Role of aldehyde dehydrogenase in hypoxic vasodilator effects of nitrite in rats and humans

Short running title: ALDH and nitrite-mediated vasodilatation

Sayqa Arif[1], Alessandra Borgognone[1], Erica Lai-Sze Lin[1], Aine G O'Sullivan[1], Vishal Sharma[4] , Nigel E Drury[1], Ashvini Menon[1], Peter Nightingale[3], Jorge Mascaro[2], Robert S Bonser[2], John D Horowitz[5], Martin Feelisch[6],Michael P Frenneaux[7]*, Melanie Madhani[1]*

[1]Centre for Cardiovascular Sciences, College of Medical and Dental Sciences, University of Birmingham, UK
[2]Queen Elizabeth Hospital Birmingham, Edgbaston, Birmingham, UK
[3]Wellcome Trust Clinical Research Facility, Edgbaston, Birmingham, UK
[4] Royal[i] Liverpool University Hospital, Liverpool, UK
[5]Basil Hetzel Institute, Queen Elizabeth Hospital, University of Adelaide, Adelaide, Australia
[6]Clinical and Experimental Sciences, Faculty of Medicine, University of Southampton, Southampton, UK
[7] School of Medicine and Dentistry, University of Aberdeen, Aberdeen, UK

* These authors contributed equally to this publication

Address for Correspondence
Dr Melanie Madhani
Centre for Cardiovascular Sciences,
College of Medical and Dental Sciences
University of Birmingham, B15 2TT, UK
E-mail: m.madhani@bham.ac.uk
Tel: +44(0) 121 414 6897

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Abstract

Background and purpose: Hypoxic conditions favour the reduction of nitrite to nitric oxide (NO) to elicit vasodilatation, but the mechanism(s) responsible for bioconversion remains ill defined. In the present study, we assess the role of aldehyde dehydrogenase 2 (ALDH2) in nitrite bioactivation under normoxia and hypoxia in the rat and human vasculature.

Experimental approach: The role of ALDH2 in vascular responses to nitrite was studied using rat thoracic aorta and gluteal subcutaneous fat resistance vessels from patients with heart failure (HF; 16 patients) *in vitro* and by measurement of changes in forearm blood flow (FBF) during intra-arterial nitrite infusion (21 patients) *in vivo*. Specifically, we investigated the effects of (1) ALDH2 inhibition by cyanamide or propionaldehyde and the (2) tolerance-independent inactivation of ALDH2 by glyceryl trinitrate (GTN) on the vasodilator activity of nitrite. In each setting, nitrite effects were measured *via* evaluation of the concentration response relationship under normoxic and hypoxic conditions in the absence or presence of ALDH2 inhibitors.

Key Results: Both in rat aorta and human resistance vessels dilatation to nitrite was diminished following ALDH2 inhibition, in particular under hypoxia. In humans there was a non-significant trend towards attenuation of nitrite-mediated increases in FBF (2.48±0.36 to 1.72±0.15 in pre-and post GTN infusion group, respectively; P=0.08).

Conclusions and implications: In human and rat vascular tissue *in vitro*, hypoxic nitrite-mediated vasodilatation involves ALDH2. In patients with heart failure *in vivo* the role of this enzyme in nitrite bioactivation is at most modest, suggesting the involvement of other more important mechanisms.

Abbreviations:

Blood pressure (BP)

Endothelial nitric oxide synthase (eNOS)

Heart rate (HR)

Heart failure (HF)

Nitrite (NO_2^-)

Nitric Oxide (NO)

Sodium nitrite ($NaNO_2$)

Spermine-NONOate (Sper/NO)

Forearm blood flow (FBF)

Glyceryl Trinitrate (GTN)

Ethylenediamine Tetraacetic Acid (EDTA)

Aldehyde dehydrogenase (ALDH)

Mean arterial blood pressure (MABP)

Methaemoglobin (Met Hb)

Xanthine oxidoreducatse (XOR)

Introduction

Nitrite can be chemically reduced *in vivo* to nitric oxide (NO) to elicit vasodilatation (Lundberg *et al.*, 2011; Totzeck *et al.*, 2012; Bailey *et al.*, 2014). However, *in vitro* this conversion process is relatively slow, perhaps explaining why relatively high, supraphysiological nitrite concentrations are required to relax pre-constricted isolated blood vessels (Maher *et al.*, 2008; Ormerod *et al.*, 2011; Furchgott & Bhadrakom, 1953). We and others have demonstrated that infused nitrite acts as a vasodilator in healthy volunteers (Maher *et al.*, 2008; Gladwin *et al.*, 2000; Larsen *et al.*, 2006; Dejam *et al.*, 2007; Cosby *et al.*, 2003), with greater potency as an arteriolar vasodilator in forearm resistance vessels of patients with heart failure (HF) compared to those of normal subjects (Maher *et al.*, 2013). While several candidate mechanisms have been postulated, the exact mechanism(s) responsible for bioactivation of nitrite in man remain ill defined.

Under conditions of profound hypoxia and/or acidosis, the conversion of nitrite to NO may occur *via* acid disproportionation. Under less extreme conditions, various factors have been reported to convert nitrite to NO. These include xanthine oxidoreductase (XOR), aldehyde oxidase, endothelial nitric oxide synthase (eNOS), and heme proteins (Lundberg *et al.*, 2011;Doyle *et al.*, 1981;Tiso *et al.*, 2011;Basu *et al.*, 2007;Totzeck *et al.*, 2012;Pinder *et al.*, 2009;Li *et al.*, 2008). Previous studies have demonstrated that mitochondrial aldehyde deyhydrogenase (ALDH2) may be an important additional source of nitrite-derived NO in rat heart (Perlman *et al.*, 2009) and the vasculature (Golwala *et al.*, 2009) , but whether the oxidative environment in the vasculature of HF patients may alter the role for ALDH2 remains unknown. Interestingly, ALDH2 is also known to act as a reductase of organic nitrates, such as glyceryl trinitrate (GTN; Chen *et al.*, 2002), and currently GTN is used to treat HF patients. However, prolonged exposure of the vasculature to GTN leads to inactivation of ALDH2. While it has been argued that ALDH2 inactivation is the principal mechanism for the development of nitrate tolerance (Chen *et al.*, 2002;DiFabio *et al.*, 2003), other investigators have demonstrated hysteresis between the onset/offset of ALDH2 inactivation by GTN and the time course of GTN tolerance (D'Souza *et al.*, 2011), potentially permitting the utilization of GTN pre-exposure as an ALDH2-inactivating manoeuvre without induction of GTN tolerance.

Herein, we first explored the putative role of ALDH2 as a contributor to nitrite bioactivation under normoxic and hypoxic conditions, utilizing *in vitro* isolated rat vessels. To translate our findings into the clinical setting we explored the role of ALDH2 in a proof-of-principle study in HF patients *in vitro* (isolated vessel) and *in vivo* forearm blood flow (measured by venous occlusion plethysmography). We chose to evaluate HF patients, first because of the potential therapeutic relevance, and second because we have previously shown that *in vivo* arteriolar responses to nitrite are increased in patients with heart failure *vs* healthy controls (Maher *et al.*, 2013).

