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Nitric oxide inhibits creatine kinase and regulates rat heart contractile reserve

(nitric oxide synthase/cardiac myocyte/phosphorus-31 nuclear magnetic resonance/adenosine triphosphate/creatine phosphate)

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ABSTRACT **Cardiac myocytes express both constitutive and cytokine-inducible nitric oxide synthases (NOS). NO and its congeners have been implicated in the regulation of cardiac contractile function. To determine whether NO could affect myocardial energetics, ^{31}P NMR spectroscopy was used to evaluate high-energy phosphate metabolism in isolated rat hearts perfused with the NO donor *S*-nitrosoacetylcysteine (SNAC). All hearts were exposed to an initial high Ca^{2+} (3.5 mM) challenge followed by a recovery period, and then, either in the presence or absence of SNAC, to a second high Ca^{2+} challenge. This protocol allowed us to monitor simultaneously the effect of SNAC infusion on both contractile reserve (i.e., baseline versus high workload contractile function) and high-energy phosphate metabolism. The initial high Ca^{2+} challenge caused the rate-pressure product to increase by 74±5% in all hearts. As expected, ATP was maintained as phosphocreatine (PCr) content briefly dropped and then returned to baseline during the subsequent recovery period. Control hearts responded similarly to the second high Ca^{2+} challenge, but SNAC-treated hearts did not demonstrate the expected increase in rate-pressure product. In these hearts, ATP declined significantly during the second high Ca^{2+} challenge, whereas phosphocreatine did not differ from controls, suggesting that phosphoryl transfer by creatine kinase (CK) was inhibited. CK activity, measured biochemically, was decreased by 61 ± 13% in SNAC-treated hearts compared to controls. Purified CK in solution was also inhibited by SNAC, and reversal could be accomplished with DTT, a sulfhydryl reducing agent. Thus, NO can regulate contractile reserve, possibly by reversible nitrosothiol modification of CK.**

Recent evidence documents that endogenous nitric oxide (NO) production, exogenous NO donors, or agents that inhibit endogenous generation of nitrogen oxides can exert inotropic and lusitropic effects on cardiac myocytes, on isolated hearts, and on intact hearts *in situ* in several species including humans (1–6). Endogenous NO generated by the "high output" inflammatory cytokine-inducible NO synthase (iNOS or NOS2) in myocytes causes a reduction in contractile responsiveness to positively inotropic stimuli, including β-adrenergic agonists (7–15). Even in the absence of cytokine-induced NO production, endogenous release of NO by cardiac myocytes, and possibly by microvascular endothelial cells as well, has been shown to diminish the influx of Ca^{2+} into myocytes through L-type voltage-sensitive ion channels and to blunt their responsiveness to β-adrenergic agonists (16–19). Both cell types have been shown recently to express the endothelial constitutive isoform of NO synthase (ecNOS or NOS3) (16, 17).

Many of the actions of endogenous NO are known to be mediated by activation of soluble guanylate cyclase and an increase in intracellular cGMP (20–23). cGMP analogues have been shown to inhibit $I_{Ca\text{-}L}$ and to produce a positive lusitropic effect in isolated mammalian cardiac myocytes (24). Also, intracellular dialysis with an agent such as methylene blue, which inhibits the activation of guanylyl cyclase by NO, blocks muscarinic cholinergic agonist attenuation of $I_{Ca\text{-}L}$ in whole-cell patch clamp experiments of ventricular myocytes exposed to a β-adrenergic agonist (17). However, it has recently been shown that activation of cGMP signaling pathways in skeletal muscle accounts for only a modest portion of the suppressive effect of increased endogenous NO production on the force-frequency relationship of electrically stimulated muscle fibers (25, 26). Potential non-cGMP-mediated actions of NO and related congeners include formation of peroxynitrite ($OONO^-$), modification of enzymes and nuclear regulatory factors via binding to transition metals or sulfhydryl groups, and formation of nitrotyrosines influencing regulatory or catalytic sites (20, 27, 28). It has been proposed that either S-nitrosylation of Ca^{2+} regulatory proteins in the sarcoplasmic reticulum or a direct suppression of mitochondrial respiration by NO could account for some of the actions of NO (25). Indeed, NO is known to rapidly and reversibly decrease mitochondrial membrane potential ($\Delta\Psi$) in isolated mitochondria, suggesting that NO could inhibit electron transport through the respiratory chain, probably at cytochrome *c* or cytochrome oxidase (29, 30). Shen *et al.* (31) have also shown that whole animal oxygen consumption could be increased in resting, chronically instrumented conscious dogs following systemic infusion of the NO synthase inhibitor L-nitroarginine. Similar findings have been reported for an isolated hindlimb preparation, suggesting that endogenous NO, among other functions, might regulate the energy metabolism of both resting and contracting muscle (32).

