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THEMED ISSUE ARTICLE

Inorganic nitrate attenuates cardiac dysfunction: roles for xanthine oxidoreductase and nitric oxide

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Background and purpose: NO is a vasodilator and independent modulator of cardiac remodelling. Commonly, in cardiac disease (e.g., heart failure), endothelial dysfunction (synonymous with NO deficiency) has been implicated in increased BP, cardiac hypertrophy and fibrosis. Currently, no effective therapies replacing NO have succeeded in the clinic. Inorganic nitrate (NO_3^-), through chemical reduction to nitrite and then to NO, exerts potent BP lowering, but whether it might be useful in treating undesirable cardiac remodelling is not known.

Experimental approach: We analysed demographics in a nested age- and sex-matched case–control study of hypertensive patients with or without left ventricular hypertrophy (NCT03088514) and assessed the effects of dietary nitrate in mouse models of cardiac dysfunction.

Key results: Lower plasma nitrite concentrations and vascular dysfunction accompanied cardiac hypertrophy and fibrosis in patients. In mouse models of cardiac remodelling, restoration of circulating nitrite levels using dietary nitrate improved endothelial dysfunction through targeting the xanthine oxidoreductase-driven increase in levels of H_2O_2 and superoxide, and decreased cardiac fibrosis through NO-mediated block of SMAD phosphorylation leading to improvements in cardiac structure and function.

Conclusions and implications: Dietary nitrate offers easily translatable therapeutic options for delivery of NO and thereby treatment of cardiac dysfunction.

KEYWORDS
heart failure, hypertension, nitrate, NO, oxidative stress, xanthine dehydrogenase

Abbreviations: ADI, acceptable daily intake; ANGII, angiotensin II; DPI, diphenylene iodonium; FMD, flow-mediated dilatation; HF, heart failure; HFpEF, heart failure with preserved ejection fraction; LVH, left ventricular hypertrophy; PWV, pulse wave velocity; SNO, spermine-NO; XDH, xanthine dehydrogenase; XO, xanthine oxidase; XOR, xanthine oxidoreductase.

Lorna C. Gee, Gianmichele Massimo and Clement Lau contributed equally to this work.

1 | INTRODUCTION

Decreased **NO** bioavailability is considered the primary pathophysiological characteristic of endothelial dysfunction, a phenomenon involved in the pathogenesis of most forms of cardiovascular disease, and due at least in part to the scavenging of NO by the free radical superoxide (O_2^-). O_2^- is thought to reduce NO levels, by reacting to form peroxynitrite ($ONOO^-$), as well as by the oxidation of tetrahydrobiopterin (**BH_4**), a crucial cofactor for endothelial NOS (**eNOS**/NOS3) activity, leading to decreased constitutive NO generation in the cardiovascular system (Laursen et al., 2001; Stuehr et al., 2005).

Increased O_2^- in cardiovascular disease is a consequence of upregulation of expression and activity particularly of **NAD(P)H oxidases** and xanthine dehydrogenase (**XDH**)/xanthine oxidase (XO) often collectively termed xanthine oxidoreductase (XOR) (Cai & Harrison, 2000) and is highly relevant, in the context of hypertension and left ventricular hypertrophy (LVH) (Brunner et al., 2005), both significant risk factors for heart failure (HF) (Brunner et al., 2005; Katholi & Couri, 2011; Panza et al., 1990; Treasure et al., 1993). Despite this, treatments restoring NO levels, through directly delivering NO or via antioxidant treatments to reduce oxidative stress, remain an unmet need (Ponikowski et al., 2016). Importantly, both hypertension and LVH are significant risk factors, and patients with HF exhibit decreased flow-mediated dilatation (FMD) responses (Drexler et al., 1992; Kubo et al., 1991) and express reduced levels of NO metabolites and **cGMP** (a biomarker of NO activity), compared with healthy age-matched individuals and controls (Bhushan et al., 2014; Katz Stuart et al., 1999). Studies in dogs have suggested that decreased eNOS activity mediated these outcomes (Smith Carolyn et al., 1996), although such deficiencies in NO are also coupled with increased oxidative stress. Whereas there is much evidence for a role for NAD(P)H oxidases in driving oxidative stress in the heart (Münzel et al., 2015), in vivo models attempting to simulate chronic HF also demonstrate progressive increases in myocardial XOR levels, as contributors to cardiac oxidative stress, as disease worsens (Amado et al., 2005; Ekelund et al., 1999).

In addition to NO deficiency increasing the risk of LVH development indirectly (due to reduced vasodilation, contributing to chronically raised BP), constitutive NO is also considered a direct negative modulator of cardiac hypertrophy. Both neuronal NOS (**nNOS/NOS1**) and eNOS knockout mice develop cardiac hypertrophy spontaneously, with double-knockout mice exhibiting a more severe phenotype (Barouch et al., 2002, 2003). Numerous preclinical studies demonstrate worsening cardiac function, hypertrophy and fibrosis with NOS inhibition, including in the setting of hypertension, such as in angiotensin II (ANGII)-induced cardiac dysfunction in rats (Hou et al., 1995). In patients with HF, reduced levels of NO metabolites have been demonstrated, particularly in those with heart failure with preserved ejection fraction[HFpEF], although there was no clear discrimination of which NO metabolites particularly were reduced [Chirinos & Zamani, 2016; Franssen et al., 2016; van Heerebeek et al., 2012]) and HF patients with a genetic variant of eNOS have reduced enzyme activity associated with increased mortality (McNamara Dennis et al., 2003).

What is already known

- Dietary nitrate lowers BP.
- Whether this leads to reductions in consequent cardiac dysfunction and the mechanisms involved are unknown.

What does this study add

- Dietary nitrate elevates plasma nitrite concentrations in mouse models of cardiac dysfunction.
- Nitrite is converted to NO by xanthine oxidoreductase, simultaneously reducing superoxide generation, attenuating cardiac fibrosis/dysfunction.

What is the clinical significance

- Dietary nitrate is a potential therapeutic agent for diseases characterised by cardiac dysfunction.

Preclinical studies, attempting to restore NO levels in the setting of cardiac dysfunction, have shown beneficial effects (Bhushan et al., 2014; Fraccarollo et al., 2008; Chang et al., 2005). However, in the clinical setting, the delivery of NO is not straightforward. This is due to the rapid development of tolerance with the organic nitrates (Münzel et al., 2014) and the short half-life of inorganic nitrite (NO_2^-) (Hunault et al., 2009). However, recent evidence suggests that dietary inorganic nitrate (NO_3^-), processed through the enterosalivary circuit (non-canonical pathway), provides a reliable and safe method for sustained elevation of NO levels in patients with chronic disease (Kapil et al., 2015). Recent translational studies in patients with HF have demonstrated positive effects of acute treatments with dietary nitrate and nitrite upon surrogate markers of disease, including increases in submaximal exercise capacity (Eggebeen et al., 2016; Zamani et al., 2015). Such benefits have been proposed to be linked to improved muscle contractile function (Coggan et al., 2015; Zamani et al., 2015), as well as improvements in haemodynamics in patients with HFpEF (Simon et al., 2016). In contrast, a recent study in patients with HFpEF demonstrated no beneficial effect of exogenous nitrite, inhaled three times daily for 4 weeks, on exercise capacity (Borlaug et al., 2018). However, due to the short half-life of nitrite, of approximately 30 min (Kapil et al., 2020), it is likely that sustained elevation of circulating nitrite was not achieved with this intervention. To date, whether *sustained* elevation of circulating nitrite might effect improvements in cardiac fibrosis and structure to improve function, and thus provide long-term sustained benefits useful in the treatment of chronic disease, remains unknown.

Thus, we examined plasma nitrite concentrations in hypertensive patients with and without cardiac hypertrophy in a matched case–control study (NITRATE-TOD) to determine, whether LVH in the hypertensive setting was associated with lower levels of this anion. In addition, in preclinical models of cardiac hypertrophy and hypertension, we tested whether restoring or raising nitrite levels might improve cardiac remodelling and performance and whether such effects occur through BP-dependent or BP-independent mechanisms.

2 | METHODS

2.1 | Matched case–control clinical study in patients with hypertension and LVH

A matched case–control study using a cohort from the ongoing NITRATE-TOD study (NCT03088514) was performed. Nitrate-TOD was approved by the London – City and East Research Ethics Committee (10/H0703/98) and the study conducted according to the principles of the declaration of Helsinki. All patients provided their informed consent prior to recruitment in the trial. Patients in this cohort had treated, but as yet uncontrolled, hypertension. Cases were defined as those with cardiac hypertrophy at baseline. Age- and sex-matched controls without cardiac hypertrophy were included from the same NITRATE-TOD cohort. For each patient, measurements were used from pre-randomisation baseline assessments of plasma nitrite and uric acid concentration, arterial stiffness, endothelial function, BP and cardiac MRI (CMR) parameters. All biochemical analyses were conducted blind to group allocation.

2.2 | Animal sourcing and husbandry

All animal experiments were conducted according to the Animals (Scientific Procedures) Act 1986 and the EU Directive 2010/63/EU and the revising Directive 86/609/EEC on the protection of animals used for scientific purposes. Animal studies are reported in compliance with the ARRIVE 2.0 guidelines (Percie du Sert et al., 2020) and with the recommendations made by the British Journal of Pharmacology (Lilley et al., 2020). Male C57Bl6 mice (6–7 weeks of age), from Charles River Laboratories (Harlow, UK), were housed in individually ventilated cages (3-6 mice per cage) with food (Labdiets PicoLab Mouse diet 20 EXT diet 5R58, International Product Supplies Ltd, London UK) and water, available ad libitum. Room lighting (7:00 AM to 7:00 PM), humidity (55 ± 10%) and room temperature (19–23°C) were monitored according to Home Office regulations. A total of 361 animals were used.

2.3 | Dietary nitrate supplementation

Mice were randomly assigned to receive 15-mM KCl (equimolar salt control) or 15-mM KNO_3 (treatment) in the drinking water provided ad libitum, using a dose of KNO_3 in mice previously validated and shown to raise circulating nitrite levels commensurate with those achieved in patients and with important and functionally relevant beneficial activity (Khambata et al., 2017). Mice were treated either 2 weeks prior to (pre-treatment) or 2 weeks following (reversal) induction of cardiac hypertrophy, induced by ANGII infusion with implanted osmotic minipumps (see below). As controls, some mice received 15-mM KCl or 15-mM KNO_3 water ad libitum for 5 weeks, with no osmotic minipump implantation (Figure S2).

