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Continuous Electrochemical Monitoring of Extracellular Lactate Production from Neonatal Rat Cardiomyocytes following Myocardial Hypoxia

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ABSTRACT: Continuous monitoring of lactate production from cardiomyocytes is of great physiological and pathological importance since the level of lactate in extracellular fluid is closely associated with myocardial energy metabolism with implication in the diagnosis and therapeutics of myocardial hypoxia and ischemia. This study demonstrates an electrochemical approach to continuous monitoring of lactate production from neonatal rat cardiomyocytes following myocardial hypoxia with a dehydrogenase-based electrochemical biosensor and a negative pressure driven culture sampling. To eliminate the effect of pH variation occurring following the cardiomyocyte hypoxia on the biosensor response and to supply nicotinamide adenine dinucleotide (NAD^+) cofactor necessary for the enzymatic reaction of lactate dehydrogenase (LDH), artificial cerebrospinal fluid (aCSF) containing NAD^+ cofactor is externally perfused and mixed online with cell culture before the culture goes to the detector. The method exhibits a high selectivity against the electrochemically active species endogenously existing in the extracellular culture of cardiomyocytes and a high tolerance against the variation of pH following cardiomyocyte hypoxia. The dynamic linear range for lactate detection is from 0.20 to 10 mM (I (nA) = 25.6 C_{Lactate} (mM) + 20.1, γ = 0.996) with a detection limit of 0.16 mM (S/N = 3). The physiological level of the extracellular lactate of neonatal rat cardiomyocytes is determined to be 1.1 ± 0.1 mM (n = 3) with the cell density of about 0.5×10^3 cells/mm^2. When the cardiomyocytes are subject to hypoxia induced with anoxic reagents, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), the extracellular lactate increases to 255 ± 30.3 % (n = 3), in relative to the physiological level, following 20 min of the hypoxia. This study essentially offers a new and effective electrochemical platform for investigating energy metabolism during cardiac physiological and pathological processes.

INTRODUCTION

As the leading cause of death worldwide, coronary artery disease has drawn much attention over the last several decades because it greatly threatens human health and increases the public health burden.[1,2] Increasing evidence has demonstrated that understanding of chemical essences involved in myocardial processes, such as energy failure, anoxic depolarization, peri-infarct depolarization and oxidative stress in the early stage of myocardial ischemia, is of great physiological and pathological importance because these chemical events in the acute ischemic period essentially trigger the pathologic damage and eventually cause heart injury and finally lead to heart death.[3,4]

Lactate is the salt form of lactic acid which was first discovered in sour milk by the Swedish chemist Karl Wilhelm Scheele in 1780.[5] As the end product of glycolysis, lactate is constantly produced from pyruvate in the cytoplasm of cells during normal metabolism. As one kind of the most important cellular metabolites in glycolysis, lactate presents in small amounts during aerobic respiration, but is excessively produced following the processes of hypoxia/ischemia to supply ATP to the organisms.[6,7] Information on the dynamic production of lactate from living cardiomyocytes remains very essential for the study of myocardial energy metabolism, which is of great importance for the clinical diagnosis and therapy of the anoxia injury.

Thanks to the excellent high throughput and robust techniques such as capillary electrophoresis, microfluidics, microarrays, microelectrodes and various imaging techniques, the past two decades have witnessed a great progress in single cell analysis both in the methodological development and in the neurochemical investigations.[8-19] To understand the molecular mechanism of diseases, the information on the dynamic changes in the extracellular neurochemicals produced from a group of isolated cells (i.e., single cells) such as neurons and cardiomyocytes remains essential for the pathophysiological investigations

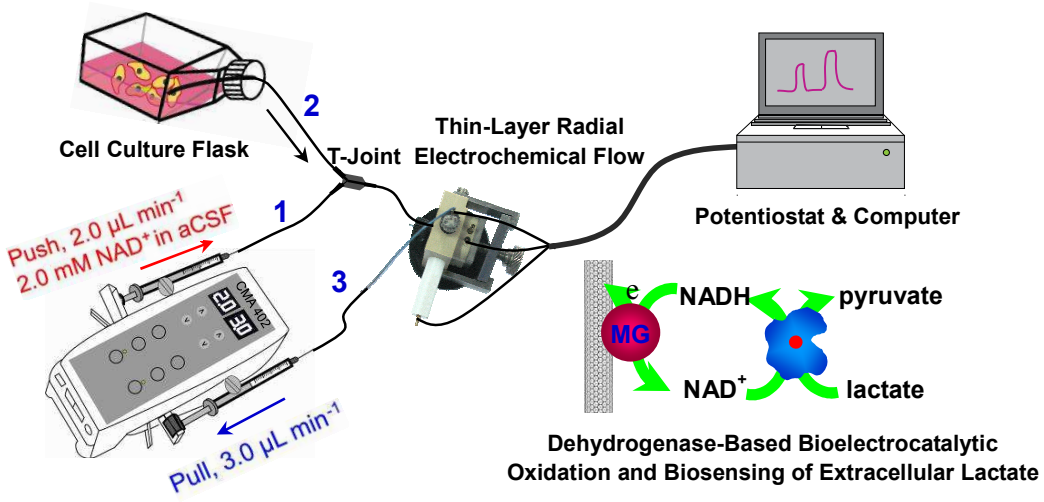
since this mode well preserves the cell's communication and regulation.[20-22] Moreover, vast production of lactate from the cells not only influences the intracellular homeostasis of a single cell, but also creates a hostile environment for the neighboring cells because exported lactate from cells by co-transporting with protons with the help of monocarboxylate transporter to the microenvironment leads to lowing of the extracellular pH.[23,24] In addition, elevated blood lactate levels in thermodynamically unstable subjects have been used as index for circulatory shock, arterial hypoxemia or both. Therefore, quantitative monitoring of extracellular lactate production from cardiomyocytes following cell hypoxia could offer straightforward information on myocardial energy metabolism, with implication in diagnosis and therapeutics of myocardial hypoxia and ischemia.

