Heat shock regulates the respiration of cardiac H9c2 cells through upregulation of nitric oxide synthase

Govindasamy Ilangoovan, Sola Osinbowale, Anna Bratasz, Mary Bonar, Arturo J. Cardounel, Jay L. Zweier, and Periannan Kuppusamy

Center for Biomedical EPR Spectroscopy and Imaging, The Davis Heart and Lung Research Institute, and Division of Cardiovascular Medicine, Department of Internal Medicine, The Ohio State University, Columbus, Ohio 43210

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Heat shock regulates the respiration of cardiac H9c2 cells through upregulation of nitric oxide synthase. Am J Physiol Cell Physiol 287: C1472–C1481, 2004; doi:10.1152/ajpcell.00103.2004.—Mild and nonlethal heat shock (i.e., hyperthermia) has been shown to protect the myocardium and cardiomyocytes against ischemic injury. In the present study, we have shown that heat shock regulates the respiration of cultured neonatal cardiomyocytes (cardiac H9c2 cells) through activation of nitric oxide synthase (NOS). The respiration of cultured cardiac H9c2 cells subjected to mild heat shock at 42°C for 1 h was decreased compared with that of control. The O2 concentration at which the rate of O2 consumption is reduced to 50% was increased in heat-shocked cells, indicating a lowering of O2 affinity in the mitochondria. Western blot analyses showed a fourfold increase in the expression of heat shock protein (HSP) 90 and a twofold increase in endothelial NOS (eNOS) expression in the heat-shocked cells. Immunoblots of eNOS, inducible NOS (iNOS), and neuronal NOS (nNOS) in the immunoprecipitate of HSP90 of heat-shocked cells showed that there was a sevenfold increase in eNOS and no changes in iNOS and nNOS. Confocal microscopic analysis of cells stained with the NO-specific fluorescent dye 4,5-diaminofluorescein diacetate showed higher levels of NO production in the heat-shocked cells than in control cells. The results indicate that heat shock-induced HSP90 forms a complex with eNOS and activates it to increase NO concentration in the cardiac H9c2 cells. The generated NO competitively binds to the complexes of the respiratory chain of the mitochondria to downregulate O2 consumption in heat-shocked cells. On the basis of these results, we conclude that myocardial protection by hyperthermia occurs at least partly by the pathway of HSP90-mediated NO production, leading to subsequent attenuation of cellular respiration.

nitric oxide synthase activation; electron paramagnetic resonance oximetry; nitric oxide

MILD HEAT SHOCK, in TERMS OF HYPERTHERMIA, has been shown to protect the myocardium and cardiomyocytes from injuries such as oxidative stress, ischemia-reperfusion damage, or lethal heat shock (25). In a typical experiment, cultured cells, an isolated organ, or the whole body is exposed to an elevated temperature for a defined period and then brought back to normal conditions. Such treatment induces heat shock proteins, which can accumulate with time and play protective roles against various injuries. Studies of isolated rat hearts revealed that hearts subjected to elevated perfusate temperatures were protected from ischemic injury (2, 8). Similar studies using the whole body also showed that the hearts of rats exposed to elevated temperatures were more resistant than those of controls to ischemic injury (9). Despite the fact that the hyperthermia-induced stress proteins are involved in various cellular signaling activities (23, 24), the complete mechanism of such protection is not yet understood.

The predominantly induced heat shock proteins (HSP), namely, HSP27, HSP70, and HSP90 (named after the molecular mass of each in kilodaltons), act as chaperones. Detailed investigations into the roles of these chaperones in the process of apoptosis have been performed (25). These proteins are involved in different stages of caspase-9-mediated apoptosis (25). HSP27 prevents the release of cytochrome c from the mitochondria, while HSP90 has been shown to prevent cytochrome c-Apf-l complex formation. HSP70 prevents the recruitment of pro-caspase-9 elements. On the other hand, HSP90 has been identified as a signaling molecule in the activation of all the isoforms of nitric oxide synthase (NOS) (14, 15). HSP90 binds with NOS and facilitates its phosphorylation, and this in turn increases NO production from the enzyme. Many studies in which isolated and purified NOS and HSP90 were used have demonstrated the effect of HSP90 on NOS function (33, 34, 37, 38). With regard to cellular studies on the effect of HSP90 on NOS activity, only very limited information has been reported. A recent study of bovine aortic endothelial cells indicated that moderate heat shock induced the expression of endothelial NOS (eNOS) and increased NO production (14). Studies such as the one by Harris et al. (14) with cellular systems or isolated organs rather than purified enzymes assume importance because the activation of NOS results in a higher level of NO production, which can play important roles as a physiological regulator in vivo.

