CHAPTER FOUR

In Vivo Imaging of Nitric Oxide and Hydrogen Peroxide in Cardiac Myocytes

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Abstract

Nitric oxide (NO) and hydrogen peroxide (H_2O_2) are synthesized within cardiac myocytes, and both molecules play key roles in modulating cardiovascular responses. However, the interconnections between NO and H_2O_2 in cardiac myocyte signaling have not been properly understood. Adult mouse cardiac myocytes represent an informative model for the study of receptor-modulated signaling pathways involving reactive oxygen species and reactive nitrogen species. However, these cells typically survive for only 1–2 days in culture, and the limited abundance of cellular protein undermines many biochemical analyses. We have exploited chemical sensors and biosensors for use in *in vivo* imaging studies of H_2O_2 and NO in adult cardiac myocytes. Here we describe detailed methods for the isolation of cardiac myocytes suitable for imaging studies. We also present our methods for the generation of recombinant lentiviral preparations encoding the H_2O_2 biosensor HyPer2 that permit analysis of intracellular H_2O_2 levels using fluorescence microscopy in living cardiac myocytes following tail vein

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injection and in cultured endothelial cells following infection. We also describe our protocols for using the NO chemical sensor Cu_2(FL2E) in living adult mouse cardiac myocytes to study the effects of agonist-modulated H_2O_2 production on NO synthesis. Using these techniques, we have demonstrated that receptor-stimulated increases in intracellular H_2O_2 modulate NO levels in living cardiac myocytes. These and similar approaches may facilitate a broad range of studies in other terminally differentiated cells that involve the interaction of NO- and H_2O_2-regulated signaling responses.

1. INTRODUCTION

Cardiac myocytes are critical determinants of the heart's contractile function (Bers, 2000; Brady, 1991). Nitric oxide (NO) is a free radical signaling molecule that has been shown to play key roles in modulating cardiac responses (Balligand, Feron, & Dessy, 2009). Both the endothelial (eNOS) and the neuronal (nNOS) isoforms of NO synthase are robustly expressed in cardiac myocytes. eNOS and nNOS are Ca^{2+}-calmodulin-dependent enzymes that can be regulated by diverse extracellular stimuli acting both via cell surface receptors and by mechanochemical signals. NO synthases are also modulated by diverse protein kinases and phosphoprotein phosphatases (Dudzinski, Igarashi, Greif, & Michel, 2006). At least some of these phosphorylation pathways are influenced by cellular oxidants. Under specific pathophysiological conditions, NOS enzymes can be "uncoupled" from NO synthesis, leading to the synthesis of superoxide anion instead of NO. Clearly, there is a complex interplay between NO and oxidant pathways in cardiac cells (Maron & Michel, 2012).

Cell-derived reactive oxygen species (ROS) oxidize a broad array of biomolecules and are implicated in pathological states ranging from neurodegeneration to atherosclerosis (Stocker & Keaney, 2004; Storz, 2006). However, not all effects of ROS are deleterious: endogenously generated ROS have been implicated in posttranslational protein modifications that subserve critical physiological roles in cellular signaling (Rudolph & Freeman, 2009). H_2O_2 is one such ROS that has been identified as a key signaling molecule in many cell types (Cai, 2005; D'Autreaux & Toledano, 2007; Sartoretto, Kalwa, Pluth, Lippard, & Michel, 2011; Sartoretto et al., 2012). Whereas the physiological role of NO in the heart has been extensively characterized, the physiological role of H_2O_2 is less well understood, and much remains to be learned about the interplay between H_2O_2 and the reactive nitrogen species (RNS) in cardiac myocytes. Diverse

cell surface receptor-modulated pathways activate eNOS, and yet other extracellular stimuli enhance H_2O_2 synthesis, but the relationships between NO and H_2O_2 in cardiac myocyte signaling are incompletely understood. We have recently shown that H_2O_2 is a critical intracellular mediator that modulates eNOS phosphorylation and enzyme activation in adult cardiac myocytes (Sartoretto et al., 2011, 2012).

The isolation and analysis of primary adult ventricular cardiac myocytes are of particular importance due to the lack of a representative and reliable cell line for studying the redox signaling pathways that modulate cardiac myocyte function. Moreover, the analysis of cardiac responses in transgenic mouse models involving ROS and RNS requires the isolation and culture of primary adult mouse cardiac myocytes. While adult mouse cardiac myocytes represent an informative model for studies involving ROS and NO, the isolation of suitable cells and their subsequent culture under physiological conditions for *in vivo* imaging studies can be problematic. The challenge for us was to adapt established adult cardiac myocyte isolation protocols (Liao & Jain, 2007; O'Connell, Rodrigo, & Simpson, 2007; Schluter & Schreiber, 2005) to permit the rapid and reproducible isolation of healthy cardiac myocytes suitable for *in vivo* imaging analyses. Moreover, the cells need to survive overnight without a significant loss of response to agonists. Freshly isolated myocytes need to be suitable for both biochemical studies (such as measurement of protein phosphorylation) and imaging studies to quantitate endogenous levels of ROS or RNS. Below we describe our protocols for the isolation and culture of adult mouse cardiac myocytes to detect receptor-modulated changes in H_2O_2 or NO levels in living cells by the use of the HyPer2 biosensor or of the Cu_2(FL2E) chemical sensor, respectively. We also describe the production of recombinant lentiviruses expressing HyPer2 and the method used to infect adult mice with the HyPer2 lentivirus. These methods facilitate studies of the dynamic regulation of NO and H_2O_2 metabolism in cardiac myocytes as a basis for understanding the interplay of ROS and RNS in cardiac physiology and pathophysiology.

