Reversal of Isoflurane-Induced Depression of Myocardial Contraction by Nitroxyl via Myofilament Sensitization to Ca^{2+}

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ABSTRACT

Isoflurane (ISO) is known to depress cardiac contraction. Here, we hypothesized that decreasing myofilament Ca^{2+} responsiveness is central to ISO-induced reduction in cardiac force development. Moreover, we also tested whether the nitroxyl (HNO) donor 1-nitrosocyclohexyl acetate (NCA), acting as a myofilament Ca^{2+} sensitizer, restores force in the presence of ISO. Trabeculae from the right ventricles of LBN/F1 rats were superfused with Krebs-Henseleit solution at room temperature, and force and intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) were measured. Steady-state activations were achieved by stimulating the muscles at 10 Hz in the presence of ryanodine. The same muscles were chemically skinned with 1% Triton X-100, and the force- Ca^{2+} relation measurements were repeated. ISO depressed force in a dose-dependent manner without significantly altering $[\text{Ca}^{2+}]_i$. At 1.5%, force was reduced over 50%, whereas $[\text{Ca}^{2+}]_i$

remained unaffected. At 3%, contraction was decreased by ~75% with $[\text{Ca}^{2+}]_i$ reduced by only 15%. During steady-state activation, 1.5% ISO depressed maximal Ca^{2+} -activated force (F_{max}) and increased the $[\text{Ca}^{2+}]_i$ required for 50% activation (Ca_{50}) without affecting the Hill coefficient. After skinning, the same muscles showed similar decreases in F_{max} and increases in Ca_{50} in the presence of ISO. NCA restored force in the presence of ISO without affecting $[\text{Ca}^{2+}]_i$. These results show that 1) ISO depresses cardiac force development by decreasing myofilament Ca^{2+} responsiveness, and 2) myofilament Ca^{2+} sensitization by NCA can effectively restore force development without further increases in $[\text{Ca}^{2+}]_i$. The present findings have potential translational value because of the efficiency and efficacy of HNO on ISO-induced myocardial contractile dysfunction.

Introduction

The effects of inhalational anesthetic agents on myocardial contraction have been the focus of extensive investigations for many years, and the depression of force development by different inhalational agents has been repeatedly shown. At the cellular level, altered cardiac excitation-contraction coupling has been identified as a major vehicle of impaired cardiac performance. In fact, inhalational anesthetics have been shown to decrease sarcolemmal L-type Ca^{2+} channel

function (Terrar and Victory, 1988), decrease sarcoplasmic reticular Ca^{2+} storage (Davies et al., 2000), Ca^{2+} release (Jiang and Julian, 1998), and decrease myofilament Ca^{2+} sensitivity (Bosnjak et al., 1992; Davies et al., 2000). More importantly, it appears that the impact of inhalational agents on myocardium is agent- and dose-dependent, with decreasing Ca^{2+} availability being viewed as the major mode of action based on previous studies (Rusy and Komai, 1987; Housmans and Murat, 1988; Housmans et al., 2000).

However, other recent studies have shown that, at clinical dosages (i.e., 0.5–1.5 minimum alveolar concentration), isoflurane (ISO) or sevoflurane differentially decrease contractile force development while producing little inhibition of Ca^{2+} release. This point is proven by the lack of change in the amplitudes of intracellular Ca^{2+} transients (Hanley and Loisel, 1998; Bartunek and Housmans, 2000; Davies et al.,

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ABBREVIATIONS: ISO, isoflurane; NCA, 1-nitrosocyclohexyl acetate; HNO, nitroxyl; K-H, Krebs-Henseleit; ANOVA, analysis of variance; F_{max} , maximal Ca^{2+} -activated force; Ca_{50} , Ca^{2+} required to achieve 50% of F_{max} ; Levo, levosimendan; $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} ; $[\text{Ca}^{2+}]_o$, extracellular Ca^{2+} ; Tn, troponin; TnC, troponin C.

2000; Graham et al., 2005). This clearly hints at the possibility that ISO directly decreases myofilament Ca^{2+} responsiveness. Nonetheless, early studies that used skinned cardiac muscle preparations yielded variable results; some failed to show any effect of ISO at low concentrations (i.e., clinically relevant doses: 1–3%) (Su and Kerrick, 1980; Su and Bell, 1986), and others showed either increased or decreased myofilament Ca^{2+} sensitivity (Murat et al., 1988; Herland et al., 1993). The contention was that the presence of a cellular membrane system could influence the modulation of cardiac inotropy by inhalational agents (Herland et al., 1993). Thus, it remains unclear whether ISO affects myofilament function per se, i.e., independently from changes induced at the sarcolemmal level.

Nitroxyl (HNO), the protonated one-electron reduction product of nitric oxide (Tocchetti et al., 2011), augments cardiac inotropy and improves relaxation in dog hearts, both under normal and heart failure conditions (Paolocci et al., 2001, 2003). The positive inotropic and lusitropic effects of HNO are independent of β -adrenergic stimulation but are sensitive to the redox states of the cardiac cell (Tocchetti et al., 2007; Froehlich et al., 2008). Recently, we found that HNO augments cardiac contractility by increasing Ca^{2+} responsiveness of the myofilaments (Dai et al., 2007). This enhancement of force is not associated with an increase in diastolic tension or myofibrillar Mg-ATPase activities. Subsequently, we have confirmed that HNO, by interacting with specific, highly reactive thiols in myofilament proteins, modifies the redox states of key contractile proteins, thus promoting an increase in force production after Ca^{2+} binds to troponins (Tn) (Murray et al., 2009).

