Increased Superoxide Generation and Decreased Stress Protein Hsp90 Expression in Human Umbilical Cord Vein Endothelial Cells (HUVECs) from Pregnancies Complicated by Preeclampsia

Yang Gu¹, David F. Lewis¹, Yanping Zhang¹, Lynn J. Groome¹, and Yuping Wang¹,²
¹Department of Obstetrics and Gynecology, Louisiana State University Health Sciences Center, Shreveport, Louisiana
²Department of Cellular and Molecular Physiology, Louisiana State University Health Sciences Center, Shreveport, Louisiana

Abstract

Objective—Endothelial dysfunction is associated with increased oxidative stress in the vascular system in women with preeclampsia (PE), a hypertensive disorder occurring during human pregnancy. However, due to the nature of the disease, direct evidence of increased endothelial oxidative stress in the maternal vascular system at an in vivo situation is still lacking. We previously reported that primary cultured endothelial cells (ECs) from umbilical cords (HUVECs) from pregnancies complicated by PE exhibit phenotypic changes compared to those from normal pregnancies such as reduced eNOs expression associated with disorganized endothelial junction protein distribution and increased endothelial permeability. In this study, we sought to determine whether increased oxidative stress was also present in primary cultured HUVECs from women with PE.

Methods—HUVECs were isolated from normal and PE pregnancies and EC oxidative stress was examined by superoxide generation using positive nuclear dihydroethidium (DHE) staining as an indicator. Since Hsp90 is believed to have protective effects on endothelial function, we also determined mRNA and protein expression for Hsp90. Using Hsp90 inhibitor geldanamycin (GA), we further determined the potential role of Hsp90 in superoxide generation, eNOs expression, and prostacyclin production of altered EC function associated with PE pregnancies.

Results—We found that primary cultured ECs from PE pregnancies showed an increase in DHE positive cells, p < 0.01. Hsp90 protein expression was significantly decreased in ECs from PE compared with that from normal pregnancies, p < 0.05. Inhibition of Hsp90 by GA resulted in an increase in superoxide generation and a decrease in eNOs protein expression. Decreased prostacyclin production was also found in ECs treated with GA.

Conclusion—These in vitro HUVEC data suggest that increased endothelial oxidative stress may also occur in the fetal compartment during preeclampsia.

Keywords
Endothelial oxidative stress; Heat shock protein; eNOs; Prostacyclin; Preeclampsia
INTRODUCTION

During pregnancy, the maternal cardiovascular system undergoes significant changes in order to provide adequate uteroplacental perfusion for fetal development without compromising maternal function, including increased cardiac output and blood volume, reduced systemic vascular resistance and decreased perfusion pressure (1). Although these vascular physiological adaptations represent a complex interplay between the humoral, nervous, and systemic organic functional and structural coordination, the well behaved function of vascular endothelium is extremely critical for a healthy pregnant outcome of both the mother and the baby.

Preeclampsia is a hypertensive and multiple-system disorder, but occurs only during human pregnancy. Often, pregnancies complicated by preeclampsia are delivered preterm because of an uncontrollable hypertensive syndrome. Compelling evidence supports the notion that endothelial activation/dysfunction associated with increased oxidative stress is a central pathophysiological event in the maternal vascular system in preeclampsia. It has been demonstrated that maternal circulating levels of lipid peroxides and oxidized low-density lipoproteins (LDL) are increased during preeclampsia. In contrast, decreased antioxidant levels and activities including superoxide dismutase, ascorbic acid, selenium, and α-tocopherol, have been found in preeclampsia (2–4). Enhanced nitrotyrosine staining in maternal vessels from pregnancies complicated by preeclampsia has also been reported (5). Consistent with the maternal oxidative changes in the vascular system, endothelial cells isolated from umbilical cords (HUVECs) from preeclamptic pregnancies display various cellular and functional alterations associated with enhanced oxidative stress, such as increased neutrophil-endothelial interaction, perturbed endothelial junction protein VE-cadherin and occludin distribution, and altered endothelial NO synthase (eNOs) expression (6–8). These cellular and functional changes in HUVECs suggest that increased oxidative stress may also occur in the fetal compartment in pregnancies complicated by preeclampsia.