Here we report the effects of an exogenous NO donor, *S*-nitrosoacetylcysteine (SNAC), on high-energy phosphate metabolism and cardiac contractile reserve in isolated rat hearts.§ SNAC markedly suppressed the ability of these hearts to recruit contractile reserve in response to a high Ca^{2+} challenge. This was accompanied by a fall in ATP and the unexpected maintenance of phosphocreatine (PCr) content at control levels, suggesting that phosphoryl transfer between PCr and ATP by creatine kinase (CK) had been inhibited. This finding, together with biochemical measurements of CK activity from the same hearts and of the isolated enzyme,

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Abbreviations: NOS, nitric oxide synthase; SNAC, *S*-nitrosoacetylcysteine; RPP, rate-pressure product; PCr, phosphocreatine; CK, creatine kinase; KH buffer, Krebs–Henseleit buffer.

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supports the view that NO can modulate contractile reserve in the heart through modification of CK activity.

MATERIALS AND METHODS

Rat Heart Preparation. Male Sprague–Dawley rats (Charles River Breeding Laboratories) weighing between 350 and 450 g were anesthetized by i.p. injection of 20 mg of pentobarbital sodium. Their hearts were excised quickly and placed in cold phosphate-free Krebs–Henseleit (KH) buffer containing (118 mM NaCl/4.7 mM KCl/1.75 mM $CaCl_2$/4.0 mM $MgSO_4$/0.5 mM EDTA/25 mM $NaHCO_3$/11.0 mM glucose) (all reagents from Sigma). The aorta was cannulated and hearts were perfused in the isovolumic Langendorff mode at a constant temperature of 37°C and a constant flow rate of 22–26 ml/min. The root of the pulmonary artery was pierced to allow right ventricular outflow, and a small polyethylene tube was placed through the apex of the left ventricle to drain flow from the Thebesian veins. A water-filled Latex balloon was inserted into the left ventricle and connected to a pressure transducer (Viggo Spectramed P23XL; Oxnard, CA) for continuous measurement of heart rate and left ventricular pressure (Hewlett–Packard system 7754B). Left ventricular end-diastolic pressure was set at 6–8 mmHg. The heart was placed into a 20-mm glass NMR tube. Contractile performance was estimated as rate-pressure product (RPP), the product of left ventricular developed pressure and heart rate. Cardiac contractile reserve was defined as the increase of RPP from baseline to peak performance during inotropic stimulation with a high Ca^{2+}-containing buffer.

Experimental Protocol. Two perfusate buffers were used. The first was the phosphate-free KH buffer described above ("regular KH buffer"). The second perfusate was identical, except that it contained 3.5 mM $CaCl_2$ ("high Ca^{2+} KH buffer"). Both perfusates were equilibrated with 95% O_2/5% CO_2, yielding a pH of 7.4, and were prepared daily. The NO donor, SNAC, at a concentration of 5 mM, was made from $NaNO_2$ and *N*-acetylcysteine (Sigma) (see below) and was prepared daily (pH 7.4 at 37°C) just before being added to the perfusate through a separate line. This concentration of SNAC was chosen based on preliminary experiments in which hearts (n = 3) were perfused at constant pressure over a range of SNAC concentrations (100 μM, 1 mM, 5 mM, and 10 mM). Neither the 100 μM nor the 1 mM concentrations had any vasodilatory effect. However, 5 mM SNAC resulted in a submaximal (27 ± 6%) increase in coronary flow. Coronary flow was doubled by exposure to 10 mM SNAC.

