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Nitric oxide donor, NOC7, reveals biphasic effect on contractile force of isolated rat heart after global ischemia

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Abstract
Purpose. Our purpose was to investigate whether the NO donor,3-(2-hydroxy-1-methyl-2-nitroso-hydrazino)-N-methyl-1-propanamine (NOC7), restored cardiac function following global ischemia in an isolated rat heart model and whether intracellular messengers were involved in its effect.
Methods. Isolated rat hearts ($n = 36$) were randomly divided into six groups. The sham control group was perfused with modified Krebs-Henseleit bicarbonate buffer (KHB) alone. The ischemic control group and the NOC7 groups were subjected to 35 min of global ischemia, followed by 30 min of reperfusion with KHB alone, or reperfusion with KHB including NOC7 at 0.2, 2, 20, or 200 μM, respectively. Left ventricular developed pressure (LVDP), the maximum and the minimal rate of rise in LVP ($\pm dP/dt$), and coronary flow were measured continuously. Cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) levels were measured in myocardium homogenate, using enzyme immunoassay (EIA) methods.
Results. NOC7 at 2 and 20 μM rescued myocardial performance (LVDP, 111.9 ± 10.5% and 124.3 ± 12.5% of baseline, respectively; $P < 0.05$ vs ischemic control) at 30 min after reperfusion. However, NOC7 at 200 μM reduced the LVDP to 55.3 ± 6.0% of baseline. Coronary flows remain unchanged. The cAMP levels increased significantly from 0.83 ± 0.44 pmol·mg^{-1} protein in the ischemic control group to 1.79 ± 0.39, 1.86 ± 0.25, and 2.63 ± 0.24 pmol·mg^{-1} protein, in the groups with NOC7 at 2, 20, and 200 μM, respectively ($P < 0.05$). The cGMP level increased from 1.49 ± 0.61 pmol·mg^{-1} protein in the ischemic control group to 3.92 ± 0.66 pmol·mg^{-1} protein in the group with NOC7 at 200 μM alone ($P < 0.05$).
Conclusion. NOC7 appeared to exert a biphasic effect on the contractile force of the isolated rat heart after 35-min global ischemia. The balance between intracellular cAMP and cGMP levels seemed to be involved in its mechanism.

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Key words Myocardial contractility · Ischemia/Reperfusion · Post-resuscitation period · Nitric oxide (NO) donor · Phosphodiesterase inhibition · Cyclic nucleotides

Introduction

Sudden unpredictable cardiac arrest can cause myocardial dysfunction even when cardiopulmonary resuscitation is successful. Preconditioning can be an effective method of reducing cardiac dysfunction after global ischemia in organized clinical settings, such as cardiac surgery or cardiac catheterization, but it is not applicable for sudden unpredicted cardiac arrest [1]. The hemodynamic instability during the post-resuscitation period is generally treated with fluid infusion, vasoactive agents such as norepinephrine, positive inotropic agents such as dobutamine, and inodilator agents such as phosphodiesterase (PDE) 3 inhibitors. However, these tools are not necessarily satisfying, and unfortunately, the strategies used, even those in the 2005 American Heart Association Guidelines for Cardiopulmonary Resuscitation and Emergency Cardiovascular Care, seems not to be changed remarkably compared to the previously recommended strategies [2]. Thus, we were encouraged to seek a new strategy that could restore cardiac function after global ischemia.

Nitric oxide (NO) donors are promising candidates to relieve these situations, because NO is known to have inotropic effects under some conditions [3]. However, there is no obvious evidence that NO donors actually restore cardiac function, as a whole organ, after global ischemia. Instead, several studies report that NO donors exhibit biphasic contractile effects on the myocardium; i.e., low concentrations of NO donors reinforce cardiac contractility, whereas higher concentrations cancel such a benefit [4]. One explanation for this phenomenon is that NO donors affect the intracellular concentrations

of both cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) [5].

The objective of this study was to investigate whether the NO donor, 3-(2-hydroxy-1-methyl-2-nitroso-hydrazino)-N-methyl-1-propanamine (NOC7), restored cardiac function following global ischemia in an isolated rat heart model, and whether intracellular messengers were involved in its effect. We also measured cAMP and cGMP concentrations, and we discuss their roles in the regulation of cardiac contractility, including their relationship to the concentration-effect of NOC7.

