Prediction of Stroke Lesions in Stroke-prone Spontaneously Hypertensive Rats by Glutathione Peroxidase in Erythrocytes

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Received January 26, 1995

The incipient timing of cerebral strokes in the stroke-prone spontaneously hypertensive rats (SHRSP) was biochemically determined by investigating the relationship between the glutathione peroxidase (GSH-Px) activity in erythrocytes and the extent of stroke lesions. When the blood pressure of SHRSPs was maintained at over 240 mmHg, the GSH-Px activity fell, and the body weight also decreased. In SHRSP whose GSH-Px activity in erythrocytes had dropped below 23 units/ml of blood, the incidence of cerebral strokes was 98% (n=88/90). The hematocrit level did not change even after the GSH-Px activity had dropped to 23 units/ml of blood. The reduced GSH-Px activity in erythrocytes observed during continued hypertension was found to be due to a decrease in GSH-Px protein, and not to any inactivation of the enzyme, as evident from immunochromatography. At the moment when the GSH-Px activity had dropped to 23 units/ml of blood, and the control diet was changed to one based on fish or a hydralazine treatment given, the activity recovered, and an increase in body weight and prolongation of the life-span were observed. It was deduced from these findings that tracing the GSH-Px activity in erythrocytes in SHRSP would serve as an indicator for predicting and prognosing stroke lesions.

Hypertension lasting for a long period results in hypertrophy or necrosis of the arterial walls, which is known to cause ischemic changes in such essential organs as the brain, heart, and kidneys. In particular, the central nervous system is susceptible to the influence of ischemia or hypoxia, and nerve cells are easily denatured or necrosed, so that a stroke (cerebral hemorrhage or softening) may be caused in the severest cases. It is hence important to elucidate the mechanism for cell damage by ischemia to prevent or treat strokes, but little has been learned so far. It was recently noticed that the free radical generated by reperfusion after ischemia was related to the cause of cell damage by ischemia.1-5) The lipid peroxidative reaction by active oxygen or a free radical is regarded as one of the risk factors for the onset of cerebrovascular diseases. In the normal metabolic pathway, no accumulation of lipid peroxide occurs, but when the defense mechanism against lipid peroxide production is broken, a morbid state occurs, which is likely to accelerate aggravation of the pathological state. Tomita et al.3) have investigated the change in the content of lipid peroxide in blood during stroke development in the stroke-prone spontaneously hypertensive rat (SHRSP), and reported that a sudden elevation of the lipid peroxide level in blood immediately before a stroke was related to the cause inducing the stroke. In humans, the lipid peroxidative reaction by radicals is known as one of the factors for aggravating cerebrovascular disease.7,8) The present authors9,10) have recognized that the glutathione peroxidase (GSH-Px) activity in erythrocytes of SHRSP was dramatically reduced at the time of a stroke, this being slightly earlier than the elevation of serum lipid peroxide. This change of the GSH-Px activity in erythrocytes was considered to be potentially useful as a biochemical index for predicting the incidence of cerebral strokes in SHRSP.

In the present studies, the erythrocyte GSH-Px activity before and after the onset of a stroke in SHRSP was traced, and its fluctuation by nutritional improvement and by treating with an antihypertensive drug were also studied.

Materials and Methods

Diet and drug. The control diet used was commercially available (Funabashi SP, Funabashi Farm Co., Ltd., Chiba, Japan). The diet was composed of 7.0% moisture, 21.8% crude protein, 4.5% crude fat, 3.4% crude fibre, 5.7% crude ash and 57.6% nitrogen-free extract. White fish meal (cod) and skim milk, as animal proteins, and defatted soybean, as vegetable protein, were the protein sources in the Funabashi SP diet. As the protein source of the fish diet also used this experiment, dried fish flakes of horse mackerel and mackerel (Yamaki Co., Ltd., Ehime, Japan) were used. The chemical composition of the dried fish flakes was 72.8% crude protein, 3.4% crude fat, 5.3% crude ash, 17.7% moisture, and 0.8% nitrogen-free extract. The fish diet consisted of 30.2% fish meal, 5% soybean oil (Wako Pure Chemical Industries Ltd., Osaka, Japan), 4% AIN-76-prescribed salts (CLEA Japan Ltd., Tokyo, Japan), 1% AIN-76-prescribed vitamin mixture (CLEA Japan Ltd.), 0.24% choline chloride (Wako Pure Chemical Industries Ltd.), 3% cellulose powder (type E, Advantec Toyo Co., Ltd., Tokyo, Japan), and 56.54% corn starch (CLEA Japan Ltd.). The protein content in the fish diet was adjusted to 22% as in the control diet (Funabashi SP).

