Chronic Administration of the Nitroxy1 Donor 1-NitrosoCYclo-Hexyl Acetate Limits Left Ventricular Diastolic Dysfunction in a Mouse Model of Diabetes In Vivo

Cao et al: HNO Prevents Diabetic Cardiomyopathy

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Abstract

Background—Nitroxyl (HNO), a redox congener of nitric oxide (NO•), is a novel regulator of cardiovascular function, combining concomitant positive inotropic,lusitropic and vasodilator properties. HNO moreover exhibits myocardial antihypertrophic and superoxide-suppressing actions. Despite these favorable actions, the impact of chronic HNO administration has yet to be reported in the context of cardiomyopathy. Diabetic cardiomyopathy is characterized by early diastolic dysfunction and adverse left ventricular (LV) structural remodeling, with LV superoxide generation playing a major causal role. We tested the hypothesis that the HNO donor 1-nitrosocyclohexylacetate (1-NCA) limits cardiomyocyte hypertrophy and LV diastolic dysfunction in a mouse model of diabetes in vivo.

Methods and Results—Diabetes was induced in male FVB/N mice using streptozotocin (STZ). After 4 weeks, diabetic and nondiabetic mice were allocated to 1-NCA therapy (83mg/kg/day i.p.) or vehicle, and followed for a further 4 weeks. Diabetes-induced LV diastolic dysfunction was evident on echocardiography-derived E and A wave velocities, E:A ratio, deceleration and isovolumic relaxation times (IVRT); LV systolic function was preserved. Increased LV cardiomyocyte size, hypertrophic and pro-fibrotic gene expression, and upregulation of LV superoxide were also evident. These characteristics of diabetic cardiomyopathy were largely prevented by 1-NCA treatment. Selectivity of 1-NCA as an HNO donor was demonstrated by sensitivity of acute 1-NCA to L-cysteine but not to hydroxocobalamin (HXC) in the normal rat heart ex vivo.

Conclusions—Our studies provide the first evidence that HNO donors may represent a promising strategy for treatment of diabetic cardiomyopathy, and implies therapeutic efficacy in settings of chronic heart failure.

Key Words: diabetes; diastolic function; cardiac hypertrophy; cardiomyopathy; nitrate
Diabetes mellitus is a widespread chronic disease affecting ~285 million adults worldwide, projected to reach 439 million by 2030\(^1\). Cardiovascular disease is among the most common causes of mortality in diabetic patients. Further, diabetes increases heart failure (HF) risk in humans 2.5-fold, even when adjusted for comorbidities such as concomitant coronary artery disease, obesity, dyslipidemia or age\(^2-4\). Diabetic cardiomyopathy is characterized by early onset of LV diastolic dysfunction, often preceding systolic dysfunction\(^2-4\), accompanied by adverse structural changes such as LV hypertrophy, increased cardiac fibrosis and cardiomyocyte apoptosis. These characteristics are a prognostic indicator of mortality in diabetic patients\(^4\) hence development of new pharmacological targets to treat LV dysfunction and remodeling in the diabetic heart is paramount.

HNO, the one electron-reduced congener of NO\(_x\), is a novel regulator of cardiovascular function\(^5-7\). Until recently, potential sources of HNO for pharmacological investigations have been limited to its prototypical donor Angeli’s salt (also generating nitrite) and isopropylamine-NONOate (IPA-NO). Both are relatively short-acting (t\(_{1/2}\) ~2.5mins), and their highly-alkaline vehicle (10mM NaOH) restricts utility in chronic therapeutic studies \textit{in vivo}. Like NO\(_x\) donors, HNO donors elicit potent vasodilator actions \textit{in vitro} and \textit{in vivo}\(^8-12\). In contrast to NO\(_x\), HNO is resistant to ROS scavenging, and does not develop vascular tolerance\(^9,12\). Moreover, HNO enhances LV systolic and diastolic function, which persists in the failing heart and is independent, at least in part, from soluble guanylyl cyclase (sGC)-mediated vasodilator actions\(^8,11,13-17\). HNO moreover exhibits antihypertrophic and superoxide-suppressing actions in the myocardium\(^18,19\), but these have yet to be extended to an \textit{in vivo} setting. HNO donors may thus offer favorable actions in a range of HF etiologies. Indeed, the recent reports that a pure HNO donor CXL-1020 reproduces these acute inotropic and lusitropic benefits in failing canine and human myocardium highlight the exciting therapeutic potential offered by HNO\(^20,21\). CXL-1020 is superior to classical donors, as it
only releases HNO (without generation of other by-products) and does not require an alkaline vehicle.\textsuperscript{20}

