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Nitroxyl (HNO) a Novel Approach for the Acute Treatment of Heart Failure

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Abstract

Background—The nitroxyl (HNO) donor, Angeli's salt (AS), exerts positive inotropic, lusitropic, and vasodilator effects *in vivo* that are cyclic AMP-independent. Its clinical utility is limited by chemical instability and co-generation of nitrite that itself has vascular effects. Here we report on effects of a novel, stable, pure HNO donor (CXl-1020) in isolated myoctyes, and intact hearts in experimental models and in patients with heart failure (HF).

Methods and Results—CXL-1020 converts solely to HNO and inactive CXL-1051 with a $t_{1/2}$ of 2 minutes. In adult mouse ventricular-myocytes, it dose-dependently increased sarcomere shortening by 75–210% (50–500 μM), with a ~30% rise in the peak Ca^{2+} transient only at higher doses. Neither protein-kinase-A or soluble guanylate-cyclase inhibition altered this contractile response. Unlike isoproterenol, CXL-1020 was equally effective in myocytes from normal or failing hearts. In anesthetized dogs with coronary microembolization-induced HF, CXL-1020 reduced LV end-diastolic pressure and myocardial oxygen-consumption while increasing ejection fraction from 27 to 40% and maximal ventricular power index by 42% (both $p<0.05$). In conscious

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Disclosures

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dogs with tachypacing-induced HF, CXL-1020 increased contractility assessed by end-systolic elastance, and provided veno-arterial dilation. Heart rate was minimally altered. In patients with systolic HF, CXL-1020 reduced both left and right heart filling pressures and systemic vascular resistance, while increasing cardiac and stroke volume index. Heart rate was unchanged, and arterial pressure declined modestly.

Conclusions—These data show the functional efficacy of a novel pure HNO donor to enhance myocardial function, and show first-in-man evidence for potential utility in heart failure.

Clinical Trial Registration—URL: http://www.clinicaltrials.gov. Unique identifiers: NCT01096043, NCT01092325.

Keywords

nitroxyl; cardiomyopathy; contractility; myocyte; pharmacology; human; canine

Patients with acute decompensated heart failure (ADHF) present a complex and often life threatening clinical syndrome. New therapeutic advances remain scant and patients are at major risk for recurrent hospitalizations and suffer a high mortality rate[1–3]. The initial thrust of therapy focuses on decongestion and hemodynamic stabilization, with removal of excess fluid by diuresis or ultrafiltration[4], and use of arterial and venous dilators to reduce preload and afterload[3, 5]. In a substantial number of patients, these approaches prove insufficient or cannot be adequately employed due to renal dysfunction and hypotension. In such individuals, inotropes are often considered[6] though this avenue has been historically limited by difficulties in separating therapeutic benefit from unwanted toxicity. The most commonly used agents are dobutamine or milrinone, but both confer adverse myocardial effects, including tachycardia and arrhythmia linked to cAMP-dependent signaling[7], and can worsen long-term outcomes[8, 9]. Several new strategies are being pursued, including omecamtiv mercarbil, an activator of myosin ATPase[10, 11], and istaroxime that is thought to impact calcium handling[12]. Safe and effective therapies that enhance LV function and also aid in decongestion, remain lacking.

Nitroxyl (HNO) is a reactive-nitrogen species that while related to nitric oxide (NO), displays many unique biochemical and pharmacological features[13, 14]. HNO improves myocardial function by direct positive cAMP-independent lusitropic and inotropic effects, and by combined venous and arterial dilation[15–19]. HNO targets selective cysteine residues (negatively charged, or thiolates) resulting in covalent bonding and/or formation of a reversible disulfide. In myocytes, HNO enhances sarcoplasmic reticular calcium uptake and release via cysteine modifications on SERCA2a[19, 20], phospholamban[21, 22] and the ryanodine receptor[19], and also improves myofilament calcium sensitivity[15, 23]. HNO does not alter L-type calcium channel current nor total SR calcium load[16]. Unlike its myocyte effects, vasodilation from HNO has been attributed to soluble guanylate cyclase activation[24, 25] though other pathways remain possible. Importantly, the effects of HNO on the heart are a) independent of cAMP or cGMP; b) similar in normal and failing myocardium; c) minimally impacted by β-adrenergic receptor blockade; and d) are additive with agents stimulating cAMP/PKA pathways (e.g. beta-receptor agonists), unlike NO [17].

The compendium of pharmacological effects of HNO donors has suggested potential for treating both congestion and hemodynamic insufficiency in ADHF. However, major limitations in available HNO donors have impeded progress in the field. Virtually all prior studies have utilized the inorganic compound Angeli's salt (AS, $Na_2N_2O_3$)[26] that is chemically unstable and thus unsuitable for clinical use. AS also co-generates nitrite which itself has potent vascular effects[27]. To circumvent these limitations, we developed a novel Piloty's acid cogener, CXL-1020, which non-enzymatically decomposes to produce pure HNO and an inactive organic by-product (CXL-1051). We tested the impact of CXL-1020 on isolated myocyte function and calcium transients, determined its dose-dependent efficacy *in vivo* in two canine models of cardiac failure, and performed the first clinical study of an HNO donor testing proof-of-concept for patients with decompensated HF. The results support the potential utility of HNO donors as a novel HF treatment.

Methods

Pharmacology of CLX-1020

CXL-1020 (Cardioxyl Pharmaceuticals, NC) was synthesized as a pure HNO donor that chemically decomposes to HNO and an organic byproduct (CXL-1051). CXL-1051 has no cardiovascular pharmacological activity, and is not metabolized in vivo but rather excreted unchanged in the urine. In PBS buffer, the decay half-times of CXL-1020 and generation of HNO and CXL-1051 measured by reverse phase HPLC were 1.9, 1.5, and 2.1 minutes, respectively (Figure 1A, conditions for HPLC analysis provided in Supplemental Table 1, quantitation shown in Supplemental Figure 1). At high concentrations, HNO rapidly dimerizes in aqueous solution to HON-NOH which decomposes to nitrous oxide (N_2O) and water. Thus, in the test tube, HNO generation is measurable by quantifying N_2O by gas chromatography headspace analysis. The disappearance of CXL-1020 and appearance of N_2O and CXL-1051 were highly correlated (Figure 1A), and importantly 100% degradation of CXL-1020 yielded 100% appearance of N_2O and CXL-1051, confirming CXL-1020 did not generate other NO-species such as NO or nitrite. Quantitative conversion of CXL-1020 to CXL-1051 was also documented in EDTA-treated whole human blood (Figure 1B), with $t_{1/2}$ for loss of CXL-1020 and formation of CXL-1051 being 2 min. CXL-1020 is stable (>95%) in aqueous solution at pH < 4.5 for at least 24 hrs, and soluble to ~1 mg/mL in H_2O for injection, 5% dextrose, and 0.9% saline, and 100 μM Citrate pH 4.0. Higher concentrations (up to 30 mg/mL) were achieved by formulation with a β-cyclodextran.