Because HSP90 is known to enhance NO production by NOS (34), we hypothesized that the level of NO should be higher in cardiomyocytes subjected to heat shock than in the untreated control. Under such conditions, NO is expected to interfere with cellular respiration. We further hypothesized that the higher levels of NO might cause reversible and competitive binding to cytochrome c oxidase (complex IV) directly or that the downstream products of NO, for example, peroxynitrite (ONOO−) and nitrite (NO2−), could irreversibly block the other complexes of the respiratory chain in the mitochondria, thereby reducing O2 consumption (VO2) in the heat-shocked cardiomyocytes. To verify this hypothesis, we performed VO2 studies in heat-shocked and control cardiac H9c2 cells using electron paramagnetic resonance (EPR) oximetry (19). This technique...
is capable of yielding high-resolution O$_2$ data similar to the data normally obtained with high-resolution respirometry (13) or phosphorescence quenching-based optical probes (32). We used this technique because it is capable of providing measurements in small volumes of cell suspensions (10–20 μl) containing as few as 10,000 cells (19).

The cultured cardiac H9c2 myocytes used in the present work were from a cloning muscle cell line derived from embryonal rat hearts. Although these cells display certain features of skeletal muscle (16, 21), they retain some features of cardiac muscle, such as expression of a cardiac isoform of creatine phosphokinase, L-type Ca$^{2+}$ channels, and the tissue-specific splicing protein Smn (12, 17). Previous studies in the literature have used this cell line as a model system to evaluate various characteristics of cardiomyocytes, including the cardiotoxicity caused by the antitumor drug doxorubicin (7, 26). Also, previous studies with this cell line revealed that all HSP can be induced by heat shock (28, 29), pharmacological induction (31), or viral vector transfection (5). In the present work, we have used this technique because it is capable of providing measurements in small volumes of cell suspensions (10–20 μl) containing as few as 10,000 cells (19).

In the present work, the cells were grown on glass-bottomed 3-cm-diameter round-bottomed culture dishes or on glass slides for confocal microscopic experiments, the cells were grown on glass slides. MATERIALS AND METHODS

Materials

Nitro-l-arginine methyl ester (l-NAME), geldanamycin (GA), and bradykinin (BK) were obtained from Sigma. 4,5-Diaminofluorescein diacetate (DAF-2DA) was purchased from Alexix Biochemicals (San Diego, CA). Myxothiazole was purchased from Fluka Biochimica (Buchs, Switzerland). The monoclonal antibodies for Western blot and immunoblot analyses were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Methods

Cell culture. Cardiac H9c2 cells (undifferentiated neonatal rat cardiomyoblasts) were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Monolayer subcultures were performed in full-growth medium (ATCC). The growth medium contained Dulbecco’s modified Eagle’s medium, 10% FBS, and 1% antibiotic (streptomycin + penicillin). For oximetry and Western blot experiments, cells were grown in regular 75-cm$^2$ culture dishes, and for confocal microscopic experiments, the cells were grown on glass-bottomed 3-cm-diameter round-bottomed culture dishes or on glass coverslips. Experiments were performed when the cultures reached 80–90% confluence.

Heat shock treatment. One day before the experiments, the culture dishes were incubated in a water bath at 42°C for 1 h and then brought back to the incubator and kept at 37°C. The 42°C temperature was chosen because it has been reported that HSP90 is more specific at this temperature and that at higher temperatures HSP70 is predominantly induced (2, 16).

Cell viability. Cell viability was determined by Trypan blue (TB) dye uptake. TB dye is excluded by the viable cells with intact cell membranes. Previous studies with this cell line indicated that the cell viabilities, measured by TB assay and lactate dehydrogenase (LDH) assay (released by ruptured cell membrane of dying cell through oncosis), were parallel (26). Thus, in the present work, cell viability was assessed by TB exclusion because it is a more rapid procedure for assessing the development of onotic cell death. The cells were incubated for 2 min in the medium added with 0.4% TB and then observed under a light microscope. Viable cells that were able to exclude the dye were counted using a hemocytometer. The percentage of the viable cells over the total cell count was expressed as cell viability. The cell density mentioned throughout this article is the viable cell count.

Measurement of cellular respiration. EPR-based oximetry was used for O$_2$ measurements in the present study (19). EPR oximetry is capable of determining O$_2$ concentration ([O$_2$]) with a resolution of submicromolar concentration in small volumes (10–20 μl) (19). This technique is based on the principle of EPR line broadening by molecular O$_2$. Lithium phthalocyanine (LiPc) was used as the oximetric probe in the present study. The EPR line width vs. [O$_2$] calibration curve was constructed using known ratios of premixed O$_2$ and N$_2$ gases (18). The slope of the calibration curve was 8.6 mG/mmHg. Thus, by measuring the EPR line width, the [O$_2$] in the solution was obtained at any given time.