The chronic effects of any HNO donor, either CXL-1020 or older donors (Angeli’s salt, IPA-NO), have never been reported in any etiology, likely due to short-acting effects \textit{in vivo}. Moreover, efficacy of HNO in the distinctive context of diabetic cardiomyopathy also remains unresolved. Given the diabetic heart is characterized by cardiac remodeling and diastolic dysfunction, downstream of LV superoxide upregulation\textsuperscript{4,22,23}, we hypothesized that chronic administration of an HNO donor limits cardiomyocyte hypertrophy and LV diastolic dysfunction in a mouse model of diabetes \textit{in vivo}. An alternative putative HNO donor, 1-NCA, has been described, reportedly releasing HNO at physiological pH. Vasodilation and cardiomyocyte contractility are enhanced by 1-NCA \textit{in vitro}\textsuperscript{24-26}; both HNO and NO\textsuperscript{-} may however contribute to its actions. Although the half-life of 1-NCA has only been reported in cell-free settings, this has been reported as \textasciitilde{}13h in a methanol:neutral buffer mixture\textsuperscript{24} (considerably longer than that offered by CXL-1020 or conventional HNO donors). On this basis, we selected 1-NCA for this \textit{in vivo} study.

\textbf{Methods}

\textbf{Animal Models}

Studies were conducted in accordance with National Health and Medical Research Council of Australia (NHMRC) guidelines, and approved by Alfred Medical Research and Education Precinct (AMREP) Animal Ethics Committee. The majority of studies utilized male FVB/N mice \textit{in vivo} (AMREP Animal Services, Melbourne, Australia). At 6-7wks old, mice received five consecutive daily i.p. injections of citrate (0.1M pH 4.5) or STZ (55 mg/kg, in 0.1M citrate)\textsuperscript{22,23}. Diabetes was confirmed by fortnightly blood glucose from saphenous vein (ACCU-CHEK Advantage; Roche, Basel, Switzerland). In mice, blood glucose \textgtr{}26mM was

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{circulation.png}
\caption{Circulation Heart Failure}
\end{figure}
considered diabetic, and <12mM nondiabetic; mice with blood glucose 12-25.9mM were
excluded. Upper limit of detection for blood glucose readings was 33.3mM; HI readings were
recorded as 33.3mM. After 4wks, 1-NCA (83mg/kg/day EpiChem Pty Ltd, Murdoch, WA) or
equivalent vehicle (0.25% methanol, ~200μl/day) were administered i.p. to nondiabetic and
diabetic mice for 4wks. After total 8wks diabetes, LV function was determined, prior to
tissue collection27. Treatment groups in vivo comprised (i) vehicle-treated nondiabetic mice
(n=8), (ii) vehicle-treated diabetic mice (n=9), (iii) 1-NCA-treated nondiabetic mice (n=8)
and (iv) 1-NCA-treated diabetic mice (n=8). Thirteen adult male Sprague-Dawley rats (350-
450g, AMREP Animal Services) were also used, for acute ex vivo studies.

Echocardiography for analysis of LV function in mice in vivo
Mice were anesthetized (ketamine/xylazine/atropine, 60/6/0.6mg/kg i.p. respectively) at study
endpoint, and echocardiography performed (Philips iE33 ultrasound machine, North Ryde,
NSW)22,23,27-29. LV function and dimensions were assessed via two-dimensional M-mode and
Doppler echocardiography (using 15MHz linear array and 12MHz sector transducers
respectively). Doppler transmitral echocardiography allowed assessment of LV diastolic
filling (LV E:A, the ratio of peak early, E, and late atrial, A, transmitral blood flow
velocities), deceleration time of early (E) velocity and IVRT22,23,27-29. LV dimensions
measured on M-mode echocardiography included external LV dimension, LV end-systolic
dimension (LVESD) and LV end diastolic dimension (LVEDD). We then derived LV mass,
fractional shortening (FS=(LVEDD-LVESD)/(LVEDD×100%)) and heart-rate-corrected
velocity of circumferential fiber shortening (Vcfc)22,23,27-29. Aortic systolic (SBP) and
diastolic blood pressures (DBP) were determined via micromanometer-tipped catheter (1.4 F;
Millar Instrument Co., TX, USA)22,23,27-29.
**Tissue collection and histology**