2.4 | Models of cardiac dysfunction

Mice were surgically anaesthetised using 5% isofluorane/0.4 L min^{-1} O_2 in an induction box and maintained using approximately 2.5% isofluorane/0.4 L min^{-1} O_2 through a nose cone, with the mouse in a prone position. Vetergesic (100 μL of 3 μg mL^{-1} s.c.) was administered upon anaesthesia induction to give sufficient post-surgery analgesia. ALZET® 1004 osmotic minipumps (DURECT Corporation, USA), delivering 1.3-$\mu g \cdot kg^{-1} \cdot min^{-1}$ angiotensin II (**ANGII**) for 3–5 weeks or 10-$\mu g \cdot kg^{-1} \cdot min^{-1}$ isoprenaline hydrochloride, were implanted subcutaneously. Some mice also underwent surgery to implant BP telemetry probes (TA11PA-C10; Data Sciences International, USA) for conscious BP measurement or were subjected to tail-cuff BP measurement (BP CODA mouse BP set-up [Kent Scientific Corporation, USA]) at the end of study. Telemetry-derived BP was normalised to activity. At 3 or 5 weeks after implantation, mice were subjected to echocardiography and blood samples collected via intracardiac puncture and cardiac tissue samples collected for further biochemical analysis.

2.5 | Biochemical assessments

To assess potential mechanisms of action, available tissue samples were collected and subjected to a range of methodologies described below.

2.5.1 | Histology and immunohistochemistry

Whole hearts were fixed in 10% Formalin solution (5mL) and embedded in paraffin. Hearts were randomly assigned to be sectioned in either the transverse or dorsal plane. 4 μm sections were cut using a microtome (Thermo Scientific, UK) and heart sections stained with picrosirius red (PSR) to identify areas of connective tissue deposition. Images were acquired using a NanoZoomer slide scanner (Hamamatsu) and viewed using NDP View software. Three left ventricle, three intraventricular septum and three right ventricle fields of view were randomly selected at x20 magnification (see supplement Figure S14) and PSR staining quantified using manual thresholding techniques in ImageJ software.

Wheat germ agglutinin fluorescent staining was conducted in order to determine cardiac myocyte area. Sections were stained with

wheat germ agglutinin conjugated to Alexaflour 647 (1:500 Molecular Probes, Invitrogen W32466) and Prolong gold DAPI mountant. Images were acquired using a NanoZoomer slide scanner (Hamamatsu) and viewed using NDP software. Four images were taken and the cardiac myocyte area was analysed using ImageJ, with a minimum of 300 cells analysed per sample.

Sections were treated with anti-Smad2 + anti-Smad3 primary antibody (1:500, ab217553) or anti-Smad2 + Smad3 (phospsho T8) (1:50, 63399 RRID:AB1142934) using the Ventana Discovery Ultra system. Three left ventricle (LV), three intraventricular septum (IVS) and three right ventricle (RV) fields of view were randomly selected at x20 magnification and staining quantified and averaged to give an n=1. For the isoprenaline study, seven left ventricles (LV) were selected for KCl and KNO_3 groups and images acquired as before. All quantification was conducted blind.

2.5.2 | Organ bath pharmacology

In a subset of study mice, at the end of the study, thoracic aortae were used in organ bath assays to characterise vasoreactivity in response to various vasodilators and vasoconstrictors. Thoracic aortae were dissected from mice and cut into rings ≈3 mm long. Rings were mounted in 10 ml organ baths containing Krebs solution heated to 37 °C and bubbled with 95% O_2 in CO_2. Tension of aortic rings was set at 0.3 g and rings left to equilibrate for 45 minutes. A cumulative contraction concentration response curve to phenylephrine (10^{-9} – 3 x 10^{-5} M), or in phenylephrine-pre-contracted vessels, a relaxation curve to ACh (10^{-9} – 3 x 10^{-5} M) or spermine-NO (SNO; 10^{-9} – 3 x 10^{-5} M) was then constructed. For phenylephrine contraction the concentration was titrated until vessel tension reached EC_{75} of KCl maximal contraction.

2.5.3 | Oxidative stress quantification by lucigenin-enhanced chemiluminescence (LECL)

Hearts were homogenised in a 1:5 ratio (tissue weight (mg) and protein concentration determined using a Pierce® BCA Protein Assay Kit (Thermoscientific, UK). For LECL 100 μg protein was used per well, incubated with experimental substrate(s) xanthine/NADPH/NADH added at 100 μM per well, followed by 10 μM lucigenin (Sigma Aldrich, UK) in the absence or presence of various inhibitors listed below. Plates were immediately analysed using a Perkin Elmer Wallac 1420 Victor2 plate reader (Perkin Elmer, USA), Superoxide (O_2^-) anion production was read for 50 repeat readings with 30 seconds of interval between each repeat. Each sample was tested in duplicate and the results averaged to give an n=1.

2.5.4 | Xanthine oxidase (XO) activity

XO activity was measured in liver homogenates using a commercially available colorimetric XO assay kit (abcam, UK). Liver homogenate samples were diluted 1:3 (50 μl sample: 150 μl XO assay buffer) and 50 μl of this added to the plate in triplicate. The plate was measured using a Dynex MRX Revelation plate reader (Dynex Technologies Limited, UK) at 25°C, taking readings every 2 minutes for 3 hours at 570 nm. The point at which XO activity was observed during this period was determined by establishing when absorbance readings rose above that of the negative control. Each sample was tested in duplicate and the results averaged to give an n=1.

2.5.5 | Plasma nitrite and nitrate quantification by ozone chemiluminescence

Plasma was deproteinated using Vivaspin 500 filter columns (Sartorius Stedim Biotech, SA) at 14,000 g for 60 mins at 4 °C, before ozone chemiluminescence was conducted on samples using a 280i Nitric Oxide Analyzer (Sievers, USA) as previously described (Ghosh et al., 2013; Ignarro et al., 1993).

2.5.6 | Nitrite reductase activity

Nitrite reductase activity was measured in both heart and liver homogenates using gas phase chemiluminescence as we have done previously[2]. Heart and liver samples were homogenized in a 1:5 and 1:2 ratio respectively (tissue weight (mg): buffer volume (μL)) in buffer consisting of PBS with 5.7 μM benzamidine, 1.5 μM antipain, 0.15 μM aprotinin, 4.2 μM leupeptin, 1.5 μM pepstatin A and 400 μM AEBSF protease inhibitors. Resulting homogenate was centrifuged at 4°C for 2 mins at 9000 RCF and supernatant collected. The protein concentration of supernatant was determined using a Pierce® BCA Protein Assay Kit (Thermoscientific, UK). Experiments were performed in a sealed 10 ml glass reaction chamber containing citric acid/Na_2HPO_4 buffer at pH 7.4 (physiological levels) or pH 6.8 (representing acidosis), and KNO_2 (10-1000 μM) in a total volume of 1 ml. This solution was bubbled with nitrogen gas (100%) by an NO scrubbing air filter (Sievers, USA). Headspace NO concentration was measured in parts per billion by continuous sampling using a 280A Nitric Oxide Analyzer (Sievers, USA). The effects of biological tissue on NO production from NO_2^- was determined by the addition of heart or liver supernatant (150 and 200 μg of protein respectively) and measurement of NO over 2 min, calculating the rate of NO production (nmol per g of tissue per s) from the area under the curve.

2.6 | Immunoblotting

Heart, liver and aortic samples were subjected to SDS/PAGE (0.1% w/v) immunoblotting analysis using the following antibodies to XOR (1:2000, 133268 RRID: AB_11154903), SMAD2/3 (1:1000, 8685 RRID: AB_10889933), p-SMAD2 (Ser465-467)/SMAD3 (Ser423/425) (1:1000, 8828 RRID: AB_2631089), Nox2 (1:1000; 180642), β-actin

(1:5000, 16039 RRID: AB_956497), and GAPDH (1:5000, RRID: AB_2536381) and visualized using Clarity™ Western ECL Substrate (Bio-Rad). Chemiluminescence was automatically recorded and analyzed with the FluorChem E imager. Primary and secondary antibodies were purchased from Abcam, Cell Signalling, Thermofisher (UK). The Immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology*.

2.7 | Quantitative real-time PCR

mRNA expression was determined in mouse heart tissue using RT-PCR. The primer sequences used are shown in supplement Table S2. Whole mouse hearts were homogenized using a Precellys®24 homogeniser and then RNA extracted as per the manufacturer's instructions using the RNeasy Fibrous Tissue Minikit (Qiagen Ltd, UK). RNA was converted to cDNA and RT-PCR assays conducted using SYBR green ROX mix (Thermo Scientific Abgene, UK) in 384 well plates, with the relevant primers sourced from Thermo Fischer Scientific. An ABI7900 HT Realtime PCR System (Applied Biosystems®, Life Technologies, UK), with SDS 2.3 computer software, was used to run and analyse plates. Each sample was measured in triplicate and the average taken to represent n=1. Gene expression was measured relative to internal control 18S, and expressed relative to KCl control samples using ΔΔCT analysis. See supplement for primer sequences (Table S1) and further methodology.

2.8 | Data and statistical analysis

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018).

2.8.1 | Clinical study

Descriptive data are presented as mean ± SD or median and IQR. Statistical significance was taken at $P < 0.05$. All statistical analyses were conducted on GraphPad Prism v 8.0 and STATA v15.1. Baseline characteristics were examined by Student's *t* test. In the matched case–control study, matching was performed using a propensity score based on age and sex. Conditional logistic regression models were created using a priori confounders (duration of hypertension diagnosis, baseline daytime systolic ambulatory BP, body mass index and renal function) to investigate the relationship between variables of interest (baseline plasma nitrite concentrations, FMD and arterial stiffness measures) with cases and controls in this adjusted model. We also investigated the influence of these variables of interest with each other when included together in our multivariable adjusted model, if found to improve the model. All models were tested for assumptions of linearity using squared terms.

2.8.2 | Preclinical studies

Values shown are mean ± SEM of *n* values where *n* refers to the number of animals. In assays conducted with replicates, results of replicates were averaged to give an $n = 1$ for each mouse. The sample size was determined using a preliminary echocardiographic left ventricular (LV) wall thickness dataset of eight animals in each group. The analysis was conducted blind to the intervention. We found that in ANGII-treated animals, LV wall thickness after 3 weeks of treatment with KCl was 1.297 mm (SD = 0.41), whereas in KNO_3-treated mice, it was 0.813 mm (SD = 0.14). Using these values with an α of 0.05 and a power of 95%, 12 animals in each group were required for statistical significance. To account for technical loss, the *n* value was increased to 15 for 3-week intervention studies. For the longer duration reversal study, we increased the *n* value by 6 to account for potential technical loss. Datasets were tested for outliers using Rout's test. All mechanistic biochemical analyses were conducted blind to treatment allocation. For comparison of two groups, unpaired Student's *t* test was used. For comparisons of more than two groups, one-way ANOVA was used followed by an appropriate post-test, only if *F* achieved $P \leq 0.05$ and there was no significant variance inhomogeneity.

2.9 | Materials

All reagents were purchased from Sigma-Aldrich (Poole, UK), unless otherwise stated. Isoprenaline was supplied by Merck Life Science (Dorset, UK)

2.10 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY (http://www.guidetopharmacology.org) and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander, Christopoulos et al., 2021; Alexander, Fabbro et al., 2021a, 2021b).