Materials and methods

The Institutional Animal Care Committee approved all experimental procedures and protocols used in the present study.

Isolated perfused rat heart and perfusion solution

Langendorff preparations of an isolated rat heart model and the perfusion solution used were basically as previously described, with minor modifications [6]. Briefly, 36 male Sprague-Dawley rats, weighing 250 to 280 g, were used. The animals were anesthetized with pentobarbital (50 $mg \cdot kg^{-1}$), administered intraperitoneally, and heparin (500 $IU \cdot kg^{-1}$), was administered intravenously via the caudal vein. After the heart was isolated, the aorta was cannulated within 1 min. The heart was then perfused at a constant pressure of approximately 80 cmH_2O. The perfusion solution was maintained at 37°C. A collapsed latex balloon was inserted into the left ventricular cavity via a left atrial incision, and we used an LB-2 latex balloon (Nihon Koden, Tokyo, Japan) for the measurement of pressure variables; the intra-balloon pressure was adjusted to 5–10 mmHg. Pressure parameters (left ventricular systolic pressure [LVSP], left ventricular end-diastolic pressure [LVEDP], maximum rate of rise in LVP [dP/dt_{max}], and the minimal rate of rise in LVP [dP/dt_{min}]) were recorded continually. LV developed pressure (LVDP) was calculated as follows:

$$LVDP = LVSP - LVEDP \text{ (mmHg)}$$

Coronary flow was measured at a constant pressure and temperature by a transit-time, in-line ultrasound flow meter (MFV3200; Nihon Kohden).

The perfusion solution was a modified Krebs-Henseleit bicarbonate buffer (KHB) that was composed as follows (in $mmol \cdot l^{-1}$): NaCl, 118; $NaHCO_3$, 25; KCl, 4.7; $CaCl_2$, 1.25; $MgSO_4$, 1.2; KH_2PO_4, 1.2; and glucose, 11. The perfusion solution was gassed continuously with 95% oxygen and 5% carbon dioxide gas at 37°C to maintain P_{O_2} at more than 500 mmHg and pH approximately at 7.4.

Experimental protocol

After preparation, all hearts were subjected to an equilibration period of 20 min before baseline measurements. Those hearts in which LV systolic pressure was greater than or equal to 45 mmHg at the end of the equilibration period were the subject of the study. Subsequently, the hearts were randomly divided into six groups: 1. A sham control group, which was perfused with oxygenated KHB solution alone until the end of experiment, without global ischemia; the hearts in the other five groups were subjected to 35 min of global ischemia by stopping perfusion with the oxygenated KHB solution, and then reperfused for 30 min [reperfusion period]. 2. An ischemic control group, which was perfused with KHB solution alone during the reperfusion period. 3–6. Four NOC7 groups, in which the hearts were perfused with KHB solution containing NOC7, at final concentrations of 0.2, 2, 20, and 200 μM, during the reperfusion period (Fig. 1). Myocardial function was assessed as the LVDP in the pre- and post-ischemic periods (5, 10, 20, and 30 min of reperfusion). Myocardium from the heart apex was collected at the end of the reperfusion period, frozen immediately in liquid nitrogen, and stored at –80°C until cyclic nucleotide analysis.

NOC7 (Dojindo Laboratories, Tokyo, Japan) was prepared in 10 mM NaOH, to make a 200-mM solution, and stored at 5°C. This solution was diluted to the final concentrations of 0.2, 2, 20, or 200 μM just before use.

