Optical mapping of myocardial reactive oxygen species production throughout the reperfusion of global ischemia

Long-Sheng Lu
National Taiwan University
Graduate Institute of Pharmacology
Taipei, Taiwan

Yen-Bin Liu
National Taiwan University Hospital
Department of Internal Medicine
Taipei, Taiwan

Chia-Wei Sun
Industrial Technology and Research Institute
Biomedical Engineering Center
Hsinchu, Taiwan

Lung-Chun Lin
National Taiwan University Hospital
Department of Internal Medicine
Taipei, Taiwan

Ming-Jai Su
National Taiwan University
Graduate Institute of Pharmacology
Taipei, Taiwan

Chau-Chung Wu
Eda Hospital
Department of Internal Medicine
Kaohsiung, Taiwan
and
National Taiwan University
College of Medicine
Departments of Primary Care Medicine
and Internal Medicine
Taipei, Taiwan
E-mail: cwu@ha.mc.ntu.edu.tw

Abstract. Reactive oxygen species (ROS) are short-lived, highly reactive chemical entities that play significant roles in all levels of biology. However, their measurement requires destructive preparation, thereby limiting the continuous measurement of ROS in a living tissue. We develop an optical mapping system to visualize ROS production in an isolated and perfused rat heart. By staining the heart with dihydroethidium (DHE), a 532-nm laser beam is directed to the epicardial surface, where we collect the red fluorescence (>600 nm) for semiquantitative analysis. With this system, ROS production as well as ventricular pressure and ECG in isolated perfused rat hearts are monitored throughout the reperfusion of global ischemia. Ischemia would decrease myocardial ROS production, while reperfusion would immediately result in sustained ROS overproduction. Optical mapping would provide information regarding the spatial distribution and temporal evolution of myocardial ROS production, which would enhance knowledge of the role of free radicals in cardiovascular biology. [DOI: 10.1117/1.2186321]

Keywords: reactive oxygen species; optical mapping; reperfusion; global ischemia.

Paper 05169SSRR received Jul. 1, 2005; revised manuscript received Nov. 26, 2005; accepted for publication Dec. 1, 2005; published online Mar. 24, 2006.

1 Introduction

The heart is an energy-demanding organ. Experimental estimation of myocardial mechanical power ranged between 0.1 to 3 W from rodents to humans.[1,2] The delicacy of energy balance of the heart relies heavily on adequate perfusion via the coronary arteries. Insufficient oxygen supply, as in the case of coronary artery disease, could result in a 34% increase of myocardial waste heat production and 14% loss of mechanical efficiency.[3] On the other hand, oxygen surge can also result in excessive reactive oxygen species (ROS) production, with myocardial damage, as in the case of reperfusion after prolonged ischemia. As ROS might modify biologically important lipids and proteins, reperfusion injury manifests as impaired mechanical performance, ventricular arrhythmia, and permanent myocardial damage. However, the highly reactive nature of ROS makes direct observation of their kinetics at the tissue level very difficult.

Recently, optical imaging methods became attractive techniques for myocardium characterization. In such techniques, the light-tissue interactions are measured with a light source, including photon scattering,[4] absorption,[5] polarization,[6] coherence,[7] and fluorescence.[8–10] Fluorescence measurements could be applied to indicate many physiologic parameters in

Address all correspondence to Chau Chung Wu, National Taiwan University, Department of Internal Medicine, No. 7 Chung-Shan South Road, Taipei, Taiwan 108 Taiwan. Tel: 888–2–23123456#5408; Fax: 886–2–23914853; E-mail: cwu@ha.mc.ntu.edu.tw