3 | RESULTS

3.1 | Matched case–control patient study

The matching of patients of a case–control cohort on the basis of age, sex and duration of hypertension in the larger NITRATE-TOD patient cohort was successful as indicated in Table 1—with equal numbers of women, similar age and hypertension duration between the LVH and non-LVH groups. As expected, LV mass indexed to body surface area (LVMi) and BP were greater and FMD was lower in hypertensive patients with LVH compared with those without LVH (Table 1 and Figure 1). Importantly, this difference was accompanied by lower

TABLE 1 Baseline characteristics of the matched case–control clinical study population

	Control (no LVH)	Cases (LVH)	Significance (*P*)
Demographics			
n (female)	32 (8)	32 (8)	1.00
Age (years)	55.1 ± 13.5	55.8 ± 13.5	0.83
BMI ($kg{\cdot}m^{-2}$)	26.4 ± 3.8	28.9 ± 3.9	0.01
BSA (m^2)	1.95 ± 0.23	1.99 ± 0.19	0.59
Duration of HTN (years)	8.9 ± 6.9	12.4 ± 8	0.07
Haemodynamic assessments			
Systolic ABP (mmHg)	142.6 ± 8.9	151.3 ± 9.8	<0.001
Diastolic ABP (mmHg)	88.1 ± 9.8	90.3 ± 9.8	0.38
Heart rate (bpm)	70.8 ± 7.9	71.4 ± 10.3	0.78
Central SBP (mmHg)	136.8 ± 12.7	141.8 ± 15.0	0.16
Central DBP (mmHg)	82.7 ± 8.4	86.4 ± 11.9	0.16
Aortic pulse pressure (mmHg)	54 ± 13.6	54.9 ± 15.0	0.80
Augmentation index (%)	23.5 ± 6.4	24 ± 5.7	0.72
PWV ($m{\cdot}s^{-1}$)	9.4 ± 2.2	9.5 ± 1.9	0.85
Hypertension medications (*n*)	1.84 ± 0.97	2.31 ± 1.03	0.06
Number of antihypertensives (1/2/3/4)	15/9/6/2	8/11/8/5	0.27
Patients on medications (*n*)			
ACE-i	12	10	
ARB	9	13	
CCB	17	21	
Diuretics	8	14	
Aldosterone antagonist	2	5	
Alpha blocker	5	7	
CMR measures			
LV EDV index ($ml{\cdot}m^{-2}$)	70.1 ± 13.4	76.6 ± 17.7	0.11
LV ESV index ($ml{\cdot}m^{-2}$)	23.2 ± 9.8	25.9 ± 9.6	0.28
LV EF (%)	67.5 ± 7.7	66.8 ± 7.5	0.69
LV mass index (g^2)	68.9 ± 9.6	91.7 ± 12.4	<0.0001
Native T1 myocardium (ms)	1014 ± 26	1040 ± 28	0.0005
T1 blood (ms)	1576 ± 68	1587 ± 57	0.49
ECV (%) by MOLLI	26.6 ± 2	27.3 ± 2.7	0.28
T2 myocardium (ms)	49.1 ± 2.4	49.7 ± 2.9	0.40
LGE (*n*)	8	8	0.76
Vascular measures			
Baseline diameter (mm)	4.15 ± 0.69	4.44 ± 0.73	0.11
Peak shear rate (per s)	1518 ± 466	1377 ± 441	0.22
Biochemistry			
eGFR ($ml{\cdot}min^{-1}$)	79.8 ± 13	75.7 ± 12.2	0.20
Haematocrit (%)	0.43 ± 0.03	0.43 ± 0.03	0.91

Note: Data are presented as mean ± SD. Statistical analyses conducted as *t* test for continuous data and chi-squared for categorical variables.

Abbreviations: ABP, ambulatory BP; ACE-i, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; BMI, body mass index; BSA, body surface area; CCB, calcium channel blocker; CMR, cardiac MRI; DBP, diastolic BP; ECV, extracellular volume; EDV, end-diastolic volume; EF, ejection fraction; eGFR, estimated GFR; ESV, end-systolic volume; HTN, hypertension; LGE, late gadolinium enhancement; LV, left ventricular; LVH, left ventricular hypertrophy; MOLLI, modified look-locker inversion recovery; PWV, pulse wave velocity; SBP, systolic BP.

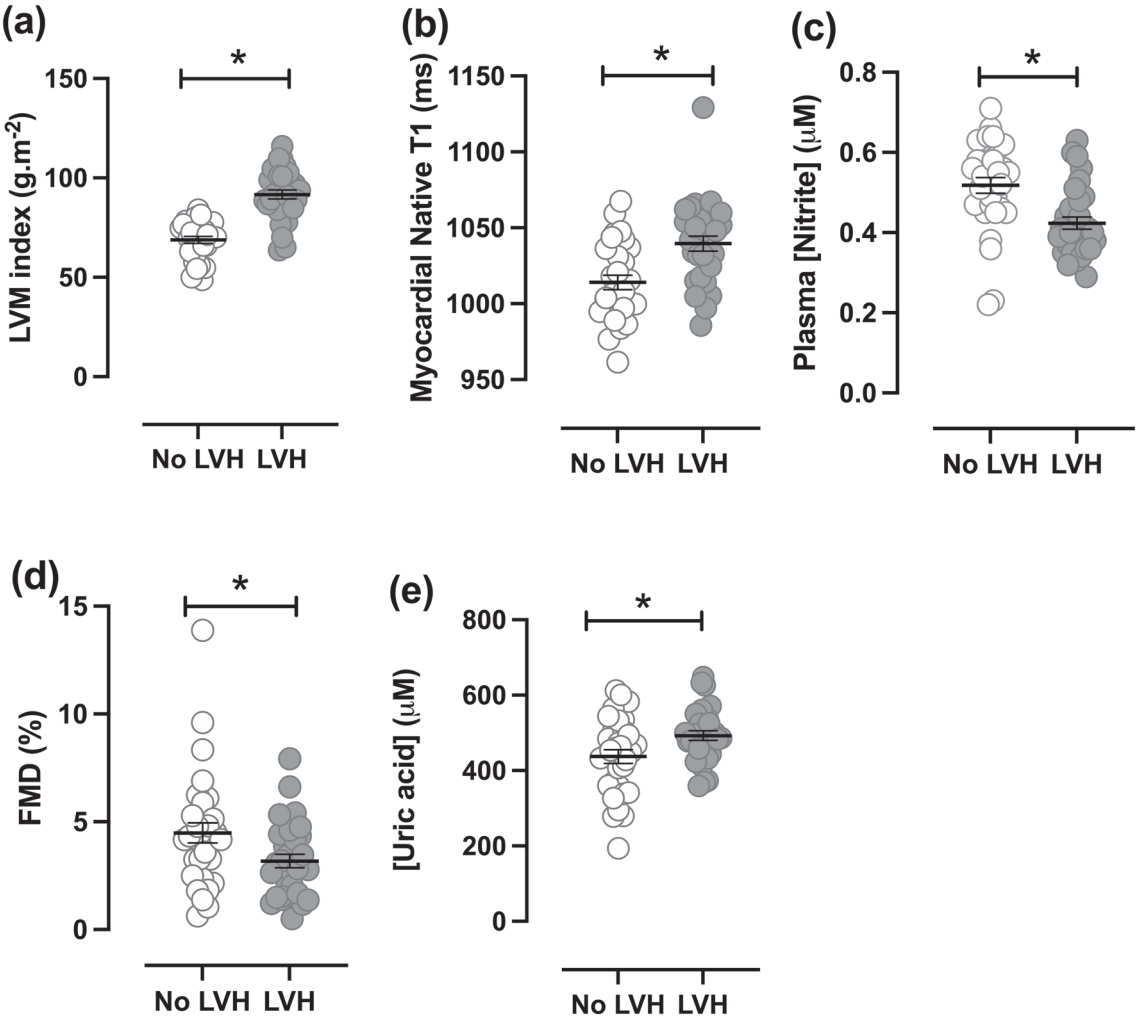

FIGURE 1 Matched case–control patients from NITRATE-TOD with or without left ventricular hypertrophy. Differences in (a) left ventricular mass (LVM) index by cardiac MRI, (b) diffuse myocardial fibrosis by cardiac MRI, (c) plasma [nitrite] (d) brachial artery flow-mediated dilation (FMD) measured by ultrasound, and (e) uric acid measured using a commercially available ELISA in patients with ($n = 32$) or without ($n = 32$) left ventricular hypertrophy (LVH) matched for age, sex and duration of hypertension. Data shown are individual values with means ± SEM. *$P < 0.05$; significantly different as indicated; unpaired t test

levels of circulating nitrite, measured in venous blood (Figure 1c). Because nitrite is thought to be an accurate reflection of endothelial NO generation (Kleinbongard et al., 2003; Lauer et al., 2001), these findings support the view that worsening disease is associated with decreased levels of bioavailable NO.

Pulse wave velocity (PWV), central BP and aortic pulse pressure (all measures of arterial stiffness) were not different between cases and controls (Table 1). Advanced CMR (Figure S1) tissue characterisation techniques showed a statistically significant higher native T1 (a signal versus time MRI measure thought to represent the degree of diffuse cardiac fibrosis) in those hypertensives with LVH, but measures of extracellular volume (ECV) were the same (Table 1). T2 mapping values were used to estimate oedema (and by inference inflammation) and found to be similar between groups suggesting that inflammatory load within the myocardium was not driving differences between the two groups. FMD was significantly lower in the LVH group (β coefficient −1.36; 95% confidence interval [CI] −2.63 to −0.09) in the fully adjusted model (Figure 1 and Table S1). To test whether this relationship with FMD and LVH was driven by circulating nitrite concentrations, the model was adjusted for this, and this resulted in a loss of the statistically significant association of FMD with LVH (Table S2). These analyses suggest that circulating nitrite levels are likely to drive the relationship between FMD and LVH and support the rationale for identifying interventions that might restore nitrite levels.

3.2 | Preclinical models of cardiac hypertrophy

Because the worse cardiac hypertrophy in patients with LVH correlated with lower circulating levels of nitrite, we tested whether raising levels of nitrite using a dietary nitrate approach might improve these measures in two distinct preclinical models, mimicking human cardiac hypertrophy (Figure S2). The models chosen were ANGII-induced

(hypertension-driven) and isoprenaline-induced (tachycardia-driven) cardiac hypertrophy (Figure S2). ANGII treatment caused a prominent rise in BP (Figure S2B), whereas isoprenaline treatment resulted in a pronounced elevation of HR, as expected (Figure S2D). These observations confirm that increased BP and increased heart rate respectively are the primary drivers for the different models of the cardiac hypertrophy.