Cyclic nucleotide determination

Myocardial samples were homogenized and suspended in modified Krebs-hydroxyethylpiperazine ethanesulfonic acid (HEPES) buffer (pH 7.4; 30°C). After the reactions were terminated with the addition of ice-cold trichloroacetic acid (TCA; final concentration, 6%), the samples were centrifuged at 2000 *g* for 15 min at 4°C. The supernatants were collected, frozen, and stored at –80°C until cAMP and cGMP were determined, using an acetylation protocol for cAMP and cGMP (Biotrak enzyme immunoassay system [RPN225 and RPN226]; Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions. Levels of cyclic nucleotides in the samples were calculated as pmol cyclic nucleotide·mg^{-1} protein. The pellets were submitted for measurement of protein using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

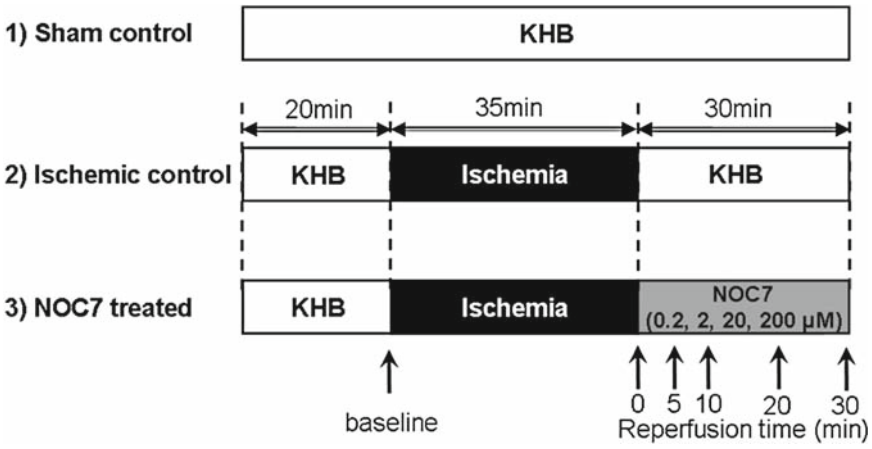

Fig. 1. Experimental protocol. After an equilibration period of 20 min, the sham control group did not undergo global ischemia, and instead, was perfused with modified Krebs-Henseleit bicarbonate buffer (*KHB*) alone until the end of the experiment. In the other groups, the hearts were subjected to 35 min of global ischemia, followed by 30 min of reperfusion. During the reperfusion period, in the ischemic control group, the hearts were perfused with KHB alone. In the 3-(2-hydroxy-1-methyl-2-nitroso-hydrazino)-N-methyl-1-propanamine (*NOC7*) groups, the hearts were perfused with KHB solution containing different concentrations of NOC7 (0.2, 2, 20, or 200 μM as final concentrations); $n = 6$ in each group

Statistical analysis

The values for results for each group were expressed as means ± SD. Differences within and between groups were determined by one-way analysis of variance and multiple comparisons, using the Tukey test with SPSS statistical analysis software 11.5 (SPSS, Chicago, IL, USA). A P value of less than 0.05 was regarded as statistically significant.

Results

Hemodynamic function

Hemodynamic variables are shown in Figs. 2 and 3 and Table 1. The baseline LVDP values were identical in all groups. Thirty-five min of global ischemia reduced the LVDP to 13.0 ± 4.5% at 10 min of reperfusion, which represented an 85% reduction from the sham control value ($P < 0.05$). While 30-min reperfusion with KHB alone partially restored cardiac function, recovery was not complete; LVDP increased only slightly during reperfusion, resulting in a value of only 48.8 ± 6.1% of baseline ($P < 0.05$; Fig. 2).

NOC7 improved myocardial performance, and significantly restored LVDP, to 62.0 ± 6.0% and 67.3 ± 9.7% at 2 and 20 μM, respectively, with 20-min reperfusion from 36.8 ± 7.3% in the ischemic control group ($P < 0.05$). This effect of NOC7 was more prominent after 30 min of reperfusion, and the mean percent LVDP recovered to 111.9 ± 10.5% and 124.3 ± 12.5% of the

