Heat shock-induced attenuation of hydroxyl radical generation and mitochondrial aconitase activity in cardiac H9c2 cells


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Submitted 18 July 2005; accepted in final form 4 September 2005

Heat shock (hyperthermia) induces a family of stress proteins called heat shock proteins (Hsps), such as Hsp25/27, Hsp60, Hsp70, Hsp90, etc., which are known to act as molecular chaperones to perfect the nascent protein folding and refolding of damaged proteins. Earlier studies (32) have identified the involvement of these proteins in various cellular signaling cascades, apart from acting as molecular chaperones. For example, the smaller Hsps, namely Hsp25 and its homologue Hsp27, are known to play protective roles against apoptosis (9, 35, 63) and oxidative stress (14). The role of Hsp90 has been extensively studied using both purified enzymes and cellular systems and it has been shown that it is involved in the activation of nitric oxide (NO) synthase (NOS) (16, 52, 64). It was reported that NOS can be activated by Hsp90 in endothelial cells (21, 31). By using cardiac H9c2 cells, we recently reported that the heat shock-induced Hsp90 activates the endothelial NOS to increase NO generation and the excess NO, produced by such an activation, can block the mitochondrial electron transport chain (ETC) by binding at the cytochrome c oxidase site of the complex IV (26). Hence, the heat shock-induced adaptation of myocytes, to be able to survive at lower oxygen concentration, could be one of the benefits of the heat shock pretreatment. Thus the reduced respiration of myocytes in the intact myocardium of heat-shocked animals might be responsible, at least in part, for the reduced ischemic injury.

Free radicals are known to play important roles in the myocardial ischemic injuries. For example, a burst of reactive oxygen species (ROS) is encountered during the reperfusion of ischemic hearts leading to various cardiovascular dysfunctions. Activation of various enzymes, such as NOS, xanthine oxidase (XO), etc., in the myocardium was also found to induce ROS (47). Recently, the question of the effect of Hsp90-induced activation of recombinant NOS on the magnitude of ROS generation was addressed (51). Another important area, where the role of ROS is vital, is chemodrug-induced cardiotoxicity. Anthracyclines, such as doxorubicin, a widely used chemotherapeutic drug, exhibits an adverse effect of cardiotoxicity, which is reportedly mediated by ROS (10, 18, 48, 55, 60). Numerous studies have been reported on the mechanism of this drug. Although this drug is believed to work through a complex mechanism in killing cancer cells, generation of ROS and the subsequent oxidative damage inevitably causes cell death in the myocardium. A recent study (10) has shown a paradoxically beneficial effect of ROS in protecting the cells. Another important study reported recently has identified that, depending on other cellular reductants, turning it over to be oxidized Fe3+ to Fe2+. These proteins, in combination with the activation of the nitric oxide synthase (NOS) enzyme, play important roles in the protection of the myocardium against a variety of diseases. In the present work we report that the generation of potent reactive oxygen species (ROS), namely -OH in cardiac H9c2 cells, is attenuated by heat shock treatment (2 h at 42°C). Western blot analyses showed that heat shock treatment induced overexpression of Hsp70, Hsp60, and Hsp25. The observed -OH was found to be derived from the superoxide (O2-) generated by the mitochondria. Whereas the manganese superoxide dismutase (MnSOD) activity was increased in the heat-shocked cells, the mitochondrial aconitase activity was reduced. The mechanism of O2- conversion into -OH in mitochondria is proposed as follows. The O2- released from the electron transport chain, oxidatively damages the mitochondrial aconitase, releasing a free Fe2+. The aconitase-released Fe2+ combines with H2O2 to generate -OH via a Fenton reaction and the oxidized Fe3+ recombines with the inactivated enzyme after being reduced to Fe2+ by other cellular reductants, turning it over to be active. However, in heat-shocked cells, because of higher MnSOD activity, the excess H2O2 causes irreversible damage to the mitochondrial aconitase enzyme, thus inhibiting its activity. In conclusion, we propose that attenuation of -OH generation after heat shock treatment might play an important role in reducing the myocardial ischemic injury, observed in heat shock-treated animals.

proteins; free radicals; spin trapping; reactive oxygen species

IT HAS BEEN PREVIOUSLY REPORTED (32) that myocardial ischemic injury is significantly reduced in animals subjected to heat shock before ischemia-reperfusion, indicating a possible therapeutic intervention by a simple heat shock treatment for cardiovascular diseases. Although such an observation of improvement in myocardial recovery was observed previously in the intact myocardium, as well as in isolated cardiomyocytes, the actual mechanism by which this reduction in injury is mediated still remains elusive. Many studies (11, 12, 32, 33, 42, 44) using various myocardial cell lines and isolated heart models have reported different hypotheses on the mechanism of such protection.

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upon the cell type, the mechanism of cell death could be potentially different (60). Thus understanding the mechanism of ROS-induced cell death by doxorubicin and its derivatives is much more complex than it was previously believed (10).