EPR oximetry was performed using a Bruker ER-300 X-band EPR spectrometer fitted with a TM110 microwave cavity. Cell suspensions were obtained in a 20-μl glass microcapillary for EPR measurements. In a typical experiment, a suspension of cardiac H9c2 cells of required cell density was saturated with room air. LiPc (≈20 μg) was added to the suspension, incubated for 10 min at 37°C in a water bath, and sampled into the 20-μl capillary. The capillary was sealed at both ends using clay (Bruker BioSpin, Billerica, MA). While sealing, care was taken to ensure that there was no air gap inside the tube that might act as an additional source of O$_2$. The sealed tube was placed inside the microwave cavity, and EPR spectral acquisitions were started immediately. To avoid the settling down of the cells and LiPc particles in the tube, the cavity was rotated to keep the capillary in the horizontal position. Data acquisition was performed using custom-developed personal computer software. Because the cells were in a closed tube and continuously consumed O$_2$, the EPR line width decreased with time. The [O$_2$] in the cell suspension was computed from the EPR line width using a standard curve.

Experimental protocol for drug treatment. Cell suspensions with appropriate concentrations of l-NAME, GA, BK, or myxothiazole were incubated at 37°C in water bath for 10 min and then drawn into the glass capillaries for V$_{O_2}$ measurements. The toxicity of these agents was evaluated using a TB exclusion method under the same experimental conditions used for the V$_{O_2}$ studies. Cell densities in the present study are expressed as the viable cell counts per milliliter estimated after the addition of a particular drug.

Determination of O$_2$ kinetics at low [O$_2$]. Although a constant rate of respiration was observed at high [O$_2$] (>5 μM), the rate of respiration was altered at low [O$_2$]. A characteristic cellular respiration parameter, p50, was defined as the concentration of O$_2$ at which V$_{O_2}$ rate was reduced to 50%. This is analogous to the K_m values in purified enzymatic reactions. The p50 value was determined from the oximetry curve using the procedure reported for the Oroboros oxigraph (Oroboros Instruments, Innsbruck, Austria) (6, 13). The change in [O$_2$] with time was converted to a V$_{O_2}$ rate vs. [O$_2$] plot, and the resultant data were used to fit the equation

$$\frac{V_{O_2}}{V_{O_2,max}} = \frac{P_{O_2}}{P_{O_2} + P_{O_2}}$$

(1)

where V$_{O_2}$ is the O$_2$ consumption rate at a given concentration (P$_{O_2}$) and V$_{O_2,max}$ is the maximum oxygen consumption rate. The parameters V$_{O_2,max}$ and p50 were obtained using nonlinear least-squares fitting of the data.

Western blot analysis. The cardiac H9c2 cells in culture were washed twice with ice-cold PBS, trypsinized, and centrifuged at 3,000 rpm. The cell pellet (1.2 × 10^7) was homogenized in 1 ml of ice-cold RIPA buffer (PBS, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml PMSF, 30 μg/ml aprotinin, and 100 mM sodium orthovanadate). The homogenates were then centrifuged at 10,000 g for 30 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. Samples were resolved on 4–12% Bis-Tris polyacrylamide gels and transferred to a nitrocellulose membrane using a Bio-Rad semidry transfer cell. After blocking with 5% nonfat milk, blots were
probed with a rabbit anti-HSP90 (1:2,500 dilution; Santa Cruz Biotechnology). Goat anti-rabbit horseradish peroxidase-conjugated antibody was used as secondary (1:2,000 dilution), and blots were developed with enhanced chemiluminescence (ECL; Amersham, Piscataway, NJ).

**Immunoprecipitation of NOS and immunoblotting.** To determine the expression of various isoforms of NOS in control and heat-shocked cardiac H9c2 cells, immunoprecipitation of different isoforms of NOS was performed using the protein A agarose. Total protein (1,000 μg) was incubated for 24 h on a rotator at 4°C, with 20 μl of protein A and respective monoclonal antibody against NOS in 50 mM Tris, pH 7.4. After incubation, the sample was centrifuged and the pellet was resuspended in 40 μl of 2× electrophoresis sample buffer, boiled, and subjected to electrophoresis as described in Western blot analysis.

**Coimmunoprecipitation and immunoblotting of NOS.** The cells were lysed and the insoluble debris was removed by centrifuging at 10,000 rpm, and the protein content was measured as described in Western blot analysis. Total cellular protein (~1,000 μg) was added to 10 μl of primary antibody against HSP90 and incubated for 24 h with protein G agarose at 4°C. The immunoprecipitate was pelleted by centrifuging for 30 s at 4°C and then carefully aspirated, and the supernatant was discarded. The pellet was washed three times with RIPA buffer, each time repeating the centrifugation step. After the final wash, the pellet was further aspirated and the supernatant was discarded. The pellet was resuspended in 40 μl of 2× electrophoresis sample buffer. The sample was boiled for 5 min and loaded onto gels. Western blotting was performed using corresponding monoclonal antibodies [eNOS, inducible NOS (iNOS), or neuronal NOS (nNOS)].