So far, some excellent methods have been developed for effectively monitoring extracellular lactate production from various cells, including cancer cells, fibroblast cells, ovary cells, and adipocytes.[25-28] While the simplicity in methodological development and experimental procedures of the lactate oxidase-based electrochemical methods make them very popular for continuous measurements of lactate, the high potentials employed for the electrooxidation of hydrogen peroxide produced from the lactate oxidase-catalyzed oxidation, unfortunately, make it difficult to apply these methods for selectively monitoring extracellular lactate production from neonatal rat cardiomyocytes following myocardial hypoxia.[29,30]

This study demonstrates an electrochemical method for continuous selective monitoring of extracellular lactate production from neonatal rat cardiomyocytes with a dehydrogenase-based electrochemical biosensor coupled with a negative pressure driven culture sampling. Dehydrogenase, rather than oxidase, is used here for lactate biosensing because, as demonstrated in our early study,[31] the less O_2 dependency and the low potentials of dehydrogenases-based biosensing schemes substantially endow the lactate biosensors with

a high selectivity against electrochemically active neurochemicals and a good tolerance against the O_2 variation occurring in response to the cell hypoxia induced by increasing N_2 supply for the cell culture medium. As shown in Scheme 1, the biosensors empolyed for extracellular lactate monitoing are prepared with lactate dehydrogenase (LDH) as the biorecognition element and methylene green (MG) confined onto single-walled carbon nanotubes (SWNTs) as the electrocatalyst for the oxidation of NADH at a relatively low potential. To avoid the influence of pH change during cardiac hypoxia and to introduce NAD^+ cofactor to the continuous electrochemical detecting system, 2.0 mM NAD^+ cofactor dissolved in aCSF was perfused into the system (Line **1**) and online mixed with the cell culture (Line **2**). The lactate biosensor is fixed into a radial thin-layer electrochemical flow cell and the cell is positioned in the downstream of a tubing sampling introduced in Line **3**. The method is selective and stable for continuous monitoring of the dynamic change in the

Scheme 1. Schematic Diagram of the Electrochemical Detecting System for Continuous Monitoring of Extracellular Lactate Production from Cardiomyocytes following FCCP-Induced Hypoxia.



lactate production form the cardiomyocytes following the myocardial hypoxia. This study essentially offers a reliable electrochemical platform for investigation of myocardial energy metabolism during myocardial hypoxia.

EXPERIMENTAL SECTION

Reagents and Solutions. Uric acid (UA), sodium ascorbate (AA), (±)-epinephrine (E), DL-noradrenaline hydrochloride (NE), D(+)-glucose, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), reduced and oxidized forms of nicotinamide adenine dinucleotide (NADH and NAD^+), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), Hanks' balanced salts, 5-bromo-2'-deoxyuridine (BrdU), L-lactic dehydrogenase (LDH, EC 1.1.1.27, type II, from rabbit muscle), lactate oxidase (LOx, EC 1.13.12.4, from *Pediococcus sp.*), collagenase (EC 3.4.24.3, type II, from *Clostridium histolyticum*) were all purchased from Sigma and used as supplied. Bovine serum albumin (BSA) was obtained from Proliant. L(+)-Lactic acid (90%) was bought from ACROS Organics. Methylene green (MG) was purchased from Beijing Chemical Company (Beijing, China). Trypsin (1:250) was obtained from Amresco. Dulbecco's modified Eagle Medium (DMEM, high glucose, 4.5 g/L) was purchased from GIBCO. Fetal bovine serum (FBS, South American Origin) was obtained from Hyclone. Single-walled carbon nanotubes (SWNTs, less than 2 nm in diameter) were purchased from Shenzhen Nanotech Port Co., Ltd. (Shenzhen, China). Prior to use, SWNTs were purified by refluxing the as-received SWNTs in 2.6 M nitric acid for 5 h, followed by centrifugation, resuspension, filtration, and air-drying to evaporate the solvent. The purified SWNTs were further heated under vacuum at 500 ^{o}C for 2 h.

Stock solutions of lactate were prepared with appropriate buffers just before use. HEPES-buffered Ringer's solution (HBRS) was prepared by mixing NaCl (125 mM), KCl

(2.6 mM), KH_2PO_4 (1.2 mM), $MgSO_4$ (1.2 mM), $CaCl_2$ (1.0 mM), and HEPES (10 mM) into doubly distilled water and was used as the perfusion solution for the continuous measurements. Artificial cerebrospinal fluid (aCSF) used to dissolve NAD^+ was prepared by mixing NaCl (126 mM), KCl (2.4 mM), KH_2PO_4 (0.5 mM), $MgCl_2$ (0.85 mM), $NaHCO_3$ (27.5 mM), Na_2SO_4 (0.5 mM), and $CaCl_2$ (1.1 mM) into doubly distilled water. Krebs−Ringer buffer (KRB) was prepared by mixing NaCl (120 mM), HEPES (20 mM), KCl (5.4 mM), NaH_2PO_4 (0.52 mM), $MgCl_2$ (3.5 mM), and $CaCl_2$ (1 mM) into doubly distilled water. The pH value of all solutions was adjusted to 7.40. Compound sodium chloride injection containing 0.85% NaCl, 0.03 % KCl, and 0.033% $CaCl_2$ was purchased from Shijiazhuang No. 4 Pharmaceutical Co., Ltd. and used as supplied. Other chemicals were of at least analytical grade and were used as received. All aqueous solutions were prepared with doubly distilled water.