Effects of heat shock treatment on ROS generation by various cell lines have been reported (3, 53, 54, 65). However, no evidence of direct participation of a particular Hsp in the generation/attenuation of ROS has been identified. In other words, no pathway has been identified as the major source of either the stimulation or attenuation of ROS in the heat-shocked cells. It is very important to address this issue in the case of cardiomyocytes as described below. The ROS activates pro-caspase signaling, resulting in apoptotic cell death. Because heat stress (treating cells at 42–45°C) is known to induce anti-apoptotic proteins such as Hsp27, we hypothesized that heat shock treatment would alter ROS generation in the cardiac myocytes. Using EPR spin trapping and flow cytometry measurements, we report here that the cardiac H9c2 cells generate -OH species in normal conditions, which is derived from superoxide (O_2^-) generated in the mitochondrial ETC. The mechanism of mitochondrial generation of -OH has been identified to be associated with the mitochondrial aconitase enzyme. Previous studies (6, 7, 58) with purified aconitase enzymes have reported that the mitochondrial aconitase generates -OH due to oxidative damage by O_2^- . The present study demonstrates that Hsps indirectly regulate this redox chemistry of aconitase in cellular systems.

In the present study, we used a myogenic cell line (cardiac H9c2 cells). Cultured cardiac H9c2 myocytes are from a clonal muscle cell line derived from embryonal rat hearts. Although these cells display certain features of skeletal muscle (22, 30), they retain some features of cardiac muscle such as the expression of a cardiac isoform of creatine phosphokinase, L-type Ca^2+ channels, and the tissue-specific splicing protein smN (17, 23). Previous studies (10, 34) have used this cell line as a model system to evaluate various characteristics of neonatal cardiomyocytes, including the cardiotoxicity effect caused by the anti-tumor drug doxorubicin. Also, previous studies on this cell line revealed that all of the Hsps can be induced by heat shock (43, 44), pharmacological induction (49, 56), or viral vector transduction (8).

**MATERIALS AND METHODS**

The EPR spin traps, namely 5,5’-dimethyl-1-pyrroline-N-oxide (DMPO) and 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide (DEPMPO), were procured from Alexis (San Diego, CA). 5,6-Chloromethyl-2,7’-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) was obtained from Molecular Probes (Eugene, OR). Rotenone, stigmasterol (ATCC, Manassas, VA; CRL-1446) and cultured as described before (26). Monolayer subcultures were carried out in full growth media (ATCC). The growth media contained Dulbecco’s modified Eagle’s medium + 10% FBS + 1% antibiotic (streptomycin + penicillin). For electron paramagnetic resonance (EPR) and Western blot experiments, cells were grown in regular 150 cm^2 culture dishes and for microscopy fluorescence experiments, the cells were grown on glass-bottomed 3 cm-diameter round-bottom culture dishes or on glass coverslips. Experiments were performed when the cultures reached 80–90% confluency.

**Heat shock treatment.** One day before the experiments, the culture dishes with 60–70% confluence were incubated in a water bath at 42°C for 2 or 4 h and then brought back to the incubator and kept at 37°C. All of the experiments described in this manuscript used cells treated for 2 h, unless otherwise stated.

**Cell viability.** Cell viability was determined by TB dye uptake. TB dye is excluded by the viable cells with intact cell membranes. Previous studies with this cell line have indicated that the cell viabilities, measured by TB assay and lactate dehydrogenase (released by ruptured cell membrane of a dying cell through oncosis) assay were in parallel (34). Thus, in the present work, cell viability was assessed by TB exclusion because it is a more rapid procedure to assess the development of oncotic cell death. The cells were incubated for 2 min in the 1× PBS medium added with 0.4% TB, and then counted under a phase-contrast inverted microscope. Viable cells that were able to exclude the dye were counted with the use of a hemocytometer. The percentage of the viable cells over the total cell count was expressed as cell viability. The cell density mentioned throughout the manuscript is the viable cell count.

**Measurement of cellular respiration.** EPR spectroscopy was used for oxygen measurements (EPR oximetry) in the present study (28). EPR oximetry is capable of the determination of O_2 concentration with a resolution of submicromolar concentration in small volumes (10–20 μl) (28). This technique is based on the principle of EPR line broadening by molecular oxygen. Lithium phthalocyanine (LiPc) was used as the oximetry probe. The EPR line width vs. O_2 concentration calibration curve was constructed using known ratios of premixed O_2 and N_2 gases (25). The slope of the calibration curve was 5.8 mG/mmHg. Thus, by measuring the EPR line width, the oxygen concentration in the solution could be obtained at any given time.

EPR oximetry was carried out using a Bruker ER-300 X-band EPR spectrometer fitted with a TM10 microwave cavity. Cell suspensions [in Hanks’ balanced solution (HBS), supplemented with 1 mg/ml glucose] were taken in a 20-μl glass microcapillary tube for EPR measurements. In a typical experiment, a suspension of cardiac H9c2 cells of required cell density was saturated with room air. About 20 μg of LiPc were added to the suspension, incubated for 10 min at 37°C using a water bath and sampled into the 20-μl capillary tube. The capillary tube was sealed at both the ends with the use of clay (Bruker BioSpin, Billerica, MA). While the tube was being sealed, care was taken to ensure that there was no air gap inside the tube that might act as an additional source of oxygen. The sealed tube was placed inside the microwave cavity and EPR spectral acquisitions were started immediately. To avoid the settling of the cells and LiPc particles in the tube, the cavity was rotated to keep the capillary in the horizontal position. Data acquisition was carried out using custom-developed computer software. Because the cells were in a closed tube and the cells continuously consumed O_2, the EPR line width decreased with time. The oxygen concentration in the cell suspension was computed from the EPR line width using a standard curve (O_2 concentration vs. EPR line width).

