Activation of Hsp90-eNOS and increased NO generation attenuate respiration of hypoxia-treated endothelial cells

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Presley T, Vedam K, Velayutham M, Zweier JL, Ilangovan G. Activation of Hsp90-eNOS and increased NO generation attenuate respiration of hypoxia-treated endothelial cells. Am J Physiol Cell Physiol 295: C1281–C1291, 2008. First published September 11, 2008; doi:10.1152/ajpcell.00550.2007.—Hypoxia induces various adaptive signaling in cells that can cause several physiological changes. In the present work, we have observed that exposure of bovine aortic endothelial cells (BAECs) to extreme hypoxia (1–5% \( O_2 \)) attenuates cellular respiration by a mechanism involving heat shock protein 90 (Hsp90) and endothelial nitric oxide (NO) synthase (eNOS), so that the cells are conditioned to consume less oxygen and survive in prolonged hypoxic conditions. BAECs, exposed to 1% \( O_2 \), showed a reduced respiration compared with 21% \( O_2 \)-maintained cells. Western blot analysis showed an increase in the association of Hsp90-eNOS and enhanced NO generation on hypoxia exposure, whereas there was no significant accumulation of hypoxia-inducible factor-1α (HIF-1α). The addition of inhibitors of Hsp90, phosphatidylinositol 3-kinase, and NOS significantly alleviated this hypoxia-induced attenuation of respiration. Thus we conclude that hypoxia-induced excess NO and its derivatives such as ONOO− cause inhibition of the electron transport chain and attenuate \( O_2 \) demand, leading to cell survival at extreme hypoxia. More importantly, such an attenuation is found to be independent of HIF-1α, which is otherwise thought to be the key regulator of respiration in hypoxia-exposed cells, through a nonphosphorylative glycolytic pathway. The present mechanistic insight will be helpful to understand the difference in the magnitude of endothelial dysfunction.

IN TISSUES, HYPOXIA is the state of insufficient \( O_2 \), caused by inadequate transport or an excess consumption of \( O_2 \). Duration, frequency, and severity of hypoxia strongly influence whether the effect is detrimental or beneficial (20, 43). Interestingly, hypoxia-exposed cells do not always undergo cell death or diminish ATP levels (11), therefore leading to cell survival and normal cell function. Many factors that are activated upon hypoxia, such as the hypoxia-inducible transcription factor (HIF-1), heat shock protein 90 (Hsp90), nitric oxide (NO) synthase (NOS), and reactive oxygen species (11, 13), determine whether the cells survive after salvage or succumb to death (2). Particularly, the role of NO during and after hypoxia in the endothelium has been found to be important in maintaining oxygen metabolism (31). NO is a multifaceted endogenous factor and is involved in many pathophysiological processes in cells. In vitro studies have shown that NO production is increased in hypoxic conditions (9, 31). NO has been found to inhibit the mitochondrial electron transport chain (ETC) (8, 14, 15). Thus it is an endogenous modulator of cellular respiration in different pathogenic conditions. While NO restrains cytochrome-c oxidase (CcO) by competing for the oxygen binding site at the heme of the enzyme, its derivative, peroxynitrite (ONOO−), blocks the other ETC complexes mainly by \( \cdot \)nitrosation (25). Moreover, intermittent or acute hypoxia exposure is known to cause an increase in Hsp90 binding to endothelial NOS (eNOS), to facilitate Ser1177 phosphorylation (4, 13, 43). Thus, the excess NO in hypoxia that is caused by an increase in Hsp90-eNOS association may inhibit the ETC; yet, this hypothesis remains untested.

Hypoxia-induced HIF-1α can also attenuate cellular respiration (47). When there is a limited presence of oxygen, HIF-1α is induced and stabilized by inhibition of prolyl hydroxylation-dependent (PHD) binding of the ubiquitin ligase von Hippel-Lindau (pVHL) tumor suppressor (28). Moreover, Hsp90 has been reported to play a role in the stability of HIF-1α in low \( O_2 \) as well as heat-induced conditions (27, 28). Previous studies have shown that hypoxia-stabilized HIF-1α transcribes a set of genes that is related to glucose transporters (GLUT-1 and GLUT-3); hence the normal oxidative phosphorylation is slowly switched to glucose metabolism by an anaerobic lactate pathway (Warburg effect) in a hypoxic state (5, 6). Although this effect has been well established in cancer cells (47), recent reports have revealed that the cells that express high NOS, such as endothelial cells, show a different behavior in terms of HIF-1α stabilization. Mechanistic studies have established that even though PHD is inhibited at a low \( P_O_2 \), the excess NO during hypoxia can induce PHD2, which accelerates the HIF-1α (7). Thus, the role of HIF-1α in the regulation of hypoxia-treated endothelial cells (which have high abundance of NOS) is not known. Despite the two distinct possible mechanisms of inhibition of cellular respiration in hypoxia-treated endothelial cells, it is not known whether the respiration in hypoxia-exposed endothelial cells is regulated by a mechanism that is dependent on NO or HIF-1.