2. ISOLATION AND CULTURE OF ADULT MOUSE VENTRICULAR CARDIAC MYOCYTES

Protocols for cardiac myocyte isolation have been previously published in detail (Liao & Jain, 2007; O'Connell et al., 2007; Schluter & Schreiber, 2005), and we have slightly modified these methods to improve the yield of cells suitable for cell imaging experiments, as described below.

For the detection of H_2O_2 and NO in living cardiac myocytes, we typically isolate cells from 8- to 10-week-old mice. The animals are placed in a glass desiccator jar and lightly anesthetized with isoflurane for 10–20 s prior to intraperitoneal injection of heparin (100 U/mL, 0.3 mL). We found that isoflurane anesthesia prior to heparin injection improves the yield of viable cardiac cells, possibly reflecting decreased stress on the animals. Ten minutes following heparin injection (the amount of time needed to effect full anticoagulation) the animals are sacrificed with isoflurane (1–2 min). Immediately after the animal dies, the peritoneal cavity and chest are opened with scissors, and the heart is exposed and lifted with forceps. Next, the descending thoracic aorta and common carotid arteries are cut, and the heart is placed in a 100-mm dish containing "perfusion buffer" (0.6 m*M* KH_2PO_4; 14.7 m*M* KCl; 0.6 m*M* Na_2HPO_4; 4.6 m*M* $NaHCO_3$; 120 m*M* NaCl; 1.2 m*M* $MgSO_4 \cdot 7H_2O$; 5.5 m*M* glucose; 30 m*M* taurine; 10 m*M* 2,3-butanedione monoxime; 10 m*M* HEPES; adjust pH to 7.0). Visualized using a dissecting microscope, the aorta is cannulated above the aortic valve and below the carotid arteries using a 25-gauge needle with a blunted tip. In order to permit perfusion of the heart, a small clip (straight micro clip, 10 mm length, 1.5 mm width, Roboz Company, Rockville, MD) is placed between the carotid arteries, and the aorta is tied below the clip to the cannula using cotton thread. The time from removal of the heart to the beginning of perfusion should not exceed 1 min; practice is essential.

The isolated heart is next perfused through the aorta using a peristaltic pump at a rate of 3–4 mL/min with perfusion buffer warmed to 37 °C for at least 5 min or until all the blood is removed. After the heart is completely free of blood, the next step is the enzymatic digestion of the heart. Collagenase type 2 (Worthington Biochemical) is added to the perfusion buffer at a final concentration of 2.4 mg/mL (now called "digestion buffer"). After 2 min of perfusion with digestion buffer, $CaCl_2$ is added at a final concentration of 37.5 μ*M* and the perfusion is continued for another 8–10 min. The time of heart digestion may vary from heart to heart, depending on the age, sex, and weight of the animals; digestion time also varies between different lots of collagenase type 2. A proper digestion is confirmed by a spongy, swollen, and pale appearance of the heart. Once the heart is thoroughly digested (the heart turns pallid, distended, and soft), the heart is removed from the cannula and placed in a 60 mm dish with 2 mL cardiac perfusion buffer containing bovine calf serum (10%, v/v) to stop the collagenase digestion. The ventricles are immediately torn into ~10 small pieces using forceps (micro dissecting tweezers, 45° angled, tip 0.05 mm × 0.01 mm, Roboz Company, Rockville, MD), and the solution is pipetted up and down using a 1-mL pipette until it

becomes a cell suspension. The cell suspension is transferred to a 50-mL conical tube attached to a cell strainer (100 μm nylon) to separate dissociated cells from undigested pieces of heart tissue. Next, the cells are allowed to sediment by gravity for 10–15 min. For the final steps, calcium is gradually reintroduced to the cells. After the cells sediment, the supernatant is removed and the cells are resuspended in cardiac perfusion buffer containing 10% bovine calf serum. Three solutions with increasing concentrations of calcium (100, 400, and 900 μ*M*) are added sequentially to the cells. Between each step, the cells are allowed to sediment by gravity for at least 15 min.

After gradually reintroducing calcium to the cells, the cardiac myocytes are plated in laminin (10 μg/mL)-coated 35 mm petri dishes (containing 10 mm Microwell #0 cover glasses) in "plating medium," which consists of Minimum Essential Medium with Hank's Balanced Salt Solution, supplemented with calf serum (10%, v/v), 2,3-butanedione monoxime (10 m*M*), penicillin–streptomycin (100 units/mL), glutamine (2 m*M*), and ATP (2 m*M*). Cardiac myocytes used for determining H_2O_2 and NO production should be plated at about 50% confluence. For *in vivo* analyses of cardiac myocyte production of H_2O_2 or NO, the quality of the cells isolated is far more important than the quantity of cells obtained per isolation. Viable cardiac myocytes have a rod-shaped appearance under the microscope, and moribund myocytes are round; the percentage of rod-shaped to round myocytes should be greater than 60%; if not, the preparation should be discarded and the myocyte isolation repeated.