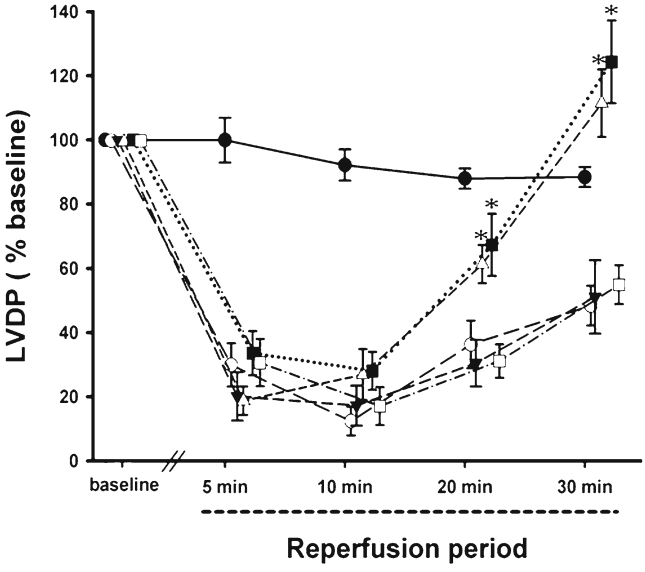

Fig. 2. Recovery of left ventricular developed pressure (*LVDP*) measured at 5, 10, 20, and 30 min of reperfusion after 35 min of global ischemia (mean percentage of baseline). Data values are presented as mean percentage changes ± SD; $n = 6$ in each group. *$P < 0.05$ compared with ischemic control. Data were analyzed using repeated-measure analysis of variance (ANOVA) followed by the Tukey test. *Closed circles*, Sham control; *open circles*, ischemic control; *closed triangles*, NOC7 0.2 μM; *open triangles*, NOC7 2 μM; *closed squares*, NOC7 20 μM; *open squares*, NOC7 200 μM

Table 1. Hemodynamic variables during experiments

	Baseline	Reperfusion time (min)			
		5	10	20	30
LVEDP (mmHg)					
Ischemic control	7.6 (0.5)	78.6 (16.5)**	87.2 (12.0)**	70.6 (11.0)**	65.6 (9.6)**
NOC7 0.2 μM	8.1 (0.5)	85.3 (7.8)**	83.6 (6.1)**	73.3 (5.2)**	68.0 (4.7)**
NOC7 2 μM	7.9 (0.4)	69.4 (5.3)**	67.4 (5.7)***	53.4 (8.7)***	41.6 (8.8)***
NOC7 20 μM	8.0 (0.5)	69.7 (4.0)**	77.8 (4.7)**	65.3 (7.9)**	45.9 (4.7)***
NOC7 200 μM	8.0 (0.7)	79.5 (9.1)**	86.8 (6.3)**	79.6 (5.0)**	68.4 (4.0)**
dP/dt_{min} ($mmHg \cdot s^{-1}$)					
Ischemic control	–2379 (296)	–361 (267)**	–199 (180)**	–666 (121)**	–643 (440)**
NOC7 0.2 μM	–2353 (124)	–490 (197)**	–419 (145)**	–767 (197)**	–1190 (297)**
NOC7 2 μM	–2427 (280)	–506 (145)**	–769 (275)**	–1536 (314)***	–3056 (490)*
NOC7 20 μM	–2449 (425)	–796 (178)**	–657 (131)**	–1367 (106)***	–2684 (291)*
NOC7 200 μM	–2498 (426)	–725 (235)**	–458 (198)**	–781 (188)**	–1419 (299)**
Coronary flow ($ml \cdot min^{-1}$)					
Ischemic control	14.5 (0.8)	13.4 (1.3)	12.9 (1.3)	11.5 (1.2)	11.5 (1.1)
NOC7 0.2 μM	13.1 (0.7)	12.0 (1.1)	12.0 (1.2)	12.4 (1.1)	11.3 (0.8)
NOC7 2 μM	12.8 (1.4)	12.3 (1.8)	12.2 (1.8)	11.6 (1.2)	11.5 (1.0)
NOC7 20 μM	12.5 (1.6)	12.5 (0.6)	13.0 (0.8)	12.0 (0.9)	11.2 (0.9)
NOC7 200 μM	13.0 (1.2)	11.1 (1.1)	11.7 (1.8)	12.1 (1.9)	10.8 (1.8)