Primary Culture of Cardiomyocytes and Immunofluorescence Identification. Neonatal Sprague-Dawley rats (24-48 hours old) were purchased from Health Science Center, Peking University. Neonatal rat cardiomyocytes were prepared as reported previously.[32] Briefly, cardiac tissue was cut into small pieces immediately after it was obtained from neonatal SD rats with the skin sterilized with 75 % alcohol before. After being washed twice with phosphate buffered saline (PBS, pH 7.40), the cardiac tissues were enzymatically digested with 0.1 % trypsin and 0.03 % collagenase II for 10 times, each for 4 min. Digestion was carried out at 37 ^oC in consecutive steps. Cells collected from the last eight times digestion were washed twice with DMEM containing 10 % FBS and re-suspended into DMEM containing 10 % FBS. Then, the cells were preplated in culture flasks and cultured in the medium of DMEM containing 10 % FBS at 310 K and 5% CO_2. After differential adhesion for 90 min, the suspension was collected and filtered through stainless steel sifter (bore diameter, 150 μm) and then plated onto culture flasks at a density

of 0.5×10^3 cells/mm^2 counted with traditional counting chamber (Thoma) in the medium of DMEM containing 10% FBS, 200,000 units/mL penicillin, 100,000 units/mL streptomycin and 0.10 mM BrdU (used to inhibit fibroblast proliferation) at 310 K and 5% CO_2 for at least 24 hours. After that, the medium was changed to HBRS containing 11 mM glucose and the cardiomyocytes were cultured for 1 hour prior to the measurements.

Cardiomyocytes were identified from cardiac fibroblasts by immunofluorescence, as described previously.[33,34] Briefly, the cells were firstly fixed for 25 min with 4% paraformaldehyde. The peroxidases in cells were removed with 3% H_2O_2. Then, the cells were permeabilized for 10 min with 0.1% Triton X-100. Following blocking with 5% bovine serum albumin for 60 min at 310 K, the cells were stained with primary antibody against α-sarcomeric actin (1:50, Boster Co.) at 277 K overnight, then incubated with secondary antibody, i.e. fluorescein isothiocyanate (FITC)-goat antimouse IgG (1:64, Boster Co.), at 310 K for 1 h. The cells were washed with PBS after each time of incubation.

Cardiomyocytes apoptosis was examined by fluorescence staining before and after treatment with 15 μM FCCP for 30 min. Briefly, the cardimyocytes were stained with 1 μg/mL propidium iodide (PI) in staining buffer (140 mM NaCl, 5 mM $CaCl_2$, 10 mM HEPES) for 15 min at room temperature. The cardiomyocytes were thereafter imaged under a fluorescence microscope.

A Leica inverted microscope (DMI 6000) equipped with a digital monochrome camera (Leica DFC 350 FX) with software provided by Leica (LAS AF) was used to visualize the cells. Phase contrast and fluorescence micrographs were obtained with a 10× objective (N PLAN).

Continuous Monitoring of Lactate Production from Hypoxic Cardiomyocytes. Electrochemical monitoring of lactate production from cardiomyocytes was performed with the LDH/MG/SWNT-based biosensor integrated into a continuous-flow electrochemical cell

(Scheme 1). Glassy carbon (GC, 6-mm-diameter) electrodes used in the thin-layer radial electrochemical flow cell for continuous electrochemical measurements were purchased from Bioanalytical Systems Inc. (BAS, West Lafayette, IN). GC electrodes were polished first with emery paper and then with aqueous slurries of fine alumina powder (0.3 and 0.05 μm) on a polishing cloth. The electrodes were finally rinsed with acetone and doubly distilled water under an ultrasonic bath, each for 5 min. The thin-layer radial electrochemical flow cell consists of a thin-layer radial flow block with a 50-μm gasket, GC electrode as working electrode, stainless steel as auxiliary electrode, and Ag/AgCl electrode as reference electrode.

MG/SWNT-modified electrodes were prepared as reported in our early study.[35] A 5 μL of the prepared MG-SWNT suspension (1 mg mL^{-1}) was dip-coated onto GC electrodes to form MG/SWNT-modified GC electrodes. To immobilize LDH onto the MG/SWNT-modified GC electrodes, 6 μL of LDH crystalline suspension was mixed with 3 μL of 1 % BSA aqueous solution, and 3 μL of 1 % glutaraldehyde. The resulting mixture was totally coated onto the MG/SWNT-modified GC electrodes to form LDH/MG/SWNT-modified GC electrodes. The electrodes were rinsed with distilled water and dried at ambient temperature and then fixed into the thin-layer radial electrochemical flow cell for the measurements of lactate production from the cardiomyocytes.

Continuous monitoring of lactate production from cardiomyocytes following hypoxia induced by anoxic reagents (i.e., FCCP) was performed with the LDH/MG/SWNT-based biosensor integrated into a continuous-flow electrochemical cell coupled with a negative pressure driven culture sampling (Scheme 1). A syringe pump (CMA 402, CMA Microdialysis AB, Stockholm, Sweden) with two channels, separately responsible for pushing or pulling the solutions, were used. The pushing channel with a gas-impermeable syringe (i.e., Line **1**) was used to perfuse 2.0 mM NAD^+ cofactor dissolved in aCSF at a flow

rate of 2.0 μL/min, while the pulling channel with the other gas-impermeable syringe connected with the outlet of the thin-layer radial electrochemical flow cell (Line **3**) was used to introduce both the NAD^+ solution (Line **1**) and the culture from the cell culture flask sampled with the tetrafluoroethylene hexafluoropropene (FEP) tubing (0.12 mm inner diamater, CMA Microdialysis AB, Stockholm, Sweden) (Line **2**), with a flow rate of 3.0 μL/min. The NAD^+ cofactor (Line **1**) and the cell culture (Line **2**) were mixed thoroughly in a T-joint and the mixture was finally introduced into the radial electrochemical flow cell. The length of the tubing from flask to T-joint (Line 2), from syringe to T-joint (Line 1), and from T-joint to the electrochemical cell was 4 cm, 2 cm and 2 cm, respectively. The LDH-based electrochemical biosensor was polarized at 0.0 V for continuous electrochemical monitoring of extracellular lactate production from the hypoxic cardiomyocytes. To ensure the air-tightness of the system, the joints among the tubes were sealed with parafilm and the tubes were ultrasonic cleaned thoroughly. The flow rate for FEP tubing sampling of the cell culture was verified to be 1.0 μL/min by calculating the average weight change of the cell culture solution per minute as the system ran for 15 min. After the culture was introduced into the flow cell to equilibrate for at least 60 min, 15 μM FCCP was mixed into the culture (3.0 mL). Continuous monitoring of lactate in the culture was carried out concomitantly following the FCCP addition. The cells morphology and beating rate were visualized with the inverted microscope.