After the cells attach to the coverslip (1 h), the plating medium is changed to "culture medium" consisting of Minimum Essential Medium with Hank's Balanced Salt Solution, supplemented with bovine serum albumin (1 mg/mL), penicillin/streptomycin (100 units/mL), glutamine (2 m*M*), 2,3-butanedione monoxime (10 m*M*), insulin (5 μg/mL), transferrin (5 μg/mL), and selenium (5 ng/mL). The cells can be studied immediately or may be cultured overnight at 37 °C in a humidified incubator at 2% CO_2. Before performing live cell imaging experiments, it is critical to use fresh medium one or two times to wash away dead cells and other cellular debris after the cells have properly settled, to avoid imaging artifacts.

3. LIVE CELL IMAGING OF CARDIAC MYOCYTES

Live cell imaging of cardiac myocytes infected with the HyPer2 H_2O_2 biosensor or loaded with the Cu_2(FL2E) NO chemical sensor requires the use of specialized equipment to maintain cells under optimal culture

conditions and to minimize thermal drift. For these imaging experiments, cells are maintained at 37 °C, 2% CO_2 in a humidified environment using a Tokai on-stage incubator mounted on an Olympus IX81 inverted fluorescence microscope equipped with a mercury arc lamp (Sartoretto et al., 2011). Whatever specific apparatus is chosen for live cell imaging, it is critical to use an environmental chamber that minimizes fluctuations in temperature, humidity, and CO_2 levels. For control of the microscope and for data acquisition and analysis, we used Metamorph software, which we found to work as a versatile platform for a wide range of imaging experiments. Of course, other combinations of hardware and software designed for live cell imaging may also be used. A detailed description of Metamorph scripting, filter selection, and control of background fluorescence can be found in the papers of Aoki and Matsuda (2009) and Pase, Nowell, and Lieschke (2012). These two informative publications also contain detailed descriptions on data analysis. Here we will focus on describing the specific considerations for using cardiac myocytes in live cell imaging experiments.

The photosensitivity of cardiac myocytes mandates that phototoxic stress during cell imaging be reduced by keeping the exposure time as short as possible and by minimizing the measurement frequency. Depending on the time course of the response being analyzed, it is usually sufficient to obtain an image every few seconds. We also use a medium level transmission neutral density filter (6–12%) to further reduce phototoxicity. If high spatial resolution is not necessary, "binning" of the data procured by the camera chip can decrease the exposure time needed to obtain a good signal-to-noise ratio. In our experiments, we used symmetrical 2× binning as previously described (Aoki & Matsuda, 2009). To minimize background fluorescence from the imaging solutions, we use phenol red-free medium, and routinely evaluate other medium components for intrinsic fluorescence. Importantly, cardiac myocytes have a very high natural fluorescence in the green and red parts of the spectrum, and it is critical to acquire the baseline fluorescence characteristics of the cells of interest. This baseline then serves as a reference to detect expression of the biosensor and/or loading of the cell with the chemical sensor used for the actual experiment.

4. IMAGING INTRACELLULAR NO WITH Cu_2(FL2E) DYE

Details of the synthesis, purification, and validation of Cu_2(FL2E) have been described in detail (McQuade, Pluth, & Lippard, 2010). We obtained this reagent from Professor Stephen Lippard at MIT; Cu_2(FL2E)

is now commercially available from Strem Chemicals (Newburyport, MA). The Cu_2(FL2E) is solubilized in DMSO and can be stored at −80 °C for no more than 2 weeks; the Cu_2(FL2E) powder is more stable, and can be stored at 4 °C for several months. Before loading the cells with Cu_2(FL2E), the plating medium is removed and replaced with Tyrode's solution (140 m*M* NaCl; 5 m*M* KCl; 10 m*M* HEPES; 5.5 m*M* glucose; 1 m*M* $CaCl_2$; 1 m*M* $MgCl_2$; adjust pH to 7.4). Cells are loaded at 37 °C and 2% CO_2 with 5 μ*M* Cu_2(FL2E) for 2 h in Tyrode's solution, washed twice with warm Tyrode's solution, and then imaged. The culture dishes are placed in an on-stage incubator (e.g., Tokai, Tokyo, Japan) on a suitable fluorescence microscope; we use an Olympus IX81 inverted microscope equipped with an UPlan 40X/1.3 oil objective in a low-volume glass-covered recording chamber. Fluorescence signals are analyzed by using a Hamamatsu Orca CCD camera (Hamamatsu, Tokyo, Japan) at 470 nm and processed using Metamorph. Viable rod-shaped cardiac myocytes with rectangular ends are selected by differential interface contrast imaging and then subjected to fluorescence imaging following agonist treatments. Variations in levels of basal fluorescence between experimental preparations are observed because of differences in cellular loading of the NO dye from day to day. The signal from the NO sensor Cu_2(FL2E) is analyzed as the slope of the fluorescence increase seen following the addition of agonist or vehicle. Analysis is done with a standard FITC/GFP exciter emitter block at 480 nm (Semrock GFP 3035-B-OMF-zero). Figure 4.1 shows results of an imaging