Cardiac puncture was performed in anesthetized mice for heparinized blood collection. Heart and lungs were excised and wet weights recorded. Tibia bones were removed to measure length. A small LV portion was used for lucigenin chemiluminescence in fresh tissue, with remaining tissue cut into 3 at the horizontal short-axis plane. These were allocated to a cryomold (Sakura Finetek USA, Torrance, USA) containing Tissue-Tek®O.C.T (Grale Scientific, Ringwood Australia) on dry ice (with subsequent storage at -80°C)\(^29\), paraffin-embedding, or snap-freezing in liquid nitrogen (stored at -80°C). Paraffin-embedded sections were H&E-stained for determination of cardiomyocyte width and cross-sectional area, measures of cardiomyocyte hypertrophy\(^22,23,27,28\).

**Analysis of LV gene expression in mice**

RNA was extracted from frozen tissues, and cDNA generated from DNase-treated RNA via reverse transcription (Taqman® Reverse-Transcription reagents, Applied Biosystems, Mulgrave, VIC, Australia)\(^27\). SYBR® Green chemistry was employed to determine expression of \(\beta\)-myosin heavy chain (\(Myh7\)), connective tissue growth factor (CTGF) and pro-oxidant mitochondrial uncoupling protein 3 (UCP3), in addition to sarcoplasmic reticulum \(\text{Ca}^{2+}\) ATPase-2a (SERCA2a), using the Applied Biosystems ABI Prism® 7700 Sequence Detection System. Ribosomal 18S was the endogenous control. Primers were generated using murine-specific sequences derived from Genbank. Relative fold increases in expression compared to vehicle-treated nondiabetic mice were calculated using the comparative delta-delta \(C_t\) method\(^27,28\).

**Detection of LV superoxide generation in mice**

Superoxide generation was quantitated firstly using lucigenin-(5\(\mu\)M)-enhanced, NADPH-driven chemiluminescence in fresh heart tissue, normalized to tissue weight\(^22,23,28,29\). Dihydroethidium (DHE) fluorescence was used as a second measure of superoxide
Detection^29. LV sections were mounted on super-frosted slides, prior to 45mins incubation, 37°C, with DHE alone (Invitrogen, Mulgrave, Australia; 2μM in ice-cold phosphate-buffered-saline) or DHE+superoxide dismutase-polyethylene glycol (PEG-SOD, Sigma-Aldrich, Sydney, Australia; 500 U/ml), to quench superoxide. Images were taken with a Zeiss LSM 510 Meta confocal microscope under 40x magnification using 568nm/585nm excitation/emission wavelengths. Three images/section were analysed using Image J software (Version 1.44, National Institutes of Health, USA)^29.

**Changes in coronary flow in the isolated rat heart**

Isolated Langendorff rat heart studies were undertaken for insight into the relative contribution of HNO vs NO• in 1-NCA actions. Hearts isolated from anaesthetized male Sprague Dawley rats (ketamine/xylazine, 100/12mg/kg i.p. respectively) were perfused with Kreb’s (pH 7.4), bubbled with 95% O2/5% CO2 at 37°C, under constant pressure^8. Coronary flow was detected using the STH Pump Controller (ADInstruments Pty Ltd). Following 30mins equilibration, U46619 (10^-5.5M 0.1-2.5ml/min) was used to preconstrict coronary vessels by ~50%. A single bolus dose of vehicle (2% methanol) was administered just above the aorta, followed by a 1-NCA dose-response curve (10^-7-10^-1 mol, in 2% methanol). In parallel series of hearts, selective scavengers of HNO (L-cysteine, 4 mM) or NO• (HXC, 50μM) were added to perfusion buffer from the last 15mins of equilibration, for the remaining duration. All materials for rat heart studies were obtained from Sigma-Aldrich (Sydney, Australia) unless indicated otherwise.

**Statistical analysis**

All data are presented as mean±SD, with n representing number of animals. Statistical analysis was performed using one- or two-way ANOVA as indicated, followed by Tukey’s *post-hoc* test for *in vivo* studies in mice (unless otherwise specified). Two-way repeated measures ANOVA followed by Bonferroni’s *post-hoc* test was used for rat isolated heart studies.
(GraphPad Prism 6, GraphPad Software Ins, La Jolla, USA). P<0.05 was considered significant.