In the present work, we elucidate the mechanism of the regulation of oxygen consumption in hypoxia-exposed bovine aortic endothelial cells (BAECs). To measure cellular respiration, electron paramagnetic resonance (EPR) oximetry was used as a quantitative tool (24, 40). EPR oximetry is a highly
sensitive technique that is accurate, requires only a microvolume of sample, and is capable of yielding high-resolution \( \text{O}_2 \) data similar to data obtained in high-resolution respirometry. In this work, we show that prolonged hypoxia exposure attenuates cellular respiration by the activation of the Hsp90-eNOS complex in BAECs. This activation was dependent on both duration and severity of hypoxia. Our results reveal that when BAECs undergo a prolonged hypoxia exposure, respiration is regulated by Hsp90-eNOS enhancement but not by HIF-1\( \alpha \)-related factors, such as increased glycolysis.

MATERIALS AND METHODS

Materials

4,5-Diaminofluorescein diacetate (DAF-2DA) was purchased from Alexis Biochemicals (San Diego, CA). Dilithium phthalocyanine, acetonitrile, nitro-L-arginine methyl ester (L-NAME), and geldanamycin (GA) were obtained from Sigma-Aldrich (St. Louis, MO). Wortmannin was purchased from Millipore (Billerica, MA). Tetraethyl ammonium perchlorate was purchased from ICN Biochemicals (Aurora, OH). The antibodies for Western blot analysis were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), Abcam (Cambridge, MA), and Cayman (Ann Arbor, MI).

Methods

Cell culture. BAECs were obtained from Cell Systems (Kirkland, WA). The BAECs were cultured in MEM (GIBCO), 10% FBS, nonessential amino acid solution, and endothelial cell growth factor. Cells were grown in regular 150-cm² culture dishes, coated with attachment factor. Cells were trypsinized and used in experiments when the cultures reached 70–80% confluency.

Cell viability. The cell viability was determined by a NucleoCounter system (New Brunswick Scientific, Edison, NJ) composed of the NucleoCounter automatic cell counter, the NucleoCassette, a cell preparation lysing buffer and a stabilizing buffer, and NucleoView software. Two aliquots of the cell suspension, for nonviable count and the total cell count, were taken. For the total cell count, equal amounts of the lysing buffer and the stabilizing buffer were added to the cell suspension. Each sample was loaded into the NucleoCassette and placed into the NucleoCounter cell counter for analysis. The nonviable count was determined first, followed by the total cell count. Using the NucleoView software, the nonviable, total cell count, viable cell count, and viability were determined. Viability of BAECs in suspension was found to be about 90% to 95%.

Hypoxia. Cell suspension was prepared and seeded in normoxic (21% \( \text{O}_2 \)) conditions and then transferred to a hypoxic incubator (prefixed with desired \( \text{O}_2 \) tension) as described in Figs. 1A and 2A. BAECs were placed into a hypoxic atmosphere, using an incubator prejudged for a desired lower value (Thermo Electron Forma Series II Water Jacketed \( \text{CO}_2 \) Incubator). The sensor in the incubator precisely measured the \( \text{PO}_2 \) inside the incubator and displayed the \( \text{PO}_2 \) as well (21). Thus, by measuring the \( \text{PO}_2 \) line width, the \( \text{PO}_2 \) in the solution can be obtained at any given time. LiPc measures the extracellular \( \text{PO}_2 \) in the cellular suspension. Since the LiPc microcrystals were comparatively larger in the oximetry measurements, the particulates remained in the bulk volume and there was no ingestion by the cells.

Mitochondrial membrane potential measurements by flow cytometry. Rhodamine 123 (Molecular Probes, Eugene, OR) was used to measure mitochondrial membrane potential using the procedure previously described (38). Both 21% \( \text{O}_2 \)-maintained cells (control) and hypoxia-exposed cells (1% \( \text{O}_2 \) for 24 h) were trypsinized and counted using the NucleoCounter system. Equal number density (5 \( \times 10^5 \)/ml) cell suspensions were obtained by resuspension in MEM medium containing 10% FBS and 10 mg/ml rhodamine 123 and incubation for 10 min. After incubation, the cell suspensions were pelleted and resuspended in ice-cold MEM-10% FBS medium and subjected to flow cytometry, using BD FACSCalibur. Fluorescence values of all 10,000 cells were displayed in log normal distribution curves and converted to quadrant plots.

