行政院國家科學委員會專題研究計畫成果報告

中文題目：過氧化物消除酵素(SOD)在高血壓形成過程所扮演角色之研究

英文題目：Comparison of Superoxide Dismutase Gene Expression and Activity in the Heart of Spontaneously Hypertensive Rats with that in Nor-motensive Rats

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中文摘要

細胞膜的異常是人類原發性高血壓的重要因素。近年來許多研究顯示，氧自由基的破壞是高血壓性血管病變的重要致病機轉。而超氧化物轉化酶 (Superoxide Dismutase, SOD) 可以清除氧自由基，是人類體內重要的天然抗氧化系統。

為了了解高血壓狀態下，心臟中超氧化物轉化酶的活性。本研究比較了自發性高血壓大白鼠 (Spontaneously Hypertensive Rats, SHR) 和正常血壓鼠 Wistar-Kyoto rats (WKY) 兩種老鼠體內主要 SOD 的活性及基因表現。

在 6 週，9 週及 12 週大的兩種大白鼠身上，分別以西方點墨方法分析 SOD 的活性，及北方點墨法分析訊息核糖核酸(mRNA)的程度。本研究發現在 SHR 的心臟中，有比 WKY 鼠更高的 Mn-SOD 含量及訊息核糖核酸的表現。在整體 SOD 的總量上，兩種實驗鼠種並無差異，表示 SHR 體內有較低的 CuZn-SOD 活性及訊息核糖核酸的表現。這些天然抗氧化系統 SOD 的改變，可能降低 SHR 心臟對抗氧自由基的傷害，亦可能是此種老鼠產生首發性高血壓的致病機轉。

ABSTRACT

Membrane abnormalities in human essential hypertension are well-established. Recent study has suggested that oxygen free radicals (OFR) play a role in the pathogenesis of hypertensive vascular disease. Superoxide dismutase (SOD) is a naturally existed antioxidant in human which has an important role in scavenging OFR. In order to determine if changes in superoxide (SOD) in the heart occur in the hypertensive state.The present study compared the levels of the two main subtypes of this enzyme in spontaneously hypertensive rats (SHR) with age-matched normotensive Wistar-Kyoto (WKY) rats using enzyme activity estimation, Western blotting analysis for enzyme contents, and Northern blotting analysis of mRNA level at 6-week, 9-week and 12-week old rats. A higher level of both Mn-SOD activity and Mn-SOD mRNA expression was found in heart of SHR as compared with WKY rats. Also, the mRNA levels of CuZn-SOD in the heart of SHR differed from WKY rats in parallel to the enzyme activities. However, the amount of SOD enzyme subtypes, determined by Western blotting analysis, was not different between SHR and WKY rats.The results indicate a lower gene expression and less activity of CuZn-SOD in SHR heart. This alternation of SOD may be one of the important factors for the vulnerability of the heart from oxygen free radicals or may be related to the pathogenesis of hypertension in this species.

INTRODUCTION

Hypertension is a key determinant of risk for premature cardiovascular morbidity and mortality associated with major cardiac disorders including small arteriolar disease of the coronary circulation, occlusive epicardial coronary arteriolar disease, and congestive heart failure; hemorrhagic and nonhemorrhagic stroke; end-stage renal disease; and dissecting aortic aneurysm and other emergent hypertensive disorders.[1]

The etiology of hypertension is multifactorial, so it is critically necessary to understand the nature of the disease pathophysiologically; by doing so, it is then possible to conceive and to develop new therapies as well as to select pharmacological treatment more rationally.

Membrane abnormalities in human essential hypertensions are well-established.[2] Toxic oxygen free radicals (OFR) such as superoxide anions have been implicated in tissue injury to the heart and other organs.[3] Topical application of free radical scavengers to the brain inhibits the development of pial arteriolar lesions in the cat during acute hypertension had been reperted.[4] In rats, infusion of free radical scavengers inhibits vascular hyperpermeability and cellular damage during acute hypertension induced by angiotensin II[5] and bolus injection of superoxide dismutase (SOD) lowers blood pressure in spontaneously hypertensive rats (SHR) but not in normotensive rats.[6] An important role of free radicals in the pathogenesis of organ damage from hypertensive disease has been suggested.[7]