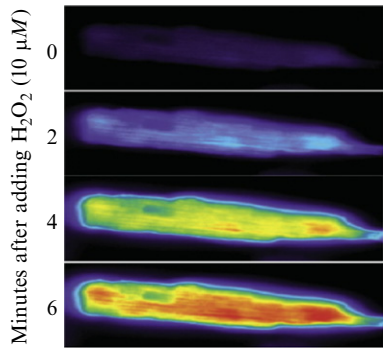

Figure 4.1 Effects of exogenous H_2O_2 on cardiac myocyte NO synthesis. Adult mouse cardiac myocytes were loaded with the NO dye Cu_2(FL2E), and then treated with hydrogen peroxide (H_2O_2, 10 μ*M*) and analyzed by fluorescence microscopy. Fluorescence images obtained at varying times after adding H_2O_2 are shown, as indicated.

experiment using cardiac myocytes loaded with the Cu_2(FL2E) chemical sensor for NO. As can be seen, the addition of a physiological concentration of H_2O_2 (10 μM) promotes a prompt increase in the fluorescence signal, indicative of an increase in intracellular NO in response to H_2O_2. This robust response of the chemical sensor permits the analysis of the signaling pathways that connect H_2O_2 and NO. For example, the experiment shown in Fig. 4.2 indicates that H_2O_2-promoted NO synthesis in cardiac myocytes can be blocked by pretreating the cells with pharmacological concentrations of the clinically important calcium channel blocker nifedipine. As the Cu_2(FL2E) NO chemical sensor becomes more widely available, we expect that this reagent will become even more broadly used to probe NO pathways in diverse tissues and disease states.

5. PRODUCTION AND *IN VIVO* EXPRESSION OF LENTIVIRUS EXPRESSING THE HyPer2 H_2O_2 BIOSENSOR

Detection of H_2O_2 using the HyPer2 biosensor has facilitated studies of ROS metabolism in a broad range of cells transfected with plasmids encoding this informative biosensor (Belousov et al., 2006; Pase et al., 2012). However, analyses of HyPer2 biosensor responses are more challenging to study in cardiac myocytes and in other cell types that are difficult to transfect and/or are unstable in cell culture. Recombinant lentiviruses represent powerful tools for the delivery of transgenes into dividing and nondividing cells *in vitro* as well as *in vivo*. Lentiviruses do not usually elicit a severe systemic immune reaction in mice and are generally safe and well tolerated by the animal. Recombinant proteins encoded by lentiviral constructs can be studied in cardiac myocytes and other cells that are not suitable for prolonged cell culture by using the lentivirus-infected mouse as a "living incubator" to allow viral protein expression in a broad range of tissues. The key element for this approach in our studies is the production of a concentrated and stabilized solution of the lentivirus with a viral titer that is high enough to permit efficient viral infection of target tissues, leading to levels of recombinant HyPer2 protein expression that are sufficient for H_2O_2 detection. To briefly summarize our approach, the coding sequence of HyPer2 was cloned into the pWPXL lentiviral expression plasmid downstream of the EF1-α promoter. Recombinant vesicular stomatitis virus-glycoprotein pseudo-typed lentivirus particles were generated in HEK293-T cells by transfection of the envelope:packaging:transgene plasmids at a 1:1:1.5 ratio