**Results**

**Systemic characteristics**
Successful diabetes induction was shown by significantly higher blood glucose in vehicle- and 1-NCA-treated diabetic, compared to nondiabetic, mice (Table 1, P<0.0001 two-way ANOVA). There were no significant differences in final bodyweight, tibia length, SBP or DBP induced by diabetes and/or 1-NCA.

**1-NCA protected the heart against diabetes-induced cardiac dysfunction**
Representative transmitral pulsed-wave Doppler echocardiography flow patterns are shown in Figure 1A. Diabetes significantly increased peak A wave velocity and reduced peak E wave velocity (Figure 1B,1C). 1-NCA treatment for the last 4wks of diabetes significantly improved peak A wave velocity. On two-way ANOVA, each of diabetes (P=0.0036), 1-NCA (P=0.0427) and their interaction (P=0.0165) were associated with significant differences in peak A wave velocity. A similar trend for 1-NCA to ameliorate impaired peak E wave velocity was evident (P=0.06 vs vehicle-treated diabetic mice, unpaired t-test), but the interaction was not significant on two-way ANOVA. Moreover the additional markers of diastolic function, reduced E:A ratio (P=0.0027, Figure 2A), and prolonged deceleration time (P=0.0005, Figure 2B) and IVRT (P=0.0084, Figure 2C) were observed, in diabetic compared to nondiabetic mice on two-way ANOVA. These diabetes-induced impairments in diastolic function were all significantly ameliorated by 1-NCA (which had no impact on diastolic function in nondiabetic mice). Each of diabetes (P<0.005), 1-NCA (P<0.05) and their interaction (P<0.05) were associated with significant differences on all three parameters, suggesting 1-NCA prevented diastolic dysfunction.
Diabetes did not elicit any significant differences in LV systolic function (using M-mode echocardiography, Table 2), consistent with both previous studies using this model and with the clinical context of earlier stages of diabetic cardiomyopathy. 1-NCA significantly reduced LVESD (P=0.0121) and increased FS (P=0.0009), with a similar trend on Vcfc, in diabetic mice selectively. There were no significant differences in heart rate or in other LV dimensions, induced by diabetes and/or 1-NCA (Table 2).

1-NCA protected against diabetes-induced cardiomyocyte hypertrophy

As shown in Figure 3A, diabetes induced a modest but significant increase in lung weight normalized to tibia length; this was prevented by 1-NCA (P=0.0094). Neither diabetes nor 1-NCA affected echocardiography-derived LV mass, or heart weight (both normalized to tibia length, Figure 3B,3C). Despite the absence of net cardiac hypertrophy, diabetes significantly induced cardiomyocyte hypertrophy (Figure 4), on cardiomyocyte width and area; 1-NCA significantly prevented these changes. Diabetes-induced cardiomyocyte hypertrophic gene expression was also observed (P=0.0043, Figure 5A), accompanied by a trend for increased pro-fibrotic gene expression (Figure 5B) and downregulated SERCA expression (P=0.0175, Figure 5C). All three parameters of LV remodeling were significantly ameliorated by 1-NCA in diabetic mice, but the HNO donor had no impact in nondiabetic mice.

Impact of 1-NCA on diabetes-induced LV superoxide generation

Diabetes significantly increased LV superoxide generation, on both lucigenin-enhanced chemiluminescence (P=0.0145, Figure 6A) and PEG-SOD-sensitive DHE fluorescence (P=0.0001, Figure 6B); expression of mitochondrial UCP3 was also markedly upregulated (P=0.0196, Figure 6C). On two-way ANOVA, diabetes was associated with a significant difference on all 3 parameters. None of these diabetes-induced increases were evident following treatment with 1-NCA; the HNO donor tended to nonsignificantly reduce all three
parameters in diabetic mice (all $P \leq 0.1$ vs diabetic vehicle on unpaired t-test), but had no impact in nondiabetic mice.

**Relative selectivity of 1-NCA as a donor of HNO**

The relative contributions of HNO and NO• to the acute actions of 1-NCA were determined in the normal, Langendorff-rat heart. As shown in Figure 7, dose-dependent coronary vasodilator responses induced by serial 1-NCA was significantly attenuated by L-cysteine ($P<0.0001$ on two-way repeated measures ANOVA), but was unaffected by HXC, suggesting a greater contribution of HNO than NO• to the actions of 1-NCA.

