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Placental Heat Shock Protein 70 Overexpression Confers Resistance Against Oxidative Stress in Preeclampsia

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Aims: Preeclampsia is a hypertensive disorder of human pregnancy and a leading cause of premature delivery and fetal growth retardation. The purpose of this study was to evaluate the expression of heat shock protein 70 (HSP70), both constitutive (HSC70) and induced (HSP70) forms, along with oxidative stress status in the placental tissues of normotensive (control group) and preeclamptic pregnancies.

Materials and Methods: Placental samples were collected after delivery from normotensive pregnancies and preeclamptic patients (n = 20, each). Lipid peroxidation product malondialdehyde (MDA) and nitrite and nitrate levels, along with antioxidants such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) and glutathione were estimated in the placental homogenate to evaluate the oxidative stress.

Results: Placental tissue MDA levels and concentrations of nitrite and nitrate were significantly higher in the preeclamptic group (P < 0.001) than the control group. Placental SOD and GPx activities and the reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio were significantly lowered in the preeclamptic (P < 0.001) group than the control group. Both placental HSC70 and HSP70 were in significantly higher concentrations in the preeclamptic (7.25 ± 0.76, 27.67 ± 2.32 ng/mg protein; P < 0.001) than the control (5.23 ± 0.64, 17.47 ± 1.22 ng/mg protein) group, respectively.

Conclusions: The above data provide the first evidence that high levels of HSP70 are associated in the placenta of pre-eclamptic women. This enhanced expression is probably to exhibit its multiple protective effects on the cell's response to stress.

Key Words: Preeclampsia, peroxynitrite, superoxide dismutase, placenta, HSP70, oxidative stress, antioxidants

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Preeklampside Artmış Plasental Heat Shock Protein 70 Ekspresyonu Oksidatif Strese Karşı Direnç Göstergesidir

Amaç: Preeklampsia gebelik sırasında görülen ve fütüsda gelişme geriliği veya premature doğumlara yol açabilen bir hipertansif hastalıktır. Bu çalışmada, normotansif ve preeklampatik gebeliklerde plasental dokuda oksidatif stress düzeyi ve hem yapısal hem de indüklenmiş heat shock protein 70 (HSP70) ekspresyonunun ölçülmesi amaçlanmıştır.

Yöntem ve Gereç: Normotansif ve preeklampatik doğum sonrası plasental örnekler (n = 20) alındı. Plasental dokuda sırasıyla MDA, SOD, GPx, GSH ve GSSG ve nitrit/nitrat düzeyleri çalışıldı.

Bulgular: Preeklampatik grupta plasental dokuda HSC70 ve HSP 70, MDA ve nitrit/nitrat konsantrasyonları kontrol grubundan daha yüksekti (P < 0.01). Plasental SOD, GPx aktiviteleri ve GSH/GSSG oranı preeklampatik grupta kontrol grubundan daha düşüktü (P < 0.001). Hem HSC70 hem de HSP70 düzeyleri preeklampatik grupta kontrol grubundan daha yüksekti (sırasıyla 7.25 ± 0.76, 27.67 ± 2.32 ng/mg vs 5.23 ± 0.64, 17.47 ± 1.22 ng/mg protein).

Sonuç: İlk kez bu çalışma ile preeklampatik kadınların plasentalarında HSp70 düzeylerinde artış olduğu saptanmıştır. Bu artmış ekspresyon muhtemelen hücrenin strese karşı bir çok korunma yanıtı nedeniyledir.

Anahtar Sözcükler: Preeklampsia, peroksinitrit, superoksit dismutaz, placenta, HSP70, oksidatif stress, antioksidanlar

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Introduction

Preeclampsia is a pregnancy-specific multisystem disorder that is characterized by the development of hypertension and proteinuria after 20 weeks of gestation. Preeclampsia is associated with increased risk of placental abruption, acute renal failure, cerebrovascular complications and maternal death (1). Several studies carried out during recent years have clearly demonstrated the pathophysiological basis of the disease (2). The symptoms of the pathophysiological changes that occur in the disorder are the

diagnostic criteria for preeclampsia (hypertension and proteinuria); however, the development of the disease and the risk factors that render a pregnant woman more susceptible to develop preeclampsia are not clear (3,4).

