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NORMAL SUPEROXIDE DISMUTASE (SOD) GENE IN PREGNANCY-INDUCED HYPERTENSION: IS THE DECREASED SOD ACTIVITY A SECONDARY PHENOMENON?

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Reactive oxygen species (ROS) have been implicated in the pathogenesis of pregnancy-induced hypertension (PIH). A genetic factor is also thought to be associated with the disease. The aim of the present study was to investigate whether decreased superoxide dismutase (SOD) activity in PIH resulted from gene abnormalities. Fourteen patients with PIH were enrolled in the study. Normal pregnant women and normal nonpregnant women served as controls. Genomic DNA and mRNA were isolated from white cells and subjected to Southern and Northern blot analysis with a 600 bp CuZn-SOD probe. SOD activity was also determined in the white blood cells and red blood cells. The results showed that SOD activity was significantly reduced in patients with PIH compared to both control groups. There were no significant differences in the size of the CuZn-SOD gene and its expression between the patients with PIH and the controls. This study confirmed that there was a decreased SOD activity in PIH but revealed neither major structural changes in the genomic DNA nor mRNA size of CuZn-SOD. Our results suggest that the decreased SOD levels in PIH are not due to abnormalities in the CuZn-SOD gene and are an acquired phenomenon which occurs during the development of the disease.

KEY WORDS: Hypertension, pregnancy, antioxidant, gene, enzyme activity.

INTRODUCTION

Superoxide dismutase (SOD) catalyses the dismutation of the superoxide anion into oxygen and hydrogen peroxide and SOD is thought to be an important component of the cellular defense repertoire against oxidative damage mediated by superoxide radicals produced as a by-product of oxygen metabolism[1,2].

In eukaryotic cells, there are two distinct types of SOD[1,2]. The predominant SOD is a soluble cytosolic enzyme containing copper and zinc at the catalytic site (CuZn-SOD). The second type is the manganese-containing SOD (Mn-SOD) found in the matrix of the mitochondria. The Mn-SOD differs substantially from the CuZn-SOD in primary structure[3]. Although extracellular SOD, which is similar to

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CuZn-SOD, has been reported, the physiological significance of this type of SOD remains unknown[4]. The gene for human CuZn-SOD is on chromosome 21 and that for the Mn-SOD on chromosome 6; the chromosomal location for the extracellular SOD gene has not yet been reported.

In recent years, increased superoxide formation or lipid peroxidation products have been described in patients with pregnancy-induced hypertension (PIH)[5-7]. We have previously found decreased SOD activity in this disorder[8]. Therefore, an imbalance between pro-oxidation and anti-oxidation is suggested to be implicated in the pathogenesis of PIH. Considering that PIH is well known to be familial and a genetic predisposition has been proposed[9-11], one might speculate that a mutation in a critical region of SOD gene or its abnormal expression could result in an alteration of SOD activity. We thus analysed the genomic DNA and mRNA expression in patients with PIH.

MATERIAL AND METHODS

Subject

Fourteen PIH patients with mean age 26.9 ± 5.1 years and mean gestational age 36.5 ± 5.2 weeks were included in the study. Five of these patients had proteinuria and nine did not. Twelve normal pregnant women with mean age 26.3 ± 5.8 years and mean gestational age 36.3 ± 5.6 weeks and thirteen normal nonpregnant women with mean age 26.5 ± 6.3 were served as controls.

PIH is defined as a persistent or recurrent diastolic blood pressure of $\geqslant$90 mmHg developing during pregnancy after 20 weeks of gestation, and resolving by six weeks post partum. Proteinuria is defined as the persistent presence of protein in the urine detected by urine 'dipstick' testing, or >300 mg excreted in 24 hours. The latter measurement is preferred where available.

White Cells Preparation

10 ml of peripheral blood was collected into a sterile conical tube containing EDTA (1 mg/ml blood) and a total of 30 ml of blood was used. 10 parts EDTA blood were mixed with 1 part 6% (w/v) Dextran 500 (Pharmacia Ltd., Bucks, UK) and this was left to settle at room temperature. When the white cell band in the upper plasma layer was visible, it was transferred to another tube.

