Hyperthermia-induced Hsp90-eNOS Preserves Mitochondrial Respiration in Hyperglycemic Endothelial Cells by Down-regulating Glut-1 and Up-regulating G6PD Activity*

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Tennille Presley, Kaushik Vadam, Lawrence J. Druhan, and Govindasamy Ilangoval

From the Biophysics Program, Division of Cardiovascular Medicine, Department of Internal Medicine, Davis Heart and Lung Research Institute, and the Department of Anesthesiology, The Ohio State University, Columbus, Ohio 43210

Uncoupling of NO production from NADPH oxidation by endothelial nitric-oxide synthase (eNOS) is enhanced in hyperglycemic endothelium, potentially due to dissociation of heat shock proteins 90 (Hsp90), and cellular glucose homeostasis is enhanced by a ROS-induced positive feedback mechanism. In this study we investigated how such an uncoupling impacts oxygen metabolism and how the oxidative phosphorylation can be preserved by heat shock (42 °C for 2 h, hyperthermia) in bovine aortic endothelial cells. Normal and heat-shocked bovine aortic endothelial cells were exposed to normoglycemia (NG, 5.0 mM) or hyperglycemia (30 mm). With hyperglycemia treatment, O2 consumption rate was reduced (from \( V_{O_2,max} = 7.51 \pm 0.54 \) to 2.3 \( \pm 0.27 \) mm Hg/min/10^6 cells), whereas in heat-shocked cells, \( O_2 \) consumption rate remained unaltered (8.19 \( \pm 1.01 \) mm Hg/min/10^6 cells). Heat shock was found to enhance Hsp90/endothelial NOS interactions and produce higher NO. Moreover, ROS generation in the hyperglycemic condition was also reduced in heat-shocked cells. Interestingly, glucose uptake was reduced in heat-shocked cells as a result of decrease in Glut-1 protein level. Glucose phosphate dehydrogenase activity that gives rise to NADPH generation was increased by hyperthermia, and mitochondrial oxidative metabolism was preserved. In conclusion, the present study provides a novel mechanism wherein the reduced oxidative stress in heat-shocked hyperglycemic cells down-regulates Glut-1 and glucose uptake, and fine-tuning of this pathway may be a potential approach to use for therapeutic benefit of diabetes mellitus.

Heat shock proteins (Hsps) play critical roles in endothelial function in the hyperglycemic state, although their exact function is not clearly established. Along with hyperglycemia, the production of superoxide, insulin resistance, and a decline in vascular bioavailability of nitric oxide (NO) contribute to extreme rates of morbidity and mortality (1–4). Various factors regulate endothelial nitric-oxide synthase (eNOS) signaling in endothelial cells by modulating its phosphorylation/de-phosphorylation dynamics and the coupling/uncoupling of its redox reactions. Protein kinase B (Akt) phosphorylates eNOS on serine 1177 and increases the production of NO (5), and it is found to be very critical in overall glucose metabolism and cell survival (6). Hsp90 binding to eNOS was found to be a prerequisite for successive Akt-mediated stimulation of eNOS, and indeed, Hsp90 can be considered as a scaffold between eNOS and Akt (5, 7, 8). Vascular endothelial growth factor leads to Akt-dependent phosphorylation of eNOS, which results in increased NO production. NO generation is reduced under diabetic conditions due to NOS uncoupling (i.e. incomplete redox transformation of NADPH, arginine, and O2 to NO, citrulline, and H2O, leading instead to the generation of reactive oxygen species) resulting in the generation of more superoxide, and Hsp levels, especially Hsp90, are reduced in hyperglycemic conditions (2, 6, 9, 10). This loss of NOS activity in the hyperglycemic state contributes to the vasculopathy observed in diabetic patients (11), and one of the mechanisms proposed is that an increased production of reactive oxygen species (ROS) and reduced NO result in an impairment of cellular respiration with metabolic disruption.

Hsp90 is the most abundant cytosolic heat shock protein under non-stressed circumstances, and it is widely dispersed in the cytoplasm and nucleus (12); its function during various stresses is critical to maintain the structure/function of many proteins. In the event that the eNOS-Hsp90 interaction is decreased, the production of NO from eNOS is reduced. Upon a particular stress such as heat shock or hyperthermia (~42 °C), Hsp90 is overexpressed, and its association with eNOS is increased. Recent studies have proven that the association between eNOS and Hsp90 is increased during the beginning stage of high glucose exposure followed by a decreased association (6). Furthermore, it has been demonstrated that hyperthermia augments the interaction of Hsp90 and eNOS in cells (13).

Glucose homeostasis in endothelial cells is regulated by the plasma membrane-bound glucose transporters, mainly Glut-1.
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(14–16). Expression, stability, and function of Glut-1 are influenced by many factors, including glucose metabolites such as advanced glycation end product-induced oxidative stress and the ROS that is generated by enzymatic reactions such as NADPH oxidase and NOS (17–19). Indeed, ROS has been proven to affect the regulation of glucose transport through Glut-1 and favor increased uptake of glucose by endothelial cells. Thus, alteration of oxidative stress in hyperglycemic endothelial cells was found to directly affect glucose homeostasis (17, 18). Recently we demonstrated that non-lethal heat shock (hyperthermia) reduced superoxide generation (13).

