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Nitroxyl, a New Generation of Positive Inotropic Agent for Heart Failure

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1 Introduction

Heart failure (HF) not only presents a formidable challenge to clinicians but also constitutes an enormous economic burden on society today. The current clinical treatment of severely decreased contractility relies either on mechanical support or on conventional positive inotropic agents, which increase contraction by increasing intracellular Ca^{2+}. However, the prognosis of HF remains poor. Genetic manipulation (i.e., gene transfer) or the use of stem cells to reverse HF offers an alternative possibility for a cure. So far, gene therapy has failed※. Cell regeneration techniques are still in their experimental stages and are not without risks or limitations. In the meantime, new knowledge on the regulation of force generation by myofilaments has revolutionized our search for new ways to boost cardiac contractility. It is clear that post-translational modifications (PTMs) of myofilament proteins are important modulatory mechanisms of cardiac force development. Calcium sensitizers, a class of drugs that increase the effectiveness of a given amount of calcium in generating force, have been a recent focus of investigation [1]. Mechanistically, most calcium sensitizers produce PTMs by introducing themselves into the molecular structure of the regulatory proteins in order to increase the proteins' affinity to Ca^{2+}. Somewhat disappointingly, these kinds of PTMs increase diastolic force, thus worsening diastolic dysfunction in HF or causing cardiac arrhythmias. By interacting with critical cysteine residues of the contractile proteins, nitroxyl (HNO) alters the redox states of the proteins and produces unique PTMs to increase cardiac contractility. The growing popularity of HNO as a novel inotropic agent is evidenced by recent studies that show that the new intravenous HNO donor, CXL-1020, significantly improves contractile function in HF dogs [2, 3], and by the fact that CXL-1020 has entered into phase I/II clinical trial※※.

2 What is HNO?

Nitroxyl or nitrosyl hydride (HNO) is the one-electron reduction product of nitric oxide ($NO^{\bullet}$), and has a distinctive chemistry from that of $NO^{\bullet}$ [4]. Currently, there are no definitive methods available to unequivocally detect the "footprints" of HNO in a biological system because of its relatively short half-life (i.e., < 2.7 min, based on current HNO donors in a biological buffer system). However, there is plenty of evidence for its generation in *in vitro* studies, most of which are nitric oxide synthase (NOS)-dependent pathways [5]. At present, the endogenous generation of HNO (pathways) is speculative; however, some recent evidence shows that endogenous molecules can act as substrates for HNO formation, rendering its endogenous biosynthesis even more likely [5].

3 What cardiac effect does HNO exert?

The first report of HNO cardiovascular action was by Fukuto et al. [6], which showed that the HNO donated by Angeli's salt (AS, $Na_2N_2O_3$) could relax rabbit aortas and bovine intrapulmonary arteries via an sGC-dependent mechanism. We and our collaborators have investigated the biological effects of HNO during the past few years. Dr. Paolocci et al. [7] have shown that the administration of HNO causes an increase in left ventricular contractility with concomitant reduction in preload, in the presence of peripheral vasodilation. Importantly, the positive inotropic action of HNO can be prevented by thiol-donating compounds such as *N*-acetyl-*L*-cysteine [7], while HNO displays full cGMP- and β-receptor independency *in vivo* [8].

3.1 Effect of HNO on cardiac excitation-contraction (EC) coupling in a normal heart

Contraction and force generation in cardiac muscle are triggered by the generation of membrane action potential, which allows the entry of a small amount of Ca^{2+} into the myocyte via the sarcolemmal voltage-dependent Ca^{2+} channels (L-type Ca^{2+} channels). This entry triggers the release of a bigger pulse of Ca^{2+} from the sarcoplasmic reticulum (SR) through specialized Ca^{2+}-gated channels (i.e., ryanodine receptors (RyR2)). This event is known as Ca^{2+}-induced Ca^{2+} release [9].