3.2.1 | Dietary nitrate increases plasma nitrite levels and telemetric measures of activity with reduced BP

Using a validated dietary nitrate supplementation protocol for mice (15-mM KNO_3 in the drinking water; Khambata et al., 2017), plasma nitrate and, crucially, plasma nitrite concentrations were elevated in control, ANGII- and isoprenaline-infused mice compared with KCl-treated control mice (Table S3). These findings confirm an intact enterosalivary circuit, irrespective of model utilised. It is noteworthy that ANGII-treated mice consumed more water per day, than the untreated or isoprenaline-treated mice (Table S4). This resulted in plasma levels of both nitrate and nitrite after dietary intervention ~10-fold and ~2-fold greater, respectively, than those seen in control mice or those treated with isoprenaline. ANGII induces polydipsia, likely due to stimulation of thirst receptors in the brain (Rowland & Fregly, 1988), and this is likely to underlie this effect on water intake. However, the volume consumed was identical between KNO_3 and corresponding KCl controls enabling comparison of the effects of treatment within each model, according to the design in the a priori sample size estimations.

Dietary nitrate treatment reduced telemetric measures of mean arterial and diastolic, but not systolic, BP in control and ANGII-treated mice (Figure S3). Interestingly, in both control and ANGII-treated mice, KNO_3 treatment caused a significant increase in activity compared with those treated with KCl (Figure S3E,J). This increase in activity was evident both during the night when mice are active and during the day when, in the main, mice usually are at rest (Figure S4). Because activity in mice, as in humans, triggers acute increases in systolic BP (SBP) (Adlam et al., 2011), and because an elevated resting BP is associated with exaggerated rises in SBP in response to activity (Miyai et al., 2002; Miyai et al., 2021), this may explain the absence of a lowering of absolute SBP with dietary nitrate treatment. To account for the influence of activity, BP responses were normalised to activity levels (Figure S4A–E).

3.2.2 | Dietary nitrate reduces ANGII-induced cardiac hypertrophy and LV wall thickening and improves LV volumes

LV mass, assessed by echocardiography (Figure 2a,b), increased ~65% with ANGII treatment, an effect almost abolished with dietary KNO_3 treatment (Figure 2c). This was associated with reductions in LV posterior and anterior wall thickening (Figure 2d,e) and improvements in wall:lumen ratios (Figure 2i). Although ANGII infusion resulted in increases in LV internal dimensions (LVIDs) and LV volumes and decreases in ejection fraction and fractional shortening, KNO_3 pretreatment did not alter these parameters (Figures 2f–h and S5D–F). In addition, ANGII treatment resulted in an increase in cardiomyocyte cell area, determined with WGA staining quantification, which was not altered by dietary KNO_3 treatment (Figure 2j).

Importantly, for a closer model of the clinical condition, we conducted a separate experiment where KNO_3 or KCl treatment was initiated 2 weeks following development of ANGII-induced cardiac dysfunction in a reversal protocol. In this setting, KNO_3 treatment also improved measures of cardiac structure and function but did not alter cardiomyocyte cell area (Figures 3 and S6).

3.2.3 | Dietary nitrate reduces isoprenaline-induced cardiac hypertrophy and improves ejection fraction

Although isoprenaline treatment caused only a modest 28% increase in LV mass (compared with ANGII), this increase was significantly diminished with KNO_3 treatment (Figure 4a–c), and was accompanied by restoration of LV volumes to control levels (Figures 4f and S7), thus improving wall:lumen ratios (Figure 4i) but not altering cardiomyocyte area (Figure 4j). LVEF was also improved with KNO_3 treatment (Figure 4h), and it is noteworthy that these improvements occurred in the first week of measurement, suggesting that the effects of KNO_3 developed rapidly (Figure S8).

3.2.4 | Dietary nitrate attenuates expression of cardiac markers of fibrosis

Pretreatment with KNO_3 caused a statistically significant reduction compared with KCl control in connective tissue reflected by reduced Picrosirius Red (PSR) immunostaining of the heart (Figure 5a–c). This effect was associated with reductions in cardiac mRNA expression for connective tissue growth factor (CTGF; Figure 5g,i), collagen 1a (Col1a) but not Col3 (Figures 5k,l and S9). There was no statistically significant effect of KNO_3 treatment on mRNA expression of TGF-β (Figure 5d–f), BNP, AT_1 receptors, NLRP1 or NLRP3 (Figure S9), although it is noteworthy that the mRNA for TGF-β was only elevated after ANGII, whereas BNP was elevated by both ANGII and isoprenaline (Figure S9). Interestingly, the beneficial effects of KNO_3 on PSR staining were not evident when nitrate was administered as a reversal treatment in ANGII-treated mice (Figure 5).

Because relatively low levels of NO interfere with SMAD phosphorylation, we assessed levels of SMAD and pSMAD in heart tissue from isoprenaline-treated mice. Our findings showed significant decreases in pSMAD2 and a trend towards a decreased expression of pSMAD3 (Figure 5m–p). These findings suggest that, at least in the

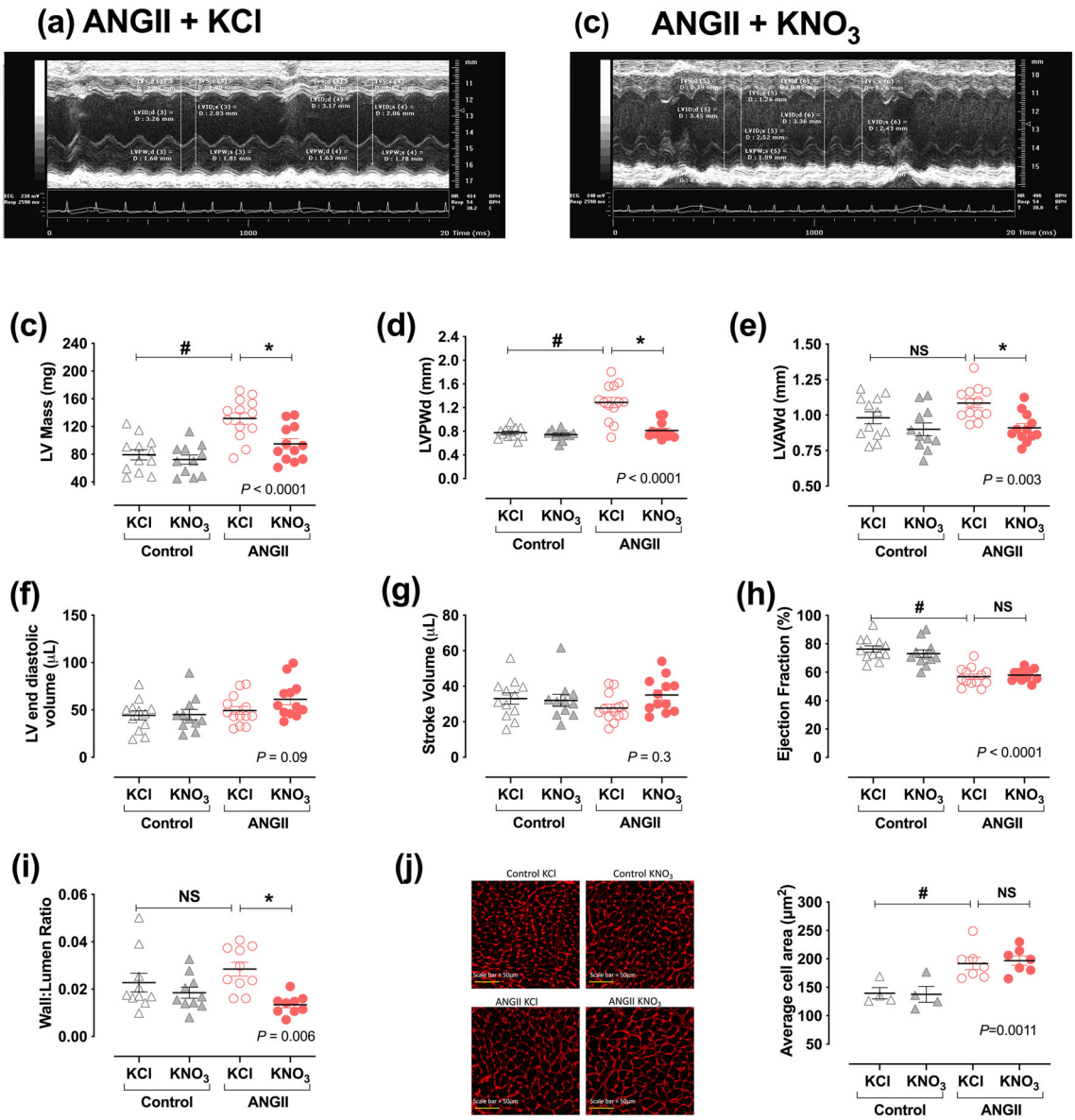

FIGURE 2 The effects of dietary nitrate on cardiac structure and performance in ANGII-infused mice. The effects of treatment of 15 mM KNO_3 versus KCl on (a, b) representative M-mode images and quantification of (c) left ventricular (LV) mass, (d) LV posterior wall in diastole (LVPWd), (e) LV anterior wall in diastole (LVAWd), (f) LV end-diastolic volume, (g) stroke volume, (h) ejection fraction and (i) wall:lumen ratio. (j) The estimated average cardiomyocyte area using ImageJ analysis of wheatgerm agglutinin staining of cardiac sections. Control animals received KNO_3 or KCl for 5 weeks without ANGII. Data shown are individual values with means ± SEM; $n = 13$ for each group for echocardiographic data and $n = 6$ for wheatgerm agglutinin immunostaining. $^{\#}P \leq 0.05$; significant difference between control and ANG II; $^{*}P \leq 0.05$, significant difference between KCl and KNO_3; one-way ANOVA with Sidak's post hoc comparisons for multiple-group testing where applicable. Unequal *n* values relate to data loss through technical failure ($n = 2$)

setting of cardiac hypertrophy not driven by BP, the TGF-β pathway is a target for the antihypertrophic actions of dietary nitrate.

3.2.5 | Xanthine oxidoreductase acts as a primary nitrite reductase in vivo in the setting of cardiac hypertrophy

XOR has been proposed to act as a key nitrite reductase in CVD (Kapil et al., 2020). There is evidence that XOR is raised in patients with cardiac dysfunction, and indeed, in our patients, raised levels of uric acid (the final product of XOR action) were found in hypertensive patients with LVH, compared with those in patients without LVH (Figure 1). Similarly, in the mouse models, treatment with ANGII, but not with isoprenaline, increased hepatic expression and activity of XOR (Figure 6a,b), a finding in accord with previous work demonstrating raised expression of this enzyme, in the setting of hypertension (Berry & Hare, 2004; Feig et al., 2013). KNO_3 treatment did not alter the levels of mRNA for XDH (Figure S9) or protein expression (Figure 6a).