* $P < 0.05$ vs ischemic control group; ** $P < 0.05$ vs baseline
Results were reported as mean values ± (SD); $n = 6$ in each group. Differences within and between groups were determined by one-way analysis of variance and multiple comparisons using the Tukey test

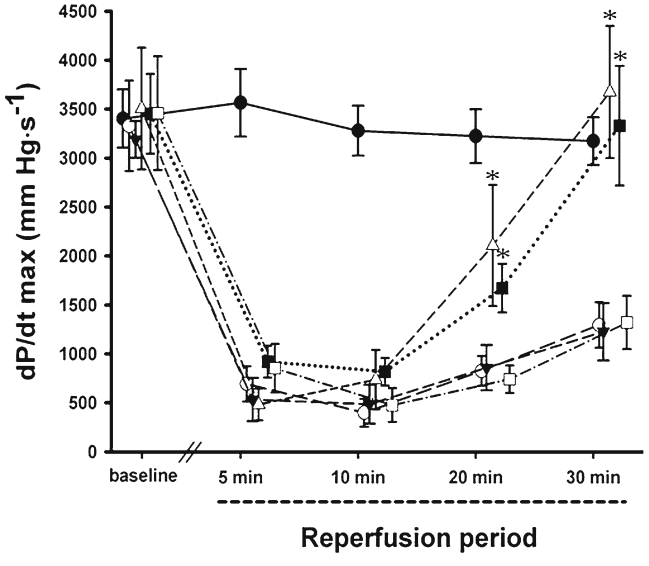

Fig. 3. Recovery in the maximum rate of rise in left ventricular pressure (dP/dt_{max}). Data values are presented as means ± SD; $n = 6$ in each group. *$P < 0.05$ versus ischemic control. Data were analyzed using repeated-measure ANOVA followed by the Tukey test. *Symbols*, As in Fig. 2

baseline at 2 and 20 μM, respectively. These values were obviously greater than the 48.8 ± 6.1% in the ischemic control, and rather close to the sham control level of 88.4 ± 3.1% ($P < 0.05$; Figs. 2 and 3). The highest concentration of NOC7, however, reversed this positive inotropic effect, and LVDP deteriorated again to 55.3 ± 6.0% of the baseline, similar to the value of 51.0 ± 11.5% with 0.2 μM NOC7 or the 48.8 ± 6.1% in the ischemic control.

During ischemia, LVEDP increased in all groups, indicating the development of myocardial contracture. With the onset of reperfusion, there was a further increase in LVEDP to values between 69 and 85 mmHg (reperfusion contracture). NOC7 at 2 and 20 μM significantly recovered LVEDP, to 41.6 ± 8.8 and 45.9 ± 4.7 mmHg, respectively, in comparison to the ischemic control value of 65.6 ± 9.6 mmHg at 30 min of reperfusion ($P < 0.05$; Table 1).

In all groups, the recovery of dP/dt_{max} as a variable of myocardial contractility paralleled the recovery of LVDP (Fig. 3). The mean stabilized dP/dt_{max} at baseline was 3393 ± 131 $mmHg \cdot s^{-1}$. NOC7 at 2 and 20 μM significantly restored dP/dt_{max} at 20 min compared with values in the ischemic control group (2129 ± 651 and 1672 ± 247 versus 844 ± 151 $mmHg \cdot s^{-1}$, respectively; $P < 0.05$), and also at 30 min (3690 ± 671 and 3330 ± 610 $mmHg \cdot s^{-1}$ versus 1311 ± 232 $mmHg \cdot s^{-1}$, respectively; $P < 0.05$). As a variable of diastolic function, dP/dt_{min} changed in a similar manner and was improved by NOC7 at 2 and 20 μM (Table 1). Coronary flow remain unchanged.

