Free Rad. Res., Vol. 33, pp. 471-476
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Plasma Protein Carbonyls in Nonpregnant, Healthy Pregnant and Preeclamptic Women

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Accepted for publication by Prof. B. Halliwell

(Received 21 February 2000; In revised form 20 March 2000)

Increased reactive oxygen species (ROS) and lipid peroxidation may be implicated in the pathogenesis of preeclampsia by causing cell (membrane) damage and impaired endothelial function. Carbonyl derivatives of proteins, or protein carbonyls, may be sensitive biomarkers of ROS-mediated damage. The aim of the study was to compare levels of protein carbonyls in plasma of preeclamptic, healthy pregnant and healthy nonpregnant women.

Plasma protein carbonyls were measured in 47 preeclamptic, 45 healthy pregnant and 22 healthy nonpregnant women by using a sensitive ELISA-method. ANOVA, the unpaired *t*-test and Pearson's correlation were used for statistical analysis.

Preeclamptic women had significantly higher plasma protein carbonyl levels than healthy pregnant women ($P < 0.0001$). Healthy pregnant women showed significantly higher protein carbonyl levels ($P < 0.001$) as compared to nonpregnant controls.

The higher levels of protein carbonyls as compared to nonpregnant controls suggest that increased oxygen free radical damage occurs in normal pregnancy and to a much higher extent in preeclampsia.

Keywords: Protein carbonyls; ROS; Oxidative stress; Preeclampsia

INTRODUCTION

Reactive oxygen species (ROS) and lipid peroxides have an important function in normal physiology, in the defence against bacteria, fungi and viruses. However, overproduction of these compounds as well as a deficiency in the protective antioxidant systems can be associated with a number of diseases such as arteriosclerosis and cancer[1]. ROS and lipid peroxidation products are elevated in women with uncomplicated pregnancy[2,3], where they are counterbalanced by an increased activity of antioxidant systems[4]. However, even high levels of ROS and lipid peroxidation metabolites, as products of an altered oxidative stress, are observed in preeclampsia[5–7], whereas certain antioxidant systems are comprised[8–11].

The pathological cascade of events related to preeclampsia is mainly associated with endothelial cell damage. Increased ROS and lipid peroxides can cause cell membrane or other cellular damage and lead to impaired endothelial func-

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tion[12]. 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA) are frequently used as biomarkers of lipid modification as a result of lipid peroxidation reactions[7,9,13]. However, under oxidative stress lipids as well as carbohydrates, proteins, and DNA are major targets of ROS. Protein modification elicited by direct oxidative attack to amino acid side chains, or by modification of side chains with lipid peroxidation products as well as glycation and glycoxidation products can all lead to the formation of protein carbonyls[14]. Therefore protein carbonyls may serve as biomarker of general oxidative stress. ROS mediated modification of proteins may lead to loss of biological function and to modified proteins that are rapidly degraded[15]. The objective of this study was to compare concentrations of plasma protein carbonyls in nonpregnant, healthy pregnant and preeclamptic women and to investigate whether these levels correlate with factors indicating the severity of the disease.

MATERIALS AND METHODS

Patients

Three groups of women were investigated: a control group of 27 healthy nonpregnant women, a group of 45 healthy women with an uncomplicated pregnancy, and 47 preeclamptic patients. Eight of the pregnant controls also contributed to another study on glutathione levels in plasma[16]. The institutional review board of the University Hospital Nijmegen approved the protocol. All subjects were randomly selected between March 1998 and August 1999. All preeclamptic patients were severely ill and either had severe preeclampsia ($n = 23$) or moderate preeclampsia complicated with the syndrome of hemolysis, elevated liver enzymes, low platelets (HELLP) ($n = 24$). The healthy pregnant women were randomly selected among the women attending the outpatient clinic for routine antenatal control. Both preeclamptic and healthy pregnant women were not in labour and had a singleton pregnancy with a live foetus when they entered the study. The healthy nonpregnant women were randomly recruited among the employees of a governmental institution located in the same area. None of the nonpregnant women had conceived during the last antedating year or had experienced complications during previous pregnancies. Seven of the nonpregnant controls used oral contraceptives. Women with primary underlying diabetes, or pre-existing renal, hypertensive, or cardiovascular disease were excluded from participation. Preeclampsia was defined as a diastolic blood-pressure over 90 mm Hg on two or more consecutive occasions, each more than 4 hours apart, and concordant proteinuria (urinary protein over 0.3 g / 24 h). Severe preeclampsia was defined as a diastolic blood pressure over 110 mm Hg on two or more consecutive occasions, each more than 4 hours apart, and concordant proteinuria. The HELLP syndrome was defined as hemolysis (lactic dehydrogenase (LDH) over 600 U/L), elevated liver enzymes (both serum aspartate aminotransferase and serum alanine aminotransferase over 70 U/L), and low platelets (platelet count under 100×10^9/L)[17]. Blood pressure was taken with a sphygmomanometer while the patient was in sitting position. Diastole was recorded at phase V Korotkoff sound.