1083-3668/2006/11(2)/021012/7/$22.00 © 2006 SPIE

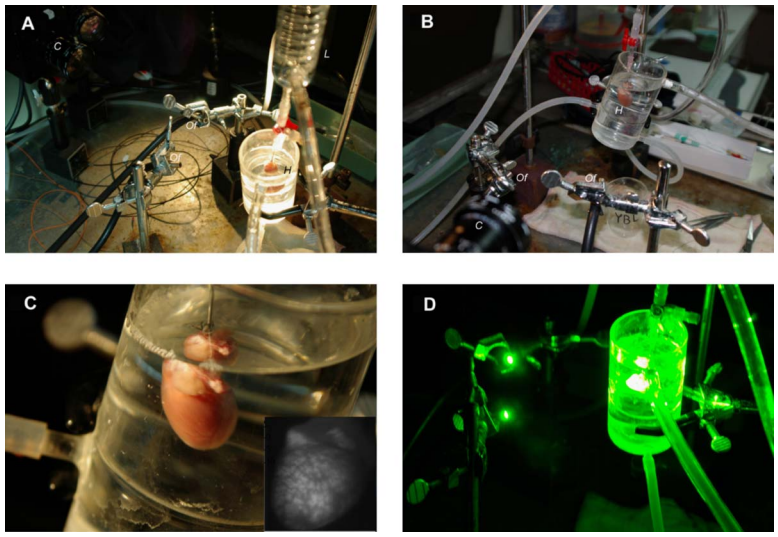

Fig. 1 Setup of optical mapping system. (a) Side and (b) front views of the optical mapping system, consisting of a high-speed CCD camera with a 600-nm long-pass filter (C), a pair of optical fibers to deliver laser (Of), and heart (H) mounted on a Langendorf perfusion apparatus (L). The left ventricle constituted a major part of (c) epicardial mapping surface (d) during the experiment.

cells and tissues, such as intrinsic fluorescence changes,[11] oxygenation,[12] membrane potential,[13] and ROS.[14,15]

In this work, we demonstrated an optical imaging technique based on recordings of myocardial oxidative metabolism with an intracellular fluorescent indicator [dihydroethidium (DHE)] in a 2-D format. We applied this method to study the spatial distribution and temporal evolution of ROS dynamics in an isolated perfused rat heart throughout global ischemia reperfusion. In addition, we determined the time sequence of intracellular ROS dynamics and cardiac performance with simultaneous recordings of ECG and intracardiac pressure.

2 Methodology

The work was conducted in accordance with Taiwan's. Animal Protection Law (Scientific Application of Animals) of 1998. For preparation of isolated perfused rat hearts 300-g male Wistar rats were heparinized (500 IU) and anaesthetized with an intraperitoneal bolus of pentobarbital (65 mg/kg). The heart was quickly removed and placed on a Langendorf perfusion apparatus. We used standard Tyrode's solution with supplementation of oxygen and CO_2 to perfuse the heart at a constant flow (3-ml/100-g body weight) with a rolling pump. The heart was then submerged in a warm tissue bath at 37.5°C throughout the experiment.

The optical mapping setup is shown in Fig. 1. A Verdi laser was used to provide 0.5 W at 532 nm as a light source. The laser beam was directed on the anterior surface of the heart with an optic fiber. The reflection of longer wavelengths (>600 nm) from the heart were collected through a long-pass filter and captured with a charge-coupled device (CCD) camera. An image stream contained 1000 128×128-pixel frames and was recorded at an average rate of 400 frames/s. The spatial resolution was 300×300 μm in each image. Image streams were processed with an in-house developing software based on a LabVIEW platform.

To record intracellular ROS dynamics throughout the global ischemia reperfusion, we adopted dihydroethidium (DHE) for probing intracellular ROS generation. DHE was reported to reflect enhanced oxidative metabolism in hypothermic guinea pig hearts.[16] Direct penetration of the chemical into the cell results in cytoplasmic distribution. ROS would oxidize DHE to yield ethidium, which intercalates DNA and stains cell nuclei with red fluorescence (absorption peak of 530 nm, emission peak of 620 nm). Ethidium bound to genomic DNA from calf thymus at an apparent Kd of 2.3 μM[17] has been reported. Since the redox equilibrium between DHE and ethidium is determined by cellular ROS, the ethidium-DNA complex-derived red fluorescence would dynamically reflect the concentration of cellular ROS, given that the cellular concentration of ethidium approaches the Kd of the ethidium-DNA interaction.