In terms of cellular protective regulation, heat shock protein induction is one of the most basic defense mechanisms against cellular injury induced by oxidative stress. This response involves the expression of a set of proteins, originally referred to as “heat shock” proteins and now generally called “stress” proteins. Heat shock protein 90 (Hsp90) is the most abundant chaperone, constituting about 1% to 2% of total protein, within a cell (9). As a major cellular chaperone in mammalian cytoplasm, Hsp90 exerts protective effect against cellular stress mainly by mediating conformational regulation of a wide variety of client proteins involved in signal transduction, cell proliferation and apoptosis (10). It was reported that the binding of Hsp90 to eNOs in endothelial cells enhances the activation of eNOs, which is an important cellular protective component of maintaining the normal bioavailability of NO and vascular homeostasis (11). We previously reported that increased endothelial permeability is associated with reduced eNOs expression in HUVECs (8). However, it is not known whether reduced eNOs expression is associated with increased endothelial oxidative stress in endothelial cells from patients with preeclampsia. In this study, we sought to determine whether increased oxidative stress was present in HUVECs from preeclamptic pregnancies. Primary cultured HUVECs were studied. We also determined Hsp90 expression in HUVECs from preeclamptic pregnancies and compared with that from normal pregnancies. Using Hsp90 inhibitor geldanamycin (GA), we further examined the potential role of Hsp90 in superoxide generation, eNOs expression, and prostacyclin production of altered endothelial function associated with the fetal syndrome of preeclampsia.
MATERIALS AND METHODS

Patient Information

Umbilical cords were collected following normal and preeclamptic pregnancies after delivery at the Louisiana State University Health Sciences Center in Shreveport (LSUHSC-S). Normal pregnancy is defined as a pregnancy in which the mother had normal blood pressure ($\leq 140/90$ mm Hg) and absence of medical and obstetrical complications. Mild preeclampsia is defined as maternal systolic blood pressure $\geq 140$ mm Hg and/or diastolic blood pressure $\geq 90$ mm Hg on two occasions, separated by 6 hours and proteinuria $>1+$ or $>300$ mg in a 24-hour period. Severe preeclampsia is defined as maternal systolic blood pressure $\geq 160/110$ mm Hg with proteinuria $>2+$ qualitative on two separate readings. In this study, a total of 42 umbilical cord specimens were used, 12 from preeclamptic pregnancies and 30 from normal pregnancies. For the preeclampsia group, one was diagnosed with mild preeclampsia and 11 with severe preeclampsia. The clinical information for the preeclampsia group was: mean maternal age 24 ± 4 years old; gestational age 33 ± 4 weeks, blood pressure 179 ± 9/110 ± 7 mm Hg, with three vaginal deliveries and nine cesarean sections. All the umbilical cords used for the normal group were from normal term pregnancies. Smokers were excluded from the study. This study was approved by the Institutional Review Board (IRB) for Human Research at LSUHSC-S, LA.

Endothelial Isolation and Culture

Endothelial cells were isolated by collagenase digestion from umbilical vein as previously described (6,12). Isolated endothelial cells were incubated with endothelial cell growth medium (EGM) purchased from BioWhittaker (A Cambrex Company, Walkersville, MD). Only the primary and first passage (P1) cells were used in this study. Primary isolated cells grown in 24 well/plates were used for immunofluorescent staining of dihydroethidium. P1 cells grown in 25 cm$^2$ culture flasks were used for protein and RNA extraction. P1 cells grown in 12-well plates were used to determine endothelial prostacyclin production.