Methods

Blood samples were obtained by venepuncture into sterile siliconized K3 EDTA (15%) vacutainer tubes and processed within 30 min at $1200 \times g$ for 10 min for plasma collection. Immediately after centrifugation plasma was stored at –30°C until analysis.

Protein carbonyls were measured using an Enzyme Linked Immunosorbent Assay (ELISA) that was a modification of the method described by Buss et al.[18]. All samples were measured in a single assay. Standards and plasma samples

were diluted in phosphate buffered saline (PBS, 8 mM sodium phosphate buffer containing 140 mM NaCl, pH 7.4) to a protein concentration of 4 mg/ml. Protein derivatization was carried out in 1.1 ml reaction tubes, with 150 µl of dinitrophenylhydrazine (DNP) solution added to 50 µl of plasma (4 mg/ml) or standard. Samples were mixed and incubated at room temperature for 45 minutes. Then 5 µl of this solution was added to 1 ml PBS. After mixing, triplicate aliquots were added to wells of white Maxisorb FluoroNunc microtiter plates (Nalge Nunc International, Rochester, NY). The plates were incubated overnight at 4°C. The next day the plates were washed 5 times with PBS and the wells were blocked with 0.1% (w/v) bovine serum albumin (BSA) in PBS (200 µl per well) for 1 h at room temperature. After washing the plates, 100 µl/well biotinylated anti-DNP antibody (Molecular Probes Inc., Eugene, OR) was added (1:1000 dilution in 0.1% (w/v) BSA, 0.05% (v/v) Tween 20 solution) and incubated for 1.5 h at 37°C. Subsequently the plates were washed again and incubated for 1 h at room temperature with 100 µl Europium labelled streptavidin (Wallac Oy, Turku, Finland; 1:3000 dilution in PBS supplemented with 0.1% (w/v) BSA, 0.05% (v/v) Tween 20 solution). After 5 final washes 100 µl Enhancement solution (Wallac Oy, Turku, Finland) was added to each well and after 5–10 minutes of mildly shaking, plates were read in a Wallac Victor2 multi label counter in the standard Europium mode. A nine-point standard curve made by mixing various amounts of reduced BSA (0 nmol/mg protein carbonyls by definition)[18] and oxidised BSA (7.0 nmol/mg protein carbonyls)[18] was included with each plate. The ELISA assay used has a detection limit of 0.1 nmol carbonyls per mg protein, and an intra-assay coefficient of variation of 14 % when measuring control plasma samples.

Statistical Analysis

The Kruskal-Wallis, Mann-Whitney U, or Chi square tests, as appropriate, were used to assess statistical significance of differences in the population characteristics between groups. Data of carbonyls were analysed by ANOVA or the unpaired *t*-test, as appropriate. Pearson correlation coefficient was used for correlations between protein carbonyls with factors indicating the severity of preeclampsia. All statistical analyses were performed with SPSS statistical software package (SPSS Inc., Chicago, USA). A probability level of $P \leq .05$ was considered significant. Results are reported as mean (± standard deviation).

RESULTS

Population characteristics are presented in Table I. Median diastolic blood pressure was significantly higher in preeclamptic women than in healthy pregnant and nonpregnant women ($P < 0.001$). There were significantly more nulliparous preeclamptic women than nulliparous healthy pregnant women included in the study ($P < 0.0001$). Gestational age and maternal age were not different between preeclamptic women and healthy pregnant women.