In vitro myocyte studies

Adult left ventricular cardiomyocytes were isolated from male 3–6 month old C57Bl/6 mice (Jackson Laboratory, ME) with either normal or failing hearts (latter induced by 9-wks transverse aortic constriction)[28]. Details are provided in supplemental methods. Cells were studied at room temperature, superfused in Tyrode's solution, and stimulated at 0.5 Hz. Sarcomere shortening and twitch kinetics were measured by inverted fluorescence microscopy (Ellipse TE2000, Nikon, Inc) using Fourier-image analysis (MyoCam, IonOptix, MA). Cells were pre-incubated with Fura-2/AM (Molecular Probes, 3 μM for 10 min, de-esterification 20 minutes) to measure whole cell Ca^{2+} transients. Cells were then exposed to

CXL-1020 (50–500 μM), prepared from a 100 mM stock solution in 100% DMSO (final concentration of DMSO of 0.05–0.5%).

In-vivo canine studies

Two canine models of cardiac failure were studied. All studies followed procedures approved by the respective institutional Animal Care and Use Committee of the Johns Hopkins Medical Institutions, or Henry Ford Hospital.

Group A: dogs had ischemic cardiomyopathy generated by serial coronary microembolization[29] (supplemental methods). An initial dose-finding study (n=3, CXL-1020 3–100 μg/kg/min × 40 min) identified two doses (n=6, 3 or 10 μg/kg/min × 4 hours) for subsequent hemodynamic analysis. CXL-1020 was mixed with 7% Captisol® in sterile water at pH=4 (vehicle), the latter then used as vehicle control. Data were obtained under general anesthesia induced by i.v. hydromorphone (0.22 mg/kg) and diazepam (0.17 mg/kg), and maintained with 1–2% isofluorane. Cardiac function was assessed by micromanometer arterial and left ventricular pressures, right heart catheterization pressures, contrast ventriculography (LV volumes and echoDoppler cardiography, as previously reported[29]. LV peak power index ($P_{max}I$) was estimated by (peak aortic flow velocity × peak systolic pressure)/EDV^2 ([30]). Diastolic function was assessed by deceleration time of mitral inflow velocity (DT) and ratio of early to late filling time integrals (E_i/A_i). Myocardial oxygen consumption was assessed at baseline and after 4 hours as previously described[31] (details in supplemental methods). At the highest dose, blood samples were obtained to determine Nt-pro BNP, pro atrial natriuretic peptide (proANP), and troponin-I (TnI) by ELISA assay per manufacture's instructions (supplemental methods).

A separate group of animals (n=6) were subjected to programmed ventricular stimulation after receiving 5 μg/kg/min CXL-1020 × 40 min or vehicle. Each study was terminated when extra-stimuli provoked sustained monomorphic ventricular tachycardia (VT) for >30 seconds or ventricular fibrillation (VF).

In Group B dogs, heart failure was induced by 3-week tachypacing. Conscious dogs were chronically instrumented to obtain LV pressure-volume relations, including an LV micromanometer (P22; Konigsberg Instruments, Pasadena, CA), right atrial and descending aortic catheters, 3-pairs of orthogonal endocardial sonomicrometers to assess LV volume, and inferior vena caval cuff occluder[17]. Epicardial pacing leads sutured to the LV free wall were connected to an implanted pacemaker (Spectrax, Medtronics, Minneapolis). Data were recorded in conscious animals both at initial baseline when the heart was normal, and following induction of HF. Pressure-volume relations were obtained and used to assess end-systolic elastance (Ees) and preload-recruitable stroke measures of contractile function, and steady state data used to assess chamber volumes and pressures and isovolumic relaxation time constant[32]. Baseline data were obtained during vehicle infusion, and then CXL-1020+vehicle was administered at doses ranging 3–100 μg/kg/min. Data were digitally recorded (200 Hz) at each dose after reaching a steady-state response (~10 minutes).

Human Heart Failure Studies

A prior Phase 1–2a pilot study in patients with stable heart failure (Clinicaltrials.gov NCT01092325) identified 4 hour exposure to CXL-1020 at 1–30 μg/kg/min as safe and potentially active.[23] The present study (Clinicaltrials.gov NCT010960430) examined the hemodynamic effects and safety of CXL-1020 at doses of 1–20 μg/kg/min in patients hospitalized for hemodynamic assessment of HF prior to transplantation or for treatment of decompensated HF requiring hemodynamic monitoring. IRB approval was obtained at each institution involved with the trial, pursuant to federal guidelines, and all subjects provided informed consent. Measurements were obtained within 72 hours of hospitalization. Inclusion criteria required a mean CI ≤ 2.5L/min and a mean PCWP or PAD > 20 mmHg at baseline, based on 3 consecutive CI and PCWP measurements within 10% agreement and measured in the hour preceding drug administration. Baseline diuretic and oral vasodilator therapy was withheld for at least 3 hours prior to baseline recordings, and no parenteral hemodynamically-active agents were allowed within 12 hours of baseline measurements. Patients with a HR <50 or ≥ 100 BPM, a systolic blood pressure of <100 or >150 mmHg or a diastolic BP of >95 mmHg at baseline prior to randomization were excluded. Also excluded were patients with atrial fibrillation without adequate rate control, or with evidence of clinically significant non-sustained VT (10 beats or at a rate >120 bpm) in the preceding 12 hours. CXL-1020 was administered intravenously using a placebo-controlled (4:1 active-to-placebo randomization ratio) 6 hour forced titration design, with up-titration at 2 hour intervals. An overall dose range of 1 – 20 μg/kg/min was studied using over-lapping dose ranges in 2 cohorts (Cohort 1 = 1, 3 and 10 μg/kg/min; Cohort 2 = 3, 10 and 20 μg/kg/min).

Statistical Analysis

For myocyte studies, data were analyzed using paired responses for a given set of cells that were exposed to a particular dose of test drug. As these differences were often not normally distributed, we tested the null-hypothesis (%change=0) using a Wilcoxan sign-rank test. For intact canine studies Group A: within group comparisons were made using repeated measures analysis of variance (ANOVA) with alpha set at 0.05. If significance was attained, then pairwise comparisons were made using the Student-Newman-Kuels test with $p<0.05$ considered significant. For Group B: parameters were assessed by repeated measures analysis of covariance, with a Tukey test for multiple comparisons, and normal versus failing data compared by non-parametric (Wilcoxan) test. For clinical studies, change from baseline of a hemodynamic parameter measured at a given time point following drug infusion (or placebo) was determined, and these differences were then compared by t-test to to determine if drug-response differed from placebo (placebo-corrected response). Data are reported as the mean ± SEM.

