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Ischemia/reperfusion-induced death of cardiac myocytes: possible involvement of nitric oxide in the coordination of ATP supply and demand during ischemia.

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Running Head: nitric oxide and ATP preservation during ischemia

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Abstract

Nitric oxide (NO) has been known to play various functional and pathological roles as an intracellular or intercellular messenger in the heart. In this study, we investigated whether NO produced during ischemia was involved in the coordination of ATP supply and demand, and also in protection from cell death using cultured cardiac myocytes. Unexpectedly, the survival rate of myocytes for 3 h simulated ischemia (SI) was increased as compared with that for 2 h SI at 24 h after reperfusion. The cellular ATP level at 3 h after the start of SI was increased compared with that at 2 h, and was almost the same as that before the start of SI. The cellular ATP level at 3 h SI was significantly reduced by either the inhibition of nitric oxide synthase (NOS) or scavenging of NO. Either the inhibition of NOS or the scavenging of NO during SI for 3 h also resulted in a significant decrease in the survival rate of myocytes. Immunocytochemical and Western blot analyses revealed that the expression of nNOS was most evident in cardiac myocytes, but no significant change was observed in the expression of all three NOS isoforms at 2 h SI and at 3 h SI. The fluorescent intensity of DAF-FM was significantly increased at 3 h SI as compared with that at 2 h SI, and
the increase in DAF fluorescence during SI was almost completely suppressed by treatment with L-VNIO, a potent specific inhibitor of nNOS. In addition, treatment with L-VNIO decreased the cellular ATP level and survival rate. This study suggested that the enhanced production of NO was critical in balancing ATP supply and demand during ischemia, and also in protecting cells from ischemia/reperfusion injury.

**Key words:**

ischemia, reperfusion, cultured cardiac myocytes, nitric oxide, ATP, lactate
Introduction

Normal myocardium generates more than 90% of its ATP by oxidative metabolism and less than 10% by anaerobic glycolysis [1]. During severe cardiac ischemia, the heart is deprived of oxygen and energy substrates such as glucose and fatty acids. Metabolic arrest achieved by means of a reversed or negative Pasteur effect (reduced or unchanging glycolytic flux) is an important cardioprotective response to ischemic stress [2]. This means that cardiac myocytes must drastically reduce ATP demand or utilization to meet the needs for survival, and thus balance reduced ATP supply with reduced demand during severe ischemia. In fact, severe hypoxic stress reduces the demand for ATP by decreasing a variety of ATP-consuming cell functions including the activities of muscle contraction [3], protein synthesis, and RNA synthesis [4,5] which are the major ATP-consuming process in cardiac myocytes.

Nitric oxide (NO) is a ubiquitous signaling molecule produced from L-arginine by nitric oxide synthase (NOS), and is known to play a variety of functional and/or pathophysiological roles in living tissues or cells [6,7]. The activity of NOS in the heart is stimulated by ischemia, and the activated NOS produces NO after the onset of
ischemia [8]. Therefore, NO presumably contribute to ischemia-induced dysfunction of the heart such as increased vulnerability to ventricular tachyarrhythmias [9], and cardiac cell death [10,11]. In fact, treatment of adult ventricular myocytes with an NO donor resulted in a marked increase in p53 expression, and induced apoptosis [12]. However, we have recently found that NO produced during ischemia is crucial to the development of preconditioning-induced ischemic tolerance of neurons in culture [13]. It has also been suggested that production of NO represents one paracrine/autocrine mechanism coordinating energy supply and demand in tissues [14]. Thus, the pathological as well as functional roles of NO produced during ischemia in cardiac functions are not clear.

Here we provide experimental evidence that the enhanced production of NO was critically involved in balancing ATP supply and demand during ischemia, and also in protecting cells from ischemia/reperfusion-induced injury.
Methods

Culture of cardiac myocytes

The animal experiments conformed to the “Principles of laboratory animal care” (NIH publication No. 85-23, revised 1996), as well as the “guide for the care and use of laboratory animals”, Hokkaido University School of Medicine (Hokkaido, Japan).