Selectively permeabilized cells for m-aconitase activity measurement. Previous studies (13, 19) have established that m-aconitase activity can be conveniently studied by respiration measurements with the use of selectively permeabilized cells using digitonin. We used this technique in the present study for the cardiac H9c2 cells. The cells were removed from the culture dish by the usual trypsinization and treated with a required concentration of digitonin (in the range of 0.001–0.01% final concentration) for 10 min on ice. After 10 min, the
permeabilized cells were centrifuged and the supernatant was removed to remove the digitonin. The pellet was resuspended in respiratory buffer composed of 230 mM mannitol, 70 mM sucrose, 30 mM Tris, 4 mM MgCl2, 5 mM KH2PO4, 1 mM EDTA, and 0.1% BSA; final pH 7.4, washed three times (by repeating the centrifuge and removal of supernatant procedure), and finally used in the respiration studies. To measure the effectiveness of permeabilization, the cells were counted with the use of the TB exclusion method.

**EPR spin trapping**. The cells were collected from the culture dishes by trypsinization and the viability was evaluated using TB exclusion method. The cells were resuspended in HBS containing 1 mg/ml glucose, 1 mg/ml bovine serum albumin, and 0.1 mM DTPA, and stored on ice. The required amount of sample was taken from the bulk and incubated at 37°C in a water bath for 15 min and added to 50 mM of spin trap (DMPO or DEPMPO) and transferred immediately into an EPR flat cell and used in the measurements.

**Experimental protocol for drug treatment**. Cell suspensions with the appropriate concentration of drugs were incubated at 37°C in a water bath for 10 min and then drawn into glass capillaries for oxygen consumption measurements or transferred into EPR flat cell. The toxicity of all the agents used in the present work was evaluated by TB exclusion method, at the same experimental conditions used in the experiment. The cell density mentioned in the present work is the viable cell count per milliliter estimated after the addition of a particular drug.

**Western blot analysis**. The cardiac H9c2 cells in culture were washed twice with ice-cold PBS, trypsinized, and centrifuged at 1,500 rpm (5 min). The cell pellet (1.2 x 10^7 cells) was lysed in 1 ml of ice-cold RIPA buffer (PBS, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 10 μl/ml PMSE, 30 μg/ml aprotinin, and 100 mM sodium orthovanadate). The homogenates were then centrifuged at 10,000 rpm for 10 min at 4°C. After pelleting the insoluble debris, the protein content in the supernatant was measured using a Bio-Rad DC protein assay kit. Equal amount of protein samples (100 μg) were loaded in each well. Samples were resolved on 4–12% Bis-Tris PAGE gel and transferred to a nitrocellulose membrane using a Bio-Rad semidry transfer cell. After being blocked with 5% nonfat milk, the blots were probed with rat anti-Hsp70, Hsp60, and Hsp25 (1:2,500 dilution, Stressgen). Goat anti-rabbit horseradish peroxidase-conjugated antibody was used as secondary (1:2,000 dilution) and blots were developed with an enhanced chemiluminescence technique (Amersham). The quantitation of blots was performed with PDQuest (Bio-Rad).

**Flow cytometry**. Apoptosis or necrosis induced by heat shock treatment was studied with the use of Annexin V-propidium iodide (PI) apoptosis kit (Sigma). Cells (H9c2) were subjected to heat shock treatment for 2 h. After 24 h, the cells were trypsinized and suspended in 1.0 ml of 1 X binding buffer. To the suspension were added 5 μl of Annexin V-FITC, followed by the addition of 10 μl of PI in the dark. Dual color analysis was done with the use of FACScalibur flow cytometry by analyzing 10,000 cells (FL1-H filter for Annexin V-FITC and FL3-H filter for PI). This combination detects cells, which are in early apoptotic phase (annexin V positive, PI negative), necrotic cells (annexin V positive, PI positive), and viable cells (annexin V negative, PI negative). As a positive control, Jurkat cells treated with camptothecin (10 μM) for 4 h and double stained with annexin V-FITC and PI, as described above, were used.

**Fluorescence measurements.** ROS generation in H9c2 cells was also analyzed using a fluorescence staining method. CM-H2DCFDA is a cell-permeable colorless compound, which is transformed into a fluorescent dye upon intracellular oxidation. DCFDA is activated by cellular esterases, and then the DCF is converted by hydrogen peroxide, hydroxyl radicals, and peroxidases to the dichlorofluorescein, a fluorescent derivative (10). Subconfluent H9c2 cells were heat shocked at 42°C as per the protocol and incubated at 37°C for 18–24 h. Heat shocked and control cells were washed with ice-cold PBS and loaded with CM-H2DCFDA (10 μM) suspended in serum-free DMEM medium and incubated at 37°C for 45 min. The cells were washed with ice-cold PBS, trypsinized (2.0 ml of 0.25% trypsin/EDTA), followed by the addition of an equal volume of trypsin inhibitor, and centrifuged at 1,500 rpm for 5 min. The cell pellets were resuspended in 1.0 ml of PBS. Fluorescence intensity was measured with the use of a fluorescence-activated cell sorter scan at wavelength 535 nm on the FL1-H channel.

**Manganese superoxide activity.** Manganese SOD (MnSOD) activity in the whole cell lysate was measured using spectrophotometric ferricytochrome c assay (40). The control and heat-shocked cells (2 x 10^6 cells/ml) were lysed using a RIPA buffer and the total protein concentration was determined using a standard curve. The activity of MnSOD was measured in terms of the change in the absorption at 550 nm due to the oxidation of ferricytochrome c by the O_2^- generated by X/O system. The Cu-ZnSOD activity was blocked by adding KCN (5 mM) to the lysate.