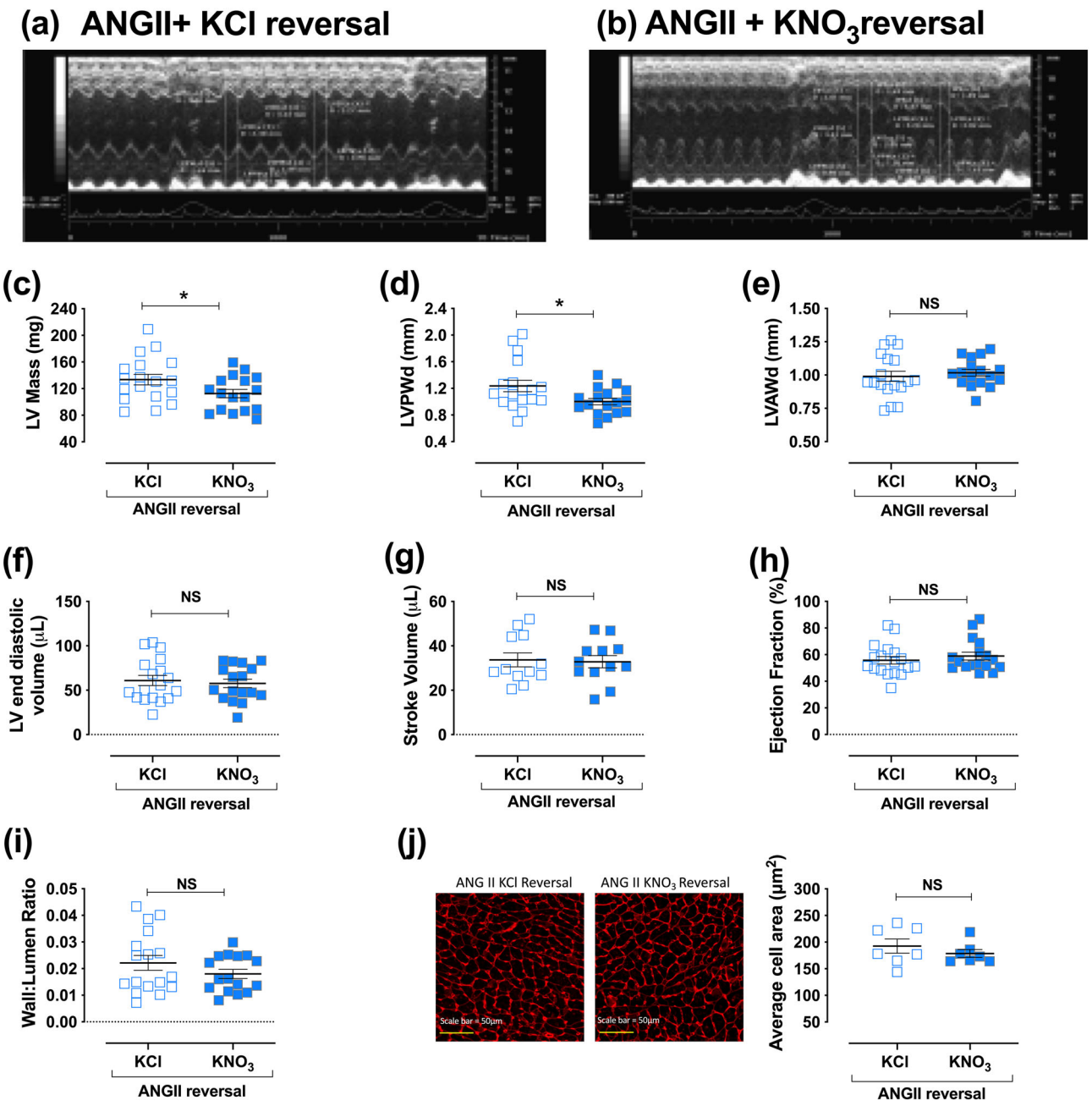

FIGURE 3 The effects of dietary nitrate reversal treatment on cardiac structure and performance in ANGII-infused mice. The effects of ANGII after 5 weeks of 1.3-µg·kg^{-1}·min^{-1} ANGII infusion and treated with 15-mM KCl ($n = 18$) or KNO_3 ($n = 17$) feeding during the final 2 weeks of infusion on (a, b) representative M-mode images and quantification of (c) left ventricular (LV) mass, (d) LV posterior wall in diastole (LVPWd), (e) LV anterior wall in diastole (LVAWd), (f) LV end-diastolic volume, (g) stroke volume, (h) ejection fraction and (i) wall:lumen ratio. (j) The estimated average cardiomyocyte area using ImageJ analysis of wheatgerm agglutinin staining of cardiac sections. Data shown are individual values with means ± SEM; $n = 18$ mice for each group for echocardiographic data and $n = 7$ for wheatgerm agglutinin immunostaining. *$P \leq 0.05$, significantly different as indicated; Student's t test. Unequal n values relate to data loss through technical failure ($n = 1$)

3.2.6 | Dietary nitrate reduces oxidative stress and enhances nitrite reductase activity

Levels of H_2O_2, as a measure of XOR-driven oxidative stress, were increased ~4-fold in the ANGII-treated mouse model but not in the isoprenaline-treated model (Figure 6b). KNO_3 treatment attenuated the rise in H_2O_2 levels in ANGII-treated mice by ~50% (Figure 6b). This effect was confirmed in cardiac tissue where KNO_3 treatment significantly attenuated O_2^- levels (Figure 6c). The importance of XOR and NADPH oxidase in mediating this oxidative stress and being a target for KNO_3 treatment was provided by observations that KNO_3 treatment reduced ex vivo determined

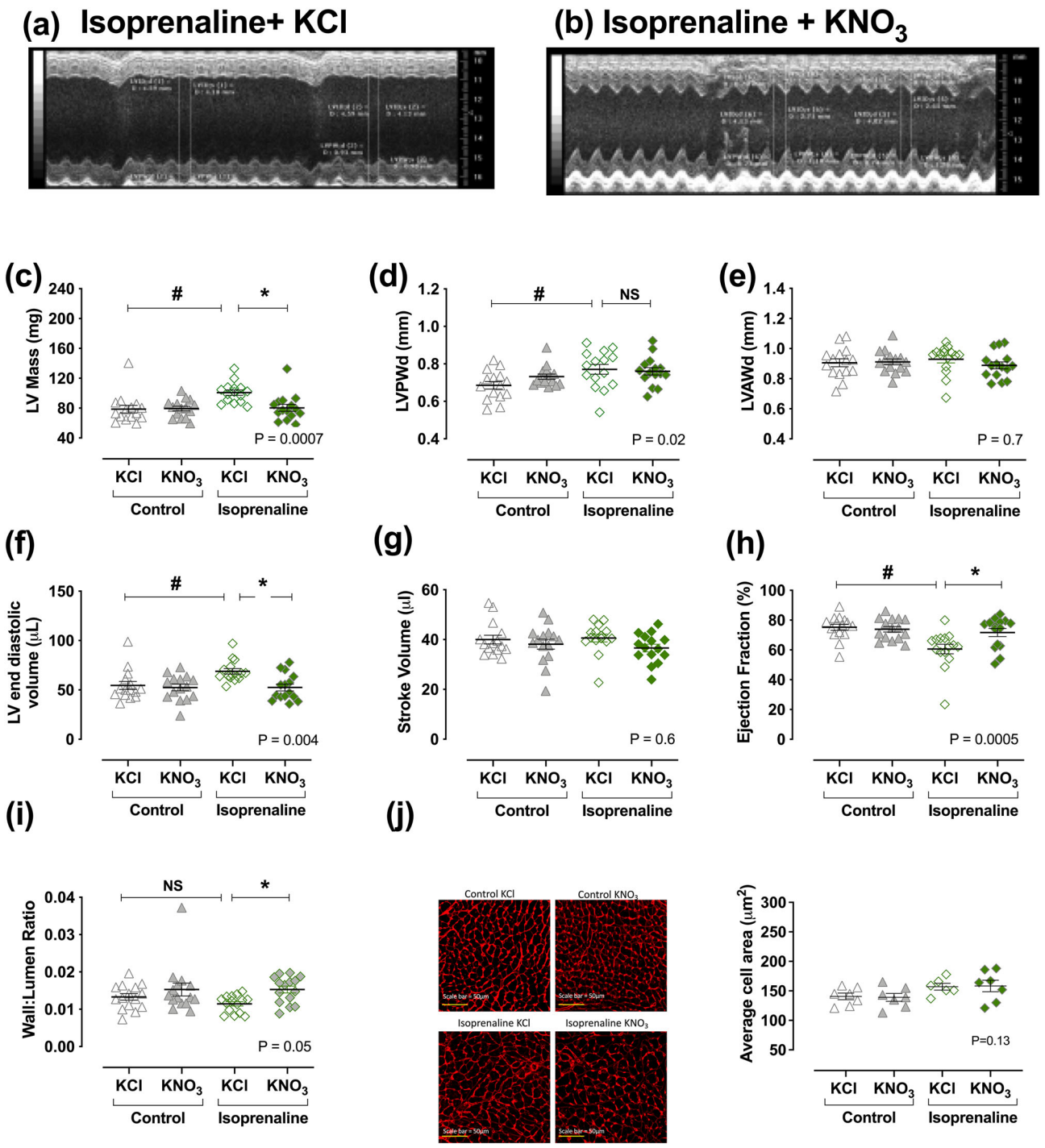

FIGURE 4 The effects of dietary nitrate treatment on cardiac structure and performance in isoprenaline-infused mice. Mice were pretreated for a 2-week run-in treatment of 15-mM KCl or KNO_3, followed by 3 weeks of 10-µg·kg^{-1}·min^{-1} isoprenaline infusion with concomitant KCl or KNO_3 feeding. (a, b) Representative M-mode images, (c) left ventricular (LV) mass, (d) LV posterior wall in diastole (LVPWd), (e) LV anterior wall in diastole (LVAWd), (f) LV end-diastolic volume, (g) stroke volume, (h) ejection fraction and (i) wall:lumen ratio. Control animals received KNO_3 or KCl for 5 weeks without isoprenaline. Data shown are individual values with means ± SEM of $n = 15$ mice for each group for echocardiographic data and $n = 7$ for wheatgerm agglutinin immunostaining. $^{\#}P \leq 0.05$; significant difference between control and ANG II; $^{*}P \leq 0.05$, significant difference between KCl and KNO_3; one-way ANOVA with Sidak's post hoc comparisons for multiple-group testing where applicable. Unequal *n* values relate to data loss through technical failure ($n = 2$)

O_2^- generation driven by xanthine (Figure 6d) or NADPH (Figure 6f) but not NADH (Figure 6e) in the hearts of ANGII-treated mice, and only NADPH-driven O_2^- generation in the hearts of isoprenaline-treated mice (Figure 6f). DPI markedly attenuated O_2^- generation driven by all substrates in homogenates of ANGII-treated hearts, whereas the XOR inhibitors (allopurinol or