**Discussion**

Compelling epidemiological and clinical evidence has confirmed the existence of diabetic cardiomyopathy, a disorder characterized by early diastolic dysfunction and adverse structural remodeling\(^2\)\(^-\)\(^4\), with pre-clinical evidence implicating a major causal role of upregulated myocardial ROS generation. Although net LV dysfunction in diabetic patients likely reflects both increased coronary heart disease (secondary to atherosclerosis) as well as a specific ‘diabetic cardiomyopathy’, diabetes not only escalates risk of HF, but also increases its incidence >2.5-fold, independent of age or concomitant obesity, dyslipidemia or coronary heart disease\(^4\). Diabetic patients account for up to one third of patients in clinical HF trials, with diabetes an independent predictor of poor outcome. Despite these statistics, there is no specific therapy for the diabetic heart\(^4\). With their concomitant positive inotropic, lusitropic and vasodilator properties, short-acting HNO donors have recently attracted significant interest for clinical management of acute HF\(^8,11,13-17,20,21\). Their impact with chronic administration has however yet to be reported in the context of cardiomyopathy. The objective of this study was to elucidate whether the putative HNO donor 1-NCA prevents LV diastolic dysfunction and cardiomyocyte hypertrophy induced by diabetes. We provide the
first evidence of beneficial myocardial actions of an HNO donor over the longer-term in vivo, demonstrating that chronic treatment with 1-NCA prevents diabetes-induced diastolic dysfunction, cardiomyocyte hypertrophy and LV superoxide production in a mouse model of diabetes. These beneficial actions appear to be a direct effect on the myocardium, as 1-NCA was without BP effects. In addition, evidence supporting 1-NCA as an HNO donor was also obtained.

**1-NCA prevents diabetes-induced LV diastolic dysfunction**

Doppler echocardiography revealed a significantly decreased E:A ratio, with increased peak A wave velocity, deceleration time and IVRT in diabetic mice (with a trend for reduced peak E wave velocity), indicative of diastolic dysfunction. This was accompanied by downregulated SERCA2a expression. Reduced resequestration of Ca^{2+} into the sarcoplasmic reticulum following contraction, as a result of impaired SERCA2a activity, is a key mechanism of slowed cardiac relaxation in diabetic heart\(^4,30,31\). This dysfunction can result from diabetes-induced post-translational modifications to SERCA2a, including oxidative (e.g. at \(^674\)Cys), advanced glycation or other modifications of SERCA2a-thiol oxidation\(^4,32-34\).

In the present study, 1-NCA prevented all impairments in both diastolic function and LV SERCA2a expression in diabetic mice compared to vehicle control. Although elucidating the impact of 1-NCA on SERCA2a function was beyond the scope of the current study, HNO is known to undergo thiol-mediated interaction with SERCA2a to acutely enhance its function\(^17,35\). Our observations here represent the first report to suggest HNO may also preserve SERCA2a expression as part of its protection of cardiac relaxation.

In the present study, systolic dysfunction was not evident following 8wks diabetes, on FS or Vcfc, consistent with previous preclinical studies, where diastolic dysfunction is either evident without systolic dysfunction, or precedes its onset, in the diabetic heart\(^4,22,23,27,28,36,37\). The modest increase in lung weight index induced by diabetes, suggestive of pulmonary
congestion (and perhaps an early indication of pending systolic dysfunction), was however prevented by 1-NCA. Interestingly, a trend for enhanced FS and Vcfc with chronic 1-NCA treatment was evident in diabetic mice, consistent with the acute positive inotropic effects observed with conventional HNO donors in HF. Indeed, cardiomyocyte hypertrophy is a common characteristic of diabetic cardiomyopathy that likely further impairs cardiac relaxation. In the present study, diabetes-induced cardiomyocyte hypertrophy was evident on cardiomyocyte width and area, as well LV β-myosin heavy chain expression, consistent with previous studies. Increased cardiomyocyte size however was not reflected in net LV or heart weight at study endpoint (possibly as a result of cardiomyocyte apoptosis). The increased size of the remaining surviving cardiomyocytes may counterbalance reduced cardiomyocyte numbers, resulting in an absence of net increased heart mass. In the current study, all markers of diabetes-induced cardiomyocyte hypertrophy were attenuated by 1-NCA, consistent with previous short-term findings with classical HNO donors in isolated cardiomyocytes. To our knowledge, this is the first evidence to demonstrate antihypertrophic actions of HNO in a chronic setting in vivo. The clear, diabetes-induced cardiomyocyte hypertrophy was accompanied by a trend for increased pro-fibrotic CTGF expression, which was absent in 1-NCA-treated diabetic mice. There were however no significant differences in cardiac collagen deposition between the four experimental groups (results not shown), perhaps due to relatively shorter duration of diabetes studied here (8wks) vs longer studies where significant cardiac fibrosis is evident.