The method of culturing cardiac myocytes was described elsewhere in detail [15]. In short, cardiac myocytes were prepared from 1- to -3 day old neonatal Wistar rat ventricles removed after decapitation. The ventricles were rinsed in a 25 mM HEPES buffered minimum salt solution (MSS) to remove contaminating blood cell components and then minced with scissors into fragments to be digested with 0.1% collagenase (Wako Chemical, Tokyo, Japan) in MSS at 37 °C for 10 min. The cell components were suspended in MCDB 107 (Research Institute for Functional Peptides, Yamagata, Japan) containing 5% FCS (MBL, Nagoya, Japan), and then passed through a wire mesh screen (90 µm porosity) to remove large aggregates of cells; the filtered suspension contains cardiac myocytes and fibroblasts. To separate cardiac myocytes
from fibroblasts based on the selective adhesion technique [16], the cell suspension was poured in petri dishes (60×15 mm, Falcon), and incubated for 60 min at 37 °C, in 5% CO₂ and 95% air. By virtue of the procedure, most of the fibroblasts adhere to the dish. After the incubation, the suspension, in which most cell components were cardiac myocytes, was collected. The cells were resuspended in MCDB 107 containing 5% FCS, transferrin (10 µg/ml, Sigma, St. Louis, MO), and insulin (10 µg/ml, Yamanouchi, Tokyo, Japan). The cell suspension was passed through a fine wire mesh screen (26 µm porosity) to remove remaining small aggregates of myocytes, and finally the isolated myocytes remaining were cultured at a density of about 3.0 × 10⁵ cells/ml in a petri dish coated beforehand with fibronectin (10 µg/ml, Sigma). Cardiac myocytes cultured for 5 days were used in this study.

**Simulated ischemia/reperfusion**

The experimental protocol used to simulate ischemia/reperfusion was a modified version of the method proposed by Rakhit and his colleagues [17]. This procedure is described elsewhere in detail [18]. In short, serum-containing incubation medium was exchanged with serum-free DMEM (Gibco BRL, Invitrogen Corp., Carlsbad, CA) 2 h
before the start of experiments. Cells were then treated with ischemic buffer solution (in mM: 118 NaCl, 24 NaHCO$_3$, 1 NaH$_2$PO$_4$H$_2$O, 2.5 CaCl$_2$2H$_2$O, 0.5 sodium EDTA•2H$_2$O, 20 sodium lactate, and 16 KCl, pH 6.2). Near anoxic conditions were achieved using an Anaero-Pack System (Mitsubishi Gas Chemical, Tokyo, Japan). After pre-gassing with 95% N$_2$-5% CO$_2$ for at least 5 min, ischemia buffer was added to the cells, which were then placed in a sealed chamber containing the deoxygenation reagent (Kenki for Cells, Mitsubishi Gas Chemical). The catalytic reaction of the reagent resulted in the consumption of O$_2$ and in the production of CO$_2$. This Anaero-Pack System provided near anaerobic conditions with an O$_2$ concentration of < 1% and a CO$_2$ concentration of about 5% within 1 h of incubation at 37 °C. This hypoxia/anoxia stress to cardiac myocytes is defined as “simulated ischemia”. Cells were exposed to these conditions for 2 h or 3 h, and then incubated again in glucose-containing DMEM at 37 °C in 95% air-5% CO$_2$ (reperfusion).

**ATP and lactate measurement**

ATP was quantified in cardiac myocyte cultures 2 h and 3 h after the start of SI
using a commercially available luciferin-luciferase assay kit (Total ATP Rapid Biocontamination Detection Kit, Promega, WI, USA) [19]. The cells were placed in 400 μl of Tris-HCl buffer, and lysis reagent was added 20 min later. The lysate was mixed with a micropipette, and transferred to a microcentrifuge tube. The diluted lysate (100 μl) was added to a polystyrene tube containing 300 μl of Swab buffer, and then rL/L reagent was added. The tube was placed in a Luminometer (TD-2020, TURNER DESIGN). To confirm whether the quantification of cellular ATP by this method was valid, cellular respiration was inhibited by treatment of cultures with carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP, 1 μM), an uncoupler of mitochondrial respiration, for 10 min.

The L-lactic acid concentrations in the culture medium were quantified using a commercially available assay kit (F-kit L-Lactate, JK International, Tokyo, Japan) 2 h and 3 h after the start of SI [20].

**Immunohistochemical analyses using anti-NOS antibodies**

The nitric oxide synthases (NOS) in cultured cells were detected by
immunostaining with anti-neuronal NOS (nNOS) (Euro-Diagnostica, Malmö, Sweden),
anti-endothelial NOS (eNOS) (Transduction Lab., Lexington KY), or anti-inducible
NOS (iNOS) (Transduction Lab.). For the labeling of nNOS, eNOS, and iNOS, the
cardiac myocytes were fixed with 4% paraformaldehyde for 5 minutes at 4 °C, followed
by 95% methanol in PBS for 10 minutes at –20 °C. The cells were then incubated
with a primary antibody over a 24 h period using a dilution of 1:2,000 for nNOS,
1:2,000 for eNOS, and 1:10,000 for iNOS. After being washed with
phosphate-buffered saline (PBS), the cells were incubated with a secondary antibody
containing 1.0% goat serum for 30 minutes. For labeling, a 1:600 dilution of
biotinylated goat antibody against mouse IgG (Vector Laboratories, Burlingame, CA)
was used. Bound antibodies were detected by the avidin-biotin-peroxidase complex
(ABC) method, using a commercial ABC kit (Vector Laboratories). Observation of
peroxidase activity was made possible by incubation with 0.1% 3,3’-diaminobenzidine
tetrahydrochloride (DAB) in a 50 mM Tris-HCl buffer (pH 7.4) supplemented with
0.02% H₂O₂. The cells were dehydrated in 70 – 100% ethanol, cleared in xylene, and
mounted on glass coverslips in Permount (Fisher Scientific, Fair Lawn, NJ) for light
microscopic observation.