Cyclic nucleotide concentrations

The average myocardial cAMP level, in the sham control group was 2.34 ± 0.21 $pmol \cdot mg^{-1}$ protein, and this was

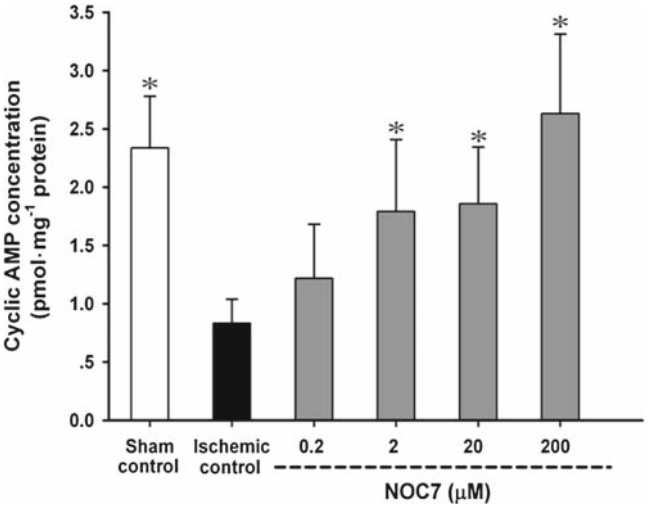

Fig. 4. The effects of NOC7 on intracellular cyclic adenosine monophosphate (*AMP*). *$P < 0.05$ versus ischemic control. Data were analyzed using repeated-measure ANOVA followed by the Tukey test

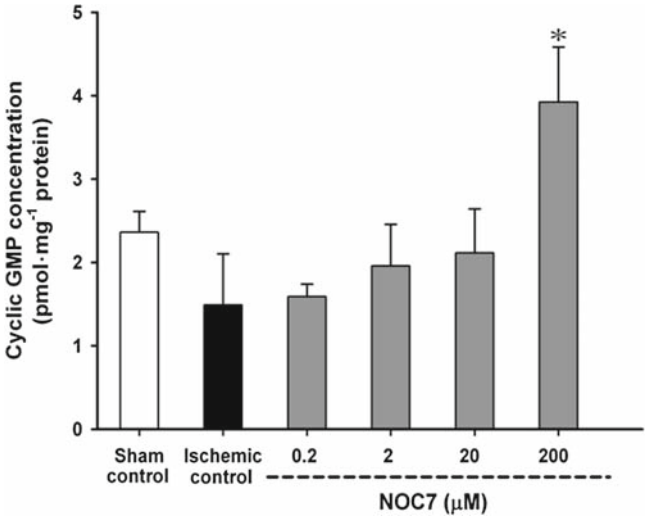

Fig. 5. The effects of NOC7 on intracellular cyclic guanosine monophosphate (*GMP*). *$P < 0.05$ versus ischemic control. Data were analyzed using repeated-measure ANOVA followed by the Tukey test

significantly reduced to 0.83 ± 0.44 pmol·mg^{-1} protein after 35-min ischemia (Fig. 4). NOC7 at 2, 20, and 200 μM significantly restored the average cAMP level, to 1.79 ± 0.39, 1.86 ± 0.25, and 2.63 ± 0.24 pmol·mg^{-1} protein, respectively ($P < 0.05$).

The cGMP level in the sham control group was 2.36 ± 0.25 pmol·mg^{-1} protein, whereas that in the ischemic control group was 1.49 ± 0.61 pmol·mg^{-1} protein (Fig. 5). Following treatment with NOC7 at 2 and 20 μM, the cGMP level changed slightly, but the change did not reach statistical significance. NOC7 at 200 μM significantly increased the cGMP level to 3.92 ± 0.66 pmol·mg^{-1} protein ($P < 0.05$ versus ischemic control).

Discussion

In the present study, we used the Langendorff-perfused isolated rat heart model. This experimental setting excludes the influence of systemic hemodynamics such as preload and afterload, as well as excluding most humoral effects and sympathetic/parasympathetic control from the central nervous systems, which can be affected by NO donors. We regard this method as the best choice among of heart-model to examine the direct myocardial effects of NOC7.

Our results demonstrated that NOC7 at concentrations of 2 and 20 μM restored cardiac function after ischemia, as assessed by LVDP, dP/dt_{max}, and dP/dt_{min}. However, NOC7 at 200 μM, the highest concentration in our present study, lost this positive inotropic effect. This finding corresponds with a previous report that demonstrated a biphasic effect of NO donors in papillary muscle [4]. Our present report is the first one to reveal a similar effect of an NO donor in isolated whole heart; i.e., a biphasic effect on contractile force after global ischemia.