Detection of Superoxide Generation

Endothelial superoxide generation was determined by staining of cells with fluorescent-labeled dihydroethidium (DHE; Molecular Probes, Eugene, OR). DHE is cell permeable and reacts with superoxide to form ethidium, which in turn intercalates with DNA and produces nuclear fluorescence. Only, primary cultured endothelial cells (usually 3 to 4 days after isolation) were used in this experiment. Cells were treated with 10 μmol/L DHE in phosphate buffered saline for 30 min at 37°C as previously described (13). Positive nuclear DHE staining is an indicator of superoxide generation in cells. In general, 5 to 6 images of cells were captured randomly by a phase-contract microscope (XI 71 Olympus, Tokyo, Japan) with a setting of 40× magnification. With this setting, one imaging with an area of 65,000 μm$^2$ contained approximately 40 to 60 cells. Total number of cells in all images (approximately 200 to 300 cells/per cover slip) was counted and the percentage of nuclear DHE positive cells was calculated. Superoxide generation was expressed as a percentage of DHE positive cells.

mRNA Expression for Hsp90β and eNOs

Total RNA was isolated from endothelial cells by Tri reagent (Molecular Research Center Inc, Cincinnati, OH). Two micrograms of total RNA from each endothelial sample were reverse transcribed by using the SuperScript first-Strand Synthesis system (Life Technologies, Rockville, MD). The product was subjected to polymerase chain reaction (PCR), using the primers that were specific for Hsp90β and eNOs. The primers for Hsp90β (CAG GTG ACA ATC TCC AAT AGA CTT GTG TCT and GAC CTA GCT TGA TCA GTG) and eNOs (AAT AGA CTT GTG TCT and GAC CTA GCT TGA TCA TCA)
TGC GAT AGA TGC GGT) yield a 304-bp fragment. The primers for eNOs (CAA CGC TAC CAC GAA GAC ATT TTC GGG CTC and GAG AGG GAA AAG AGG CGT TTT GCT CCT TCC) yield a 332-bp fragment. Actin expression was used as an internal standard. All primers were synthesized by Ransom Hill Bioscience, Inc (Ramona, CA). PCR products were analyzed on 2.0% agarose gels and recorded on Polaroid films. The density was scanned and analyzed by NIH Image 1.16.

Western Blot Analysis

Total endothelial cell protein extract (10 μg per sample) was subjected to electrophoresis on 12% polyacrylamide gels by use of the Mini-protein 3 gel running system (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membrane. The membranes were probed with a primary antibody against Hsp90β (Santa Cruz, CA), eNOs (BD Biosciences, San Jose, CA), or actin (Sigma, St. Louis, MO). The second antibody was a horseradish-linked anti-mouse antibody. The bound antibodies were visualized with an enhanced chemiluminescent ECL detection kit (Amersham Corp, Arlinton Heights, IL) and exposed to a Kodak X-Omart x-ray film. Nitrocellulose membranes were stripped with strip buffer containing 100mM β-mercaptoethanol, 2% (w/v) sodium dodecyl sulphate, 62.5 mM Tris-HCl (pH 6.7), following ECL kit instructions (Amersham Pharmacia Biotech) and blocked before they were probed again with different primary antibodies. The x-ray film was scanned and then the density of protein expressions was analyzed by NIH Image 1.16.

Measurement of Prostacyclin Production

Endothelial culture media were collected and prostacyclin production was measured in the culture medium by its stable metabolite 6-keto prostaglandin F1α (6-keto PGF1α) by ELISA (Oxford Biomedical Research, Michigan). All samples were measured in duplicate. The levels of 6-keto PGF1α were expressed as pg/mg protein.

Statistical Analysis

The experimental data are presented as mean ± SE and clinical data are presented as mean ± SD. Statistical analyses were performed with non-parametric Mann-Whitney test for superoxide generation, mRNA and protein expression between normal and preeclamptic HUVECs (for data in Figures 1, 2, and 3). Data for Figures 4 and 5 were analyzed by ANOVA and Student-Newman-test was used as the post-hoc test; and data for Figure 6 was analyzed by paired t-test, respectively. p < 0.05 was considered statistically significant, using StatView (Cary, NC).