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※※ http://www.cardioxyl.com

The released Ca^{2+} binds to troponin C (TnC), one of the three regulatory troponins (TnC, TnI, and TnT). Ca^{2+} binding to the single site in the NH_2 domain of cardiac TnC triggers a strong binding of this domain with the COOH terminal and with the central regions of TnI [10]. This binding between the two troponin subunits alters their molecular conformations [11, 12], and causes: ① release of the TnI inhibitory region (residues 136–147) from its interaction with F-actin-tropomyosin (Tm) and ② weakening of the interaction between the C-terminal region of TnI and Tm. As a result, Tm moves over the actin surface, exposing the sites on actin for the myosin head to bind, resulting in contraction.

Recent studies have shown that HNO enhances cardiac Ca^{2+} cycling by stimulating RyR2 to enhance SR Ca^{2+} fractional release without recruiting extracellular Ca^{2+} from L-type channel activity [13, 14]. Interestingly, the induced inotropy was sustained by stimulating the activity of SR Ca^{2+} ATPase (SERCA2a). This is because HNO activates SERCA2a in a manner that resembles the β-adrenergic/PKA-dependent removal of inhibition exerted by phospholamban (PLN) due to SERCA2a activation by the selective modification of cysteines in PLN [15]. We have also shown that HNO increases force relative to Ca^{2+} in cardiac muscle as a result of increased responsiveness of myofilament to Ca^{2+} [16, 17]. At fixed external Ca^{2+}, HNO increases more force than intracellular Ca^{2+} transients. Thus, HNO is a Ca^{2+} sensitizing agent. The effects of HNO on increasing intracellular Ca^{2+} transients and myofilament Ca^{2+} responsiveness are synergistic, as we have recently shown in phospholamban-deficient mice (unpublished results). At the molecular level, HNO induces the formation of a disulfide bond between cysteines.

The positive inotropic effect is also prevented and reversed by dithiothreitol (DTT), confirming HNO sensitivity to redox conditions. Thus, HNO uniquely modifies the redox state of the contractile proteins such that a positive inotropic effect is achieved. This effect is in contrast to the "overhaul" of redox state by reactive oxygen species (ROS), which results in significant depression of contractility [18–20]. Another important aspect of HNO's action is that HNO does not deplete glutathione [21].

3.2 Effect of HNO on cardiac EC coupling in a failing heart

A prominent feature of failing myocardium is increased oxidative stress [22, 23]. Increased oxidative stress (or "redox imbalance") can result in key events that determine or are associated with the onset and progression of congestive heart failure (CHF) disease. These events include altered EC coupling, cardiomyocyte maladaptive hypertrophy, extracellular matrix remodeling, abnormal tissue energetics, loss of viable myocardium, vascular and capillary alterations, and inflammation. All of these events will eventually lead to significant remodeling of the heart, signified by increased chamber dilation and loss of contractility. There are two featured changes in failing myocardium worth mentioning: ① The β-adrenergic system (which adjusts cardiac force development to increased work demand) is downregulated in CHF due to β-receptor desensitization to and uncoupling from signaling molecules [24]; ② oxidation of myofilament proteins has been found and implicated in the depressed contractility in HF [20, 25–27]. These proteins include actin, Tm, and myosin light chain 1 (MLC1). Oxidation of myofilaments can result in the formation of carbonyl groups and protein-protein cross-linkages [28]. Since the decreased contractility is reversible and redox-dependent, cysteine residues appear to be the primary targets, as supported by the formation of Tm-Tm and actin-actin dimers.

As discussed above, HNO also targets cysteines with similar redox-dependency and reversibility; however, it augments contractility. Can HNO still increase force development in failing myocardium in which some cysteine residues have already been oxidized? The answer is yes. In a dog with pacing-induced HF, Paolocci et al. [8] were able to show that HNO improved both contractility and relaxation to a similar extent as in control preparations. Further, when HNO was administered concomitantly with β-agonist mimetics such as dobutamine, they were additive in supporting myocardial contraction; as opposed to NO/nitrite, which blunted dobutamine-induced enhancement in function. The new intravenous HNO donor, CXL-1020, significantly improved contractile function in both HF dogs and in patients with HF [29]. In isolated cardiac muscles from mice models of HF, HNO also increased force development directly (unpublished data). However, the exact mechanism by which HNO augments force development in failing myocardium with increased oxidative stress (or altered redox signaling) is still an area of intense investigation.