Results

CXL-1020 improves myocyte systolic and diastolic function

Figure 2A shows example sarcomere length and Ca^{2+} tracings before and after exposure to CXL-1020 (50 μM). Sarcomere shortening rose substantially in a dose-dependent manner, reaching ~210% over baseline at 500 μM. Peak Ca^{2+} transients rose more modestly (+30% at the highest dose), displaying little change at lower doses (Figure 2B). Diastolic Ca^{2+} was

little altered (≤3%) at any of the doses (data not shown). Systolic functional changes were accompanied by a faster rate of sarcomere re-lengthening and shortening of the Ca^{2+} transient decay (Figure 2C). The decomposition product CXL-1051 had no direct impact on the cells (Supplemental Figure 2A).

To test if functional effects of CXL-1020 required protein kinase A or cGMP dependent signaling, cells were co-incubated with either Rp-cAMPs (PKA inhibitor, 100 μM × 30 min) or 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 μM×30 min) to block soluble guanylate cyclase (Figure 2D). Neither intervention impacted CXL-1020 modulation of sarcomere shortening. Rp-cAMPs or ODQ incubation alone had no effects on basal cell shortening at the concentrations studied (% shortening 3.4±0.3% with Rp-cAMPs, 3.4±0.4% with ODQ, both $p>0.22$ versus 3.0±0.19% for non –treated cells). As first demonstrated with AS [18], CXL-1020 mediated contractility was also redox sensitive, being suppressed by pre-incubating cells with n-acetyl cysteine (5 mM) (Supplemental Figure 2B).

We next tested whether CXL-1020 influences myocyte contractility in cells from failing hearts. In contrast to the blunted response to the beta-adrenergic agonist isoproterenol (2.5 nM) in failing cells, CXL-1020 induced changes similar to those in normal cells (Figure 2E). In controls, whole cell Ca^{2+} transients rose (+21±9% and +21±5%) and declined faster (−13±5% and −11±2%) similarly with stimulation by ISO or CXL-1020 (each $p<0.05$ vs baseline). However, in failing cells, the ISO Ca^{2+} response was $1/3^{rd}$ that with CXL-1020, and while the decay rate of the Ca^{2+}-transient was unaltered by ISO it shortened −15±5% with CXL-1020. These data support independence of CXL-1020 effects from cAMP/PKA-dependent inotropy or lusitropy that are blunted in failing myocytes.

In vivo effects of CXL-1020 in canines with ischemic cardiomyopathy (Group A)

We next assessed integrative cardiovascular effects of a 4-hour CXL-1020 infusion at 3 or 10 μg/kg/min (doses derived from preliminary dose-ranging study). Compared with vehicle control, LV end-diastolic volumes declined by ~15% and ejection fraction rose from 27 to 40% ($p<0.05$) at the higher CXL-1020 dose (Figure 3A). Arterial blood pressures declined slightly over the course of the procedure. The fell 8–9 mmHg more (diastolic and mean) at 3 μg/kg/min but were unaltered from control at higher doses (Table). End-systolic volume, end-diastolic pressure, and systemic vascular resistance, all declined. Heart rate was unchanged, and systolic contractility indexed by maximal power index[30] rose 42±2% ($p<0.001$) (Table). Early diastolic function reflected by E_i/A_i filling ratio (Figure 3A) and E-wave deceleration time improved, and CXL-1020 also reduced plasma NT-pro BNP and pro-ANP (Table). Plasma troponin I was unchanged.

In vivo effects of CXL-1020 in conscious canines using PV loop analysis (Group B)

To more directly test if CXL-1020 enhanced in vivo contractility, pressure-volume analysis was performed in conscious dogs before and after the induction of heart failure due to tachypacing. HF was more severe in Group B than Group A animals (Supplemental Table 2). In failing hearts, CXL-1020 (3–100 μg/kg/min) lowered end-systolic pressure and volume, and end-diastolic pressure (declined nearly 30%) in a dose-dependent manner, whereas HR was little altered (Figure 4A). Systemic vascular resistance and time-constant of

relaxation both declined whereas contractile function indexed by load-insensitive end-systolic elastance (Ees) increased. Figure 4B displays example PV relations before and after CXL-1020, showing a rise in Ees.

Many of these responses were also observed when CXL-1020 was administered to the dogs in the control state prior to inducing HF (Supplemental Table 3), however there were some differences. At the maximal CXL-1020 dose, there was a greater percent decline in preload volume (EDV) yet less reduction of systemic vascular resistance in the normal versus HF state (Figure 4C), with ejection fraction consequently rising more in dogs with HF. A similar disparity has been observed with AS[17, 18]. Heart rate tended to rise in controls likely reflecting a baro-reflex response, but declined slightly in HF dogs. The percent change in end-systolic elastance was similar in both conditions.

Electrophysiology and CXL-1020

Neither canine model revealed electrophysiological instability in association with CXL-1020 infusion. There were no changes in QTc interval (Supplemental Figure 3). In animals subjected to programmed ventricular stimulation, CXL-1020 did not alter the threshold for inducing ventricular tachycardia or fibrillation, nor impede cardioversion when either were induced (Supplemental Table 4).

Hemodynamic effects of CXL-1020 in patients with decompensated heart failure

The demographics, co-morbidities, and medications of the 31 study patients are provided in Supplemental Table 5. Subject age, gender, HF etiology, and function class were similar between the treatment groups. Baseline hemodynamics (Supplemental Table 6) demonstrated severe systolic heart failure with a depressed cardiac index (1.9–2.2 L/m/m^2), and elevated right and left heart filling pressures (15–17, 25–30 mmHg, respectively). CXL-1020 was infused at rates of 1–20 μg/kg/min in a placebo-controlled, forced titration protocol in which 2 patient groups received overlapping dose ranges (low dose group (N=12) = 1, 3 and 10 μg/kg/min; high dose group (N=12) = 3, 10 and 20 μg/kg/min) and matching placebo (N=7).

Heart rate was unchanged at all doses of CXL-1020, and there were no statistically significant changes in any hemodynamic measure at 1 or 3 μg/kg/min. Hemodynamic effects of CXL-1020 infusion at 10 and 20 μg/kg/min are shown in Figure 5. At 10 μg/kg/min, pulmonary artery diastolic and mean arterial pressure declined. At 20 μg/kg/min, PCWP also declined, accompanied by increased cardiac index and stroke volume index, whereas the mean arterial pressure change was insignificant. Systemic vascular resistance (SVR) tended to fall modestly at both 10 and 20 μg/kg/min doses, reaching significance at the higher dose, while right atrial pressure (RAP) fell significantly at both doses.

CXL-1020 was also evaluated in several echocardiography cohorts (online supplement) at doses up to 20μg/kg/min. After 6 hours of infusion, MAP was unchanged at any dose, however at the highest dose, HR declined from baseline relative to placebo ($p<0.01$). There were non-significant trends for decreases in LV end-diastolic and end-systolic volumes, an increase in ejection fraction, and increase in stroke volume (Supplemental Figure 4).