Evidence points to the placenta as a key source of factors that lead to these changes in preeclampsia (5). It is generally hypothesized that placental changes are induced by hypoxia. This may be a primary event or a secondary effect of altered placental oxygenation in preeclampsia. Oxidative stress may be the point at which multiple etiological factors converge, resulting in endothelial cell dysfunction and the consequent clinical manifestations of preeclampsia.

Lipid peroxidation products produced by vascular endothelial cells are candidate factors that may mediate disturbance of maternal vascular endothelium. They can deplete enzymatic and low molecular weight cellular antioxidants, which in turn reduce the termination of free radical lipid propagation reaction. The placenta is also known to produce nitric oxide (NO), which has pronounced effects on placental function including trophoblast proliferation, differentiation and vascular reactivity. NO is an unstable but multifunctional molecule that mediates a number of diverse physiological and pathological processes in endothelial cells. For example, NO[•] outcompetes superoxide dismutase (SOD) for O₂^{•-}. Peroxynitrite formation is favored while the peroxynitrite anion is capable of nitrating proteins and inducing lipid peroxides (6). Oxidant metabolites of NO can also deplete enzymatic and low molecular weight cellular antioxidants. There is experimental support for the role of oxidative stress as a mediator of cell dysfunction and cell death (7).

In normal pregnancies, there is an increase in free radical production and lipid peroxidation towards the end of pregnancy when compared with non-pregnant women. At the same time, the antioxidant capacity gradually increases during pregnancy, leading to oxidative balance maintenance during pregnancy (8). In pathogenic pregnancies like preeclampsia, excessive production of reactive oxygen species (ROS) may occur at certain windows in placental development leading to reperfusion injury. This leads to accumulation of placental debris through apoptosis (9). However, the protective role adopted by placental cells under these stressed conditions is unknown.

Heat shock proteins (HSPs) have been recognized for some years to protect the cells from various cytotoxic

insults. HSPs are a group of inducible proteins, some of which are constitutively expressed and increase in response to stress, whereas others are expressed only after stress. The constitutively expressed proteins act as chaperones for other cellular proteins, binding to nascent polypeptide to prevent premature folding and to translocate proteins in to organelles (10). The induction of increased levels of the stress proteins is associated with the exposure to stress agents. The induced stress proteins can act to protect cells from stress-induced damage by preventing protein denaturation and/or by repairing such damage. The aim of the present investigation was to evaluate the oxidative stress and nitrative stress along with HSP70 expression in the placenta of normal pregnant and preeclamptic pregnant women. The results can provide information about the defensive mechanism adopted by the placenta during preeclamptic pathophysiology.

Materials and Methods

Subjects

Patients registered in the Department of Obstetrics and Gynecology of a private hospital in Chennai were enrolled in this study. The study was carried out for a period of one year. The sample consisted of 20 patients aged 20-40 years. Patients with preeclampsia were defined on the basis of the following clinical and laboratory criteria: systolic blood pressure ≥ 140 mmHg and diastolic blood pressure ≥ 90 mmHg but blood pressure less than 160/110 mmHg noted on at least two occasions; proteinuria levels > 300 mg/dl found in at least two random specimens; and the activity of xanthine oxidase of about 2.6 units/mg protein. Age-matched healthy volunteers who were normotensive, of similar gestational age, race, and body mass index (BMI) and without maternal and fetal complications during the pregnancy period were selected as control subjects. Health was defined as the absence of major medical or surgical disease and no need for regular medication. Gestational age was defined based on last menstrual period and ultrasonography. Furthermore, all the patients were consulted by the internal medicine department. There were 18 vaginal deliveries and 2 cesarean sections in the control group and 15 vaginal deliveries and 5 cesarean sections in the preeclamptic group. The clinical characteristics of the preeclamptic patients were tabulated and compared with the

normotensive pregnant subjects and the data are presented in Table 1.