In the present work we tested the hypothesis that heat shock can down-regulate Glut-1 due to the reduced oxidant stress and, hence, down-regulate glucose uptake in hyperglycemic conditions. Higher intracellular glucose affects oxidative metabolism. Thus, we studied oxygen metabolism in hyperglycemic conditions to prove this hypothesis. We utilized electron paramagnetic resonance (EPR) oximetry to measure cellular respiration. We found that the attenuation of oxygen metabolism due to the higher glucose uptake and utilization in hyperglycemic states is prevented by heat shock (42 °C for 2 h followed by 37 °C incubation for 24 h) in bovine aortic endothelial cells. This prevention was found to occur via heat shock-induced attenuation of ROS and subsequent reduction in Glut-1 protein level. Moreover, the hyperthermic effect was found to increase the level of Hsp90 and increase its interaction with eNOS to the extent where it may actually inhibit eNOS uncoupling and production of superoxide. Last, heat shock was found to increase the activity of glucose-6-phosphate dehydrogenase (G6PD), the enzyme that regulates the generation of NADPH, thus, increasing the production of substrate necessary for cellular antioxidant enzymes.

EXPERIMENTAL PROCEDURES

Materials—Dilithium phthalocyanine, acetonitrile, and d- (+)-glucose were obtained from Sigma. Tetrabutyl ammonium perchlorate was purchased from ICN Biochemicals (Aurora, OH). The antibodies for Western blot were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture—Bovine aortic endothelial cells (BAECs) were obtained from Cell Systems (Kirkland, WA). The BAECs were cultured in MEM (Invitrogen), 10% FBS, non-essential amino acid solution, and endothelial cell growth factor.

Heat Shock Treatment—One day before experiments, the BAECs were placed in a 42 °C incubator for 2 h to apply heat shock. Then they were returned to 37 °C overnight, the time lag that is required for gene expression induced by heat shock (13). 42 °C was preferred because it has been reported that Hsp90 induction is more specific at this temperature, whereas higher temperatures will primarily induce Hsp70 (13).

Cell Viability—Viable cell count over total was essential to ensure equal cell density in each respiration measurement of EPR oximetry. The cell viability was determined by a NucleoCounter system (New Brunswick Scientific Co., Inc, Edison, NJ) composed of the NucleoCounter automatic cell counter, the NucleoCassette, a cell preparation lysing buffer and a stabilizing buffer, and NucleoView software. This system uses propidium iodide as a detecting agent to identify the plasma membrane integrity in viable cells. Two aliquots of the cell suspension for non-viable count and the total cell count were taken. The non-viable count was determined first followed by the total cell count. Using the NucleoView software, the non-viable, total cell count, viable cell count, and viability were determined (13, 20–23).

Glucose Treatment—Cells were cultured to 80–90% confluence and subjected to either normoglycemia (NG) of 5 mm or hyperglycemia (HG) of 30 mm D-glucose for 2–24 h. For osmotic control of HG, a 5 mm glucose and 25 mm mannitol mixture was used. These glucose concentrations were chosen based on previous publications (24, 25). For heat-shocked cells, BAECs were exposed to heat shock (42 °C) for 2 h and 24 h at 37 °C and 8 h of 30 mm glucose at 37 °C for the desired time.

Glucose Uptake by BAEC Cells—Both control and heat-shocked BAEC cells, which were subjected to normoglycemia or hyperglycemia, were incubated overnight with media containing a fluorescent, noncleavable glucose analog 2-[n-97-nitro-benz-2- 0xa-1,3-diazol-4-yl)amino-2-deoxyglucose (2-NBDG, Invitrogen, 0.5 mg was dissolved in 15 ml of MEM medium) (26). The cells were trypsinized, washed with the MEM medium, counted, and finally suspended in the MEM medium. The relative fluorescence intensity from 2-NBDG-loaded cells was measured using a FACS Calibur Flow cytometer, and histograms were analyzed using WinMDI.

O₂ Kinetics—Quantitative EPR oximetry was performed using the recently described procedure (21). Briefly, there are three phases of cellular respiration that can be analyzed from a single run of pO₂ versus time using EPR oximetry: pO₂-dependent, pO₂-independent, and a steady state respiration. These levels of cellular respiration were obtained by adopting the following equation.

\[ V_{O_2}/V_{O_2_{max}} = \frac{(pO_2) - p_0}{(p_{50} + (pO_2 - p_0))} \] 

(Eq. 1)

From this equation, the \( V_{O_2_{max}} \), \( p_0 \), and \( p_{50} \) values were acquired (21). The \( V_{O_2_{max}} \) is defined as the maximum oxygen consumption rate, \( p_0 \) is the equilibrium \( pO_2 \), and \( p_{50} \) is the concentration at which the \( V_{O_2_{max}} \) is reduced to 50%. This half-maximum value is analogous to the \( K_m \) value in enzymatic reactions and provides an indication of the oxygen affinity. Specifically, \( p_{50} \) is the inverse of the mitochondrial oxygen affinity to cytochrome c oxidase in complex IV of the electron transport chain (ETC) (21). Because lithium phthalocyanine measures the extracellular \( pO_2 \) around each cell, the \( p_0 \) provides an indication of the potential intracellular \( O_2 \) content.