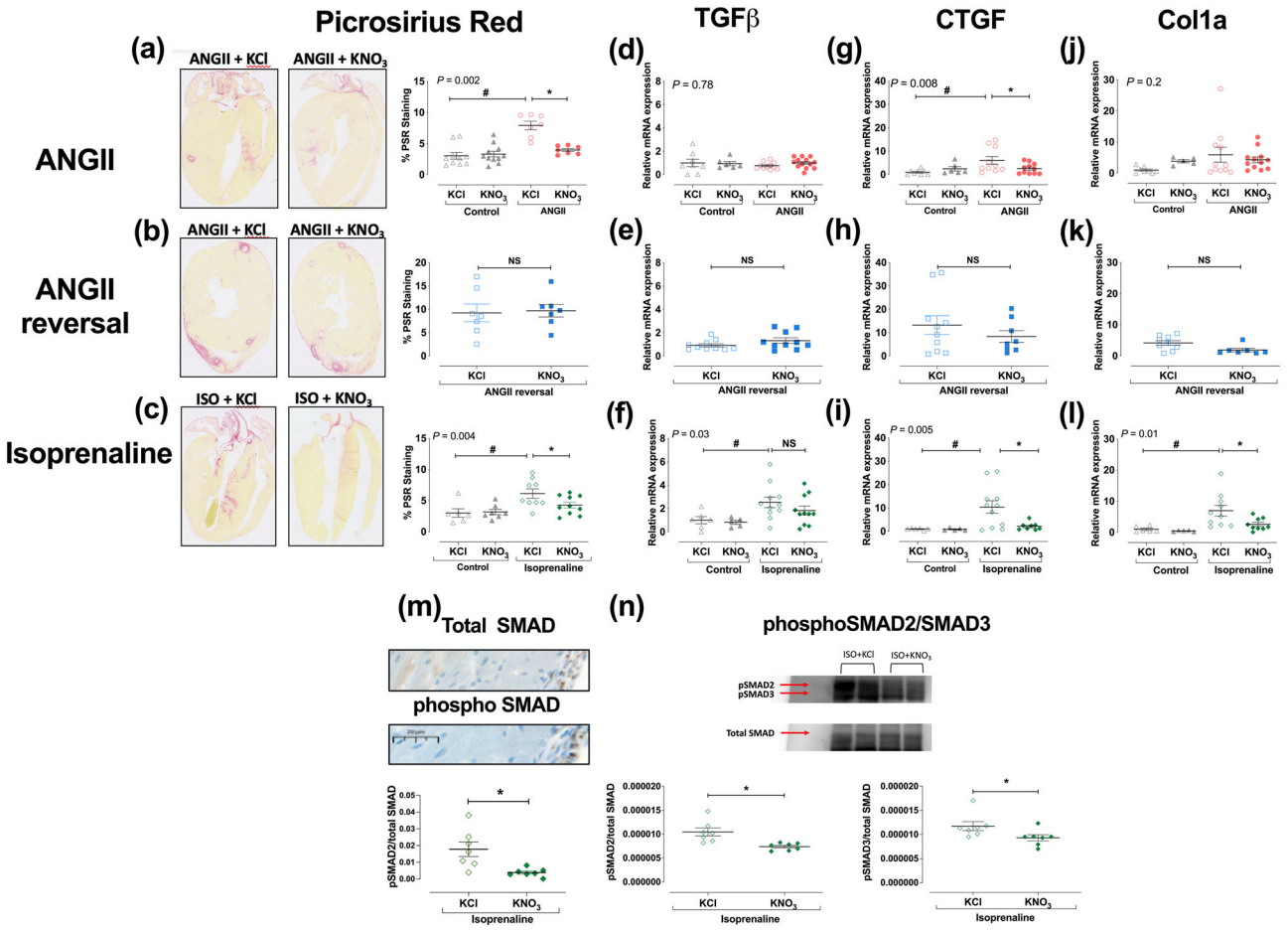

FIGURE 5 The effects of dietary nitrate on markers of fibrosis in ANGII and isoprenaline-infused mice. (a–c) Fibrosis as quantified by Picrosirius Red (PSR) staining in 4-μm sections of formalin-fixed hearts, embedded in paraffin. Three left ventricular, three intraventricular septum and three right ventricular fields of view were randomly selected at ×20 magnification and PSR staining quantified using manual thresholding techniques in ImageJ software. mRNA expression of (d–f) TGF-β, (g–i) connective tissue growth factor (CTGF) and (j–l) type I collagen (Col1a), immunohistochemical staining of (m) total SMAD and phospho-SMAD in 4-μm sections of formalin-fixed hearts, embedded in paraffin, and (n) protein expression of phosphorylated SMAD2 and SMAD3 over the total SMAD2/3 detected via immunoblotting. Data shown are individual values with means ± SEM. For mRNA expression of tissues, results were expressed relative to KCl-fed controls using the ΔΔCT method. $^{\#}P \leq 0.05$; significant difference between control and ANG II; $^{*}P \leq 0.05$, significant difference between KCl and KNO_3; one-way ANOVA with Sidak's post hoc comparisons for multiple-group. For ANGII reversal mice and isoprenaline SMAD expression levels, results were compared using unpaired Student's *t* test

febuxostat) blocked xanthine-driven O_2^- generation only (Figure 6h) with no significant effect on O_2^- generation driven by NADH or NADPH (Figure 6i,j).

KNO_3 treatment, irrespective of whether the mice had received ANGII or isoprenaline, enhanced nitrite reductase activity (Figure 7a–c).

3.2.7 | Dietary nitrate prevents vascular dysfunction

The maximum contractile response to a KCl-depolarising stimulus was not different between treatment groups (Table S6). Phenylephrine caused concentration-dependent contraction in all groups. Treatment with KNO_3 caused a modest but significant leftward shift of the concentration–response curve to both the endothelium-dependent vasodilator ACh and the endothelium-independent NO-donor compound, spermine NO (Figure S10). This effect was not evident in isoprenaline-treated mice (Figure S10 and Table S7).

4 | DISCUSSION

Inorganic nitrate, through its sequential chemical reduction to nitrite and then to NO, exerts positive effects in the clinical setting of HF (Eggebeen et al., 2016; Zamani et al., 2015). However, the mechanisms contributing to these observations remain uncertain. Here, we show that patients with hypertension and LVH experience vascular

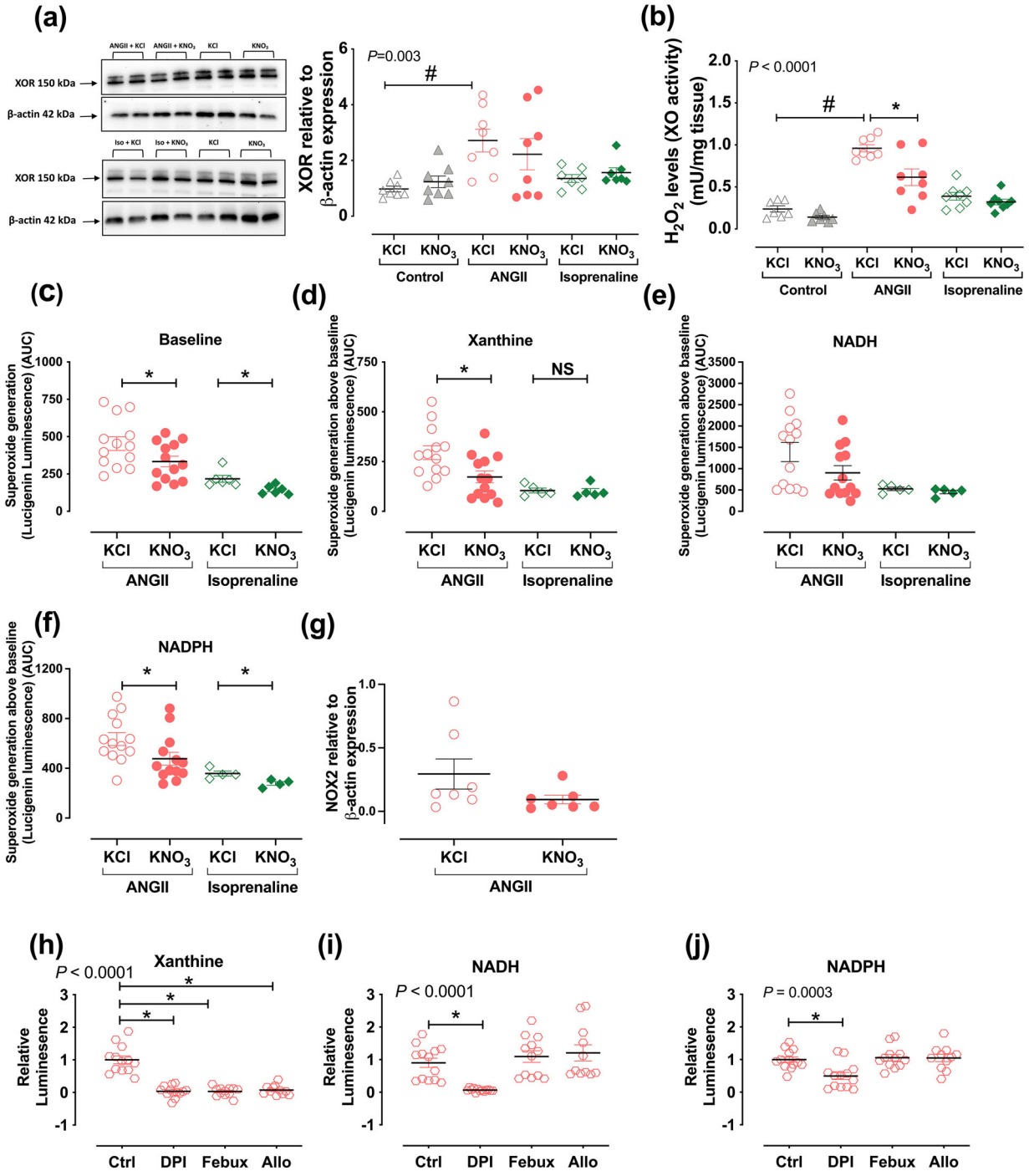

FIGURE 6 The effects of dietary nitrate on XOR protein expression and oxidative stress in angiotensin II and isoprenaline-induced models of cardiac hypertrophy in mice. (a) XOR protein expression measured in liver homogenates by Western blotting analysis and (b) levels of xanthine-driven H_2O_2 production and thus XOR activity in mouse liver homogenates. (c–e) O_2^- generation in mouse heart homogenates of mice treated with 15-mM KCl or KNO_3 for 2 weeks, followed by a 3-week infusion of either 1.3-μg·kg^{-1}·min^{-1} ANGII or 10-μg·kg^{-1}·min^{-1} isoprenaline, with concomitant KCl or KNO_3 feeding. O_2^- generation was measured by (c) quantifying lucigenin chemiluminescence in cardiac homogenates alone and following incubation with (d) xanthine, (e) NADH or (f) NADPH. (g) NOX2 expression in ANGII-treated mice and the effect of inhibitors upon (h) xanthine-, (i) NADH- or (j) NADPH-driven superoxide generation in ANGII-treated mice. For (a) and (b), one-way ANOVA was conducted with post hoc comparison comparing KCl control with KCl treatment in ANGII- or isoprenaline-treated. Data shown are individual values with means ± SEM. $^{\#}P \leq 0.05$; and $^{*}P \leq 0.05$, between KCl and KNO_3 within each model. Unpaired *t*-test comparisons were conducted for data in (g) and for all other analyses one-way ANOVA with Sidak's post hoc test for the comparison of KCl with KNO_3, within models (c–f) and Dunnett's test for the comparisons of control with treatments for (h)–(j)