Methods

The animal studies were approved by the UK Home Office and conducted according to the Animals (Scientific Procedures) Act 1986 and European Commission

guidelines. The human investigations conformed to the Declaration of Helsinki and were approved by the South Birmingham Research Ethics Committee (10/H1207/50) and registered with the UK Clinical Research Network (UK CRN 9587).

Tension Myography

Male Sprague Dawley rats (250-300g) were anaesthetized by an intraperitoneal injection of sodium pentobarbital (100mg/kg). Thoracic aorta were carefully removed and placed immediately in cold Krebs-bicarbonate buffer (pH 7.4, 95% O_2; 5% CO_2) of the following composition (mmol/L): NaCl (119), KCl (4.7). $CaCl_2$ (2.5), KH_2PO_4 (1.18), $MgSO_4$ (1.19), $NaHCO_3$ (25.1), glucose (11). Thoracic aortas were cleaned from adhering connective tissue and cut into three to four ring segments. Aortic rings were mounted in a myograph (Multi-Myograph 610M,Danish Myotechnology, Denmark) containing 5 ml Krebs-bicarbonate buffer (37°C, pH7.4) and gassed with (95% O_2/5% CO_2). After an equilibration period of 45 min, vessels were normalized, as previously described (Madhani *et al.*, 2003). Following normalization, each vessel was primed with KCl (4.8 mmol/L) before a supramaximal concentration of phenylephrine (PE; 10 µmol/L; Sigma Aldrich, UK) was added. Once the contractile tone had stabilized, acetylcholine (ACh; 1 µmol/L; Sigma Aldrich, UK) was added to the organ bath to assess the integrity of the endothelium. If the constrictor responses to PE were not maintained or ACh elicited relaxations <50% of the PE-induced pre-contractile tone the preparation was discarded. Tissues were then washed for 30 min (by addition of fresh Krebs-bicarbonate buffer at 15 min intervals) after which cumulative concentrations of PE (0.001-1 µmol/L) were added to the myograph. These tissues were then washed over 60 min to restore basal tone before contracting to approximately 80% of the maximum PE-induced response. Once a stable response to PE was achieved, a cumulative concentration response curve to sodium nitrite (Sigma Aldrich, UK) was constructed (0.001-100 µmol/L) under normoxic conditions (95% O_2/5% CO_2). Under hypoxic conditions (95% N_2/5% CO_2; resulting in ≈1% tissue bath O_2 (Maher *et al.*, 2008), the vessels were incubated for 30 minutes before exposure to EC_{80} PE (to achieve similar tension observed at 95% O_2) and administration of sodium nitrite.

To delineate the role of ALDH2 in nitrite-mediated vasorelaxation, concentration-response curves to sodium nitrite were constructed before and after 30-minute incubation with cyanamide (1 mmol/L; ALDH2 inhibitor (DiFabio *et al.*, 2003); Sigma Aldrich, UK), or proprionaldehyde (1 mmol/L; ALDH2 substrate that acts as a competitive inhibitor (DiFabio *et al.*, 2003); Sigma Aldrich, UK) under normoxic and hypoxic conditions.

To determine whether GTN pre-exposure of tissues leads to an attenuation of the response to nitrite (*via* inactivation of ALDH2), aortic rings were exposed to hypoxic conditions as described above and then incubated in the presence or absence of 30 or 100 µmol/L of GTN for a period of 1 hour (Keith *et al.*, 1982). Thereafter, blood vessels were washed every 15 minutes for 1 hour. After pre-contraction to EC_{80} PE, a concentration-response curve to sodium nitrite was constructed.

***In vitro* and *in vivo* analysis in HF patients**

The effect of ALDH2 inhibition on nitrite-mediated vasorelaxation was investigated in HF patients: (1) *in vitro* in isolated resistance vessels obtained from gluteal

subcutaneous fat tissue and (2) *in vivo* by measuring changes in forearm blood flow (FBF) during intra-arterial infusion of nitrite with and without GTN pre-treatment (to decrease ALDH activity).

Patient demographics

Patients were grouped as follows: (1) Biopsy group (*in vitro* myography; n=16), (2) plethysmography study: saline group (n=8) and GTN group (n=13); Table 1). *Inclusion criteria:* systolic HF (LVEF <40), aged between 40-80 years, and non-smoker. *Exclusion Criteria:* treatment with long-acting nitrates, past history of adverse reactions to organic nitrates, hypotension (systolic BP<110mmHg), concomitant warfarin/clopidrogel therapy or with bleeding diathesis, and obstructive sleep apnoea. All subjects were asked to refrain from alcohol, and foods with a high nitrate/nitrite content for 24 hours, and caffeine for 12 hours before the study.

Effect of ALDH2 inhibition in isolated resistance vessels

In a sub-group of nitrite/nitrate naïve HF patients (i.e. no infusions or treatment of sodium nitrite and/or GTN), subcutaneous gluteal fat biopsies were obtained under local anesthetic (2% lidocaine) and placed in cold Krebs-bicarbonate buffer, as previously described (Greenstein *et al.*, 2009). Immediately after harvesting the subcutaneous fat biopsies, small resistance arteries (~250μm ID; 2mm-long) were isolated from the fat, and mounted onto a myograph (Greenstein *et al.*, 2009). In most cases, two vessels from each biopsy were studied. The myograph protocol for normalization, priming of vessels with KCl, the assessment of supramaximal concentration of PE and the endothelial integrity was assessed as above. Cumulative concentration-response curves to sodium nitrite were constructed (0.001-100 μmol/L) under normoxic and hypoxic conditions, as described above.

To investigate whether attenuation of nitrite efficacy during hypoxia was caused by the effects of ALDH2 inhibition on downstream signaling in the NO cascade, concentration-response curves to the NO donor, spermine-NONOate (N-(2-Aminoethyl)-N-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine (Sper/NO); 0.001-1 μmol/L; Calbiochem (EMD Millipore)) were constructed in the absence and presence of the ALDH2 inhibitor, cyanamide, under hypoxic conditions.

The effect of ALDH2 inactivation by GTN on forearm blood flow

To determine the effects of ALDH2 inactivation by GTN on arteriolar responsiveness in patients from HF, forearm vascular responsiveness was assessed using the plethysmography. The ~~plethysmography~~ studies on HF patients were performed in a quiet temperature controlled (22-24°C) laboratory. All subjects were placed in a semi-recumbent position enabling the administration of hypoxia to participants. Changes in FBF were determined in both arms by venous occlusion plethysmography (DE Hokanson, USA) and blood pressure, heart rate (HR) and oxygen saturation were monitored as previously described (Maher *et al.*, 2008). A 27-gauge arterial needle (Coopers Engineering, UK) mounted onto a 16-gauge epidural catheter and sealed with dental wax was then inserted aseptically into the brachial artery of the non-dominant arm and was kept patent by the continuous infusion of normal saline (Baxter Healthcare, USA; 0.9%). An intravenous cannula (20-gauge) was inserted in the

contralateral arm for venous blood sampling. Saline was infused *via* the intra-arterial line at 1 ml/min for 10 min for baseline measurements before infusion of sodium nitrite. Data were acquired from both arms, and any changes observed were corrected for those occurring in the contralateral control arm, and presented as a ratio of FBF (FBF-R) during infusion compared to baseline (Maher *et al.*, 2008).

