High Levels of Oxidative Stress Exist in the Brain than Serum or Kidneys in Stroke-Prone Spontaneously Hypertensive Rats at Ten Weeks of Age

Akihiro Michihara,* Mai Shimatani, Makoto Anraku, Hisao Tomida, and Kenji Akasaki

Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University; Fukuyama, Hiroshima 729–0292, Japan.
Received December 1, 2009; accepted December 18, 2009; published online December 22, 2009

In the present study, we examined levels of oxidative stress in the serum, brain and kidneys of normotensive Wistar Kyoto rats (WKY) and stroke prone spontaneously hypertensive rats (SHRSP) at 10 weeks of age. Levels of advanced oxidation protein products (AOPP), oxidized albumin and oxidized proteins, markers of oxidative stress, were significantly decreased in serum among SHRSP as compared with WKY. Levels of oxidized proteins determined by immunoblotting were significantly increased in the brain, but not kidney, of SHRSP. The mRNA level of super oxide dismutase (SOD) determined by real time polymerase chain reaction (PCR) and the protein level of catalase assessed by immunoblotting were significantly increased in the brain of SHRSP. From these results, it was suggested that levels of oxidative stress were higher in the brain than serum or kidneys of SHRSP at 10 weeks of age, but are not caused by decreases in the expression of SOD and catalase.

Key words oxidative stress; stroke prone spontaneously hypertensive rat; serum; brain; kidney

The stroke prone spontaneously hypertensive rat (SHRSP) is a useful model of human malignant hypertension, cerebral hemorrhage, and more serious hypertensive renal injury.[1—4] The serum cholesterol level of this rat is lower than that of the normotensive Wistar Kyoto rat (WKY).[5] Human epidemiological studies have indicated a negative association between serum cholesterol levels and the incidence of cerebral hemorrhage.[6] Recent experimental findings suggest that oxidative stress in the vasculature and kidneys contributes to hypertension, a major risk factor for cardiovascular disease.[7—9] Free radicals were also found to contribute to the maintenance of hypertension in SHRSP at 16 weeks of age.[10] However, in SHRSP exposed to oxidative stress at an early stage, the subsequent increase in DNA damage was not a secondary effect of hypertension.[11] Therefore, not only hypertension and lower serum cholesterol levels but also oxidative stress was responsible for the cerebral hemorrhage in SHRSP.

It has been suggested that 8-hydroxy-2′-deoxyguanosine (8-OHdG) is a product of oxidative DNA damage following specific enzymatic cleavage after 8-hydroxylation of the guanine base.[12] The amount of urinary 8-OHdG, a biomarker of systemic oxidative stress *in vivo*, in SHRSP did not differ from that in WKY at 6 weeks of age, but increased beyond control values after the development of severe hypertension at 14—17 weeks of age.[11] Other groups reported that total superoxide dismutase (SOD) activity in the rostral ventrolateral medulla was decreased in SHRSP compared with WKY.[13] Namely, the increase in reactive oxygen species (ROS) in the brain of SHRSP at 16 weeks of age is caused by a decrease of SOD. It was reported that SHRSP exhibits salt-sensitivity, increased vascular release of superoxide, and decreased total plasma antioxidative capacity.[14] Amounts of oxidized proteins were also remarkably enhanced in the aorta, heart and kidney from SHRSP as compared with WKY.[15] Thus hypertension, cerebral hemorrhage, renal damage, and the decrease in plasma antioxidative capacity in SHRSP may be caused by oxidative stress. However, although oxidative stress may be involved in brain damage in SHRSP, it is not clear whether the effect is intrinsic (a direct result of oxidative stress in the brain itself) or extrinsic (a secondary effect of oxidative stress in serum or the kidneys), as hydrogen peroxide (H_2O_2), a type of ROS, passes through the plasma membrane in tissues and cells. Thus, we examined oxidative stress in the serum, brain and kidneys of WKY and SHRSP at 10 weeks of age (cerebral hemorrhage does not occur at this age in SHRSP). The results indicated higher level of oxidative stress in the brain than serum or kidneys of SHRSP of 10 weeks of age.