Immediately after delivery, the placental tissue samples (2 to 3 g each) about 2-3 cm from the margin were collected from 20 each preeclamptic patients and normal pregnant women from the maternal side of the placenta in a sterilized container and processed for tissue homogenate preparation. The proposed plan of work was approved by the Ethical Committee of the private hospital in Chennai and the samples were collected with the consent of each patient.

Preparation of Tissue Homogenates

About 1 g of placental tissue piece was weighed, washed with ice cold physiological saline and cut in to 1-2 mm pieces and homogenized in ice-cold homogenization buffer (150 mM NaCl, 50 mM Tris HCl of pH 7.4, 1 mM EDTA, 1 mM PMSF, 1% Triton X-100, 1% sodium deoxycholic acid, 0.1% SDS, 5 µg/ml aprotinin and 5 µg/ml of leupeptin) with the use of a Teflon homogenizer. Homogenates were stored at -20 °C for later western blotting. Protein concentration of these samples was determined by the method of Bradford with the use of bovine serum albumin as the standard (11). Aliquots of homogenates were then used for the analysis of lipid peroxides, estimation of NO₂⁻ and NO₃⁻,

assay of SOD and glutathione peroxidase (GPx) activity and the western blotting of HSP70.

Analysis of Oxidative Stress Markers

Estimation of malondialdehyde by HPTLC

The placental malondialdehyde (MDA) level, which is the end product of lipid peroxidation, was measured by high performance thin layer chromatography (HPTLC) analysis. The HPTLC analysis of MDA was performed according to the method of Carbonneau et al. (12) using CAMAG TLC software.

The HPTLC analysis of MDA was performed using the MDA/TBA (thiobarbituric acid) chromogen prepared according to the method of Lykkesfeldt (13), which is as follows: 40 µl of placental tissue homogenate was suitably diluted with H₂O and mixed with 20 µl of 2.8 mmol/L BHT (butylated hydroxy toluene) in ethanol, 40 µl SDS and 600 µl of TBA reagent pH 3.5. The mixture was immediately heated (60 min at 95 °C) and cooled with running water. After vigorous mixing with 200 µml of H₂O, the clear supernatant was separated by centrifugation. The product was then analyzed on a 2 mm thick Silica gel G coated aluminium plate (Merck, Silica Gel 60F254, Germany). The plate was developed using chloroform: methanol: water 65:25:4 and dried at room

Table 1. Clinical characteristics of the normotensive pregnant women and preeclamptic patients.

Characteristics	Normal Pregnancy (n = 20)	Preeclamptic Pregnancy (n = 20)
Maternal Age (Years)	24.7 ± 2.7	21.5 ± 1.4
Gestational Age	37.8 ± 0.4	32.3 ± 2.4
Prepregnancy Weight (kg)	50.1 ± 4.2	60.8 ± 4.8
Pregnancy Weight (kg)	54.3 ± 7.6	69.1 ± 8.1
Pre-pregnancy BP (mmHg)		
Systolic	113.4 ± 5.8	115.2 ± 6.2
Diastolic	76.8 ± 5.2	77.1 ± 4.8
Pregnancy BP at 20 weeks (mmHg)		
Systolic	114.7 ± 7.8	151.4 ± 18.1
Diastolic	77.5 ± 4.1	101.52 ± 9.3
Pregnancy BP at term (mmHg)		
Systolic	120.8 ± 8.2	165.8 ± 8.2
Diastolic	80.4 ± 7.1	109.1 ± 7.3
Parity	1.9 ± 0.85	1
Infant Birth Weight	3.28 ± 0.20	2.42 ± 0.68

temperature and the naturally colored spot was then scanned with a Camag TLC Scanner II (Camag, Muttenz, Switzerland) in the reflectance mode at 366 nm coupled with an SP 4100 integrator. The results were expressed as nanomoles of MDA formed /mg protein.