In order to aleviate oxidative damage to living cells, they possess three major scavenger enzymes, SOD, catalase, and glutathione peroxidase,[8] which play a protective and regulatory role in the defence of cells against a variety of exogenous and endogenous oxidants.[9] Application of exogenous SOD to improve the disorders in acute hypertension has been documented,[10] but the changes of endogenous defence enzymes in response to hypertensive stress remained obscure. In an attempt to know whether the changes of SOD in rats have correlation with hypertension, the present study examines the changes of SOD, both Mn-SOD and CuZn-SOD subtypes, in hearts of 6, 9, and 12-week old SHR in comparison to age-matched normotensive Wistar-Kyoto rats (WKY) by measuring the free radical scavenging activity, Western blotting of enzyme content and Northern blotting analysis of mRNA level.

METHODS

Animals

Male SHR and WKY rats, aged 6-12 weeks, were obtained from the animal center of National Cheng Kung University Medical College. They were housed four per

cage at 23 ± 1℃ with a 12 hr light-dark cycle. The light-cycle lasted between 0700 and 1900 hr. Food and water were available ad libitum.,

Determination of blood pressure

Systemic mean blood pressure was measured in conscious SHR and WKY at 6, 9, and 12-week old following a baseline stabilization period. An indirect tail-cuff method reading in a programmed autodetector (UR-5000; Ueda Seisaku Co. , Tokyo, Japan) was performed, as described previously.[11] Recording on the laser computerized digital program was carried out at ambient temperature (28 ± 1℃). The indicated blood pressure values were calculated from the means for each rat measured in triplicate before sacrifice.

Tissue preparations

After recording of the blood pressure, rats hearts of 6, 9, 12-week old were obtained under ether anesthesia. Rats heart were under complete dissection of tissues other than myocardium. Heart sections were than immediately homogenized (5: 1, V/W) in 10 mmol/L Tris/HCI buffer (pH 7.0) containing 0.25 mol/L sucrose. Tissues for Western and/or Northern blotting were frozen in liquid nitrogn and stored at - 80℃ until further analysis.

SOD activity assay

The homogenates were sonicated and centrifuged (10000 xg) at 4℃ for 30 min. The supernatant was employed for assay using the commercial kit (SOD kit, Wako; Tokyo, Japan), as described previously.[12] In brief, nitroblue tetrazolium (NBT) can be reduced to the insoluble blue formazan by superoxide anion produced from the reaction of xanthine oxidase with hypoxanthine. The reduced NBT dye is extracted and read at 560 nm (Hitachi U-3210, Tokyo, Japan). The activity of Mn-SOD was distinguished from total SOD activity by an addition of potassium cyanide (1 mmol/ L) into the assay buffer to inhibit the activity of CuZn-SOD. The activity of CuZn-SOD was calculated by subtracting Mn-SOD activity from total SOD activity. The SOD activity was quantified from a standard curve using purified SOD from bovine erythrocytes (S-2515; Sigma). Results are expressed as units per mg of protein, which was determined by the method of Bradford[13] using a commercial kit (Protein Assay 5000006, Bio-Rad).

Immunoblotting of SOD enzyme

The presence of SOD in heart was assessed by immunoblotting using polyclonal antibodies (K90077C & K90096C) purchased from Biodesign International (Kennebunk, Maine 04043, U.S.A.). The tissue preparations were lysed in buffer containing 1 % Triton X-100. Discontinuous slab gels (1.0 mm thickness) containing 0.1 % SDS were prepared according to Laemmli[14] with acrylamide concentrations of 12 % in the separation gel and 5 % in the stacking gel. Protein samples were fractionated by gel electrophoresis at 4℃ run at 40 and 100 V during the stacking and separation steps, respectively. The separated proteins were blotted onto nitrocellulose as described previously.[15] After treatment with anti-CuZn-SOD antibodies (43 mg/ml) or anti-Mn-SOD bodies (33 mg/ml), imrnunostaining was performed for peroxidase activity by incubation in Tris-buffer (10 mmol/L) using the enhanced chemiluminescence (ECL) development system (Amersham International Inc., England). This antibody is purified from human liver and shown to be specific by the supplier using 2D-IEP and double diffusion. In the preliminary screening, it was found to react with both subtypes of SOD in rat spleen. Identification of this response was observed at 25 Kds for Mn-SOD and at 32 Kds for CuZn-SOD. The obtained Western immunoblots were then quantified densitometrically using a laser densitometer.