MATERIALS AND METHODS

Materials The ECL Western blotting detection kit was obtained from Amersham Pharmacia Biotech (Tokyo, Japan), Cholesterol E-test Wako kit from Wako (Osaka, Japan), BioMasher® tissue homogenizer from Nippi, Inc. (Tokyo, Japan), Quick Gene RNA tissue kit S II from Fujifilm (Tokyo, Japan), and SYBR Ex Script reverse transcription-polymerase chain reaction (RT-PCR) kit from TaKaRa (Tokyo, Japan). Sheep anti-catalase (bovine) immunoglobulin G (IgG) was purchased from The Binding Site (Birmingham, U.K.) and donkey anti-sheep IgG conjugated to horseradish peroxidase was from Cortex Biochem, Inc. (San Leandro, CA, U.S.A.). The oxidized protein detection kit was obtained from Serologicals Corporation, Norcross, U.S.A. All other chemicals were of reagent grade and purchased commercially.

Animals Male (9—10 weeks old) SHRSP/Izm and WKY/Izm were obtained from the Disease Model Co-operative Research Association, Japan.

Cholesterol Levels in Serum Cholesterol concentrations in serum were determined using the Cholesterol E-test Wako kit.

Protein Assay Protein levels were measured by the method of Lowry *et al.* using bovine serum albumin (BSA) as the standard.[16]

Measurement of a Dvanced Oxidation Protein Products Advanced oxidation protein products (AOPP) were measured with oxi-rat serum albumin using a semi-automated method.[17]

* To whom correspondence should be addressed. e-mail: mitihara@fupharm.fukuyama-u.ac.jp

Measurement of Oxidized Albumin High-performance liquid chromatography (HPLC) was used to analyze serum albumin as described previously and the oxidized albumin ratio was estimated.[18] Samples obtained from WKY and SHRSP were immediately frozen and stored at −80 °C until used for analysis. Then 5 μl aliquots of serum were analyzed on a Shodex Asahipak ES-502N column (Showa Denko Co., Ltd., Tokyo, Japan). From the HPLC profile, the amount of each albumin fraction (rat mercaptalbumin, f[RMA]; rat nonmercaptalbumin-1, f[RNA-1]; rat nonmercaptalbumin-2, f[RNA-2]) was estimated as the area of the RNA fraction divided by RMA fraction of the serum albumin peak.[19]

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting SDS-PAGE was performed on 10% slab gels according to the method of Laemmli.[20] Proteins on the SDS-slab gel were transferred to a nylon membrane (NEN) by electrophoresis, using a modified version of the procedure of Towbin *et al.*[21] Positive bands were visualized using ECL Western blotting detection kits (Amersham Pharmacia, Amersham, U.K.) that contained a sensitive chemiluminescent substrate for horseradish peroxidase.

Measurement of Oxidized Proteins Oxidized protein in serum and tissues of WKY and SHRSP was detected using an oxidized protein detection kit, as described previously.[15] The Oxyblot kit provides reagents for the sensitive immunodetection of carbonyl groups. Chemiluminescence was detected with an ECL western blot detection kit. Signals of bands were measured using an Intelligent Quantifier (Bio Image).