In the experiment, the heart was loaded with 3-μM DHE in 250 ml of Tyrode's solution for 30 min, and allowed to stand for 20 to 25 minutes for stable fluorescence measurements. To test the temperature modulation of ROS production, we cooled down the temperature of the perfusate and water bath to 27°C for 20 min, and then rewarmed them to 37°C for another 10 min. Image streams were recorded in baseline, hypothermia, and rewarm periods. To record ROS production throughout global ischemia reperfusion, the rolling pump was turned off for 30 min of global ischemia and turned on again for 30 min of reperfusion. Image streams were recorded at 30, 60, 120, 240, 360, 480, 600, 900, 1200, 1500, and 1800 s in both ischemia and reperfusion periods. In the control experiment, isolated perfused hearts were subjected to the same imaging scheme for 60 min.

To study the relationship of DHE fluorescence and cardiac function, we performed another series of experiments to simultaneously record epicardial DHE fluorescence, myocardial electrical activity, and left ventricular pressure. After the heart was mounted to a Langendorf apparatus, a pair of platinum electrodes was positioned in the posterior wall of the left ven-

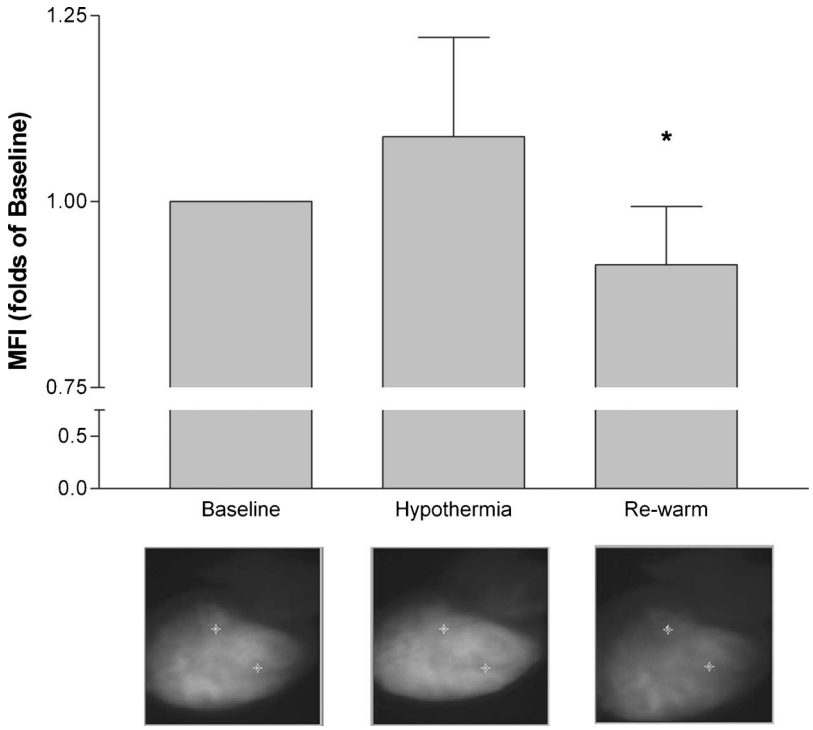

Fig. 2 Dynamic response of DHE to temperature modulation. DHE would dynamically reflect the intracellular level of reactive oxygen species (ROS). Temperature modulation of ROS production in isolated perfused heart could be demonstrated with DHE staining. Induction of ROS surge, as indicated by enhanced fluorescence intensity, with hypothermic perfusion (27°) could be reversed with reperfusion with normothermic Tyrode's solution (see upper row for representative maps, and lower bar diagram for MFI quantification. * indicates $p<0.05$ using the student's *t*-test. Data were obtained from four rat hearts).

tricle (LV) to record cardiac electrical activity. A microtip pressure transducer was inserted into the LV via an apical stab wound. Both signals were sampled at 1 KHz, digitized with a digitizer (Digidata 1322, Axon Instruments) and stored for further analysis.

In the data analysis, the mean fluorescence intensity (MFI) was used to estimate relative ROS production. MFI is defined as the pixel adjusted average of fluorescence intensity within an image. The average of MFI from 100 images in a stream was assigned to represent that series. The fluorescence signals from a single heart were normalized to their own baseline conditions and expressed as a percentage of baseline. Statistical significance was defined as $p<0.05$ using the student's *t*-test.

