

# Fluctuation of Lipid Peroxides and Related Enzyme Activities at Time of Stroke in Stroke-Prone Spontaneously Hypertensive Rats

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**SUMMARY** The levels of lipid peroxides, determined as thiobarbituric acid reactive substances (TBARS), and the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), were examined in the blood from stroke-prone spontaneously hypertensive rats (SHRSP), with and without cerebral lesions, and normotensive Wistar Kyoto (WK) rats. The levels of TBARS in the blood from healthy SHRSP were not significantly different from those of WK rats, while the values of SHRSP (male) with stroke were more than twice as high as those of healthy SHRSP. The activities of SOD and GSH-Px in stroke SHRSP were also statistically different from those of healthy SHRSP.

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EVIDENCE has accumulated that lipid peroxides levels in tissues increase with age and may cause cell death and/or damage with a consequent change of cell permeability.<sup>1,2</sup> Thus, it may be possible that lipid peroxides could be a primary etiologic factor in the genesis of stroke.

Lin and Horning<sup>3</sup> reported that  $\alpha$ -tocopherol concentration and the linoleic acid/oleic acid ratio in the plasma of patients with stroke were lower than those of adults with no history of atherosclerotic vascular disease. Similarly, Kibata et al.<sup>4</sup> observed that the levels of free fatty acids and thiobarbituric acid reactive substances (TBARS) (malonaldehyde and its precursor, lipid peroxides) in the serum of patients with stroke were high and that a close correlation ( $r = -0.56$ ,  $P < 0.01$ ) existed between  $\alpha$ -tocopherol concentration and TBARS in the serum.

We have reported in previous papers<sup>5,6</sup> that the levels of TBARS in the brain and serum of stroke-prone spontaneously hypertensive rats (SHRSP) at 7 months of age were significantly higher than those of spontaneously hypertensive rats (SHR) and Wistar Kyoto (WK) rats of the same age. There was a positive correlation between the values of serum TBARS and blood pressure ( $r = 0.63$ ,  $P < 0.05$ ) at the time close to the onset of stroke. The present study was designed to investigate the fluctuation of lipid peroxides and related enzyme activities in the blood from SHRSP with and without cerebral hemorrhage, and from WK rats as control.

## Method

SHRSP and WK rats were kindly provided by Dr. K. Okamoto of Kinki University and raised in our laboratory. The rats were kept in a room with a con-

stant temperature ( $22 \pm 1^\circ$ ) and humidity (50 ~ 55%), fed a pellet diet (CMF from Oriental Yeast Co.) and given tap water *ad libitum*. Most of SHRSP developed signs of stroke such as repetitive lifting of a limb, convulsions or paralysis of paws, ears and shoulders about 7 months after birth. The cerebral lesions; cerebral hemorrhage and/or softening in the anteromedial cortex, the occipital cortex or the basal ganglia, were confirmed by autopsy. An intraperitoneal injection of 1% Evans blue shortly before the sacrifice made possible visualization of the lesion with increased permeability. In NaCl loading experiments, tap water was replaced by drinking water containing 1% NaCl. Male SHRSP 10 weeks of age were used for this study. Most of the rats developed signs of stroke within 2 to 3 weeks after the start of the experiment. Diagnosis of stroke was made by the susceptibilities of the animals to stimuli such as sound and soft touch. Animals with stroke were also recognized by decrease of body weight and increase in volumes of fluid consumed and urine excreted.

Blood samples were taken in a heparinized syringe from the abdominal or tail aorta. Whole blood (0.1 ml) was added to 1.9 ml of isotonic NaCl solution and then centrifuged for 3 min at 3000 rpm. A portion (0.5 ml) of the supernatant was treated with 1/12N  $H_2SO_4$  (4.0 ml) and 10% phosphotungstic acid (0.5 ml) to precipitate protein. The precipitates were then treated with 1 mM EDTA (1.0 ml) and 3% sodium dodecyl sulfate (0.5 ml) to dissociate lipids reactive to TISA.<sup>7</sup> TBARS were measured by the fluorometric method (Ex 515 nm, Em 555 nm) of Yagi.<sup>8</sup> The activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were examined using erythrocytes which had been washed with isotonic NaCl solution and then hemolyzed by suspension in distilled water at  $0^\circ$  for 30 min. GSH-Px activity was determined with the hemolyzate in the presence of excess glutathione and  $H_2O_2$  according to Hafeman et al.<sup>9</sup> SOD activity was assayed according to the method of Elstner et al.;<sup>10</sup> the supernatant which was obtained by adding the mixture of  $CHCl_3$  and EtOH to the erythrocyte

