations (0, 0.01, 0.05, 0.1, & 1 mM) in the presence of cysteine (100µM), NaHS (100µM) in 50mM Tris/HCL pH 7.5. Similar results were obtained with glutathione/sulfide mixtures, although final concentrations of GSSH achieved were less compared to the corresponding cysteine analogue. All reactions were also accompanied by the formation of thiosulfate ( $\text{S}_2\text{O}_3^{2-}$ ) and small concentrations of the corresponding trisulfide (data not shown) **C.** Interaction of cysteine (100µM) or **D.** Glutathione (GSH; 100µM) in the presence of NaHS (100µM),  $\text{H}_2\text{O}_2$  (100µM) in 50mM Tris/HCL (pH 7.5). Data presented are from one single independent experiment for each condition.

**Figure 7.** Proposed mechanism of action involved in nitrite-mediated vasorelaxation, including the canonical nitrite reduction pathway (left – blue panel) and the novel nitrite oxidation pathway (right – red panel).

Figure 1

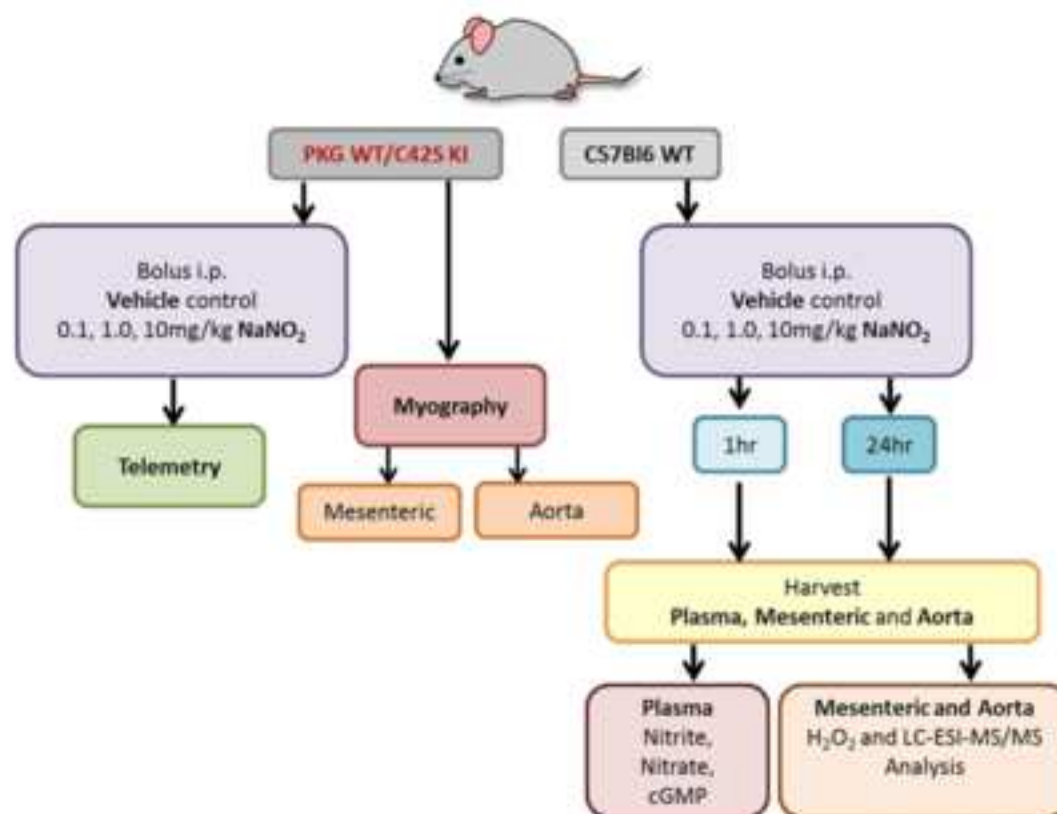




Figure 2

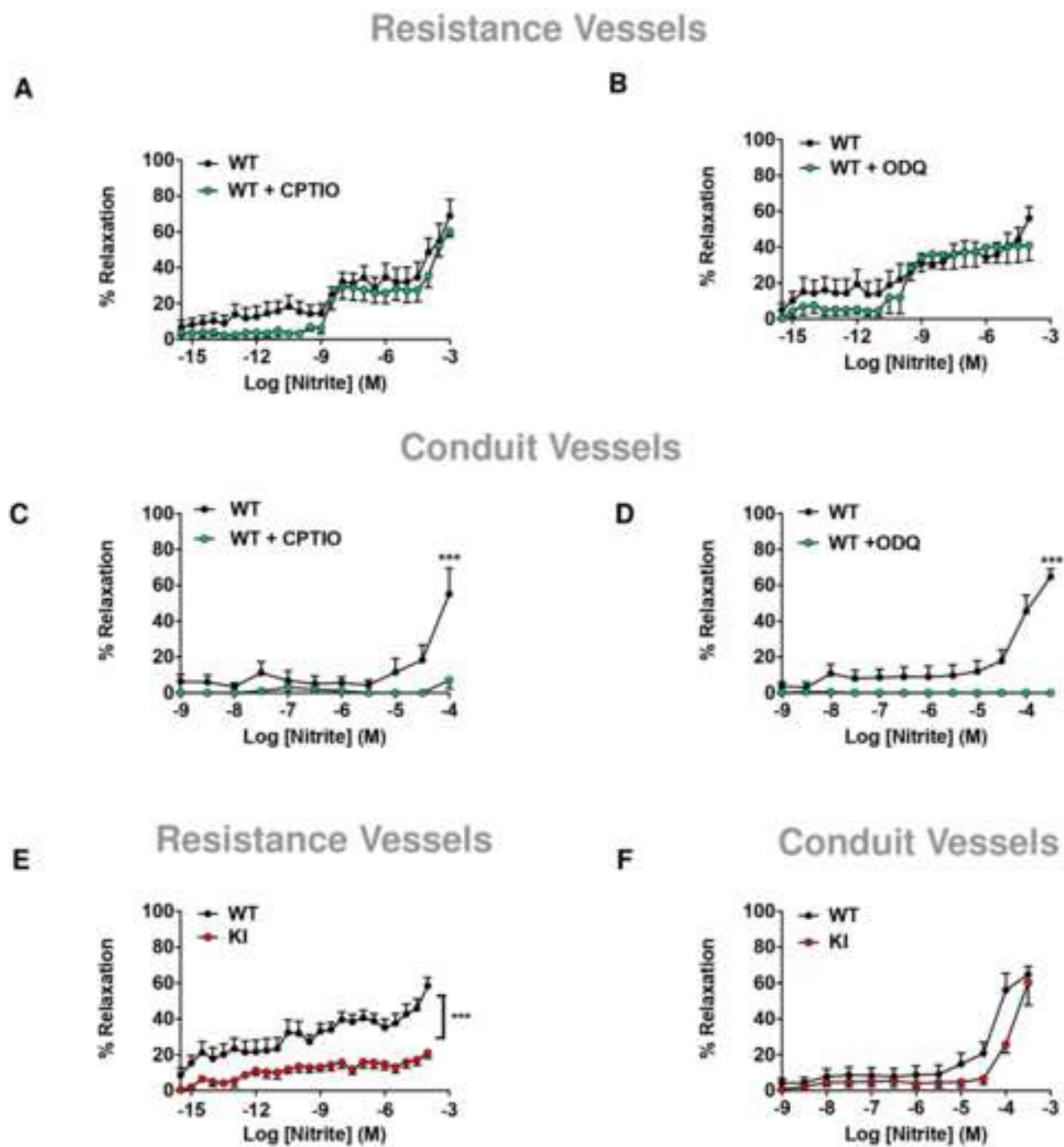


Figure 3

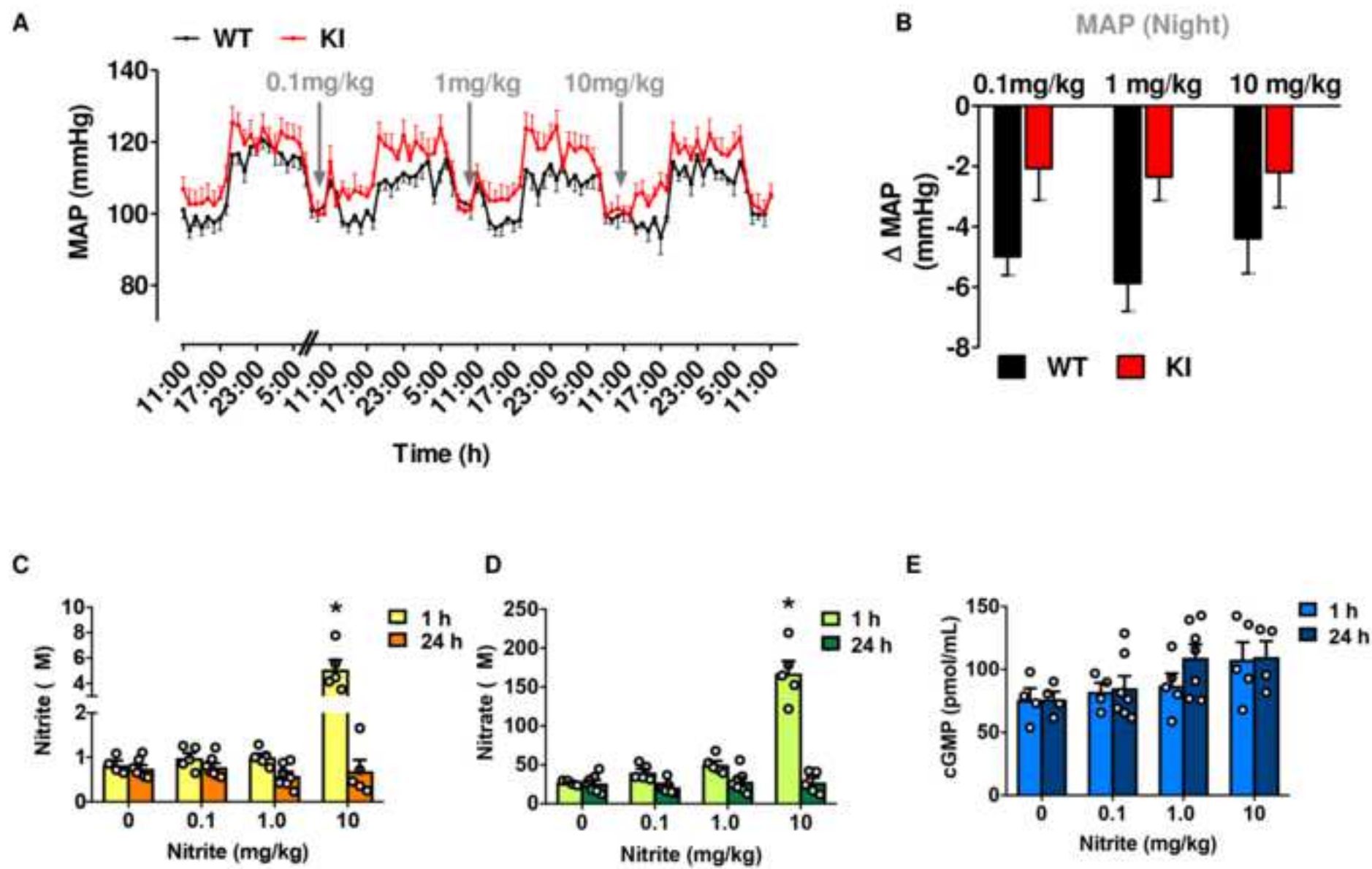


Figure 4

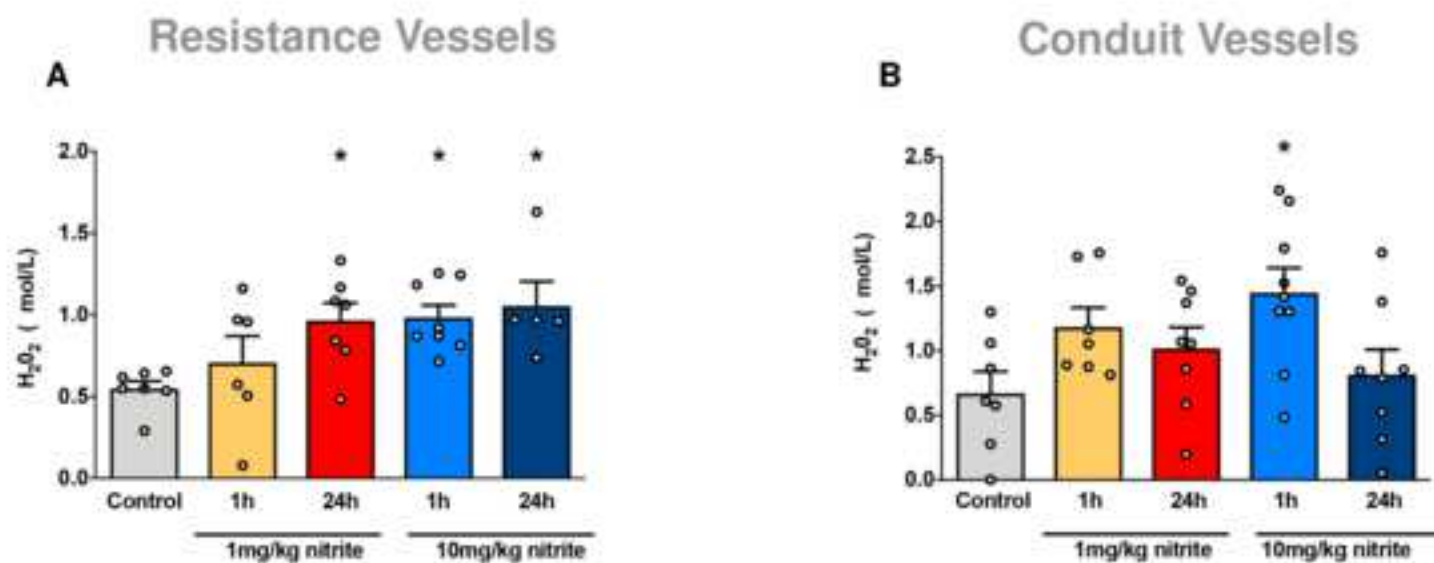
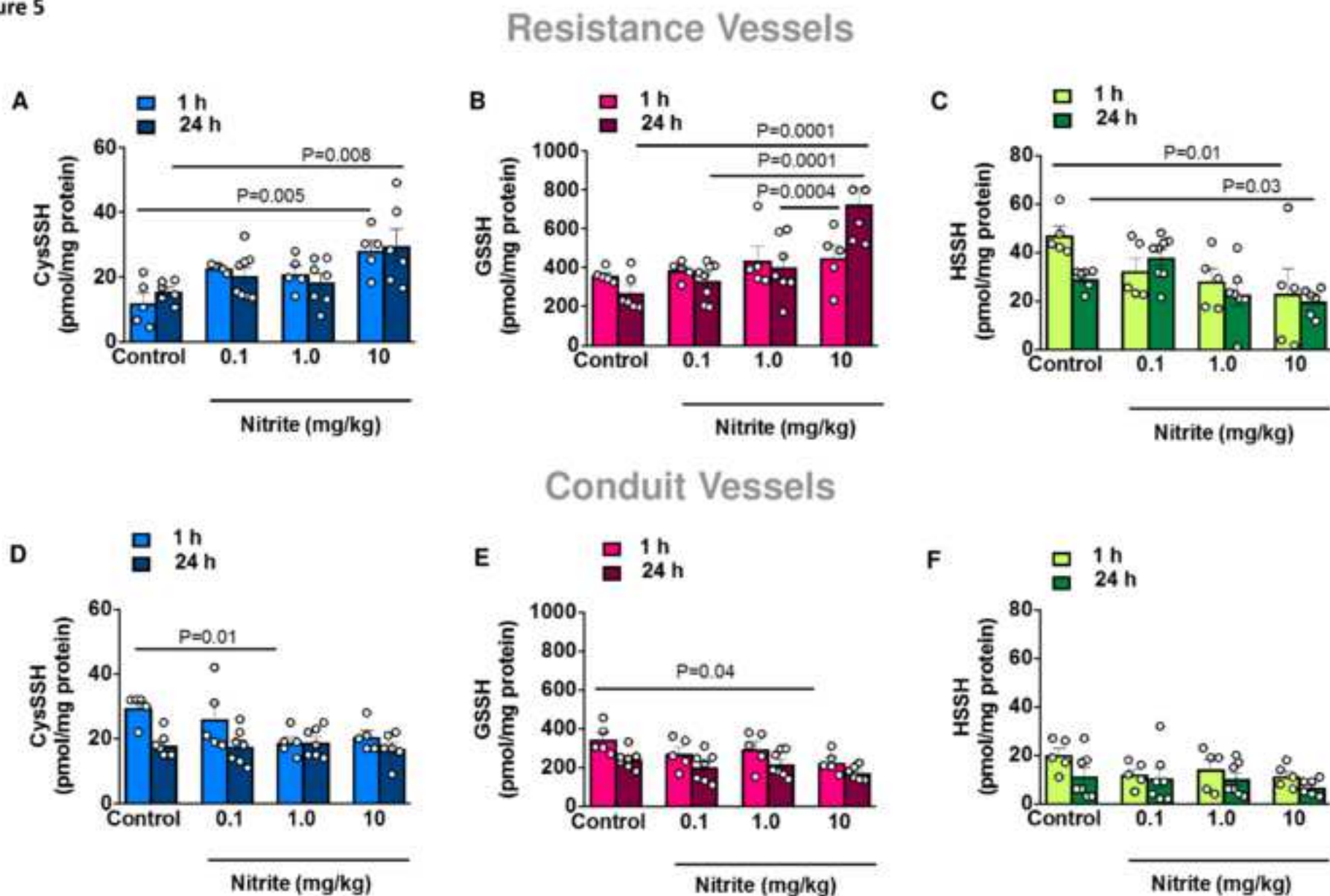