Characterization of mRNA for SOD

Isolation of RNA

Hearts taken for RNA isolation were frozen immediately in liquid nitrogen after removal and then stored at - 80℃. Total RNA was isolated by a single step acid guanidinium thiocyanate-phenol-chloroform extraction method.[16] Aliquots (15-20 μg) of RNA were denatured with 48% formamide, 6.3% formaldehyde at 55℃ for 15 min and size-fractionated by electrophoresis in 1.2 % agarose gels containing 2 % formaldehyde and 0.5 μg/ml ethidium bromide (Sigma). RNA was transferred to Hybond N nylon membranes (Amersham) by Northern blot capillary transfer overnight using 2 vol of saline sodium citrate (2 X SSC) as the transfer medium. The transfer was controlled on

an LTV transilluminator and alternatively by staining the blot membrane with 0.05% methylene blue according to Herrin and Schmidt.[17] Filters were rapidly prehybridized at 65℃ in hybridization solution (Quikhyb, Stratagene; U.S.A.)

cDNA probes

Plasmids with cDNA of rat Mn-SOD and rat CuZn-SOD were obtained as kind gifts from Dr. Ye-Shih Ho (Institute of Chemical Toxicology, Wayne State University, Detroit, Michigan 48201, U.S.A.). Transformation in *Escherichia coli,* plasmid preparation and cDNA purification were performed by standard molecular biology methods.[18] Radioactive probes were prepared by the multiprime DNA labeling system suggested by the supplier (Amersham). The prepared cDNA inserts and GAPDH probes were added directly into the prehybridization solution (Quikhyb, Stratagene) at a radioactivity of 1×10^6 CPM/ml.

Hybridization procedure

Hybridization was performed at 60℃ for 1.5 h. After hybridization, blot membranes were washed twice for 15 min at room temperature with 2 × SSC/0.1 % SDS (2.5 ml/cm^2) followed by a 30-min high stringency wash with 0.1 × SSC/0.1 % SDS at 60℃. Wet blot membranes were sealed in plastic foil and exposed to medium-sensitive medical X-ray film (Fuji, Japan) at -70℃ using intensifier screens. Exposure times were 2 - 3 days for Northern blots. Hybridization intensity of autoradiographic signals was measured using a two-dimensional densitometry. The obtained density (optical unit) is calculated versus the value of slot blot for GAPDH to make the quantification of mRNA.

Statistical analysis

Results are given as the mean ± SEM from numbers of animals. Student's *t*-test was used to compare two mean values for paired and/or unpaired observation; a probability of 0.05 or less was considered significant. Where two or more treatment means were compared to one control mean, determinations of the differences were carried out with Dunnett' s multiple comparison. [19]

RESULTS

Changes of SOD activity in adult SHR

The systemic mean blood pressure in SHR was 184 ± 12 mmHg (n=8) which was significantly higher ($p<0.001$) than that in WKY (132 ± 11 mmHg ; n=8). Activity of Mn-SOD in SHR was significantly higher ($p<0.01$) than WKY (Table 1). The activity of CuZn-SOD of SHR hearts was also higher ($p<0.05$) than WKY although the level of elevation was not as high as Mn-SOD.

The mRNA level of SOD in adult SHR

The mRNA of SOD was characteristized by Northern blotting analysis using cDNA probes for both subtypes (Figure 1). The level of Mn-SOD mRNA was significant increased in adult SHR ($p< 0.0001$) (Table 2). Interestingly, the level of CuZn-SOD mRNA showed no significant difference when compored with WKY.

Identification of SOD antibody binding density by Western blotting analysis

Presence of SOD enzyme, both Mn-SOD and CuZn-SOD subtypes, was identified by Western blotting analysis using specific antibodies although broad bands of Mn-SOD were observed (Figure 2). Recognition sites of SOD enzyme, obtained by comparison of antibody ability, showed a similar pattern in SHR compared to WKY and there was no difference in the density of response between SHR and WKY (Table 3).