3 Results

We loaded the heart with DHE and did not find any significant changes in ventricular pressure, diastolic pressure, or R-R interval. Epicardial coronary tributaries were unstained, and as a result, the mapping surface had a mosaic pattern. However, no obvious spatial inhomogeneity could be found within the cardiac muscle. Also, the photobleaching effects of the laser on the DHE stain in isolated perfused rat hearts were tested. We did not find any significant fluctuation (>2% of baseline MFI) of red fluorescence within 1 h of continuous excitation.

Since the perfusate temperature was reported to modulate cardiac ROS production,[16] we adopted this method to test the reversibility of DHE staining. We cooled down the perfusate to 27°C for 5 min and observed a significant increase of fluorescence intensity. Warming up the perfusate to 37.5°C brought the fluorescence intensity back to baseline (Fig. 2). Thus, DHE could dynamically reflect intracellular ROS levels in isolated rat heart preparations.

We applied this technique to record temporal sequences of ROS production throughout the global ischemia reperfusion (Fig. 3). Ischemia would result in a gradual decrease of MFI. After 15 min of ischemia, the MFI reached a steady state (90.9±5.1% of baseline, $p<0.05$). Reperfusion would immediately elicit a ROS burst. The peak of burst (107.0±3.0% of baseline, $p<0.05$) lasted for 6 min and gradually faded out. After 30 min of reperfusion, there were no significant differences of MFI compared with the baseline.

Simultaneous monitoring of ventricular function and ROS production throughout the reperfusion of global ischemia could give us further insight into the role of ROS in the pathogenesis of reperfusion injury. Global ischemia rapidly resulted in decreased developed pressure and left ventricular end systolic pressure (LVESP) within 3 min. Meanwhile, left ventricular end diastolic pressure (LVEDP) gradually increased. The effect was accompanied with a prolonged R-R interval and finally electrical silence after 5 min.

DHE fluorescence also gradually decreased [Fig. 3(b)], but the process took longer to reach a steady state in spite of the earlier cessation of spontaneous electrical activity and contraction. On reperfusion, the heart immediately broke electrical silence into fibrillating activity and a homogenous DHE fluorescence elevation could be seen immediately. At the end of 30 min of reperfusion, the restoration of ventricular con-