Figure 5





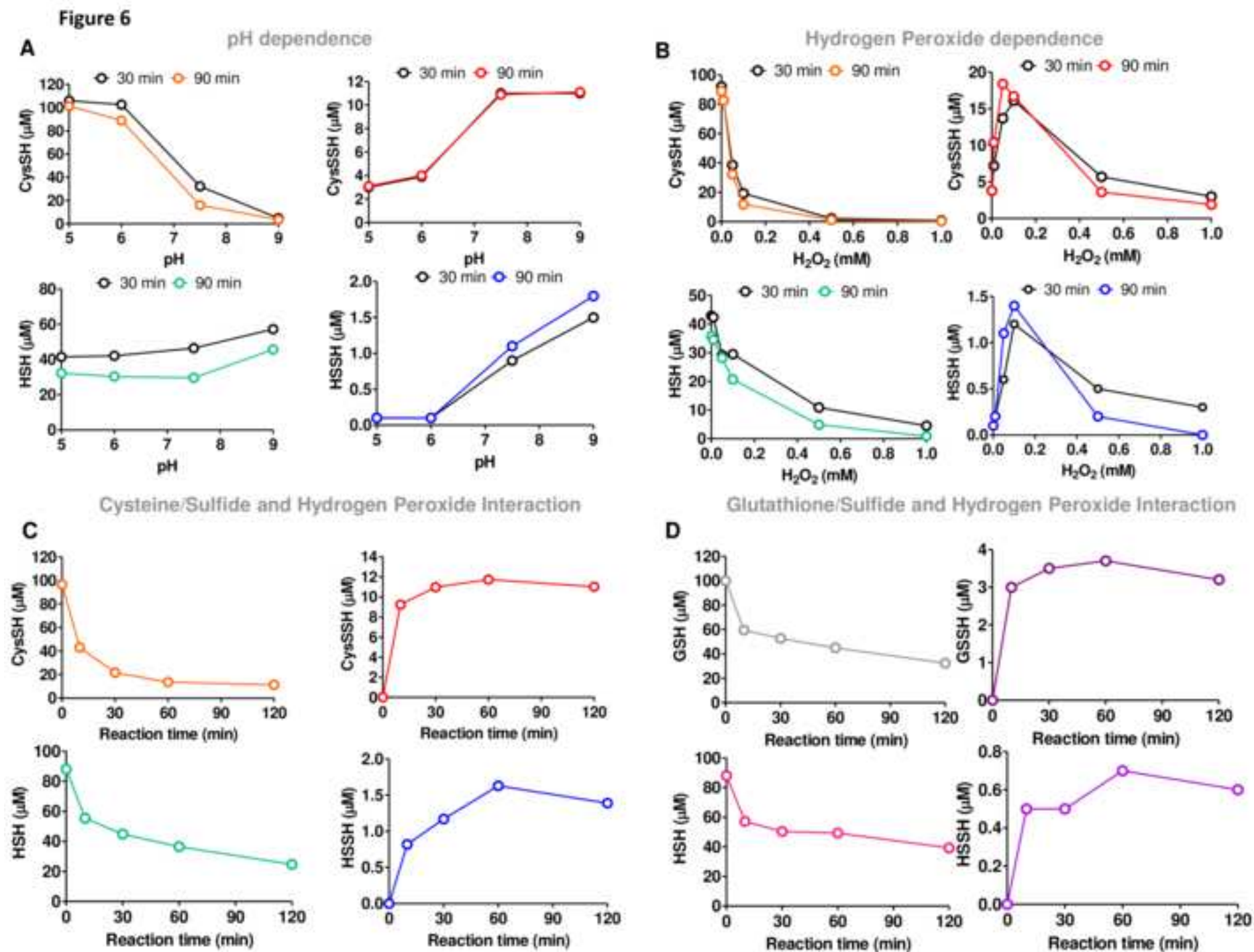