Hydralazine hydrochloride (Sigma Chemical Co., St. Louis, MO, U.S.A.) was used as a peripheral vasodilator11) as a hypotensive drug.

Animals and feeding

(1) SHRSP raised in our laboratory were used throughout the experiments, and were fed with commercial diet pellets (Funabashi SP). When the blood pressure of SHRSP had risen to over 240 mmHg, blood sampling was started, and the GSH-Px activity in erythrocytes was measured. After confirming that the GSH-Px activity had changed from a high to low level, the animals were sacrificed at various activity levels of GSH-Px, and the presence or absence of a cerebral stroke lesions was examined.

(2) Male SHRSP were fed the Funabashi SP diet. When the blood pressure was being maintained at over 250 mmHg, the activity of GSH-Px in erythrocytes of SHRSP was measured, and when it had dropped to 23-25 units/ml of blood, the rats were divided into three groups. The first group was fed the Funabashi SP diet continuously, the second group was

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Abbreviations: SHRSP, stroke-prone spontaneously hypertensive rat; GSH-Px, glutathione peroxidase.
given the fish diet, and the third group was administered with hydralazine. Hydralazine was dissolved in drinking water at a concentration of 60 mg/liter, and given together with the Funabashi SP diet.

(3) Nine male SHRSP were fed with the control diet (Funabashi SP). When the blood pressure was being maintained at over 250 mmHg, and when the GSH-Px activity in erythrocytes of SHRSP had fallen to 22–25 units/ml of blood, the control diet was switched to the fish diet (n = 3) or the hydralazine treatment was started (n = 3), and the rats of both groups were then fed throughout a 3-week period. The diet and hydralazine dose for the hydralazine-treated group were the same as those used in experiment 2. After 3 weeks, the rats were anesthetized with ether, and whole blood samples were obtained from the abdominal aorta.

The animals were raised in a room with the temperature set at 23 ± 1°C, relative humidity at 55 ± 5% and lighting for 12 h a day (lighting from 07:00 to 19:00 h). The diet and drinking water were given ad libitum. The systolic blood pressure was measured without anesthesia by the tail-cuff method (KN-210-1, Natsune Co., Ltd., Tokyo, Japan) after warming the rat in a chamber maintained at 40°C for 5–10 min. The rats that naturally died and those that were sacrificed were given an autopsy, and the tissues fixed in 10% formaline. The presence or absence of cerebral stroke lesions (cerebral hemorrhage and cerebral softening) was then macroscopically examined.

Measurement of GSH-Px activity in erythrocytes. In 1.9 ml of physiological saline, 100 μl of blood was suspended, and the suspension centrifuged (3000 rpm, 5 min, 4°C) to obtain the erythrocyte fraction. To this fraction, 2.9 ml of distilled water was added to hemolyze it, and after centrifugal separation (3000 rpm, 10 min, 4°C), the supernatant was obtained as a crude extract for measuring the GSH-Px activity. The GSH-Px activity was measured by a modification of the method of Whanger et al.12) The assay for GSH-Px activity coupled the reduction of hydrogen peroxide (0.24 μM) to the oxidation of NADPH by glutathione reductase, and this coupled reaction was monitored at 340 nm. One unit of GSH-Px activity is expressed as the amount of GSH-Px required to oxidize 1 μmol of NADPH per minute.

Measurement of hemoglobin and hematocrit. Hemoglobin was measured by a commercially available kit (Hemoglobin-test Wako; Wako Pure Chemical Industries Ltd., Osaka, Japan). The hematocrit level was calculated after centrifuging blood sampled with a heparin-treated capillary by using a hematocrit RC-24 BN centrifuge (11,000 rpm, 5 min; Tomy Seiko Co., Ltd., Tokyo, Japan).