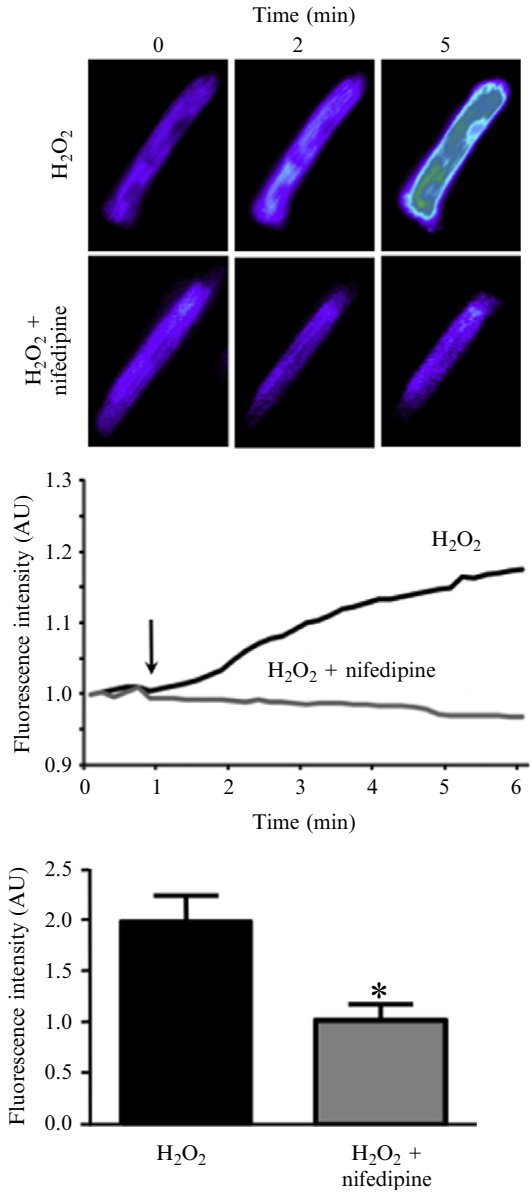

Figure 4.2 Nifedipine treatment abrogates H_2O_2-promoted NO synthesis in cardiac myocytes. Mouse cardiac myocytes were loaded with the NO chemical sensor Cu_2(FL2E), and then treated with nifedipine (100 μM) or vehicle followed by hydrogen peroxide (H_2O_2, 10 μM) treatment. The upper panel shows representative fluorescence images at 0, 2, and 5 min following treatments as indicated. The middle panel shows representative fluorescence tracings of single cells treated with H_2O_2 or H_2O_2 in the presence of nifedipine. The lower panel shows the results of pooled data analyzed from greater than three independent experiments; $^*p < 0.05$. *Adapted with modifications from Sartoretto et al. (2012).*

with Lipofectamine (Invitrogen) according to the manufacturer's protocol. The viral titer was determined with Lenti-X GoStix (Clontech), and virus particles were concentrated by polyethylene glycol precipitation with PEG-it solution (SBI Bioscience), according to the manufacturer's protocol. The virus pellet is resuspended in PBS and used immediately for tail vein injections. For the purposes of cell infection, the lentiviral titer does not need to be quite so high, and the viral suspension may be stored at −80 °C with nominal loss of efficacy.

Below we describe our protocols for construction and injection of the HyPer2 lentivirus, and for the analysis of H_2O_2 responses in cardiac myocytes.

5.1. Detailed protocol

The protocol is based on the second-generation lentivirus system developed by Trono (Zufferey, Nagy, Mandel, Naldini, & Trono, 1997). Viruses are generated by the simultaneous transfection of three plasmids: one consisting of an HIV-1 backbone into which the cDNA of interest is cloned; a second plasmid encoding the vesicular stomatitis virus glycoprotein; and a third plasmid expressing the (VSV-G) envelope protein (which determines the viral tropism). This lentiviral system is well suited to generate and deliver the required viral titers. The plasmids are transfected into a suitable producer cell line, typically HEK293-T cells. After transfection, the viral particles are secreted into the cell culture supernatant. This supernatant is then purified, enriched, and stabilized by precipitation with polyethylene glycol. This viral suspension is ready for injection into various animal models or for the infection of cells in culture. The system separates the viral genome into three independent plasmids, thereby ensuring safety and modularity, generating only replication-deficient viral particles; these procedures are performed in Level 2 biological containment facilities. This is a well-established method that has been widely used.

The three lentivirus construction plasmids are all available from Addgene; the HyPer2 plasmid is from Evrogen. The pWPXL plasmid vector comes with an eGFP insert; methods for exchanging the eGFP insert for the gene of interest can be found in standard molecular biology protocol reference. The vector comes in two easy to confound variations (pWPXL and pWPXLd); the difference is in the loxP acceptor sides flanking the gene of interest in the pWPXLd plasmid. This might render the construct unsuitable for the transduction of inducible knockout mice. The inverted terminal repeat regions in this plasmid are prone to bacterial recombination.

Therefore it is not advisable to use classical bacterial strains (e.g., DH5α) for cloning and plasmid preparation, but instead use bacteria designed for the propagation of unstable inserts (e.g., Invitrogen "One Shot Stbl3," or other similar strains). High viral titers are required for efficient expression following mouse tail vein injection: each mouse receives 250 μL of a freshly made virus suspension containing at least 10^8 infectious particles per mL (pfu/mL). To reach these high levels, it is important to implement optimization and quality control procedures along the viral production process.

Time line

The overall production time is 6 days from start to finish, with a total working time of approximately 45 min per day.

Day 1: seeding of producer cells (HEK293-T)

Day 2: transfection of lentiviral constructs

Day 3: visual assessment of transfection efficiency

Day 4: virus collection

Day 5: virus collection

Day 6: virus concentration and injection

5.2. Materials needed for virus production

5.2.1 Plasmid preparation

The viral system can be requested via Addgene (www.addgene.org)

pWPXL (Plasmid 12258), for carrying the transgene of interest

psPAX2 (Plasmid 12260) HIV helper genome

pMD2.G (Plasmid 12259) VSV-G

One Shot® Stbl3 Chemically Competent *Escherichia coli* (Invitrogen C7373-03)

HiSpeed Plasmid Maxi Kit (Qiagen 12662)

5.2.2 Cell culture

HEK293-T cells (ATCC cell line. # CRL-11268)

DMEM high glucose with glutamate (Invitrogen 11995073)

Lipofectamine 2000 (Invitrogen 11668019)

Optimem (Invitrogen 31985070)

5.2.3 Virus titration, concentration, and infection

Lenti-X GoStix (Clontech 631243)

PEG-it Virus Precipitation Solution (SBI LV810A-1)

Hexadimethrine bromide (H9268 Sigma)

5.2.4 Lentivirus production

5.2.4.1 Day 1: Seeding HEK293-T cells

Seed HEK293-T cells by splitting a subconfluent 10 cm plate (p100) 1:5. Pooling the supernatant of 10 virus-producing plates usually generates enough material for one experiment. The seeding should be done ~24 h before transfection into 10 cm plates, each with 8 mL complete growth medium (DMEM high glucose, 10% FBS) and incubated at 37 °C, 5% CO_2.