**Confocal microscopy.** NO production in cardiac H9c2 cells was analyzed using confocal microscopic fluorescence imaging. DAF-2DA, a green fluorescence probe specific for intracellular NO or its oxidation products, was used. DAF-2DA (10 μM) was added to control and heat-shocked cells, suspended in serum-free culture medium, incubated for 15 min at 37°C, and then trypsinized and pelleted. The cells were resuspended in the medium, plated on a glass slide, and covered with a glass coverslip. The fluorescence measurements were obtained using a Zeiss LSM510 multiphoton confocal inverted microscope (excitation, 492 nm; emission, 515 nm). Quantitative analysis of image intensity was performed using a Zeiss image examiner.

**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 performing a TB exclusion test. The viability, expressed as the percentage of viable cells among all cells, did not show any significant difference between the control and heat-shocked cells. The mean cell viabilities were 84.2 ± 2.8% and 81.6 ± 3.1% (n = 11) for control and heat-shocked cells, respectively. These results show that the heat shock treatment at 42°C for 1 h was not lethal to cardiac H9c2 cells.

**Expression of HSP90 after Heat Shock Treatment**

Expression of HSP90 in heat-shocked cardiac H9c2 cells was determined after 24 h of heat shock treatment. It has been reported that the expression of HSP90 reaches a maximum at 17–24 h (2, 16). Figure 1 illustrates the Western blot analysis of HSP90 in control and heat-shocked cells. The data show a fourfold higher level of HSP90 in heat-shocked cells compared with control cells.

**Effect of Heat Shock on VO₂**

VO₂ rates in control and heat-shocked cells, equilibrated with room air, were determined in three different cell densities, namely, 1, 2, and 4 × 10⁶ cells/ml. The [O₂] decreased linearly up to 10 μM in all three cases, as shown in Fig. 2A. The rate of O₂ depletion was determined from the slope of the time-course data. The normalized VO₂ values are plotted in Fig. 2B. VO₂ rates were significantly higher in 2 and 4 × 10⁶ cells/ml than in 1 × 10⁶ cells/ml. The VO₂ rates in the case of heat-shocked cells were consistently lower than those of the controls (Fig. 2, A and B), suggesting that heat shock treatment attenuated VO₂ rate.

**VO₂ Kinetics at Very Low [O₂]**

Figure 2C shows the change in the concentration of O₂ with time at very low [O₂] (<5 μM). Although a linear decrease is observed at higher concentrations, a nonlinear decrease in the concentration of O₂ is noted at very low [O₂] (Fig. 2C), indicating the reduced VO₂ rates at these lower [O₂] levels. Because EPR oximetry exclusively measures extracellular [O₂], the data in Fig. 2C can be converted to rate vs. concentration by the first derivative with respect to time (−dO₂/dt). The reduction in rate at lower [O₂] is clearly demonstrated in the −dO₂/ dt plot vs. [O₂] plots (Fig. 2D). The data shown in Fig. 2D were fit to Eq. 1, and VO₂max and p50 were evaluated. The VO₂max values were 9.98 ± 0.55 and 7.11 ± 0.64 μM/min for control and heat-shocked cells, respectively. The p50 val-

![Image](https://www.ajpcell.org/Downloaded_from/http://ajpcell.physiology.org/)

Fig. 1. Expression of heat shock protein (HSP) 90 in heat-treated H9c2 cells. A: representative Western blot analysis of HSP90 in the control and heat-shocked (HS) cardiac H9c2 cells (heat treated at 42°C for 1 h). Heat shock treatment was administered 24 h before the measurements. B: quantitative analysis of the protein content (n = 3). The protein level in control cells was used as an internal standard to normalize the protein content in the HS cells. A 4-fold increase in HSP90 was observed in HS cells.
Respiration of H9c2 cells and the effect of heat shock treatment. A: change in O$_2$ concentration ([O$_2$]) with time for various cell densities. Cells were subjected to heat shock treatment for 1 h at 42°C, and the measurements were performed 24 h later. Controls cells: ○, 1 x 10^6 cells/ml; ▲, 2 x 10^6 cells/ml; and ●, 4 x 10^6 cells/ml. HS cells: ■, 1 x 10^6 cells/ml; ●, 2 x 10^6 cells/ml; ■, 4 x 10^6 cells/ml. B: dependence of the O$_2$ consumption (V$_{O2}$) rate on cell density for control and HS cells. V$_{O2}$ rates were determined from the slopes of the [O$_2$] change vs. time plots. Error bars represent SE of 3 independent measurements. C: change in [O$_2$] at very low concentration range in HS and control cells (2 x 10^6 cells/ml). D: rate vs. concentration plot for control and HS cells (converted from C). Data in the plots were analyzed using Eq. 1 to estimate V$_{O2}^{max}$ and p50, and the theoretical lines calculated by the fit are shown as solid lines.