As depicted in Figure 1, following 10 minutes of rest, two doses of sodium nitrite (Martindale Pharmaceuticals, UK) were infused into the brachial artery of the non-dominant arm (784 nmol/min then 7.84 µmol/min for 20 min each; the infusion rate was 1 ml/min as described previously; Maher *et al.*, 2008). FBF was measured in both arms ~~(Figure 4)~~. Following the second dose of sodium nitrite (7.84 µmol/min), the patients switched from breathing room air to inspiring 12% oxygen via a facemask connected to a 2-way valve. Upon achieving target oxygen saturation of 83-88% the study proceeded with the 7.84 µmol/min infusion of nitrite for a further 10 minutes. Thereafter, patients were randomised to receive either an intravenous infusion of 10 µg/min GTN (to decrease ALDH2 activity; Lipha Pharmaceuticals Ltd, UK) as described previously (Philpott A *et al.*, 2007) or saline (the placebo group; active ALDH2) for 4 hours (15 ml/hr). Following a 30-minute washout period (saline at 1 ml/min), repeat infusions of sodium nitrite was administered during normoxia and hypoxia, as described above.

Upon completion of the plethysmography protocol, the intra-arterial needle was removed. Participants were allowed to rest for 10 minutes following which a subcutaneous fat biopsy was obtained (Figure 1), as described above. Philpott and colleagues have previously reported that low dose GTN infusion rapidly inactivates ALDH2 and that this inactivation occurs prior to the development of significant nitrate tolerance (Philpott A *et al.*, 2007). Therefore, to determine whether infusion of GTN had induced significant nitrate tolerance, GTN vasodilator concentration-response curves were compared in resistance vessels taken *via* gluteal biopsy in patients from the saline (placebo group; active ALDH2) and ~~with/without prior~~ GTN (inactive ALDH2) infusion group. Briefly, the isolated tissue resistance vessels were mounted in a myograph, the vascular rings were assessed for endothelial integrity, and then contracted sub-maximally with PE and cumulative concentration-response curves were constructed for GTN (0.001-1 µmol/L) from the saline and GTN infusion HF patient group, respectively.

Blood samples

Venous blood samples were taken at eight time points during the plethysmography study (Figure 4) for determination of venous methaemoglobin (MetHb) and pH (blood gas analyser Bayer Rapidlab 865, Siemens, NY), and determination of total plasma 8-iso prostaglandin F2α (8-Isoprostane EIA assay kit protocol, Cayman Chemical, MI).

~~**Assessment of potential induction of GTN tolerance.**~~

~~To determine whether infusion of GTN had induced significant nitrate tolerance, GTN vasodilator concentration-response curves were compared in resistance vessels taken *via* gluteal biopsy in patients with/without prior GTN infusion. The vascular tissue was mounted in a myograph as described above, and cumulative concentration-response curves were constructed for GTN (0.001-1 µmol/L).~~

Isolation of mitochondrial fraction

Rat aortic vessels that were treated as described above in the tension myography studies were immediately snap frozen at the end of the protocol for isolation of the mitochondrial fraction. Frozen thoracic aorta were suspended in the mitochondrial buffer containing 10 mmol/L MOPS (pH 7.2), 10 mmol/L KCl, 1.5 mM $MgCl_2$, 1 mmol/L EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 0.25 mol/L sucrose, and gently homogenized with a Dounce homogenizer (30 strokes), as previously described (Paneni *et al.*, 2013). The homogenate was centrifuged at 750g for 10 minutes at 4°C to remove nuclei and unbroken cells, and the supernatant was subsequently centrifuged at 10,000g for 15 minutes. The resultant mitochondrial pellet was used for the ALDH2 assay kit (see ALDH2 activity assay for details).

Mitochondrial ALDH2 activity assay

ALDH2 activity was determined in mitochondria isolated from rat thoracic aorta following solubilisation and extraction as specified in the manufacturer's recommendations (mitochondrial aldehyde dehydrogenase 2 activity assay kit, Abcam). The homogenate was then incubated on ice for 20 minutes, centrifuged at 16,000 x g for 20 minutes at 4°C. Protein concentration of the supernatant was determined and 20 μg protein was used to detect ALDH2 activity. In this assay, the generation of NADH is coupled to the 1:1 reduction of a reporter dye to yield reaction product concentration of which is monitored by measuring the absorbance increase at 450nm.

Statistical Analysis

All data are expressed as mean±SEM, and significance was accepted with $P<0.05$. For the myography analysis, concentration-response curves were analysed using 2-way ANOVA. For the *in vivo* FBF analysis, 1-way ANOVA repeated measures coupled with a Bonferroni post-hoc test was used to compare effects of pre-saline/GTN or post-saline/GTN infusion treatment. A paired non-parametric test (Wilcoxon signed ranks test) was used to compare FBF following hypoxia between pre-GTN infusion and post-GTN infusion. Statistical analysis was undertaken using Prism software (version 4.0, GraphPad Software, CA).

Results

Evaluation of the role of ALDH2 in nitrite-mediated bioactivation in rat aorta

As depicted in figure 2, the vasorelaxant response to sodium nitrite displays a biphasic concentration-response relationship. As shown in Figure 2A, following incubation of vessels with the ALDH2 inhibitor cyanamide during normoxic conditions, vasorelaxation by sodium nitrite was paradoxically enhanced at nanomolar concentrations and significantly inhibited at higher sodium nitrite concentrations (10 and 30μM) when compared to controls ($p<0.05$; by two-way ANOVA). Hypoxia enhanced nitrite-induced relaxation, but pre-treatment with cyanamide during hypoxia caused a significant attenuation of nitrite's efficiency to relax blood vessels at all concentrations tested when compared to the control ($p<0.05$; by two-way ANOVA; Figure 2B). Similar results were obtained using the ALDH2 substrate, propionaldehyde. Incubation of vessels with propionaldehyde under normoxic conditions blunted the

vasorelaxant effect of low concentrations of nitrite without affecting its efficacy at higher concentrations, but these differences did not reach statistical significance ($p>0.05$; by two-way ANOVA; Figure 2C). However under hypoxic conditions, propionaldehyde significantly attenuated the response to nitrite in the nano- to micromolar concentrations range when compared to control ($P< 0.05$; by two-way ANOVA; Figure 2D) without affecting maximal relaxation induced by the highest concentration of nitrite.