**Western blotting**

Cells were washed three times with ice-cold PBS. The cells were lysed in lysis buffer (10 mM Tris-HCl, pH 7.5, 25% glycerol, 0.82 M NaCl, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 1.5 mM MgCl₂, 0.5 mM EDTA, 2 mM sodium pyrophosphate, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 1.25 μg/ml pepstatin A, 10 μg/ml leupeptin, 2.5 μg/ml aprotinin, 0.1% Triton X-100, and 0.5% Nonidet P-40) and centrifuged at 12,000 g for 20 min at 4 °C. The supernatant was used for analysis. The protein concentration of samples was determined with BCA protein assay reagent (Pierce, Rockford, IL). Thirty micrograms of protein was electrophoresed on 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). After the transfer, the membrane was incubated with Tris-buffered saline buffer (TBST) containing 5% nonfat dry milk for 1 h at room temperature and then with polyclonal antibody to nNOS (1:2,000) (Euro-Diagnostica, Malmö, Sweden) at 4 °C overnight. After the incubation,
the membrane was washed with TBST for 10 min three times at room temperature and incubated with horseradish peroxidase-labeled anti-rabbit IgG antibody (1:2,000) (New England Biolabs, Beverly, MA). The immunoreactive bands were detected with an enhanced chemiluminescence (ECL) detection kit (Cell Signalling technology, Beverly, MA). Relative expression levels of NOS were determined from densitometric scanning of the ECL films produced by 2 independent experiments with Scion Image Bata 4.0.2 (Scion Corporation).

**Evaluation of cell viability**

Cell viability was analyzed following visualization of the nuclear morphology with the fluorescent DNA-binding dyes, Hoechst 33342 (bisbenzimide) and propidium iodide (PI). Cells were incubated with these dyes for 15 min at 37 °C. Individual nuclei were visualized using fluorescent microscopy (Olympus, IX70) and analyzed; PI was used to identify nonviable cells. An average of 450-500 cardiac myocytes from random fields were analyzed in each experiment. The survival rate of myocytes (percentage of viable myocytes) was determined by placing images of nuclear staining
on phase-contrast images, and calculated as (viable myocytes/total myocytes before the onset of SI) × 100, since some myocytes came off from the dishes at the time of inspection. Six independent experiments (n=6 different cultures) were performed and analyzed.

**Monitoring of cytosolic nitric oxide production**

Changes in the cytosolic NO concentration were monitored using a fluorescent NO probe, DAF-FM [21]. Cells were loaded with NO indicator by incubation with 10 μM of DAF-FM DA for 4 h (0.5% DMSO). High concentrations of DAF-FM, more than 20 μM, seemed toxic to cultured cardiac myocytes, since loading of cultures with a high concentration of DAF-FM sometimes resulted in morphological changes to the myocytes. Fluorescent images were acquired at 2 s intervals with a cooled CCD camera (C4880-80; Hamamatsu Photonics, Hamamatsu, Japan). An analysis of the acquired images was made with an image processing and measuring system (AQUACOSMOS, Hamamatsu Photonics). Free cytoslic NO was monitored by comparing the changes in fluorescence intensity at an excitation wavelength of 490 nm with the initial fluorescence intensity (F/F0), using an emission wavelength of 515 nm.
Because DAF-FM is not a quantitative probe like DAF-2 [21], no attempt was made to calibrate DAF-FM fluorescence.

**Experimental protocol**

In all experiments, cell viability was analyzed 24 h after the start of reperfusion, since delayed death (apoptosis) of cardiac myocytes could be observed long after the ischemic insult in some cases [22,23].