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TABLE TBA Reactive Substances (TBARS) GSH-Px and SOD Activities in Blood from SHRSP and WK Rats

Rats	Sex	TBA as malonaldehyde n moles/ml blood	GSH-Px Units $\times 10^{-3}$ /ml blood	SOD Units/ml blood
SHRSP	Healthy	Male 11.40 $\pm$ 0.35* (29)	12.38 $\pm$ 0.31 (29)	765.45 $\pm$ 39.97 (29)
		Female 12.46 $\pm$ 0.36 (18)	11.40 $\pm$ 0.40 (18)	958.41 $\pm$ 79.71† (17)
	Stroke	Male 25.05 $\pm$ 1.67† (9)	7.91 $\pm$ 0.54† (9)	595.51 $\pm$ 40.81† (8)
WK rats	Healthy	Male 12.86 $\pm$ 0.93 (7)	12.10 $\pm$ 1.15 (4)	608.60 $\pm$ 78.15 (5)
	Female	12.62 $\pm$ 1.20 (11)	11.38 $\pm$ 0.41 (18)	858.28 $\pm$ 45.25 (18)

\*Mean  $\pm$  SE; †(p < 0.001); ‡(p < 0.05): Significantly different from the mean of healthy SHRSP. Figures in parentheses indicate number of rats (3~7 months in age).

hemolyzate followed by the centrifugation and dialysis for 12 hr was incubated at 25° with xanthine, xanthine oxidase and hydroxyl ammonium chloride. Nitrite, as the oxidation product of hydroxyl ammonium chloride, was determined by adding  $\alpha$ -naphthylamine.

### Results

The table shows the levels of TBARS, the activities of GSH-Px and SOD in the blood from SHRSP and WK rats of 3 to 7 months in age. The values of TBARS, which were determined as the amount of malonaldehyde in the blood from SHRSP with stroke (male, n = 9), were significantly higher ( $P < 0.001$ ) than those from healthy SHRSP (male, n = 29). The values from healthy SHRSP, both male (n = 29) and female (n = 18), however, were not significantly different from those found in WK rats, both male (n = 7) and female (n = 11). The GSH-Px activities in the blood from SHRSP with stroke (male, n = 9) were significantly lower ( $P < 0.001$ ) than that of the healthy groups (both male and female), and there were no significant differences between the mean values from healthy SHRSP and WK rats. The SOD activities of SHRSP with stroke (male, n = 8) were also significantly lower ( $P < 0.05$ ) than those of healthy SHRSP (male, n = 29) or WK rats (male, n = 5). The SOD activities of female rats, both SHRSP and WK rats were higher than those of male rats and may

be related to the lower incidence of stroke in females.<sup>11</sup> While there was a significant correlation between the values of TBARS and GSH-Px activity in 23 samples of male SHRSP at 7 months, of which 8 samples were from rats with stroke ( $r = -0.73$ ,  $P < 0.02$ , fig. 1), no correlation was observed between the values of TBARS and SOD activity. Figure 2 indicates the change of TBARS, GSH-Px and SOD activities in the blood from SHRSP given 1% NaCl as drinking fluid *ad libitum*. These animals began to show signs of stroke on the 11th day after beginning NaCl loading. TBARS showed a tendency to increase before the time of stroke. A distinct and remarkable increase of TBARS occurred immediately after the stroke. GSH-Px and SOD activities likewise decreased significantly at the time of the stroke as shown in figure 2.