In our study, NOC7 at concentrations of 2, 20, and 200 μM increased myocardial cAMP levels (Fig. 4). In contrast with the changes in myocardial cAMP, NOC7 at 2 and 20 μM changed myocardial cGMP levels slightly, with the changes did not reaching statistical significance (Fig. 5). NO, donors produce NO, which activates a guanylyl cyclase to derive cGMP. Even a moderate increase in cGMP is reported to inhibit myocardial PDE3, and cAMP degradation is then suppressed, which results in an elevated intracellular cAMP level in the myocardium [4]. Furthermore, NO was reported to activate adenylyl cyclase (AC) without the cGMP mediated pathway [7]. This cGMP-independent activation of AC may have induced the increased cAMP levels in the myocardium found in our study. Elevated cAMP acts through a protein kinase A (PKA) to positively affect myocardial function. The cGMP produced in the myocardium may also act directly on the myocardium, through protein kinase G (PKG) activation, to negatively affect myocardial function [8]. In our study, with NOC7 at concentrations of 2 and 20 μM, however, the negative inotropic effect exerted by cGMP may have been masked by the positive effect of the elevated cAMP levels [9,10].

On the other hand, NOC7 at 200 μM, the highest concentration used in the present study, resulted in a significant increase in cGMP (Fig. 5). In spite of the

elevated cAMP levels induced by NOC7 at 200 μM, the positive inotropic effect of NOC7 was reversed, and cardiac function was seen to be similar to that in the ischemic control group. In such, an instances, it has been shown that exogenous NO donor increased the intracellular cGMP level and that under this condition, activated PKG phosphorylated troponin I, which led to myofilament relaxation [11]. In our study, this negative effect on myocardial function of increased cGMP seems to have been dominant, and it overcame the positive effects of cAMP. Of note, NO can reversibly reduce myocardial contractility through a cGMP-independent pathway, i.e., by binding to the oxygen-binding site of cytochrome oxidase in competition with oxygen in the presence of hypoxia. Furthermore, NO or its derivative N_2O_3 or S-nitrosothiols, may inactivate complex I, one of the components of the mitochondrial respiratory chain, by S-nitrosation, and thus inhibit ATP production and susceptibility to cell death [12–14]. These direct negative effects of NO, which are independent of the cGMP-PKG pathway, may be considered to have induced the cardiac dysfunction seen at 200 μM of NOC7 in the present study.

As for different possibilities to explain the observed phenomenon of the reversal of cardiac function by NOC7 at 200 μm, free radical production and calcium overload are considered to be implicated in the development of myocardial ischemia and reperfusion injury [15–17]. Furthermore, elevated NO may lead to the concomitant production of $ONOO^-$, which contributes to cardiac dysfunction [18]. Although we did not measure these factors, they may also have contributed to the reversal of cardiac function induced by NOC7 at 200 μM. Further study is necessary to elucidate these mechanisms.

Several authors report that NO donors increase coronary flow and improve cardiac function. For instance, L-arginine, at 25–1000-fold higher potency for NO production than that in our study, increased post-ischemic coronary flow [19–21]. In the present study, however, coronary flows were maintained at constant levels regardless of the NOC7 concentration (Table 1). The contractile recovery in our model is not likely to have been due to recovered oxygen supply as a result of increased coronary flow.

In the clinical situation, organic NO donors (sodium nitroprusside, nitroglycerine) or authentic NO are currently being used as vasodilators for critical/intensive care. Following our present results, the clinical application of NO donors as inotropic agents, in patients with cardiac dysfunction during post-resuscitation period can be considered. Although further investigations will be necessary before this practical use of NO donors can be implemented, we can expect a considerable benefit when they are employed at appropriate concentrations.

Conclusion

In summary, the NO donor, NOC7, exerted biphasic effect on the contractile force of the isolated rat heart after global ischemia. The balance between intracellular cAMP and cGMP levels seemed to be involved in this mechanism of NOC7. The use of a specific concentration range of NO donors may be one option for the restoration of cardiac function.

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