**Data analysis.** Data are presented as means ± SE. Statistical analysis was performed using Student’s t-test and one-way ANOVA. The general acceptance level of significance was P < 0.05.

**RESULTS**

**Viability of cells after heat-shock treatment.** The viability of cardiac H9c2 cells subjected to heat shock was determined by TB exclusion test and flow cytometry. The viability, measured by TB assay and expressed as the fraction of viable cells to total cells, did not show any significant difference between the control and heat-shocked cells. The cell viabilities were 84.2 ± 2.8% and 81.6 ± 3.1% (n = 11) for control and heat-shocked cells, respectively. Heat stress at 42°C might also cause apoptosis that cannot be identified by TB assay, which typically detects cells with impaired membrane permeability, a characteristic not present in early apoptotic cell death. Thus flow cytometry with annexin-V-PI staining was performed for both control and heat-stressed cells and obtained results are shown in Fig. 1. There is no significant difference between the control and heat-shocked cells in cell viability. These results showed that the heat shock treatment at 42°C for 2 h was not lethal to cardiac H9c2 cells.

**Generation of ROS in H9c2 cells.** Figure 2A shows the EPR spectrum of DMPO spin adduct obtained from a suspension containing 2 x 10^6 cells/ml. The spectrum contained four characteristic EPR lines with intensity ratio 1:2:2:1, which is a typical EPR spectrum of DMPO-OH spin adduct (5). The amplitude of this EPR spectrum increased with time up to 80 min and then decreased after 80 min (data not shown). The DMPO-OH spectrum, observed in the system containing H9c2 cells, could be the result of direct trapping of the O_2^- or decomposition of the O_2^- spin-adduct DMPO-OOH. This is because the lifetime of the DMPO-OOH is very short [half-life ~1 min (59)], resulting in the decomposition of the spin adduct forming DMPO-OH as one of the breakdown products. To infer more decisively on the actual species formed, we used another spin trap, namely DEPMPO, which gives O_2^- adduct, DEPMPO-OOH, with higher half-life time (~14 min (59)) than that of DMPO. Moreover, the DEPMPO-OOH adduct does not decompose to give DEPMPO-OH adduct. Figure 2B shows the EPR spectrum obtained with DEPMPO under the same experimental conditions as in the case of DMPO. The EPR spectrum of the DEPMPO spin adduct clearly showed a characteristic DEPMPO-OH spin adduct, as shown in Fig. 2. This confirmed that the DMPO-OH spin adduct observed with DMPO is a direct product of O_2^- free radicals generated from the cells.
cellular systems, the \(-\text{OH}\) is derived from \(\cdot\text{O}_2^+\), which is generated from various intra- and extracellular sources.

The source of \(-\text{OH}\) radical generation in cardiac H9c2 cells. Further experiments were designed to identify the source of the \(-\text{OH}\) radicals. As a first step, the effect of PMA, which is a stimulant for increased NADPH oxidase activity, was studied. A dose of 400 ng/ml of PMA did not show any significant change in the DMPO-OH adduct signal intensity (Fig. 3). However, the addition of DPI (5 \(\mu\)M), a flavoprotein binder known to inhibit the NADPH oxidase activity, decreased the signal intensity by 10%. Even this 10% decrease in DMPO-OH adduct signal cannot be attributed to a possible inhibition of NADPH activity because at this dose, DPI is known to inhibit the complex I of the mitochondrial ETC through binding to the flavoprotein in the complex I (36, 37). To further verify this, we carried out the respiration studies. The oxygen consumption in the presence of DPI (5 \(\mu\)M) was decreased by 10%, suggesting that the observed 10% decrease in the DMPO-OH signal intensity is actually due to the inhibition of complex I. Thus the contribution of NADPH-induced \(\cdot\text{O}_2^+\) was not likely the source for the observed \(-\text{OH}\) adduct. The other two major enzymatic sources of \(\cdot\text{O}_2^+\) generation are xanthine oxidase and NOS systems. In another set of experiments, the effect of inhibitors of these enzymes was studied (Fig. 3). The addition of inhibitors of these enzymes, namely, oxypurinol and L-NAME, in the concentration range of 20–100 \(\mu\)M, was not found to significantly alter the DMPO-OH spectral amplitude.

Another important observation is that the addition of 500 U/ml of SOD reduced the signal intensity by \(-\text{OH}\) by \(-\text{OH}\) 20% (Fig. 3). Similarly, the addition of Tiron (40 \(\mu\)M), an intracellular \(\cdot\text{O}_2^+\) scavenger, did not alter the magnitude of the DMPO-OH spin adduct spectrum much and showed only 10% reduction in the amplitude (data not shown). These results indicate that the source of the observed \(-\text{OH}\) formation is mainly the \(\cdot\text{O}_2^+\) generated in the ETC and its conversion to \(-\text{OH}\) in the mitochondria as confirmed below.

Effect of inhibitors of mitochondrial ETC. To identify the role played by mitochondrial ETC in the observed \(-\text{OH}\) generation, the effect of different mitochondrial ETC blockers was evaluated. Figure 4 illustrates the effect of the addition of rotenone (complex I blocker), stigmatellin (complex II blocker), and KCN (complex IV blocker) on the DMPO-OH spin adduct and respiration. The addition of 100 \(\mu\)M KCN abolished 47.3 \(\pm\) 8.7\% (\(n = 3\)) of the DMPO signal intensity, as well as, reduced the respiration by about the same extent. However, addition of complex I and complex II blockers showed different trends in \(\cdot\text{O}_2^+\) consumption and \(-\text{OH}\) genera-

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**Fig. 1.** Viability measured by flow cytometry. A representative histogram of analysis of control (A) and heat-shocked cell (B) stained with Annexin V-FITC and propidium iodide (PI). Bottom left, the gated percentages indicate the viability.