### Discussion

Lipid peroxides in cells have been implicated as one of the principal factors causing age-related damage to cells.<sup>1, 2, 12</sup> Peroxides are produced from unsaturated fatty acids by superoxide anion radical,  $O_2^{\cdot-}$ , and destroyed by GSH-Px.<sup>13, 14</sup> A superoxide anion radical is generated by several enzyme systems<sup>15, 16</sup> and deactivated by SOD.<sup>17, 18</sup> Both SOD and GSH-Px play important roles in the protection of various cells from damage promoted by the intracellular process.<sup>19, 20</sup>

The present investigation has demonstrated that the levels of TBARS, presumably lipid peroxides and the resultant (malonyldialdehyde) malonaldehyde, and the activities of both enzymes, SOD and GSH-Px, in the blood from SHRSP with stroke were significantly different from those of healthy SHRSP. The levels of TBARS do increase slightly in the organs of aging animals.<sup>21, 22</sup> In our experiment, however, the values of TBARS in SHRSP with cerebral hemorrhage were markedly elevated compared to animals of the same age without hemorrhage. NaCl loading was performed at a time to induce stroke in SHRSP shortly, and hence to avoid, as much as possible, age-related increases in TBARS. It has been demonstrated that the levels of TBARS increased gradually before the time of a stroke and then increased dramatically soon after the stroke. A rapid increase of TBARS is largely an associative and not a causative change in cerebral hemorrhage and infarction. Along with the elevation of TBARS in the blood, the activities of both GSH-Px and SOD concomitantly decreased significantly with

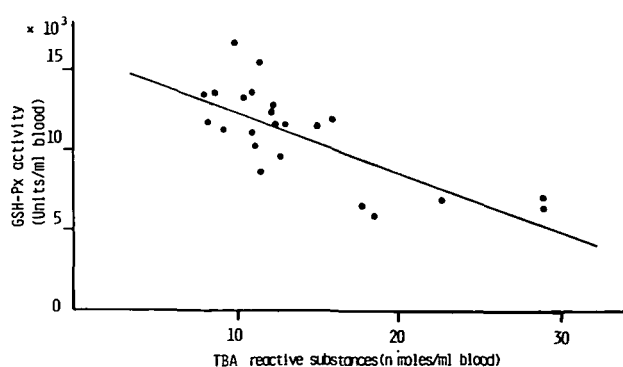


FIG. 1. Relationship between TBA reactive substances (TBARS) levels and GSH-Px activity in the blood from male SHRSP 7 months old. A common regression line ( $Y = -0.352X + 15.310 \times 10^3$ ,  $r = -0.73$ ,  $P < 0.02$ ) was obtained.

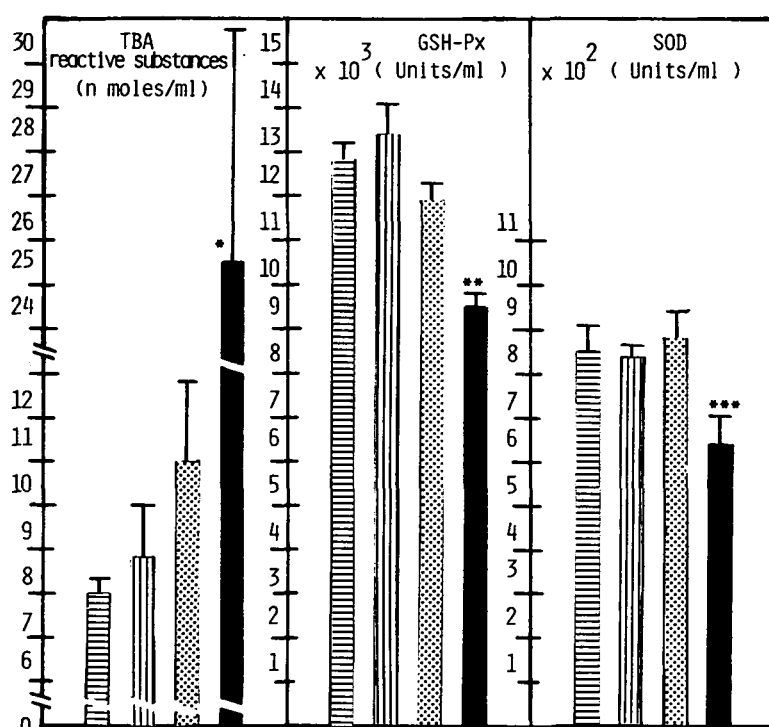


FIG. 2. Fluctuation of TBARS levels, GSH-Px and SOD activities in the blood from each 4 male SHRSP given tap water (control group) and 1% NaCl as drinking fluid (1% NaCl group). Control; (□), 1% NaCl group; at the stage of 5 ~ 10 days (▨) and 2 ~ 3 days (▤) before the time of stroke and immediately after the stroke (■).