Real-Time PCR Total RNA (50 μl) in the brain was isolated from the cells using Quick Gene RNA cultured cell kit S (total RNA extract kit) and Gene-810 (Nucleic Acid Isolation System; Fujifilm, Tokyo, Japan). The concentration of total RNA was calculated by a Qubit™ fluorometer (Invitrogen). Two-microgram samples of total RNA from each group of cells were subjected to reverse transcription (RT) using reverse transcriptase in a 50 μl reaction volume. After the RT reaction, the cDNA template was amplified by polymerase chain reaction with a SYBR Ex Script RT-PCR kit. SYBR Green was used for the real-time PCR analysis of tyrosinase. Real-time PCR was performed using an ABI7500 system (Applied Biosystems Japan, Tokyo, Japan). Relative gene expression was quantified using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The primer pairs (GAPDH, F-5′-TGTGTCCGTCGTGGATCTGA-3′ and R-5′-TTGCTGTTGAAGTCGCAGGAG-3′; SOD, F-5′-CAAGTACAGGGATCGGCCAAC-3′ and R-5′-GGTGCATTGGCTTCTGGGTAA-3′) were designed using the primer Select program of TaKaRa. The cDNA products generated by RT-PCR were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining under UV light.

Statistics Statistical analysis was carried out using Student's *t*-test. Data are presented as the mean±S.D.

RESULTS

Oxidative Stress in Serum, Brain and Kidney First, we determined the concentrations of cholesterol and protein in serum at 10 weeks of age. As shown in Fig. 1, concentrations of protein were similar between SHRSP and WKY.

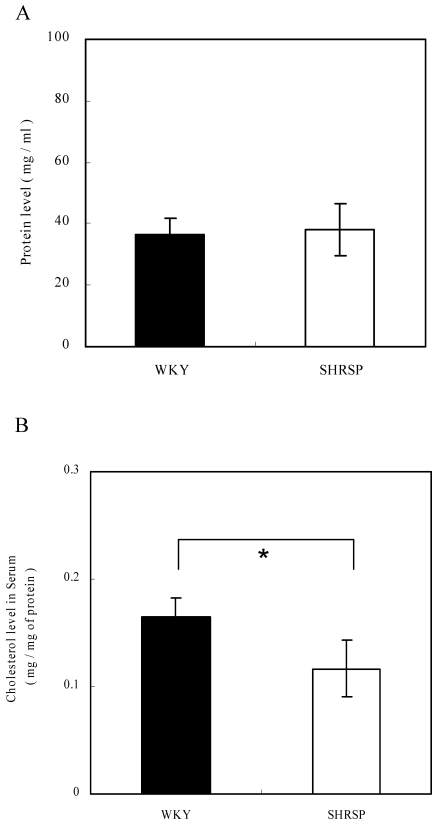

Fig. 1. Protein and Cholesterol Concentrations in the Serum of WKY and SHRSP

Protein (A) and cholesterol (B) levels in the serum of WKY and SHRSP were measured as described in Materials and Methods. Data are means for four identical experiments. Significant differences: $*p<0.05$.

Similar results were reported previously.[2] Also, serum cholesterol levels were significantly lower in SHRSP than WKY.

Next, to examine the effect of oxidative stress in serum at 10 weeks of age, we measured the levels of AOPP, oxidized albumin and oxidized proteins, markers of oxidative stress. As shown in Fig. 2, significant decreases in AOPP, oxidized albumin and oxidized proteins were found in SHRSP as compared with WKY.

Furthermore, to examine the effect of oxidative stress in the brain and kidney, we measured the amount of oxidized protein using immunoblotting. As shown in Figs. 3A, C, the level of oxidized protein in the brain was significantly increased in SHRSP as compared with WKY. However, that in the kidney did not differ significantly, although it was reduced in SHRSP (Figs. 3B, D). These results (Figs. 2, 3) indicated that the marker of oxidative stress in the brain was higher among SHRSP than WKY, but that in the serum and kidney was lower among SHRSP than WKY.