All hearts were allowed to stabilize for 20 min in the NMR magnet while being perfused with KH buffer. Baseline data defining heart performance, intracellular pH, and high-energy phosphate content were then collected. Hearts were then divided into two groups. In the experimental group (n = 7), hearts were perfused for 6 min with the high Ca^{2+} KH buffer followed by 16 min of recovery in the regular KH buffer. Subsequently, these hearts were exposed to SNAC for 6 min. After 6 min, the perfusate was switched again to high Ca^{2+} KH buffer for a 6-min interval in the continuing presence of SNAC. At this point, the SNAC was discontinued, the high Ca^{2+} KH buffer was turned off, and hearts were perfused without interruption with regular KH buffer for 16 min until termination of the experiment. A second group of hearts (control group 1, n = 4) was exposed to both high Ca^{2+} KH buffer challenges as described above, but was not exposed to SNAC.

Two other groups of control hearts were studied. In control group 2, hearts were exposed both to high Ca^{2+} KH buffer challenges as described above, except that either $NaNO_2$ (5 mM) or *N*-acetylcysteine (5 mM), pH 7.4 at 37°C, were substituted for SNAC as reagent controls (n = 2 for each condition). In control group 3, hearts served as time controls and were not exposed to high Ca^{2+} KH buffer, but were perfused in the magnet with regular KH buffer for the duration of the experiment (n = 2). At the end of each experiment, hearts were freeze-clamped with aluminum tongs precooled in liquid nitrogen, and were then stored at −80°C for biochemical assays.

^{31}P NMR Spectroscopy. ^{31}P NMR spectra were obtained at 161.94 MHz on a GE-400 Omega spectrometer (Fremont, CA). The heart was inserted into a ^{1}H/^{31}P double-tuned probe inside of a 20-cm bore, 9.4 Tesla superconducting magnet (Oxford Magnet Technology, Oxford, U.K.). Spectra were accumulated sequentially over 2-min periods, averaging data from 52 free induction decays using a pulse width of 27 μs, a pulse angle of 60°, recycle time of 2.14 sec, and sweep width of 6000 Hz. Each spectrum was analyzed with zero and first-order phase correction using 20-Hz exponential multiplication. Resonance peaks were fitted to Lorentzian function parameters and areas were computed using the NMR1 (New Methods Research, Syracuse, NY) program. Saturation correction factors, as previously determined (33), were 1.0 for [β-P]-ATP, 1.2 for PCr, and 1.15 for inorganic phosphate. The intracellular pH was determined directly from individual spectra by comparing shifts of inorganic phosphate and PCr peaks to values from a standard curve.

Determination of CK Activity in Frozen Tissue and in Solution. Following freeze-clamping at specified points in each protocol, frozen ventricular tissue (15–20 mg) was thawed and homogenized for 10 sec at 4°C in imidazole buffer (see below) to achieve a final tissue concentration of 5 mg of tissue per ml. Aliquots were removed for protein assay (34) and for assay of CK activity, as described below.