\*P < 0.02, \*\* P < 0.01, \*\*\*P < 0.05

stroke. The levels of lipid peroxides increased by about 220% while GSH-Px and SOD activities decreased by 26% and 24% respectively. A direct correlation between the levels of TBARS and the enzyme activities, however, has not always been observed. Masugi et al.<sup>23</sup> reported that the activities of GSH-Px and SOD of rat liver were unchanged while levels of TBARS were significantly high in vitamin E deficient rats. Chow<sup>24</sup> reported that SOD activity was not significantly altered but the activity of GSH-Px was decreased significantly ( $P < 0.05$ ) in the blood of vitamin E deficient rats. Though the accumulation of lipid peroxides would partly result from decreased activities of GSH-Px and SOD as a consequence of membrane disorders following cerebral hemorrhage, the mechanism must await further study.

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## Quantitation of Carotid Stenosis with Continuous-Wave (C-W) Doppler Ultrasound

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**SUMMARY** Two methods for determining the degree of stenoses developing on the origin of the internal carotid were tested using non-invasive Doppler ultrasonic imaging (DOPSCAN) of the carotid bifurcations. Spectral analysis of Doppler audio recordings was utilized in determining the maximum frequencies found within the stenosis, as well as the ratio of the frequency downstream to the stenosis, to the frequency within the stenosis. The theoretical relationships between blood flow, velocity, and pressure drop are defined for all grades of stenosis and they predict that carotid flow will not be reduced unless the lumen diameter is less than 1.5 mm. At critical diameter reductions, below 1 mm, the frequencies in human carotids do not exceed 16 KHz because turbulence limits peak velocities. If the maximum systolic frequency exceeds 5 KHz, when 5 MHz probes are directed at a 30° angle from the body axis, there is always present stenosis up to diameters of less than 3.5 mm by x-ray angiographic measurements. Frequency ratio studies confirm that plaque growth is not symmetrical but they did not improve x-ray angiography correlations because of the limitations of x-ray in measuring cross sectional areas from projection films and limitations of the spot size of x-ray tubes.

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**THE ADVERSE CLINICAL EFFECTS** of atherosclerotic plaques on the carotid artery are manifest in the patient's eye and brain through reduction of blood perfusion following stenosis of the channel or by embolization from the site of the plaque. It is generally agreed that more than one-third of strokes result from cervical arterial disease and primarily from plaques occurring on the origin of the internal carotid artery. For stroke prevention the identification of carotid plaques and quantitation of stenosis is of primary importance.

Non-invasive diagnostic methods are needed to evaluate patients with symptoms of cerebrovascular insufficiency because of the inherent dangers and costs of the alternative, x-ray contrast angiography. In addition, they are needed for medical or surgical follow up in the study of the natural history of the atherosclerotic plaque. With the general availability of non-invasive Doppler ultrasound, which provides blood velocity signals from the carotid arteries, it is important to fully utilize this information to evaluate the

degree of stenosis and the attendant collateral circulation. This paper presents a system for determining the degree of stenosis using the increased Doppler audio frequencies within the stenotic segment.

### Methods

Carotid blood velocity was measured with 5 MHz continuous-wave (C-W) directional Doppler ultrasonic equipment designed and built in the Bioengineering Center of this Institute.<sup>1</sup> The ultrasonic probe consists of a dual-crystal lens-focusing transducer mounted on a position sensing arm and directed toward the carotid arteries at a 60° angle from the body axis. With this equipment and procedure, a 1 KHz Doppler frequency shift represents a blood velocity of 30 cm/sec. The probe is placed against the neck with intervening coupling jelly and the Doppler shifted frequencies are recorded. A Doppler image of the carotid bifurcation (DOPSCAN),\* including the common carotid and its external and internal

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\*Obtainable from Carolina Medical Electronics, King, North Carolina.