Effect of ALDH2 inhibition on nitrite-mediated vasorelaxation in resistance vessels from HF patients

Under both normoxic and hypoxic conditions, sodium nitrite caused concentration-dependent relaxation of resistance vessels from HF patients (Figures 3A and B). During normoxic conditions, pre-treatment with cyanamide tended to shift the sodium nitrite concentration-response curve to the right but this was not statistically significant (Figure 3A). Under hypoxic conditions, pre-treatment with cyanamide caused a marked and concentration-dependent attenuation of the relaxation responses to sodium nitrite when compared to control ($p<0.05$; by two-way ANOVA; Figure 3B). To assess whether this effect was nitrite-specific, concentration-response curves to the NO donor Sper/NO, were constructed in the presence or absence of cyanamide. Cyanamide had no inhibitory activity on the vasorelaxant responses to Sper/NO under hypoxic conditions (Figure 3C).

Effect of GTN pretreatment on nitrite-mediated vasorelaxation in rat aorta

In addition to the above inhibitor/substrate experiments we also assessed the effects of GTN (used as a tool to decrease ALDH2 activity) on nitrite-mediated vasorelaxation. Using a well-established *in vitro* model of nitrate tolerance in rat isolated aorta (Keith *et al.*, 1982;Irvine *et al.*, 2007), blood vessels were pre-treated with GTN (30 or 100 µmol/L) for 1 hour followed by construction of a concentration-response curve to sodium nitrite under hypoxic conditions. Pre-treatment with GTN (30 and 100 µmol/L) significantly attenuated the response to sodium nitrite compared to controls ($p<0.05$; by two-way ANOVA; Figure 4A), particularly at higher concentrations. As shown in Figure 4B, mitochondrial ALDH2 activity was significantly decreased following pretreatment with GTN (30 and 100 µmol/L) when compared to control (sodium nitrite; $p<0.05$; by one-way ANOVA).

Effects of GTN-induced ALDH2 inactivation on nitrite-mediated vasodilation in the human forearm

Forearm blood flow corrected for changes in the non-infused arm (FBF-ratio) increased dose-dependently with sodium nitrite in the saline (placebo) and GTN groups, respectively (Figure 5A and B). As depicted in Figure 5A, 1-way ANOVA repeated measures showed that 7.84 µmol/min nitrite significantly increased FBF in the pre-saline infusion group from 0.94±0.09 at baseline to 1.69±0.21 during normoxic ($P<0.05$ compared to baseline) and 1.76±0.22 during hypoxic conditions ($P<0.05$ compared to baseline; Figure 5A). Following 4 hour i.v saline infusion (placebo group; active ALDH2), a similar profile in FBF-R response to sodium nitrite was observed when compared to pre-saline infusion. 7.84 µmol/min nitrite significantly increased FBF in the post-saline infusion group from 1.02±0.04 at baseline to 1.93±0.14 during normoxic

(P<0.001 compared to baseline) and 2.05±0.18 during hypoxic conditions (P<0.001 compared to baseline).

In the GTN group (Figure 5B), prior to administration of i.v. GTN, 1-way ANOVA repeated measures showed that 7.84 μmol/min nitrite infusion significantly increased FBF from 1.24±0.08 at baseline to 2.34±0.29 during normoxic (P<0.05 compared to baseline) and 2.48±0.36 (P<0.01 compared to baseline) during hypoxic conditions. Following 4hr infusion of GTN to inactivate ALDH2, although 7.84 μmol/min nitrite infusion significantly increased FBF from 1.20±0.11 (baseline) to 1.95±0.15 during normoxic conditions (P<0.001 when compared to baseline) and 1.72±0.15 during hypoxia (P<0.05 when compared to baseline). However, GTN treatment tended to attenuate the increase in FBF following nitrite infusion (7.84 μmol/min) when compared to the pre-GTN infusion. Using a paired non-parametric test (Wilcoxon signed ranks test) we compared pre- and post-GTN infusion for each of baseline, 784 nmol, and 7.84 μmol at normoxic conditions and at hypoxic conditions, respectively. There was no significant difference between pre-GTN *vs* post-GTN infusion at baseline (1.24±0.09 and 1.20±0.11, respectively; P=0.79), pre-GTN *vs* post-GTN infusion with 784 nmol/min (2.16±0.72 and 1.35±0.10, respectively; P=0.34), or at pre-GTN *vs* post GTN infusion with 7.84 μmol/min (2.34±0.29 and 1.95±0.15, respectively; P=0.28). During hypoxic conditions, GTN treatment attenuated the increase in FBF following nitrite infusion (7.84 μmol/min) but this effect did not reach statistical significance (2.48±0.36 and 1.72±0.15, pre- and post-GTN infusion, respectively; P=0.08; Figure 5C).

GTN infusion does not induce tolerance in resistance vessels

To confirm that our low dose GTN infusion protocol did not induce systemic nitrate tolerance, a gluteal biopsy was obtained at the end of the plethysmography protocol and isolated resistance vessels were used for myography experiments. There was no attenuation of the vasodilator response to GTN compared to that in the control vessels isolated from the saline infusion group (P=0.87; Figure 5D).

Assessment of haemodynamics, venous pH, 8-isoprostanes and methaemoglobin formation during nitrite infusion in HF patients

Heart rate (HR) and mean arterial blood pressure (MABP) did not alter significantly from baseline following administration of sodium nitrite in the saline and GTN groups (Table 2). Arterial oxygen saturations remained stable throughout the normoxia period in the saline group, but fell significantly following administration of 12% oxygen in both the saline and GTN groups (from 97±0.5% to 87±1.1%,p<0.001 and from 97±0.3 to 87±0.6 P<0.001 respectively). There was no significant change in either venous pH (saline group n=5-7; GTN group n=9-11; P>0.05) or 8-isoprostane levels (saline group n=4-6; GTN group n=7-9; P>0.05) in both the saline and GTN groups.

Following 4-hours saline infusion, venous MetHb levels increased during escalating sodium nitrite dose infusions from 0.31±0.04% to 1.39±0.21% (baseline and 7.84 μmol/min nitrite, normoxia) of total haemoglobin. Highest levels were reached following 7.84 μmol/min nitrite infusion during hypoxic conditions (1.63±0.17%; P<0.001; n=5-7). In the GTN group, a similar trend was observed, MetHb levels increased from 0.37±0.06% to 1.51±0.18% (baseline and 7.84 μmol/min nitrite,

respectively, normoxia) with the highest levels of MetHb (1.57±0.15%; P<0.001; n=9-11) reached following 7.84 µmol/min nitrite infusion during hypoxic conditions. There was no significant difference between the two groups. Pre-infusion data were also similar for the haemodynamics and MetHb pre-saline/GTN (data not shown).

Discussion

We previously demonstrated that intra-brachial artery infusion of nitrite causes forearm vasodilatation in both healthy subjects and in HF patients, with greater potency in the latter (Maher *et al.*, 2013), but the mechanism(s) underlying these effects remain incompletely understood. Based on these observations, we here explored the role of ALDH2 in nitrite bioactivation under normoxia and hypoxia in both rats and human vasculature. Our findings suggest that ALDH2 plays a major role in nitrite-mediated vasorelaxation *in vitro*. However, this effect was clearly less marked *in vivo*, and we postulate that this could be due to the presence of multiple *in vivo* mechanisms.