Safety and tolerability of CXL-1020 in patients with heart failure

CXL-1020 was well tolerated with few apparent side effects. Both Adverse and Significant Adverse Events for the study are provided in Supplemental Table 7. Drug treatment was not terminated for adverse experiences in any patient in this study. There were no adverse trends in routine laboratory parameters for hematology, chemistry or urinalysis.

Discussion

We report on a novel pure HNO donor, CXL-1020, and demonstrate direct positive inotropic and lusitropic effects in cardiac myocytes from normal and failing hearts, and positive contractile and lusitropic effects and mild vasodilatory effects in failing canine hearts *in vivo*. Importantly, we translate these findings for the first time to the clinic, finding CXL-1020 enhances cardiac performance while unloading the left ventricle in patients with decompensated systolic heart failure. CXL-1020 effects were stable over infusion periods ranging from 4–6 hours, and did not alter heart rate or induce arrhythmia. This first cell-to-human evaluation of a pure HNO donor suggests the potential efficacy and utility of this pharmacological approach to improve the function of the failing heart.

Despite being discovered over a century ago[26], Angeli's salt and the chemistry and physiologic role of HNO have only recently received attention. HNO's chemical cousin, nitric oxide, has been far more studied, and its signaling roles mediated by chemical modification of cysteines (S-nitrosylation)[33] and activation of sGC[34] is widely appreciated. Nonetheless, the two molecules are chemically and physiologically distinct, and they do not interconvert under normal physiological conditions. While the chemistry of HNO also involves post-translational modifications of selective reduced cysteines (thiolates), those modifications result in either a single modified residue (sulfinamide) or induction of a reversible disulfide between neighboring cysteines[13, 21, 23]. While NO synthases can produce HNO under conditions of oxidative stress, the endogenous production of HNO remains the subject of considerable speculation and debate. The lack of a bioassay has prevented definitive elucidation of this question. However, there is a growing body of data regarding physiological/pharmacological effects of HNO donors, and this has spawned considerable interest in understanding its biochemistry and potential therapeutic potential.

Paolocci and colleagues originally reported that AS augmented cardiac contractility and relaxation in a cAMP-independent manner[18]. This study also first suggested a link between HNO and secretion of the neuropeptide calcitonin gene related peptide (CGRP). However, CGRP signaling is coupled to cAMP stimulation, and in subsequent studies, we showed vivo modulation of contractility by CGRP depended on local sympathetic activation rather than a direct effect on cardiomyocytes[35]. Furthermore, the inotropic response to CGRP was markedly blunted in the failing heart[35], consistent with down regulation of sympathetic stimulation responses in this syndrome. Since AS had direct activity on cardiomyocytes and its impact was not blunted in the failing heart[17], an alternative to a CGRP mechanism was sought.

More direct mechanistic insights followed the discovery that AS directly improves Ca^{2+} uptake and release from the SR in a manner independent of cAMP or cGMP generation.

This cellular behavior was also very different from that induced by the NO donor, DEA/NO. Importantly, HNO does not alter L-type calcium channel current[16] nor augment SR calcium load[19], in contrast to agents that generate inotropy by elevating cAMP. Subsequent work revealed a direct impact on enhancing myofilament calcium sensitivity[15]. More recent studies have begun identifying the molecular targets of HNO that could underlie these myocardial effects. Glutathiolation at cysteine 674 has been proposed to link AS inotropy to enhanced SERCA2a activity, and formation of an internal disulfide in phospholamban by AS was shown to enhance SR-Ca^{2+} uptake as well[21]. Recently, Sivakumaran et al.[22] showed PLN is required to observe HNO augmentation of both inotropy and Ca^{2+} transients, and enhance SR Ca^{2+} uptake and Ca2+-dependent SERCA2a conformational flexibility. This was achieved by stabilizing PLN in an oligomeric disulfide bond-dependent configuration, decreasing the amount of free monomeric (inhibitory) PLN. HNO-induced disulfide links between actin-tropomyosin and myosin heavy chain and myosin light chain have been linked to increased myofilament Ca^{2+} sensitivity[23]. Vascular studies have reported vasodilation attributed to activation of soluble guanylate cyclase and/or voltage-gated potassium channels[25]. However, it remains possible that smooth muscle SR calcium cycling is involved, and the potential role of NO_2^- co-generated by AS decomposition remains unresolved.

The present study of CXL-1020 addresses a number of prior critical limitations of AS research. First, it isolates the response to HNO alone, excluding potential effects of the nitrite released by AS. Second, it enables studies of sustained exposure (e.g. 4–6 hours), whereas AS instability made such infusion experiments difficult to impossible. The findings with CXL-1020 at both cellular and intact organ levels – in mammalian species ranging from mouse to man – are remarkably compatible with prior data with AS. We observed modest vasodilator effects from HNO that contributed to the integrative functional improvement (e.g. stroke volume, EF) observed. However, using measures that were less load-dependent, such as peak power index or Ees, we showed significant increases in contractile function from CXL-1020. The decline in arterial pressure at high doses indicates a direct vasodilator impact of HNO, though CXL-1020 either did not change heart rate or even resulted in a slight decline at these doses, likely a result of improved contractile performance. As with AS, the onset of hemodynamic effects with CL-1020 were rapid, though recovery was somewhat slower. Lastly, our data show that sustained intravenous administration of a pure HNO donor is not arrhythmogenic. The ischemic HF model (Group A) displays easily inducible malignant ventricular arrhythmias[36], and neither rate, rhythm or QT interval were altered by CXL-1020.

Our clinical data for CXL-1020 corroborates the experimental animal data, providing the first-in-man demonstration of the hemodynamic effects of an HNO donor. At the doses and administration studied, CXL-1020 was found to be well tolerated, and a decline in diastolic filling pressures, modest fall in SVR, and rise in cardiac output resulting from increased stroke volume with no change in heart rate, were consistent with inotropic and vasodilator actions observed in dogs, and positive inotropy measured in myocytes.

The clinical study also identified a threshold dose of CXL-1020 for hemodynamic effects at 10–20 μg/kg/min. As this dose range did not induce maximal responses in dogs, higher or

more prolonged doses were considered. However, in a subsequent longer duration (12–24 hours, at a dose of 20μg/kg/min) study, CXL-1020 was found to produce an inflammatory irritation at the intravenous insertion site. Based on this, it was not felt a viable candidate for further development as a human therapeutic. However, the results with CXL-1020, AS (and other novel HNO donors) confirm that the hemodynamic effects of HNO are a class phenomenon independent of the donor. Second generation HNO donors have since been developed that abrogate the venous irritation experienced with CXL-1020, while preserving the full spectrum of HNO's hemodynamic effects in animal models, thus providing a viable option for further investigation of the HNO class.