Figure 7

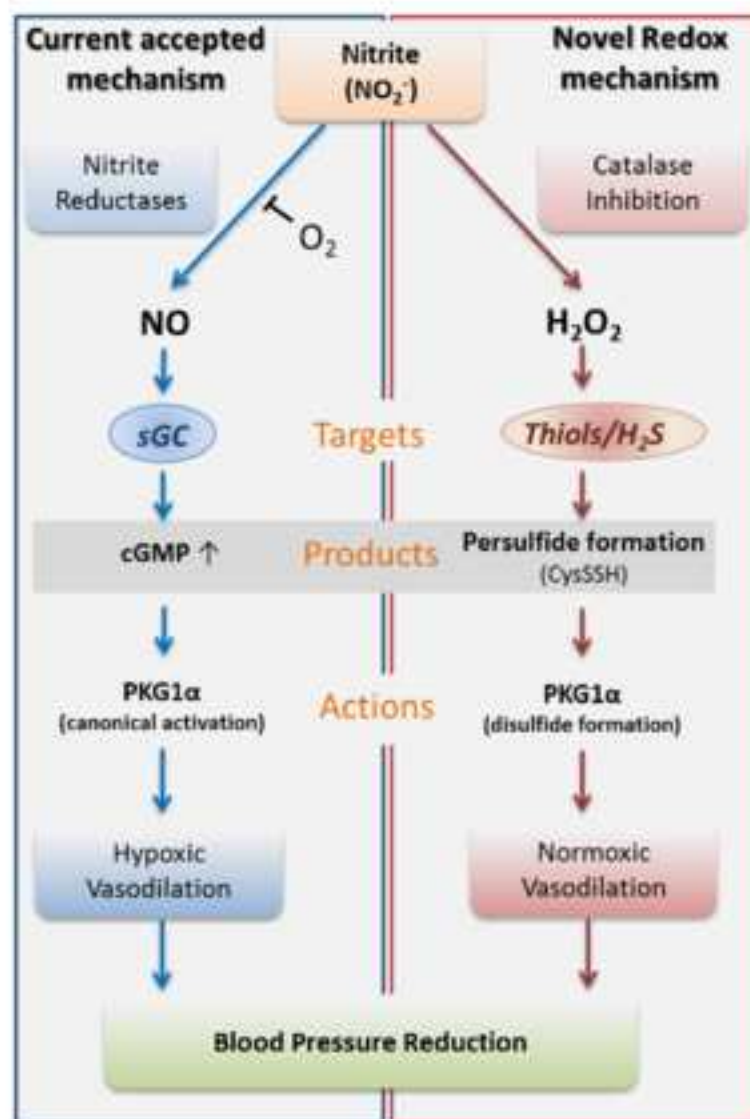


Figure 7