**Fig. 2.** Electron paramagnetic resonance (EPR) spectra of the hydroxyl radical adduct with spin traps. A: 5,5'-dimethyl-1-pyrroline-N-oxide spin adduct (DMPO-OH) formed in H9c2 cells (2 \(\times\) 10^6 cell/ml) in glucose (1 mg/ml)-supplemented Hanks’ balanced solution (HBS) buffer containing 50 mM of DMPO. The experimental conditions were the following: frequency, 9.872 GHz; receiver gain, 5.0 \(\times\) 10^5; modulation frequency, 100 kHz; modulation amplitude, 1.12 G; microwave power, 20 mW; time constant, 0.08192 s; sweep time, 15 s; number of scans, 40. B: EPR spectrum of 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide (DEPMPO)-OH spin adduct measured at the same experimental conditions as those shown in A. The spectral features in both cases indicate the hydroxyl spin adduct. C: EPR spectrum measurement at identical experimental conditions as in A without the cells.
consumption by complex II inhibition by stigmatellin reduced the oxygen to produce O2. Similar studies of DMPO and DEPMPO spin trapping of 
OH generation was carried out with cells subjected to heat shock treatment at 42°C for 2 h, 24 h before the experiment. The viability of the heat-shocked cells was not significantly different from that of the control, suggesting that it is nonlethal, as reported previously (26). The EPR spectral intensity of DMPO-OH observed in the case of heat-shocked cells was significantly lower than the control cells in normal conditions (Fig. 5A), indicating that heat shock treatment attenuated the 
OH generation in the cardiac H9c2 cells. The addition of PMA or DPI did not affect the magnitude of the DMPO-OH signal (Fig. 5B). However, the addition of oxypurinol and L-NAME (20 μM) significantly increased the DMPO-OH signal amplitude in the case of heat-shocked cells (Fig. 5B). Thus the HspS induced after heat shock treatment and NOS play important role in attenuating the 
OH flux from the mitochondria in H9c2 cells.

Heat shock proteins. We estimated the heat-induced increase in the levels of HspS known to be present in mitochondria. Western blots were used to measure the HspS, namely, Hsp70, Hsp60, and Hsp25. These proteins were estimated in the whole cell lysate because it has been reported that even cytosolic HspS can be translocated into the mitochondria and vice versa. Figure 6 shows the Western blots for the controls and cells that were heat shocked for 2 h and 4 h at 42°C. These protein levels in the heat-shocked cells were increased significantly both in 2- and 4-h-treated cells. The quantitative plots, obtained from densitometry as shown in Fig. 6, illustrate that the increase in these protein levels varied by different magnitude. The Hsp70 expression was increased slightly (1.26 ± 0.07) in the 4-h treated sample, whereas not much difference was noticed in the 2-h sample. The expression of Hsp60 was increased 1.59 ± 0.04 times in the 2-h heat-treated sample, whereas it was 1.85 ± 0.06-fold higher for the 4-h heat-shocked samples. Interestingly, in the case of Hsp25, a 3.2 ± 0.05-fold increase for the 2-h heat-shocked cells and a 4.93 ± 0.10 for 4-h heat-shocked samples were noticed (Fig. 6). Overall, the magnitude of HspS was dependent on the duration of the heat shock treatment.

Total ROS measurements. Flow cytometry was used as an alternative method for qualitative characterization of the ROS generation in H9c2 cells. All of the ROS species (O2•−, 
OH, and H2O2) are known to give green fluorescence with DCF. Figure 7 shows the flow cytometry data obtained for control and heat-shocked cells. The comparison between the control and heat-shocked cases showed that the DCF fluorescence intensity was slightly right shifted in the case of heat-shocked cells. This indicates that, the total ROS, including H2O2, is slightly higher in the case of heat-shocked cells. This is likely due to the increased MnSOD activity, as discussed later.

Aconitase activity. The mitochondrial source of 
OH has been identified to be the aconitase, which is involved in the TCA cycle (58). The O2•− leaked in the mitochondrial ETC oxidatively inactivates the m-aconitase by releasing solvent exposed Fe-α (7). The released Fe2+ has been shown to be involved in the Fenton type reaction with H2O2 also formed in such O2•−-induced inactivation of m-aconitase (6, 7, 58). Because this mechanism has been well established, we carried out experiments to verify whether there is any change in the mitochondrial aconitase activity in heat-shocked cardiac H9c2 cells. For this purpose, we used the procedure previously applied to tumor cells and neoplastic cells, where the respiration of the mitoblast is measured in the presence of different conditions.
respiratory substrates. Permeabilization of H9c2 cells were carried out by using digitonin, as described in MATERIALS AND METHODS. When eukaryotic cells are treated with digitonin, the plasma membrane and the outer membrane of the mitochondria are selectively permeabilized due to the high cholesterol-to-phospholipid ratio. Once permeabilized, the cytosolic proteins are released by centrifuging the permeabilized cells and thus allowing the mitochondrial substrates to permeate into the inner mitochondrial membrane for respiration. The cardiac cells were treated with various concentrations of digitonin (0.001–0.01%) and TB exclusion was used to measure the cell permeability. At digitonin concentrations beyond 0.002%, almost 100% of the cells were permeabilized. However, the respiration control index, measured at higher digitonin concentrations (>0.003%), was low indicating the damage to the mitochondria. Thus the digitonin concentration of 0.002% was used in further studies.