In this study, we tested two main hypotheses. First, we aimed to verify whether ISO is able to exert a direct effect on myofilament sensitization to Ca^{2+} . To do so, we determined the steady-state force- Ca^{2+} relations of individual muscles, before and after skinning. Our second aim was to test whether the HNO donor, 1-nitrosocyclohexyl acetate (NCA) (Sha et al., 2006; Shoman et al., 2011), can restore cardiac force development in the presence of ISO by virtue of its Ca^{2+} -sensitizing effect (Dai et al., 2007). We hope to demonstrate that HNO, donated by NCA, can restore myocardial contractility during ISO-induced anesthesia and that HNO donors are probably able to provide an inotropic counterbalancing effect in patients displaying compromised cardiac function due to inhalation of volatile anesthetics such as ISO, thus justifying the use of HNO donors for patients with heart failure undergoing inhalational anesthesia.

Materials and Methods

Animals. LBN/F1 rats (250–300 g; Harlan, Indianapolis, IN) were used in these experiments. Animal care and experimental protocols were approved by the Animal Care and Use Committee of The Johns Hopkins University School of Medicine.

Trabecular Muscle Preparation. The rats were anesthetized via intra-abdominal injection with pentobarbital (100 mg/kg); the heart was exposed by mid-sternotomy, rapidly excised, and placed in a dissection dish. The aorta was cannulated, and the heart was perfused in a retrograde fashion (~ 15 ml/min) with dissecting Krebs-Henseleit (K-H) solution equilibrated with 95% O_2 , 5% CO_2 . The dissecting K-H solution was composed of 120 mM NaCl, 20 mM NaHCO_3 , 5 mM KCl, 1.2 mM MgCl_2 , 10 mM glucose, 0.5 mM CaCl_2 , and 20 mM 2,3-butanedione monoxime, pH 7.35 to 7.45 at room

temperature (21–22°C). Trabecular muscle from the right ventricle of the heart was dissected and mounted between a force transducer and a motor arm, superfused with K-H solution without 2,3-butanedione monoxime at a rate of ~ 10 ml/min, and stimulated at 0.5 Hz.

Force was measured by a force transducer system (KG7; Scientific Instruments GmbH, Heidelberg, Germany) and expressed in millinewtons per square millimeter of cross-sectional area. The muscles underwent isometric contractions with the resting muscle length set, such that resting force was 15% of total force development (i.e., optimal muscle length). This resting muscle length, corresponding to resting sarcomere length of 2.20 to 2.30 μm as determined by laser diffraction (Gao et al., 1996), was maintained throughout the experiments. ISO was delivered via an ISO-specific vaporizer (calibrated by a vapor analyzer) to the K-H solution along with the 95% O_2 , 5% CO_2 gas mixture at a constant flow rate (1.0 l/min). The K-H solution was bubbled through a fine-porosity gas distribution tube with desired doses (vol %) of ISO for at least 15 min before use. Because of the volatile nature of ISO, the K-H solution was constantly bubbled with the gas mixture saturated with ISO, and the reservoir was covered to maintain the desired percentage of ISO throughout the experiments. In this design, the muscles were superfused with K-H buffer saturated with desired percentage of ISO (i.e., not via inhalation). NCA was dissolved in dimethyl sulfoxide to make a stock solution (0.06 M) and stored in -20°C until use. All experiments were performed at room temperature (20°C–22°C).

Measurement of $[\text{Ca}^{2+}]_i$. Intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) was measured by using the free acid form of fura-2 as described previously (Gao et al., 1994; Dai et al., 2007). Fura-2 potassium salt was micro-injected iontophoretically into one cell and allowed to spread throughout the whole muscle (via gap junctions). The tip of the electrode (~ 0.2 μm in diameter) was filled with fura-2 salt (1 mM), and the remainder of the electrode was filled with 150 mM KCl. After a successful impalement into a superficial cell in nonstimulated muscle, a hyperpolarizing current of 5 to 10 nA was passed continuously for approximately 15 min. In some muscles, multiple injections (up to 3–4) were applied at different sites, with duration of the injection limited to <10 min at each site to achieve an optimal signal-to-noise ratio. As previously established, this loading did not affect force development. Fura-2 epifluorescence was measured by excitation at 380 and 340 nm. Fluorescent light was collected at 510 nm by a photomultiplier tube (R1527; Hamamatsu Photonics, Hamamatsu City, Japan). The output of the photomultiplier was collected and digitized. $[\text{Ca}^{2+}]_i$ was given by the following equation (after subtraction of the autofluorescence) (eq. 1):

$$[\text{Ca}^{2+}]_i = K'_d(R - R_{\min})/(R_{\max} - R) \quad (1)$$

where R is the observed ratio of fluorescence (340 nm/380 nm), K'_d is the apparent dissociation constant, $R_{\max}$ is the ratio of 340 nm/380 nm at saturating Ca^{2+} , and $R_{\min}$ is the ratio of 340 nm/380 nm at 0 Ca^{2+} . The values of K'_d , $R_{\max}$, and $R_{\min}$ were determined by in vivo calibrations as described previously (Gao et al., 1994, 1998).

