Effects of Benidipine Hydrochloride on Antioxidant Enzyme Activity in Stroke-prone Spontaneous Hypertensive Rats (SHR-SP)

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Effects of benidipine hydrochloride or triple therapy (hydralazine, reserpine, and hydrochlorothiazide) on renal cortical and medullary intrinsic antioxidant enzyme (AOE) activity were evaluated in stroke-prone spontaneously hypertensive rats (SHR-SP) as an animal model for human essential hypertension with cerebral stroke. This study showed a significant decrease of renal intrinsic glutathione peroxidase (GSH-Px) activity in untreated SHR-SP. Renal GSH-Px activity in untreated SHR-SP was significantly lower than that in Wistar Kyoto rats (WKY) as a normotensive reference strain.

GSH-Px activity in SHR-SP was significantly improved after benidipine hydrochloride therapy. Levels of urinary albumin excretion or creatinine clearance (Ccr) in SHR-SP were also improved after the therapy. Glomerular sclerosis index was slightly improved in SHR-SP treated with benidipine hydrochloride according to light microscopic analysis. It appears that hypertension may influence the renal intrinsic GSH-Px activity, albuminuria, and Ccr in SHR-SP. Thus it is indicated that control of blood pressure may improve the GSH-Px activity in SHR-SP. *J. Clin. Lab. Anal.* 11:158–162, 1997. © 1997 Wiley-Liss, Inc.

Key words: renal AOE activity; benidipine hydrochloride; SHR-SP

INTRODUCTION

Essential hypertension, which causes renal, cardiovascular, and/or cerebral complications, is considered the so-called national disease of Japan, since the number of patients with this disease has been increasing (> 20% of the Japanese population). Stroke-prone spontaneous hypertension rats (SHR-SP) are used for determining the pathogenesis and developing new drugs for human essential hypertension. Reactive oxygen species (ROS) has been proposed as a key intermediate factor in the progression of renal diseases (1, 2). Malinski et al. (3) reported that cultured endothelial cells from stroke-prone spontaneous hypertensive rats (SHR-SP) produce less nitric oxide (NO) than the same cells from Wistar Kyoto rats (WKY), the normotensive reference strain. Grunfeld et al. (4) also showed that excess superoxide radical (O_2^-), by scavenging endothelial nitric oxide, could contribute to increased vascular smooth muscle contraction and hence elevated total peripheral resistance of these rats. Among several ROS defense systems, superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase are the most important antioxidant enzymes (AOEs). However, details of the defense mechanisms, such as the role of renal intrinsic AOE, remain unclear. Recently, the authors reported that hypertension may influence renal AOE activities and the control of blood pressure by an angiotensin converting enzyme (ACE) inhibitor

may improve these activities, especially GSH-Px, in SHR-SP (5). Several reports indicate that nifedipine, verapamil, and diltiazem exert antioxidative effects on membrane lipids (6). It was suggested that the antiatherosclerotic effects of calcium channel blockers might be related to their antiperoxidative activity. Benidipine hydrochloride is a potent Ca channel blocker with long-lasting antihypertensive and antianginal activities (7). The authors reported that the administration of benidipine hydrochloride may decrease the expansion of Bowman's area and glomerular tuft area and reduce the excretion of urinary protein in the puromycin aminonucleoside induced nephrotic syndrome in rats (8). It appeared that benidipine hydrochloride might have preventive effects on glomerular injury. However, direct antioxidant activity of benidipine hydrochloride has not yet been demonstrated.

The purpose of this study was to determine the effects of benidipine hydrochloride on the activity of renal AOE in SHR-SP.

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MATERIALS AND METHODS

Experimental Design

Twenty-seven male SHR-SP and 10 normotensive Wistar Kyoto (WKY) rats (Charles River, Japan), weighing 250–280 g and 10 weeks of age, were used in the present study. SHR-SP were treated with 640 mg/kg of benidipine hydrochloride (SHR+benidipine therapy group) in their chow for 20 weeks. Benidipine hydrochloride was kindly provided by Kyowa Hakko Kogyo Co. (Tokyo, Japan). Other SHR were treated with triple therapy (80 mg/l hydralazine, 25 mg/l hydrochlorothiazide, and 1.4 mg/l reserpine in drinking water) (SHR+triple therapy group). The dosages of antihypertensive drugs were chosen after preliminary studies (8). All these groups were caged and fed a regular rat chow containing 20% protein. Measurement of body weight and systolic blood pressure (SBP) was performed every 4 weeks. Urinary albumin excretion for 24 hr in metabolic cages was measured at sacrifice.