Several nitrite-reductases have been identified, including eNOS, XOR, aldehyde oxidase, and heme proteins such as deoxyhaemoglobin and myoglobin to bioactivate nitrite under hypoxia (Tiso *et al.*, 2011;Lundberg *et al.*, 2011;Doyle *et al.*, 1981;Basu *et al.*, 2007;Baliga *et al.*, 2012;Ghosh *et al.*, 2013;Pinder *et al.*, 2009). All of these agents alone and/or in combination are capable of bioconverting nitrite to NO *in vitro*, but their physiological role remains incompletely understood. Previous studies have reported that ALDH2 may be an important source of nitrite-derived NO in the heart (Perlman *et al.*, 2009) and vasculature (Badejo, Jr. *et al.*, 2010), but whether the oxidative environment in the vasculature alters the effects of ALDH2 on nitrite-mediated vasorelaxation remains unclear. Therefore, in the present study we first explored the role of ALDH2 in nitrite-mediated vasorelaxation *in vitro* in isolated rat conduit vessels (aorta) during normoxic and hypoxic conditions. Our *in vitro* data shows that the ALDH2 inhibitor, cyanamide and the ALDH2 substrate, propionaldehyde significantly reduced the potency of nitrite under hypoxic conditions. These data indicate the potential for ALDH2 to be involved in hypoxic nitrite vasodilatory responses.

Much of our current understanding about nitrite's mode of action as a vasodilator is based on animal experimental work and considerably less information is available on the mechanism of vasodilatation by nitrite in human tissue. Nevertheless, studies in healthy human subjects have shown that nitrite causes marked venodilation and moderate dose-dependent arteriolar dilatation, and these effects are augmented by hypoxia or exercise in healthy subjects (Maher *et al*., 2008; Cosby *et al*., 2003). We have previously shown that *in vivo* arteriolar responses to nitrite are increased in patients with heart failure *vs* healthy controls (Maher *et al.*, 2013). Due to the potential therapeutic relevance of these findings we now chose to translate these observations to the clinical setting by evaluating the role of ALDH2 in HF patients *in vitro* and *in vivo.* Since the vascular responses to intra-arterial nitrite are most accurately assessed by measuring changes in forearm blood flow (i.e. resistance vessel effects) we first investigated whether the effects of ALDH2 inhibition on nitrite-mediated vasorelaxation observed in the isolated rat conduit vessels were replicated *in vitro* in resistance vessels from patients with HF. We confirmed that the inhibition of ALDH2 significantly attenuates nitrite-mediated vasorelaxation during hypoxic conditions. Importantly, cyanamide did not alter the potency of the NO-donor Sper/NO, ruling out non-specific effects of this inhibitor on NO bioactivity. These observations concur with

functional data obtained by Huellner and colleagues, where pharmacological inhibition of ALDH2 did not alter the potency to the NO-donor DEA/NO in isolated human veins (Huellner *et al.*, 2008). To our knowledge, this is the first study in man to assess the effects of sodium nitrite in isolated resistance vessels from HF patients. Interestingly, the magnitude difference in vasorelaxation response to sodium nitrite observed in the normoxic versus hypoxic group of blood vessels from HF patients was similar. Moreover, the response to sodium nitrite when comparing rat thoracic aorta versus HF patient resistance vessels was also different. This might be related to different vascular beds, species differences or the heterogeneity in vascular response between healthy and pathophysiology tissues. Regarding the latter, based on our previous findings (Maher *et al.*, 2013), we believe that the reason why the magnitude difference in nitrite-induced relaxation is not that different between normoxia *vs* hypoxia in isolated resistance vessels from HF patients when compared to conduit vessels (thoracic aorta) from healthy rats is because firstly, the vasomotor response to nitrite is altered and enhanced and therefore the magnitude is not that different when compared to hypoxia as suggested from our previous *in vivo* studies (Maher *et al*., 2013).

Previous studies have reported that prior GTN exposure induces nitrate tolerance in association with attenuated ALDH2 activity in the vasculature (DiFabio *et al.*, 2003;D'Souza *et al.*, 2011;Huellner *et al.*, 2008). Therefore, using a well established *in vitro* model of nitrate tolerance in isolated rat aorta (Keith *et al.*, 1982;Irvine *et al.*, 2007), we demonstrated attenuation of nitrite-mediated vasorelaxation following pre-treatment with GTN during hypoxic conditions. To substantiate the role of ALDH2 as a key effector in nitrite-mediated vasorelaxation, we here show that this attenuation in vascular response is associated with reduced mitochondrial ALDH2 activity.

It is important to recognize that there are 19 known human ALDH isoenzymes, of which only a few, such as ALDH2 (Koppaka *et al.*, 2012) have been thoroughly characterised biochemically and by susceptibility to pharmacological inhibition. To date, no antagonists have been developed to specifically inhibit each ALDH isoenzyme without affecting others. Moreover, clear sensitivity differences to different ALDH2 inhibitors have been reported, for example GTN bioactivation in rat liver was sensitive to chloral hydrate but not to daidzin (Kollau *et al.*, 2005). In the current study, we chose to use cyanamide (an irreversible inhibitor) and propionaldhyde (a reversible competitive inhibitor) as both are capable of inhibiting ALDH and have been used to examine the role of ALDH2 in organic nitrate-induced vasorelaxation in rat aorta (Chen *et al.*, 2002;DiFabio *et al.*, 2003). Notwithstanding possible species differences in sensitivity to both inhibitors and vasodilators (including further differences in bioactivation mechanisms), we further complemented these results by a mechanism-based ALDH2 inhibition approach employing an *in vitro* tolerance model and have reached the same conclusion, i.e. that ALDH2 inhibition exerts inhibitory actions of sodium nitrite-mediated vasorelaxation *in vitro*.

Assessing the involvement of specific enzymes in mediating the vasodilator effects of nitrite in man *in vivo* remains a challenge. The ALDH2 inhibitor disulfiram is commonly used in clinical studies without ill effect (Johansson, 1992;Mackenzie *et al.*, 2005), but its use in HF patients is contra-indicated (Huffman & Stern, 2003). Therefore, in the present study we elected to use a low dose GTN infusion as it has previously been reported to rapidly inactivate vascular ALDH2, and that this inactivation occurs prior to the development of significant nitrate tolerance and prior to

detectable impairment of GTN bioconversion (Philpott A *et al.*, 2007). We measured the vasodilator activity of nitrite in HF patients and tested whether pre-infusion of GTN (in order to decrease ALDH2 activity) attenuates forearm vasodilatory response to nitrite during normoxic and hypoxic conditions. Despite the well-recognized presence of both endothelial dysfunction and NO resistance in HF patients (Marti *et al.*, 2012) adequate vascular activity in response to nitrite was observed in our study. During normoxic conditions, we found an approximate 2-fold increase in forearm vasodilatory response following 7.84 μmol/min of sodium nitrite in both the saline and GTN group compared to their baseline responses. These results are similar to our previous findings in healthy volunteers (Maher *et al.*, 2008). Under hypoxic conditions inhibition of ALDH2 with GTN (post-GTN infusion group) tended to reduce forearm vasodilatory response but was not significant when compared to the pre-GTN infusion group. Our observation in gluteal resistance vessels taken at the end of the *in vivo* study confirmed that the low dose GTN regime had not induced systemic tolerance, which is consistent with a previous study demonstrating that the same regime inhibited ALDH2 without inducing GTN tolerance (Philpott A *et al.*, 2007).