RESULTS AND DISCUSSION

Continuous Measurements of Lactate. In our earlier report,[31] we developed an electrochemical strategy for online and continuous monitoring of lactate in the rat brain through integrating in vivo microdialysis with online selective electrochemical detection. While this method was very responsive toward cerebral lactate with a high selectivity toward

the species endogenously existing in the rat brain and with a good tolerance against the variation of brain O_2 and pH induced by cerebral ischemia, the validation of such an electrochemical mechanism for continuously monitoring the extracellular lactate production from the living cardiomyocytes actually necessitates serial investigations, for example, on the response of the electrochemical biosensor for lactate in the cell culture and on the interference of the substances in the cardiac system towards the biosensor, due to the difference between cerebral systems and cell culturing media. Considering the composition difference between aCSF and cell cultures, of which the former solution has been normally used to simulate the extracellular fluid (ECF) of the cerebral system, we first investigated the electrochemical response of the LDH-based biosensor toward lactate in different kinds of cell cultures. Interestingly, we found that the biosensor shows very poor responses toward lactate in FBS, KRB and compound sodium chloride injection, which might be due to the enzyme (i.e., LDH) fragileness in these buffers. Whereas, a good response was obtained at the biosensor toward lactate when HBRS was used as the cell culture and aCSF was used as the solution to dissolve NAD^+ cofactor, as depicted in Figure 1. With the electrochemical detecting system displayed in Scheme 1, a well-defined amperometric response toward lactate in HBRS was obtained with the current increases as increasing the lactate concentration. The current was linear with the concentrations of lactate (inset, Figure 1), within a dynamic linear range from 0.20 to 10 mM (I (nA) = 25.6$C_{lactate}$ (mM) +20.1, γ =0.996). The detection limit, based on a signal-to-noise ratio of 3, was calculated to be 0.16 mM. The stability and reproducibility of the electrochemical method for continuous lactate measurements were studied with standard solutions of lactate. With aCSF containing 2.0 mM NAD^+ perfused from Line **1** and lactate standard in HBRS (2.0 mM) introduced from Line **2**, the system produces a good current response for lactate in the continuous-flow system that was quite stable for at least 2 h, as typically displayed in Figure 2 A. Further, the

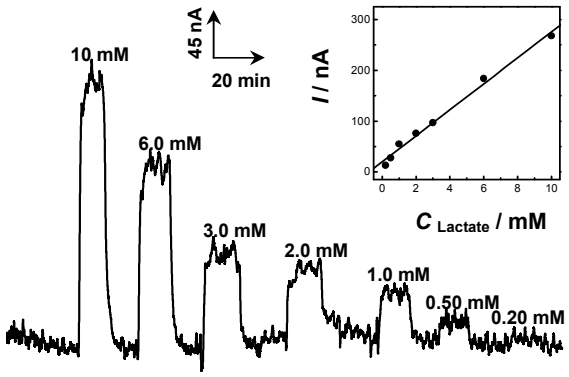


Figure 1. Typical amperometric current-time responses obtained with the electrochemical detecting system with the LDH/MG/SWNT-based biosensor as the detector. Lactate standards dissolved in HBRS were introduced from Line **2** (1.0 μL/min) and online mixed aCSF containing 2.0 mM NAD^+ perfused from Line **1** (2.0 μL/min). The resulting mixtures were introduced through Line **3** (3.0 μL/min) into the electrochemical cell for the detection (Scheme 1). The concentrations of lactate were indicated in the figure. Potential applied, 0.0 V. Inset, calibration plot of the current versus the lactate concentration. The noise in the current response was from the pumping system.

electrochemical detecting system was also stable for discontinuously biosensing lactate; the current responses for lactate were almost maintained for lactate (2.0 mM) for at least 8 days with continuous monitoring of lactate for at least 2 h each day. In addition, the analytical system was quite reproducible for the repeated measurements of lactate, as typically displayed in Figure 2 B. The relative standard deviation of the repeated measurements of lactate (2.0 mM) was 1.53% (n = 5). The good stability and high reproducibility of the as-established electrochemical method actually validate its application for continuous monitoring extracellular lactate production from cardiomyocytes, as will be described later.

Interference. Very similar to those used for continuously monitoring physiologically important species in the cerebral systems,[36-45] the electrochemical method developed by

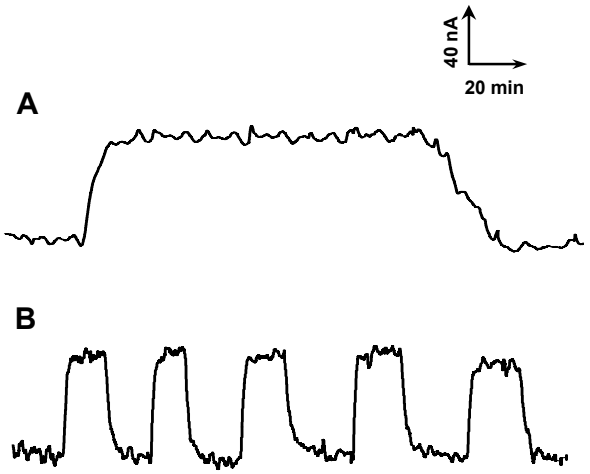


Figure 2. Typical amperometric current-time responses continuously (A) and repeatedly (B) recorded for 2.0 mM lactate in HBRS with the electrochemical detecting system with the LDH-based biosensor as the detector. Other conditions were the same as those in Figure 1.

efficiently integrating online selective detection with cell culture sampling for continuously monitoring chemical events in the cultured cells may also bear advantages over other kinds of electrochemical methods for discontinuous electrochemical measurements in terms of the simplification of the operation mechanism and procedures, maintenance of sample freshness, improved temporal resolution and, as such, physiological and pathological usefulness. However, the abrogation of sample collection and separation actually requires the electrochemical detecting system to have a high selectivity. As documented in the previous studies,[46-48] a variety of electrochemically active species, such as UA, E, NE and AA are endogenously coexisting in the cardiac systems and are also expected to be present in the culture of cardiomyocytes. These species could be readily electrochemically oxidized at the LDH-based biosensors and may thus potentially interfere with the monitoring of extracellular lactate production in this study. This is because both the redox dye (i.e., MG) and carbon nanostructure (i.e., SWNTs) used as the electronic transducers for the lactate

biosensor exhibit excellent electrocatalytic activities toward the oxidation of some kinds of small biological molecules, as reported previously.[32,41,42,49,50]