Preparation of the anti-GSH-Px antibody. Commercial bovine serum GSH-Px (Toyobo Co., Ltd., Tokyo, Japan) was used as an antigen. In a 10 mm sodium phosphate buffer (pH 7.0), 200 μg of GSH-Px was dissolved and mixed with an equivalent amount of Freund's complete adjuvant (Wako Pure Chemical Industries Ltd., Osaka, Japan) to obtain a homogeneous emulsion. This emulsion was subcutaneously administered to 6-month-old male New Zealand white rabbits. On day 7 and day 14 after the first injection, 200 μg each of GSH-Px suspended in incomplete adjuvant was injected. Three weeks later, a third injection was administered as a booster. After confirming an elevation of the antibody titer by the ring test13) after overnight fasting, whole blood was collected from the carotid artery. The collected blood was left for an hour at room temperature, and the antiserum was isolated (3000 rpm, 10 min, 4°C), which was purified by using Protein A Sepharose CL 4B (Sigma Chemical Co., Ltd., St. Louis, MO, U.S.A.). The protein content in the anti-GSH-Px antibody was measured by the Lowry method.14)

Immunoprecipitation and immunochemical titration. A crude GSH-Px extract was prepared from erythrocytes before changing to the fish diet and hydralazine treatment and 3 weeks after the change. The crude GSH-Px extract prepared from rat erythrocytes was fixed at 20 μl by immunoprecipitation, and various amounts of the anti-GSH-Px antibody were added. In the case of immunochemical titration, the amount of anti-GSH-Px antibody (28.6 μg of protein) was fixed at 20 μl, and various amounts of the crude GSH-Px extract were added. After incubating (37°C, 60 min), the antigen-antibody reaction complexes were removed by centrifuging (11,000 rpm, 10 min), and the remaining GSH-Px activity in the supernatant was determined.

Statistical processing. The experimental data are presented as the mean ± standard deviation. After the variance of values to be compared had been assayed, the results were analyzed by Student's t-test; the significant difference of data not showing equal variance was judged by Welch's t-test. The MUSCOT statistical analysis program (YUKMS Co., Ltd., Tokyo, Japan) was used for the assay, p < 0.05 being defined as significant.

Results
GSH-Px activity and stroke incidence
Figure 1 shows the presence and absence of stroke onset after sacrificing SHRSP during the process of reduced erythrocyte GSH-Px activity while the blood pressure was maintained above 250 mmHg. There was no sign of a stroke in male SHRSP, even when the GSH-Px activity was reduced to 23 units/ml of blood. Below 23 units/ml of blood, however, the stroke incidence was 98% (n = 78/80). In female SHRSP, on the other hand, the elevation of blood pressure was slow, rarely reaching 230 mmHg or higher, so that age-matching with male SHRSP was not possible. Accordingly, after selecting female SHRSP of 18 to 40 weeks of age that showed a blood pressure of 230 mmHg or higher, the GSH-Px activity was measured, and then the animals were sacrificed to examine the presence and absence of strokes. Similar to male SHRSP, even when the GSH-Px activity was reduced to 23 units/ml of blood, no strokes were observed, but with lower GSH-Px activity, strokes were observed in all 14 animals. These cases were with advanced-age SHRSP.

GSH-Px activity in erythrocytes and hematomatological findings
Shortly after reaching a blood pressure of 230 to 250 mmHg, the GSH-Px activity in erythrocytes started to drop in SHRSP, and the body weight was reduced when the enzyme activity had been reduced to about 23 units/ml of blood. The relationship between the reduction in GSH-Px activity and the hemoglobin and hematocrit levels was then investigated (Fig. 2). SHRSP with GSH-Px activity reduced to 22–25 units/ml of blood showed hemoglobin and hematocrit levels of 15 ± 1 g/dl and 47 ± 4%, respectively. These values were nearly same as those in the animals with
high enzyme activity. However, in SHRSP with enzyme activity lower than 22 units/ml of blood, decreased food intake and extremely decreased body weight were observed, and the hematocrit level was found to be very low.

**Fall in GSH-Px activity and variation of the amount of enzyme protein**

Immunochemical titration was performed in order to examine whether the fall in GSH-Px activity was caused by a decrease of the enzyme protein or by inactivation of the enzyme. When the activity of GSH-Px before and after the activity fall was inhibited in crude enzyme extracts, the relationship between the residual activity in the supernatant and the activity prior to the addition of the anti-GSH-Px antibody was the same before and after the activity fall (Fig. 3). Therefore, the reduced GSH-Px activity in erythrocytes, which was observed with persistent hypertension, was found to be due to the decrease of GSH-Px protein, and not to inactivation of the enzyme.

**Variation of erythrocyte GSH-Px activity in SHRSP treated by improved nutrition or administered with a hypotensive drug from initial stage of stroke**

When the GSH-Px activity in erythrocytes had dropped to 25 units/ml blood, feeding of the fish diet and administration of hydralazine were started. In the group continuously fed with the Funabashi SP diet, the GSH-Px activity was markedly lowered gradually, and all animals died within 24 days. In the fish diet group and hydralazine-treated group, on the other hand, the GSH-Px activity had re-

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**Fig. 2.** Relationship between the Hemoglobin or Hematocrit Level and Glutathione Peroxidase Activity in Erythrocytes of SHRSP.