Western Blotting—Cells were washed twice with ice-cold PBS, trypsinized, and centrifuged at 1500 rpm for 5 min. The cell pellet was homogenized in ice-cold radioimmuno precipitation assay buffer (1× TBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide, 1× Protease Inhibitor, 1 mM PMSF, and 1 mM sodium orthovanadate) for 45 min on ice. The protein concentrations of the supernatants were measured by the BCA method and normalized to 20 μg per sample. The samples were resolved on 4–12% Bis-Tris polyacrylamide gels and transferred to PVDF membranes. After blocking with 5% nonfat milk, blots were probed with the desired antibody (1:1000 dilution). Goat anti-rabbit

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horseradish peroxidase-conjugated antibody was used as the secondary antibody, and blots were developed with enhanced chemiluminescence.

Immunoprecipitation and Immunoblotting—The total cell lysates were prepared as described in the Western blot procedure and incubated with either anti-eNOS or anti-Hsp90 polyclonal antibody overnight at 4 °C while rotating. To immunoprecipitate eNOS and/or Hsp90, protein A/G-agarose was added to the lysates and rotated at 4 °C for 2 h. The immunoprecipitates were centrifuged at 10,000 rpm for 30 s at 4 °C. The supernatant was carefully aspirated and discarded. The pellet was washed with 500 µl of radioimmunoprecipitation assay buffer 3 times and centrifuged as described. After the final wash, the supernatant was removed, and the pellet was suspended in 40 µl of SDS-PAGE sample buffer. The samples were heated at 98 °C for 8 min and subjected to electrophoresis. The PVDF membrane was immunoblotted with anti-Hsp90 or anti-eNOS to determine the amount of association of Hsp90/eNOS.

Measurement of ROS, NO, and NADPH Oxidase—ROS generation in control or heat-shocked cells that were treated with various experimental condition were determined using 2′,7′-dichlorofluorescein diacetate with emission at 520 nm with excitation at 485 nm. Non-specific oxidation of the probe was removed by using a time control, where the probe alone was exposed to the same time, and the resultant fluorescent intensity was subtracted from all experimental samples. NO production in BAECs was analyzed using fluorescence microscopic imaging with an inverted light Nikon TE2000-U microscope and DAF-2DA, a green fluorescence NO-specific probe. BAECs were cultured on coverslips and treated with NG or HG for the desired time. Then the medium was changed to Hanks’ balanced solution and incubated with 0.1 mM L-arginine. 10 µM concentration of DAF-2DA was added directly to the medium, incubated at 37 °C for 20 min, and washed twice with PBS. The fluorescence microscopy measurements were immediately performed. MetaMorph software was used to calculate the average fluorescence intensity of individual cells. For NADPH oxidase, determination cells were grown and treated with L-arginine in regular culture dishes and stimulated with 5 µM of A23187, and the medium was collected for Griess reaction.

Glucose-6-phosphate Dehydrogenate (G6PD) Activity—G6pDH activity measurements were carried out as described before (27). Both normal and heat-shocked BAECs that were subjected to either NG or HG were lysed in assay buffer (50 mM Tris, pH 8.1, 1 mM MgCl2) by freeze-thaw cycles, and the lysates were incubated with reaction buffer containing 100 µM NADP+ and 200 µM glucose 6-phosphate. Enzyme activity was determined by measuring the rate of increase in absorbance at 340 nm due to conversion of NADP+ to NADPH.

Lactate Assay—Control and heat-shocked cells were subjected to normal and hyperglycemia treatment and lysed using freeze-thaw cycles. Lactate was determined using EnzyChromTM lactate assay kit (ECLC-100, Bioassay Systems) as per the manufacturer’s instructions.

Mitochondrial ETC Enzymatic Activities—Mitochondrial electron transfer chain complexes I-IV activities were measured as described before (22, 23). Normoglycemia and hyperglycemia-treated control and heat-shocked BAECs were homogenized in ice-cold 3 mM HEPES buffer (pH 7.2), 0.5 mM EGTA, 0.25 mM sucrose, and 2.5% protease inhibitor mixture. The protein concentration was measured using a BCA assay kit using BSA as the standard. The mitochondrial ETC complex activities were acquired by analyzing the supernatant using a Varian UV-visible spectrophotometer Cary 50. For Complex IV activity, the enzyme activity of cytochrome c oxidase was assayed by measuring cytochrome c oxidation where the assay mixture contained 50 mM NaK/PO4 buffer and 50 µM reduced cytochrome c (pH = 7.4). The cytochrome c was reduced by adding a few microcrystals of sodium ascorbate. The activity of cytochrome c oxidase was obtained by evaluating the decrease in absorption at 550 nm. Complex I activity was determined by measuring the decrease in absorbance at 340 nm of reduced nicotinamide adenine dinucleotide (NADH) initiated by ubiquinone 1. The assay mixture included 20 mM potassium phosphate buffer, pH 8.0, 2 mM NaN3, phospholipid (0.15 mg/ml), 0.1 mM ubiquinone 1, and 0.15 mM NADH. To determine complexes II/III activity, the increase in absorbance at 550 nm was measured. The activity of succinate-cytochrome c reductase was assayed by assessing ferricytochrome c reduction. The assay mixture contained 50 mM phosphate buffer, pH 7.4, 0.3 mM EDTA, 50 µM KCN, 19.8 mM succinate, and 50 µM ferricytochrome c (22).