5.2.4.2 Day 2: Transfection

Before starting transfection of the HEK293-T cells, change to fresh media and check the level of cell confluency under the tissue culture microscope. The cells should be subconfluent (~90% confluent). DNA and Lipofectamine solutions should be prepared separately in 1.5 mL test tubes with a total volume of 50 μL per plate for each transfection. The following plasmids are combined: 1 μg psPax (gag-pol expresser); 1 μg pMDG (VSV-G expresser); and 1.5 μg pWPXL (containing the cDNA of interest). Optimem is then added to a total of 50 mL. For each transfection reaction, add 40 μL Optimem to a second tube and add 10 μL Lipofectamine 2000 into the center of the tube without touching the sides. Do not vigorously vortex or pipet the mix, as this may decrease the transfection efficiency. Incubate the solutions for 15 min at room temperature, then add the DNA to the Optimem/Lipofectamine mix, and stir with the pipet tip until a homogenous suspension is formed. After incubating at room temperature for 15 min, add the transfection mix to the cells in a dropwise manner while agitating the plates by orbital shaking. After 6 h, change the medium a second time and incubate cultures overnight at 37 °C, 10% CO_2.

5.2.4.3 Day 3: Visual assessment of transfection efficiency

Approximately 24 h after transfection, check the efficiency of viral infection via fluorescence microscopy, which permits identification of fluorescent (HyPer2-expressing) cells as well as showing the formation of cell aggregates, which reflect viral infection of the HEK293-T cells. If the infection efficiency is not high enough (fewer than three fluorescent syncytial cell aggregates per field of view at 20 ×), it is unlikely that an adequate viral titer will be obtained; in this case, it is best to discard the cells and start over, possibly with new plasmid preparations.

5.2.4.4 Day 4/Day 5: Virus collection

Harvest the lentivirus by pooling the supernatant from day 4 and day 5. Collect the media into 50 mL tubes, and check the viral titer using Lenti-X

GoStix (Clontech) according to the manufacturer's protocol. If this test is negative, discard the supernatant and start over. If the titer quality is sufficient, remove cellular debris by centrifugation ($1500 \times g$ for 15 min) and add 10 mL of cold PEG-it solution to the supernatant. Mix well, and refrigerate the supernatant at 4 °C for up to 48 h.

5.2.4.5 Day 6: Virus concentration and injection

Centrifuge supernatant/PEG-it mixture at $1500 \times g$ for 30 min at 4 °C. After centrifugation, the virus particles appear as a beige or white pellet at the bottom of the tube. Carefully aspirate the supernatant and resuspend the pellets in 1/100 of the original volume using cold sterile PBS. Pool the virus suspensions and add hexadimethrine bromide solution to a final concentration of 10 μg/mL. Load individual syringes with 250 μL of virus suspension and proceed with the injections, as reviewed below. After adding the hexadimethrine bromide, perform the tail vein injection in less than 30 min. Do not freeze the viral supernatants for subsequent use in tail vein injections, as this may result in a reduction of viral titer. Obviously, this method does not allow time for quantitative viral titer determination, but the semiquantitative assessment of viral titer using Lenti-X GoStix is sufficient. Because it is important to proceed with the injections shortly after the viral enrichment process, a detailed analysis of the viral titer by serial dilution or qPCR is not feasible. We do not use supernatants giving a weak fluorescence signal on day 3 or a negative signal in the "Quicktest." It is better to discard the cells and start over than work with a suboptimal virus stock. For purposes of troubleshooting and quality control, it is useful to save an aliquot of the injected virus solution for quantitative titer determination. For infection of tissue culture cells (when the viral titer does not need to be so high), the virus can be frozen at −80 °C and stored for up to 6 months before use. The key to a high titer virus solution is optimal and healthy HEK293-T cells. Even slight variations in culture conditions can lead to significant fluctuations of virus quality and quantity. Split the cells three times a week at a ratio of around 1:4–1:6, making sure that cells are completely trypsinized to disrupt cell aggregates. Cells should be monitored for mycoplasma infection regularly, as mycoplasma can interfere with transfection efficiency.