Steady-State Activation of Trabeculae. Ryanodine (1.0 μM) was used to enable steady-state activation. After the muscles were exposed to ryanodine for 15 min, different levels of tetanizations were induced briefly (~ 4 –8 s) by stimulating the muscles at 10 Hz at various concentrations of extracellular Ca^{2+} ($[\text{Ca}^{2+}]_o$; 0.5–20 mM). The steady-state force- $[\text{Ca}^{2+}]_o$ relations were fit with a function of the Hill equation (eq. 2):

$$F = F_{\max}[\text{Ca}^{2+}]^n/(\text{Ca}_{50}^n + [\text{Ca}^{2+}]^n) \quad (2)$$

where F is the steady-state force at various Ca^{2+} concentrations, $F_{\max}$ is the maximal Ca^{2+} -activated force, Ca_{50} is the Ca^{2+} concentration required to achieve 50% $F_{\max}$, and n is the Hill coefficient.

Skinned Trabeculae. After the steady-state experiments, the same trabeculae were immediately skinned by 15 to 20 min of exposure to 1% Triton X-100 in relaxing solution containing 100 mM KCl, 25 mM HEPES, 10 mM K_2EGTA , 15 mM creatine phosphate sodium

Fig. 1. A, raw recordings of the force development (top) and the corresponding $[Ca^{2+}]_i$ transients (bottom) of a trabecular muscle in the presence of varying doses of ISO. The muscle was stimulated at 0.5 Hz in the presence of 1.0 mM $[Ca^{2+}]_0$ at room temperature (22°C). B, pooled data of trabecular force development and amplitudes of $[Ca^{2+}]_i$ transients in the presence of varying doses of isoflurane. Force development became significantly less than baseline at doses greater than 0.75%. Amplitudes of $[Ca^{2+}]_i$ transients remained unchanged at all of the doses tested. Temperature = 22°C; $[Ca^{2+}]_0$ = 1.0 mM; stimulation rate = 0.5 Hz. *, $P < 0.001$; $n = 10$.

salt (Na_2CrP), 5 mM Na_2ATP , 5.15 mM $MgCl_2$, and 0.5 mM leupeptin, pH 7.2 with KOH. Activating solution contained 10 mM Ca^{2+} -EGTA, 100 mM KCl, 25 mM HEPES, 15 mM Na_2CrP , 5 mM Na_2ATP , 4.75 mM $MgCl_2$, and 0.5 mM leupeptin, pH 7.2. Different Ca^{2+} concentrations were achieved by mixing the activating solution and relaxing solution at different ratios. Skinning was considered to be complete when the preparation lost its pink color and sarcomeres were visible. Diastolic sarcomere length was determined by direct visualization under 100× magnification and was set at $\sim 2.2 \mu m$. Resting force was usually 10 to 15% of maximal activated force at this sarcomere length. The force- Ca^{2+} relationship was obtained by exposing the skinned muscles to activating solutions with various Ca^{2+} concentrations and were fit with the Hill equation as described above.

Statistical Analysis. Paired Student's t test and multivariate ANOVA were used for statistical analysis of the data (Systat 10.2.01; Systat Software Inc., San Jose, CA). A value of $P < 0.05$ was considered to indicate significant differences between groups. Unless otherwise indicated, pooled data are expressed as means $\pm$ S.E.M.

Results

Isoflurane Depresses Twitch Force without Affecting $[Ca^{2+}]_i$ Transients. We initially studied the effect of ISO on force development and $[Ca^{2+}]_i$ transients in isolated intact trabecular muscles. Figure 1A depicts a representative trace of the typical raw recordings of force and $[Ca^{2+}]_i$ transients at different ISO concentrations, whereas Fig. 1B shows the pooled data of all of the muscles tested. ISO depressed force development in a dose-dependent manner without significantly altering the amplitude of $[Ca^{2+}]_i$ transient. Moreover, at doses between 0.5 and 1.5%, developed force

was reduced by more than 50% (from 18.0 ± 1.2 to 8.6 ± 0.5 mN/mm^2 , $P < 0.001$), whereas $[Ca^{2+}]_i$ transient amplitudes remained unaffected ($[Ca^{2+}]_i$ = $0.58 \pm 0.06 \mu M$ at 0.5% and $0.55 \pm 0.06 \mu M$ at 1.5% ISO, $P > 0.05$). At 3% ISO, contraction was decreased by approximately 75% (to 4.9 mN/mm^2), whereas $[Ca^{2+}]_i$ was reduced by only 15% (to $0.5 \pm 0.05 \mu M$, $P > 0.05$). Thus, after treatment with ISO, the reduction of force seen seems to stem from changes in the properties of the myofilament (i.e., decreased myofilament Ca^{2+} responsiveness), rather than from decreased Ca^{2+} cycling/availability. In addition, relaxation of twitch force, as measured by time to half-relaxation from peak force, was faster in the presence of ISO (Table 1).

Isoflurane Decreases Myofilament Ca^{2+} Responsiveness. The data presented above only indirectly supports the contention that myofilament properties change under the influence of ISO given that, during activation and contraction, peak force development and peak amplitude of the $[Ca^{2+}]_i$ transient are not at equilibrium. Thus, to unequivocally prove that the anesthetic has a primary effect on myofilaments, we measured the steady-state force- $[Ca^{2+}]_i$ rela-

TABLE 1

Effect of isoflurane exposure and 1-nitrosocyclohexyl acetate exposure on time to peak (T_p) and time to 50% relaxation from peak (T_r) of contractions

	Control	Isoflurane (1.5%)	NCA (5 μM)
T_p (ms)	216 $\pm$ 14	185 $\pm$ 13	201 $\pm$ 10
T_r (ms)	147 $\pm$ 14	127 $\pm$ 14*	137 $\pm$ 18

* $P < 0.001$, paired t test vs. control. ms, millisecond. $n = 6$ in each group.