Data Analysis—Data are presented as the means ± S.E. Statistical analysis was performed using Student’s t test and one-way analysis of variance. The general acceptance level of significance was p < 0.05. The EPR spectra, collected during the cellular respiration measurements, were analyzed as formerly described (13). 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 before (21).

RESULTS

Attenuation of Oxygen Consumption Rate (OCR) in BAECs at Hyperglycemic Conditions—At 37 °C, BAECs were cultured to 80–90% confluence. The cultured cells were treated with 5 mM glucose (NG) for 2, 4, 8, 12, and 24 h, as illustrated in Fig. 1A, trypsinized, and re-suspended in respiration medium (Krebs-Ringer solution) containing a concentration of 5 mM glucose. Care was taken to ensure that equal numbers of viable cells were used for each experiment. Respiratory data by EPR oximetry were obtained at 5-s time intervals for up to 60 min for 10 × 106 cells treated at 5 mM glucose for 2–24 h. Fig. 1B, left panel, shows the pO2 versus time data for cells treated with 5 mM glucose for 8 h and control cells, showing that there is no difference in OCR produced by treatment with 5 mM glucose. These data were transformed into dpO2/ dt (i.e. OCR) versus pO2 to get the OCR (defined as VO2max) as a function of prevailing O2 concentration as shown in Fig. 1C (13, 21). From these data, three distinct phases of respiration are observable: a constant region equivalent to the maximum OCR in the pO2 range >10 mm Hg, a curved region representing the prevailing pO2-dependent OCR, and a constant value at which the cellular respiration ceases (Fig. 1C). Once converted into OCR versus pO2 (Fig. 1C), the data were fit as previously described, and the relevant parameters were determined. For 8 h of 5 mM glucose,
Reduced O$_2$ metabolism in endothelial cells at hyperglycemic conditions. A, shown is a schematic illustration of hyperglycemia treatment. BAECs were grown to ~70% confluence and exposed to normoglycemia (5 mM glucose) or hyperglycemia (30 mM glucose) for 2–24 h. B, shown is O$_2$ consumption of BAECs in normoglycemia and hyperglycemia. BAECs were treated with 5 mM (normoglycemia) or 30 mM (hyperglycemia) glucose for different times as indicated, lysed, and blotted for Hsp90 and eNOS. The quantitative plots show that both Hsp90 and eNOS are significantly reduced by hyperglycemia in BAECs but only after more than 8 h treatment. The eNOS level and its association with Hsp90 were assessed. Fig. 2, right panel, illustrates an apparent reduction in the overall respiration rate in the 30 mM glucose-treated cells. The $V_{O_{2,max}}$ showed a value of 4.30 ± 0.23 mm Hg/min/10$^6$ cells ($n = 5$), and the $p_{50}$ reached a value of 2.35 ± 0.27 mm Hg, whereas the $V_{O_{2,max}}$ for the equi-osmotic control was 7.51 ± 0.54 mm Hg/min/10$^6$ cells, and $p_{50} = 4.51 ± 0.58$ mm Hg. In NG the OCR was reduced to approximately half of the value of NG. By taking the inverse of the $p_{50}$ value, the hyperglycemic cells treated for 8 h show a value of 0.45 ± 0.08 mm Hg$^{-1}$ (control = 0.35 ± 0.04 mm Hg$^{-1}$), implying there is no change in the mitochondrial affinity of oxygen (21–23) in the presence of 30 mM glucose despite the reduced respiration. The osmotic control (5 mM glucose + 25 mM mannitol) showed OCR and parameters close NG (Fig. 1D).

Decreased Hsp90-eNOS in Hyperglycemic BAECs—To determine whether the attenuation in OCR correlated to a deterioration of eNOS in hyperglycemic conditions, $V_{O_{2,max}} = 6.37 ± 0.90$ mm Hg/min/million cells, $p_{50} = 3.37 ± 0.25$ mm Hg, and $p_{0} = 0.86 ± 0.15$ mm Hg ($n = 4$). Similarly, the control cells reached a maximum OCR of 7.51 ± 0.54 mm Hg/min/10$^6$ cells, a $p_{50} = 4.51 ± 0.58$ mm Hg, and $p_{0} = 0.23 ± 0.04$ mm Hg. These results demonstrate that the normoglycemic state did not affect either mitochondrial respiration or oxygen affinity at cytochrome $c$ oxidase of complex IV in the ETC.
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8 h. Immunoprecipitation of eNOS and immunoblotting of Hsp90 (and vice versa, data not included) were also carried out as summarized in Fig. 2B to determine whether there is any change in association of eNOS-Hsp90. The immunoblot intensity plotted as the Hsp90/eNOS ratio in eNOS immunoprecipitates, as shown in quantitative plots, decreased between 8 and 24 h of hyperglycemia treatment. This may be a result of the change in these proteins levels in these conditions and/or enhanced dissociation due to hyperglycemia. In any case there is a significant decrease in the eNOS-Hsp90 complex produced after 8 h of hyperglycemic treatment.