Study limitations

Despite strict inclusion/exclusion criteria to standardise experimental procedures, a degree of heterogeneity will exist in the population studied in terms of cause and severity of HF. Furthermore, we did not measure plasma levels of nitrite/nitrate as the results are likely to have been affected by the local and systemic metabolism of GTN to nitrite and nitrate, masking any changes to circulating endogenous nitrite/nitrate levels that may have occurred as a result of ALDH2 inhibition. Finally, we were unable to confirm in our *in vivo* studies whether ALDH2 activity was actually attenuated by GTN.

Conclusions

Our *in vitro* data confirms a role for ALDH2 in nitrite-mediated vasorelaxation during hypoxic conditions in both rat aorta and human resistance vessels. Since nitrite is under consideration as a therapeutic agent, the role for ALDH2 in the bioactivation of nitrite during hypoxic vasodilatation is of importance. Our *in vitro* experiments demonstrate that nitrite could directly affect vasorelaxation without necessitating interaction with blood constituents. However, we cannot discount other non-enzymatic and/or nitrite reductase species (e.g. heme proteins, eNOS, XOR and aldehyde oxidase), which may contribute to the reduction of nitrite during hypoxic conditions *in vitro* (Baliga *et al.*, 2012;Ghosh *et al.*, 2013;Li *et al.*, 2008;Pinder *et al.*, 2009;Totzeck *et al.*, 2012). Furthermore, although our *in vivo* results demonstrate a (non-significant) trend towards attenuation of forearm vasodilatory response to nitrite following GTN treatment, we suggest that this is probably due to a decrease in ALDH2 activity. However, no firm conclusion can be drawn about the mechanism sub-serving this effect *in vivo*. The fact that this effect was non-significant (P=0.08) may reflect the presence of multiple *in vivo* mechanisms. In particular, we cannot exclude the role for deoxyhemoglobin *in vivo* since blood-borne NO species derived from nitrite may contribute to its vasodilatory effects (Angelo *et al.*, 2006). In addition, previous work by Gladwin's group has demonstrated that the inhibition of eNOS or XOR does not attenuate nitrite-induced vasodilatation in healthy volunteers (Dejam *et al.*, 2007;Cosby *et al.*, 2003). However, although it seems that XOR may not contribute to nitrite reduction in health, a recent

work by Ghosh et al. reported that XOR does mediate nitrite reduction during pathological conditions (hypertensive animals and patients) (Ghosh *et al.*, 2013). Thus, their relative contribution to the hypoxic vasodilatory effect of nitrite in man *in vivo*, in particular in patients with HF, remains unresolved and is yet to be fully explored.

Moreover, the observations of the current study suggest that pre-exposure to GTN (even without inducing tolerance) might attenuate nitrite vasodilatory effects. This finding may be of clinical importance when considering nitrite as a therapeutic intervention on patients who may have received recent therapy with organic nitrates. Further studies are required to elucidate the extent to which ALDH2 is involved in nitrite reduction to NO *in vivo* in man.

Author Contribution

SA, AB, LL & AGO – performed the research, data acquisition, and analysis; VS, NED, AM, JM - performed the research; PN – data analysis; RSB, JDH – design of the work and interpretation; MF, MPF, & MM: design of the work, data interpretation, article drafting and revision; MPF & MM – supervision of the study concept, article drafting and final revision.

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Conflict of interest

The authors disclose no conflict of interest.

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Tables

Table 1. Patient Demographics. NYHA, New York Heart Association classification; MABP, mean arterial blood pressure; ACEI, angiotensin converting enzyme inhibitors; ARB, angiotensin II receptor blockers. Data expressed as mean±SEM.

Table 2. Assessment of haemodynamic, venous pH, 8-isoprostane and methaemoglobin. Heart rate (HR), mean arterial blood pressure (MABP), arterial oxygen (O_2) saturation, pH, plasma 8-isoprostane, methaemoglobin (MetHb%) measurements following sodium nitrite infusion in the saline or GTN group. Data expressed as mean±SEM. P*<0.0001

Figure Legends

Figure 1. Schematic protocol of the plethysmography study.

Figure 2. ALDH2 inhibition attenuates nitrite-induced vasorelaxation in rat aortae Concentration-response curves to sodium nitrite in the presence or absence of ALDH2 inhibitor, cyanamide during (A) normoxia (n ≥ 5), (B) hypoxia (n ≥ 5), and ALDH2 substrate, propionaldehyde during (C) normoxia (n ≥ 5) and (D) hypoxia (n ≥ 5). Relaxation is expressed as mean±SEM percentage reversal of PE-induced tone. *P<0.05, **P<0.01 and ***P<0.001 control versus cyanamide or propionaldehyde by two-way ANOVA.

Figure 3. ALDH2 inhibition decreases nitrite-induced vasorelaxtion in resistance vessels from HF patients. Concentration-response curve to sodium nitrite in the presence or absence of cyanamide during (A) normoxia (n=7) and (B) hypoxia (n=9). (C) Concentration-response curve to Sper/NO in the presence or absence of cyanamide during hypoxic conditions (n ≥6). Relaxation is expressed as mean±SEM percentage reversal of PE-induced tone. *P< 0.05 and **P<0.01 control versus cyanamide by two-ANOVA.

Figure 4. Tolerance-independent inactivation of ALDH2 attenuates vasorelaxation in rat aortae (A) Concentration-response curve to $NaNO_2$ in the presence or absence of GTN during hypoxic conditions. Relaxation is expressed as mean±SEM percentage reversal of PE-induced tone (n=10); *p<0.05, ***P< 0.001 versus control 2-way-ANOVA. (B) The effect of sodium nitrite (control) in the presence or absence of GTN during hypoxic conditions on mitochondrial ALDH2 activity (mean±SEM from n 4-6 animals; *p<0.05 versus control by 1-way-ANOVA).

Figure 5. GTN infusion attenuates nitrite-induced vasorelaxation in the resistance vasculature of HF patients Forearm vasodilatory measurements following nitrite infusion in HF patients subjected to 4 hrs infusion of (A) saline (n=8 patients) or (B) GTN (n=11 patients) treatment. *P<0.05 compared to baseline; **P<0.01 compared to baseline; ***P<0.001 compared to baseline. (C) Comparison of pre- and post-GTN infusion following 7.84 µmol/min sodium nitrite during hypoxic conditions (n=8 and n=11 patients, respectively; P=0.08). (D) Concentration-response curve to GTN in isolated resistance vessels from HF patients (saline n=7; GTN n=11 patients).