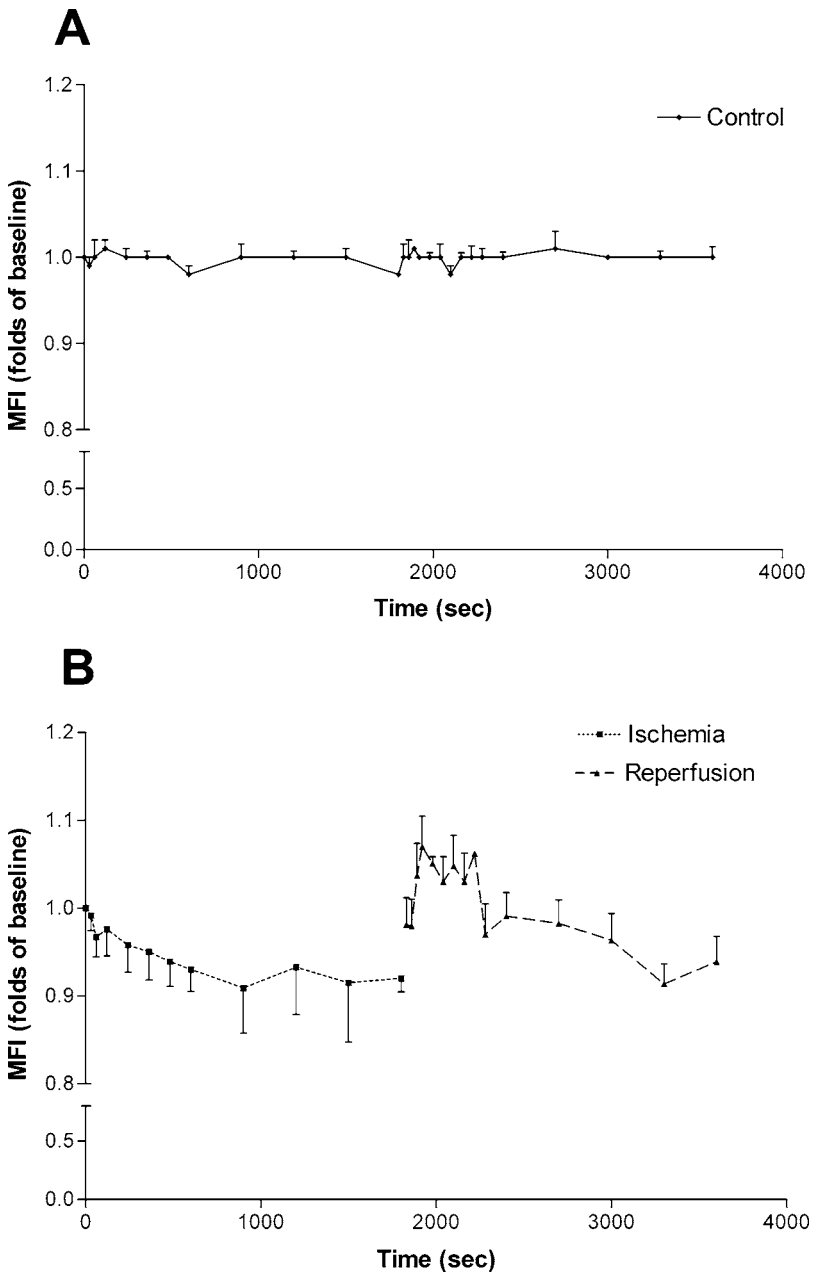

Fig. 3 DHE fluorescence response to ischemia reperfusion. Results of (a) control experiment were obtained from three hearts, showing resistance to photobleaching and small fluctuation in the 60-min recording period. Throughout global ischemia and reperfusion, (b) ischemia would depress epicardial DHE fluorescence, while reperfusion would immediately induce a transient surge. Image streams were recorded at 30, 60, 120, 240, 360, 480, 600, 900, 1200, 1500, and 1800 s in both ischemia and reperfusion periods. Mean fluorescence intensity was normalized to baseline and expressed in relative folds. Data were acquired from eight hearts.

traction and the organization of ventricular tachycardia were accompanied with the normalization of DHE fluorescence. A typical series is shown in Fig. 4.

4 Discussion

In the present study, we demonstrated that optical mapping of myocardial ROS production is feasible. Also, we demonstrated the time sequence of cardiac functional alteration and ROS production throughout global ischemia reperfusion in isolated perfused rat hearts. We found that after reperfusion, the initiation of ventricular arrhythmia was accompanied with an elevation of ROS production. Attenuated ROS surge could be seen after 30 min of reperfusion, and acute cardiac dysfunction, such as deterioration of pressure generation and electrical instability, was also partially alleviated.

ROS played an important role in cardiovascular biology. All of the cell types in the cardiovascular system are capable of generating ROS. The source of cellular ROS includes mitochondrial oxidative phosphorylation, activity of NADPH oxidase and xanthine oxidase, auto-oxidation of catecholamines, and uncoupling of NO synthase.[18] Their reactive nature could result in oxidative modification of biomolecules