Reversal of OCR in Hyperglycemia-induced Attenuation of Respiration in Heat-shocked BAECs—BAECs were subjected to heat shock for 2 h at 42 °C followed by 24 h at 37 °C as described in the schematic illustration in Fig. 3A. Because we observed a maximum attenuation in the OCR at 8 h of hyperglycemic treatment of non-heat-shocked cells (see Fig. 1), the following studies were restricted to 8 h of HG treatment. As shown in Fig. 3B, the heat-shocked cells consumed oxygen slower than control in NG as determined previously (25). However, heat-shocked cells that were also subjected to HG did not show any attenuation of OCR. Indeed the $V_{\text{O}_2,\text{max}}$ and $P_{\text{aw}}$ were restored to values comparable with the control when exposed to hyperthermia before hyperglycemia (Fig. 3B and C).

In previous publications, the deterioration of eNOS function due to a lack of Hsp90 association was attributed to a significant dysfunction of hyperglycemic endothelial cells, and in the present study we also found such a reduction in the Hsp90 association (see Fig. 2). Thus, further measurements were made to evaluate whether the heat shock-induced change in the eNOS-Hsp90 complex formation before HG altered the OCR and, thus, implicates eNOS function as the cause of the observed restoration of OCR produced by heat shock in the presence of hyperglycemia (Fig. 2). Measurement of OCR with incubation of the Hsp90 antagonist, geldanamycin (GA), and/or a NOS inhibitor, l-NAME, were carried out. BAECs were incubated with the inhibitors for 30 min before heat shock (HS) and high glucose exposure. As shown in Fig. 3C, the l-NAME treatment of the HS and high glucose exposure cells decreased the $V_{\text{O}_2,\text{max}}$ to a value comparable with the BAECs under hyperglycemic conditions alone. Similarly, when we incubated the HS and high glucose-exposed cells with GA, the $V_{\text{O}_2,\text{max}}$ was of the same magnitude of that observed with the hyperglycemia-treated cells. Interestingly in cells treated with l-NAME, acute bolus addition of 20 μM DETANO (Cayman), an NO-releasing agent, did not show any improvement in the respiration in hyperglycemic conditions (Fig. 3C). These results indicate that eNOS-derived NO and the interaction between Hsp90 and eNOS are key factors in the mechanism of heat-shock restoration of the decrease in respiration rate produced by hyperglycemia.

Increased Hsp90/eNOS Interactions in Heat-shocked Cells and Higher NO Generation at Hyperglycemic Conditions—To directly demonstrate that the Hsp90/eNOS complexes were enhanced by heat shock during HG exposure, immunoprecipitation and immunoblotting of eNOS and Hsp90 and vice versa were carried out. Heat-shocked BAECs were exposed to 5 and 30 mM glucose for the same times as described in Fig. 2A. In the case of the heat-shocked and NG-treated BAECs, there was an observable increase in the level of both Hsp90 and eNOS (Fig. 4A). Moreover, in heat-shocked cells, treatment with 30 mM glucose for >8 h did not affect either Hsp90 or eNOS levels, unlike the non-heat-shocked HG-treated cells that showed a significant decrease (see Fig. 3A). Additionally, the interaction between Hsp90 and eNOS was retained in heat-shocked hyperglycemic BAECs (Fig. 4B).

To demonstrate the functional consequences of the observed increase in the eNOS-Hsp90 interaction, NO generation was measured with fluorescence microscopy for normal and heat-shocked cells that were exposed to 30 mM glucose. DAF-2DA staining, which yields green fluorescence by reacting with NO and its derivatives, was used to quantify NO generation. Fig. 5A illustrates the fluorescence images of DAF-2DA-stained control and heat-shocked cells that were subjected to hyperglycemia. When compared with the control BAECs, a distinct intensification in fluorescence can be seen in the heat-shocked HG-treated cells (Fig. 5A), and this observed increase in NO generation was inhibited by preincubation with GA (before heat shock). Based on the quantitation of the intensity mea-
measurements, HG significantly reduced NO generation, but heat-shocked BAECs showed an average of about 2-fold higher intensity, compared in the HG exposed BAECs (Fig. 5B). These data confirm that NO generation is increased in HS cells even in hyperglycemia, illustrating that in HS cells, NOS function is not inhibited. To confirm the fluorescence measurements, we determined the concentration of the nitrate species (NADPH oxidase) under the same conditions (Fig. 5C). Similar to the fluorescence imaging, NADPH oxidase was consistently higher in heat-shocked hyperglycemia-treated cells (about a 2-fold increase), and this increase was prevented by treatment with GA.

**ETC Activities and ROS Generation**—To directly demonstrate that the observed decrease in OCR was indicative of decrease mitochondrial respiration, we measured the activity of the mitochondrial ETC complexes. Activities measured for all the complexes (I-IV) showed a significant lowering of activity when treated with HG over a period of >8 h (Fig. 6A). Notably, heat shock prevented the HG-induced decrease in activity (Fig. 6A). ROS measurements showed a similar pattern (Fig. 6B). Upon treating with hyperglycemia, a strong DCF fluorescence was observed (Fig. 6B). However, in heat-shocked BAECs that were treated with either NG or HG, there was no increase in fluorescence. Interestingly, GA-incubated heat-shocked cells did show higher fluorescence upon HG treatment (Fig. 6B), illustrating that the association of Hsp90 with eNOS prevents the heat shock-induced decrease ROS in HG.