Glucose uptake by BAECs. Both control and hypoxia-treated BAECs were incubated overnight with media containing a fluorescent, noncleavable glucose analog [2-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino-2-deoxyglucose]; 2-NBDG; Invitrogen; 0.5 mg is dissolved in 15 ml MEM medium](16). The cells were trypsinized, washed with MEM medium, counted, and finally suspended in the MEM medium. The relative fluorescence intensity was measured using the FACSCalibur flow cytometer, and histograms were analyzed using WinMDI software.

Drug treatments. To understand the effect of Hsp90 and eNOS on hypoxic respiration, various inhibitors of each protein were used. BAECs were treated with GA (10 \( \mu \text{M} \)), L-NAME (0.5 mM), or wortmannin (1 \( \mu \text{M} \)) before or following hypoxia. In the first set of experiments, cells were placed in a 1% \( \text{O}_2 \) hypoxic environment for 24 h and treated with each respective inhibitor for 30 min. In the second condition, each drug was added to the cultured cells for 30 min and placed in 1% \( \text{O}_2 \) for 24 h. Following each experimental condition, the cells were trypsinized for experiments.

Oximetry probe. Lithium phthalocyanine (LiPc) microcrystals were used as the oximetry probe. The probe was synthesized electrochemically, using the established procedure (23). The synthesized microcrystals were subjected to various physicochemical characterizations, such as X-ray diffraction, EPR, and microscopy, to ensure the purity of the material. These microcrystals were found to be in the pure \( \lambda \)-isofrom (or equivalently known as X-form), which has been characterized to yield a \( \text{PO}_2 \)-dependent EPR line width. For oximetry measurements, we used approximately 20–30 \( \mu \text{g} \) of LiPc.

Measurement of cellular respiration. The oxygen measurements were performed using EPR oximetry (21, 24, 40). From the EPR line width, the \( \text{PO}_2 \) in the cell suspension was determined using the calibration curve. The EPR line width vs. \( \text{PO}_2 \) calibration curve was constructed using known ratios of premixed \( \text{O}_2 \) and \( \text{N}_2 \) gases. The slope of the calibration curve was 5.8 mG/mmHg. Although this calibration curve was constructed using gas mixtures, we have previously demonstrated that this curve is applicable in aqueous solutions as well (21). Thus, by measuring the EPR line width, the \( \text{PO}_2 \) in the solution can be obtained at any given time. LiPc measures the extracellular \( \text{PO}_2 \) in the cellular suspension. Since the LiPc microcrystals were comparatively larger in the oximetry measurements, the particulates remained in the bulk volume and there was no ingestion by the cells.

EPR oximetry experimental setup. The respiration studies have been carried out using an X-band (9.7 GHz) EPR spectrometer fitted with a TM110 microwave cavity. A 50-\( \mu \)l microcapillary tube was used to hold the cells in the horizontal EPR cavity. In a typical experiment, the cell suspension of the desired cell density was maintained in respiration medium (in mM: 117.3 \( \text{NaCl} \), 4.7 KCl, 1.3 MgSO\(_4\), 1.2 CaCl\(_2\), 1.2 KH\(_2\)PO\(_4\), 25 NaHCO\(_3\), and 20 glucose; \( \text{pH} 7.4 \)) and saturated with room air (\( \text{PO}_2 \approx 160 \text{ mmHg} \)). The cell suspension was incubated for 10 min in a 37°C water bath. LiPc microcrystals (20 \( \mu \)g) were added to the cells and sampled into 50-\( \mu \)l capillary tubes. The tube was then closed off at both ends with tube sealing clay (Chase Scientific Glass, Rockwood, TN). While sealing, care was taken to ensure that there were no air gaps present inside the tube, since such a gap may act as an additional source of \( \text{O}_2 \). The tube was placed inside the horizontal microwave cavity, and EPR spectral acquisitions of the LiPc were immediately started. During measurements, the modulation amplitude was adjusted to always be less than one third of the line width to avoid modulation-induced broadening.

\( \text{O}_2 \) kinetics. Quantitative EPR oximetry was performed using recently described procedures (40). Briefly, there are three phases of cellular respiration that can be analyzed from a single run of \( \text{PO}_2 \) vs. time using EPR oximetry; \( \text{PO}_2 \)-dependent, \( \text{PO}_2 \)-independent, and a
These levels of cellular respiration were obtained by adopting the following equation:

\[
V_{O_2}/V_{O_2max} = \left[ (P_{O_2} - p_0) p50 + (P_{O_2} - p_0) \right]
\]

From this equation, \(V_{O_2max}\), \(p_0\), and \(p50\) values were acquired (40). The \(V_{O_2max}\) is defined as the oxygen consumption rate (\(V_{O_2}\)) in coupled state, when oxygen is not limiting; \(p_0\) is the equilibrium PO2; and \(p50\) is the concentration at which the \(V_{O_2max}\) is reduced to 50%.