Genomic DNA Preparation

DNA was isolated by mixing white cells with an equal volume of 2x lysis buffer (0.1 M Tris pH 7.9, 1.0 mM EDTA, 20 mM NaCl and 4% SDS) for 30 minutes to lyse the cells. This preparation was extracted twice with an equal volume of water saturated phenol. DNA was precipitated with ethanol. The nucleic acid precipitate was then dissolved in lx lysis buffer (0.05 M Tris pH 7.9, 0.5 mM EDTA and 10 mM NACl) and treated with ribonuclease A (RNase A, BRL, Gibco Ltd, UK) and proteinase K (Boehringer Mannheim GmbH., German) digestion followed by phenol:chloroform:isoamyl alcohol (25:24:1) extraction and ethanol precipitation. Finally DNA was dissolved in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0).

Southern Blot Analysis

15 μg of DNA was digested with restriction enzyme *EcoRI* (2 unit/μg, BRL) at 37°C overnight and the fragments separated on 1.0% agarose gel (6 μg/lane). The molecular weight markers were φX174 *Hae* III and λ DNA *Hind* III (BRL). The fragments were transferred onto nitrocellular membrane (Hybond-C; Amersham International, Amersham, UK) by the method of Southern[14] after denaturation and neutralization and baked under vacuum at 80°C for 2 hours. A 0.6 Kb *Pst* I insert from pSOD1, a plasmid containing a CuZn-SOD cDNA, was used as the probe[15,16] and radiolabelled with ^{32}P-dATP using the random primer extension method[17,18]. The membranes were prehybridized for at least 1 hour and then hybridized overnight at 68°C in DNA hybridization solution (5x SSC, 4x Denhardts, 10% dextran sulphate, 0.1% SDS, 0.1% sodium pyrophosphate, 100 μg/ml denatured salmon sperm DNA). After hybridization the filters were washed in a mixture of 2x SSC, 0.1% SDS, 0.1% sodium pyrophosphate (PPi) at 68°C and then in 0.1x SSC, 0.1% SDS, 0.1% PPi at 68°C. After washing, filters were subjected to autoradiography using Kodak XAR5 film and an intensifying screen at −70°C.

RNA Preparation and Northern Blot Analysis

Total RNA from the cells was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method[19]. 10 μg of total RNA was applied to 1.2% formaldehyde-agarose gels[13]. A 0.24–9.5 Kb RNA Ladder (BRL) was used as molecular weight marker. Samples from the gel were transferred to nitrocellular membranes and baked under vacuum at 80°C for 2 hours. The filters were prehybridized for 1 hour in RNA hybridization solution (5x SSC, 4x Denhardts, 0.1% SDS, 0.1% PPi, 100 μg/ml denatured salmon sperm DNA) and then hybridized to a ^{32}P-dATP labelled CuZn-SOD probe at 68°C. The washing and autoradiographic conditions were the same as Southern blot analysis. The relative amount of each specific mRNA band was quantified by densitometric scanning using a soft laser scanning densitomer.

SOD Activity Assay

SOD activity in white blood cells and red blood cells was determined by using a SOD-525 spectrophotometric assay kit (Bioxytech S.A., Cedex, France) and the assay was performed according to the manufacturer's instructions. The possible interfering agents such as hemoglobin and albumin in the samples were eliminated by the ethanol/chloroform method recommended in the manufacturer's instructions. The results were expressed in SOD-525 activity units per ml of sample (U/ml).

Statistical Analysis

Data are expressed as mean ± S.D., with differences between groups being assessed by Student's *t* test.