Fig. 2. Steady-state force Ca^{2+} relations in the presence and absence of ISO (1.5%) in isolated intact and skinned cardiac trabecular muscles. The steady-state forces were plotted against amplitudes of $[\text{Ca}^{2+}]_i$ that were grouped into bins in 0.5 μM ranges. A, in intact trabeculae, F_{max} was significantly reduced, whereas Ca_{50} was significantly increased by ISO. B, the same muscles in which steady-state relations were first obtained were chemically skinned and activated with various Ca^{2+} concentrations. The effect of ISO on F_{max} and Ca_{50} persisted in these skinned muscles. Temperature = 22°C; $n = 7$.

TABLE 2
Effect of isoflurane on steady-state force- Ca^{2+} relationships in isolated cardiac muscles

	Intact			Skinned		
	F_{max}	Ca_{50}	Hill	F_{max}	Ca_{50}	Hill
	mN/mm^2	μM	n	mN/mm^2	μM	n
Control	70.0 ± 4.5	0.60 ± 0.20	3.75 ± 1.27	69.0 ± 4.1	1.16 ± 0.06	3.0 ± 0.9
Isoflurane	$60.0 \pm 3.0^*$	$0.90 \pm 0.09^*$	4.72 ± 1.52	$41.8 \pm 3.8^*$	$2.28 \pm 0.5^*$	3.3 ± 1.2

* $P < 0.05$ from respective controls; paired t test between control and ISO-treated muscles, $n = 7$.

tion before and during 1.5% ISO exposure. In the presence of ISO, F_{max} decreased, whereas the $[\text{Ca}^{2+}]_i$ required for 50% activation (Ca_{50}), was increased (Fig. 2A; Table 2). The Hill coefficient was not affected in these muscles. Overall, there is a significant difference between the two relationships ($P < 0.001$) when multivariate ANOVA was performed among the parameters of F_{max} , Ca_{50} , and Hill coefficient. Hence, the action of ISO in the intact muscle was to decrease F_{max} and increase Ca_{50} .

In the intact muscle, the myofilaments are bathed in a cellular milieu that contains many factors that modulate the steady-state force- $[\text{Ca}^{2+}]_i$ relationship (Gao et al., 1997). ISO could potentially modify these factors, thereby decreasing myofilament Ca^{2+} responsiveness. Therefore, to rule out all potential confounding factors, we skinned the same trabecular muscle in which the steady-state force- $[\text{Ca}^{2+}]_i$ relations had been obtained. This enabled us to obtain force- $[\text{Ca}^{2+}]$ relations devoid of potential soluble confounding factors in these muscles. Likewise, in these skinned muscles, the F_{max} and Ca_{50} were significantly depressed (Fig. 2B; Table 2) in the presence of ISO. Multivariate ANOVA also showed significant difference between the two relationships ($P < 0.001$). Thus, the depressing effect of ISO on the steady-state force- Ca^{2+} relation persisted in skinned muscles. We also performed experiments in which trabeculae were chemically skinned immediately after stabilization and the force- Ca^{2+} relationship was obtained in the absence or presence of 1.5% ISO. These experiments served as internal checkpoints for the tetanization protocol performed to verify that the protocol itself did not affect the force- Ca^{2+} in the (same) skinned muscles afterward. Indeed, these experiments showed identical force- Ca^{2+} relations as in Fig. 2B, indicating that tetanization protocol had no effect on subsequent force- Ca^{2+} relations in skinned muscles (Fig. 3). Thus, all together these data strongly support our initial hypothesis that the ISO-

Fig. 3. Effect of ISO on steady-state force- Ca^{2+} relations in skinned trabeculae. After stabilization, these muscles were immediately skinned chemically with 1% Triton X-100. Different levels of forces were obtained by activating the muscles with varying Ca^{2+} concentrations. ISO decreased F_{max} from 66.6 ± 2.7 to 47.8 ± 4.3 mN/mm^2 , $P < 0.05$, paired t test) and increased Ca_{50} from 1.45 ± 0.12 to 2.35 ± 0.37 μM , $P < 0.05$, $n = 5$.

induced negative effect on myocardial contraction largely stems from a direct adverse impact on the myofilament.

Reversal of the Negative Inotropic Effect of Isoflurane. If ISO inhibits force generation by directly decreasing myofilament Ca^{2+} responsiveness, agents that increase myofilament Ca^{2+} responsiveness (so-called Ca^{2+} sensitizers) should be able to restore force in the presence of this anesthetic agent. We tested this hypothesis by exposing the muscles to the new HNO donor, NCA (Sha et al., 2006; DuMond and King, 2011; Shoman et al., 2011). As we learned earlier,

Fig. 4. Effect of ISO on force and amplitudes of Ca^{2+}_i transients in the presence of NCA, a newly discovered HNO donor. A, raw recording of force development in the presence of ISO and NCA. B, pooled data. ISO (1.5%) significantly decreased force development without affecting $[\text{Ca}^{2+}]_i$ transients. NCA (5 μM) restored force development despite the presence of ISO, with minimal increases in amplitudes of $[\text{Ca}^{2+}]_i$ transients. Temperature = 22°C; stimulation rate = 0.5 Hz; $[\text{Ca}^{2+}]_o = 1.0 \text{ mM}$. *, $P < 0.05$; $n = 4$.