In the first set of experiments, the citrate-dependent respiration (aconitase activity) was measured in the presence of 5 mM citrate alone and in the presence of 5 mM citrate and 1 mM ADP as substrates. Figure 8 illustrates the respiration (O2 concentration vs. time) of mitoblasts under these conditions. As shown in Fig. 8, in the case of control mitoblasts, a significantly increased respiration was observed in the presence of ADP. On the other hand, in the heat-shocked case, there was no increase in the respiration in the presence of ADP, indicating that the aconitase activity was reduced by the heat shock treatment and thus the observed decrease in the \( \cdot \text{OH} \) generation in the case of heat-shocked cells was indeed due to the decrease in the activity of aconitase in the heat-shocked cells.

Complex I and complex II activities. The complex I activity was also measured for the mitoblasts, prepared for aconitase activity, by supplementing isocitrate as a substrate, which bypasses the aconitase enzyme in the TCA cycle, as illustrated in Fig. 8. In these studies, the concentrations of the substrates were 5 mM isocitrate alone, and 5 mM isocitrate and 1 mM ADP combined. There was no significant difference between the heat-shocked and controls, observed in the complex I...
activity as shown in Fig. 8. Similarly, the complex II activity was also measured using succinate with and without ADP (state IV and state III) respirations. The results showed no significant difference between the control and heat-shocked cases (data not shown).

MnSOD activity. The mitochondrial SOD, namely MnSOD, activity has been previously shown to be sensitive to the induction of Hsps, such as Hsp70 (57) and Hsp25 (63). In the present work, the MnSOD activity was measured to determine the effect of heat shock. Figure 9 shows the relative MnSOD activity for control and heat-shocked cells. The MnSOD activity was about three times higher in the case of heat-shocked cells compared with control.

DISCUSSION

The present study established that a mild and nonlethal heat shock treatment to the cardiac H9c2 cells at 42°C for 2 h reduced the free radicals flux from the mitochondrial ETC, perhaps as a protective mechanism of oxidative stress. This is an important observation in the sense that cardiomyocyte deaths are either initiated or mediated by the potent ·OH in many pathophysiological conditions. Previous studies on the effect of heat shock on the generation of ROS in various cells have reported conflicting results. Zuo et al. (65) reported that intra- and extracellular ROS (from mitochondria and NADPH oxidase as sources, respectively) are produced during heat stress in the diaphragm muscle. Other reports with H9c2 cells (54), CCL39 (53), and rat intestinal epithelial cells (15) have also observed a similar increase in ROS after heat-shock treatment. On the other hand, the observation of the inhibition of ROS generation by human neutrophils after heat shock treatment has also been reported (38, 39). These studies were carried out either immediately after the heat shock or during heat stress. However, to show the role of heat shock proteins, the experiments need to be carried out 24 h after heat shock treatment, because, as mentioned before, the induction of heat shock proteins after hyperthermia requires 18 h. The present study has investigated the role of Hsps in the ROS generation and shows evidence that Hsps are regulating the mitochondrial ·OH generation. The EPR spin trapping study and the microscopic fluorescence studies have demonstrated that the superoxide generated from the ETC is converted to hydroxyl free radicals by a mechanism, which is influenced by Hsps.

The EPR spin trapping with both DMPO and DEPMPO have shown spectral characteristics corresponding to ·OH species. Considerable ambiguity is generally expressed on the resulting DMPO-·OH spin-adduct spectrum, especially when used in cellular systems (59). Questions such as whether these spin traps can penetrate the plasma membrane to get into the cells, or whether the free radicals can leak out of the membrane and

![Figure 5](http://www.ajpcell.org/)
any evidence that any one of these enzymes could be a source of \( \cdot \text{OH} \) radicals (Fig. 3). On the other hand, our studies with different ETC blockers have shown that when the ETC complexes I and II were blocked, the \( \cdot \text{OH} \) generation increased. Superoxide leak in ETC has been known and documented in the literature. However, the present study with cardiac H9c2 cells showed that \( \cdot \text{OH} \) is formed in the mitochondria. Thus it is evident that \( \text{O}_2^{\cdot} \) is converted into \( \cdot \text{OH} \) within the mitochondria. Recently, it was reported that \( \text{O}_2^{\cdot} \) formed in the mitochondria can be converted to \( \cdot \text{OH} \) by free \( \text{Fe}^{2+} \), released by the mitochondrial aconitase, resulting m-aconitase as a permanent source of \( \cdot \text{OH} \) species (58). According to this mechanism, aconitase is subjected to oxidative damage by \( \text{O}_2^{\cdot} \) and the solvent exposed Fe-\( \alpha \) is released from the [4Fe-4S]\(^{2+} \) cluster, resulting in [3Fe-4S]\(^{1+} \), a free \( \text{Fe}^{2+} \) and a \( \text{H}_2\text{O}_2 \) molecule (7). The released free \( \text{Fe}^{2+} \) reacts with the \( \text{H}_2\text{O}_2 \), also formed in the inactivation process, to generate \( \cdot \text{OH} \) and \( \text{Fe}^{3+} \), which can then be reincorporated into the cluster after reduction to \( \text{Fe}^{2+} \) by cellular reductants, as illustrated in Fig. 10 (7, 58). Such \( \text{O}_2^{\cdot} \)-induced damage is completely reversible, suggesting that m-aconitase may be an intramitochondrial sensor of redox status. The externally added bolus \( \text{H}_2\text{O}_2 \) was also observed to cause similar oxidative damage releasing Fe-\( \alpha \), but the damage, however, is irreversible (58). Extending this mechanism to the present study, we propose that the observed \( \cdot \text{OH} \) in the H9c2 cells is the result of the mitochondrial \( \text{O}_2^{\cdot} \)-induced oxidative damage to the aconitase and the subsequent reaction between \( \text{Fe}^{2+} \) and \( \text{H}_2\text{O}_2 \) formed during the aconitase inactivation. However, the decrease in \( \cdot \text{OH} \) generation and reduced m-aconitase activity, observed in heat-shocked cells, could be explained from the difference in the Mn-SOD activity as follows. The Mn-SOD activity in heat-shocked H9c2 cells was higher than controls. This leads to a higher (Fig. 9) concentration of \( \text{H}_2\text{O}_2 \) in these cells, and might cause irreversible damage to the m-aconitase. The Hsp25 and Hsp70 family of proteins have been reported to increase the induction and activity of Mn-SOD (45, 57, 63). It can be noticed that the Hsp25 level is increased in the heat-shocked cells, as demonstrated in Fig. 6. However, the Hsp70 measured in the present work did not show any significant difference between the control and heat-shocked cells (Fig. 6). This is consistent with the results reported previously that 42°C heat shock does not.