For the creation of stable cell lines expressing lentivirus-encoded HyPer2, we infected p100 dishes of HEK293-T cells with the HyPer2 lentivirus ($\sim 5 \times 10^6$ pfu), prepared as described above. After 6 h, media was changed for DMEM containing 10% FBS without phenol red, and cells were incubated for 24 h before splitting. With this procedure, we generated

a stable cell line of HEK293-T cells expressing the HyPer2 biosensor; levels of HyPer2 protein expression are stable through multiple passages, and cells can be frozen down for storage and the thawed cells still retain robust HyPer2 expression. To validate the response of the HyPer2 cell line to H_2O_2, HEK293-T cells stably expressing HyPer2 biosensor were plated in 24-well dishes precoated with polyethylenimine (25 μg/mL) to improve cell attachment. Before starting measurements, media was replaced with dPBS containing 5 m*M* glucose. Fluorescence emission at 520 nm was measured using a fluorescence plate reader (Omega Fluostar, BMG) with 400 and 485 nm excitation filters (Fig. 4.3A). After a period of base line stabilization (due to photoactivation of the YFP fluorophore in HyPer2), the enzyme glucose oxidase (Calbiochem, cat # 345386) was added to the extracellular media to generate fluxes of H_2O_2 (0–15 μ*M*/min). Glucose oxidase activity was determined using commercially available colorimetric kits (e.g., Cayman Chemical). The increase in HyPer2 ratiometric fluorescence (485 nm/400 nm) corresponds to the oxidation of HyPer2, and initial slopes correlate well with H_2O_2 fluxes.

We also utilized HyPer2 lentivirus to infect bovine aortic endothelial cells (BAEC) using the same procedures as described above. BAEC expressing Hyper2 were plated in gelatin-coated 35 mm petri dishes containing 10 mm Microwell #0 coverglasses. After incubating for 4 h in phenol red-free DMEM with 10% FBS to allow cell attachment, the cell media was replaced with Hank's Buffered Salt Solution and live cell imaging was obtained as described before (Pase et al., 2012). Figure 4.3B shows imaging results obtained in BAEC encoding HyPer2 biosensor after the addition of hydrogen peroxide (H_2O_2, 100 μ*M*) to the extracellular media. Once the baseline is established, the addition of H_2O_2 results in a robust increase in fluorescence, monitored and quantitated as described above. BAEC expressing HyPer2 can be used to study the mechanisms of H_2O_2 modulated signaling pathways in endothelial cells. Because BAEC are primary cells, the lentivirus-infected cells are only used for one or two passages following infection with the HyPer2 lentivirus.

For infection of cardiac tissues, the HyPer2 lentivirus is infused through the tail vein (250 μL of 10^8 pfu/mL) of adult male mice (8–10 weeks old). One or two weeks after injection of virus (or saline control), mice are euthanized, and cardiac myocytes are isolated, cultured, and analyzed as described above. Although mouse tail vein injection is a commonly used technique, it takes practice to perform it correctly. Great care has to be taken to confirm

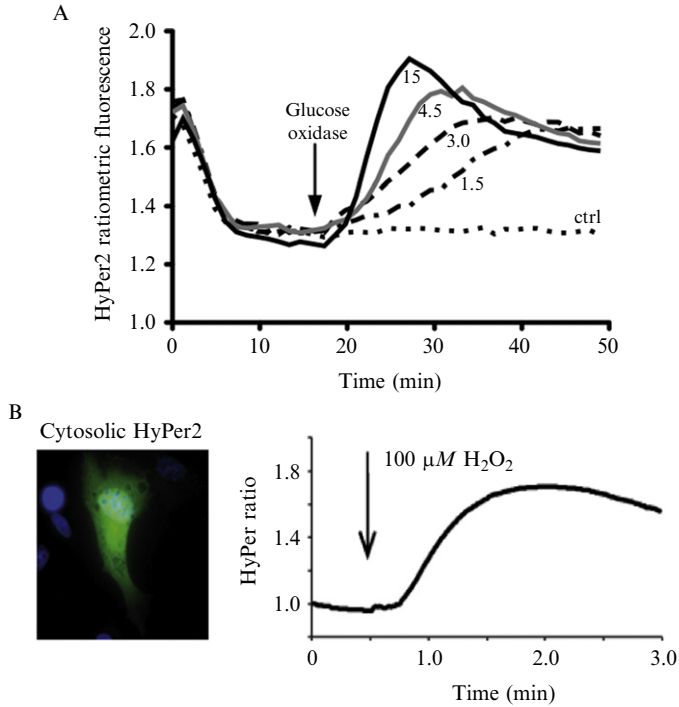

Figure 4.3 Imaging H_2O_2 in cells infected with Hyper2 lentivirus. (A) The results of cellular imaging of HEK293-T cells stably expressing the HyPer2 biosensor. Fluorescence emission at 520 nm was measured using a fluorescence plate reader with 400 and 485 nm excitation filters. After stabilization of the base line, the glucose oxidase was added to the extracellular media to generate H_2O_2 flux (0–15 μ*M*/min), as described in the text. (B) Results obtained in BAEC infected with HyPer2 lentivirus and treated with H_2O_2 (100 μ*M*); images were taken every 5 s. The image shows HyPer2 fluorescence in untreated BAEC that had been infected with the HyPer2 lentivirus; the graph shows a time course of the ratiometric fluorescence change (YFP_{500}/YFP_{420} ratio), revealing a rapid increase in HyPer2 oxidation after adding H_2O_2.