Measurement of Blood Pressure, Kidney Weight, Ccr and NAG Activity, and Urinalysis

Systolic blood pressure (SBP) was measured by indirect tail cuff plethysmography in the prewarmed conscious state. Kidney weight was calculated. Urinary albumin excretion was measured by ELISA. Levels of serum creatinine (s-Cr) were measured by Jaffe's method (Eiken Chemical Co., Tokyo, Japan) and then creatinine clearance (Ccr) was determined.

Measurement of Renal Antioxidant Enzyme (AOE) Activity

Kidneys were perfused with 0.9% NaCl solution and removed. Kidneys were homogenized by Sonifier® (Sonifier cell disruptor 200, Branson) and centrifuged at 20Kg for 30 min at 4°C. The supernatant was used for measurement of AOE activity (9). The protein in the pellet was measured by the BIO-RAD method (Bio-Rad Laboratories, CA). The activity of SOD in each sample was measured by a modification of the Oyanagi's method as described previously (10). In brief, 0.1 ml of supernatant with 0.5 ml of distilled water for total SOD measurement or 0.5 ml of 0.2 mM KCN for Mn-SOD measurement was added to a reaction mixture containing 0.1 ml of 3 mM sulfanilic acid. Then 25% acetic acid (Wako Pure Chemical, Osaka, Japan) solution was added and the mixture was left at room temperature for 30 min. The activity of SOD was measured at 550 nm in a DU-64 Spectrophotometer (Beckman Instrument). The activity obtained by this method was defined as total or Mn-SOD activity. The amounts of SOD required to inhibit the rate of reduction by 50% was defined as 1 unit of activity. The Cu/Zn-SOD activity was obtained by subtracting Mn-SOD from the total SOD. The activity was expressed as U/mg protein.

The activity of GSH-Px in each sample was detected by a

modification of Flohe's method (11). In brief, the reaction mixture consisting of 0.1 ml of supernatant, 0.5 ml of 0.1 M phosphate buffer (pH 7.0), 0.1 µl of glutathione reductase (GSSH) (0.24 U), and exactly 0.1 ml of 10 mM GSH (Boehringer Mannheim, Mannheim, Germany) was preincubated in a semimicro cuvette kept at 37°C for 10 min. Thereafter, 0.1 ml of NADPH solution was added, and the hydroperoxide-independent consumption of NADPH was monitored for 3 min. The overall reaction was started by adding 0.1 ml of prewarmed hydrogen peroxide solution and the decrease in absorption at 340 nm was measured for 5 min. The nonenzymatic reaction rate was assessed by replacing the enzyme sample by buffer. The activities (A) of GSH-Px were obtained according to Eq: $A = 0.868 (\Delta \text{NADPH}) / [\text{GSH}_0] (\text{Vi}/\text{Vs})$. ΔNADPH was the decrease in NADPH concentration 1 min after the reaction started. $[\text{GSH}_0]$ was the initial GSH level, and Vi and Vs were the volumes of the incubation mixture and the supernatant sample. The GSH-Px activity was expressed as mMol NADPH/min/mg/protein.

The activity of catalase in each sample was measured by a modification of Aebi's method (12). In a 1.6 ml quartz cuvette, 50 µl of the sample was added to 700 µl of a mixture containing 7.7 mM H₂O₂ in 10 mM phosphate buffer (pH 7.0). Changes in the absorbance at 240 nm were measured. The rate constant of a first-order reaction (K) was used as the unit in the equation: $K = (1/\Delta t) \times \ln(A_1/A_2)$, where Δt is the measured interval (sec.), and A₁ and A₂ are the absorbance at the initial and final measurement points, respectively.