RESULTS

The Effect of Hypoxia on BAEC Respiration

BAECs were cultured at various O2 contents, namely, 21%, 5%, 3%, and 1% for 24 h, as shown in Fig. 1A. At the end of hypoxia exposure, the cells were trypsinized and resuspended in the respiratory medium for oxygen consumption measurements. The oxygen consumption of BAECs was followed by EPR oximetry. An equal density (5 \( \times \) 10^6 cells/ml) of cells was used for respiration measurements in each case presented in Fig. 1A, B and C. EPR spectra were obtained at 15-s intervals for steady-state respiration. The EPR spectra, collected during the cellular respiration measurements, were analyzed as formerly described (40). The correlation coefficient of 0.98 was set as the standard of acceptance of the results. The PO2 data conversion, differentiation, and curve fit were carried out as described previously (40).

Fig. 1. Hypoxia exposure and cellular respiration. Effect of different percentages of O2. A: schematic illustration of hypoxia exposure of cells. The cells were seeded in normoxic conditions and taken to normoxic (21% PO2) or hypoxic incubator with preset PO2 (5%, 3%, or 1% PO2) and cultured for desired time as indicated. At the end of hypoxic treatment, the cells were taken out and suspended in normoxic respiration medium, and electron paramagnetic resonance (EPR) oximetry was performed on a fixed cell density (5 \( \times \) 10^6 cells/ml). B: EPR spectra were obtained for both hypoxic and control cells, and the EPR line width data were converted into PO2 data. As the percentage of O2 reduces, the cells seem to require more time to consume O2. C: data from B were converted into rate of oxygen consumption (dPO2/dt) and plotted with respect to PO2. Three distinct phases of respiration can be observed. The 1% O2 cells have a substantial decrease in the maximum oxygen consumption rate.
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EPR OXIMETRY TO STUDY CELLULAR RESPIRATION UNDER LOW O2

Table 1. Quantitative analyses of EPR oximetry measurements, in which 5 x 10⁶ cells were treated with various O2 concentrations at a range of time points

<table>
<thead>
<tr>
<th>Time</th>
<th>3% O₂</th>
<th>1% O₂</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>VO₂max, mmHg/min</td>
<td>p₀, mmHg</td>
</tr>
<tr>
<td>2 h</td>
<td>5.72±0.73</td>
<td>0.35±0.06</td>
</tr>
<tr>
<td>4 h</td>
<td>4.65±0.18</td>
<td>0.49±0.13</td>
</tr>
<tr>
<td>8 h</td>
<td>4.43±0.46</td>
<td>0.44±0.09</td>
</tr>
<tr>
<td>16 h</td>
<td>3.45±0.34</td>
<td>0.45±0.16</td>
</tr>
<tr>
<td>24 h</td>
<td>3.01±0.69</td>
<td>0.45±0.09</td>
</tr>
</tbody>
</table>

Values are means ± SE. The control values are as follows (n = 7): VO₂max = 4.07 ± 0.18 mmHg/min; p₀ = 0.37 ± 0.03 mmHg; p5₀ = 2.86 ± 0.25 mmHg; and 1/p5₀ = 0.36 ± 0.03 mmHg⁻¹. EPR, electron paramagnetic resonance.
2-NBDG uptake by these cells was found to be the same (Fig. 3C), indicating that the functional levels of GLUT-1 and -2 are the same both in control and in hypoxia-treated cells. These results together prove that the observed attenuation of respiration is not due to higher glycolysis in 1% O2-exposed cells. Considering these facts, additional experiments were carried out to determine whether the activation of eNOS is responsible for the observed attenuation in the hypoxia-exposed BAECs.

Increased Production of NO in Hypoxia-Treated Cells

Observing that there was no significant accumulation of HIF-1 in hypoxia treated BAECs, NO generation was measured with fluorescence microscopy at each time exposure to 1% O2 hypoxia. DAF-2DA staining, which yields green fluorescence by reacting with NO and its derivatives, was used to quantify NO generation on hypoxia exposure. BAECs were cultured in glass cover slides in normoxia (21% O2) and hypoxia (1% O2) for 24 h and treated with DAF-2DA for 20 min. Figure 4, A and B, illustrates the fluorescence images of control and hypoxic cells stained with DAF-2DA. When compared with the control BAECs, a distinct intensification in fluorescence can be seen in the hypoxia-exposed cells. The quantitative determination of fluorescence intensity was carried out and is illustrated in Fig. 4C. On the basis of the intensity measurements, the cells that were exposed to 24 h of 1% hypoxia had an average intensity of 722.8 ± 23.6 arbitrary units (AU; n = 12), whereas the control BAECs had an average intensity of 186.5 ± 6.9 AU (n = 24), demonstrating nearly a fourfold increase (Fig. 4C). These data confirm that NO generation is greatly augmented in hypoxic cells versus normal BAECs. Further studies were carried out to determine whether the higher NO results in nitration of proteins in 1% O2-exposed cells. Figure 4D shows the Western blots of nitrotyrosine proteins in whole cell lysates, probed with 3-nitrotyrosine-specific antibodies. Higher tyrosine-nitrated proteins are observed in hypoxia-treated samples than in 21%
O$_2$-maintained cells, indicating that the protein nitration in mitochondrial proteins may be responsible for the observed attenuation of respiration. However, treating hypoxia-exposed cells with L-NAME (NOS inhibitor) reduced the nitrosylation of proteins (Fig. 4D).