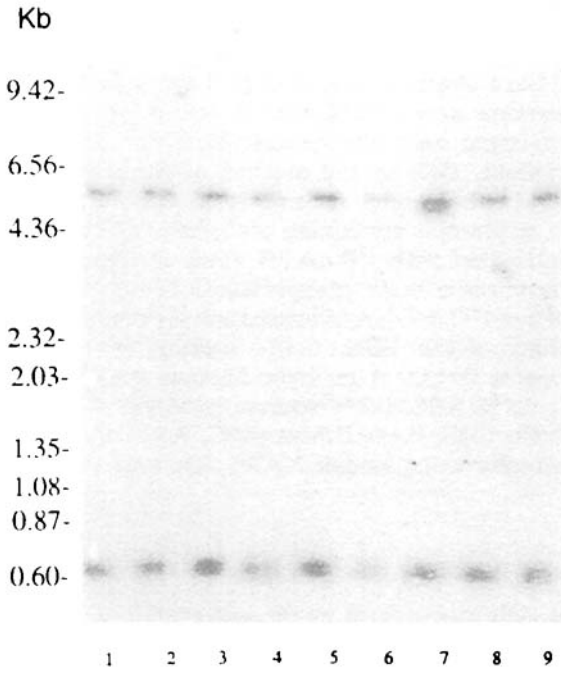

FIGURE 1 Southern blot analysis of genomic DNA from white cells of PIH patients (lane 3–7), normal pregnant women (lane 8, 9) and normal nonpregnant women (lane 1, 2). DNA was digested with *EcoRI* restriction enzyme and the filter was hybridized using a human CuZn-SOD probe. The frequencies of 5.26 and 0.74 kb fragments did not differ between PIH patients and the control groups.

RESULTS

The SOD activity in both white blood cells and red blood cells was significantly decreased in patients with PIH as compared to either normal pregnant women or normal nonpregnant women (Table 1). There was no difference between the normal pregnant women and normal nonpregnant women.

Genomic DNA from white cells of the eight women with PIH was hybridized to a CuZn-SOD probe. Representative experiments are shown in Figure 1. The occurrence of the 5.26 and 0.74 Kb fragments did not differ between patients with PIH and the control groups as all the three group showed both of these bands. However,

TABLE 1
SOD activity in patients with PIH and controls

	n	SOD (U/ml) in white cells	*n*	SOD (U/ml) in red cells
Normal pregnancy	12	0.359 ± 0.16**	10	1.745 ± 0.43*
Normal nonpregnancy	13	0.370 ± 0.19**	10	1.728 ± 0.31**
PIH	14	0.126 ± 0.04	10	1.090 ± 0.29

*$p < 0.01$, **$p < 0.001$ compared with PIH.

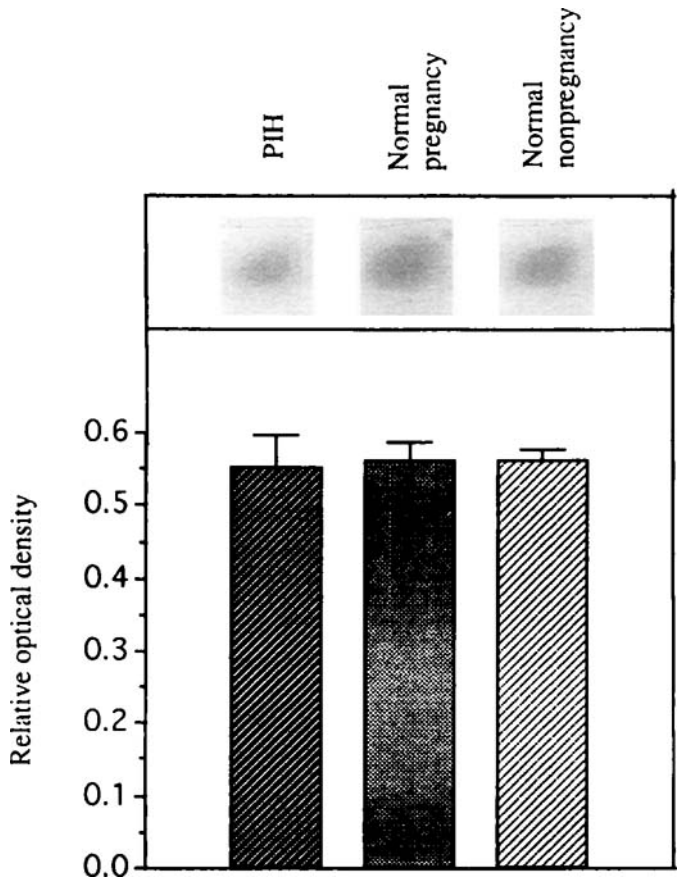

FIGURE 2 Northern blot analysis of RNA from white cells of PIH patients ($n = 7$), normal pregnant women ($n = 7$) and normal nonpregnant women ($n = 5$). Blots were hybridized with a human CuZn-SOD probe. The intensities of autoradiographic images were quantified by the Microcomputer Imaging Device. The upper panel shows the autoradiography of a representative experiment. The graph represents the mean ± SD of the individual experiments.

there seems to be an extra band in lane 7 whose size is close to 5.26 Kb. This patient suffered from asthma and nephrotic syndrome in addition to PIH.