Histological and Statistical Analyses

A portion of the right kidney was fixed in buffered formalin (pH 7.4) for light microscopic examination. Renal sections (3 µm) were stained with periodic-acid-Schiff (PAS). Fifty cortical glomeruli from each specimen were examined. Fifty glomeruli for each rat were used for determining the glomerular sclerosis index. Glomerular sclerosis index was obtained by scoring each glomerulus from 0–4 as described by Raiji et al. (13).

Statistical analysis was performed by one-way analysis of variance. *P* values of < 0.05 was regarded as significant.

RESULTS

Clinical Parameters

Levels of systolic blood pressure (SBP), urinary albumin, and Ccr are shown in Table 1. The ratio of kidney to body weight is also shown in Table 1. Levels of SBP in the SHR-SP+benidipine, SHR-SP+triple, or WKY group were significantly lower than those in the untreated SHR-SP group (*P* < 0.01). There were no significant changes in the ratio of kidney to body weight among these treated groups and the untreated SHR-SP group. Levels of urinary albumin in the SHR-SP+benidipine group were significantly lower than those

TABLE 1. Laboratory Data

Group		Blood pressure mmHg	KW/BW %	Urinary albumin mg/day	Ccr ml/min
WKY	n = 10	141.9 ± 1.02**	0.618 ± 0.030	5.72 ± 1.731**	1.018 ± 0.088**
SHR-SP	n = 7	221.1 ± 4.77	0.638 ± 0.016	44.01 ± 13.056	0.416 ± 0.085
SHR-SP+benidipine	n = 10	176.9 ± 3.35**	0.704 ± 0.022	9.07 ± 6.427*	0.690 ± 0.088*
SHR-SP+triple	n = 10	142.0 ± 1.35**	0.689 ± 0.028	28.94 ± 10.75	0.663 ± 0.090

(mean±SE).

P* < 0.05 vs. SHR-SP.*P* < 0.01 vs. SHR-SP.

in the untreated SHR-SP group (*P* < 0.01). Marked decrease of SBP was not observed in the SHR-SP+benidipine group. However, the amounts of urinary albumin excretion in the SHR-SP+benidipine group were lower than those in the SHR-SP+triple group. Levels of Ccr in the untreated SHR-SP group were significantly lower than those in the WKY group (*P* < 0.01). The levels of Ccr in the SHR-SP+benidipine group were significantly increased compared with those in the untreated SHR-SP group (*P* < 0.05).

Renal Cortical and Medullary Antioxidant Enzyme (AOE) Activities

Activity of Cu/Zn-SOD, Mn-SOD, GSH-Px or catalase in renal cortical and medullary tissues is summarized in Tables 2 and 3. There were no significant changes in the activity of Cu/Zn-SOD, Mn-SOD, or catalase in the renal cortical tissues among the treated groups and untreated SHR-SP group. Renal cortical GSH-Px activity in the untreated SHR-SP group was significantly lower than that in the SKY group (*P* < 0.05). The renal cortical GSH-Px activity in the SHR-SP+benidipine or SHR-SP+triple group was significantly higher than that in the untreated SHR-SP group (*P* < 0.01, *P* < 0.05).

There were no significant changes in the activity of Cu/Zn-SOD, Mn-SOD or catalase in the renal medullary tissues among these treated groups and the untreated SHR-SP group. The renal medullary GSH-Px activity in the untreated SHR-SP group was slightly lower than that in the WKY group. The renal medullary GSH-Px activity in the SHR-SP+benidipine

group was significantly increased compared with that in the untreated SHR-SP group (*P* < 0.05).

Renal Histopathological Findings

No histopathological findings in renal tissues were observed in the WKY group. Mild thickening of the extraglomerular vessels was observed in the untreated SHR-SP group. There were no significant changes in the thickening of extraglomerular vessels among the untreated SHR+SP, SHR+benidipine, and SHR+triple groups. The glomerular sclerosis index in the SHR-SP+benidipine of SHR+triple group (74.5 ± 38.4, 68.8 ± 52.3, respectively) was significantly lower than that in the untreated SHR-SP (185.1 ± 60.3) (*P* < 0.01). However, there were no significant changes in the glomerular sclerosis index between the SHR-SP+benidipine and the SHR-SP+triple groups.