Table 1

	In vitro analysis	*In vivo* analysis	
	Biopsy only group (n=16)	**SALINE group (n=8)**	**GTN group (n=13)**
Age (years)	64.5±3.8	62±4.0	66±3.3
Male gender, n (%)	13 (81)	7(88)	12(92)
Mean weight (kg)	78.3±3.8	74.6±2.5	82.3±3.3
Body mass index (kg/m^2)	27.8±1.5	25.4±0.5	27.1±0.9
Ejection fraction (%)	26.4±2.3	25.1±2.5	27±2.1
NYHA class			
I	2	1	1
II	6	4	10
III	8	3	2
Heart rate (bpm)	72±2.8	62±4.4	62±2.0
MABP (mmHg)	95±4.1	89±2.9	88±2.2
Aetiology, n(%)			
Dilated cardiomyopathy	8 (50)	5 (62)	6 (46)
Ischaemic cardiomyopathy	6(38)	3 (38)	6 (46)
Other	2 (13)	0	1 (8)
Medication, n (%)			
ACEI/AT2 antagonists	15 (94)	8 (100)	12 (92)
Beta blockers	10 (63)	5 (62)	10 (77)
Spironolactone/eplerenone	10 (63)	3 (38)	3 (23)
Loop diuretic	12 (75)	4 (50)	8 (62)
Aspirin	12 (75)	4 (50)	10 (77)

Table 2

	Saline Group				GTN group			
	BL	**784nmol/min**	**7.84µmol/min**	**Hypoxia + 7.84µmol/ min**	**BL**	**784nmol/min**	**7.84µmol/min**	**Hypoxia + 7.84µmol/ min**
HR (BPM)	62±4.1	64±4.1	65±4.8	68±5.7	62±2.3	62±2.2	60±1.6	6.4±0.9
MABP (mmHg)	84±1.9	85±2.9	78±2.5	80±2.6	83±2.8	83±2.6	86±2.5	85±2.6
Arterial O_2 Sat (%)	97±0.5	97±0.5	97±0.5	87±1.1*	97±0.3	98±0.3	98±0.3	87±0.6*
pH	7.37±0.02	7.39±0.01	7.40±0.01	7.41±0.01	7.37±0.01	7.38±0.01	7.38±0.01	7.39±0.01
Isoprostanes (ng/L)	5.96±0.55	7.42±0.93	7.13±0.39	7.29±0.88	7.61±1.00	9.14±1.75	7.54±0.83	9.45±2.45
MetHb (% of total Hb)	0.31±0.04	0.61±0.08	1.39±0.21*	1.63±0.17*	0.37±0.06	0.58±0.07	1.51±0.18*	1.57±0.15*