**Hsp90-eNOS Association in Hypoxic BAECs**

The interaction of Hsp90 and eNOS has been reported to be enhanced in stressful conditions such as heat shock and hypoxia, in which this association leads to an increase in NO production (13, 22). To determine if Hsp90-eNOS association is an important factor in the attenuation of respiration in hypoxia-exposed cells, the induction and association of Hsp90 and eNOS and its activity were studied. BAECs were exposed to 5% O$_2$, 3%, and 1% O$_2$ for the same times as used in EPR oximetry studies of respiration (Fig. 1). At the end of hypoxia exposure, the cells were trypsinized, lysed, and used for either Western blot analysis or immunoprecipitation (IP). Two sets of experiments were carried out: In the first set, eNOS was immunoprecipitated and Hsp90 and eNOS were immunoblotted (IB); in the second set, IP of Hsp90 and IB of eNOS and Hsp90 were performed. In both cases of 5% and 3% O$_2$-exposed cells, there was no change in the expression of Hsp90 or eNOS at each time of treatment (data not shown). These results are consistent with the respiration measurements that no significant changes occurred in the respiration by exposing the cells to 5% or 3% O$_2$. On the other hand, a significant difference in the expression of Hsp90 and eNOS, as well as the IP of Hsp90, obtained from IB of eNOS, was observed for cells exposed to 1% O$_2$ for 24 h. *P < 0.05 vs. control.
treated at 1% O₂. As seen in Fig. 5A, the induction of both Hsp90 and eNOS increased up to 16 h of 1% O₂ hypoxia exposure, followed by a moderate decline at 24 h. Likewise, there is an increased expression and association of Hsp90 to eNOS, between 8 and 16 h of exposure to 1% O₂, and a slight decrease at 24 h (Fig. 5B). This increased association may contribute to the increased NO production in hypoxia-exposed cells. Such an increased NO production may cause both reversible and irreversible inhibition of the ETC, resulting in attenuation of respiration. Also in the case of hypoxia-treated cells, the confluence was less, indicating that the cells are still actively proliferating. This in agreement with previous reports that such active proliferation increases Hsp90/eNOS association in endothelial cells (36). Thus it is difficult to decide whether the observed activation of Hsp90/eNOS association is due to hypoxia alone. However, when compared with control (which had also not reached 100% confluency at the time of the experiment), the reduced respiration of hypoxia-treated cells is likely due to hypoxia. Moreover, it is also possible that detaching the cells from culture dishes may promote Hsp90/eNOS and trigger apoptosis. However, the DNA laddering studies did not show any significant fragmentation of DNA (Fig. 7B), confirming that there was no apoptosis due to detachment of cells.

Mitochondrial integrity was determined to infer whether the observed attenuation in respiration is due to physical or functional changes of mitochondria. Rhodamine 123 uptake, a measure of mitochondrial membrane potential (38), was determined for control and 1% O₂-treated cells, and the obtained results are included in Fig. 4E. The rhodamine fluorescence measured, using flow cytometry, was not different for the samples, and, indeed, both samples showed >99% positive staining for rhodamine 123. This result indicates that the observed attenuation in respiration in hypoxic cells is not due to physical changes and is indeed due to functional changes in mitochondria. This is most likely due to the nitration of mitochondrial proteins, as observed in Fig. 4D.