DISCUSSION

Recently, the authors reported that reactive oxygen species (ROS) has been proposed as one of the key factors in the progression of renal diseases such as chronic glomerulonephritides, diabetic nephropathy, and hypertensive nephrosclerosis (5, 9, 14). The authors also indicated the increase of SOD-like activity and lipid peroxide in sera from patients with essential hypertension (unpublished data). ROS scavengers, i.e., the antioxidant defense mechanism, are present in glomerular and tubular epithelial cells and may play a major role in cellular protection in the kidney. Recent reports indi-

TABLE 2. Renal Cortical Antioxidant Enzyme Activity

Group		Cu/Zn-SOD U/mg.pro	Mn-SOD U/mg.pro	GSH-PX mMol.NADPH/ min/mg.pro	Catalase K/mg.pro
WKY	n = 10	24.89 ± 1.331	0.071 ± 0.021	61.54 ± 2.987**	1.061 ± 0.087*
SHR-SP	n = 7	19.87 ± 1.206***	0.145 ± 0.056	44.90 ± 2.997	1.756 ± 0.295
SHR-SP+benidipine	n = 10	20.72 ± 1.594***	0.237 ± 0.051****	63.08 ± 3.887**	1.864 ± 0.238****
SHR-SP+triple	n = 10	23.70 ± 1.495	0.141 ± 0.040	57.07 ± 2.169*	1.750 ± 0.193***

(mean±SE).

P* < 0.05 vs. SHR-SP.*P* < 0.01 vs. SHR-SP.****P* < 0.05 vs. WKY.*****P* < 0.01 vs. WKY.

TABLE 3. Renal Medullary Antioxidant Enzyme Activity

Group		Cu/Zn-SOD U/mg.pro	Mn-SOD U/mg.pro	GSH-PX	Catalase K/mg.por
				mMol.NAPDH/Catalase min/mg.pro	
WKY	n = 10	45.334 ± 4.261	1.430 ± 0.320	29.05 ± 1.916	0.631 ± 0.105
SHR-SP	n = 7	18.51 ± 3.004***	3.498 ± 1.019	25.32 ± 0.685	0.343 ± 0.045**
SHR-SP+benidipine	n = 10	17.41 ± 2.919***	4.409 ± 1.209**	35.70 ± 4.559*	0.444 ± 0.067
SHR-SP+triple	n = 10	18.89 ± 2.666***	4.414 ± 0.818**	33.54 ± 1.782	0.486 ± 0.037

(mean±SE).

P* < 0.05 vs. SHR-SP.*P* < 0.05 vs WKY.****P* < 0.01 vs WKY.

cate that structurally unrelated calcium blockers, including nifedipine, verapamil, and diltiazem, may possess lipid antioxidative properties (15, 16). The reports indicate that calcium blockers behave as weak antioxidants in membrane systems. Janero et al. (17) reported that the antioxidant property of nifedipine appeared to be ineffective compared to that of nisoldipine, felodipine, and nicardipine. Active dihydropyridine derivatives were thought to act as lipophilic chain-breaking antioxidants (17). Benidipine hydrochloride is a dihydropyridine derivative that has a strong and long-lasting antihypertensive effect as well as an antianginal effect (6). Recently, Yamakado (18) reported that treatment with benidipine hydrochloride increases the SOD-like activity and decreases the serum lipid peroxidase (LPO) activity and urinary albumin excretion in Japanese patients with essential hypertension.

The present study showed a decrease of renal cortical or medullary intrinsic GSH-Px activity in unrelated SHR-SP, although severe thickening of intrarenal vascular vessels was not observed. Renal cortical or medullary GSH-Px activity in untreated SHR-SP was lower than those in the WKY group. Improvement of renal GSH-Px activity was achieved by control of hypertension using antihypertensive drugs, i.e., benidipine hydrochloride or triple therapy (hydralazine, hydrochlorothiazide, and reserpine). However, it is unknown why the improvement of renal AOE activities after the control of hypertension mainly occurred in GSH-Px activity. Levels of urinary albumin excretion or Ccr were significantly improved after treatment with benidipine hydrochloride therapy. The levels of renal GSH-Px activity, urinary albuminuria, and Ccr in the SHR-SP+benidipine group were improved compared with those in the SHR-SP+triple group, but there was no significance. The glomerular sclerosis index was also improved after such treatment. Thus it is premature to conclude that these findings are due to the direct antioxidant activity of benidipine hydrochloride in this study. It appears that hypertension may influence renal AOE activities and the control of blood pressure may improve GSH-Px activity in stroke-prone spontaneous hypertensive rats (SHR-SP). At present, the authors are measuring the levels of AOE activities in serum and urinary samples in patients with essential

hypertension (in preparation). It is necessary to determine if treatment with benidipine hydrochloride might have preventive effects on renal injury in patients with essential hypertension.