**Down-regulation of Glut-1 and Up-regulation of G6PD Activity in Heat-shocked Cells at Hyperglycemia**—To investigate the mechanism of the observed reversal of the HG-dependent attenuation of respiration by heat shock, we examined how heat shock affected the processes involved in glucose metabolism. First, we investigated glucose uptake. Both control and heat-shocked cells were treated with 30 mM 2-NBDG, a fluorescent glucose analog, for 8 h and analyzed in flow cytometry (23, 26). As shown in Fig. 7A, as expected the non-heat-shocked hyperglycemic cells showed higher fluorescence (with 97.55% of the counted cells positive for the 30 mM 2-NBDG with mean fluorescence 14.30 ± 1.03). Conversely, in heat-shocked cells the fluorescence was much lower (36.66% of the counted cells showed the mean value of 8.32 ± 0.86), indicating that the glucose uptake is significantly reduced in the heat-shocked cells. Additionally, preincubation of BAECs with GA before heat shock prevented the attenuation of 2-NBDG uptake (Fig. 7A) produced by heat shock. Next we determined whether there is any difference in glucose transporters in these cells. It has been previously established that Glut-1 and Glut-2 are the major glucose transporters found in BAECs (14, 17, 18, 23). The Western blots of Glut-1 and Glut-2 showed that the Glut-1 is very much reduced in heat-shocked cells, whereas there was no change in Glut-2 (Fig. 7B). The quantitative estimations showed that Glut-1 is reduced to one-third in heat-shocked cells and heat-shocked and hyperglycemia-treated cells (Fig. 7B), whereas there was little increase in control cells that were treated with higher glucose. Increased glucose uptake in hyperglycemia has been attributed to an oxidative stress-induced auto-regulation of glucose transport in endothelial cells (18); although we saw no change in Glut-1 in HG-exposed cells (Fig. 7B), we did see a significant decrease of Glut-1 level in the heat-shocked HG cells that can be correlated to the observed heat-shock induced reduction of ROS (as illustrated in Fig. 6), leading to the decrease in glucose uptake.

Next we determined how heat shock affected the intracellular metabolism of glucose. First, we determined the intracellular concentration of lactate, the product of the generation of ATP via the anaerobic catabolism of glucose. The intracellular accumulation of lactate can lead to the inhibition of glycolysis (28), as such heat shock could affect oxidative phosphorylation via the inhibition of glycolysis by...
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A

**p = 0.001

![Graph showing mitochondrial ETC complexes activities and ROS generation in hyperglycemic BAECs.](Image)

B

![Graph showing mitochondrial ETC complexes activities and ROS generation in hyperglycemic BAECs.](Image)

FIGURE 6. Mitochondrial ETC complexes activities and ROS generation in hyperglycemic BAECs. A, activities of the ETC complexes in BAECs determined in NG, HG, HS + HG, and isotonic osmotic control (OC) (25 mm mannitol + 5 mm glucose) are shown. B, shown are ROS measurements in NG, HG, HS + HG along with samples that were preincubated with GA (0.1 mM) before hyperthermia and hyperglycemic treatments (HG + HG + GA). Background-corrected (see “Experimental Procedures”) quantitative plots of the fluorescence intensities from individual cells were obtained using the Metamorph software. At least 10 cells were used as minimum for averaging from each individual experiment, and each bar represents three independent experiments. Error bars represent ± S.E.

Inhibiting lactate export. However, as shown Fig. 8A, there was in fact a decrease in the intracellular lactate concentration produced by heat shock. Moreover, there was no difference between the lactate concentration of heat-shocked cells with or without HG treatment. Thus, heat shock does not inhibit glycolysis by altering lactate export. However, heat shock does decrease lactate indicative of a decrease in the necessity for regeneration of NADPH via the conversion of pyruvate to lactate, in agreement with our observed heat shock-induced increase in oxidative phosphorylation in HG-treated cells. Next we determined the activity of G6PD, the rate determining enzyme in the pentose phosphate pathway, which utilizes glucose for the production of NADPH. In hyperglycemia, several studies have reported that the activity of this enzyme is altered and that such changes in activity could regulate glycolysis (23, 25, 29, 30). The G6PD assay in the present study showed a reduction in the G6PD activity in hyperglycemic non-heat-shocked cells, whereas in heat shock alone and in hyperglycemic heat-shocked cells, G6PD activity was found to be increased 2- and 3-fold, respectively (Fig. 8B). Thus, heat shock stimulates the utilization of G6P for the production of NADPH and as such could potentially decrease the total through-put of glycolysis in HG via a decrease in the total phosphorylated hexose pool (25).

Moreover, the heat shock-induced increase in G6PD activity allows for an increase in the activity of the NADPH-dependent anti-oxidant enzymes, in agreement with our observed decrease in ROS produced by heat shock.

DISCUSSION

In the present work we report a new approach, namely hyperthermia, to minimize oxidative stress in diabetes by increasing the eNOS-Hsp90 interaction and NO generation. Classically the increase in NO in a diabetic setting would be beneficial due to the restoration of cGMP signaling and the prevention of high blood pressure. In this work we demonstrate that heat shock-induced NO modulates endothelial OCR and that this is associated with a change in glucose homeostasis, as indicated by an up-regulation of G6PD activity and down-regulation of Glut-1. As such, heat shock decreases both glucose uptake and oxidative stress and restores mitochondrial O2 metabolism in HG to a level that is comparable with NG. Recent studies have shown that Metformin, a well known drug used to treat type 2 diabetes, augmented Hsp90 and eNOS, increased NO bioactivity, and reduced endothelial apoptosis, supporting our present hypothesis that the heat shock-induced increase in eNOS-Hsp90 is able to ameliorate endothelial dysfunction, induced by HG (11).