To investigate whether the NOS-derived NO is involved in the preservation of cellular ATP during SI and in the protection from reperfusion-induced cell death, cultures were pre-treated with 1 mM L-NMMA, a wide-spectrum NOS inhibitor, for 2 h, with 100 μM carboxy-PTIO, a scavenger of NO, for 30 min, and with 100 μM vinyl-L-NIO (L-VNIO), a potent nNOS-specific inhibitor [24], for 30-60 min before the onset of SI. To elucidate whether the reduction in cellular ATP or survival of myocytes caused by the inhibition of NOS activity by either L-NMMA or L-VNIO treatment was antagonized by the application of NO, cultures were co-treated with 10 μM SNAP, an NO donor, or with 10 μM of decomposed SNAP during SI.
Chemicals

$\text{N}^G\text{-monomethyl-L-arginine (L-NMMA), S-nitroso-N-acetylpenicillamine (SNAP), carbonylcyanide 4-trifluoromethoxyphenylhydrazone (FCCP), bisbenzimide (Hoechst 33342), and propidium iodide (PI) were obtained from Sigma (St. Louis, MO). Decomposed SNAP (decSN) was prepared by incubating SNAP at room temperature for 48 h to completely liberate NO [7]. SNAP was dissolved in DMSO and the final concentration of DMSO was set at less than 0.1%. 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl3-oxide (Carboxy-PTIO) was obtained from Dojindo Lab., Inc. (Kumamoto, Japan). N5-(1-imino-3-butenyl)-L-ornithine (L-VNIO) was obtained from ALEXIS Japan (Tokyo, Japan). The other chemicals were from Wako Chem. (Tokyo, Japan).}$

Statistics

Data are represented as the mean±S.D. Inter-group comparisons were performed using the one-way analysis of variance (ANOVA) followed by a paired t-Test. Differences with a value of $P<0.05$ were considered significant.
Results

*Ischemia/reperfusion-induced death of cardiac myocytes*

We first investigated whether the exposure of cultured cardiac myocytes to simulated ischemia (SI) and 24 h reperfusion evoked cell death while changing the duration of SI to 1, 2, and 3 h (Fig. 1). Surprisingly, exposure to 2h SI resulted in the significant death of cardiac myocytes at 24 h after the start of reperfusion (Fig. 1C & E), but exposure to 3 h SI did not (Fig. 1D & E). The survival rate of cardiac myocytes when the cultures were exposed to 3 h SI was almost the same as that of controls without SI or with 1h SI. This means that as the duration of the ischemia increased, the survival rate of cardiac myocytes rose. This unexpected result raises the question as to what mechanisms are responsible for the ischemia/reperfusion-induced death of cardiac myocytes. We therefore investigated the metabolic status of cardiac myocytes during SI.

*Nitric oxide involved in the coordination of ATP supply and demand*

Exposure of cardiac myocytes to SI stress tended to decrease the cellular ATP
level at 2 h after the onset of SI (Fig. 2A & B). Interestingly, cellular ATP level at 3 h SI was increased compared with that at 2 h SI, and was almost the same concentration as that of the control before the onset of SI, suggesting that cellular ATP was strictly regulated for the close matching of supply and demand at this time. SI increased lactate output at both 2 and 3 h after the onset of SI, but there was not significant difference between the outputs at 2 and 3 h SI (Fig. 2C & D).

A previous study has suggested that production of NO represents one paracrine/autocrine mechanism coordinating energy supply and demand in tissues [14]. Thus, we investigated whether the NO produced during SI changed the metabolic status of cardiac myocytes. Treatment of the cultures with either L-NMMA (1 mM) or carboxy-PTIO (100 μM) during SI did not change the cellular ATP level at 2 h SI. However, at 3 h SI, treatment with either L-NMMA or carboxy-PTIO resulted in a significant decrease in the cellular ATP level (Fig. 2A & B). SI increased lactate output, but treatment of the cultures with either L-NMMA or carboxy-PTIO did not change the lactate level significantly (Fig. 2C & D). Thus, an NO-dependent change in the cellular ATP level was observed only at 3 h after the onset of SI.
We then confirmed that the decrease in cellular ATP on treatment of the cultures with either L-NMMA or carboxy-PTIO observed at 3 h SI was not caused by a decrease in the number of cardiac myocytes; that is, cell death. At 3 h after the onset of SI, a significant loss of cardiac myocytes was not observed (Fig. 2E), indicating that the decrease in cellular ATP on treatment of the cultures with either L-NMMA or carboxy-PTIO observed at 3 h SI was not due to myocyte death. This result also suggested that reperfusion after SI was necessary for the significant induction of cell death among myocytes, and cardiac myocytes probably adapted to this ischemic environment and survived to at least 3 h SI.

We then confirmed that NO is responsible for the coordination of ATP supply and demand at 3 h after the start of SI (Fig. 3A). Treatment of cultures with L-NMMA (1 mM) significantly decreased the cellular ATP level at 3 h SI. Co-treatment with L-NMMA (1 mM) and SNAP (10 μM) significantly antagonized the L-NMMA-induced decrease in cellular ATP, but co-treatment with decomposed SNAP did not.