Effect of Heat Shock on the NOS Expression

The expression of different isoforms of NOS enzymes in cardiac H9c2 cells was evaluated by immunoprecipitation. Figure 3 shows the Western blots and the quantitative measurements of eNOS, iNOS, and nNOS expression. The quantitative estimations indicated that the expression of iNOS and nNOS was not significantly altered by the heat shock treatment. However, a more than twofold increase in eNOS expression was observed in heat-shocked cells. This is in agreement with the previous report by Harris et al. (14) that eNOS is a heat-sensitive protein.

NOS-HSP90 Complex Formation

Figure 3 shows the immunoblots and the quantitative plots of all three isoforms of NOS detected in the immunoprecipitate of HSP90. In these experiments, HSP90 was immunoprecipitated with the HSP90 antibody, and the immunoprecipitated isoforms of NOS were probed using appropriate antibodies. Interestingly, the eNOS was observed to be prominent, because there was a sevenfold increase in the HSP90 immunoprecipitates of heat-shocked cells, while only a slight increase (~2-fold) was detected with iNOS and no trace of nNOS was observed. These results demonstrate that the eNOS forms a complex with HSP90 prominently. HSP90 complexation with eNOS, and more recently with iNOS, has been reported in HEK-293 cells (38). However, previous studies with lysate from rat cerebellum revealed that nNOS does not form a complex with HSP90 (11). This is consistent with the present results. Thus HSP90-activated eNOS predominantly contributes to the observed increase in NO production in the heat-shocked cardiac H9c2 cells.

Upregulation of NO Production

Enhancement of NO production by the increased expression of HSP90 and its binding to NOS was previously reported (14). To determine whether NO production was increased in the cardiac H9c2 cells after heat shock treatment, NO generation was measured by fluorescence microscopic imaging using DAF-2DA. Figure 4 shows the confocal fluorescence images of DAF-2DA-stained control and heat-shocked cells. The images of the heat-shocked cells show more than threefold greater fluorescence image intensity than the control cells (Fig. 4C), confirming the increased production of NO in the case of heat-shocked cells.

Effects of L-NAME on V$_{O2}$

The NO generated by HSP90-activated eNOS (14) can bind either reversibly or irreversibly on the active sites in the mitochondria. Thus L-NAME, a nonspecific NOS blocker, is expected to alter V$_{O2}$ rate in heat-shocked cells. V$_{O2}$ rates of control and heat-shocked cells at various concentrations of L-NAME are shown in Fig. 5. For control cells, V$_{O2}$ rate remained the same at lower L-NAME concentrations, and a decrease in V$_{O2}$ rate was observed at higher concentrations. Although no change in V$_{O2}$ rate was expected in the control cells, the observed decrease in V$_{O2}$ rate at higher concentration (significant only at 1 mM; Fig. 5) could be due to the toxicity at higher concentrations, as confirmed by an independent cell viability study using the TB exclusion method. The inefficaciousness of added L-NAME in the 0–100 μM concentration range was determined by its inability to alter NO production.
range is consistent with previously published results in myocardial tissue VO₂ studies indicating that l-NAME did not alter basal VO₂ (27). In the case of heat-shocked cells, VO₂ rate increased with an increase in l-NAME concentration. That the addition of l-NAME reversed the observed NO-induced attenuation of VO₂ suggests that NO may bind to cytochrome c oxidase in the mitochondria (3). Thus the inhibition of NO production and the removal of its competitive binding to the cytochrome c oxidase site can reverse the attenuation of respiration. It also indicates an important notion that blocking NO generation can shift the equilibrium back toward more O₂ binding.

**Effect of GA on VO₂**

HSP90 activity is reported to be inhibited by the constitutive binding of the antagonist GA (15). If HSP90 is involved in the attenuation of respiration in heat-shocked cells, the respiration is expected to increase upon addition of GA. To the cell suspension, the desired concentration of GA (prepared in DMSO, with ~1% final concentration of DMSO in the cell suspension) was added and incubated for 10 min in a 37°C water bath, and the VO₂ rate was followed. A dose-response experiment was performed in the 0–100 μM concentration range. At higher concentrations of GA, typically >50 μM, a significant extent of toxicity and cell death was observed as confirmed by TB exclusion cell viability studies. The dose-response study was therefore restricted to 20 μM. The VO₂ rates observed at the concentrations of 0 and 10 μM are displayed in Fig. 6. In the control cells, VO₂ rate remained the same at 0 and 10 μM concentrations of GA. The VO₂ rate of the heat-shocked cells (Fig. 6) increased in the presence of 10 μM GA, and the rate was similar to that of the control cells. Such
An increase in \( \dot{V}O_2 \) rate is due to the inhibition of HSP90 and the downregulation of NO production.