Alleviation of NO-Induced Inhibition of Respiration in Hypoxia-Exposed Cells

To ensure the role of Hsp90 and eNOS in the observed attenuation of respiration in 1% O₂-exposed cells, the effects of various inhibitors of Hsp90, phosphatidylinositol 3-kinase (PI3K), and NOS on the respiration of hypoxia exposed cells were studied. GA was used as the Hsp90 inhibitor, l-NAME as the NOS inhibitor, and wortmannin as the PI3K inhibitor. Wortmannin is a cell-permeable irreversible inhibitor of PI3K that blocks the catalytic activity of PI3K without influencing upstream signaling events. Two sets of experiments were carried out with these inhibitors. In the first set of experiments, BAECs were treated for 30 min with 10 μM GA, 1 μM wortmannin, or 0.5 mM l-NAME and were returned to regular culture medium and maintained for 24 h at 1% O₂. The cells were trypsinized and resuspended in regular respiration medium for oxygen consumption measurements. The oxygen consumption of BAECs was followed by EPR oximetry as described above. Briefly, EPR spectra were obtained at 15-s intervals for a period of 90 min for 5 × 10⁵ cells/mL. The PO₂ data were obtained, plotted with respect to time (Fig. 6A), and further transformed into dPO₂/dt vs. PO₂ data (Fig. 6B) (40). In Fig. 6, A and B, it is clear that the cells treated with GA have an enhanced maximum respiration rate in comparison to the hypoxic cells. The VO₂max was recovered from 1.96 ± 0.35 mmHg-min⁻¹·5 × 10⁻⁶ cells to 3.23 ± 0.43 mmHg-min⁻¹·5 × 10⁻⁶ cells; n = 2 (Table 2). Similarly, the addition of l-NAME also significantly increased the VO₂max value; however, there was no relevant difference with the treatment of wortmannin (Fig. 6, C and D). Although GA and l-NAME recovered the maximum O₂ rate, there was no significant change in the p50
or mitochondrial affinity between all of the inhibitors. A complete analysis of the data was performed as described in MATERIALS AND METHODS, and the relevant parameters have been summarized in Table 2. A second set of experiments was done in which the cells were cultured at 1% O$_2$ for 24 h and then treated for 30 min with each drug treatment before trypsinization. There was no significant difference between the control cells and the BAECs treated with the inhibitors, implying that the NO inhibition at this stage was not effective to recover respiration (data not shown).

Finally, experiments were also carried out to determine whether there was any significant difference in Hsp90 binding to eNOS upon treatment with these inhibitors. BAECs were treated with each inhibitor for 30 min before 24 h of 1% O$_2$ and returned to regular medium. Cells were trypsinized and lysed, and the protein estimation was carried out. Normalized cell lysates were used to measure the association of Hsp90 and eNOS for each condition, and the results are summarized in Fig. 7. In comparison to the control, there is an increased association of Hsp90 to eNOS for the cells that were exposed to 1% O$_2$ for 24 h. The addition of GA slightly reduced the Hsp90 immunoblotted from the IP of eNOS (Fig. 7). Knowing that the PI3K/Akt pathway can interact with Hsp90, BAECs were treated with wortmannin. Wortmannin blocked the association of eNOS and Hsp90. t-NAME, a NOS inhibitor, also exhibited the same effect of suppressing the Hsp90 and eNOS interaction (Fig. 7).

**DISCUSSION**

The primary finding of the present work is that prolonged hypoxia exposure of BAECs attenuates respiration by a mechanism involving Hsp90 and eNOS. More importantly, this reduced respiration in endothelial cells appears to be independent of HIF-1α, which is otherwise thought to be a key factor influencing respiration. Earlier reports have demonstrated reduced respiration, caused by HIF-1, CcO, and AMP kinase, in hypoxia-treated cells such as hepatocytes and carcinomas (10, 12, 30, 47). Although hypoxia has been previously shown to increase eNOS mRNA and protein expression, as well as augment basal and bradykinin-stimulated NO production in BAECs (20), to our knowledge, the present work is the first to reveal the influential role of Hsp90 and eNOS in posthypoxic cellular respiration. A number of experiments were carried out, in which BAECs were exposed to various percentages of O$_2$ in the range of normoxia ($21\%$) to extreme hypoxia ($1\%$) for different durations. Under these conditions, the expression of eNOS and Hsp90 and their association and NO production were determined. Together, these results prove the proposed mechanism that the activation and association of Hsp90 and eNOS increase NO under hypoxia, leading to an observable attenuation of respiration. Overall, no accumulation of HIF-1α was found upon exposure of BAECs to hypoxia. However, a significant upregulation of the association of Hsp90 with eNOS occurred (Fig. 5), and increased NO generation (Fig. 4) was

### Table 2. Quantitative analyses of EPR oximetry measurements, in which 5 x 10$^6$ cells were incubated with each inhibitor for 30 min before 1% O$_2$ hypoxia for 24 h

<table>
<thead>
<tr>
<th>Drug Treatment</th>
<th>V$\Delta$O$_{2max}$, mmHg·min$^{-1}$·5 x 10$^{-6}$ cells</th>
<th>p$_{50}$, mmHg</th>
<th>p$50$, mmHg</th>
<th>O$_2$ Affinity, mmHg$^{-1}$</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geldanamycin</td>
<td>3.23±0.43*</td>
<td>0.61±0.29</td>
<td>2.89±0.40</td>
<td>0.35±0.09</td>
<td>2</td>
</tr>
<tr>
<td>Radicicol</td>
<td>3.03±0.35</td>
<td>0.62±0.23</td>
<td>2.86±0.38</td>
<td>0.33±0.05</td>
<td>3</td>
</tr>
<tr>
<td>t-NAME</td>
<td>2.88±0.47**</td>
<td>0.64±0.17</td>
<td>2.35±0.32</td>
<td>0.43±0.05</td>
<td>2</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>2.23±0.31</td>
<td>0.62±0.25</td>
<td>3.00±0.29</td>
<td>0.33±0.08</td>
<td>2</td>
</tr>
<tr>
<td>None</td>
<td>1.96±0.35</td>
<td>0.59±0.19</td>
<td>2.43±0.35</td>
<td>0.41±0.07</td>
<td>3</td>
</tr>
</tbody>
</table>

Values are means ± SE. t-NAME, nitro-t-arginine methyl ester. *P < 0.05, significant tests compared with the 24-h 1% O$_2$ cells.