Fig. 6. Western blot analyses of Hsps in control and heat-shocked (HS) cells. Hsp70, Hsp60, and Hsp25 were blotted from lysates of control, 2- and 4-h heat-shocked cells. In the case of heat-shocked cells, the cells were heat shocked at 42°C for 2 or 4 h, followed by a 24-h incubation at 37°C. Heat shocked HeLa cells (StressGen) was used as positive control (PC). The quantitative plots were obtained from the densitometry measurements. The quantitative bar charts presented as relative intensity with respect to control (H9c2 cells not subjected to heat shock). The error bars were obtained from 3 independent measurements.

get trapped by the spin traps, are still being debated. A recent study (1) has pointed out that at appropriate concentrations (e.g., 50 mM, as in the case of present study), DMPO as well as DEPMPO can penetrate into the cells and get equilibrated in the organelles within 10 s. Another study, in which a new spin trap, namely BMPO, was used to trap the intracellular \( \cdot \text{OH} \) generated in doxorubicin-treated bovine aortic endothelial cells, demonstrated that the hydroxyl spin adduct is intracellularly formed (29). Thus with the use of spin trapping, it is possible to trap the \( \cdot \text{OH} \) radicals generated in the intracellular organelles, such as mitochondria. The DEPMPO-\( \cdot \text{OH} \) adduct spectrum observed in the present work is a clear indication that \( \cdot \text{OH} \) is generated in cardiac H9c2 cells (Fig. 2).

Inhibitors/stimulators of various enzymes, which can produce ROS like NADPH oxidase, XO, and NOS, failed to show
Studies (61, 62) have shown that heat shock also induces various stress-induced protein kinases, such as MAP kinases, which can phosphorylate the Hsp25 to functionalize. We have carried out elaborate studies on the phosphorylation status of Hsp25, using 2D gel electrophoresis and

Fig. 8. Measurements of aconitase activity. The cardiac H9c2 cells were selectively permeablized using digitonin (mitoblasts) and used for respiration studies. Top, schematics of role of aconitase in the TCA cycle. Middle, oxygen concentration change with time for mitoblast prepared from control cells with citrate alone and citrate and ADP (left) and for the mitoblasts obtained from heat-shocked cells (center). Quantitative measures of the activity for control and heat-shocked cells from three independent measurements (right). Aconitase activity is reduced in the case of heat-shocked cells. Bottom, complex I activity measurements for mitoblast prepared from control and heat-shocked cells. Respiration was measured in the presence of isocitrate alone and isocitrate + ADP as indicated in the figure; quantitative measurements of the activities measured over 3 independent measurements. There is no significant difference in the complex I activity between control and heat-shocked cells.

induce Hsp70 (2, 22). Studies (61, 62) have shown that heat shock also induces various stress-induced protein kinases, such as MAP kinases, which can phosphorylate the Hsp25 to functionalize. We have carried out elaborate studies on the phosphorylation status of Hsp25, using 2D gel electrophoresis and

Fig. 9. Activity of MnSOD measured by spectrophotometric assay. The activity of MnSOD was measured as described in the experimental procedure. The activity was significantly higher in the heat-shocked cells (\(*P < 0.05\)). Error bars indicate means ± SE of 3 independent runs.

Fig. 10. Schematic illustration of m-aconitase inactivation by superoxide and \(-\)OH generation (data adapted from Ref. 58). Fe^{2+} and H_{2}O_{2}, formed as a by product in the O_{2}^-/superoxide-induced oxidative stress to the m-aconitase, generate the \(-\)OH species. In the heat-shocked cells, higher levels of Hsp70, Hsp60, and Hsp25 increase the activity of MnSOD, leading to higher concentration of H_{2}O_{2}, resulting in irreversible damage, as shown by less aconitase activity in these cells. RSH, thiol.
Matrix-assisted laser desorption/ionization-Time of flight and the results have confirmed phosphorylation of Hsp25 by heat shock (Cardounel AJ, Zweier JL, Venkatakrishnan CD, Kuppusamy P, and Ilangoan G, unpublished observations). Thus the activation of Hsps and heat shock induced excess activity of MnSOD, which generates increased H$_2$O$_2$ (which is known to irreversibly inactivate the m-aconitase), are likely responsible for the observed attenuation of ·OH species in heat-shocked cells.