In many pathophysiological conditions, Hsp90 association with eNOS is lessened, and subsequently, eNOS or its co-factors become targets for many oxidants, potentially leading to inactivation of eNOS and less NO generation (5, 31–35). In diabetes Hsp90 dissociation from eNOS has also been implicated in the inactivation of eNOS. However, the mechanism by which the eNOS-Hsp90 interaction is decreased during HG is less understood. Various mechanisms have been proposed, such as competitive binding of IKK (36) and protein kinase A-dependent translocation to the outside of endothelial cells (37). Our results, demonstrating that heat shock increases the eNOS-Hsp90 association, would support the IKK competitive binding mechanism because it has been shown that heat shock can deplete IKK in the myocardium (38). The association between eNOS and Hsp90 has been previously shown to be regulated during the beginning stages of high glucose exposure (<6 h) and dissociated by longer exposures (2, 6, 9), and our results corroborate these findings.

We applied EPR oximetry, an emerging oxygen sensing technique (39) for cellular respiration measurements in microliter volumes, to very accurately study the OCR and changes in O2 metabolism. During HG, we found that V_{O2,max} was similar to that of the non-heat-shocked cells up to 8 h of exposure, i.e. no significant change in OCR, consistent with previous work indicating that acute exposure to hyperglyceremia does not attenuate O2 metabolism (24, 25). However, the BAECs that were exposed for >8 h to HG demonstrated significantly reduced OCR (Fig. 3). This observation recapitulates the results reported previously that mitochondrial respiration as a source of ATP generation becomes submaximal at high glucose levels and, by extension, that other non-mitochondrial pathways of glucose metabolism such as glycolysis or pentose shunting predominate in hyperglycemic cells (25).

The novel finding that heat shock treatment of HG-treated BAECs restored the OCR (in terms of V_{O2,max}) to a level that was
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**FIGURE 7.** Glut-1, Glut-2, and glucose uptake in hyperglycemic BAECs. A, shown are histograms of fluorescent cell (due to uptake of fluorescent 2-NBDG, a glucose analog) population obtained by FACS analyses. The heat-shocked cells (HS + HG + 2-NBDG) showed significant reduction in fluorescent cell population compared normal cells that were treated with HG (HG + 2-NBDG) as indicated in the figure. Preincubation with 0.1 mM GA (HS + GA + HG + 2-NBDG) inhibited the observed HG-dependent reduction. B, Western blots of Glut-1 and Glut-2 in normoglycemia- and hyperglycemia-treated normal and heat-shocked cells are shown. The hyperglycemic treatment was for 8 h. The quantitative plots were obtained from three independent experiments, and the error bars indicate S.E.

**FIGURE 8.** Lactate determination and G6PD activity measurements in hyperglycemia subjected BAECs. A, lactate concentration was measured in the lysates of BAECs, treated as described in the figure. In both HS and HS + 30 mm glucose-treated cells, the lactate concentrations were slightly lower than the control cells. B, G6PD activity; determined in whole cell lysate of BAECs that were treated as in A. The values represent the mean obtained from three independent experiments; error bars represent S.E.

comparable with the NG-treated cells indicates that the predominance of glycolysis in HG as a source of ATP (akin to the Crabtree effect) is abolished in the heat-shocked cells, resulting in the restoration of mitochondrial respiration. We found an enhanced interaction in heat-shocked cells; thus, we hypothesized that the mechanism of such a recovery of OCR and retention of maximal mitochondrial function was a result of the heat shock-dependent conservation of the Hsp90-eNOS interaction, which would result in protection of eNOS from uncoupling leading to an increase in NO. Confirmatory results for this hypothesis were obtained by demonstrating that the inhibition of the Hsp90-eNOS interaction and the direct inhibition of eNOS activity prevented the heat shock-induced restoration of OCR.

We and others have demonstrated previously that heat shock enhanced NO generation in normoglycemic conditions by increasing the eNOS-Hsp90 interaction (13, 23, 32). Such an enhanced NO generation was demonstrated to have serious implications in cellular respiration, and we have elucidated a possible mechanism of this Hsp90-eNOS-induced modulation of cellular respiration involving both irreversible/reversible inhibitory roles of NO on the mitochondrial oxidative metabolism (22). In this work we demonstrate that in a hyperglycemic setting the same hyperthermia-induced eNOS activation by Hsp90 also increases NO generation and reduces ROS, but this is accompanied by an increase in mitochondrial respiration. The two seemingly opposing NO-dependent effects, namely attenuation of mitochondrial respiration by NO and its derivatives in heat-shocked normoglycemic endothelial cells and restoration of mitochondrial respiration in hyperglycemic heat-shocked endothelial cells appear to be controlled by mechanisms that depend upon the cellular respiration state and/or glucose metabolism. Indeed, NO seems to be a modulator of respiration to protect the cells from metabolic stress.