*Nitric oxide and ischemia/reperfusion-induced cell death*
We next investigated whether the NO produced during ischemia was protective of cardiac myocytes when the cultures were exposed to 3 h SI and 24 h reperfusion (Fig. 3B-E). The inhibition of the nitric oxide synthase (NOS) activity with L-NMMA (1 mM) during SI resulted in a significant decrease in the survival rate of myocytes at 24 h after the start of reperfusion. The decrease in the rate of survival caused by treatment with L-NMMA during SI was significantly antagonized by co-treatment with SNAP (10 μM), an NO donor, but not with decomposed SNAP (10 μM). These results suggested that NO produced during SI protected myocytes from ischemia/reperfusion-induced death.

Cellular ATP at 3 h SI was maintained at almost the same concentration as that of the control before the onset of SI, and NO produced during SI was critically involved in this preservation of cellular ATP. An NO-dependent change in the cellular ATP level was unexpectedly observed at 3 h after the onset of SI, suggesting that the production of NO was enhanced at this time. Thus, we then investigated whether the expression of NOS isoforms (nNOS, iNOS, and eNOS) changed at 3 h after the onset of SI by conducting immunocytochemical and Western blot analyses (Fig. 4). The expression
of nNOS was most evident in cardiac myocytes, but no significant change was observed in the expression of any of the isoforms at 2 h SI or at 3 h SI (Fig. 4A-C).

**Increased production of nitric oxide during ischemia**

Therefore, we next investigated whether NO production really increased during the present protocol of simulated ischemia (SI), especially at 3 h SI. To do this, the fluorescent NO probe DAF-FM DA was used to estimate the changes in the cytosolic NO concentration of cultured cardiac myocytes (Fig. 5). DAF-FM fluorescence in myocytes gradually increased with time after the onset of SI, especially at 3 h SI (Fig. 5A & C). The fluorescent intensity at 3 h SI was significantly higher than that at 2 h SI (Fig. 5D). The increase in intracellular DAF-FM fluorescence was significantly suppressed by treating the cultures with 100 μM vinyl-L-NIO (L-VNIO), a potent specific inhibitor of nNOS [24] (Fig. 5B-D). DAF-FM fluorescence was also significantly suppressed by treatment with 1 mM L-NMMA, a non-specific inhibitor of NOS (data not shown). These results suggested that SI resulted in an increase in the production of NO, probably via the activation of nNOS, in cultured cardiac myocytes,
especially at 3 h SI. The relatively rapid time course of the increase in the DAF fluorescent intensity reflecting increased NO production during SI suggested that NO was produced by constitutive NOS (cNOS), not by iNOS [17].

**Involvement of nNOS-derived nitric oxide**

We finally investigated whether the nNOS-derived NO produced during SI was involved in the preservation of cellular ATP at 3 h SI as well as in the protection of cardiac myocytes from reperfusion-induced cell death (Fig. 6). Treatment of cultures with 100 μM L-VNIO resulted in a significant decrease in cellular ATP. Co-treatment with SNAP (10 μM) significantly antagonized the L-VNIO-induced reduction in cellular ATP, but co-treatment with decomposed SNAP (10 μM) did not (Fig. 6A). Treatment of cultures with L-VNIO also resulted in a significant decrease in the survival rate of cardiac myocytes at 24 h after reperfusion (Fig. 6B-E). Co-treatment with SNAP (10 μM) significantly antagonized the L-VNIO-induced reduction in the rate of survival of myocytes, but co-treatment with decomposed SNAP did not. These results suggested that enhanced production of nNOS-derived NO was involved in the
preservation of cellular ATP during SI, and the protection of cardiac myocytes from ischemia/reperfusion-induced death.
Discussion

The present study revealed that the increase in the production of NO during simulated ischemia (SI) was critically involved in balancing ATP supply and demand during ischemia, and in protecting cells from ischemia/reperfusion-induced injury.

Reperfusion, not simulated ischemia, induces death of cardiac myocytes

No significant death of cardiac myocytes was observed for 3 h SI as compared with control cultures without ischemic stress even when cultures were treated with either L-NMMA or carboxy-PTIO (Fig. 2), suggesting that almost all the myocytes survived during a prolonged ischemia of at least 3 h and reperfusion is necessary for the induction of myocyte death. This finding is in agreement with the report that chick embryonic cardiomyocytes exposed to a prolonged SI for 4 h exhibited only a marginal increase in cell death [25]. These findings have raised the question as to what mechanisms were responsible for the increase in cell death when cultured myocytes were treated with L-NMMA or carboxy-PTIO during SI. Co-treatment with L-NMMA and SNAP during SI increased the survival rate of myocytes at reperfusion (Fig. 3E),
suggesting that NO produced during SI rescued myocytes from reperfusion-induced death.