Figure 1

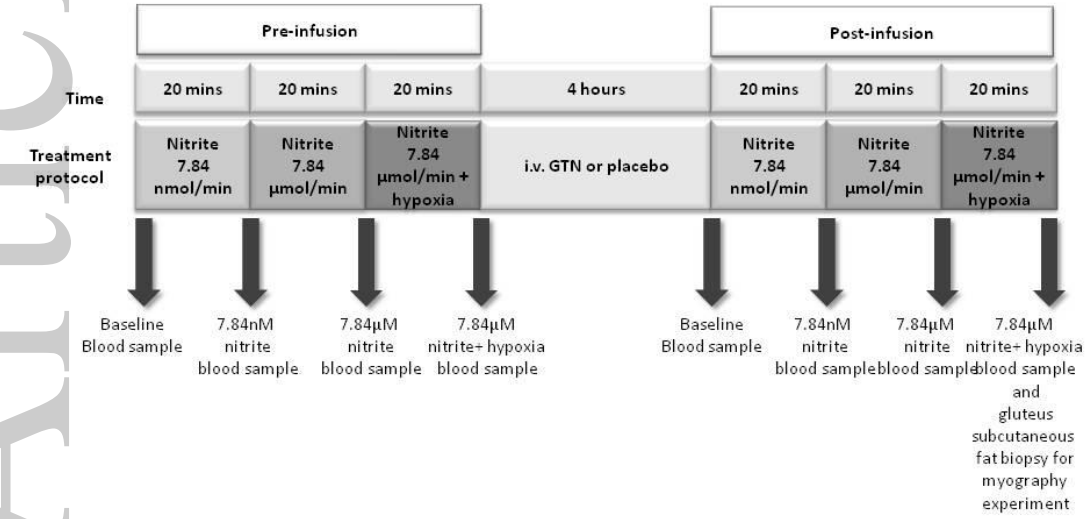

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Figure 2

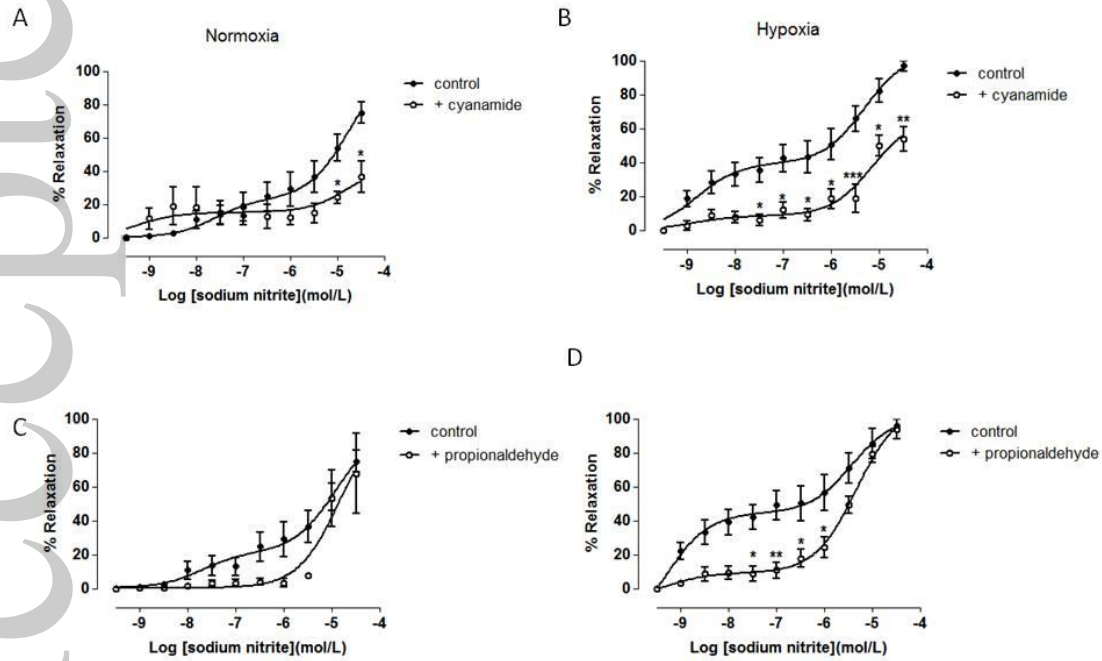

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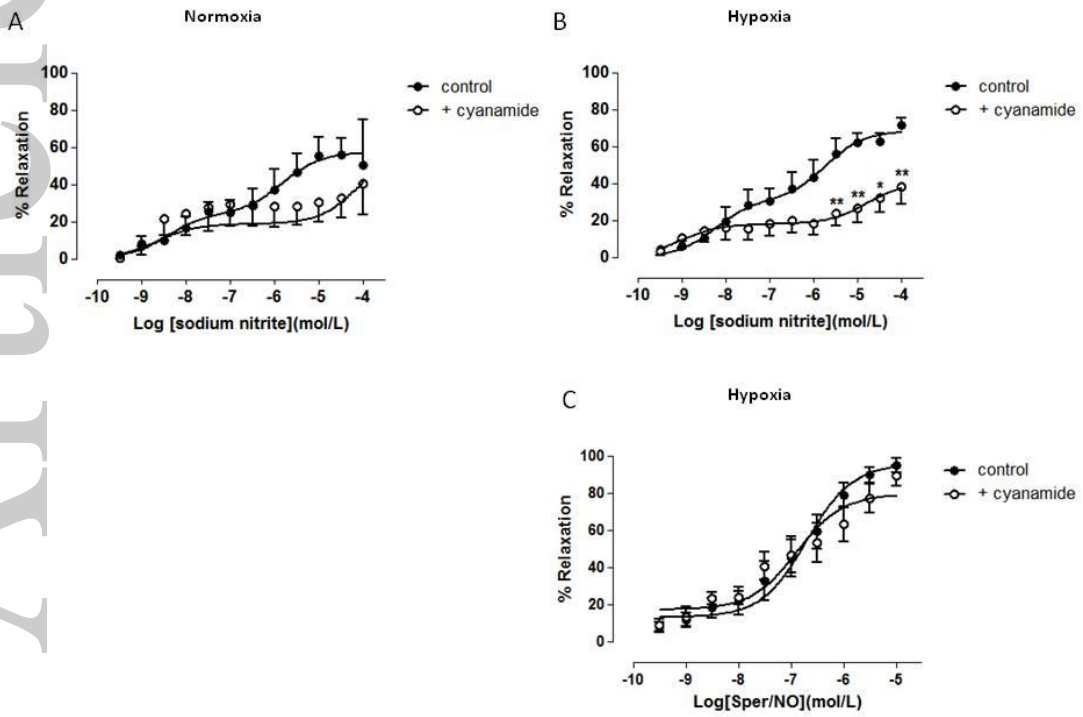
Figure 3



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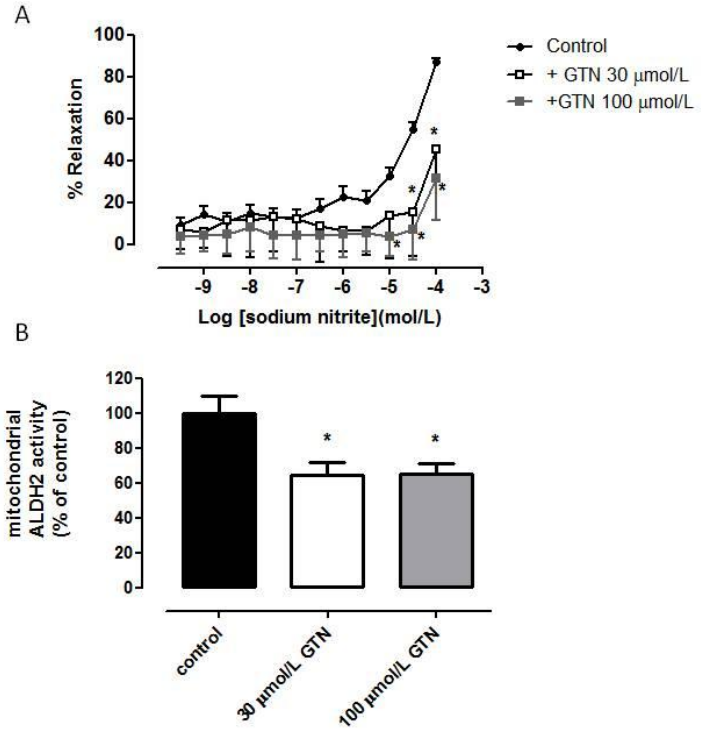

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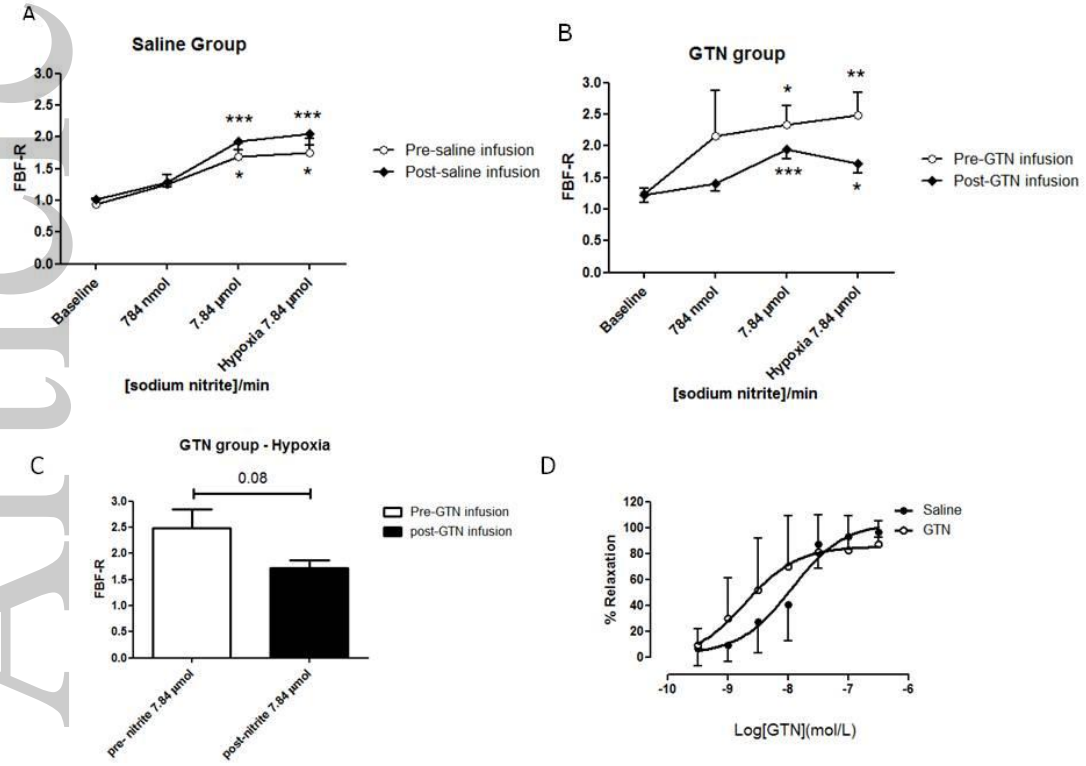
Figure 5



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