In conclusion, we show that a novel pure HNO donor enhances cardiac systolic function while reducing arterial and venous tone, and without increasing heart rate. Direct contractile enhancement from CXL-1020 is observed in isolated myocytes and experimental hearts *in vivo*, and supported by improved systolic function in humans with congestive heart failure. The combination of effects differentiates HNO donors from other classes of inotropes or ino-dilators, and provides a strong rationale for continuing studies to develop donors with optimized pharmacological and clinical efficacy for the treatment of congestive heart failure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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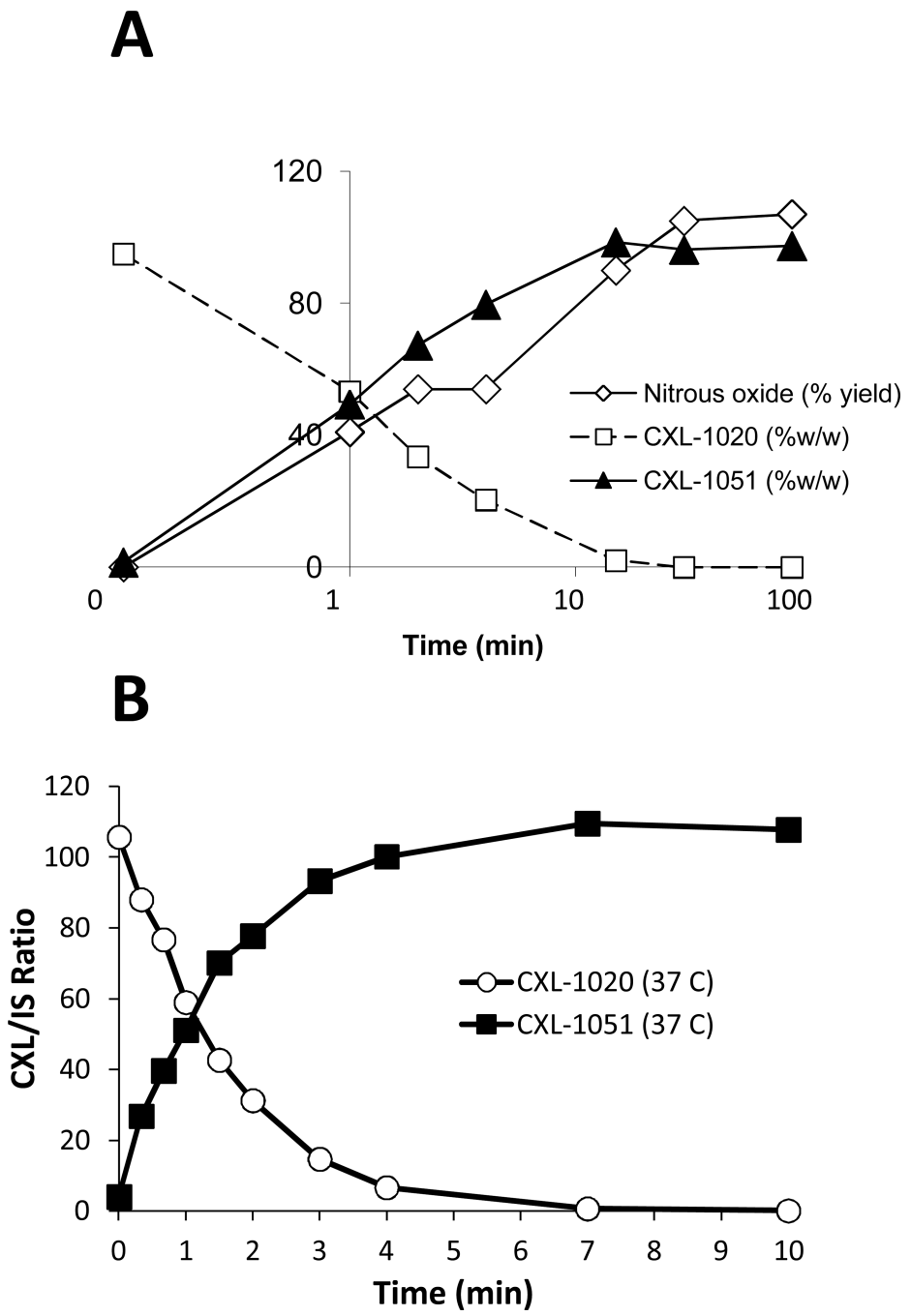

Figure 1. Pharmacological decomposition of CXL-1020

A) Decomposition of CXL-1020 in aqueous solution into HNO (measured by nitrous oxide) and CXL-1051. Conversion is rapid, and virtually complete by 15 minutes, with stoichiometry confirming pure generation of HNO and CLX-1051 in equal parts. B) Decomposition of CXL-1020 in human whole blood shows similar rapid pharmacokinetics. IS: internal standard, see supplemental methods.