DISSCUSSION

The results of this study indicate that SOD increased significantly in SHR when compared to WKY normotensive rats. The increase in SOD seemed to be correlated to the elevation of blood pressure. Rats used in the present study cannot be considered as old because old age is generally regarded as 24-25 months or more in this species.[20] However, the age of experimental animals employed seemed to be appropriate since 3 months old rats were generally regarded as an adult model.[21]

For the assay of SOD activity in rat hearts, the present study found that SHR had significantly lower activity found that SHR had significantly lower activity of Mn-SOD than WKY rats. This result is similar to that observed in the brain of SHR reported

recently,[22] and also in stroke-prone strain SHR.[23] The possible explanation that SOD increase may be due to SHR matured with a stable response to hypertension. Previous studies have indicated that myocardial damage in SHR is caused by lipid peroxidation of plasma membranes,[24] and it has been shown that OFR may be related to the pathogenesis of essential hypertension.[5] However, changes of SOD in the SHR remain obscune except the report that SHR liver has a higher Mn-SOD activity and a lower CuZn-SOD activity than that in WKY.[25]

In human and other mammals, SOD can be divided into three subtypes, Mn-SOD (mitochondrial SOD), CuZn-SOD (cytosolic SOD) and EC-SOD (extracellular SOD), EC-SOD is a secreted CuZn-containing glycoprotein, which is highly homologus to cytosolic CuZn-SOD.[26] The activity of SOD has been determined mainly according to the principle of McCord and Fridovich[27] and the activity of Mn-SOD was generally suggested to be changed easily than that of CuZn-SOD, probably due to mitochondria are more oxygen-dependent and more susceptible to ischemic damage.[28] The present data are also compatible with this phenomenon.

As in previous studues,[29] EC-SOD activity was not measured separately in the present study because it is highly homologus to cytosolic CuZn-SOD.[25] It is possible that EC-SOD activity contributes to the activity we obtained for CuZn-SOD. Moreover, due to differences in substrates or other factors, results regarding the changes of SOD activity in hypertension or with aging are very variable.[29,30]

In addition to the gene expression, formed SOD was also identified using specific antibodies. The results of Western blotting analysis showed that recognizin sites by antibodies for SOD, both the Mn-SOD and CuZnSOD subtypes, were similar in SHR to the WKY rats and no statistical difference could be found between SHR and WKY for the amount of both Mn-SOD and CuZn-SOD subtypes in rat hearts (Table 3). The content of Mn-SOD has previously been quantified by the enzyme-linked immunosorbent assay (ELISA) method in other tissues.[31] The basic principle of Western blotting analysis is similar to ELISA in the application of specific antibodies. Our findings represent the changes of formed enzyme protein depending on the recognition sites for antibodies. Thus, changes in gene expression in SHR seem unable to alter the amounts of SOD enzyme protein, both the Mn-SOD and CuZn-SOD subtypes in the hearts. It may be due to a rapid degeneration of SOD enzyme in response to the high amount of free radicals in SHR and the persistence of enzyme in a less active form that can still be recognized by the antibodies. In addition to less rapid turnovers of protein, factors involved in the post-translation control of SOD enzyme biosynthesis can not be ruled out. Further investigation is required to elaborate the detailed mechanisms.

A recent report by Sagar et al.[3] shows that a significant inverse correlation was obtained between systolic and diastolic blood pressure on one hand and SOD on the other. Hypertensive patients showed global membrane abnormality with an increased chemiluminescence generation by neutrophils, which is an indirect evidence of OFR production. Is OFR generation by neutrophils in severe hypertension significant enough to result in endothelial damage? Kontos has implicated OFR in the pathogenesis of hypertensive cerebral vascular injury in experimental animals.[4] What exactly is the role of OFR and OFR scavengers such as SOD in hypertension is still far form clear.

In conclusion, the present study indicate that gene expression and enzyme activity of SOD subtypes show a different pattern in the heart of SHR compared with age-matched WKY rats. This alternation of SOD may represent one of the important factors for the vulnerability of the heart against oxygen free radicals or may be relevant to the pathophysiology of hypertension in these rats.

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Table1. The in vitro activity of superoxide dismutase (SOD) in the hearts from spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY) at different stages.