As our first endeavor, we investigated the current responses of these species with the electrochemical detecting system simply by introducing the standard solutions of these species in HBRS into the electrochemical flow cell from Line **2**. We found that the introducing of 10 μM UA, 10 μM E, 10 μM NE or 5 μM AA into the system did not produce a recordable current response (Figure 3 A), as compared with that for lactate, indicating that these species do not interfere with the electrochemical monitoring of the extracellular lactate production from cardiomyocytes under the conditions employed in this study.

To further investigate the possible interference from the electrochemically active species endogenously existing in the cell culture, the selectivity of this method was investigated in vivo by adding 2 U lactate oxidase (LOx, 0.25 mg mL^{-1}) into the culture of cardiomyocytes and the culture was sampled from Line **2** for subsequent measurements with the electrochemical system (Scheme 1). LOx specifically catalyzes the chemical oxidation of lactate in the presence of dissolved O_2 and the addition of such a kind of oxidase is anticipated to deplete lactate in the cell culture. The addition of LOx into the cell culture leads to the decrease in the current response to the background level, i.e., the level before switching to the cell culture from HBRS containing 11.0 mM glucose (Figure 3 B). This result demonstrates that the endogenous species existing in cardiomyocytes could not be electrochemically oxidized or reduced at the lactate biosensor under the present conditions. Since the oxidation of lactate under the catalysis of LOx-catalyzed produces H_2O_2, the result also suggests that the electrochemical method bears a good selectivity against H_2O_2. To further investigate the selectivity of the electrochemical detecting system against the species produced following cardiac hypoxia, such as reactive oxygen species (ROS), xanthine, hypoxanthine, and malondialdehyde (a marker for oxidative stress), [51,52] 2 U LOx was added

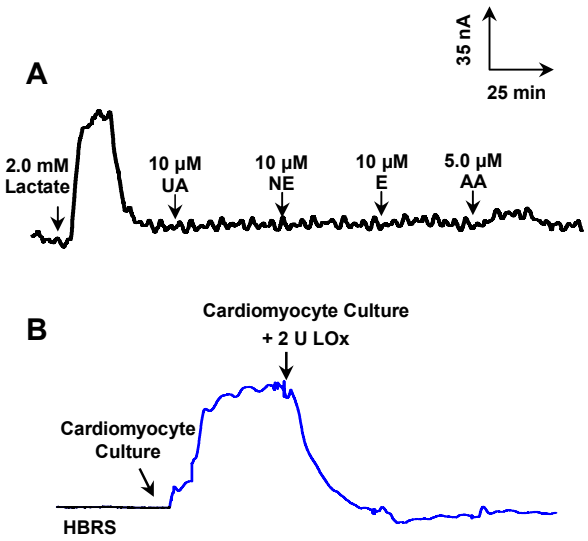


Figure 3. Amperometric current-time responses with the electrochemical detecting system for the standards of lactate, UA, NE, E and AA in HBRS containing 11 mM glucose (A) and for the cardiomyocyte culture (B). The concentrations of the species were shown in the A. In Figure B, the current responses were recorded first in HBRS containing 11 mM glucose without cardiomyocytes, then in the HBRS containing 11 mM glucose with the presence of cardiomyocytes for 35 min, and finally in the HBRS containing 11 mM glucose in the presence of cardiomyocytes with the addition of 2 U lactate oxidase. Other conditions were the same as those in Figure 1.

into the anoxic cardiomyocytes medium. The current response almost restored to the basal level when added 2 U LOx in the hypoxia cell medium (data not shown), illustrating that the observed current increase was due to the increase in the lactate level. In the other words, the substances produced in the anoxic process of the cardiomyocytes do not influence the electrochemical detecting system for lactate production. Moreover, consistent with our earlier report,[31] the electrochemical detecting system for lactate with LDH as recognition elements and with methylene green (MG) as the electrocatalyst for the oxidation of NADH was found to bear a good tolerance against the variation of pH occurred in response to the hypoxia (data not shown). These demonstrations substantially demonstrate that the

electrochemical detecting system developed in this study is very selective for continuous monitoring the dynamic lactate production during cardiomyocytes hypoxia.

Lactate Production from Hypoxic Cardiomyocytes. Prior to the lactate measurements, the cells cultured were identified with contrast and fluorescent images. Generally, the cardiomyocytes characteristically express α-sarcomeric actin, and they show green fluorescence after immunofluorescent assay of α-sarcomeric actin, while the fibroblasts show no fluorescence.[33,34] As typically displayed in Figure 4, all the cells cultured in this study show green fluorescence, demonstrating that the cells are cardiomyocytes, rather than fibroblasts. At the native state, cadiomyocytes with pseudopodiums connect with each other. The cells adhere onto the culture flask and are beating at a frequency of 50-80 times per minute. As a model for cellular anoxia, in this study cardiomyocytes were subject to the treatment of FCCP added into the culture. FCCP simulates the anaerobic conditions by inhibiting oxidative phosphorylation by uncoupling the mitochondrial respiratory chain and promoting hydrolysis of ATP.[53-55] In independent experiments, we found that the addition of

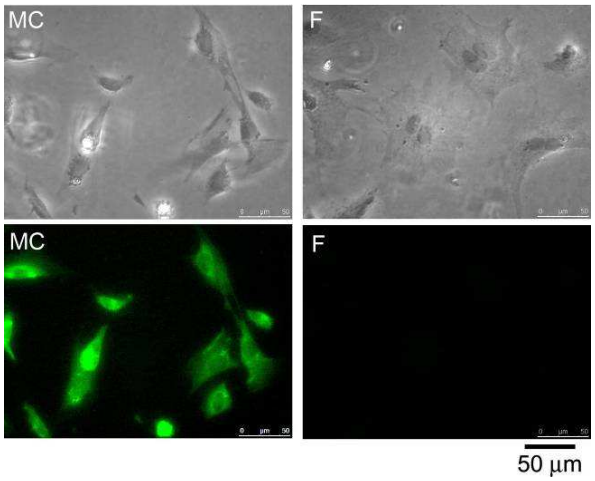


Figure 4. Identification of cardiomyocytes with the immunofluorescent assay of α-sarcomeric actin. Left: Contrast (upper) and fluorescent (lower) images of cardiomyocytes; Right: Contrast (upper) and fluorescent (lower) images of cardiac fibroblasts.