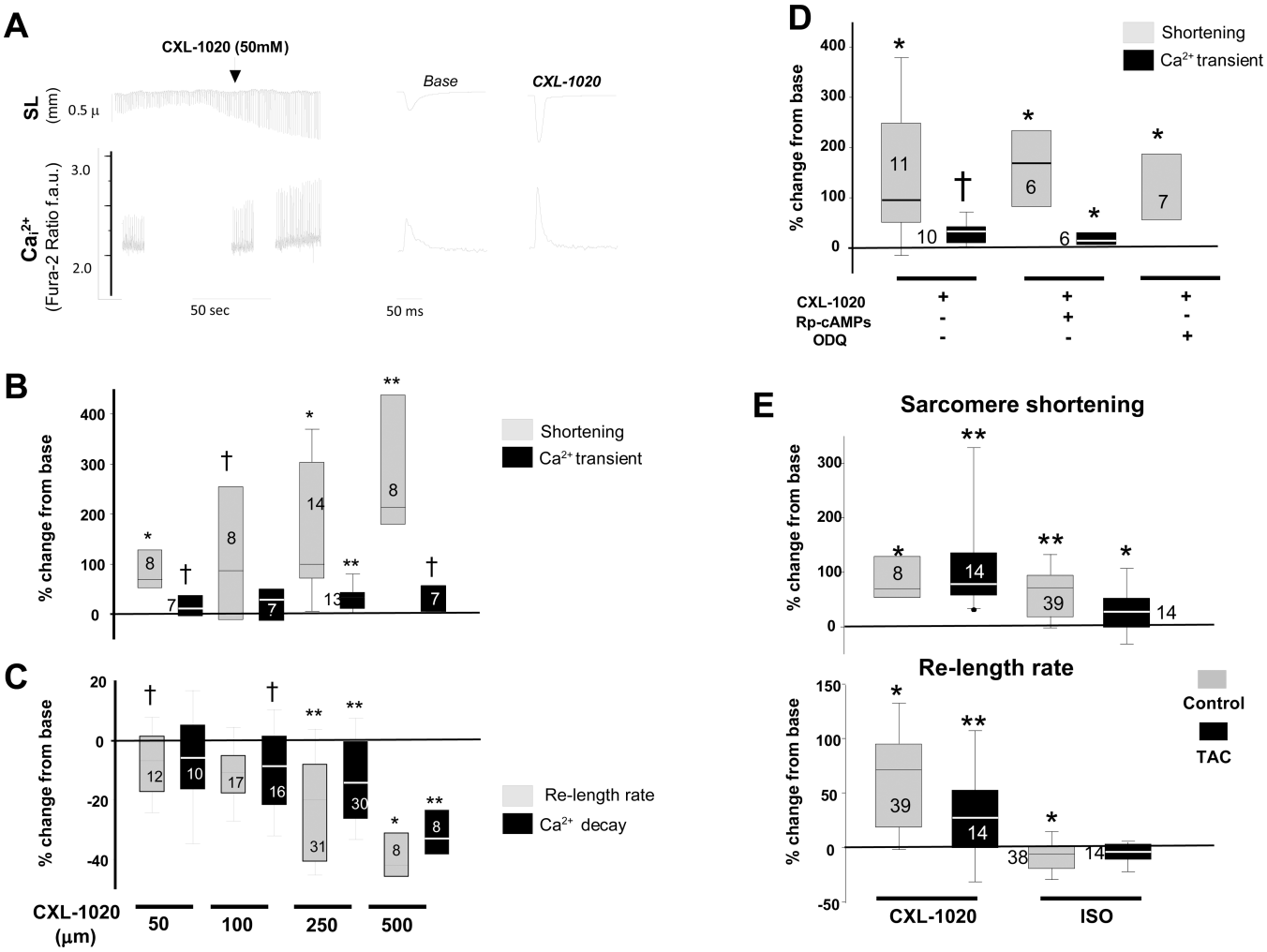

Figure 2. Influence of CXL-1020 on isolated cardiac myocytes from normal and failing heart

A) (Left) Isolated myocyte sarcomere length (SL, upper tracing) and calcium transients (Ca^{2+}, lower tracing) after exposure to CLX-1020. There is a marked rise in sarcomere shortening (SS) and a rise in the peak Ca^{2+} transient, as well as acceleration of the time for re-lengthening and calcium decline. B) Box-plots show percent change in SS and peak Ca^{2+} transient relative to baseline with incremental CXL-1020 dose. Only one dose was tested per myocyte; the sample size at each dose is provided in the plots. C) Percent reduction in sarcomere relengthening and calcium decay time. D) Percent change in SS and peak Ca^{2+} transient following CXL-1020 with or without co-inhibition of PKA (Rp-cAMPs) or soluble guanylate cyclase (ODQ). E) Percent change in SS and re-lengthening rate in myocytes isolated from control or failing hearts that are then exposed to either isoproterenol (ISO, 2.5 nM) or CXL-1020 (50 µM). † - $p<0.05$; * $p<0.01$; ** $p\leq0.001$ versus respective (pre-CXL1020, or ISO) baseline, by Wilcoxan Sign-rank Test.

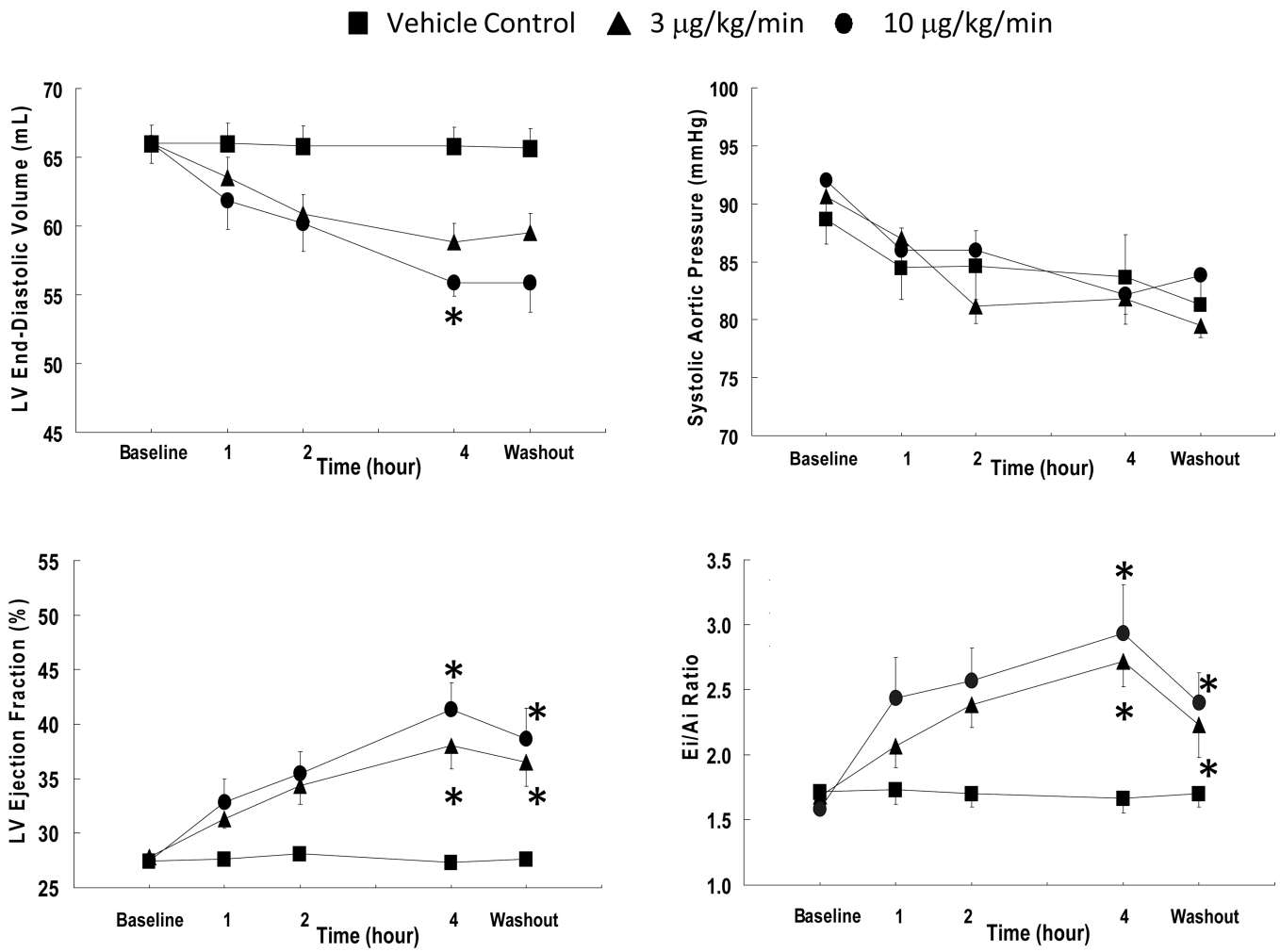

Figure 3. Influence of CXL-1020 in anesthetized dogs over 4-hour infusion

A) Left ventricular and systemic hemodynamics at either 3 or 10 μg/kg/min CXL-1020, with 1 hour of washout (n=6). Ei/Ai= ratio of early to late (atrial) mitral inflow. * P<0.05 versus vehicle control by multiple comparisons test (Student-Newman-Keuls) following repeated measures ANOVA.