RESULTS

Superoxide Generation by HUVECs from Preeclampsia

In this experiment, primary cultured HUVECs from 12 normal and 6 preeclamptic pregnancies were used. Figure 1 shows a representative DHE staining in cells from one normal (A) and one preeclamptic (B) subject. Nuclear DHE staining was observed in cells from preeclamptic, but barely visible to HUVECs from normal pregnancies. The percentage of positive nuclear DHE staining cells was significantly higher detected in HUVECs from preeclamptic (9.16 ± 3.96%) than from normal (0.20 ± 0.13%) pregnancies, p < 0.01 (Figure 1C). These results suggest that HUVECs from preeclamptic pregnancies were still in a state of oxidative stress within days of in vitro culture.

Reduced Hsp90β Expression in HUVECs from Preeclampsia

Figure 2 shows mRNA expression for Hsp90β in HUVECs from normal (n = 6) and preeclamptic (n = 6) pregnancies (Figure 2A). The relative mRNA expression for HUVECs
from preeclamptic pregnancies was slightly, but not significantly, decreased compared with that in normal pregnancy HUVECs, 0.26 ± 0.02 vs. 0.38 ± 0.07, respectively (Figure 2B). Using total cellular protein from the same endothelial samples, Hsp90β protein expression (Figure 3) was decreased in HUVECs from preeclamptic pregnancies (n = 6) compared with that from normal pregnancies (n = 5), 0.32 ± 0.14 vs. 1.11 ± 0.33, p < 0.05 (Figure 3B). Total protein from one normal sample was insufficient for Western blots. The relative mRNA and protein expression for Hsp90β was normalized by actin expression. These findings indicate that reduced Hsp90β expression occurs at protein, but not at mRNA, level in HUVECs from preeclampsia.

**Effects of Hsp90 Inhibition**

To determine whether increased superoxide generation was associated with altered Hsp90 function in HUVECs from preeclamptic pregnancies, cells from normal pregnancies (n = 12) were treated with geldanamycin (GA, a Hsp90 inhibitor) in culture and superoxide generation was detected. As shown in Figure 4, nuclear staining of DHE is barely visible in control cells (Figure 4A). In contrast, numerous cells exhibit positive nuclear staining in cells treated with 10 μM GA for 30 minutes (Figure 4B), which could be blocked by pre-incubating cells with 30 μg of superoxide dismutase for 30 minutes (Figure 4C), control EC: 0.20 ± 0.13%; ECs treated with GA: 6.06 ± 0.66%; and ECs pre-treated with SOD and then GA: 1.23 ± 0.69%, p < 0.01, respectively (Figure 4D).

Effects of Hsp90 inhibition on endothelial function was further determined by eNOS expression and prostacyclin production in HUVECs treated with GA. We found eNOS protein expression was down-regulated in a concentration dependent manner in cells treated with GA, Figure 5A and B. However, eNOS mRNA expression showed no change (data not shown), which indicate that Hsp90 regulates eNOS at post-transcriptional or translational level. Figure 6 shows prostacyclin production as measured by its stable metabolite 6-keto pros-taglandin F1α. HUVECs treated with GA produced less prostacyclin than those of controls, 0.52 ± 0.06 vs. 0.68 ± 0.07pg/mg protein, p < 0.05.

**DISCUSSION**

In this study, by detection of nuclear staining of ethidium, we, for the first time, observed an increase in superoxide generation in primary cultured HUVECs from pregnancies complicated by preeclampsia. This observation suggests that HUVECs may experience an oxidative stress and phenotypic modification during preeclampsia.