Either the inhibition of NOS activity or the scavenging of NO during SI for 3h resulted in the significant decrease in cellular ATP (Fig. 2). This suggests that NO produced during ischemia was involved in the preservation of cellular ATP during ischemia. Tsukube et al. demonstrated in isolated rabbit hearts that the preservation of high-energy phosphates is essential for the reduction of reperfusion-induced Ca\(^{2+}\) overload [26]. A deficiency of ATP at the onset of reperfusion may inhibit the activity of Na\(^{+}/K^{+}\)-ATPase (Na\(^{+}\)-pump), and cause Na\(^+\) to accumulate in cardiac myocytes during reperfusion [27]. A loss of Na\(^+\) homeostasis during reperfusion results in the dysfunction of secondary ionic active transporters such as the Na\(^{+}/H^{+}\) or Na\(^{+}/Ca^{2+}\) exchanger. Reversed uptake of Ca\(^{2+}\) via the Na\(^{+}/Ca^{2+}\) exchanger produces Ca\(^{2+}\) overload in myocardium upon reperfusion [28,29]. Ca\(^{2+}\) overload and a resultant fall in mitochondrial membrane potential (ΔΨ\(_m\)) may induce mitochondrial permeability transition, which is an initiating step for apoptosis and necrosis [30].
**NO is involved in the coordination of ATP supply and demand during ischemia**

The cellular ATP concentration was almost the same at 3 h SI as before the onset of SI, although it decreased at 2 h SI (Fig. 2A & B). In addition, cellular ATP at 3 h SI was significantly decreased by treatment with either L-NMMA or carboxy-PTIO during SI, but not at 2 h SI (Fig. 2A & B). Cellular ATP at 3 h SI was also decreased by treatment with vinyl-L-NLO, a potent specific inhibitor of nNOS [24] (Fig. 6). The decrease in cellular ATP at 3 h SI caused by either L-NMMA or vinyl-L-NIO was significantly antagonized by co-treatment with an NO donor SNAP, but not with decomposed SNAP (Fig. 3 & Fig. 6). These results suggested that NO produced during SI by NOS, especially by nNOS, was crucially involved in the coordination of ATP supply and demand during SI. It has been generally considered that the maintenance of the energy balance and of stable steady-state ATP concentrations in hypoxia-tolerant cells is a sign of an effective defense against anoxia or ischemia [31,32]. Thus, the cultured cardiac myocytes from neonatal rats used in this study seem to be “hypoxia tolerant”, not “hypoxia sensitive” cells, since one of the features of “hypoxia sensitive” cells is that ATP synthesis cannot meet energy requirements [2].
Cellular ATP at 3 h SI tended to increase as compared with that at 2 h SI, but lactate output remained about the same between 2 h and 3 h SI (Fig. 2). In addition, lactate output during SI was not significantly changed by treatment with either L-NMMA or carboxy-PTIO. These results suggested that anaerobic glycolysis was not enhanced markedly at 3 h SI as compared with 2 h SI, and was probably independent of the NO produced during SI. Therefore, the NO produced during SI may suppress ATP utilization in order to match the decreased ATP supply due to ischemic stress to cardiac myocytes.

The question then arises as to what mechanisms are responsible for the NO-induced suppression of ATP utilization of cardiac myocytes during ischemia. Exact mechanisms are currently unknown, but one possibility is that NO produced during SI may contribute to a down-regulation of proton leak in the mitochondria. Mitochondrial membrane potential cannot be abolished completely during anoxia since it is crucially needed to import proteins that ensure mitochondrial maintenance [33]. During normoxia, the futile cycle of mitochondrial proton pumping and proton leak across the inner mitochondrial membrane is estimated to make up about 20% of the
standard metabolic rate of mammals [34]. During anoxia or ischemia, the inverse operation of F1,F0-ATPase attempts to maintain the mitochondrial membrane potential by using ATP to translocate protons back into the intermembrane space. This means that mitochondrial F1,F0-ATPase is a ATP consumer, not a ATP producer, in the absence of oxygen [35]. Since rat cardiac myocytes contain small amounts of higher affinity F1-ATPase inhibitory subunit (IF1)[36], there is a possibility that the most effective strategy of cardiac myocytes reducing ATP utilization during ischemia is a down-regulation of proton leak in the mitochondria. Further studies will be needed to clarify this.
Acknowledgments