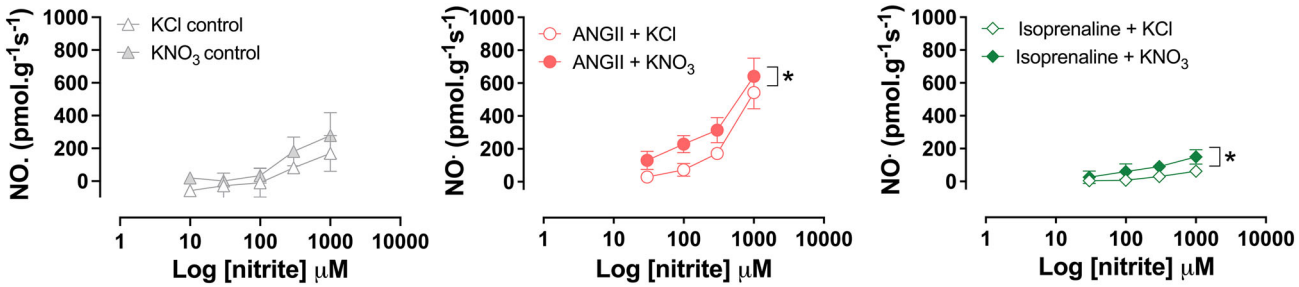

FIGURE 7 Cardiac nitrite reductase activity is increased in ANGII-induced hypertrophy and further improved with dietary nitrate treatment. Cardiac nitrite reductase activity of whole heart homogenates measured using ozone chemiluminescence. Data shown are individual values with means ± SEM. *$P \leq 0.05$; significantly different as indicated; two-way ANOVA

dysfunction associated with decreased levels of circulating nitrite, when compared with those without LVH. Our data from the mouse models also demonstrated that dietary nitrate, by elevating circulating nitrite levels, improved cardiac function by lowering BP and by exerting direct effects upon cardiac structure and function. The former effect, in part, is likely to relate to improved vascular function together with decreased oxidative stress and that the latter effects relate to anti-fibrotic mechanisms driven predominantly by altered TGF-β signalling. Our findings support the view that, irrespective of the underlying cause of cardiac dysfunction, dietary nitrate treatment may have potential as a safe, long-term approach to improving cardiac function, through direct inhibition of TGF-β signalling pathways and damaging ROS production in the heart, as well as improved vascular function, particularly in the setting of raised BP.

In patients with treated, yet uncontrolled, hypertension, the lower plasma nitrite levels were associated with pathological cardiac hypertrophy. Plasma nitrite levels are inversely correlated to baseline BP (Kapil et al., 2010), and changes in plasma nitrite concentrations correlate strongly with changes in BP in both directions. Indeed, interfering with the non-canonical pathway for NO is associated with significant elevation of BP (Bondonno et al., 2015; Kapil et al., 2013), whereas increasing plasma nitrite concentration (through provision of dietary inorganic nitrate) is associated with reductions in BP (Kapil et al., 2015). We matched patients for age and sex, but importantly, the associations with the NO metabolite remained when the data were adjusted for the known confounders of baseline ambulatory daytime BP, duration of hypertension and renal function (all of which influence the likelihood of developing cardiac hypertrophy). This fact suggests that additional beneficial mechanisms, beyond BP lowering, exist for nitrite. Vascular dysfunction plays a pivotal role in precipitating cardiac dysfunction and HF (Akiyama et al., 2012; Lam & Brutsaert, 2012). Previous clinical studies have demonstrated that elevation of plasma nitrite improves FMD in patients with vascular dysfunction caused by cardiovascular risk factors (Rammos et al., 2014; Velmurugan et al., 2013). Exploratory assessment of cardiac imaging biomarkers demonstrated a small but significant increase in native T1 (thought to reflect diffuse fibrosis) but not T2 or ECV. These changes are hard to interpret, suggesting perhaps a lack of sufficient sample size, but are not likely to represent inflammation (Rodrigues et al., 2016; Treibel et al., 2015). Analysis of the full powered dataset will be of value to ascertain the robustness of the findings particularly whether dietary nitrate treatment changes these measures. Our findings in preclinical models of cardiac hypertrophy discussed below, however, would suggest that fibrosis is a potential target of a dietary nitrate treatment, at least in mice.

Dietary nitrate, as expected, increased the circulating levels of nitrate and nitrite, confirming that dysfunction per se does not impair operation of the non-canonical pathway. More importantly, our observations demonstrate functionally active concentrations of nitrite were achieved and sustained with the chosen and validated dosing regimen (15 mM in drinking water) (Khambata et al., 2017) demonstrating the efficacy of this approach to provide sustained elevations in circulating nitrite.

By measuring ambulatory BP, we confirmed that dietary nitrate reduces BP in both untreated and ANGII-treated mice, aligning with observations in healthy volunteers (Webb et al., 2008) and hypertensive patients (Kapil et al., 2015). Importantly, in this study, only diastolic BP (DBP) was decreased but this was coupled with pronounced increases in activity. Normalisation of BP measures to activity, however, exposed significant decreases in both SBP and DBP in KNO_3-treated mice compared with those after KCl treatment. Because dietary nitrate improves energy metabolism (Larsen et al., 2011), exercise capacity (Eggebeen et al., 2016; Hirai et al., 2017; Zamani et al., 2015; Zamani et al., 2017), haemodynamics and muscle performance (Borlaug et al., 2016; Coggan et al., 2015; Ferguson et al., 2013; Ferguson et al., 2016; Hernández et al., 2012; Pironti et al., 2016), it is possible that this effect of nitrate on metabolism stimulates mice to perform increased spontaneous activity, in an unchallenging home environment.

Dietary nitrate reduced echocardiographic measures of LV mass in both experimental models and perhaps, most importantly, also in reversal experiments where treatment was initiated following establishment of cardiac dysfunction. Arguably, this latter effect is of greater relevance to the clinical setting of HF where patients present with disease. With ANGII treatment, there was a prominent LV wall thickening (LVPW > IVS) indicating a maladaptive concentric hypertrophy, characteristic of BP-driven cardiac dysfunction (i.e., hypertensive heart disease) (Gaasch & Zile, 2011; Hunter &

Chien, 1999). Despite favourable restoration of the wall:lumen ratio, ejection fraction was not improved by nitrate pretreatment in this model. There was however evidence of favourable trends in LV volumes, after KNO_3 treatment, and improved cardiac output, suggesting improved heart function.

In patients with pathological concentric hypertrophy, progression towards a dilated LV with wall thinning is often observed and accompanies transition from pathological cardiac hypertrophy to HF, with concurrent worsening of cardiac function (de Simone, 2004). This pattern of ventricular dilatation with wall thinning is commonly regarded as a marker of poor clinical outcome. However, eccentric hypertrophy *can* be a positive physiological adaptation to a dilating and failing ventricle, as seen in dilated cardiomyopathy. The nature and context of cardiac remodelling is therefore critical. In hypertensive heart disease, concentric, rather than eccentric, remodelling has been associated with worse clinical outcome. Regression of wall thickening (reverting to eccentric remodelling), in contrast, has been associated with improved prognosis (Muiesan et al., 2004). In the mouse experimental study, the lack of improvement in ejection fraction might relate to a lag in the effects of the structural changes upon ejection fraction that with longer treatment become evident, and this issue is worthy of further investigation.

Dietary nitrate also reduced LV mass in isoprenaline-treated mice. Isoprenaline caused changes in cardiac structure and function typical of eccentric remodelling. The increased mass was associated with increased volumes but had no evidence of wall thickening. These characteristics are similar to those of volume overload, in a scenario mimicking aspects of the increased β-adrenoreceptor stimulation that occurs in advanced HF (see S. C. Chang et al., 2018). Treatment with dietary nitrate essentially prevented all of these outcomes, maintaining ejection fraction at control levels without any effects upon BP. These results, together with those in the ANGII model, suggest that whether the maladaptive remodelling is concentric (ANGII) or eccentric (isoprenaline), characteristic of early cardiac dysfunction or in the later stages of worsening disease, sustained delivery of NO with dietary nitrate was effective in improving cardiac function. The efficacy of nitrate in this setting can be explained by the sustained elevation of circulating nitrite levels. In a recent trial with exposure to inhaled nitrite, three times daily, no improvement of cardiac function was reported in patients with HFPEF (Borlaug et al., 2018). However, it is unlikely that a sustained elevation of circulating nitrite levels was achieved in the patients with this regimen because the half-life of nitrite is in the region of 30 min and the authors did not demonstrate sustained circulating levels of the anions. Because inorganic nitrate has a half-life of 6–8 h and elevation of circulating levels of nitrate is paralleled by sustained elevations of circulating nitrite (Kapil et al., 2020), dietary nitrate provision is likely to be more effective as an intervention to deliver NO.

In addition to the effects upon cardiac function, dietary nitrate also exerted anti-fibrotic effects. Our data fit well with previous data demonstrating that low levels of NO interfere with SMAD phosphorylation and its consequent nuclear localisation (Saura et al., 2005), an effect linked with elevations in cGMP and **PKG** activity (Sandner & Stasch, 2017), most likely due to actions on NO-sensitive fibroblasts, rather than directly on cardiac myocytes (Broekmans et al., 2020; Menges et al., 2019) and fits with the data demonstrating no change in cardiomyocyte size with dietary nitrate in either intervention, despite the decreases in LV mass. Recent studies in rats have demonstrated that inorganic nitrite treatment reduces L-NAME-induced cardiac fibrosis (Sonoda et al., 2017), an effect linked to reductions in ANGII and AT_1 receptor expression—a key remodelling pathway that is activated particularly in hypertensive heart disease (Berk et al., 2007). However, we found no effect, in our models, of dietary nitrate on AT_1 receptor expression. The reasons for this difference may relate to the dosing regimens used. In the Sonada study, rats were treated with very high doses of sodium nitrite in the water: 10 and 100 $mg{\cdot}L^{-1}$ equating to 0.1–1 mM. Clinically, such high concentrations of nitrite in the setting of chronic disease are unlikely to be useful due to concerns regarding toxicity particularly cancer (Ahluwalia et al., 2016) as well as acute poisoning and cyanosis (Chen et al., 2012). In contrast, our studies using dietary nitrate highlight an option with greater safety producing sustained elevation of circulating levels of nitrite that are functional and safe. Studies in those with cardiovascular risk factors using doses equivalent to the ADI (3.7 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$ equivalent to 260 $mg{\cdot}day^{-1}$) for inorganic nitrate have shown that this dose is sufficient to achieve levels of circulating nitrite that are functional (Kapil et al., 2015).