Plasma protein carbonyl levels are illustrated in Figure 1. The mean plasma protein carbonyl level was significantly higher in preeclamptic patients (0.49 ± 0.25 nmol/mg protein) as compared to pregnant (0.28 ± 0.11 nmol/mg; $P < 0.0001$) and nonpregnant (0.17 ± 0.06 nmol/mg protein; $P < 0.0001$) controls (mean ± SD). Levels in healthy pregnant women were significantly higher than in nonpregnant women ($P < 0.001$). No differences in plasma protein carbonyl levels were found between preeclamptic women who either did or did not develop concurrently the HELLP syndrome (0.50 ± 0.27 nmol/mg protein versus 0.48 ± 0.23 nmol/mg protein; $P = 0.79$). We found no differences in protein carbonyl levels in nonpregnant women with (n = 7) or without (n = 20) oral contraceptive use (0.18 ± 0.07 versus 0.17 ± 0.05 nmol/mg protein; $P = 0.89$).

TABLE I Clinical Characteristics of Preeclamptic Patients, and Healthy Pregnant and Nonpregnant Controls

	Preeclamptic women (*n* = 47)	*Pregnant women* (*n* = 45)	*Nonpregnant women* (*n* = 22)
Age (y)	30 (22 – 44)	32 (23 – 42)	28 (19 – 39)
Diastolic BP (mmHg)	110 (90 – 140)[a]	70 (55 – 85)	75 (55 – 90)
Gestational age (wk)	32 (24–39)	31 (28–36)	–
Nulliparous	81%[b]	31%	–
Proteinuria (g / 24h)	5.5 (0.3 – 33.8)	ND	ND
Serum uric acid (mmol / L	0.45 (0.23–0.69)	ND	ND
Platelet count ($^{*}10^{9}$ / L)	63 (24 – 303)	ND	ND

Values are given as median (range) or numbers (percentage), as appropriate; ND = not determined; nulliparous, women in their first pregnancy; proteinuria, urinary protein excretion over 0.3 g/24 h.

a. $P < 0.001$ (Kruskal-Wallis test) preeclampsia compared with pregnant and nonpregnant controls.
b. $P < 0.0001$ (Chi-square test) preeclampsia compared with pregnant controls.

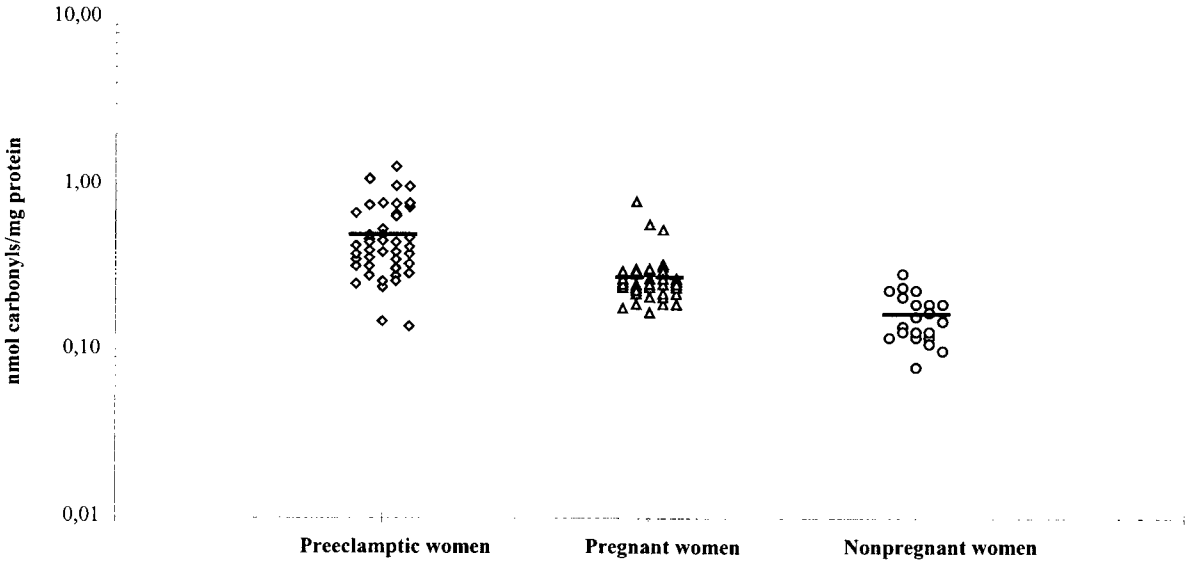

FIGURE 1 Plasma protein carbonyl levels in preeclampsia (◊), healthy pregnancy (△) and nonpregnant women (○). Horizontal lines indicate the means