Experiments designed to determine whether the activity of CK could be reversibly inhibited by an NO donor were performed with rabbit skeletal muscle CK (Sigma, Catalog no. 47–10) that had been activated with the reducing agent DTT. Lyophilized CK was dissolved in aqueous imidazole buffer (93.5 mM) at pH 6.6 at a concentration of 4 μg/liter. One ml of CK solution was preincubated with 10 μl of 1 M DTT on ice for 40 min. To remove the DTT, the activated CK solution was eluted through a Sephadex G-25 column (Pharmacia) with imidazole buffer. Activated CK was diluted 1:1 (vol/vol) with ice-cold H_2O and kept on ice until the reaction mixture was added. The reaction mixture consisted of 35 mM PCr, 21 mM glucose, 10 mM Mg acetate, 3.1 mM EDTA, 1.2 mM ADP, 0.42 mM NADP, 10 μM ADP, 1 unit/ml hexokinase, and 0.5 unit/ml G6PDH, in 93.5 mM imidazole buffer at pH 6.6. At the initiation of the reaction, 0.2 ml of ice-cold reaction mixture was mixed with 1 ml of CK solution and allowed to stand for 3 min at room temperature. After 3 min, absorbance at 340 nm was noted and again at 1-min intervals for the next 2 min. CK activity was determined as the average of these changes in absorbance readings and compared to a standard curve with known concentrations of CK. Multiple readings were calculated in this fashion over an 80-min period.

SNAC was prepared by mixing 0.5 ml of 100 mM $NaNO_2$ in 1 nmol HCl with 0.5 ml of 100 mM *N*-acetylcysteine in H_2O with 1 ml of 100 mM Tris at pH 7.4. The *N*-acetylcysteine and $NaNO_2$ control reagents were prepared by mixing 0.5 ml of 100 mM *N*-acetylcysteine (in H_2O) and 0.5 ml of 100 mM $NaNO_2$ (in 1 M HCl), respectively, with 1 ml of 100 mM Tris at pH 7.4. Both SNAC and control reagents were prepared daily and kept on ice until the reaction was initiated.

At the initiation of the CK inhibition experiment, 5 μl of the 25 mM SNAC mixture (or $NaNO_2$ or *N*-acetylcysteine mixture) were added to 0.5 ml of CK solution in 0.495 ml of ice-cold H_2O and allowed to stand (on ice) for several minutes. At the end of this time, the CK reaction mixture was added and CK activity determined as described above. In experiments where DTT was added to reverse the effects of SNAC on CK, 10 μl

of 0.1 M DTT were added at specified time points following the addition of SNAC.

Statistical Analysis. All data are presented as mean ± SEM. Between-group comparisons were performed by a Student's *t* test where data were normally distributed, or by the Mann–Whitney test for nonparametric data. All calculations were performed using MicroCal Origin software (Northampton, MA). The null hypothesis was rejected at $P < 0.05$.

RESULTS

Effects of SNAC on Contractile Reserve in Perfused Hearts. To determine the effect of an NO donor on cardiac contractile reserve, isovolumic perfused adult rat hearts were exposed to sequential challenges with a high (3.5 mM) Ca^{2+}-containing buffer in the absence and presence of 5 mM SNAC, followed by a recovery period. The baseline heart rate was 300 ± 25 beats per minute for all groups. During the course of the experiment, heart rate did not change significantly and did not differ between hearts in the control groups and hearts exposed to SNAC. Similarly, end-diastolic pressures were not significantly different in any group of hearts at any time point.

Fig. 1 depicts the changes in RPP in control hearts and hearts that received SNAC. All hearts increased their RPP during the initial high Ca^{2+} challenge, and returned to baseline during the first recovery period with regular KH buffer. When the SNAC perfusion began, experimental hearts dropped their RPP somewhat, but the decrease was not significantly greater than that of controls. During the second high Ca^{2+} challenge, control hearts demonstrated an increase in RPP that was indistinguishable from their initial response (74 ± 5% versus 78 ± 7% increase, respectively; mean ± SEM). In contrast, hearts exposed to SNAC did not increase their RPP significantly above baseline values. Upon recovery there was no difference in RPP between SNAC-treated and control hearts.