NCA increases force development by increasing myofilament sensitivity to Ca^{2+} (Murray et al., 2009). At 5 μM (slightly higher than the EC_{50} of NCA as determined in cardiac muscle (Murray et al., 2009), NCA increased force development significantly without affecting twitch dynamics (Table 1). It is noteworthy that NCA completely restored force development in the presence of 1.5% ISO, with little increase in the amplitude of the $[\text{Ca}^{2+}]_i$ transient (Fig. 4). To show that NCA was indeed effective, we compared force recovery and $[\text{Ca}^{2+}]_i$ transient amplitude when $[\text{Ca}^{2+}]_o$ was raised (Fig. 5). Although ISO-dependent force depression could be fully restored by increasing the $[\text{Ca}^{2+}]_o$, the $[\text{Ca}^{2+}]_i$ more than doubled. In contrast to Ca^{2+} , NCA produced a full recovery in force without increasing $[\text{Ca}^{2+}]_i$. It is noteworthy that levosimendan (Levo), another Ca^{2+} -sensitizing agent, which binds to cardiac TnC (Haikala et al., 1995), hardly restored force in the presence of ISO (Fig. 6), although it was able to increase force development in the absence of it as expected (Sato et al., 1998). This suggests that ISO affects steps downstream of Tn activation in myofilament contraction.

Discussion

In this study, we provide two novel findings. First, we demonstrated that ISO is able to reduce force development without affecting the intracellular Ca^{2+} transient. For the first time, we show that ISO decreased F_{max} and increased Ca_{50} of the steady-state force- Ca^{2+} relation in individual muscles, both before and after they were chemically skinned. Second, we demonstrated the ability of HNO donors such as NCA to rescue force development in ISO-treated muscles by virtue of their sensitizing action to Ca^{2+} at the myofilament level. This study extended the results from previous investigations on the effect of ISO on myocardial contractility while

Fig. 5. Bar plots showing the reversal of ISO-induced force depression by Ca^{2+} and NCA. NCA (5 μM , $n = 6$) alone caused significant increases in force development without increases in $[\text{Ca}^{2+}]_i$ transients. After ISO-induced force reduction had stabilized for 10 min ($n = 8$), either Ca^{2+} (doses titrated to achieve complete recovery of force development, $n = 4$) or NCA (5 μM , $n = 4$) was added to the perfusion solution. Force was completely restored by raising $[\text{Ca}^{2+}]_o$, but $[\text{Ca}^{2+}]_i$ transients more than doubled. NCA restored force development with little increase in $[\text{Ca}^{2+}]_i$ transients. Temperature = 22°C; stimulation rate = 0.5 Hz. *, $P < 0.05$ versus all other groups for force development; #, $P < 0.05$ versus all other groups for $[\text{Ca}^{2+}]_i$ transients. **, $P < 0.05$ versus all other groups for force development.

Fig. 6. Effect of Levo on force development in the presence and absence of ISO in a trabecular muscle. Exposure to ISO (1.5%) decreased force to ~50% of pre-exposure and remained low in the presence of increasing doses (up to 30 μM) of Levo. Levo increased force at doses of $>5 \mu\text{M}$ in the absence of ISO. Similar results were obtained in three additional muscles.

deepening our understanding of how ISO may dampen myocardial function.

The myofilament desensitizing effect of ISO (and/or sevoflurane) has been shown by a number of prior studies in intact cardiac muscles and myocytes (Hanley and Loiselle, 1998; Jiang and Julian, 1998; Graham et al., 2005). ISO may decrease myofilament Ca^{2+} responsiveness by 1) directly interacting with the contractile proteins to affect activation/contraction processes and 2) modifying soluble cytoplasmic factors that may influence the behavior of the myofilaments. These pathways can be removed by skinning the muscle preparation; thus, the environment bathing the myofilaments proteins can be controlled. By equalizing the composition of solutions bathing the myofibrils, any of the effects of ISO observed is the result of a direct interaction between the agents and the contractile proteins. On the other hand, the results of previous studies using skinned muscle preparations have been variable; some have shown that myofilament

Ca^{2+} responsiveness to inhalational anesthetics decreased (Herland et al., 1993), whereas others have shown no change or increased myofilament Ca^{2+} responsiveness (Su and Kerrick, 1980; Su and Bell, 1986; Murat et al., 1988). Taken together, these studies suggest that, at least in part, the myofilament desensitization may reflect changes in the soluble cytoplasmic factors. Therefore, we chose to try another approach to evaluate the effect of ISO on myofilament Ca^{2+} responsiveness using the same muscle before and after skinning. This is a technically challenging experimental design that allows us to neatly distinguish the relative roles of soluble cytoplasmic factors and myofilament proteins in ISO-induced force depression, directly with increased reliability. The decreased myofilament Ca^{2+} responsiveness induced by ISO in intact muscles was reproducible in the same skinned muscles, confirming a direct myofilament effect of ISO.