Fig. 7. Western blot analysis of the inhibition of extreme hypoxia. BAECs were treated with GA (10 μM), t-NAME (0.5 mM), or wortmannin (1 μM) for 30 min and placed in a 1% O$_2$ hypoxic environment. A: immunoblots of Hsp90 and eNOS obtained in the immunoprecipitates of eNOS. The bar graphs represent the ratio of Hsp90 to eNOS in respective samples that were treated with GA; wortmannin (Wort) reduced the association (**P < 0.001). The quantitative plots were obtained from 3 sets of immunoprecipitation and immunoblotting (n = 3). B: DNA laddering analysis in GA- and wortmannin-treated control and hypoxia-treated cells.
observed. The exposure of endothelial cells to hypoxia increases eNOS activity due to Hsp90-assisted increase in phosphorylation at Ser1177 by PI3K/Akt pathway, and an increase in NO generation occurs. Such an upregulation of NO in cells is known to induce adaptation of cells to the reduced oxygen content. In endothelial cells, eNOS phosphorylation at Ser1177 is necessary for this hypoxia-induced eNOS activation and NO production (13). Other factors have also been explored and shown to contribute to this attenuated respiration, such as CaCO, ATP utilization, HIF-1, and AMP kinase; however, the role of Hsp90 and eNOS in cellular respiration in a hypoxic environment remains unclear (10, 12, 30, 47).

The mechanism of NO-induced inhibition of respiration has been well elucidated by various groups (1, 8, 14, 15). NO has been found to exert two distinct types of inhibition on cellular ETC, namely, irreversible inhibition due to chemical modifications caused in the ETC complexes by ONOO− and reversible inhibition at CcO of complex IV. Moreover, CcO has also been recognized as the mitochondrial enzyme that reduces NO2 to NO (9). The reversible inhibition of CcO is due to direct competition of NO with O2 at the O2 binding site of CcO (32). In our model expression described in MATERIALS AND METHODS, the V2max decrease is attributed to the overall inhibition, and the increase in p50 is attributed to the reversible inhibition at CcO. Although there was a relevant change in the V2max for the hypoxia-exposed BAECs, there was no considerable difference in the p50 values (P02 at which the V2max is half). Such a behavior has been previously observed for BAECs, where eNOS was activated by various stimulators (32). We have found that there is no CcO inhibition at low P02 values (even in eNOS-activated conditions), because there is not an adequate amount of O2 at a low P02 to generate NO by NOS and hence there is no reversible inhibition at CcO (unpublished observation). It appears that a similar effect seems to be caused by the excess flux of NO generated during hypoxic treatment and its reactions with ETC complexes, meaning that the irreversible damage is responsible for the observed attenuation of respiration. However, the data in Fig. 4 show higher fluorescence intensity, which is likely due to nonspecific staining of NO and derivatives such as ONOO−. This argument is further supported with the results of various inhibitors used in the present work. When the cells were treated with L-NAME and GA during hypoxic exposure (even as low as 1% O2), the V2max was unaffected compared with the cells maintained in normoxia (21% O2) (i.e., the attenuation of respiration is prevented; Fig. 6). Yet, the addition of these agents following hypoxia and immediately before respiration measurements did not eliminate the attenuation of respiration (data not shown). While L-NAME indiscriminately inhibits all of the isoforms of NOS, the GA binds to Hsp90, preventing the eNOS protein association with Hsp90. Thus it appears that the increased binding of eNOS with Hsp90 during hypoxia is essential for the observed attenuation of respiration (Fig. 2) in the hypoxia-treated cells. This association leads to the increased phosphorylation of eNOS and generation of NO, which can potentially induce PHD2 for HIF-1 degradation and will inhibit the ETC. Further results of Western blotting (in terms of time course) also supported the proposed mechanism of hypoxia-induced NO upregulation. Between 8 and 16 h of hypoxia, there is a clear increase in expression of both Hsp90 and eNOS, as well as an enhanced association of Hsp90 and eNOS (Fig. 5). This directly correlates to the trend in the attenuation of respiration observed in similar experimental conditions (shown in Fig. 2).