Effect of BK on \( \dot{V}O_2 \)

BK is known to stimulate eNOS activity in the endothelial cells through B2 receptors and to reduce mitochondrial respiration (27). It also activates HSP90 through tyrosine phosphorylation of HSP90 (15, 37). Because we established in the present work that eNOS is overexpressed and that the eNOS-HSP90 complex is increased sevenfold in heat-shocked cells, we also studied whether BK could alter the mitochondrial respiration of cardiac H9c2 cells by affecting the HSP90-induced NO generation. Figure 7 shows the \( \dot{V}O_2 \) rate obtained in control and heat-shocked cells. In the case of heat-shocked cells, the \( \dot{V}O_2 \) rate was slightly increased in the presence of the BK, but the increase was not significant at higher concentrations. However, the opposite was observed in control cells. The decreasing \( \dot{V}O_2 \) rate in the case of control cells is due to the attenuation of mitochondrial respiration through the activation of basal eNOS in cardiac H9c2 cells as reported for myocardial tissue (27). If the same concept is simply extended to the heat-shocked H9c2 cells, the \( \dot{V}O_2 \) rate is expected to decrease further at all concentrations of BK studied. However, the fact that the rate more or less remains the same, except for the initial slight increase, reveals that the maximum NO-induced attenuation of mitochondrial respiration has already been reached by the increased NOS/HSP90 activity. Hence any

![Fig. 4](image-url)  
**Fig. 4.** Confocal microscopic analysis of NO production in control (A) and HS cells (B) stained with 4,5-diaminofluorescein diacetate (DAF-2DA). Cells were incubated with DAF-2DA for 30 min. Images were obtained under identical conditions. **C:** Quantitative fluorescence image intensity [arbitrary units (au)] averaged from intensities from all individual cells. *\( P < 0.05 \) vs. control.

![Fig. 5](image-url)  
**Fig. 5.** Effect of nitro-L-arginine methyl ester (L-NAME) on \( \dot{V}O_2 \) rate of control and HS cells (\( n = 3 \)). Appropriate concentration of L-NAME was added to \( 2 \times 10^6 \) cells/ml and incubated for 10 min in 37°C water bath and sampled for \( O_2 \) measurements. *\( P < 0.05 \) vs. 0 L-NAME concentration.

![Fig. 6](image-url)  
**Fig. 6.** Effect of geldanamycin (GA), a HSP90-specific blocker, on \( \dot{V}O_2 \) rate (\( n = 3 \)). Data shown are for both control and HS cells (for 1 h at 42°C). Cell density was \( 2 \times 10^6 \) cells/ml.
further addition of NO, generated by BK, may be ineffective in mitochondrial respiration.

The effect of BK in the presence of NO inhibitor L-NAME was also studied in both control and heat-shocked cells. The purpose of this experiment was to confirm further that BK-induced NO does not affect the mitochondrial respiration of heat-shocked cells. Cell suspensions with 1 mM of L-NAME were incubated in a water bath for 2 min, the appropriate concentration of BK was added in a single dose, and the incubation was continued for 10 min. The cells were sampled in 20-μl tubes for VO₂ studies. The obtained results are shown in Fig. 7B. At 1 mM L-NAME alone, there was no difference between the control and heat-shocked cells, as observed in the other experiment described in Fig. 5. In the case of control cells, the increased concentration of BK in addition to 1 mM L-NAME significantly decreased VO₂ rate. However, in the case of heat-shocked cells, VO₂ rate remained unaffected up to 15 mM BK. Taken together, the results in the two experiments above show that in the case of control cells, the increased addition of BK attenuated mitochondrial respiration by increased production of NO. On the other hand, in the heat-shocked cells, BK did not alter the mitochondrial respiration. This may have been due to the fact that the NO effect was already maximized.

**Effect of Myxothiazole, a Complex III Blocker, on VO₂**

ONOO⁻ is formed in the mitochondria by the reaction of NO with superoxide (O₂⁻). Because complex III is the major source of mitochondrial O₂⁻ generation under normal conditions, blocking complex III should lead to some difference among the control and heat-shocked cells with respect to VO₂ if an NO-derived species, ONOO⁻, plays a significant role in reducing the respiration in the heat-shocked cells. The VO₂ rate obtained for control and heat-shocked cells at the myxothiazole concentrations in the 1–10 μM range is shown in Fig. 8. The rate did not show any significant difference between the control and heat-shocked cells, indicating that respiration blocking is caused mainly by the direct binding of NO to the cytochrome c oxidase site and not by ONOO⁻.

**DISCUSSION**

The results of the present study show that HSP90 induced by mild heat shock treatment can activate NOS enzymes in cardiac H9c2 cells, resulting in NO production, which regulates the respiration of the cells. The interaction of HSP90 with different isoforms of NOS and the mechanism of increased production of NO have been studied in detail previously (14, 15, 37). Many reports in intact heart, isolated cardiomyocytes, and cultured cells have shown that NO regulates respiration (27, 30, 36). The regulation of cellular respiration is directly related to many cardiovascular diseases. For example, Alder et al. (1) recently measured the regulation of VO₂ by NO in myocardial tissues and concluded that the cardiovascular disease associated with aging is linked to the decreased abundance of NO in aged myocardial tissues. Similarly, decreased association between HSP90 and eNOS is reported to be responsible for persistent pulmonary hypertension in fetal lambs (22).