Measurement of nitrite (NO_2^-) and nitrate (NO_3^-)

Placental tissue homogenate nitrite and nitrate were estimated by the method of Yokoi et al. (14) with slight modifications using Griess reagent, which relies on a colorimetric reaction between nitrite, sulphanilamide and N-(1-naphthyl ethylene diamine dihydrochloride to produce a pink azo product, which was measured at 520 nm. For the nitrate estimation, prior to the addition of Griess reagent, all nitrate was converted to nitrite using cadmium sulphate and copper sulphate. Concentrations were determined by comparison to a standard solution of sodium nitrite. The levels of nitrate can be obtained by subtracting the value of nitrite (nitrate converted to nitrite) from total nitrite content. The levels of NO_2^- and NO_3^- were expressed as micromoles per gram tissue protein.

Analysis of Enzymatic Antioxidants

Assay of superoxide dismutase

The activity of SOD was assayed by monitoring the oxidation of epinephrine by SOD at 420 nm for 3 min at 30 sec time interval according to the procedure of Mistry et al. (15). Auto oxidation of epinephrine was also monitored in the reaction mixture without adding the enzyme source, which serves as blank. The activity of the enzyme was expressed as units of SOD/min/mg protein.

Assay of glutathione peroxidase

The activity of GPx was assayed according to the method of Rotruck et al. (16). The suitably prepared tissue extract was assayed for glutathione content using DTNB at 412 nm. The activity of GPx is expressed as micromoles of glutathione consumed /min/mg protein.

Estimation of GSH/GSSG Ratio by Spectrofluorometry

The glutathione status was assessed by the method of Hissin and Hilf (17). This method is based on the reaction of O-phthaldehyde (OPT) as a fluorescent reagent with reduced glutathione (GSH) at pH 8.0 and oxidized glutathione (GSSG) at pH 12.0, which involves excitation at 350 nm and fluorescence at 420 nm. For the GSSG estimation, GSSG was complexed to N-ethylmaleimide

(NEM) to prevent interference of GSH with the measurement of GSSG. The values were expressed as nanomoles/mg of protein.

Western Blotting of HSP70

The western blotting was performed according to the method of Towbin et al. (18) with a slight modification. The placental homogenate (100 μg of protein) was analyzed with the use of SDS-polyacrylamide gel electrophoresis (10%) according to Laemmli method (19). After electrophoresis, the gel was blotted on to a PVDF membrane (BioTrace; Germany), blocked with 5% Biotin overnight with agitation and then probed with a mouse alkaline phosphatase conjugated monoclonal antibody raised against HSP70 (SPA -820; Stressgen, Canada) diluted in PBS buffer. This antibody recognizes both the inducible and cognate forms of HSP70. The BCIP-NBT substrate (Sigma, St. Louis, MO) system was used to detect the alkaline phosphatase conjugate as described by the manufacturer. All immunoblots for HSP70 showed a doublet of bands. Apart from those bands, the antibodies did not cross-react with any protein on the membrane. The band intensities were scanned with the HP Scan Imager and quantified using the TotalLab Software, Germany.

Statistical Analysis

All data were expressed as mean value \pm SD. The values were subjected to statistical analysis using normal tests of significance. For the statistical analysis, normal subjects were compared with preeclamptic patients using Wilcoxon Mann-Whitney test. The SPSS software package version 7.0 was used to test the significance of the experiments performed.

Results

Figure 1 represents the HPTLC profiles of the lipid peroxidative product MDA in the placental tissue of the preeclamptic patients and of the normal pregnant women.

The levels of MDA and NO metabolites NO_2^- and NO_3^- in the placental tissue of the preeclamptic patients and of the normal healthy volunteers are presented in Table 2. The MDA levels were significantly higher in the preeclamptic (4.12 ± 0.32 nmol/mg protein; $P < 0.001$) than the control (2.78 ± 0.12 nmol/mg protein) group. Placental tissue nitrite and nitrate were significantly

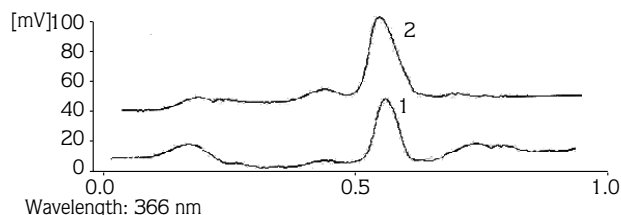


Figure 1. HPTLC analysis of MDA/TBA chromogen in the placental tissues of normotensive pregnant women and patients with preeclampsia.