Change of SOD and Catalase Levels in the Brain Major enzymes involved in the decrease in oxidative stress were SOD and catalase. It was reported that the increase in the amount of ROS in the brain of SHRSP at 16 weeks of age was caused by a decrease of SOD.[10] Thus, we examined the change in SOD and catalase (antioxidative enzymes) levels in the brain. As shown in Fig. 4, the level of SOD in the brain was significantly increased in SHRSP compared to WKY, as measured using real-time PCR. The level of catalase in the brain was also significantly increased in SHRSP, as measured

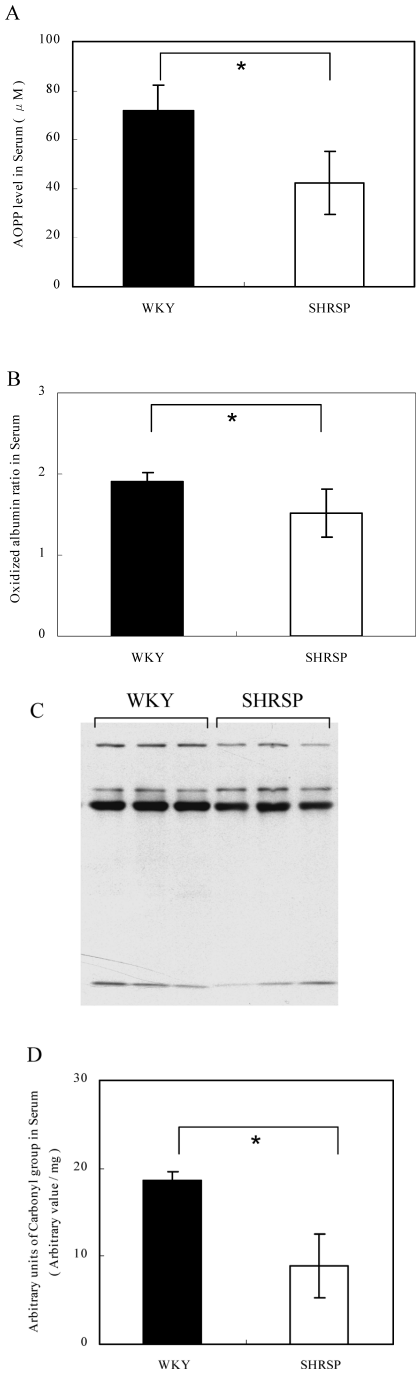

Fig. 2. AOPP, Oxidized Albumin and Oxidized Protein Concentrations in the Serum of WKY and SHRSP

AOPP (A) and oxidized albumin (B) levels in the serum of WKY and SHRSP were measured as described in Materials and Methods. Serum samples (C; 5 μl) of WKY and SHRSP were subjected to immunoblotting as described in Materials and Methods, and the signals in C were measured using an Intelligent Quantifier (D). Data are means for three identical experiments. Significant differences: $*p<0.05$.