In the present study, the VO₂ rate of the cardiac H9c2 cells was constant with time at higher [O₂] (>5 μM). The pattern of VO₂ depends on several factors, such as the type of cell line, the cell density, and the medium used. For example, in the case of maximally activated neutrophils, an exponential decay in [O₂] with time has been reported (18). In the case of FSaII fibrosarcoma tumor cell lines, linear variation (constant consumption) has been observed, as in the case of cardiac H9c2 cells in the present work (20). The VO₂ rate measured in the present work is also in the range previously reported (20). The [O₂] steadily decreased in both the control and heat-shocked cells, and thus VO₂ rate remained constant up to 5 μM [O₂]. This indicates that mitochondrial respiration is independent of prevailing [O₂] and maintains the steady state at least up to values close to 5 μM. At these higher [O₂] values, the VO₂ rate is
lower in heat-shocked cells than in control cells (Fig. 2B), indicating the attenuation of \( \dot{V}O_2 \) by heat shock treatment. However, when \([O_2]\) drops to lower values, the \( \dot{V}O_2 \) rate also decreases, as shown in Fig. 2D. Striking differences between the control and heat-shocked cells are also observed in this region, consistent with results reported for mitochondria isolated from cardiomyocytes wherein respiration was observed to be independent up to 8–10 \( \mu \)M and the rate of respiration became markedly compromised at \(<5 \mu \)M (32).

To evaluate mitochondrial respiratory control, the dependence of \( \dot{V}O_2 \) rate on prevailing low \([O_2]\) was analyzed. We found that \( O_2 \) affinity of mitochondria is reduced in the case of heat-shocked cells. The p50 was determined to be 5.82 \pm 1.24 \( \mu \)M. In the literature, p50 values have been reported in the 0.01–3.0 \( \mu \)M range (13) and higher (32). Therefore, the observed value for p50 in the case of the control is in the same range as the values reported in the literature. However, the heat-shocked cells showed a p50 value of 18.02 \pm 1.25 \( \mu \)M, which clearly suggests that the respiration is reduced in the heat-shocked cells. The values of mitochondrial affinity of \( O_2 \) (the reciprocal of p50; Ref. 6) are 0.172 and 0.056 \( M^{-1} \cdot s^{-1} \) for control and heat-shocked cells, respectively. The affinity is three times lower in the heat-shocked cells. A recent study with purified cytochrome \( c \) oxidase showed that the presence of NO could result in an increase in \( K_m \) (equivalent of p50 in cellular systems) and a decrease in the affinity of \( O_2 \) (6). Thus the results of the present study show that in the case of heat-shocked cells, NO acts as a competitive binder at the cytochrome \( c \) oxidase site.

The mechanism of lowered \( \dot{V}O_2 \) in the heat-shocked H9c2 cells is thus linked to increased NO production. Western blot analysis revealed that all three isoforms of NO (eNOS, iNOS, and nNOS) are expressed in cardiac H9c2 cells. This is in agreement with a recent report that these cells have all NO enzymes (39). It also was observed previously that the NO generated in cardiac H9c2 cells is concentrated in the mitochondria and that mitochondrial NO concentration can be modulated in response to changes in the flux of cytosolic NO. Hence it can be expected that increased NO production in heat-shocked cardiac H9c2 cells, as confirmed in the confocal microscopic analysis of the present study, should be more concentrated in the mitochondria. Thus the higher abundance of NO in the mitochondria produced by the HSP90-NOS association is linked in some way to the observed attenuation of \( \dot{V}O_2 \) in the heat-shocked cells as shown in Fig. 9. The question of what is responsible for increased NO production in the heat-shocked cells was resolved by performing immunoblot analysis. The Western blot analysis showed that eNOS increased more than twofold in the heat-shocked cells, while the other forms did not show any significant change after heat shock treatment. Recently, Harris et al. (14) reported a similar observation of elevation of eNOS level in the aortas obtained from heat-shocked rats. Our results confirm that eNOS is a heat-sensitive protein. Immunoprecipitation of HSP90 has shown that eNOS is coimmunoprecipitated because a sevenfold increase is observed in the heat-shocked cells. These results indicate that both the increased expression of eNOS as well the increased association of eNOS with HSP90 are both responsible for the increased production of NO in heat-shocked cardiac H9c2 cells. We have further confirmed that the incubation of heat-shocked cells with GA at \( \geq 10 \mu \)M concentration recovered the \( \dot{V}O_2 \) rate close to that of the control cells (Fig. 6). This result confirms that the reduced \( \dot{V}O_2 \) by the heat-shocked cells is due to the HSP90-induced NO and its interaction with the complexes of electron transport chain in the mitochondria.