^{31}P NMR Measurements of Intracellular pH, ATP, PCr, and Inorganic Phosphate. ^{31}P NMR spectroscopy was used to determine whether changes in high-energy phosphate content accompanied the observed changes in cardiac contractile reserve. Throughout the experiment, no changes in intracellular pH were noted in either experimental or control groups. As expected, during the first high Ca^{2+} challenge, as workload increased, ATP remained unchanged from baseline (Fig. 2*A*), whereas PCr fell and inorganic phosphate increased in all hearts (Fig. 2 *B* and *C*). During the first recovery period, we observed the expected PCr overshoot and then PCr content returned to levels statistically unchanged from baseline in all

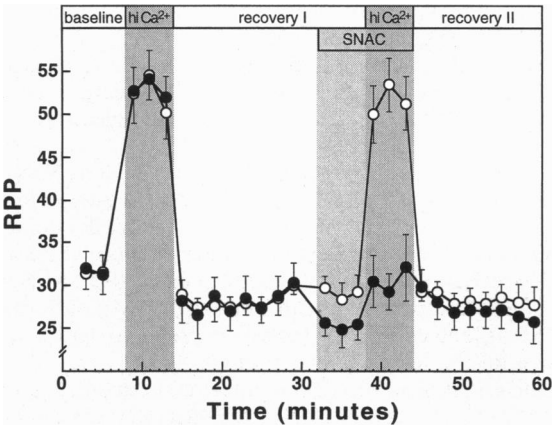

FIG. 1. Changes in RPP [heart rate (beats per minute) × systolic blood pressure (mmHg) ÷ 10^3] throughout the experimental protocols in rat hearts exposed to two high Ca^{2+} challenges without (○), or with (●) SNAC (5 mM). SNAC was initiated 6 min preceding the second high Ca^{2+} challenge. Data represent means ± SEM.

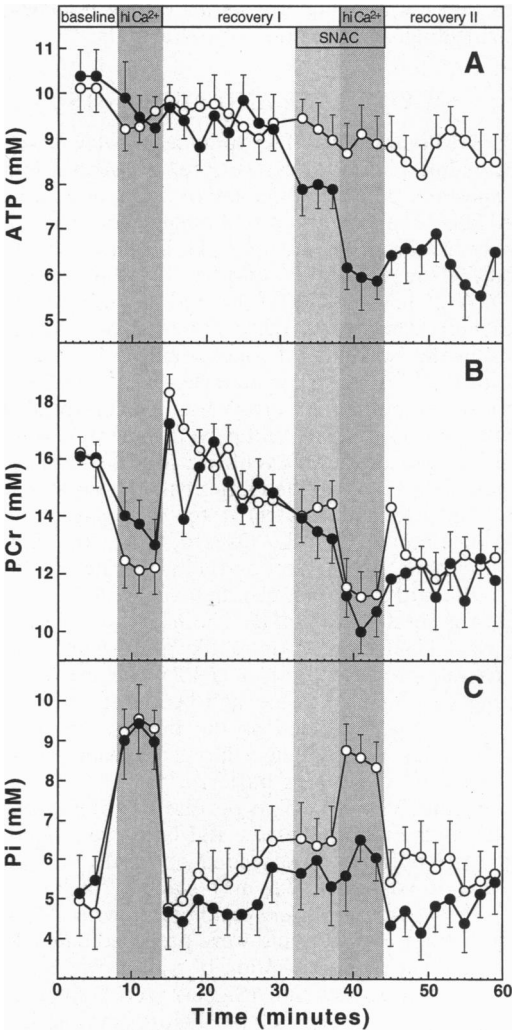

FIG. 2. Changes in ATP (*A*), PCr (*B*), and inorganic phosphate (*C*) concentrations (mM) throughout the experimental protocols in rat hearts exposed to two high Ca^{2+} challenges without (○) or with (●) SNAC (5 mM) perfusion during the 6 min preceding the second high Ca^{2+} challenge. Data represent means ± SEM.

hearts. Following the first recovery period, hearts were perfused for 6 min with either regular KH buffer plus 5 mM SNAC (experimental group), regular KH buffer alone (control group 1), or regular KH and control reagents (control group 2). There were no differences in RPP, ATP, or PCr content among hearts in control groups 1 and 2 throughout the protocol (data not shown). Therefore, these results were pooled.