Previously, several studies have correlated the magnitude of the Hsp90-eNOS association and higher NO generation to protective effects; yet, none of them reported respiration measurements, especially for hypoxia-treated cells (37). Castello et al. (9) found that NO production began when the oxygen concentration dropped below a 2% dissolved O2 concentration and maximized when O2 was not present. Shi et al. (43) have suggested that the advantages of chronic hypoxia are more closely related to how much Hsp90 associates with eNOS than to the magnitude of eNOS phosphorylation at Ser1177 alone (43). The generation of NO in central and peripheral neurons is increased during chronic hypoxia. Because of increased production of NO, the animals may develop a tolerance to the low P02 environment (39). While nearly all of these studies have proposed NO upregulation in hypoxia-exposed cells, its role in cellular respiration in the posthypoxic phase was never considered before. For example, the relationship of eNOS and Hsp90 was reported to decline in the hypoxic pulmonary artery injury (33, 35). Other studies have established that NO production is correlated to eNOS activity at the posttranslational level and not from the eNOS protein expression alone (33, 35).

Thus, activation of Hsp90 increases posttranslational modifications of eNOS in hypoxia-exposed cells, even though the eNOS protein was observed to be the same (Fig. 5). Furthermore, these results can be compared with an ischemia-reperfusion model in the heart. Through adaptation by the cells to a low O2 environment, an increase in cardiac tolerance to all critical consequences of O2 deprivation is established (44, 46). Hsp90 is considered a target to augment NO formation, significantly lessening myocardial reperfusion injury. Overexpression of Hsp90 can protect the myocardium from hazardous hypoxic and ischemic conditions (47). This allows normal oxygen consumption to occur (left). At an extreme hypoxic level (~1% O2), there is an augmented association (right). This leads to an increase in NO production and attenuation in the overall oxygen consumption rate.

![Fig. 8. Schematic representation of Hsp90-eNOS during normal and low oxygen.](http://ajpcell.physiology.org/)
effects of ischemia-reperfusion through the endothelial NO pathway (29). In the event of very high concentrations of NO, it has been proven to be detrimental. For example, extreme amounts of NO have been reported to mediate pathophysiological events in hypoxia-induced brain injuries (45). Furthermore, hypoxia activates the expression of several genes. Prabhakar et al. (39) have shown that acute hypoxia at 12 h activates the neuronal NOS gene and increases posttranscriptional neuronal NOS protein.

This work further demonstrates that there was no observable accumulation of HIF-1α (Fig. 3), therefore showing that HIF-1α does not play any role in the observed attenuation of respiration in BAECs. Recently, HIF-1 has been reported to downregulate mitochondrial O2 consumption during hypoxia through the activation of pyruvate dehydrogenase kinase (38). HIF-1 is vital in mediating cellular responses to hypoxia (3, 28). It manages oxygen consumption, angiogenesis, glycolysis, cell proliferation, and cell survival (17, 41). We observed no significant difference in the expression of HIF-1α, HIF-1α-subunits are stabilized when pyrrol hydroxylation-dependent binding of the ubiquitin ligase pVHL is inhibited. HIF-1α-subunits can interact with Hsp90, where the PAS B domain is necessary and HIF-1α stabilization occurs. Any disturbance in the function of Hsp90 defers HIF-1α accumulation (28). In its active state, the PI3K/Akt pathway is necessary for the expression of Hsp90 to protect HIF-1α from degradation in renal cell carcinoma (48). HIF-1α was also found to be stabilized by reactive oxygen species (42). It appears that the higher NO generated during hypoxia in endothelial cells enhances HIF-1 degradation (19, 34).

In summary, we have used EPR oximetry as a tool to measure changes in cellular respiration when BAECs were exposed to various O2 concentrations. EPR oximetry reports the extracellular PO2. Thus the mitochondrial PO2 could be potentially different from the measured extracellular PO2 due to oxygen diffusion barrier (18). Indeed, the gradient has been found to be ~50 µM under some conditions. At a moderate level of hypoxia (5% O2), no changes in cellular respiration were observed. However, at an extended hypoxic state of 1% O2, the overall maximum O2 consumption rate is decreased, whereas the direct NO competition with O2 at CeO (psO did not significantly differ) is not present. The stability of HIF-1α is hindered by hypoxia-induced NO. Additionally, the interaction of Hsp90 and eNOS was shown to peak around 8–16 h, followed by a moderate decline in association at 24 h. Together, these results show that the oxygen consumption of BAECs is attenuated when placed in a hypoxic environment (around 1% or less) for a considerable amount of time. The mechanism of such a reduced respiration seems to be involved with the Hsp90-eNOS pathway (Fig. 8). Overall, our results represent a new mechanism of cellular adaptation and modifications of cellular respiration that occur during hypoxia. This finding may provide insight into the different magnitude of endothelial dysfunction in various ischemic tissues. The variability could be due to the variation in the magnitude of Hsp90 association with eNOS, depending on individual cases.

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