The mechanism by which ISO interacts with myofilaments to decrease force development is not known at the moment. In cardiac muscle, force development depends on this sequence of events: Ca^{2+} binding to TnC (Krudy et al., 1994), interactions between Tn subunits (Kleerekoper et al., 1995; Dong et al., 2003), Tn-tropomyosin-actin interactions (McKillop and Geeves, 1993; Gordon et al., 2000; Lehman et al., 2001), and cross-bridge formation to generate force (Geeves and Holmes, 2005; Fujita-Becker et al., 2006). ISO has been shown to increase TnC affinity for Ca^{2+} and to promote conformational changes within Tn (Breukelmann and Housmans, 2007); however, such changes would not be expected to decrease force. In in vitro motility assays, ISO does not interfere with cross-bridge formation between isolated myosin and actin (Vivien et al., 2005). The inability of Levo, another known myofilament Ca^{2+} sensitizer that binds TnC and stabilizes the conformation of Ca^{2+} -Tn complex (Haikala et al., 1995), to restore force in the presence of ISO suggests that ISO probably targets process(es) after TnC activation. Thus, given the above considerations, actin-tropomyosin interactions are most probably targeted by ISO. The fact that HNO provides complete (and competitive) reversal of ISO-induced force depression without additional increases in intracellular Ca^{2+} provides strong support to our view that HNO increases force development by increasing myofilament Ca^{2+} responsiveness (Dai et al., 2007; El-Armouche et al., 2010). Recently, we have shown that HNO promotes formation of disulfide bridges between actin and tropomyosin. These disulfide bridges prime tropomyosin for exposing more force-generating cross-bridge binding sites on actin so that more force could be produced (Murray et al., 2009). However, the molecular nature of isoflurane's action on myofilament proteins is unknown. Ongoing studies in our laboratory are focusing on identifying the myofilament protein(s) that are targeted by ISO.

Our current findings have some important pragmatic implications. The negative inotropic effect of ISO is probably to depress contractility further in patients with heart failure under anesthesia. Routinely, vasoconstrictors (i.e., phenylephrine) and crystalloid are administered to restore blood pressure and maintain cardiac output. In spite of these therapies, the perioperative morbidity and mortality for patient with heart failure is surprisingly high (i.e., 11.7%) (Hernandez et al., 2004). It is conceivable that vasoconstrictors and fluid therapy may impair the capability of the heart to contract due to an increase in pre- and postload. On the other

hand, positive inotropic agents are expected to be more effective because they can augment cardiac contractility but they tend to produce Ca^{2+} overload. Ca^{2+} sensitizers, which represent a new class of inotropic agents, may be an effective alternative (Kass and Solaro, 2006). Their potential is especially relevant for patients with heart failure who require anesthesia. Decreased myofilament Ca^{2+} responsiveness as a result of alterations in the function of myofilament regulatory proteins is a significant contributor to the severely decreased contractility of failing myocardium (Noguchi et al., 2004; VanBuren and Okada, 2005; Murphy, 2006). Thus, inotropic agents with Ca^{2+} -sensitizing properties are more desirable and probably more efficient for restoring contractility than conventional inotropic agents that increase contractility simply by increasing the availability of Ca^{2+} (Brixius et al., 2005; Hasenfuss and Teerlink, 2011). In addition, because of the exaggerated Ca^{2+} overload, the use of conventional inotropic agents is associated with worsening morbidity and mortality in heart failure patients (Cohn et al., 1998; Cuffe et al., 2002). Another aspect that singles NCA/HNO out among other inotropes is that it does not alter ISO-induced impact on lusitropy (Table 1).

In summary, we demonstrated that, at clinically relevant doses, the inhalational anesthetic ISO directly depresses myofilament contractility by decreasing Ca^{2+} responsiveness. The force depression can be effectively and fully restored by the novel myofilament Ca^{2+} sensitizer, NCA. Ca^{2+} can also restore the loss in force depression, but only with concomitant doubling of $[\text{Ca}^{2+}]_i$. Although the molecular nature of ISO's myofilament action is still under investigation, our findings indicate that cardiac depression during anesthesia, especially in patients with heart failure, is more effectively and efficiently treated with positive inotropic agents that possess Ca^{2+} -sensitizing properties.

Authorship Contributions

Participated in research design: Ding, Li, Shen, Martin, Sivakumaran, Paolucci, and Gao.

Conducted experiments: Ding, Li, Shen, and Gao.

Contributed new reagents or analytic tools: King and Martin.

Performed data analysis: Ding, Li, Shen, and Paolucci.

Wrote or contributed to the writing of the manuscript: Ding, Li, Shen, Martin, Sivakumaran, Paolucci, and Gao.

References

- Bartunek AE and Housmans PR (2000) Effects of sevoflurane on the contractility of ferret ventricular myocardium. *J Appl Physiol* **89**:1778–1786.
- Bosnjak ZJ, Aggarwal A, Turner LA, Kampine JM, and Kampine JP (1992) Differential effects of halothane, enflurane, and isoflurane on Ca^{2+} transients and papillary muscle tension in guinea pigs. *Anesthesiology* **76**:123–131.
- Breukelmann D and Housmans PR (2007) Halothane, isoflurane, and sevoflurane increase the kinetics of Ca^{2+} -induced conformational change of recombinant human cardiac troponin C. *Anesth Analg* **104**:332–337.
- Brixius K, Hoyer HK, and Schwinger RH (2005) Ca^{2+} -sensitizers—a promising option to treat heart failure? *Cardiovasc Drugs Ther* **19**:423–428.
- Cohn JN, Goldstein SO, Greenberg BH, Lorell BH, Bourge RC, Jaski BE, Gottlieb SO, McGrew F 3rd, DeMets DL, and White BG (1998) A dose-dependent increase in mortality with vesnarinone among patients with severe heart failure. Vesnarinone Trial Investigators. *N Engl J Med* **339**:1810–1816.
- Cuffe MS, Califf RM, Adams KF Jr, Benza R, Bourge R, Colucci WS, Massie BM, O'Connor CM, Pina I, Quigg R, et al. (2002) Short-term intravenous milrinone for acute exacerbation of chronic heart failure: a randomized controlled trial. *JAMA* **287**:1541–1547.
- Dai T, Tian Y, Tocchetti CG, Katori T, Murphy AM, Kass DA, Paolucci N, and Gao WD (2007) Nitroxyl increases force development in rat cardiac muscle. *J Physiol* **580**:951–960.
- Davies LA, Gibson CN, Boyett MR, Hopkins PM, and Harrison SM (2000) Effects of isoflurane, sevoflurane, and halothane on myofilament Ca^{2+} sensitivity and sarcoplasmic reticulum Ca^{2+} release in rat ventricular myocytes. *Anesthesiology* **93**:1034–1044.
- Dong WJ, Robinson JM, Stagg S, Xing J, and Cheung HC (2003) Ca^{2+} -induced