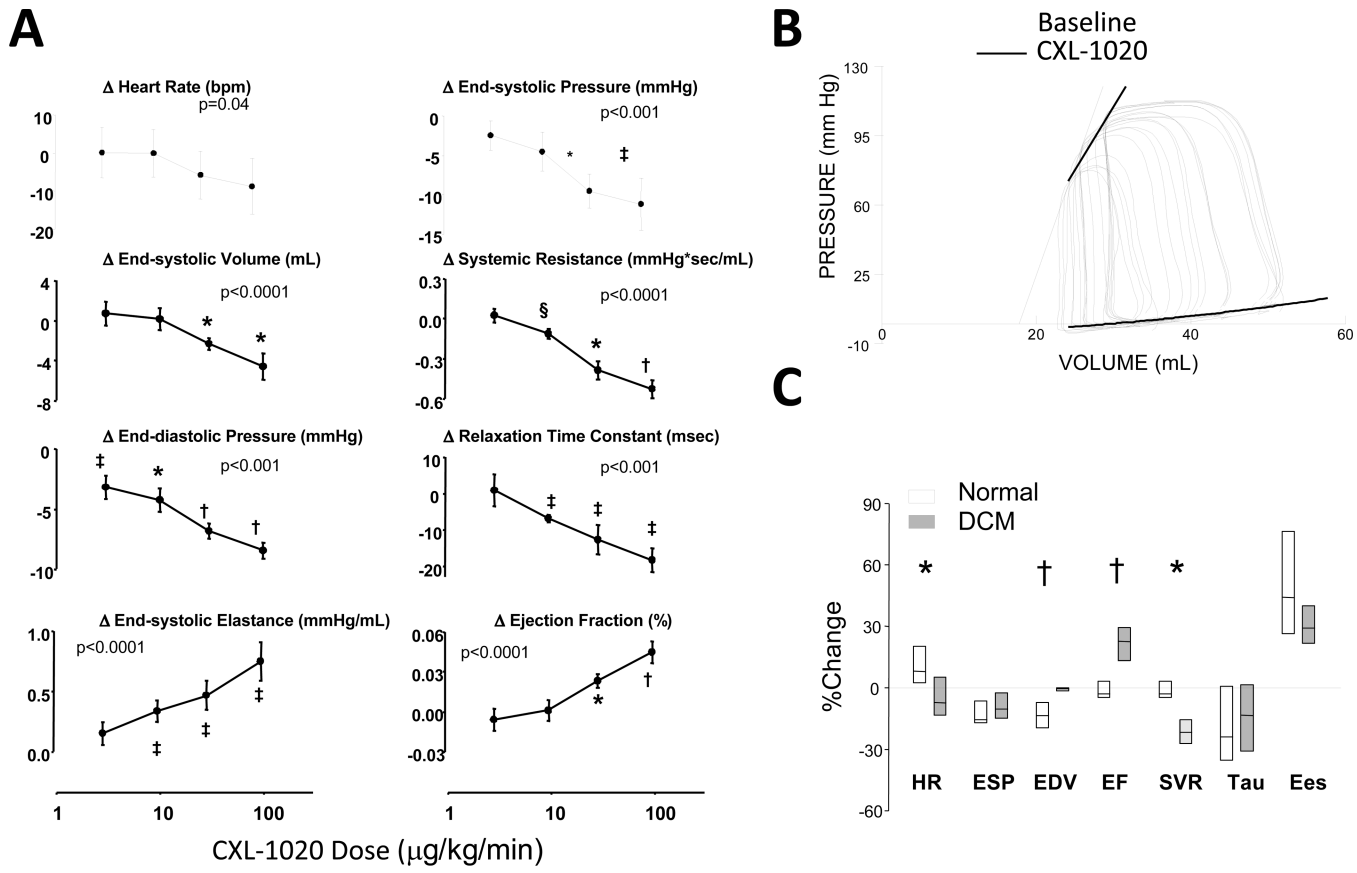

Figure 4. Influence of CXL-1020 in conscious heart failure dogs

A) Absolute change in hemodynamic parameters in Group 2 (conscious) heart failure dogs as a function of increasing CXL-1020 dose. P-values in each plot are for a repeated measures analysis of co-variance (with drug dose as the continuous variable, n=5). Post-hoc multiple comparisons test for dose response versus baseline: * - $p<0.005$; † - $p<0.001$; ‡ - $p\leq0.01$; § - $p=0.02$. **B)** Example pressure volume loops at baseline and after CXL-1020 infusion, showing an increase in the slope of the end-systolic pressure volume relationship (solid line is control, dashed after CXL-1020). **C)** Box-plot for percent change in hemodynamic parameters before and after 100 mg/kg/min CXL-1020 in normal dogs and the same dogs after inducing heart failure. * - $p<0.05$, † $p<0.01$ between groups (n=5 per group, Kruskal-Wallis used to test for effect of heart failure on the response).