We showed that heat shock of cells before HG treatment reduced glucose uptake via a decrease in Glut-1 (Fig. 7). ROS and oxidative stress are known to regulate Glut-1 (17, 18), and NO is known to have anti-oxidant properties (40). Thus, by decreasing ROS the observed increase in NO produced by heat shock would lead to the observed decrease Glut-1 and glucose uptake (Fig. 6B). We also found that heat shock was associated with an increase in G6PD activity (Fig. 8). In support of this finding, previous work has demonstrated that the deletion of HSF-1, the transcription factor that regulates expression of the heat shock proteins, led to decreased G6PD activity (41). The increase in G6PD produced by heat shock could increase OCR in our diabetic model in two ways. First, G6PD activity would increase NADPH, promoting the down-modulation of glucose uptake via the antioxidant-induced decrease in Glut-1 by providing substrate for eNOS and for the other NADPH-dependent antioxidant enzymes. Second, activation of G6PD decreases the phosphorylated hexose pool, potentially limiting the glucose available for ATP generation.

Assuming constant ATP demand, the heat shock-induced decrease in glucose uptake and availability would require more efficient production of ATP from each equivalent of glucose, and thus, the cells revert to oxidative phosphorylation. As such, our data support a model for the observed heat shock-induced increase in OCR that is driven by alterations in glucose metabolism. Although it is clear that the increase in NO produced by heat shock in high glucose-treated cells does not inhibit mito-
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... mitochondrial respiration (but does inhibit the NG setting), the definitive explanation for this remains to be determined. One possible mechanism could involve differential eNOS subcellular localization, which has been shown to alter the activity of eNOS-derived NO and to be potentially regulated by glucose uptake (42–44).

In agreement with our hypothesis that an increased G6PD activity in HG-treated cells decreases ROS (leading to a decrease in Glut-1 expression), G6PD overexpression has been shown to reduce oxidative stress (45). Conversely, higher G6PD activity in the diabetic heart has been found to lead to an increase in NADPH oxidase-derived ROS (29). Thus, higher NADPH generation could potentially act as a double-edged sword. However, there are conflicting reports as to the source of the HG-induced ROS. Whereas many studies have determined that NADPH oxidase is the main source (25, 29, 46, 47), some studies have determined that the mitochondria are the major contributors of the ROS observed in hyperglycemia (24, 48, 49).

We found that G6PD activity (indicative of NADPH production) was reduced in hyperglycemic-treated endothelial cells, which showed an increase in observed ROS. Moreover, G6PD activity was increased with heat shock with a concomitant decrease in ROS. Thus, if G6PD activity correlates with ROS production from NADPH oxidase, our data indicate that NADPH oxidase is not the source of the observed ROS, at least not in the endothelium. Our oximetry data demonstrate that the flux through the mitochondria is decreased by HG, and this decrease is restored with heat shock. The simplest interpretation of these data would lead to the conclusion that mitochondrial-derived ROS would be diminished by HG treatment and increased by heat shock. However, the observed HG-dependent decreased mitochondrial function could also be interpreted as an uncoupling of the mitochondria, which would lead to an increase in mitochondrial-derived ROS.

Normal O$_2$ metabolism in mitochondria results in the reduction of O$_2$ to H$_2$O, with very little production of ROS. However, in HG, normal mitochondrial respiration is inhibited, and the ETC leaks electrons, especially from complexes II and III, leading to increased ROS formation. This electron leak and subsequent ROS formation is much less efficient than normal respiration; thus, in HG, although the O$_2$ consumption decreased, mitochondrial ROS production could be increased. As such, our oximetry data could be interpreted as indicative of the mitochondria as the source of superoxide. Moreover, it could be postulated that either NADPH oxidase- or mitochondrial-derived ROS is an upstream activator of other ROS-producing enzymes, e.g. xanthine oxidase or NOS (50, 51). In the case of NOS, the O$_2^*$ oxidizes and depletes a critical co-factor, H$_2$B, and this leads to uncoupling and ROS generation directly from NOS (50, 52, 53). Our data indicated that heat shock attenuated the HG-dependent O$_2^*$-induced inactivation of eNOS via an enhanced association with Hsp90. Thus, the determination of the definitive source of the observed increase in superoxide produced by HG requires more study.

In summary, we have demonstrated that the HG-dependent inhibition of oxygen metabolism via mitochondrial respiration in hyperglycemic BAECs is restored by hyperthermia and that this restoration is due to an increase in NO from eNOS produced by an enhancement of the eNOS-Hsp90 interaction. The decrease of OCR in BAECs at HG is accompanied by higher glucose uptake, allowing for the adequate production of ATP via glycolysis. Concomitantly, there is a significant increase in ROS and obvious eNOS dysfunction. Hyperthermia enhanced the eNOS-Hsp90 interaction and increased G6PD activity, leading to higher NO generation and NADPH production. These actions down-regulate Glut-1 level by reducing ROS, attenuating glucose uptake, and limiting glucose available for glycolysis and, thus, restored oxidative metabolism by the mitochondrial electron transport chain. A schematic of the proposed mechanism is presented in Fig. 9. Even though we demonstrated here that hyperthermia-induced activation of the eNOS-Hsp90 interaction can alleviate the HG-induced attenuation of respiration, we cannot rule out that other approaches (such as hypoxia) that can also activate eNOS-Hsp90 will show a similar positive impact on cellular respiration. Taken together our data indicate the potential for hyperthermia (or other methods for increasing the eNOS-Hsp90 interaction) or the pharmacological inhibition of Glut-1 as a potential therapeutic approach for protecting the endothelium in diabetes mellitus.