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REFERENCES

- Stratta P, Canvese C, Dogliani M, Mazzucco G, Monga G, Vercellone A: The role of free radicals in the progression of renal disease. *Kidney Int* 17(Suppl.):33–37, 1991.
- Shav SV: Role of reactive oxygen metabolites in experimental glomerular disease. *Kidney Int* 35:1093–1106, 1989.
- Malinski T, Kapturczak M, Dayharsh J, Bohr DF: Nitric oxide synthase activity in genetic hypertension. *Biochem Biophys Res Commun* 194:654–658, 1993.
- Grunfeld S, Hamilton CA, Mesaros S, McClain SW, Dominiczak AF, Bohr DF, Malinski T: Role of superoxide in the depressed nitric oxide production by the endothelium of genetically hypertensive rats. *Hypertension* 26(part 1):854–857, 1995.
- Shou I, Wang LN, Suzuki S, Fukui M, Tomino Y: Effects of antihypertensive drugs on antioxidant enzyme activities in stroke-prone spontaneous hypertensive rats (SHR-SP). *AJMS* (submitted).
- Henry PD: Antioxidative activities of calcium antagonists and atherosclerosis. *J Cardiovasc Pharmacol* 18(Suppl 1):S6–S10, 1991.
- Fuji Y, Suzuki H, Katsuama S, Nakajima S, Saruta T: Hormonal and renal responses to oral once-daily calcium entry blocker in normotensive and hypertensive persons. *J Cardiovasc Pharmacol* 11:438–443, 1988.
- Tang Z, Fukui M, Mizokuchi M, Shirato I, Tomino Y: Effect of benidipine on decreasing glomerular expansion in the experimental nephrotic syndrome. *Nephron* 70:369, 1995.
- Wang LN, Fukui M, Shou I, Yaguchi Y, Funabiki K, Horikoshi S, Shirato I, Tomino Y: Detection of antioxidant enzyme activities in renal tissues of early stage IgA nephropathy in ddY mice. *JCLA* 10:394–398, 1996.
- Oyanagi Y: Reevaluation of assay methods and establishment of kit for superoxide dismutase activity. *Analyt Biochem* 142:290–296, 1984.
- Flohe L, Gunzler WA: Assays of glutathione peroxidase. In *Method in Enzymology*. Vol 105: Oxygen Radicals in Biological Systems. L. Packer, ed. Academic Press, London, 1984, p 114–121.
- Aebi HE, Catalase. In *Methods in Enzyme Analysis*, 3rd ed, vol III. HU Bernmeyer, ed. Verlag Chemie, Weinheim, 1982, p 273–282.
- Raiji L, Azar S, Keane W: Mesangial immune injury, hypertension, and progressive glomerular damage in Dahl rats. *Kidney Int* 26:137–143, 1984.

14. Tang Z, Shou I, Wang LN, Fukui M, Tomino Y: Effects of antihypertensive drugs or glycemic control on antioxidant enzyme activities in spontaneously hypertensive rats (SHR). *Nephron* (in press).
15. Robak J, Duniece Z: Membrane activity, antioxidant, antiaggregatory and antihemolytic properties of four calcium channel blockers. *Pharmacol Res Commun* 12:1107–1117, 1986.
16. Shridi F, Robak J: The influence of calcium channel blockers on superoxide anions. *Pharmacol Res Commun* 20:13–21, 1988.
17. Jenero DR, Burghard BI: Antioxidant effects of dihydropyridine calcium antagonists. *Biochem Pharmacol* 38:4344–4348, 1989.
18. Yamakado M: Clinical study on antiatherogenic properties of benidipine hydrochloride in patients with essential hypertension. *Therapeutic Res* 17:2733–2738, 1994 (in Japanese).