15 μM FCCP results in immediate stop of cell beating with little cell morphology change. This demonstrates that 15 μM FCCP is enough to induce hypoxia of cardiomyocytes adhered onto the plate.

On the other hand, since the anoxic reagents (i.e., FCCP) used in this study was dissolved into DMSO, rather than aqueous buffer, the effect of FCCP and its solvent (i.e., DMSO) on the current response of the LDH-based biosensor toward lactate was thus investigated. For such a purpose, 4.0 μL DMSO and 15 μM FCCP (dissolved in 4.0 μL DMSO) were independently added into the cell culture (i.e., HBRS) without the cardiomyocytes. The resulting mixtures were introduced from Line **2** and online mixed with NAD^+ solution from Line **1** and were finally detected in the electrochemical cell. As displayed in Figure 5 A, the addition of 4.0 μL DMSO or 15 μM FCCP (dissolved in 4.0 μL DMSO) into 1.5 mL cell culture did not produce a recordable current response, as compared with that of 1.0 mM lactate. Further, as observed with the confocal microscope, the addition of such a small volume of DMSO has little influence to the cells with respects to the cell morphology and contracting frequency (data not shown). These control experiments further validate the use of FCCP as the anoxic reagent to induce the cardiomyocytes hypoxia. Moreover, the use of FCCP to establish the anoxic model of cardiomyocytes made the electrochemical detecting system simpler and more controllable, as compared with the anoxic model induced by increasing N_2 supply for the cell culture medium.

Figure 5 B displays typical amperometric responses for the cell culture continuously sampled following the cardiomyocyte hypoxia induced by FCCP. Based on the demonstration mentioned above, the current responses were ascribed to extracellular lactate production from the cardiomyocytes into the cell culture. The physiological level of the extracellular lactate was determined to be 1.1 ± 0.1 mM (n = 3) with the cell density of about 0.5×10^3 cells/mm^2. When the cardiomyocytes were treated with 15 μM FCCP, the

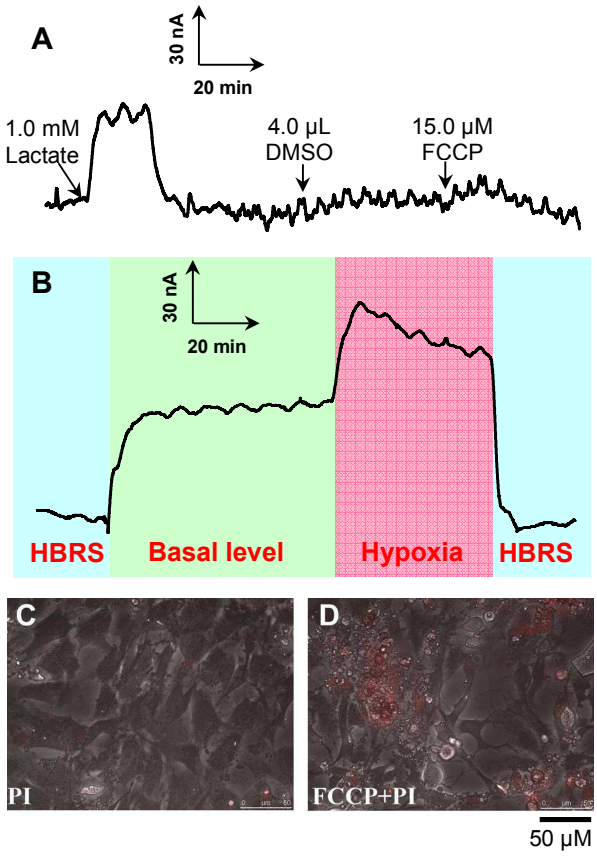


Figure 5. (A) Amperometric current-time responses recorded in HBRS without cardiomyocytes. Lactate standard, 4.0 µL DMSO, and 15.0 µM FCCP dissolved in 1.0 mL HBRS were introduced from Line **2** (1.0 μL/min) and online mixed aCSF containing 2.0 mM NAD^+ perfused from Line **1** (2.0 μL/min). (B) Amperometric current-time responses recorded for the cell culture continuously sampled from cell flask with cardiomyocytes following the hypoxia induced by 15.0 μM FCCP. For convenient comparison, the tubing of Line 2 was switched to HBRS buffer before and after the measurements in the cell culture. Other conditions were the same as those in Figure 1. (C) Fluorescent images of cardiomyocytes treated by propidium iodide (PI) to determine cell apoptosis. (D) Fluorescent images of cardiomyocytes treated by 15 μM FCCP for 30 min plus PI.

amperometric response increased immediately. According to Figures 1 and 5, the extracellular lactate was calculated to increase to 255 ± 30.3 % (n = 3) of the physiological level following the 20 min of the FCCP-induced hypoxia. In the previous study, the level of

lactate was increased to 3 times of the basal level when a single cardiomyocyte was treated with FCCP and saponine,[53] which was almost consistent with our study. By comparing Figure 5 C with D, we could see that the treatment of cardiomyocytes with 15 μM FCCP for 30 min led to cell apoptosis with loss of their three dimensional structures and increase of cell permeabilization, which could be evident by red fluorescence produced by the reaction between DNA with PI (Figure 5 D). The observed change in the level of lactate might be understood in terms of the increased demand of metabolism consumption, increased glycolysis, diminished respiration, increased cell permeabilization and so forth, following the cardiomyocytes hypoxia induced by FCCP that inhibits oxidative phosphorylation by uncoupling the mitochondrial respiratory chain and promotes hydrolysis of ATP, as discussed earlier.[53-55] This study substantially suggests that the electrochemical detecting system demonstrates here could be used as a platform to effectively probe the energy metabolism related to lactate change in the cell hypoxia process.