It is noteworthy that when dietary nitrate was initiated once cardiac dysfunction was established (with ANGII), no reversal of fibrotic markers was evident, despite clear reductions in LV mass. Fibrosis can develop rapidly following hypertension, even prior to LVH development (Muller-Brunotte et al., 2007), and it is likely that this underlies why no obvious reductions were seen. However, despite no reduction in fibrosis, the progression of hypertrophy could be slowed to some degree, because wall thickening was reduced. This suggests that increased dietary nitrate in hypertensive heart disease could lead to early improvements in cardiac function through shifting hypertrophy from a concentric to eccentric phenotype. This issue is being tested currently in our clinical trial NITRATE-TOD.

There is growing evidence suggesting that the nitrate–nitrite–NO pathway targets oxidative stress to increase NO bioavailability (Montenegro et al., 2011; Tripatara et al., 2007). Our assessments of O_2^- generation from cardiac homogenates demonstrate that, although in ANGII-treated mice, XOR and NAD(P)H oxidase-driven production of O_2^- were both decreased by dietary nitrate, in the isoprenaline-treated mice, only NAD(P)H oxidase-driven O_2^- production was affected. Previously published work suggests that NO generated via the nitrate–nitrite–NO pathway decreases the production of O_2^- through suppressing the expression of specific NAD(P)H oxidase subunits, particularly p47phox and p67phox (Zollbrecht et al., 2016). It is likely that these effects underlie the differences in NAD(P)H oxidase activity evident in our study.

There is substantial evidence implicating raised systemic pressure as the stimulus for elevated expression of XOR (principally hepatic) in both preclinical models and human hypertension (Feig et al., 2008; Laakso et al., 1998; Laakso et al., 2004), a fact supported by our

observations demonstrating increased levels of uric acid in patients with LVH compared with those without LVH. The liver is one of the main sites for XOR expression (Parks & Granger, 1986), and although low-levels of XOR are expressed within the endothelium (Kelley et al., 2006), it is likely that, in the heart, endothelial bound XOR of hepatic origin plays a major role in cardiac XOR activity. Importantly, although hypertension increased hepatic XOR expression, this was not altered by dietary nitrate, despite a clear decrease in XOR-induced O_2^- and H_2O_2 generation. These decreases contrast starkly with an up-regulation of the XOR-dependent nitrite reductase activity of the same tissues. Such findings validate the proposal that nitrite reduction occurring at the molybdenum binding site of XOR occurs at the expense of O_2^- and H_2O_2 generation at the flavin adenine dinucleotide site of the enzyme (Khambata et al., 2015).

We postulate that the beneficial effects of dietary nitrate in the ANGII model in particular are twofold. We suggest that nitrate-derived NO triggers dilation of blood vessels, through improving vascular responses to endogenously generated dilators and decreasing remodelling via direct signalling, and also that nitrite as a substrate for XOR decreases O_2^- generation due to a 'repurposing' of the enzyme (Khambata et al., 2015). It is also worth noting that the efficacy of XOR as a nitrite reductase is increased with low pH and O_2 tension (Li et al., 2001; Millar et al., 1998), two environmental conditions very much relevant to the cardiac dysfunction and HF disease scenario.

Differences in pathways for oxidative stress in the ANGII and isoprenaline models are likely to be the result of the different pathophysiological mechanisms involved. ANGII drives cardiac pathology

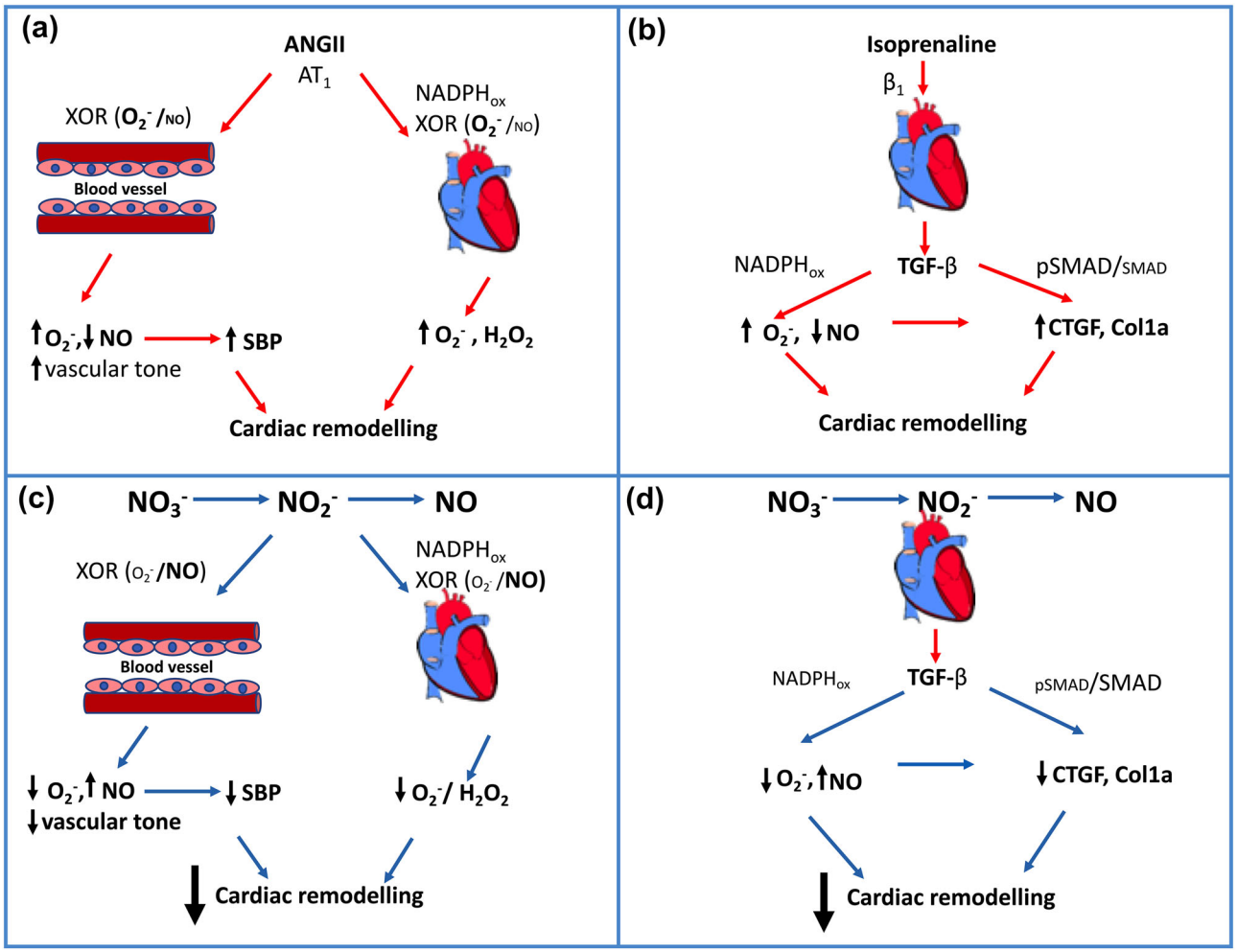

FIGURE 8 (a, b) Mechanisms of angiotensin II- and isoprenaline-induced cardiac dysfunction and (c, d) beneficial effects of the non-canonical nitrate (NO_3^-)–nitrite (NO_2^-)–NO pathway. (a) and (b) summarise the main molecular pathways responsible for cardiac remodelling in the BP-driven (ANGII) and the tachycardia-driven (isoprenaline) cardiac dysfunction. ANGII increases SBP as a consequence of increased vascular tone and by increasing oxidative stress directly in the heart via NADPH oxidase and XOR stimulation. Isoprenaline induces cardiac β_1-adrenoceptor activation, increasing NADPH oxidase-driven O_2^- production and stimulating pro-fibrotic pathways. Inorganic nitrate ameliorates the clinical outcome, irrespective of the model. (c) In the BP-dependent model, the beneficial effects of dietary nitrate are likely to be due to XOR acting as a nitrite reductase to generate NO and concomitantly reducing O_2^-, with consequent decreases in vascular tone and SBP. (d) In the BP-independent model, inorganic nitrate improves cardiac performance via decrease of NADPH oxidase-derived oxidative stress and decreasing collagen deposition

both in the vasculature and directly within the heart, but isoprenaline largely drives pathology from direct cardiac signalling and tachycardia. Differences in the triggers and sources of O_2^- are also therefore likely to influence the response to nitrate treatment. Attenuating NAD(P)H oxidase-mediated oxidative stress, for example, may have a greater effect on direct cardiac signalling and cardiac output, whereas decreasing XOR-mediated oxidative stress may have more benefit in the vasculature, for example, improving vascular function and subsequently BP-dependent wall thickening and fibrosis as we have seen in these experiments (Figure 8).

Our studies do have some limitations. For assessments of O_2^-, we used the lucigenin assay, which has known specificity issues (Lee et al., 2012). However, we confirmed our observations using fluorometric measurement of H_2O_2. In addition, the exact cellular source of the ROS was not demonstrated in our work. Further studies using cell-specific deletion mouse models would provide this information, and these studies are currently underway in our laboratory. The studies were substantially limited by the technical issues associated with the use of osmotic minipumps; limiting the duration of ANG II or isoprenaline treatment. Thus, it is possible that longer duration models of cardiac dysfunction enabling longer treatment with dietary nitrate might have evoked stronger benefit; an issue particularly relevant for the reversal experiment. In addition, we used the tail-cuff method for BP assessment which, while an excellent approach for non-invasive measurements that is in keeping with the 3Rs principles, has well described limitations that lead to variability in absolute measured values from one laboratory to another. The clinical assessments shown were made on a sample set that forms part of a larger powered clinical trial and, although we utilised robust statistical approaches to create a matched case control, the outcome of the full powered study is required. Translational studies in patients with hypertension hopefully will address this issue.

In summary, our data showed that increasing dietary intake of inorganic nitrate provides important inhibition of cardiac remodelling that is evident whether the heart disease is driven by hypertension or not. With the growing burden of HF globally, and most particularly the recent concerns regarding the worse and devastating outcome for patients with COVID-19 infection and cardiac dysfunction including those with HF (Chidambaram et al., 2020; Linschoten et al., 2020), identification of therapeutic options that are easy to deliver, safe and acceptable to patients is a current imperative. We suggest that encouraging greater inorganic nitrate consumption through the diet is an easily translatable adjunct option that is likely to appeal the international community and provide benefit in the current pandemic setting.

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AUTHOR CONTRIBUTIONS

A.A. and V.K. designed the study. J.C.M. made substantial contributions to the design of the study. L.C.G., G.M., C.L., C.P., D.F., J.C., K.S.R., A.J.P.H., F.F., R.S.K., A.K.G., G.N. and A.S.I. made substantial contributions to the acquisition, analysis and interpretation of data. All authors were involved in drafting or revising the manuscript and gave final approval of the version to be published.

CONFLICT OF INTEREST

A.A. is a co-director of a small start-up company, Heartbeet Ltd., seeking to identify therapeutic opportunities for dietary nitrate. There are no other conflicts of interest to report.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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