We also found reduced Hsp90β protein expression in HUVECs from pregnancies complicated by preeclampsia compared with those from normal pregnancies. As stated, Hsp90 is abundant and its molecular chaperone property is fundamental maintaining cellular homeostasis. To determine whether reduced Hsp90 activity in endothelial cells might contribute to increased superoxide generation seen in HUVECs from preeclampsia, geldanamycin was employed. Geldanamycin, a widely used Hsp90 inhibitor to study superoxide generation and eNOS activity (11,13–15), binds to the ATP binding site of Hsp90, inhibits the ATP/ADP cycle, and influences its function, although its specificity of inhibition was concerning (13). Our data showed that inhibition of Hsp90 by geldanamycin resulted in an increase in superoxide generation in endothelial cells, which was blocked by addition of superoxide dismutase in culture. Our results are consistent with previously published work using spin-trap to study the role of geldanamycin in superoxide generation in endothelial cells and smooth muscle cells (13). These observations implicate that down-regulation of Hsp90 may contribute to endothelial oxidative stress in an in vivo condition such as the fetal syndrome of preeclampsia. These results also suggest intracellular and
extracellular antioxidant activity and capacity to significantly influence endothelial antioxidative function.

Increased oxidative stress has long been proposed in the vascular system in pregnancies complicated by preeclampsia (16). One question which arises from our study is that since HUVECs are of fetal, not maternal, origin, the observed increased superoxide generation and reduced Hsp90 expression may not be relevant to the maternal vascular system in preeclampsia. During pregnancy, umbilical endothelial cells are exposed to circulating substances that pass into fetal blood from the maternal circulation. Consistent with this, cord blood from infants of preeclamptic women has increased concentration of fibronectin, a marker of endothelial activation present in the mother (17). In addition, as with exposure to maternal blood of preeclamptic women, exposure of endothelial cells to cord blood from their infants increases nitric oxide and prostaglandin released from endothelial cells (17). Using HUVECs from preeclamptic subjects compared with those from normal pregnancies, we found increased endothelial monolayer permeability correlates with disorganized endothelial junctional protein distribution of VE-cadherin and occludin (7). Consistently, disorganized endothelial junction protein distribution is in line with the observations of enlarged and disrupted endothelial junctions in maternal vessel samples from preeclamptic pregnancies (18,19). The fact that these cellular alterations are only present at the early passage of cells (7) supports the concept that HUVECs may suffer from hypoxia and oxidative stress as maternal vascular endothelial cells (5) in preeclampsia. Furthermore, in cases of preeclampsia, placental villi and HUVECs derived from the fetus are far more likely to have been subjected to uteroplacental insufficiency and hypoxia. This abnormal in vivo environment in preeclampsia may be an underlying reason for the altered function observed in HUVECs derived from preeclamptic pregnancies.

To study downstream effects of altered Hsp90 function on endothelial cells, we further examined eNOs expression and prostacyclin production, two events of altered endothelial function that occur in preeclampsia. Regulation of eNOs by Hsp90 has been considered an important cellular protein-protein interaction controlling endothelial function (20). It is well known that normal eNOs activity generates constant NO. Endothelial-derived NO can react with a broad variety of molecules (21). For example, NO can directly activate calcium-dependent potassium channels (22), leading to endothelial-dependent hyperpolarization of vascular smooth muscle cells, resulting in vasodilation. NO regulates leukocyte adhesion to endothelium, inhibits vascular smooth muscle cell proliferation and platelet aggregation (23,24). Our results show that inhibition of Hsp90 activity by geldanamycin results in downregulation of endothelial eNOs protein expression and reduced vasodilator prostacyclin generation. We previously reported that decreased eNOs expression contributes to increased endothelial permeability in preeclampsia (8). We and others also found reduced prostacyclin levels in the maternal blood and amniotic fluid in preeclampsia (4,25,26). Prostacyclin is a major vasodilator produced by endothelial cells. Dysfunctional endothelial cells produce less prostacyclin which, combined with platelet activation and increased thromboxane release, constitutes the imbalance of increased vasoconstrictor thromboxane and decreased vasodilator prostacyclin in preeclampsia. Altered eNOs activity is also a source of superoxide generation in cells. It has been demonstrated that Hsp90 is essential for eNOs-dependent nitric oxide production and inhibition of ATP-dependent conformational changes in Hsp90 uncouples eNOS activity and increases eNOs-dependent superoxide generation (15). Decreased eNOs expression and reduced prostacyclin production, by altering Hsp90 in endothelial cells, indicate that deficient Hsp90 activity may produce an enormous influence and multiple effects on endothelial function. These results suggest that decreased eNOs expression and prostacyclin production could be a consequence of down-regulation of Hsp90 in HUVECs from preeclampsia.
In addition, we noticed that protein expression for actin was not uniform especially for the HUVECs from preeclamptic pregnancies even though the same amount of total cellular protein from each sample was loaded for running the polyacrylamide gel. Although patients in the preeclamptic group were delivered preterm and most of them were by cesarean section, we do not believe that preterm or labor would contribute to the difference in endothelial actin or even Hsp 90 expression. Actin filaments are the primary protein that forms the cytoskeletal fiber. Whether the reduced actin expression is a phenomenon of endothelial dysfunction in preeclampsia needs to be further determined.