- conformational transition in the inhibitory and regulatory regions of cardiac troponin I. *J Biol Chem* **278**:8686–8692.
- DuMond JF and King SB (2011) The chemistry of nitroxyl-releasing compounds. *Antioxid Redox Signal* **14**:1637–1648.
- El-Armouche A, Wahab A, Wittköpper K, Schulze T, Böttcher F, Pohlmann L, King SB, DuMond JF, Gerloff C, Böger RH, et al. (2010) The new HNO donor, 1-nitrosocyclohexyl acetate, increases contractile force in normal and β -adrenergically desensitized ventricular myocytes. *Biochem Biophys Res Commun* **402**:340–344.
- Froehlich JP, Mahaney JE, Keceli G, Pavlos CM, Goldstein R, Redwood AJ, Sumbilla C, Lee DI, Tocchetti CG, Kass DA, et al. (2008) Phospholamban thiols play a central role in activation of the cardiac muscle sarcoplasmic reticulum calcium pump by nitroxyl. *Biochemistry* **47**:13150–13152.
- Fujita-Becker S, Reubold TF, and Holmes KC (2006) The actin-binding cleft: functional characterisation of myosin II with a strut mutation. *J Muscle Res Cell Motil* **27**:115–123.
- Gao WD, Atar D, Liu Y, Perez NG, Murphy AM, and Marban E (1997) Role of troponin I proteolysis in the pathogenesis of stunned myocardium. *Circ Res* **80**:393–399.
- Gao WD, Backx PH, Azan-Backx M, and Marban E (1994) Myofilament Ca^{2+} sensitivity in intact versus skinned rat ventricular muscle. *Circ Res* **74**:408–415.
- Gao WD, Liu Y, Mellgren R, and Marban E (1996) Intrinsic myofilament alterations underlying the decreased contractility of stunned myocardium. A consequence of Ca^{2+} -dependent proteolysis? *Circ Res* **78**:455–465.
- Gao WD, Perez NG, and Marban E (1998) Calcium cycling and contractile activation in intact mouse cardiac muscle. *J Physiol* **507**:175–184.
- Geeves MA and Holmes KC (2005) The molecular mechanism of muscle contraction. *Adv Protein Chem* **71**:161–193.
- Gordon AM, Homsher E, and Regnier M (2000) Regulation of contraction in striated muscle. *Physiol Rev* **80**:853–924.
- Graham MD, Bru-Mercier G, Hopkins PM, and Harrison SM (2005) Transient and sustained changes in myofilament sensitivity to Ca^{2+} contribute to the inotropic effects of sevoflurane in rat ventricle. *Br J Anaesth* **94**:279–286.
- Haikala H, Kaivola J, Nissinen E, Wall P, Levijoki J, and Lindén IB (1995) Cardiac troponin C as a target protein for a novel calcium sensitizing drug, levosimendan. *J Mol Cell Cardiol* **27**:1859–1866.
- Hanley PJ and Loiselle DS (1998) Mechanisms of force inhibition by halothane and isoflurane in intact rat cardiac muscle. *J Physiol* **506**:231–244.
- Hasenfuss G and Teerlink JR (2011) Cardiac inotropes: current agents and future directions. *Eur Heart J* **32**:1838–1845.
- Herland JS, Julian FJ, and Stephenson DG (1993) Effects of halothane, enflurane, and isoflurane on skinned rat myocardium activated by Ca^{2+} . *Am J Physiol* **264**:H224–H232.
- Hernandez AF, Whellan DJ, Stroud S, Sun JL, O'Connor CM, and Jollis JG (2004) Outcomes in heart failure patients after major noncardiac surgery. *J Am Coll Cardiol* **44**:1446–1453.
- Housmans PR and Murat I (1988) Comparative effects of halothane, enflurane, and isoflurane at equipotent anesthetic concentrations on isolated ventricular myocardium of the ferret. II. Relaxation. *Anesthesiology* **69**:464–471.
- Housmans PR, Wanek LA, Carton EG, and Bartunek AE (2000) Effects of halothane and isoflurane on the intracellular Ca^{2+} transient in ferret cardiac muscle. *Anesthesiology* **93**:189–201.
- Jiang Y and Julian FJ (1998) Effects of isoflurane on $[\text{Ca}^{2+}]_i$, SR Ca^{2+} content, and twitch force in intact trabeculae. *Am J Physiol* **275**:H1360–H1369.
- Kass DA and Solaro RJ (2006) Mechanisms and use of calcium-sensitizing agents in the failing heart. *Circulation* **113**:305–315.
- Kleerekoper Q, Howarth JW, Guo X, Solaro RJ, and Rosevear PR (1995) Cardiac troponin I induced conformational changes in cardiac troponin C as monitored by NMR using site-directed spin and isotope labeling. *Biochemistry* **34**:13343–13352.
- Krudy GA, Kleerekoper Q, Guo X, Howarth JW, Solaro RJ, and Rosevear PR (1994) NMR studies delineating spatial relationships within the cardiac troponin I-troponin C complex. *J Biol Chem* **269**:23731–23735.