Absorption spectra of MDA/TBA chromogen. Scans carried out in reflectance mode at 366 nm.

¹ MDA/TBA Chromogen of normal pregnant women.

² MDA/TBA Chromogen of preeclamptic patients.

Table 2. Levels of MDA and nitric oxide metabolites NO₂⁻ and NO₃⁻ in the placental tissue of the preeclamptic patients and of the normal healthy volunteers.

Subjects	MDA (nanomoles/g protein)	NO ₂ ⁻ (µmol/g protein)	NO ₃ ⁻ (µmol/g protein)
Normotensive pregnant women	2.78 ± 0.12	2.3 ± 0.1	3.1 ± 0.3
Preeclamptic women	4.12 ± 0.32*	5.4 ± 0.4*	7.2 ± 0.5*

Values are expressed as mean ± S.D for 20 individuals in each group. NO₂⁻: Nitrite. NO₃⁻: Nitrate.

*P < 0.001, when compared with normotensive pregnancy.

higher in the preeclamptic (5.4 ± 0.4 and 7.2 ± 0.5 mmol/gram protein; P < 0.001) than in the control (2.3 ± 0.1 and 3.1 ± 0.3 mmol/gram protein, P < 0.001) group, respectively.

As shown in Table 3, the placental SOD and GPx activities were significantly lower in the preeclamptic (0.94 ± 0.17 units/min/mg protein, 0.48 ± 0.035 mmol of glutathione consumed/min/mg protein; P < 0.001) than in the control (4.45 ± 0.22 units/min/mg protein, 1.94 ± 0.047 mmol of glutathione consumed/min/mg protein) group, respectively.

Figure 2 shows the box plot of the GSH/GSSG ratio in placental tissue of the preeclamptic patients and of the normal healthy volunteers. The GSH/GSSG ratio was significantly decreased (46.67 ± 2.41; P < 0.001) in the preeclamptic patients compared to the control group (68.23 ± 2.41).

Table 3. Levels of the activities of SOD and GPx enzymes in the placental tissue of the preeclamptic patients and of the normal healthy volunteers.

Subjects	SOD (units/min/mg protein)	GPx (µmol of glutathione consumed/min/mg protein)
Normotensive pregnant women	4.45 ± 0.22	0.94 ± 0.047
Preeclamptic women	1.94 ± 0.17*	0.48 ± 0.035*

SOD: superoxide dismutase. GPx: Glutathione peroxidase.

Values are expressed as mean ± S.D for 20 individuals in each group.

*P < 0.001, compared with normotensive pregnancy.

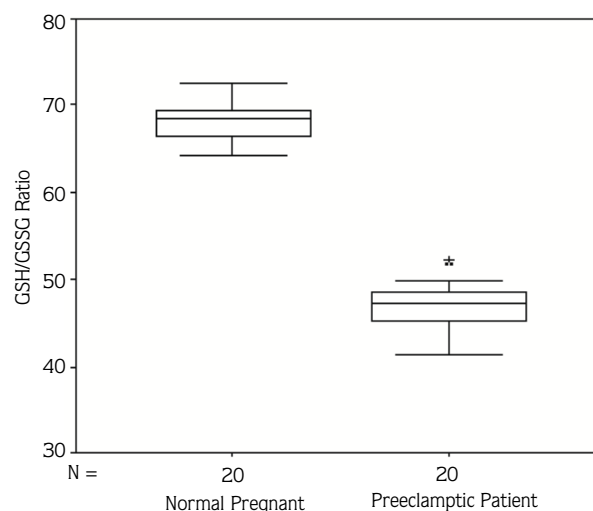


Figure 2. Box plot of the GSH/GSSG in the placental tissue of the normotensive pregnant women and preeclampsia patients.