Control hearts maintained ATP levels during the second high Ca^{2+} challenge and concomitantly showed a decline in PCr content that was indistinguishable from that observed during the initial high Ca^{2+} challenge (Fig. 2 *A* and *B*). In contrast, in the experimental group, ATP content began to decline after the initiation of SNAC perfusion. This decline became significant during the second high Ca^{2+} period. Although ATP levels fell, PCr levels remained at control levels. The ATP content of SNAC-treated hearts remained significantly lower than that of controls during the time course of these experiments. In contrast, ATP content in control hearts was not different from control hearts perfused with regular KH buffer alone for the same time duration as these experiments (data not shown).

CK Activity in Ventricular Muscle. The depletion of ATP, but not PCr, in SNAC-treated hearts implied that CK did not function to transfer phosphoryl groups from PCr to ATP. To determine whether there was a difference in CK activity between control and SNAC-treated groups, hearts were frozen

after the second Ca^{2+} infusion and at the end of the experimental protocol, and CK activity was assayed in samples prepared from frozen ventricular muscle. In tissue from hearts treated with the NO donor (SNAC), CK activity was significantly reduced to 39.7 ± 9% of control ($n = 3$; $P < 0.05$) at the end of the second high Ca^{2+} infusion. By the end of the final recovery period in SNAC-treated hearts, CK activity had recovered partially to 65.1 ± 3% of control ($n = 4$; $P < 0.05$). All control groups demonstrated comparable CK activities throughout the protocol.

NO and CK Activity In Solution. To address the etiology of the decreased CK activity in ventricular muscle of hearts exposed to 5 mM SNAC, purified CK (freeze-dried rabbit skeletal muscle, MM isoform) that had been preactivated by the thiolate-reducing compound DTT (see *Materials and Methods*) was exposed to 50 μM of SNAC. CK exposed to control buffer alone exhibited a slow but consistent decline in activity with time (Fig. 3). This decline could be prevented by adding 1 mM DTT to the CK mixture at the beginning of the experiment. CK activity in additional control buffers containing either 50 μM $NaNO_2$ or 50 μM *N*-acetylcysteine was not different from that observed with control reagents alone (data not shown). Fifty μM of SNAC reduced CK activity to less than 10% of control as quickly as the spectrophotometric assay could be completed (about 3 min), and then to 5% of control at 80 min. There was no evidence of recovery of CK activity during this period. As shown in Fig. 3, addition of 1 mM DTT following exposure to SNAC increased CK activity to control levels over a subsequent 50-min period. Addition of excess acetylcysteine also reversed the effect of SNAC on CK activity (data not shown). To confirm that the inhibition of CK we observed here could be duplicated with another pharmacologic NO donor, these experiments were repeated with SNAP. Exposure of CK in solution to SNAP under otherwise identical experimental conditions resulted in complete inhibition of CK activity that could be reversed by addition of 10 mM DTT. A somewhat higher (100 μM) concentration of SNAP was necessary, however, to completely inhibit CK activity and, importantly, the time course of inhibition was longer, requiring approximately 2 hr even at concentrations of SNAP as high as 500 μM (data not shown).

DISCUSSION

Contractile Performance of Hearts Exposed to an Endogenous NO Donor. The experiments using isolated perfused adult rat hearts and ^{31}P NMR spectroscopy were designed to determine if NO, supplied exogenously, could affect high-energy phosphate metabolism either at baseline or during short-term inotropic stimulation. Prior reports have suggested that NO itself, as well as derivatives formed from interactions with reactive oxygen intermediates, can inhibit mitochondrial respiration and enzymes involved in glycolysis (27–31). These actions are ascribed to the propensity of NO to form nitrotyrosines or S-nitrosothiols or to bind to iron–sulfur clusters, thus inactivating proteins such as mitochondrial aconitase or heme-containing proteins in the mitochondrial respiratory chain (27–30, 35, 36). NO has also been shown to inactivate glyceraldehyde-3-phosphate dehydrogenase, either by facilitating irreversible binding to NAD or by ADP-ribosylation (27, 37). Most of these actions have not been considered relevant to the physiologic regulation of cellular functions such as respiration and ATP synthesis, but as an unfavorable outcome of excessive or inappropriate NO production in response to an inflammatory stimulus. Nevertheless, recent evidence suggests that endogenous NO production in tissues not exposed to inflammatory mediators could also play a role in the regulation of oxygen consumption and energy metabolism (25, 31, 32).