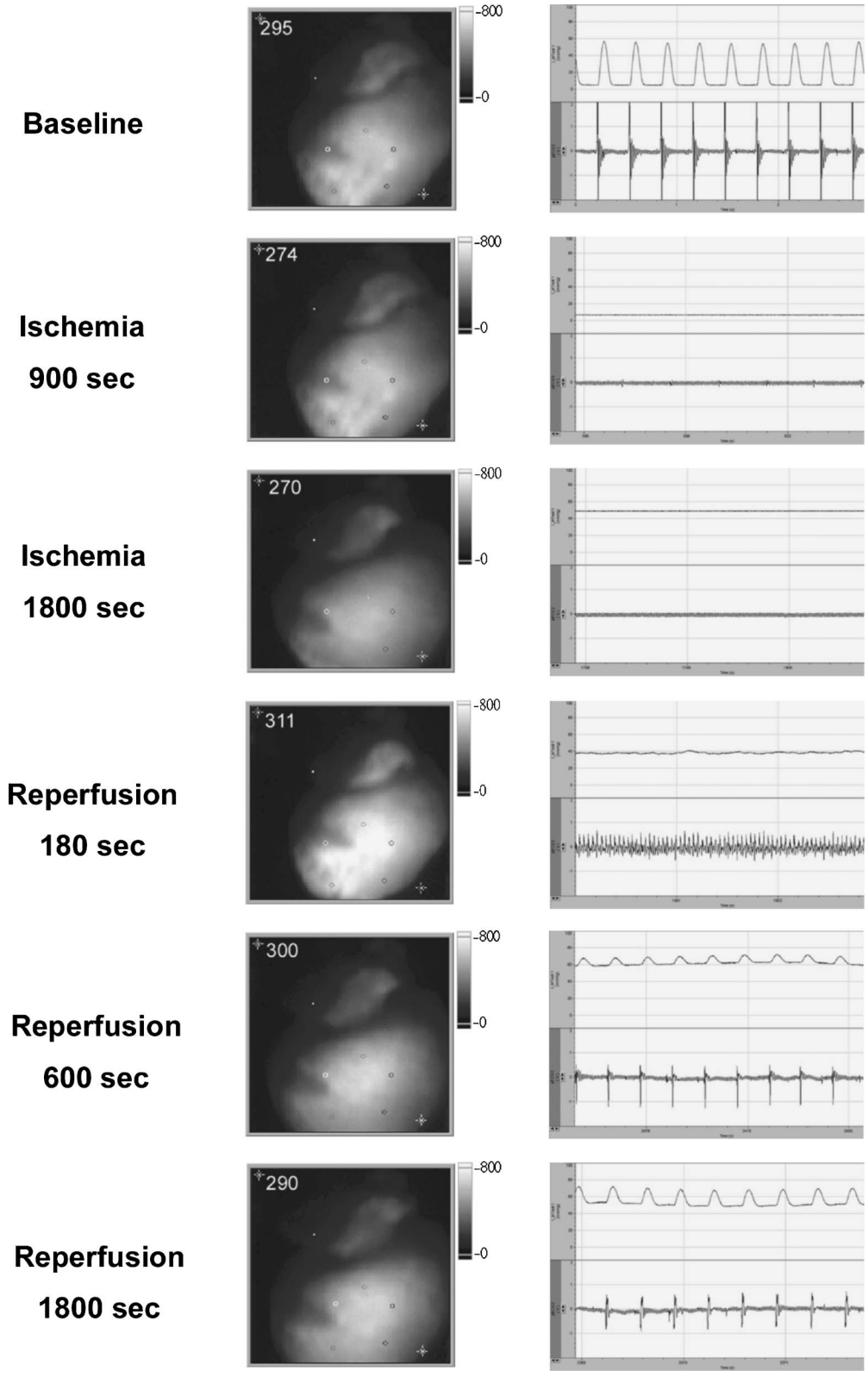

Fig. 4 Cardiac function and DHE fluorescence. A representative series of simultaneous intraventricular pressure, electric activity, and epicardial DHE fluorescence recording throughout global ischemia reperfusion was demonstrated. The left column is the timing of recording; the middle column is the DHE fluorescence intensity maps; the right column is the pressure (upper half) and ECG (lower half) traces. Reperfusion for 30 min would return DHE fluorescence to the baseline level, but it would only partially reverse the ischemia-induced acute cardiac dysfunction.

and initiate signal cascades to modify cellular function. ROS have been reported to modulate ion flux, calcium handling, energy homeostasis, vasomotor function, hypertrophy, gene expression, and the signaling pathways of growth factors and cytokines,[19] and therefore have great impacts on cardiac function.

Numerous experimental data suggested the association between an increased oxidative burden and various cardiovascular pathologies, including atherosclerosis, ischemic heart disease, myocardial stunning, reperfusion injury, and heart failure.[19] However, a lack of consistent efficacy of antioxidants in large-scale clinical trials[20–23] raised questions about the pathogenic role of ROS. To solve the controversy, direct measurement of tissue ROS production throughout the disease process would be of great value. Currently available methods to analyze tissue oxidative burdens rely on quantification of biomarkers, such as thiobarbituric acid reactive species (TBARS) and 8-epi-isoprostane. Although widely used, there are still concerns in the preparation, analysis, specificity, and sensitivity of these methods.[24] In addition, these methods could not demonstrate physiologic or pharmacologic responses in a single preparation. Optical imaging methods represent another approach to address this issue.