*P < 0.001, compared to normotensive pregnant women.

Figure 3 shows the western blot of HSP70 of the placental tissue of the preeclamptic patients and of the normal healthy volunteers. Overall there was a multi-fold increase in the expression of both constitutive and inducible forms of HSP70 in the preeclamptic patients when compared to normal healthy volunteers. The placental HSC70 and HSP70 levels were in significantly higher concentrations in the preeclamptic (7.25 ± 0.76, 27.67 ± 2.32 ng/mg protein; P < 0.001) than in the control (5.23 ± 0.64, 17.47 ± 1.22 ng/mg protein) group, respectively.

Discussion

Preeclampsia develops as a consequence of an exceptionally complex interaction between a multiplicity

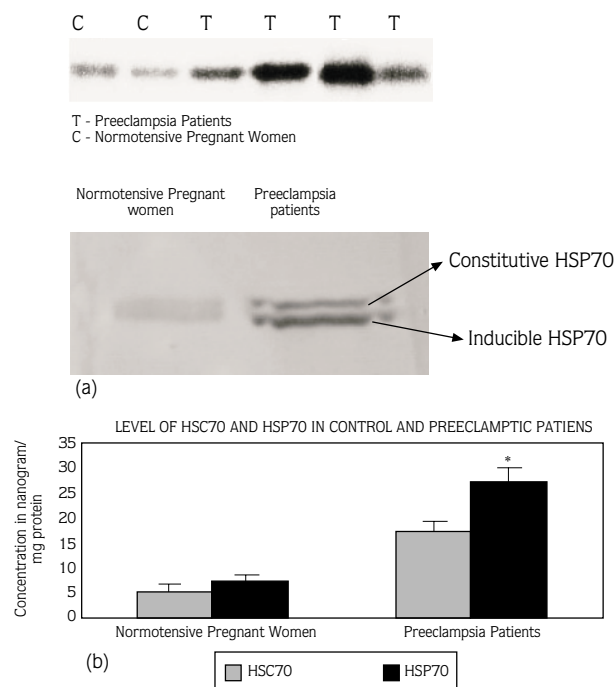


Figure 3. (a) Western blot of HSP70 of the placental tissue of the normotensive pregnant women and patients with preeclampsia, (b) Histogram of HSP70 of the placental tissue of the normotensive pregnant women and preeclampsia patients. *P < 0.001 compared to normotensive pregnant women.

of factors that originate in two genetically different individuals (the mother and the fetus). Oxidative stress may represent one group of such factors as shown from this study. In the women with established preeclampsia, lipid peroxide product MDA increased and enzymatic antioxidants SOD and GPx as well as non-enzymatic antioxidant glutathione were decreased. In healthy pregnant women, the total antioxidant capacity (enzymatic and non-enzymatic) is maintained as a compensatory balance of the injury/defense against lipid peroxidation products, allowing oxidative equilibrium to persist throughout pregnancy. Preeclamptic women fail to develop this compensatory mechanism. In normal pregnancies, there is remodeling at the placental vascular bed. Here cytotrophoblasts invade the maternal spiral arteries feeding the intervillous space involving the endothelium, making them lose their smooth muscle. This becomes sinusoids, a type of vessel with large capacity and low resistance lacking any contractibility. In preeclampsia, these physiological changes are drastically affected. The trophoblastic invasion is very shallow,

keeping the uteroplacental circulation in a state of high resistance and leading to reduced uteroplacental perfusion and focal regions of hypoxia. These changes may cause blood flow to remain under vasomotor control. We have earlier reported atherosclerosis, fibroid necrosis and infarction in the placenta of preeclamptic patients (20). This results in large fluctuations in intervillous oxygen concentration leading to ischemic reperfusion phenomenon with overproduction of ROS (21). This study confirms that in preeclampsia, lipid peroxidation is increased significantly when compared to healthy pregnant women.