that the injection is indeed into the tail vein (there is an informative demonstration of the tail vein injection technique on line at http://www.youtube.com/watch?v=8MDcyardkmw). Mice have two lateral tail veins, visible along either side of the tail. The mouse is placed in a mouse tail illuminator/restrainer (Braintree Scientific) and the lentivirus is injected using an insulin syringe with a 27-gauge needle. Lentivirus may be diluted with sterile saline to a final volume of 200–250 μL. While injecting, there should be negligible resistance. Two weeks after injection of HyPer2, mouse tissues can be harvested and analyzed for HyPer2 protein expression by probing

immunoblots with an antibody against GFP (e.g., the polyclonal GFP antibody from Cell Signaling); the HyPer2 protein migrates at $M_r = 52$ kDa.

6. IMAGING INTRACELLULAR H_2O_2 IN CARDIAC MYOCYTES AND ENDOTHELIAL CELLS EXPRESSING HyPer2

Lentiviral infection by mouse tail vein injection of the HyPer2 lentivirus leads to a heterogeneous level of HyPer2 expression in cardiac myocytes. In a typical cardiac myocyte preparation following HyPer2 tail vein injection, only approximately 5% of the myocytes express detectable HyPer2. This low proportion of HyPer2-positive cells is further confounded by the high background fluorescence present in cardiac myocytes. The intensity level of this background fluorescence has to be determined first; only cardiac myocytes showing at least twice this fluorescence level are selected for imaging. Following initiation of the live cell imaging protocol, it is important to wait until the baseline signal stabilizes: the first ~30 s of acquired data usually cannot be analyzed because of an optical artifact that arises from the initial photoconversion of YFP (seen as a rapid drop in fluorescence), as has been previously described (Aoki & Matsuda, 2009). The individual imaging conditions (e.g., exposure time and choice of neutral density filters) depend on the specific microscope setup being used. The paper by Pase et al. (2012) contains an extensive description of these considerations. Figure 4.4 shows imaging results obtained in cardiac myocytes isolated from mice after tail vein injection with the HyPer2 lentivirus (Sartoretto et al., 2011). Changes in cell-derived fluorescence were analyzed after treating the cells with hydrogen peroxide (H_2O_2, 10 μ*M*), angiotensin-II (ANG-II, 500 n*M*), or isoproterenol (ISO, 100 n*M*). HyPer2 fluorescence increases after the addition of H_2O_2, serving as a key positive control. ANG-II also promotes a significant increase in HyPer2 fluorescence, but there was no significant increase in HyPer2 fluorescence following the addition of ISO (Fig. 4.4). Both of these agonists promote NOS activation (Sartoretto et al., 2009, 2011), but H_2O_2 plays a differential role in receptor signaling: ANG-II-promoted eNOS activation is dependent on H_2O_2, whereas beta adrenergic receptor activation of nNOS is independent of changes in intracellular H_2O_2. These and similar experimental approaches can be used to probe H_2O_2-dependent signaling pathways in cardiac myocytes and other terminally differentiated cells.

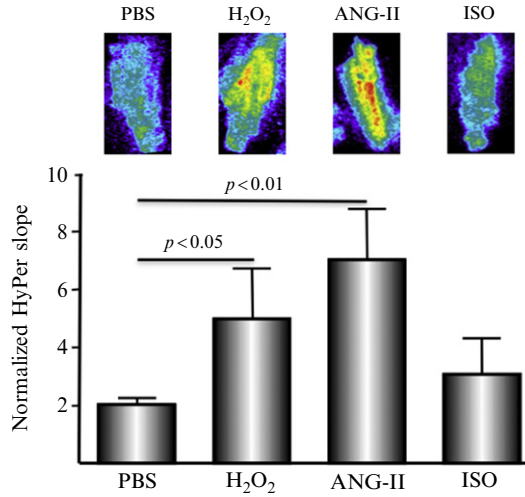

Figure 4.4 Detection of H_2O_2 in cardiac myocytes isolated from mice infected with lentivirus expressing the HyPer2 biosensor. Adult mice were injected via tail vein with lentivirus expressing the HyPer2 H_2O_2 biosensor ($\sim 10^9$ pfu); 2 weeks later the mice were euthanized, and cardiac myocytes were isolated and analyzed. The bar graph shows pooled data from three independent experiments, in which the H_2O_2 response is quantitated as the slope of the fluorescence signal in arbitrary units (AU) measured between $t = 0$ and $t = 5$ min after the addition of 10 μ*M* of H_2O_2, 500 n*M* of ANG-II (angiotensin-II), or 100 n*M* of ISO (isoproterenol). $^*p < 0.05$ compared to PBS-treated cells. Also presented are representative HyPer2 images shown in isolated cardiac myocytes treated as displayed. The HyPer2 H_2O_2 image is determined as the YFP_{500}/YFP_{420} excitation ratio; the gray scale is adjusted to improve contrast. *Adapted with modifications from Sartoretto et al. (2011).*

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