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**Figure Legends**

Fig. 1

Simulated ischemia/reperfusion-induced death of cultured cardiac myocytes. Figures A1, B1, C1, and D1 show phase-contrast photomicrographs of cultured cardiac myocytes at 24 h after the onset of reperfusion; sham control (A1), 1 h simulated ischemia (SI) (B1), 2 h SI (C1), and 3 h SI (D1). Figures A2, B2, C2, and D2 indicate photomicrographs of cultured cardiac myocytes stained with Hoechst 33342 (bisbenzimide) at 24 h after the onset of reperfusion; sham control (A2), 1 h SI (B2), 2 h SI (C2), and 3 h SI (D2). Figures A3, B3, C3, and D3 indicate photomicrographs of myocytes stained with propidium iodide at 24 h after the onset of reperfusion; sham control (A3), 1 h SI (B3), 2 h SI (C3), and 3 h SI (D3). Figure E shows the survival rate of cardiac myocytes. The scale bar (100 μm) is the same as in A-D. Data are expressed as the mean + SD (n= 6 different cultures). * : P< 0.05. Abbreviations: SI(1h), 1 h SI; SI(2h), 2 h SI; SI(3h), 3 h SI.
Nitric oxide produced during ischemia is crucial for the coordination of ATP supply and demand. Figures A and B show the changes of cellular ATP at 2 h and 3 h after the onset of simulated ischemia (SI) on treatment with and without 1 mM L-NMMA (A) or 100 μM carboxy-PTIO (B). The cellular ATP concentration at 3 h after the onset of SI was almost the same as the control level, and was significantly decreased by treatment with either L-NMMA (A) or carboxy-PTIO (B) during SI. Figures C and D show the changes of lactate output at 2 h and 3 h after the onset of simulated ischemia (SI) on treatment with and without 1 mM L-NMMA (C) or 100 μM carboxy-PTIO (D). Lactate output significantly increased at both 2 h and 3 h after the onset of SI, but did not change significantly on treatment with either L-NMMA (C) or carboxy-PTIO (D) during SI. Figure E shows that no significant death of myocytes was observed at 3 h after the onset of ischemia without reperfusion. The cultures were exposed to 3 h SI without or with 1 mM L-NMMA or 100 μM carboxy-PTIO. Data are expressed as the mean ± SD (n=6 different cultures except for cultures with FCCP treatment in B and D (n=4)). *: P< 0.05. Abbreviations: cont, control before the
onset of simulated ischemia (SI); SI(2h)NM, 2 h SI with L-NMMA treatment; SI(2h)PT, 2 h SI with carboxy-PTIO treatment; SI(3h)NM, 3 h SI with L-NMMA treatment; SI(3h)PT, 3 h SI with carboxy-PTIO treatment; FCCP, treatment with 1 μM FCCP for 10 min. Other abbreviations are the same as in Fig. 1.

Fig. 3

Exogenously applied NO reversed the decrease in cellular ATP caused by NOS inhibition during simulated ischemia. Figure A shows the changes in the cellular ATP level at 3 h after the onset of simulated ischemia (SI) on treatment with 1 mM L-NMMA, and co-treatment with 1 mM L-NMMA and 10 μM SNAP or 10 μM decomposed SNAP. Co-treatment of cultures with L-NMMA and SNAP significantly increased the cellular ATP level compared with treatment with L-NMMA alone, but co-treatment with decomposed SNAP did not. The cellular ATP concentration was normalized to the mean ATP concentration at 3 h after the onset of SI (control ischemia). Data are expressed as the mean ± SD (n= 6 different cultures). * : P< 0.05. Figures B-D show that exogenously applied NO reversed the increase in the
ischemia/reperfusion-induced death of cardiac myocytes caused by inhibition of NOS during simulated ischemia. Figures B1, C1, and D1 show phase-contrast photomicrographs of cultured cardiac myocytes at 24 h after the onset of reperfusion; 3 h SI with 1 mM L-NMMA treatment (B1), 3 h SI with L-NMMA and 10 μM SNAP (C1), and 3 h SI with L-NMMA and 10 μM decomposed SNAP (D1). Figures B2, C2, and D2 show photomicrographs of myocytes stained with Hoechst 33342 (bisbenzimide) at 24 h after the onset of reperfusion; 3 h SI with L-NMMA (B2), 3 h SI with L-NMMA and SNAP (C2), and 3 h SI with L-NMMA and decomposed SNAP (D2). Figures B3, C3, and D3 show photomicrographs of myocytes stained with propidium iodide at 24 h after the onset of reperfusion; 3 h SI with L-NMMA (B3), 3 h SI with L-NMMA and SNAP (C3), and 3 h SI with L-NMMA and decomposed SNAP (D3). Figure E shows the survival rate of cardiac myocytes. The scale bar (100 μm) is the same as in B-D. Data are expressed as the mean ± SD (n= 6 different cultures).