**Potential role of HNO-induced LV suppression**

Development and progression of cardiovascular diabetic complications is closely associated with increased oxidative stress as a result of imbalanced upregulated ROS generation and...
their ineffective elimination. NADPH oxidase is one of the major sources of LV ROS. In the current study, LV NADPH-driven superoxide generation was increased in diabetes, likely secondary to hyperglycemia, consistent with previous studies. We have previously shown that Angeli’s salt and IPA-NO suppress cardiomyocyte ROS production in response to hypertrophic stimuli in cultured cardiomyocytes. In the current study, the ability of diabetes to stimulate NADPH-driven LV superoxide generation (on lucigenin-enhanced chemiluminescence, confirmed on PEG-SOD-sensitive DHE fluorescence) was absent in 1-NCA-treated mice. This trend was reproduced on LV mitochondrial UCP3 expression.

Although sGC (in its reduced, Fe²⁺-containing form) is regarded as the receptor for NO, HNO is an equipotent sGC stimulator. HNO suppression of cardiomyocyte ROS production is mediated via sGC-dependent downregulation of NADPH oxidase. Insufficient mouse LV tissue precluded determination of LV cGMP content here. Unlike NO, HNO is resistant to ROS scavenging, does not develop tolerance to its actions and may target oxidized LV tissue precluded determination of LV cGMP content here. Unlike NO, HNO is resistant to ROS scavenging, does not develop tolerance to its actions and may target oxidized sGC. HNO also has a unique ability to enhance LV function, considered dependent on both sGC-independent and -dependent mechanisms.

**HNO contributes to the mechanism of action of 1-NCA**

1-NCA is thought to spontaneously hydrolyze to form an unstable α-hydroxy-nitroso intermediate, which then generates cyclohexanone and HNO. There is however prior evidence implicating both HNO and NO in its actions in vitro. We selected this donor here however on the basis of both its reportedly longer halflife than that offered by CXL-1020 or conventional HNO donors, as well as its stability as an HNO donor at physiological pH. In the present study, we sought to determine the relative contribution of the two redox siblings to the vasodilator properties of 1-NCA, by examining its acute sensitivity to selective scavengers of HNO (L-cysteine) and NO (HXC) in the normal isolated rat heart ex vivo. L-cysteine significantly reduced 1-NCA-induced increases in coronary flow, implicating HNO,
at least in part, in the mechanism of action of acute 1-NCA. In previous studies, the putative selective NO• scavenger, carboxy-PTIO [2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide, monopotassium salt], tended to also reduce a component of the vasodilator actions of 1-NCA. As carboxy-PTIO blunts both NO• and peroxynitrite-mediated actions\textsuperscript{48}, the current study used HXC (a more selective NO• scavenger) to investigate the possible contribution of NO to 1-NCA actions. HXC did not affect vasodilator action of 1-NCA; this suggests that the effects of 1-NCA may be more dependent on HNO rather than NO•.

**Limitations of the study**

Limited tissue availability precluded assessment of LV cGMP content, or of excitation-contraction coupling, to gain further insight into the potential mechanisms of action of 1-NCA. Moreover, the longer-term nature of our study did not permit use of HNO or NO•-selective scavengers \textit{in vivo}, to unequivocally demonstrate the role of HNO in the protective effects of chronic therapy. Our experimental design also precluded pharmacokinetic assessment of 1-NCA, to determine its plasma half-life and bioavailability \textit{in vivo}. Although 1-NCA has been reported to have a longer half-life (~13h)\textsuperscript{24} compared to other HNO donors (Angeli’s salt, IPA-NO and CXL-1020, half-life 2-3 min)\textsuperscript{5,7,20}, this has only been determined in a cell free environment \textit{in vitro}\textsuperscript{24}. Our studies however open the door for analogous longer-term studies interrogating the impact of next-generation, pure HNO donors on diabetes-induced LV diastolic dysfunction, cardiomyocyte hypertrophy and LV ROS generation, building on the promising results achieved with CXL-1020 in the clinical context of acute HF\textsuperscript{20,21}.