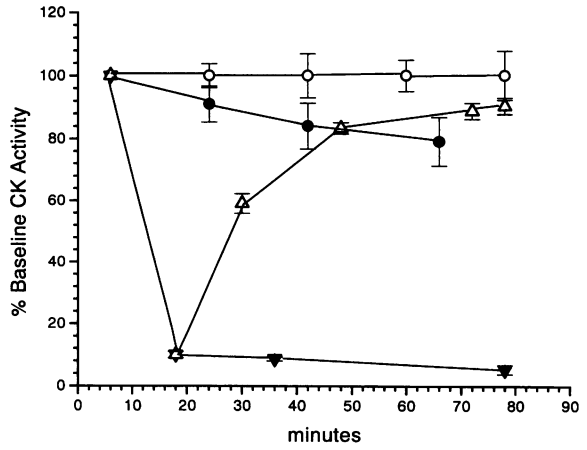

FIG. 3. CK activity in solution is reversibly inhibited by SNAC. Rabbit skeletal muscle CK in solution was prepared as described and then exposed to either control buffer alone (●) or control buffer plus 1 mM DTT (○) for 80 min, or to 50 μM SNAC (▼) at the initiation of the experiment. The decrease in SNAC-treated CK activity could be reversed by adding 1 mM DTT (Δ) after 18 min of exposure to SNAC alone. Data represent means ± SEM.

We chose to infuse SNAC, a light-stable NO donor that spontaneously generates NO, at a concentration (5 mM) that produced a modest and submaximal increase in coronary flow during retrograde perfusion of rat hearts. During the 90-min time course of the isolated heart perfusion experiments, neither hearts exposed to control buffers alone (i.e., control group 1), nor hearts exposed to the control reagents $NaNO_2$ or *N*-acetylcysteine during the second high Ca^{2+} challenge (control group 2), showed any unusual or significant change in ATP levels. As expected for small animal hearts, PCr levels fell rapidly by about 25% during each high Ca^{2+} challenge and then returned toward baseline levels at the end of the subsequent recovery periods. The magnitude of the decrease in PCr is similar to that which we have reported for isolated rat hearts with the sulfhydryl inhibitor iodoacetamide (45). In contrast to control hearts, ATP levels in SNAC-perfused hearts dropped by 40–50% with high Ca^{2+}. This was the case despite the complete absence of the expected increase in RPP with high Ca^{2+}. Unexpectedly, PCr levels did not differ between SNAC-treated hearts and control hearts at the time of ATP depletion.

PCr normally serves to buffer rapid increases in ATP consumption due to an increase in workload as reviewed by Wallimann *et al.* (39). The rate of transfer of the phosphoryl group from PCr to ATP by CK is known to be at least 10-fold higher than the maximum rate of ATP generation by oxidative phosphorylation in normal hearts (40), ensuring rapid resynthesis of ATP during short-term increases in demand. If NO released during the SNAC infusions had acted predominantly to diminish ATP generation by oxidative phosphorylation, whether by inhibiting the metabolism of Krebs cycle intermediates or by slowing electron transport in the cytochrome chain, the cytosolic reservoir of high-energy phosphates in the form of PCr pool should have been consumed to maintain ATP levels. The fact that we observed no fall in PCr as ATP content declined suggests that NO could be selectively inhibiting CK activity.