- Lehman W, Rosol M, Tobacman LS, and Craig R (2001) Troponin organization on relaxed and activated thin filaments revealed by electron microscopy and three-dimensional reconstruction. *J Mol Biol* **307**:739–744.
- McKillop DF and Geeves MA (1993) Regulation of the interaction between actin and myosin subfragment 1: evidence for three states of the thin filament. *Biophys J* **65**:693–701.
- Murat I, Ventura-Clapier R, and Vassort G (1988) Halothane, enflurane, and isoflurane decrease calcium sensitivity and maximal force in detergent-treated rat cardiac fibers. *Anesthesiology* **69**:892–899.
- Murphy AM (2006) Heart failure, myocardial stunning, and troponin: a key regulator of the cardiac myofilament. *Congest Heart Fail* **12**:32–38; quiz 39–40.
- Murray CT, Gao WD, Zhong X, Stanley BS, Foster DB, King SB, Wink DA, Van Eyk J, and Paolucci N (2009) HNO induced disulfide bond between cysteine residues on actin (Cys 257) and tropomyosin (Cys 190) increasing force development: a novel, redox-based mechanism for contractile regulation. *Circulation* **120**:S871.
- Noguchi T, Hünlich M, Camp PC, Begin KJ, El-Zaru M, Patten R, Leavitt BJ, Ittleman FP, Alpert NR, LeWinter MM, et al. (2004) Thin-filament-based modulation of contractile performance in human heart failure. *Circulation* **110**:982–987.
- Paolucci N, Katori T, Champion HC, St John ME, Miranda KM, Fukuto JM, Wink DA, and Kass DA (2003) Positive inotropic and lusitropic effects of HNO/NO⁻ in failing hearts: independence from beta-adrenergic signaling. *Proc Natl Acad Sci USA* **100**:5537–5542.
- Paolucci N, Saavedra WF, Miranda KM, Martignani C, Isoda T, Hare JM, Espey MG, Fukuto JM, Feelisch M, Wink DA, et al. (2001) Nitroxyl anion exerts redox-sensitive positive cardiac inotropy in vivo by calcitonin gene-related peptide signaling. *Proc Natl Acad Sci USA* **98**:10463–10468.
- Rusby BF and Komai H (1987) Anesthetic depression of myocardial contractility: a review of possible mechanisms. *Anesthesiology* **67**:745–766.
- Sato S, Talukder MA, Sugawara H, Sawada H, and Endoh M (1998) Effects of levosimendan on myocardial contractility and Ca^{2+} transients in aequorin-loaded right-ventricular papillary muscles and indo-1-loaded single ventricular cardiomyocytes of the rabbit. *J Mol Cell Cardiol* **30**:1115–1128.
- Sha X, Isbell TS, Patel RP, Day CS, and King SB (2006) Hydrolysis of acyloxy nitroso compounds yields nitroxyl (HNO). *J Am Chem Soc* **128**:9687–9692.
- Shoman ME, DuMond JF, Isbell TS, Crawford JH, Brandon A, Honovar J, Vitturi DA, White CR, Patel RP, and King SB (2011) Acyloxy nitroso compounds as nitroxyl (HNO) donors: kinetics, reactions with thiols, and vasodilation properties. *J Med Chem* **54**:1059–1070.
- Su JY and Bell JG (1986) Intracellular mechanism of action of isoflurane and halothane on striated muscle of the rabbit. *Anesth Analg* **65**:457–462.
- Su JY and Kerrick WG (1980) Effects of enflurane on functionally skinned myocardial fibers from rabbits. *Anesthesiology* **52**:385–389.
- Terrar DA and Victory JG (1988) Isoflurane depresses membrane currents associated with contraction in myocytes isolated from guinea-pig ventricle. *Anesthesiology* **69**:742–749.
- Tocchetti CG, Stanley BA, Murray CI, Sivakumaran V, Donzelli S, Mancardi D, Pagliaro P, Gao WD, van Eyk J, Kass DA, et al. (2011) Playing with cardiac “redox switches”: the “HNO way” to modulate cardiac function. *Antioxid Redox Signal* **14**:1687–1698.
- Tocchetti CG, Wang W, Froehlich JP, Huke S, Aon MA, Wilson GM, Di Benedetto G, O'Rourke B, Gao WD, Wink DA, et al. (2007) Nitroxyl improves cellular heart function by directly enhancing cardiac sarcoplasmic reticulum Ca^{2+} cycling. *Circ Res* **100**:96–104.
- VanBuren P and Okada Y (2005) Thin filament remodeling in failing myocardium. *Heart Fail Rev* **10**:199–209.
- Vivien B, Lecarpentier Y, Riou B, and Coirault C (2005) Halothane and isoflurane do not directly interact with cardiac cross-bridge function. *Anesthesiology* **102**:364–370.

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