CONCLUSIONS

By efficiently integrating a simple tubing sampling and selective electrochemical detection, we have successfully developed a new and effective electrochemical method for continuously monitoring lactate production from neonatal rat cardiomyocytes following myocardial hypoxia induced with anoxic reagents, FCCP. In addition to the good linearity and stability and high selectivity against the electrochemically active species both endogenously existing in the heart systems and produced following hypoxia process, the uses of dehydrogenases as the recognition elements in the biosensors and the externally perfused solution to minimize the variation of pH substantially endow the as-developed continuous electrochemical method with a good physiological relevance, bearing a high tolerance to the variation of the extracellular pH occurring in response to the cardiac hypoxia.

The method demonstrated here is reliable and durable and could be used as a new and effective electrochemical platform for the investigations on the energy metabolism during cardiac physiological and pathological processes.

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REFERENCES

(1) Couzin-Frankel, J. *Science* **2010**, *328*, 1220-1221.

(2) Harismendy, O.; Notani, D.; Song, X.; Rahim, N. G.; Tanasa, B.; Heintzman, N.; Ren, B.; Fu, X.-D.; Topol, E. J.; Rosenfeld, M. G.; Frazer, K. A. *Nature* **2011**, *470*, 264-268.

(3) Linsel-Nitschke, P.; Samani, N. J.; Schunkert, H. *N. Engl. J. Med.* **2010**, *363*, 2462-2463.

(4) Martinelli, N.; Girelli, D.; Lunghi, B.; Pinotti, M.; Marchetti, G.; Malerba, G.; Pignatti, P. F.; Corrocher, R.; Olivieri, O.; Bernardi, F. *Blood* **2010**, *116*, 5688-5697.

(5) Scheele, K. W. *Opuscula chemica et physica*; Müller: Leipzig, 1789.

(6) Suzuki, A.; Stern, S. A.; Bozdagi, O.; Huntley, G. W.; Walker, R. H.; Magistretti, P. J.; Alberini, C. M. *Cell* **2011**, *144*, 810-823.

(7) Richardson, A. R.; Libby, S. J.; Fang, F. C. *Science* **2008**, *319*, 1672-1676.

(8) Fan, Y.; Rubakhin, S. S.; Sweedler, J. V. *Anal. Chem.* **2011**, *83*, 9557-9563.

(9) Rubakhin, S. S.; Romanova, E. V.; Nemes, P.; Sweedler, J. V. *Nat. Methods* **2011**, *8*, S20-S29.

(10) Lorenz, M. A.; Burant, C. F.; Kennedy, R. T. *Anal. Chem.* **2011**, *83*, 3406-3414.

(11) Slaney, T. R.; Nie, J.; Hershey, N. D.; Thwar, P. K.; Linderman, J.; Burns, M. A.; Kennedy, R. T. *Anal. Chem.* **2011**, *83*, 5207-5213.

(12) Lin, Y.; Trouillon, R.; Safina, G.; Ewing, A. G. *Anal. Chem.* **2011**, *83*, 4369-4392.

(13) Xie, Y.; Zhang, W.; Wang, L.; Sun, K.; Sun, Y.; Jiang, X. *Lab Chip.* **2011**, *11*, 2819-2822.

(14) Yuan, B.; Li, Y.; Wang, D.; Xie, Y.; Liu, Y.; Cui, L.; Tu, F.; Li, H.; Ji, H.; Zhang, W.; Jiang, X. *Adv. Funct. Mater.* **2010**, *20*, 3715-3720.

(15) Liu, D.; Xie, Y.; Shao, H.; Jiang, X. *Angew. Chem. Int. Ed.* **2009**, *48*, 4406-4408.

(16) Takahashi, Y.; Shiku, H.; Murata, T.; Yasukawa, T.; Matsue, T. *Anal. Chem.* **2009**, *81*, 9674-9681.

(17) Takahashi, Y.; Miyamoto, T.; Shiku, H.; Asano, R.; Yasukawa, T.; Kumagai, I.; Matsue, T. *Anal. Chem.* **2009**, *81*, 2785-2790.

(18) Lin, Z.; Takahashi, Y.; Murata, T.; Takeda, Michiaki.; Ino, K.; Shiku, H.; Matsue, T. *Angew. Chem. Int. Ed.* **2009**, *48*, 2044-2046.

(19) Ueda, A.; Niwa, O.; Maruyama, K.; Shindo, Y.; Oka, K.; Suzuki, K. *Angew. Chem. Int. Ed.* **2007**, *46*, 8238-8241.

(20) Terauchi, A.; Johnson-Venkatesh, E. M.; Toth, A. B.; Javed, D.; Sutton, M. A.; Umemori, H. *Nature* **2010**, *465*, 783-787.

(21) Miyamichi, K.; Luo, L. *Science* **2009**, *325*, 544-545.

(22) Tirziu, D.; Giordano, F. J.; Simons, M. *Circulation* **2010**, *122*, 928-937.

(23) Becker, H. M.; Deitmer, J. W. *J. Biol. Chem.* **2008**, *283*, 21655-21667.

(24) Le Floch, R.; Chiche, J.; Marchiq, I.; Naiken, T.; Ilk, K.; Murray, C. M.; Critchlow, S. E.; Roux, D.; Simon, M.-P.; Pouyssegur, J. *P. Natl. Acad. Sci. USA* **2011**, *108*, 16663-16668.

(25) Zheng, X. T.; Yang, H. B.; Li, C. M. *Anal. Chem.* **2010**, *82*, 5082-5087.

(26) Eklund, S. E.; Taylor, D.; Kozlov, E.; Prokop, A.; Cliffel, D. E. *Anal. Chem.* **2004**, *76*, 519-527.