Regulation of CK Activity. The decline in cardiac contractile reserve following SNAC infusion, without a decrease in PCr/ATP ratio, is reminiscent of myocardial "stunning," the reversible decline in cardiac function that follows reperfusion of transiently ischemic muscle (41–43). Among other mechanisms, experimental evidence has been interpreted to suggest that the reversible inhibition of myofibrillar CK activity during stunning, which has been attributed to the generation of oxygen free radicals during reperfusion, could modify sulfhydryl groups essential to normal enzyme function (45). We (45) and Krause and Jacobus (44) have observed a similar rapid decline in contractile reserve with maintenance of the PCr/

ATP ratio during infusion of isolated perfused hearts with iodoacetamide, a sulfhydryl alkylating agent, at concentrations that result in a relatively selective decrease in CK activity. In those reports, the decrease in CK activity was greater and yet the decline in RPP to an acute inotropic challenge was less than observed here with an NO donor.

The observations reported here suggest that the decline in cardiac contractile reserve during perfusion with SNAC could be due, at least in part, to a decrease in CK-catalyzed transfer of the high-energy phosphate group between PCr and ATP. Whereas it is possible that NO could have modified CK activity indirectly through some intermediate signaling pathway, a direct reversible modification of the enzyme such as *S*-nitrosylation seems more likely. The decrease in CK activity assayed biochemically in muscle of hearts frozen at the end of the SNAC plus high Ca^{2+} perfusion supports this hypothesis. Cytosolic CK, which exists as functional dimers of M-CK and/or B-CK isoform subunits, contains eight -SH groups, of which two are believed to be at or near the catalytic or substrate binding sites, and appear to be essential for enzyme activity (39, 46–51). CK activity in solution is inhibited by low μM concentrations of *N*-ethylmaleimide, and by a DTT-reversible oxidation reaction catalyzed by iron, both of which are presumed to be mediated by direct covalent modification of sulfhydryl groups on the enzyme (52). The relatively selective inhibition of CK in the intact isovolumic rat heart by iodoacetamide at low concentrations emphasizes the lability of these sulfhydryl groups on this enzyme (45).

NO is known to bind covalently and to alter the activity of a number of enzymes and transcriptional regulatory factors by direct *S*-nitrosylation, by the intermediate formation of metal–NO adducts, or in combination with superoxide anion O_2^-, by peroxynitrite and by formation of nitrotyrosines (20, 27, 28). The actions of NO in a cell depend, therefore, on its concentration, the abundance of metals, thiols, and other nucleophile targets, and the cellular redox state. Many actions of NO are reversible and could serve a physiologic regulatory role: the binding of NO to the heme prosthetic group in guanylate cyclase, for example. The data in Fig. 3 indicate that the inhibitory effect of SNAC on CK activity in solution is reversible in the presence of the reducing agent DTT, suggesting that formation of an *S*-nitrosothiol does occur. These data also suggest that this reaction could be readily reversible in cells depending upon the availability of reduced thiol-containing compounds such as glutathione. The fact that ATP content in SNAC-treated hearts did not return to baseline levels at the end of the second recovery period may be due to insufficient time for recovery, to the loss of sulfhydryl reducing activity in the SNAC-treated hearts, or to the loss of diffusible products of ATP degradation.

It is possible, perhaps likely, that the actions of NO or its congeners on the regulation of high-energy phosphate metabolism differ as a function of their concentration within the cell. Under usual physiologic conditions, if the constitutive NO synthase isoform were localized to mitochondrial inner membranes as suggested in one report (26), it could act to regulate electron transport through the cytochrome chain, or it could serve to buffer oxygen free radicals generated by oxidative phosphorylation. The data reported here indicate that CK also may be a physiologically important target for NO in cardiac muscle. Reversible inhibition of CK during increased NO production deserves further study as a potential mechanism underlying cardiac energetic and functional abnormalities in clinically important settings such as myocardial stunning, systemic sepsis, and myocardial inflammatory disease (38).

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