During preeclampsia, the levels of NO in the placenta are also increased. Under conditions of oxidative stress, the oxygen free radical, superoxide anion will preferentially react with available NO, increasing the formation of peroxynitrite in tissues (22). This study evidences the reduction of antioxidant capacity in preeclamptic women. The low SOD activity shown in the preeclamptic patients may be explained by the lack of induction for production of SOD, since superoxide anion reacts with NO to produce high peroxynitrite levels (23). The major biological end products of •NO metabolism are nitrite NO₂⁻, and nitrate NO₃⁻, both of which are excreted in the urine. During metabolism to NO₂⁻/NO₃⁻, NO can undergo a series of reactions that generate reactive nitrogen species having a variety of oxidative states. NO reacts with superoxide O₂^{•-} to form peroxynitrite (ONOO⁻). ONOO⁻ is a highly reactive species that reacts with proteins, lipids, carbohydrates and DNA of subcellular organelles and cell system through oxidation and nitration mechanisms (24). Peroxynitrite is known to induce nitration of tyrosine residues on proteins, thus altering its function. Peroxynitrite formation has been observed in the maternal vasculature of women with diabetes (23).

GPx and enzymatic antioxidant decrease lipid peroxide by reducing hydroperoxides to alcohols. The reactive nitrogen species ONOO⁻ yields more nitrosonium ion NO⁺ that is responsible for oxidizing the selenocysteine residue of GPx to a selenylsulfide having a free thiol thereby inactivating the enzyme (25). Once GPx is inhibited, it can no longer scavenge peroxides and terminate propagating lipid oxidation reaction. Therefore, lipid peroxide reactions can be expected to increase as seen from our studies. In addition to inhibiting GPx, ONOO⁻ also oxidizes GPx-reducing cofactor glutathione (26). By impairing cellular defenses against lipid peroxidation through the

depletion of antioxidants and inhibition of GPx, oxidation reactions can greatly increase the rate of lipid peroxidation. The impairment of NO-mediated endothelial function and the accumulation of oxidized lipids in the vessel wall are central pathological events in endothelial dysfunction. Nitrosative stress in preeclampsia may also alter placental function via increased trophoblast apoptosis.

Overexpression of HSP is an important means of cell protection during physiological stress such as that occurring during apoptosis. As metabolically active tissues vital to the maintenance of pregnancy, it is probable that placental tissue, particularly in preeclampsia, experiences overstress and this may be the reason for the boost in the production of HSP70. NO has a powerful effect on maternal blood vessels and its release is partly mediated by the HSP90 (27). It had been reported that abundance of HSP70 may modulate uterine function in response to stress (28). Adrie et al. (29) have also demonstrated that $\cdot\text{NO}/\text{ONOO}$ represents a strong stress to human monocytes leading to increase in HSP70. Increased expression of HSP70 may allow proliferating endothelial cells in the placenta an increased chance of survival.

The data reported by Xu et al. (30) suggested that HSP could play a role in the repair of denatured proteins

modified by NO and in folding of nascent polypeptide chains. Thus, HSP70 is likely to be important in maintaining homeostasis during NO and oxidative stress.

Overall, these observations suggest that NOs and free radicals may induce an unrecognized primary line of defense via elevation of HSP70 against oxidative stresses and protect the cell from apoptosis in preeclampsia. In conclusion, these data provide the first evidence that high levels of HSP70 are associated in the placenta of preeclamptic women. This enhanced expression is probably to exhibit its multiple protective effects on the cell's response to stress.

Perspectives

The present study is the first, to our knowledge, to assess the overexpression of the constitutive and inducible forms of HSP70 during the placental oxidative stress that plays a pivotal role in preeclampsia. We have demonstrated significant expression of HSPs during placental oxidative and nitrosative stress in preeclampsia. This study raises an additional question with regard to how the overexpression of HSPs in placental cells confers resistance to hemodynamic stress. Further research for the answers will result in valuable clinical application for preeclampsia.

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