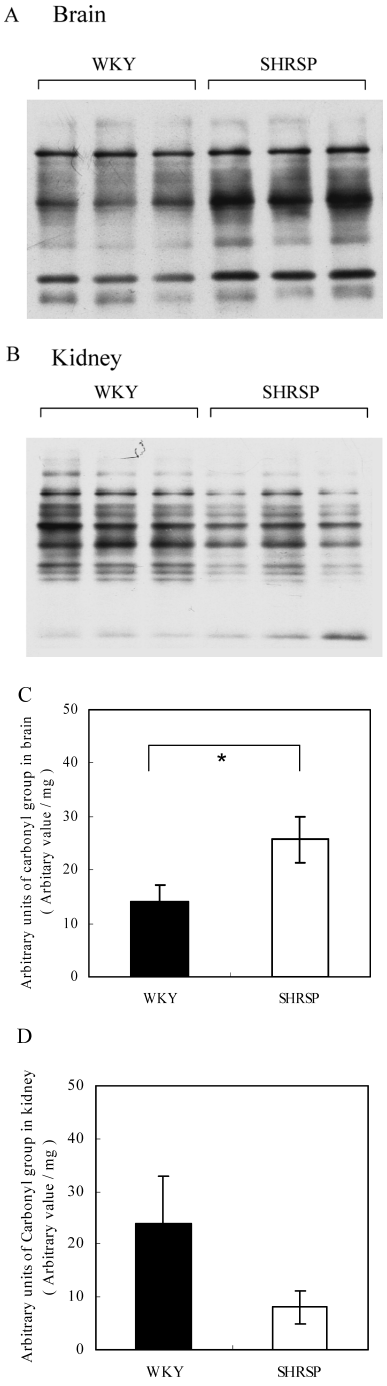

Fig. 3. Oxidized Protein Levels in the Brain and Kidney of WKY and SHRSP

Crude extract (30 μg) in the brain (A) and kidney (B) of WKY and SHRSP was subjected to immunoblotting as described in Materials and Methods, and the signals in A and B were measured using an Intelligent Quantifier (C, D), respectively. Data are means for three identical experiments. Significant differences: $*p<0.05$.

using immunoblotting. Thus, the increased oxidative stress in the brain of SHRSP can not be explained by the decreases in expression of SOD and catalase.

DISCUSSION

The damage to the brain and kidney, and decrease in plasma antioxidant capacity in SHRSP may be caused by oxidative stress.[11,13,14] However, it is not clear whether the oxidative stress in the brain is intrinsic or extrinsic. In the present study, levels of oxidative stress were higher in the brain than serum or kidneys of SHRSP at 10 weeks of age (Figs. 2, 3). Therefore, the oxidative stress in the brain was intrinsic (a direct effect of oxidative stress in the brain itself), not extrinsic (a secondary effect of oxidative stress in serum or the kidneys).

In general, oxidative stress is caused by an imbalance between the production of ROS and the removal of ROS by the

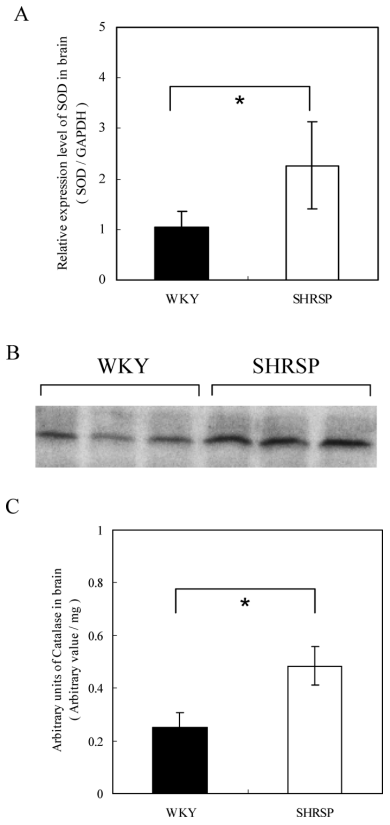

Fig. 4. The mRNA Level of SOD and Protein Level of Catalase in the Brain of WKY and SHRSP

(A) RT-PCR was performed using primer pairs for SOD or GAPDH from total RNA in the brain of WKY and SHRSP as described in Materials and Methods. cDNA products (1 μl) were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining under UV light. After each band (GAPDH or SOD) generated by RT-PCR was recognized as a single band, real-time PCR was performed as described in Materials and Methods. Crude extract (30 μg) in the brain of WKY and SHRSP was subjected to immunoblotting using anti-catalase antibody as described in Materials and Methods (B), and the signals were measured using an Intelligent Quantifier (C). Data are means for three identical experiments. Significant differences: $*p<0.005$.

antioxidant system. Next, we examined whether decreased expression of antioxidative enzymes occurred in the brain of SHRSP. The present findings demonstrated that levels of SOD and catalse in the brain were also significantly increased (Fig. 4). Therefore, the increased oxidative stress in the brain of SHRSP can not be explained by the decrease in expression of these enzymes. From these results, it was suggested that high levels of oxidative stress exist in the brain in young SHRSP (10 weeks of age), but are not caused by decreases in the expression of SOD and catalase.

We conclude that the increased oxidative stress in the brain of SHRSP at 10 weeks of age is intrinsic (high levels of oxidative stress in the brain itself), although the reason for such high levels remains unclear. Further study will be needed to understand the effect of oxidative stress on the brain in SHRSP.