The optical mapping technique was traditionally used to study the behavior of electric excitation wavefronts in the heart. As fluorescent indicators of ROS production were widely used to address free-radical biology at the cellular level,[25,26] their application in tissue levels has been explored with optic fiber spectrofluorophotometry.[15] In our study, we extended to the 2-D analysis of spatial distribution and temporal evolution of ROS production. Motion-related registration artifacts have been minimized by a high sampling rate.

Reduced energy demand for electric-contraction coupling and basal metabolism might account for decreased MFI during the 30 min of ischemia. Reoxygenation-associated mitochondrial respiratory bursts might attribute to excessive ROS production during the 30 min of reperfusion. Normalization of ROS production could only partially reverse the reperfusion-induced acute cardiac dysfunction. This suggests that factors other than excessive ROS production could be involved in the pathogenic mechanism of reperfusion injury.

There are some limitations of this study. First, the spatial resolution of the system was about 300 μm, which meant each pixel contained 10 to 30 cardiomyocytes. Reflective confocal intravital microscopy would provide much better planar resolution and would enable us to study the contribution of delicate structures, such as endothelial cells and leukocytes. Second, we excluded leukocytes and platelets in the perfusate, which might attenuate the magnitude of reperfusion injury.[27,28] Finally, ethidium was a potential carcinogen, and therefore, its application for diagnostic use in humans would be limited.

Acknowledgments

This work was supported by grants NSC92-2314-B-002-289 and NSC92-2314-B-002-220 from the Taiwan National Science Council. The authors also wish to extend their heartfelt gratitude to T. Z. Wu for her technical assistance.