	WKY Rats (n=8)			SHR Rats (n=8)		
	6wks	9wks	12wks	6wks	9wks	12wks
Mn-SOD (unit/mg protein)	35.9±1.3	33.1±0.9	33.6±0.4	56.9±1.2**	54.1±0.6**	55.9±0.8**
CuZn-SOD (unit/mg protein)	28.6±1.0	27.6±0.7	27.3±0.4	31.3±0.8*	31.7±1.1*	31.9±0.4*

Values are mean ±SEM. * P < 0.05, **P<0.001, between spontaneously hypertensive rats and Wistar-Kyoto rats

Table2. The gene expression of superoxide dismutase (SOD) using Northern blotting analysis in the hearts from spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY) at different stages

	WKY Rats (n=8)			SHR Rats (n=8)		
	6wks	9wks	12wks	6wks	9wks	12wks
Mn-SOD (%)	100	115±26	119±37	217±64*	259±82*	195±50*
CuZn-SOD (%)	100	114±13	112±20	99±16	102±23	108±22

Values are mean±SEM of the mRNA level. * P < 0.001 between spontaneously hypertensive rats and Wistar-Kyoto rats

Table3. The amount of superoxide dismutase (SOD) enzyme using Western blotting analysis in the hearts from spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY) at different stages

	WKY Rats (n=8)			SHR Rats (n=8)		
	6wks	9wks	12wks	6wks	9wks	12wks
Mn-SOD (%)	100	100	100	110.7±16.5	94.8±3.9	96.9±15.9
CuZn-SOD (%)	100	100	100	107.6±9.5	98.2±19.9	105.2±9.3

Values are mean±SEM * P < 0.05 between spontaneously hypertensive rats and Wistar-Kyoto rats

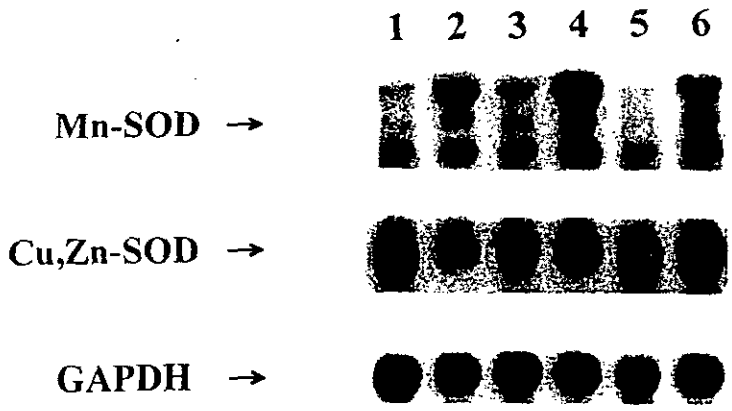

Figure 1. Representative picture of mRNA Levels for superoxide dismutase (SOD), both Mn-SOD and CuZn-SOD, in spontaneously hypertensive rats (S) and normotensive Wistar-Kyoto rats (W) determined by Northern blotting analysis using GAPDH as internal standard.

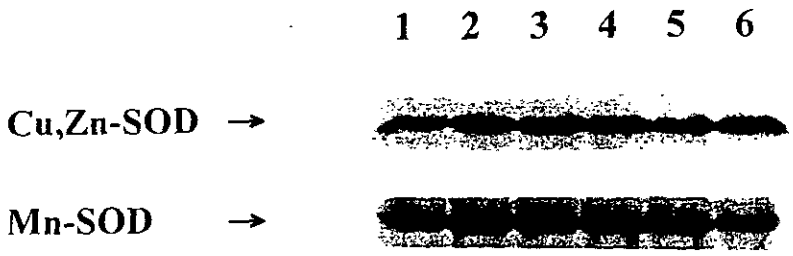

Figure 2. Identification of formed superoxide dismutase (SOD) in hupertensive (SHR) and normotensive rats (WKY) using Western blotting analysis. The bands for CuZn-SOD are indicated in upper portion and those for Mn-SOD in lower portion. Land 1,3,5 WKY 6-week, 9-week, 12-week old. Land 2,4,6 SHR 6-week, 9-week, 12-week old.