In addition to this, as we reported recently, the NO concentration is increased in the heat-shocked cardiac H9c2 cells due to the NOS activation by the heat shock induced Hsp90 (26). Thus NO or peroxynitrite-induced inactivation of ETC or m-aconitase activity cannot be completely ruled out. However, in the present work, the addition of the NOS inhibitor, namely 1-NAME, did not reduce the ·OH generation. Indeed, it significantly increased the ·OH generation (Fig. 5B). The increase in the ·OH generation can be explained as follows. Recently, we reported that heat stress-induced Hsp90 attenuated the cellular respiration (26). The Hsp90 activates the eNOS and iNOS, upregulating the NO, which, in turn, competitively binds at the cytochrome c site of complex IV in the mitochondrial ETC (26). We also noticed that the addition of 1-NAME recovered the reduced attenuation to control level. Thus the increased ·OH generation in the presence of 1-NAME might be due to the increased respiration of the cells proportionately increasing the ·OH radicals. Alternatively, it is also possible that 1-NAME eliminates the peroxynitrite-induced inactivation of m-aconitase. Moreover, the aconitase activity measured in the present studies by the respiration measurements has indicated that the heat-shock treatment reduced the activity. Thus the observed difference in the ·OH generation can be attributed mainly to the difference in the m-aconitase activity. The Hsps generated in the heat stress could result in the attenuation of m-aconitase activity indirectly by enhancing the MnSOD activity as proposed in Fig. 10. Thus the NOS contribution is less likely to be significant in the observed ·OH generation.

In the present work, we observed that although a significant reduction in the aconitase activity is noticed, interestingly, there is no difference in the complexes I and II of ETC. Sammut et al. (50) have reported that the mitochondrial complexes I–V activities are increased in postischemia reperfusion for hearts isolated from hyperthermia-subjected animals. However, they did not notice any difference between the control and heat-shocked cases in normal conditions, which is in agreement with our present results. There is no prior report in the literature on direct observation of the influence of Hsp on the m-aconitase activity. However, some studies (24) have reported that Hsp60 and Hsp10 that are located in the mitochondria can protect the ETC by significantly reducing ROS generation. The Hsp60 and Hsp10 overexpressed rat neonatal myocytes showed very good resistance to ischemia-reperfusion injury, indicating a reduced ROS generation in these cells (24).

As of now, there is no evidence showing the direct association of the m-aconitase with Hsps. However, its binding with murine hepatitis virus RNA has been found to be in association with mitochondrial Hsp70, Hsp60, and Hsp40 (46). Thus the higher concentration of H$_2$O$_2$, due to higher MnSOD activity in heat-shocked samples and its oxidative damage to m-aconitase appears to be the primary mechanism by which the ·OH formation is reduced.

In conclusion, the mitochondrial ·OH generation has been observed in cardiac H9c2 cells using EPR spin trapping and fluorescence imaging techniques. The slow generation of ·OH originates in the mitochondria by forming a Fenton-type reaction between the Fe$^{2+}$, released by the m-aconitase. This is due to the oxidative damage by the ETC leaked O$_2^\cdot$ and the H$_2$O$_2$, also formed in the inactivation process. The present study has revealed that the mitochondrial generation of ·OH in the cardiac H9c2 cells was attenuated by nonlethal heat shock treatment at 42°C. Such an attenuation of O$_2^\cdot$ generation in heat-shocked cells was due to the increased generation of H$_2$O$_2$ by the higher MnSOD activity in the mitochondria. This is known to cause irreversible oxidative damage to aconitase. Thus the m-aconitase activity in the heat-shocked cells is lesser than the control. Although the m-aconitase activity was reduced, complex I and complex II activities in the ETC remain unaltered by the heat shock treatment. The MnSOD activity was also found to be increased in the heat-shocked cells. On the basis of the results obtained in the present work and previous results reported in the literature by others (6, 7, 58), we propose that a possible binding of some of the small heat shock proteins, such as Hsp60, Hsp25, or Hsp10 (which are increased after heat stress) to MnSOD, increasing its activity to generate H$_2$O$_2$, irreversibly inactivates the m-aconitase enzyme. Further work is underway to delineate the roles of different Hsps on the MnSOD activity. The potential limitations of the present work, however, are as follows. The magnitude of ·OH radical generation is generally considered to be very weak in most cell lines, compared with the H9c2 cells used in this study, and hence any mechanism of attenuation of such a weak generation is of limited relevance. Thus this mechanism should be validated to a different model where ROS are actually generated by a well-studied mechanism. Perhaps the results presented in this study have potential applications for the use of hyperthermic treatment in the attenuation of ROS generation in such conditions as ischemia/reperfusion injury or the adverse effects of antitumoral drugs (10). Another limitation of the present study is that the mechanism of ·OH generation and attenuation suggested in the present work is based on many indirect evidences. To substantiate this mechanism, studies with transgenic cell lines, e.g., cells overexpressing MnSOD or Hsps, are required.

ACKNOWLEDGMENTS

We thank Yeong-Renn Chen for useful comments and discussion on the mitochondrial respiration studies.

GRANTS

This study was supported by National Institutes of Health Grant R21 EBO04658 and American Heart Association Grant BGIA 0365203B.

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