References

1. C. L. Gibbs, "Cardiac energetics: sense and nonsense," *Clin. Exp. Pharmacol. Physiol.* **30**, 598–603 (2003).
2. D. S. Loiselle and C. L. Gibbs, "Species differences in cardiac energetics," *Am. J. Physiol.* **237**, H90–H98 (1979).
3. J. T. Stewart, L. A. Simpson, R. E. Smith, C. Callicott, and A. I. Camm, "Left ventricular energetics: heat production by the human heart," *Cardiovasc. Res.* **27**, 1024–1032 (1993).
4. Y. M. Wang, C. W. Sun, C. K. Lee, C. W. Lu et al., "Comparisons of optical scattering properties between biological tissues and a phantom with time, aperture, and angle gating," *Opt. Express* **12**, 1157–1168 (2004).
5. S. P. Nighswander-Rempel, R. A. Shaw, B. Kuzio, and V. V. Kupriyanov, "Detection of myocardial cell damage in isolated rat hearts with near-infrared spectroscopy," *J. Biomed. Opt.* **9**, 779–787 (2004).
6. C. W. Sun, L. S. Lu, C. C. Yang, Y. W. Kiang, and M. I. Su, "Myocardial tissue characterization based on the time-resolved Stokes-Mueller formalism," *Opt. Express* **10**, 1347–1353 (2002).
7. I. K. Jang, G. L. Tearney, B. MacNeill, M. Takano et al., "*In vivo* characterization of coronary atherosclerotic plaque by use of optical coherence tomography," *Circulation* **111**, 1551–1555 (2005).
8. I. R. Efimov, V. P. Nikolski, and G. Salama, "Optical imaging of the heart," *Circulation* **94**, 21–23 (2004).
9. V. K. Ramshesh and S. B. Knisley, "Spatial localization of cardiac optical mapping with multiphoton excition," *J. Biomed. Opt.* **8**(2), 253–259 (2003).
10. G. A. MacGowan, C. Du, V. Glonty, J. P. Suhan et al., "Rhod-2 based measurements of intracellular calcium in the perfused mouse heart: Cellular and subcellular localization and response to positive inotrop," *J. Biomed. Opt.* **6**(1), 23–30 (2001).
11. G. Salama, R. Lombardi, and J. Elson, "Maps of optical action potentials and NADH fluorescence in intact working hearts," *Am. J. Physiol.* **252**, H384–H394 (1987).
12. B. Chance, "On the mechanism of the reaction of cytochrome oxidase with oxygen," *Ann. N.Y. Acad. Sci.* **244**, 163–173 (1975).
13. M. Morad and G. Salama, "Optical probes of membrane potential in heart muscle," *J. Physiol. (London)* **292**, 267–295 (1979).
14. C. P. LeBel, H. Ischiropoulos, and S. C. Bondy, "Evaluation of the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress," *Chem. Res. Toxicol.* **5**, 227–231 (1992).
15. L. G. Kevin, A. K. Camara, M. L. Riess, E. Novalija, and D. F. Stowe, "Ischemic preconditioning alters real-time measure of O2 radicals in intact hearts with ischemia and reperfusion," *Am. J. Physiol. Heart Circ. Physiol.* **284**, H566–H574 (2003).
16. A. K. Camara, M. L. Riess, L. G. Kevin, E. Novalija, and D. F. Stowe, "Hypothermia augments reactive oxygen species detected in the guinea pig isolated perfused heart," *Am. J. Physiol. Heart Circ. Physiol.* **286**, H1289–H1299 (2004).
17. N. W. Luedtke, J. S. Hwang, E. Nava et al., "The DNA and RNA specificity of eilatin Ru(II) complexes as compared to eilatin and ethidium bromide," *Nucleic Acids Res.* **31**, 5732–5740 (2003).
18. C. F. Mueller, K. Laude, J. S. McNally, and D. G. Harrison, "Redox mechanisms in blood vessels," *Arterioscler., Thromb., Vasc. Biol.* **25**, 274–278 (2005).
19. F. J. Giordano, "Oxygen, oxidative stress, hypoxia, and heart failure," *J. Clin. Invest.* **115**, 500–508 (2005).
20. C. H. Hennekens, J. E. Buring, J. E. Manson, M. Stampfer et al., "Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasms and cardiovascular disease," *N. Engl. J. Med.* **334**, 1145–1149 (1996).
21. D. D. Waters, E. L. Alderman, J. Hsia, B. V. Howard et al., "Effects of hormone replacement therapy and antioxidant vitamin supplements on coronary atherosclerosis in postmenopausal women: a randomized controlled trial," *JAMA, J. Am. Med. Assoc.* **288**, 2432–2440 (2002).
22. M. Boaz, S. Smetana, T. Weinstein, Z. Matas et al., "Secondary prevention with antioxidants of cardiovascular disease in endstage renal disease: randomised placebo-controlled trial," *Lancet* **356**, 1213–1218 (2000).
23. R. M. Salonen, K. Nyyssonen, J. Kaikkonen, E. Porkkala-Sarataho et al., "Antioxidant supplementation in atherosclerosis prevention study: Six-year effect of combined vitamin C and E supplementation on atherosclerotic progression," *Circulation* **107**, 947–953 (2003).
24. K. K. Griendling and G. A. FitzGrerald, "Oxidative stress and car-

diovascular injury," *Circulation* **108**, 1912–1916 (2003).
25. Z. Xie, P. Kometiani, J. Liu et al., "Intracellular reactive oxygen species mediate the linkage of Na+/K+-ATPase to hypertrophy and its marker genes in cardiac myocytes," *J. Biol. Chem.* **274**, 19323–19328 (1999).
26. E. A. Konorev, H. Zhang, J. Joseph, M. C. Kennedy, and B. Kalyanaraman, "Bicarbonate exacerbates oxidative injury induced by antitumor antibiotic doxorubicin in cardiomycytes," *Am. J. Physiol. Heart Circ. Physiol.* **279**, H2424–H2430 (2000).
27. A. M. Lefer, B. Campbell, and Y. K. Shin, "Effects of a metalloproteinase that truncates P-selectin glycoprotein ligand on neutrophil-induced cardiac dysfunction in ischemia/reperfusion," *J. Mol. Cell. Cardiol.* **30**, 2561–2566 (1998).
28. J. G. Kingma, Jr., S. Plante, and P. Bogaty, "Platelet GPIIb/IIIa receptor blockade reduces infarct size in a canine model of ischemia-reperfusion," *J. Am. Coll. Cardiol.* **36**, 2317–2324 (2000).