REFERENCES

1) Okamoto K., Yamori Y., Nagaoka A., *Circ. Res.*, **34/35** (Suppl. 1), 143—153 (1974).
2) Hayashi K., Koyama M., Kido H., Egi Y., Kubo Y., Shinyama H., Iwamoto M., Nakamura N., Kagitani Y., *Clin. Exp. Pharmacol. Physiol.*, **24**, 831—840 (1997).
3) Matsunaga M., Komuro T., Yamamoto J., Hara A., Morimoto K., Yamori Y., *Jpn. Heart J.*, **21**, 737—751 (1980).
4) Ogata J., Fujishima M., Tamaki K., Nakatomi Y., Ishitsuka T., Omae T., *Virchows Arch. A Pathol. Anat. Histol.*, **394**, 185—194 (1982).
5) Iritani N., Fukuda E., Nara Y., Yamori Y., *Atherosclerosis*, **28**, 217—222 (1977).
6) Neaton J. D., Blockburn H., Jacobs D., Kullern L., Lee D. J., Sherwin R., Shih J., Wentworth D., *Arch. Int. Med.*, **157**, 1490—1500 (1992).
7) Lacy F., O'Connor D. T., Schmid-Schönbein G. W., *J. Hypertens.*, **16**, 291—303 (1998).
8) Russo C., Olivieri O., Girelli D., Faccini G., Zenari M. L., Lombardi S., Corrocher R., *J. Hypertens.*, **16**, 1267—1271 (1998).
9) Shaughnessy K. S., Boswall I. A., Scanlan A. P., Gottschall-Pass K. T., Sweeney M. I., *Nutr. Res.*, **29**, 130—138 (2009).
10) McIntyre M., Hamilton C. A., Rees D. D., Reid J. L., Dominiczak A. F., *Hypertension*, **30**, 1517—1524 (1997).
11) Negishi H., Ikeda K., Sagara M., Sawamura M., Yamori Y., *Clin. Exp. Pharmacol. Physiol.*, **26**, 482—484 (1999).
12) Loft S., Vistisen K., Sagara M., Tjonneland A., Overvad K., Poulseu E., *Carsinogenesis*, **13**, 2241—2247 (1992).
13) Kishi T., Hirooka Y., Kimura Y., Ito K., Shimokawa H., Takeshita A., *Circulation*, **109**, 2357—2362 (2004).
14) Manning R. D. Jr., Meng S., Tian N., *Acta Physiol. Scand.*, **179**, 243—250 (2003).
15) Tanito M., Nakamura H., Kwon Y. W., Teratani A., Masutani H., Shioji K., Kishimoto C., Ohira A., Horie R., Yodoi J., *Antioxid. Redox. Signal.*, **6**, 89—97 (2004).
16) Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J., *J. Biol. Chem.*, **193**, 265—275 (1951).
17) Witko-Sarsat V., Friedlander M., Capeillère-Blandin C., Nguyen-Khoa T., Nguyen A. T., Zingraff J., Jungers P., Descamps-Latscha B., *Kidney Int.*, **49**, 1304—1313 (1996).
18) Anraku M., Kitamura K., Shinohara A., Adachi M., Suenga A., Maruyama T., Miyanaka K., Miyoshi T., Shiraishi N., Nonoguchi H., Otagiri M., Tomita K., *Kidney Int.*, **66**, 841—848 (2004).
19) Shimada H., Kitamura K., Anraku M., Miyoshi T., Adachi M., Tuyen D. G., Wakamatsu S., Nonoguchi H., Tanaka M., Tomita K., *Hypertens. Res.*, **28**, 987—994 (2005).
20) Laemmli U. K., *Nature* (London), **227**, 680—685 (1970).
21) Towbin H., Staehelin T., Gordon J., *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 4350—4354 (1979).