4 Future directions in HNO research as a unique positive inotropic agent

Redox imbalance has been increasingly recognized as a major mechanism of decreased contractility in HF. Oxidized myofilament proteins have also been found in experimental HF and implicated in decreases in heart function. The fact that HNO can still increase contraction under oxidative stress suggests that HNO acts in a unique way that is less affected by these changes. For example, it is not known whether cross-bridge kinetics are regulated by redox signaling. Since HNO augments force development in a redox-dependent way, it is important to find out how HNO affects cross-bridge cycling kinetics. Studies of this kind will not only uncover the molecular mechanism of HNO action in the heart, but also enrich our understanding of the PTM-induced modulation of the actin-activated myosin chemomechanical cycle that contributes to force generation, and help in the design of new therapies for all HF cases in which force is reduced, oxidative stress is increased, and other conventional inotropic agents may be limited in effectiveness.

Another area of HNO research is its effect on diastolic function. So far, no studies have focused on this topic. However, previous studies from our laboratories have indicated that HNO does not alter diastolic relaxation and ATPase activity. Diastolic function is determined by three major factors: the rate of intracellular Ca^{2+} decline, thin-filament de-activation, and the rate of cross-bridge cycling. By increasing the activities of SERCA2a, HNO can promote Ca^{2+}

uptake and thus accelerate the rate of intracellular Ca^{2+} decline [13]. It remains attractive to find out if HNO modifies the cross-bridge cycling rate such that the force generated by a cross-bridge is higher with unchanged (or faster) detachments (i.e., relaxation).

Our previous studies indirectly demonstrated that HNO enhancement of EC_{50} and F_{max} depends, at least in part, on its modification of some cysteine residues in myofilament contractile or regulatory proteins [16, 17]. However, a direct proof of that can be achieved only by replacing critical cysteine residues with alanines. Moreover, as per our previous studies, HNO can modify additional residues such as Cyst 475 in MyBPC [17]. Yet whether this modification is incidental or functionally relevant is unknown. Therefore, experiments involving mutagenesis of the targeted cysteine residues will provide direct evidence for HNO's myofilament actin at the molecular level.

Finally, no studies have tested yet whether endogenous oxidants such as H_2O_2, at predicted physiological levels, can differently affect force generation, and eventually where (H_2O_2) targets. Moreover, the effect of HNO on HF should be investigated in models of HF *in vivo*, especially where maximal Ca^{2+}-activated force is significantly depressed.

5 Concluding remarks

In treating HF, therapeutic interventions aiming at generating more force at the myofilament levels can be summarized by three classes of molecules: calcium sensitizers, myosin activators, and HNO donors. The first class of drugs increases the effectiveness of a given amount of calcium in generating force. Most calcium sensitizers produce PTMs by introducing themselves into the molecular structure of the regulatory proteins in order to increase the proteins' affinity to Ca^{2+}. However, these kinds of PTMs increase diastolic force, thus worsening diastolic dysfunction in HF or causing cardiac arrhythmias. Myosin activators are recently discovered force-promoting molecules [30], which also increase diastolic force. By interacting with critical cysteine residues of the contractile proteins, HNO donors represent a new and redox-based class of drugs that increases cardiac contractility independently of β-adrenergic stimulation, without altering diastolic tension and ATP consumption. Thus, HNO-mediated PTMs hold great promise of success for the treatment of HF: The efficacy of HNO is expected to be sustained or enhanced because of the depletion of potential intracellular thiol buffers and because of its resistance to ROS scavenging. Thus, this unique, novel, redox-related regulator of cardiac contractility will shed light in generating new strategies to treat HF patients.

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