There are many targets for NO in the mitochondria that could potentially inhibit mitochondrial respiration. NO can potentially bind to transition metal ions in the mitochondrial enzymes, such as the catalytic iron sulfur or copper clusters of cytochrome \( c \) oxidase, and cause reversible inhibition (3, 4). The competitive binding of NO at the cytochrome \( c \) oxidase site could increase the \( K_m \) for \( O_2 \). In addition, the rate of mitochondrial respiration may also be dependent on the NO:O\(_2\) ratio (10). The rate constants of \( O_2 \) and NO binding are high (\( k = 0.4–1 \cdot 10^9 \text{M}^{-1} \cdot \text{s}^{-1} \)) and of the same order. Thus NO and \( O_2 \) can competitively bind to cytochrome \( c \) oxidase. At high NO:O\(_2\) ratios, the rate of inhibition of mitochondrial respiration seems to be rapid (\(<1 \text{s}\)). Moreover, alterations in the NO:O\(_2\) ratio can change the binding equilibrium, showing that NO inhibition at complex IV is reversible and that any blocking of NO production should shift the equilibrium toward more \( O_2 \) binding and vice versa. The existence of reversible inhibition is supported by the results of the present study indicating that the \( L\)-NAME-treated heat-shocked cells showed increased respiration compared with basal levels. This indicates that the suppression of NO flux shifts the equilibrium toward more \( O_2 \) binding. Furthermore, \( L\)-NAME treatment at concentrations as high as 1 mM led to recovered respiration almost to the level of control cells (Fig. 5). This indicates that the attenuation of the respiration in H9c2 cells by NO is reversible and that it occurs by competitive binding at the cytochrome \( c \) oxidase site.

The data obtained from complex III inhibitor, namely, myxothiazole, suggest that ONOO\(^{-}\)–induced damage to the respiratory chain is not significant. The irreversible changes are caused by the NO derivatives in the mitochondria (3, 4). NO can react with \( O_2 \) to generate ONOO\(^{-}\) and NO\(_2\), which can irreversibly bind to the other complexes in the mitochondrial respiratory chain (3, 4). The respiration rates observed at various concentrations of myxothiazole did not show any

![Fig. 9. Schematics of NOS and HSP90 complexation and regulation of cellular respiration. HSP90 binds with the NOS enzyme in H9c2 cells and activates NOS to produce NO, which competitively binds to cytochrome c oxidase of mitochondria to block respiration.](http://ajpcell.physiology.org/)
significant difference between control and heat-shocked cells. The respiration rate is reduced to <20% (Fig. 8) in both control and heat-shocked cells, although there was a significant difference at the basal level.

The results obtained with respect to the effect of BK on respiration and heat shock treatment are also of particular interest. BK’s effect on VO_{2} in control and heat-shocked cells showed opposite trends. In the case of the control cells, the decreasing VO_{2} rate with increasing concentration of BK is likely due to the activation of cNOS by BK and increased production of NO (37). However, it is interesting to note that the addition of BK increased VO_{2} to some extent in the case of heat-shocked cells. This indicates that BK-induced NO generation was ineffective in controlling respiration. Recent studies of the interaction of BK with HSP90 have predicted that the binding of HSP90 to NOS can increase the effect of BK-induced activation of NOS (Fig. 7). However, we observed the reverse trend in the case of cardiac H9c2 cells. Our observation was also further supported by experiments with the addition of l-NAME. While in the case of heat-shocked cells BK + l-NAME did not show any significant change in the respiration rate, the control experiments showed decreased VO_{2}, indicating that even in the presence of 1 mM l-NAME, NO production seemed to be in excess.

CONCLUSIONS

The present study demonstrates that HSP90 generated after mild heat shock treatment regulates the respiration of cardiac H9c2 cells through the activation of NOS and increased production of NO. The EPR oximetric measurements showed attenuated VO_{2} by heat-shocked cells. Western blot analysis showed a fourfold increase in the expression of HSP90 in H9c2 cells after 24-h heat shock. Confocal microscopy showed elevated levels of NO in the heat-shocked cells. Although many pathways of respiration control by NO and its derivatives are possible, the reversible competitive binding of NO at the cytochrome c oxidase site in the mitochondria seemed to be the main mechanism of attenuation of mitochondrial respiration. This conclusion is based on the observation that l-NAME induces recovery of respiration in heat-shocked cells to the level of control cells. The increased levels of NO shift the binding equilibrium against O_{2} binding. The present study reveals that the protective effect offered by heat shock treatment in the heart might be attributable at least in part to HSP90-induced NO activation and upregulation of NO, which reduce the rate of mitochondrial respiration as a survival act.

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