Figure 2 shows the Northern blot analysis of RNA isolated from white cells. The RNA was hybridized to a cDNA probe of CuZn-SOD. A band, estimated (by the 0.24-9.5 Kb RNA Ladder) to be 0.67 Kb, was detected. This is a similar size to that of the CuZn-SOD mRNA in human cells reported by others (Lieman-Hurwitz *et al.* 1982; Sherman *et al.* 1984; Delabar *et al.* 1987). The results obtained in the Northern blot experiments were quantified by the densitometry on a Microcomputer Imaging Device (Imaging Research INC., Canada). No significant change in expression of the CuZn-SOD gene was found between the patients with PIH and controls ($p > 0.05$).

DISCUSSION

This study confirms our previous observation of decreased SOD activity in patients with PIH[8]. Although the SOD-525 spectrophotometric assay is convenient, reproductive and free of serious interference, it cannot identify the type of SOD and the measurement could be of both CuZn-SOD and Mn-SOD. However, the SOD measured in the peripheral red blood cells is considered to be the CuZn-SOD because the mature red blood cells contain no mitochondria[20], which is the home of Mn-SOD[1,2]. Therefore the decreased SOD activity found in this red cell study model should correspond to CuZn-SOD. The SOD determined in the white blood cell model could be both CuZn-SOD and Mn-SOD. However, it is reasonable to consider that the decreased SOD activity in white blood cells in the present study is at least partially due to an alteration of CuZn-SOD activity since this type of SOD activity in red blood cells is reduced in this study and CuZn-SOD comprises approximately 80% of the total cellular SOD activity[21].

CuZn-SOD is the major SOD in mammalian tissue[21]. A decreased CuZn-SOD activity in PIH is not surprising because the disease has been associated with increased superoxide production and lipid peroxidation[5-7]. Superoxide is known to exert a hypertensive effect by changing the pattern of prostaglandin production[7,8], inactivating the endothelium-derived vascular relaxing factor[22], and directly contracting smooth muscles[23]. Conversely, injection of SOD has been shown to have a hypotensive effect[24].

The inheritance of a low CuZn-SOD activity has been documented in some human diseases such as amyotrophic lateral sclerosis[25]. There is increasing evidence to show that genetic factors play a role in the pathogenesis of PIH[9-11], but the genetic study of antioxidant enzymes has not been investigated before in this disease. Although mutation of its gene or defective expression is one mechanism to explain low SOD activity, this possibility seems unlikely in the present study as both the DNA and mRNA related to CuZn-SOD in PIH did not differ from that in normal pregnancy and normal nonpregnant women. One patient had an unusual finding on Southern blotting observing an extra band with a size close to 5.26 Kb (Figure 1, lane 7). This PIH patient was also suffering from asthma and nephrotic syndrome at time of sampling. Further studies would be necessary to determine whether this possible extra band was related to asthma or/and nephrotic syndrome or whether some PIH women complicated with other diseases such as asthma and nephrotic syndrome show abnormalities of the SOD gene whereas others do not.

It is known that hydrogen peroxide, a product of SOD itself, can effectively inactivate the CuZn-SOD enzyme by a Fenton-type reaction of H_2O_2 with Cu^{++} at the centre of the enzyme, forming a reactive intermediate that destroys an essential liganding histidine residue[26,27]. Hydrogen peroxide is mainly metabolized by a selenium-dependent glutathione peroxidase at the expense of glutathione (GSH)[28]. A dietary lack of selenium has been suggested to contribute to a high incidence of PIH[29] and, furthermore, our previous study indicated that lower GSH levels occurred in patients with PIH[8]. In addition, the activation of cells, especially white blood cells, is known to induce hydrogen peroxide formation[30,31]. Neutrophils and mononuclear cells appear to be activated in PIH[32,33]. Therefore, the decreased SOD activity in PIH could be explained by the increased hydrogen peroxide formation. A number of cytokines such as tumour necrosis factor and interleukin 1 have been reported to affect SOD activity *in vitro*[34]. However, it is not known at present whether these cytokines are altered in PIH. A final possibility is that

the SOD deficiency may also occur at some time during the course of its protein synthesis.

Taken together, in the absence of major structural rearrangements in genomic DNA and size abnormality of mRNA, it is likely that a decreased SOD activity in the present study is a secondary phenomenon which develops during the course of the disease though whether there is a subtle change such as microdeletion/insertion or point mutation needs further investigation.

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