In summary, in this study we observed increased superoxide generation and decreased Hsp90 expression in HUVECs obtained from pregnancies complicated by preeclampsia. Hsp90 is an important host defense molecule and key molecular chaperone to maintain cellular homeostasis. Therefore, deficient Hsp90 in endothelial cells could promote oxidative stress by increasing superoxide generation, reducing NO availability by inhibition of eNOs, and inducing vasoconstriction by limitation of prostacyclin production. Our in vitro HUVEC data provide strong evidence that endothelial dysfunction associated with increased oxidative stress may also occur in the fetal compartment during preeclampsia.

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REFERENCES


Figure 1.
Fluorescent staining of ECs with dihydroethidium (DHE) in cells derived from normal and preeclamptic pregnancies. A: ECs from a normal pregnant woman; B: ECs from a woman with preeclampsia. Primary cultured HUVECs (usually 3 to 4 days after isolation) were used in this experiment. HUVECs from preeclamptic pregnancies showed numerous nuclear staining of ethidium indicating increased superoxide generation. This phenomenon was not observed in cells from normal pregnancies. C: % of positive nuclear DHE staining cells, $p < 0.01$, bar = 50 micron.
Figure 2.
mRNA expression for Hsp90β in HUVECs from normal or preeclamptic pregnancies.  

A: PCR products for Hsp90β in HUVECs from 6 normal and 6 PE pregnancies.  

B: the relative mRNA expression for Hsp90β after normalized by actin expression.
Figure 3.
Protein expression for Hsp90β in HUVECs from normal or preeclamptic pregnancies. A: Protein expressions in HUVECs from the same pregnant women (as shown in Figure 1). An aliquot of 10 μg of total protein was used in per sample. Since the protein volume from one normal sample was not enough, only 5 samples from the normal group were studied (A). B: the relative protein expression for Hsp90β after normalized by actin expression. Protein expression for Hsp90β was significantly reduced in ECs from preeclamptic pregnancies, *p < 0.05.
Figure 4.
Effects of Hsp90 inhibition on superoxide generation in HUVECs. 

A: control cells; B: cells were treated with GA 10 μM for 30 minutes; C: cells were pretreated with superoxide dismutase (SOD) 30 μg/well for 30 minutes before adding GA 10 μM in culture. The micrographs were representatives of 12 independent experiments. Pretreatment of cells with SOD blocked GA induced endothelial generation of superoxide radicals; bar = 50 micron.

D: % of positive nuclear DHE staining in control cells, cells treated with GA, and cells pretreated with SOD, **p < 0.01: GA vs. control; ##p < 0.01: SOD + GA vs. GA, respectively.
Figure 5. Effects of Hsp90 inhibition on endothelial eNOs protein expression. HUVECs from normal pregnancies were treated with GA for 48 hours. Downregulation of eNOs expression was in a concentration-dependent manner in cells treated with GA. The relative eNOs expression (B) was normalized by actin expression in each sample. Data are from three independent experiments, *p < 0.05.
Figure 6.
Effects of Hsp90 inhibition on endothelial prostacyclin production. Prostacyclin was measured by its stable metabolite 6-keto PGF1α by ELISA. ECs treated with GA 10 μM produced less prostacyclin than those of controls, *p < 0.05. Data are from six independent experiments with triplicate treatment in each.