(27) Ciobanu, M.; Taylor, D. E.; Wilburn, J. P.; Cliffel, D. E. *Anal. Chem.* **2008**, *80*, 2717-2727.

(28) Bairras, C.; Ferrand, C.; Atgie, C. *J. Physiol. Biochem.* **2003**, *59*, 161-167.

(29) Goran, J. M.; Lyon, J. L.; Stevenson, K. J. *Anal. Chem.* **2011**, *83*, 8123-8129.

(30) Yashina, E. I.; Borisova, A. V.; Karyakina, E. E.; Shchegolikhina, O. I.; Vagin, M. Y.; Sakharov, D. A.; Tonevitsky, A. G.; Karyakin, A. A. *Anal. Chem.* **2010**, *82*, 1601-1604.

(31) Lin, Y.; Zhu, N.; Yu, P.; Su, L.; Mao, L. *Anal. Chem.* **2009**, *81*, 2067-2074.

(32) Luo, D. ; Yang, D. ; Lan, X. ; Li, K.; Li, X.; Chen, J.; Zhang, Y.; Xiao, R.; Han, Q.; Cheng, H. *Cell Calcium* **2008**, *43*, 165-174.

(33) Inya-Agha, O.; Klauke, N.; Davies, T.; Smith, G.; Cooper, J. M. *Anal. Chem.* **2007**, *79*, 4581-4587.

(34) Sartoretto, J. L.; Kalwa, H.; Pluth, M. D.; Lippard, S. J.; Michel, T. *P. Natl. Acad. Sci. USA* **2011**, *108*, 15792-15797.

(35) Li, X.; Zhang, L.; Su, L.; Ohsaka, T.; Mao, L. *Fuel Cells* **2009**, *9*, 85-91.

(36) Zhang, M.; Yu, P.; Mao, L. *Acc. Chem. Res*. **2012**, *45*, 533-543.

(37) Zhang, Z.; Zhao, L.; Lin, Y.; Yu, P.; Mao, L. *Anal. Chem.* **2010**, *82*, 9885-9891.

(38) Li, X.; Zheng, W.; Zhang, L.; Yu, P.; Lin, Y.; Su, L.; Mao, L. *Anal. Chem.* **2009**, *81*, 8557-8563.

(39) Xiang, L.; Zhang, Z.; Yu, P.; Zhang, J.; Su, L.; Ohsaka, T.; Mao, L. *Anal. Chem.* **2008**, *80*, 6587-6593.

(40) Lin, Y.; Liu, K.; Yu, P.; Xiang, L.; Li, X.; Mao, L. *Anal. Chem.* **2007**, *79*, 9577-9583.

(41) Zhang, M.; Liu, K.; Xiang, L.; Lin, Y.; Su, L.; Mao, L. *Anal. Chem.* **2007**, *79*, 6559-6565.

(42) Zhang, M.; Liu, K.; Gong, K.; Su, L.; Chen, Y.; Mao, L. *Anal. Chem.* **2005**, *77*, 6234-6242.

(43) You, T. Y.; Niwa, O.; Tomita, M.; Hirono, S. *Anal. Chem.* **2003**, *75*, 2080-2085.

(44) Kurita, R.; Arai, K.; Nakamoto, K.; Kato, D.; Niwa, O. *Anal. Chem.* **2010**, *82*, 1692-1697.

(45) Yoshioka, K.; Sato, Y.; Murakami, T.; Tanaka, M.; Niwa, O. *Anal. Chem.* **2010**, *82*, 1175-1178.

(46) Holme, I.; Aastveit, A. H.; Hammar, N.; Jungner, I.; Walldius, G. *J. Intern. Med.* **2009**, *266*, 558-570.

(47) Basu, P.; Som, S.; Choudhuri, N.; Das, H. *Eur. J. Pediatr.* **2009**, *168*, 833-838.

(48) Kawada, T.; Yamazaki, T.; Akiyama, T.; Sato, T.; Shishido, T.; Inagaki, M.; Tatewaki, T.; Yanagiya, Y.; Sugimachi, M.; Sunagawa, K. *J. Auton. Nerv. Syst.* **2000**, *80*, 137-141.

(49) Yan, J.; Zhou, H.; Yu, P.; Su, L.; Mao, L. *Adv. Mater.* **2008**, *20*, 2899-2906.

(50) Li, X.; Zhou, H.; Yu, P.; Su, L.; Ohsaka, T.; Mao, L. *Electrochem. Commun.* **2008**, *10*, 851-854.

(51) King, N.; McGiven, J. D.; Griffiths, E. J.; Halestrap, A. P.; Suleiman, M. S. *J. Mol. Cell. Cardiol.* **2003**, *35*, 975-984.

(52) Chen, H. W.; Chien, C. T.; Yu, S. L.; Lee, Y. T.; Chen, W. J. *Br. J. Pharmacol.* **2002**, *137*, 771-781.

(53) Cai, X. X.; Klauke, N.; Glidle, A.; Cobbold, P.; Smith, G. L.; Cooper, J. M. *Anal. Chem.* **2002**, *74*, 908-914.

(54) Brennan, J. P.; Southworth, R.; Medina, R. A.; Davidson, S. M.; Duchen, M. R.; Shattock, M. J. *Cardiovasc. Res.* **2006**, *72*, 313-321.

(55) Fukumoto, G. H.; Lamp, S. T.; Motter, C.; Bridge, J. H. B.; Garfinkel, A.; Goldhaber, J. I. *Circ. Res.* **2005**, *96*, 551-557.

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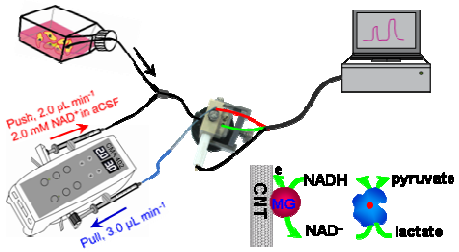
Push, 2.0 μL min-1
2.0 mM NAD+ in aCSF
Pull, 3.0 μL min-1
CNT
MG
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