* : P< 0.05. Abbreviations: NM, treatment with L-NMMA during SI; NM&SN, co-treatment with L-NMMA and SNAP during SI; NM&decSN, co-treatment with L-NMMA and decomposed SNAP during SI.
Fig. 4

Immunocytochemical and Western blot analyses on the expression of NOS isoforms. Western blot analysis of nNOS (top), eNOS (middle), and iNOS (bottom) in the sham-treated cultures without SI, and at 2 h and 3 h after the onset of SI. Lanes 1 and 2 show the nNOS, eNOS and iNOS detected in sham-treated cultures. Lanes 3 and 4 show 2h after the SI, and lanes 5 and 6 show 3 h after the SI, respectively. Figures B and C show the quantification of nNOS (B) and eNOS (C). The expression levels are normalized with the mean expression level in the sham-treated cultures. Data are expressed as the mean + SD (n=4 different cultures). * p<0.05. An immunocytochemical analysis using anti-nNOS (E), anti-eNOS (F), and anti-iNOS (G) antibodies revealed that the expression level of the NOS isoforms showed no detectable changes after 2 h (E2, F2, G2) and 3 h SI (E3, F3, G3) as compared with that before the onset of SI (E1, F1, G1). The scale bar indicates 100 µm. Figure D shows the expression and distribution of nNOS in cardiac myocytes. The scale bar indicates 100 µm.
Fig. 5

Increased nitric oxide production during simulated ischemia. Figures A1-A4 show phase-contrast images of the culture (A1), and the changes of DAF-FM fluorescence with time during simulated ischemia (SI) at 10 (A2), 120 (A3), and 180 min (A4) after the onset of SI, respectively. Figures B1-B4 indicate phase-contrast images of the culture (A1), and the DAF fluorescence when the cultures were treated with 100 \( \mu \text{M} \) vinyl-L-NIO (L-VNIO), a potent specific inhibitor of nNOS, at 10 (B2), 120 (B3), and 180 min (B4) after the onset of SI, respectively. The DAF fluorescence during SI was markedly reduced by treatment with L-VNIO. Relative fluorescence intensity reflecting intracellular NO increases from dark blue to red through yellow. Figure C shows the changes of relative DAF fluorescence intensity with time of six myocytes with and without treatment with L-VNIO (mean + SD). The fluorescence intensity was significantly increased at 3 h SI compared with that at 2 h SI (D). At 3 h SI, the DAF fluorescence intensity was significantly reduced by treatment with L-VNIO (E). Data are expressed as the mean + SD (n= 6 cells). * : P< 0.05.
Fig. 6

Reduction in the cellular ATP concentration and survival rate of cardiac myocytes caused by inhibiting the activity of nNOS during simulated ischemia. Figure A shows changes in the cellular ATP level at 3 h after the onset of simulated ischemia (SI) on treatment with 100 μM vinyl-L-NIO (L-VNIO), a potent specific inhibitor of nNOS, and co-treatment with 100 μM L-VNIO and 10 μM SNAP or 10 μM decomposed SNAP. Co-treatment of cultures with L-VNIO and SNAP significantly increased the cellular ATP concentration as compared with treatment with L-VNIO alone, but co-treatment with decomposed SNAP did not. Cellular ATP was normalized to the average ATP concentration at 3 h after the onset of SI (control ischemia). Data are expressed as the mean ± SD (n= 4 different cultures). *: P< 0.05. Figures B1, C1, and D1 show phase-contrast photomicrographs of cultured cardiac myocytes at 24 h reperfusion after 3 h SI with 100 μM L-VNIO (B1), 24 h reperfusion after 3 h SI with 100 μM L-VNIO and 10 μM SNAP (C1), and 24 h reperfusion after 3 h SI with 100 μM L-VNIO and 10 μM decomposed SNAP (D1). Figures B2, C2, and D2 show
photomicrographs of myocytes stained with Hoechst 33342 at 24 h reperfusion after 3 h SI with L-VNIO (B2); with L-VNIO and SNAP (C2), and with L-VNIO and decomposed SNAP (D2). Figures B3, C3, and D3 show photomicrographs of myocytes stained with propidium iodide at 24 h reperfusion after 3 h SI with L-VNIO (B3); with L-VNIO and SNAP (C3), and with L-VNIO and decomposed SNAP (D3). Figure E shows the survival rate of cardiac myocytes. The scale bar (100 μm) is the same as in B-D. Data are expressed as the mean ± SD (n=6 different cultures). *: P<0.05. Abbreviations: SI, 3h SI (control ischemia); SI(VN), treatment with L-VNIO during 3 h SI; SI(VN&SN), co-treatment with L-VNIO and SNAP during 3 h SI; SI(VN&decSN), co-treatment with L-VNIO and decomposed SNAP during 3 h SI.
Fig. 2

A

ATP content

B

ATP content

C

Isocitrate output

D

Isocitrate output

E

Survival rate of myocytes (%)
Fig. 3

A

ATP content

B1  B2  B3

C1  C2  C3

D1  D2  D3

E

Survival rate of myocytes (%)

SI  NM  NM&SN  NM&decSN