**Conclusions**

This study is the first to show the beneficial effects of chronic therapy with a putative HNO donor over the longer-term \textit{in vivo}, in any cardiomyopathy context. Moreover, it is the first
interrogation of the potential actions of HNO specifically in the setting of diabetes. We have demonstrated here that 1-NCA rescued LV diastolic function, together with reduced LV ROS production and cardiomyocyte hypertrophy, in a mouse model of diabetes in vivo, without adverse effects. Collectively, our studies provide the first evidence that HNO donors may represent a promising new strategy for the treatment of diabetic cardiomyopathy, and implies their therapeutic efficacy in settings of chronic HF, either as stand-alone therapy and/or as a supplement to standard care.

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Disclosures
None.

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</tbody>
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*P<0.05 and ****P<0.0001 vs nondiabetic, vehicle-treated sham; #P<0.05 vs diabetic, vehicle-treated mice.
Table 2. LV dimensions and function on M-mode echocardiography (mean±SD).

<table>
<thead>
<tr>
<th></th>
<th>vehicle-treated nondiabetic mice (n=8)</th>
<th>vehicle-treated diabetic mice (n=9)</th>
<th>1-NCA-treated nondiabetic mice (n=8)</th>
<th>1-NCA-treated diabetic mice (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heartrate (bpm)</strong></td>
<td>370±363</td>
<td>391±88</td>
<td>387±41</td>
<td>437±80</td>
</tr>
<tr>
<td><strong>External LV dimension (mm)</strong></td>
<td>5.42±0.26</td>
<td>5.54±0.35</td>
<td>5.39±0.18</td>
<td>5.37±0.29</td>
</tr>
<tr>
<td><strong>LVEDD (mm)</strong></td>
<td>3.55±0.39</td>
<td>3.65±0.32</td>
<td>3.58±0.20</td>
<td>3.45±0.29</td>
</tr>
<tr>
<td><strong>LVESD (mm)</strong></td>
<td>2.09±0.31</td>
<td>2.11±0.35</td>
<td>2.35±0.25</td>
<td>1.76±0.33*</td>
</tr>
<tr>
<td><strong>LVPW (mm)</strong></td>
<td>0.85±0.11</td>
<td>0.86±0.18</td>
<td>0.73±0.13</td>
<td>0.89±0.14</td>
</tr>
<tr>
<td><strong>FS (%)</strong></td>
<td>41.3±2.8</td>
<td>42.5±5.4</td>
<td>35.6±4.3</td>
<td>49.2±6.3*</td>
</tr>
<tr>
<td><strong>Vcfc (circ/s)</strong></td>
<td>7.4±1.5</td>
<td>7.6±2.2</td>
<td>6.0±1.2</td>
<td>9.2±3.4*</td>
</tr>
</tbody>
</table>

LVPW, LV posterior wall thickness. *P<0.05 vs vehicle-treated diabetic mice.
Figure Legends

**Figure 1.** 1-NCA prevents diabetes-induced changes in transmitral flow (over final 4wks of 8wks diabetes). A Representative mitral flow patterns from Doppler echocardiography. Diabetes significantly B increases peak A and C decreases peak E wave velocity. Both effects are absent in 1-NCA-treated diabetic mice. Results are presented as mean±SD. *P<0.05, **P<0.01 vs nondiabetic vehicle, #P<0.05 vs diabetic vehicle.

**Figure 2.** 1-NCA prevents diabetes-induced LV diastolic dysfunction. Diabetes A decreases E:A and prolongs B deceleration time, and C IVRT in mice. 1-NCA prevents these diabetes-induced changes. Results are presented as mean±SD. **P<0.01, ***P<0.001, ****P<0.0001 vs nondiabetic vehicle, #P<0.05, ###P<0.001, ####P<0.0001 vs diabetic vehicle.

**Figure 3.** Impact on net cardiac hypertrophy in diabetic mice. A Although diabetes modestly increases lung weight:tibia length (ameliorated by 1-NCA), neither B echocardiography-derived LV mass or C heart weight are significantly affected by chronic diabetes or 1-NCA in vivo (all normalized to tibia length). Results are presented as mean±SD. *P<0.05 vs nondiabetic vehicle, #P<0.05 vs diabetic vehicle.