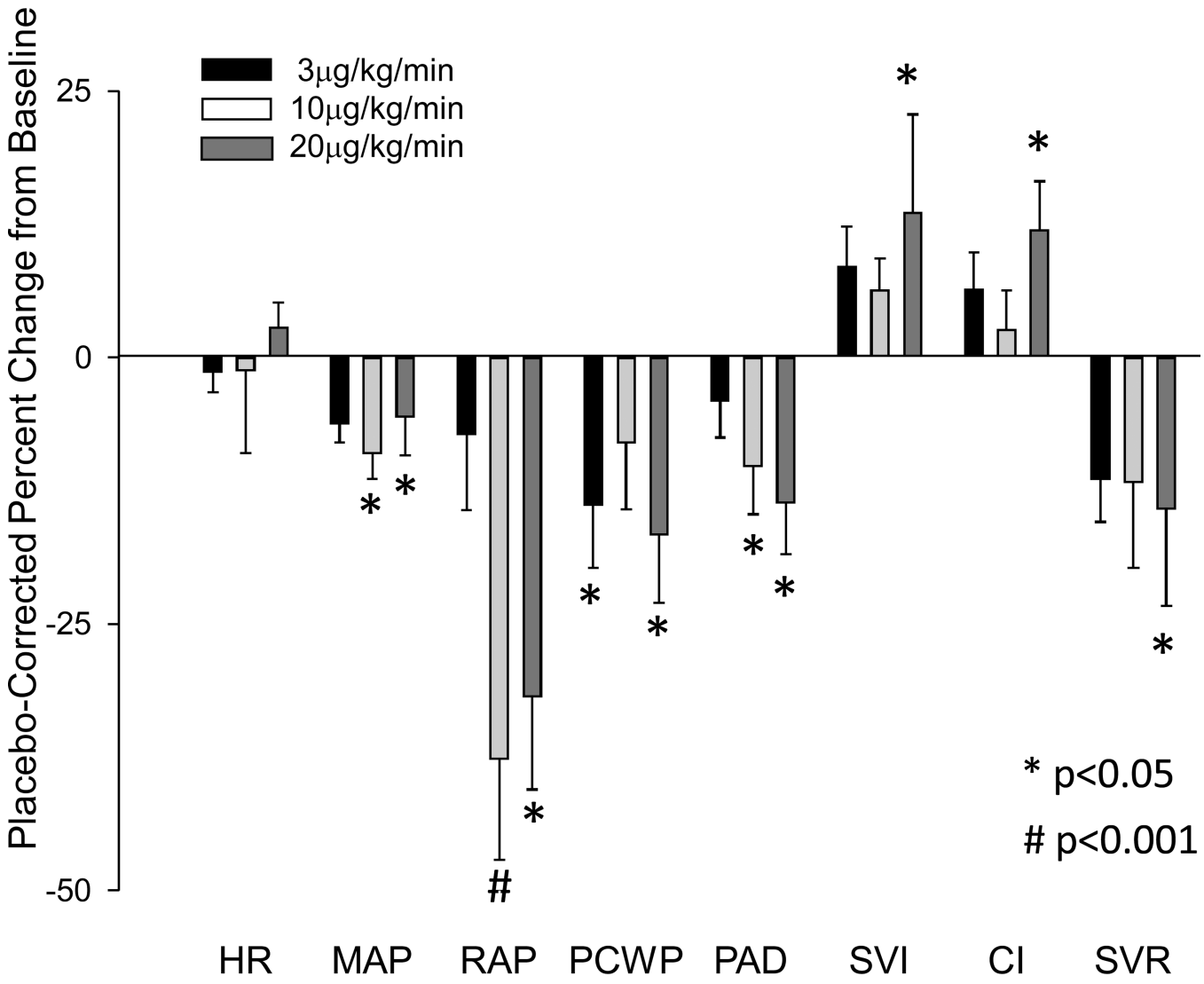

Figure 5. Hemodynamic effects of CXL-1020 in patients with symptomatic heart failure

Effects of CXL-1020 at 3, 10 or 20 ug/kg/min on heart rate (HR), mean arterial pressure (MAP), right atrial pressure (RAP), pulmonary capillary wedge pressure (PCWP), stroke volume index (SVI), cardiac index (CI) and systemic vascular resistance (SVR) in 12 patients with symptomatic heart failure ('high dose' titration group described in Methods). Data show the mean percent change ±SEM at each dose minus theresponse observed in the placebo group at the same time point (e.g. i.e. corrected to placebo). * $p<0.05$ versus baseline, # - $p<0.005$ versus baseline.

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Table

Hemodynamic variables and plasma biomarkers during 4-hr infusion of CXL-1020 in dogs with ischemic cardiomyopathy

	Vehicle Control (n=6)			3 µg/kg/min			10 µg/kg/min		
	Baseline	**4 hours**	**Washout**	**Baseline**	**4 hours**	**Washout**	**Baseline**	**4 hours**	**Washout**
Heart Rate (bpm)	75±4	69±1	71±3	74±2	75±5	65±1	74±1	77±2	75±5
Systolic AoP (mmHg)	89±3	84±4*	81±2	91±2	82±1	80±1	92±6	82±3	84±2
Diastolic AoP (mmHg)	62±3	55±3	57±4	64±2	55±2*	53±1*	64±4	50±1*	53±1
Mean AoP (mmHg)	75±3	71±4	68±3	76±2	68±2*	66±1*	77±5	63±2*	65±2*
LVEDP (mmHg)	13.7±0.8	15.3±0.7	12.3±1.0	14.5±0.7	13.3±0.3	13.8±0.7	13.8±0.3	10.8±0.9*	11.7±1.5
LVESV (mL)	47.8±1.0	47.8±1.0	47.7±1.1	47.7±1.1	36.5±2.1*	37.8±2.4*	48.0±1.2	32.8±1.9*	34.3±2.5*
LV EDV (ml)	66.0±1.3	65.8.0±1.3	65.7±1.4	66.0±1.3	58.8±2.1*	59.5±2.2*	66.0±1.5	55.8±0.9*	55.8±2.1*
LV FAS (%)	25.0±0.4	25.3±0.7	25.2±0.7	25.5±0.3	35.8±2.1*	33.5±2.3*	24.8±0.3	38.8±2.2*	35.3±2.8*
Stroke Volume (mL)	18.2±0.3	18.0±0.4	18.0±0.4	18.3±0.3	22.3±1.1*	21.7±1.0*	18.0±0.4	23.0±1.3*	21.5±1.5*
SVR (dynes.sec.cm^5)	4476±328	4640±405	4283±200	4502±142	3335±247*	3793±234*	4642±236	2904±138*	3342±311*
Deceleration Time (msec)	93.2±2.5	96.3±3.0	96.7±3.6	94.8±3.8	119.8±5.3*	103.3±2.3*	97.3±4.0	128.0±7.5*	121.3±6.9*
Peak Power Index (mmHg/sec*mL)*100	184±12	171±10	169±9	192±11	245±19*	212±14	180±14	262±16*	250±20*
MVO_2 (µmol/min)	---	---	---	---	---	---	102.5±6.3	71.5±14.5*	---
nt-proBNP (fmols/mL)	165±12	164±13	---	164±15	133±11*	---	166±13	93±16*	---
pro-ANP (pmols/mL)	0.56±0.06	0.57±0.06	---	0.57±0.07	0.45±0.05*	---	0.57±0.06	0.31±0.05*	---
TnI (ng/mL)	0.53±0.03	0.56±0.03	---	0.56±0.03	0.36±0.03*	---	0.58±0.02	0.28±0.2†	---

AoP=aortic pressure; LV=left ventricular; EDP=end-diastolic pressure; ESV=end-systolic volume; EDV=end-diastolic volume; FAS=fractional area of shortening; SVR=systemic vascular resistance,. MVO_2=myocardial oxygen consumption. nt-pro BNP=n-terminal pro-brain natriuretic peptide; pro ANP=pro atrial natriuretic peptide, TnI – troponin I.

*p<0.05 versus Baseline