We were not able to demonstrate a significant correlation of protein carbonyl levels with factors indicating severity of disease, such as: blood pressure r = 0.19 ($P > 0.05$), serum uric acid levels r = 0.05 ($P > 0.05$), proteinuria r = 0.05 ($P > 0.05$), or platelet count r = – 0.20 ($P > 0.05$).

DISCUSSION

ROS and lipid peroxidation may be implicated in the pathogenesis of preeclampsia. Important sources of ROS in preeclampsia include placental mitochondria[19]. ROS can also arise from

stimulated neutrophils, increased amounts of peroxynitrite, TNF-α and lipid peroxidation in the placenta[20]. Lipid peroxides are formed when polyunsaturated fatty acids interact with free radicals. ROS and lipid peroxides can lead to several adverse biological effects by causing cellular damage through oxidising nucleic acids, proteins and membrane lipids[1].

Protein carbonyl derivatives may be generated by three different ways: first by direct oxidation of several amino acid residues (lysine, arginine, proline, and threonine) or oxidative cleavage of proteins by either the α-amidation pathway with formation of a peptide in which the *N*-terminal amino acid is blocked by an α-ketoacyl group, or by oxidation of glutamyl side chains leading to a peptide in which the *N*-terminal amino acid is blocked by a pyruvyl group[14,15]; secondly by reaction with aldehydes, such as HNE or MDA, produced during lipid peroxidation[14,15]; and thirdly by interaction of reducing sugars or their oxidation products with lysine residues of proteins[14,15]. Therefore they may represent a good biomarker for general oxidative stress, while frequently used markers like MDA and HNE serve as biomarkers for oxidative stress from lipoxidation reactions only[21].

We found significantly higher protein carbonyl levels in preeclamptic women than in healthy pregnant women. This is in agreement with several studies investigating MDA- or HNE- levels as markers for oxidative stress in preeclampsia[3,7–9]. We also found higher protein carbonyl levels in healthy pregnant women as compared to nonpregnant women. This is in agreement with earlier results by Wickens et al. who demonstrated increased free radical activity in normal pregnancy and explained this by either increased cell turnover or a relative or absolute decline in antioxidant free radical scavenging mechanisms[3]. Data of longitudinal studies on lipid peroxidation in normal pregnancy are not consistent: one study showed that the mean levels of lipid hydroperoxides were more or less stable throughout pregnancy[23], whereas other investigators reported increased lipid peroxidation in the second and third trimester[24,25]. In addition, a cross-sectional study showed little difference among trimesters[4], while other studies showed higher values of lipid hydroperoxides in late pregnancy[3,21]. Antioxidant activity was shown to be increased in the second and third trimester[8]. We now clearly demonstrate higher protein damage in the third trimester of preeclamptic women versus healthy pregnant women.

As reviewed by Ciavatti et al[26] oral contraceptive use might modify the oxidative status in women by decreasing the antioxidant defences (as by the vitamins E and C) and increasing free radical generation due to estrogens. However, we found no differences in plasma protein carbonyl levels in nonpregnant women with or without oral contraceptive use.

We could not demonstrate any significant correlation between factors indicating severity of the disease and plasma protein carbonyl levels. This might be due to the fact that the protein carbonyl levels are the result of protein damage due to ROS generated by a wide variety of causes and contributing to the origin of the disease, whereas the parameters describing the severity of disease are totally different entities indicating the course of the disease. In addition, the preeclamptic study group was a quite homogenous group as all patients included were severely ill. In a search for a correlation between protein carbonyl levels and the severity of the disease process also mild and moderate preeclamptic patients should be studied in the future.

In conclusion, our study clearly shows increased protein damage by ROS in preeclampsia. Compared to other markers previously used, plasma protein carbonyls may serve as a useful biomarker for general oxidative stress. More research is needed to find out whether plasma protein carbonyl levels may be of any predictive value for the development of the disease, or can serve as or a good measure for its severity of the disease.

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