**Figure 4.** 1-NCA prevents diabetes-induced cardiomyocyte hypertrophy in vivo. A Representative H&E-stained LV sections. Diabetes increases B cardiomyocyte width and C cardiomyocyte area, both of which are prevented by 1-NCA. Results are presented as mean±SD. P<0.05 vs nondiabetic vehicle, #P<0.05 vs diabetic vehicle (unpaired t-test).
Figure 5. **1-NCA prevents diabetes-induced pro-remodeling LV gene expression.** Diabetes increases A β-myosin heavy chain; with a similar trend on B CTGF expression. C LV SERCA2a expression is also reduced in diabetic mice. 1-NCA tends to attenuate these diabetes-induced changes (all relative to 18S, fold nondiabetic sham). Results are presented as mean±SD. *P<0.05 vs nondiabetic vehicle, #P<0.05, ##P<0.01 vs diabetic vehicle (unpaired t-test).

Figure 6. **Impact of 1-NCA on diabetes-induced LV oxidative stress in diabetic mice.** Diabetes increases A LV superoxide generation (lucigenin chemiluminescence), B PEG-SOD-sensitive DHE fluorescence and C UCP3 expression (relative to 18S, fold nondiabetic sham). These increases were no longer evident in 1-NCA-treated diabetic mice compared to diabetic vehicle. Results are presented as mean±SD. *P<0.05 vs nondiabetic vehicle.

Figure 7. **An HNO scavenger prevents 1-NCA-induced coronary vasodilation ex vivo.** Dose-response curves to serial bolus doses of 1-NCA on coronary flow in isolated, Langendorff-perfused rat hearts, in the absence and presence of L-cysteine (4mM) or HXC (50μM); results presented as mean±SD, n=3-7/group; ****P<0.0001 for the dose-response curve to 1-NCA alone, vs that in the presence of L-cysteine, on two-way, repeated-measures ANOVA.
Figure 1

A) Vehicle and A wave Peak E Wave (cm/s) and A wave Peak A Wave (cm/s) for nondiabetic and diabetic groups with 1-NCA treatment. 

B) Distribution of Peak A Wave (cm/s) for nondiabetic and diabetic groups with 1-NCA treatment. 

C) Distribution of Peak E Wave (cm/s) for nondiabetic and diabetic groups with 1-NCA treatment.
Figure 2

A

E:A wave ratio

Vehicle 1-NCA

nondiabetic diabetic

** #

B

Deceleration time (ms)

Vehicle 1-NCA

**** ###

C

IVRT (ms)

Vehicle 1-NCA

*** ####
Figure 3
Figure 4

A

Diabetic
Nondiabetic

Vehicle 1-NCA

B

Cardiomyocyte Width (μm)

Vehicle 1-NCA

nondiabetic diabetic

C

Cardiomyocyte area (μm²)

Vehicle 1-NCA

* #
Figure 5

Panel A: β-myosin heavy chain
- Vehicle
- 1-NCA
- nondiabetic
- diabetic

Panel B: TGF expression
- Vehicle
- 1-NCA

Panel C: SERCA expression
- Vehicle
- 1-NCA

Statistical significance:
- * P=0.09
- # P<0.01
Figure 6

A

![Bar graph showing lucigenin (RLU/sec/mg) with nondiabetic and diabetic groups. The p-value is 0.09.]

B

![Bar graph showing DHE fluorescence (fold) with nondiabetic and diabetic groups.]

C

![Bar graph showing UCP3 expression with nondiabetic and diabetic groups.]

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Figure 7

Circulation

Heart Failure

Δ Coronary Flow (ml/min)

Log [1-NCA] mol

1-NCA alone

1-NCA + L-cysteine

1-NCA + HXC

****
Chronic Administration of the Nitroxyl Donor 1-Nitrosocyclo-Hexyl Acetate Limits Left Ventricular Diastolic Dysfunction in a Mouse Model of Diabetes In Vivo

Nga Cao, Yung George Wong, Sarah Rosli, Helen Kiriazis, Karina Huynh, Chengxue Qin, Xiao-Jun Du, Barbara K. Kemp-Harper and Rebecca H. Ritchie

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