**Fig. 3.** Immunochemical Titration of Glutathione Peroxidase Prepared from Erythrocytes before and after the Incidence of Stroke Lesions in SHRSP.

Crude GSH-Px extracts were prepared from erythrocytes before (○) and after (●) GSH-Px activity had dropped in erythrocytes of SHRSP. Immunochemical titration with the anti-GSH-Px antibody was carried out as described in the Experimental section. The abscissa represents the GSH-Px activity before the immunochemical reaction, and the ordinate shows the activity remaining after removing the antigen-antibody complex. Each experimental point represents the mean of three assays (95% coefficient of variation).

**Fig. 4.** Changes in Glutathione Peroxidase Activity in Erythrocytes of SHRSP Fed with the Fish Diet or Administered with Hydralazine from the Onset Period of a Stroke.

When the GSH-Px activity of SHRSP had dropped to 22–25 units/ml of blood (a) or below 22 units/ml of blood (b), the diet was switched to the fish diet or the hydralazine treatment was started. The fish diet or hydralazine were given after the GSH-Px activity had dropped in erythrocytes of SHRSP. Hydralazine was administered in drinking water (60 mg/liter) together with the control diet (commercial stock diet, Funabashi SP). The values are expressed as the mean ± S.D. (n = 5–8). Asterisks indicate a significant difference from the control (p < 0.05). ○, control diet; △, fish diet; ◇, hydralazine treatment; †, natural death.
Table Incidence of Stroke Lesions and Average Survival Period of SHRSP Fed with the Fish Diet or Administered with Hydralazine from Initial Stage of Stroke

<table>
<thead>
<tr>
<th>GSH-Px activity before diet change</th>
<th>Diets</th>
<th>No. of rats</th>
<th>Average survival period from diet change (days)</th>
<th>Incidence of stroke lesions (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>22-25 (units/ml of blood)</td>
<td>Control diet</td>
<td>7</td>
<td>24 ± 16</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Fish</td>
<td>7</td>
<td>75 ± 22*</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>Hydralazine</td>
<td>5</td>
<td>57 ± 19*</td>
<td>100</td>
</tr>
<tr>
<td>Under 22 (units/ml of blood)</td>
<td>Control diet</td>
<td>8</td>
<td>15 ± 19</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Fish</td>
<td>6</td>
<td>22 ± 12</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Hydralazine</td>
<td>5</td>
<td>22 ± 9</td>
<td>100</td>
</tr>
</tbody>
</table>

Hydralazine was administered in drinking water (60 mg/liter) together with the control diet (Funabashi SP). The values are expressed as the mean ± S.D. Asterisks indicate a significant difference from the control (p < 0.05).

![Graph](image1)

**Fig. 5.** Immunoprecipitation of Glutathione Peroxidase in the Erythrocyte Hemolysate from SHRSP before and after the Change to a Fish Diet or Hydralazine Treatment.

When the GSH-Px activity of SHRSP had fallen to 22-25 units/ml of blood, the control diet (○) was switched to the fish diet (□), or the hydralazine treatment was started (△). A crude GSH-Px extract was prepared from erythrocytes before changing to the fish diet or starting the hydralazine treatment, and 3 weeks after the change. The diet and hydralazine dose were the same as those shown in Fig. 4. Immunoprecipitation was performed with the anti-GSH-Px antibody, and a crude GSH-Px extract was obtained from erythrocytes. Vertical bars represent the remaining activity in the supernatant after the antigen-antibody reaction. Each point is expressed as the mean ± S.D. (n = 3).

covered after 3 weeks to 35.3 and 33.1 units/ml of blood, respectively (Fig. 4-a), and the body weight had also increased. The average life-span (Table) of SHRSP after the nutritional improvement or hypotensive treatment was significantly longer as compared to the animals fed with the Funabashi SP diet (24 ± 16 days). On the other hand, when either of these treatments was started after the GSH-Px activity had dropped below 22 units/ml of blood, neither recovery of activity nor extension of life-span was observed (Fig. 4-b).

**Effects of nutritional improvement and hypotensive treatment from the initial stage of stroke on the synthesis of GSH-Px protein in erythrocytes**

Immunoprecipitation was conducted by using the anti-GSH-Px antibody, and a crude enzyme extract was obtained from erythrocytes samples before and 3 weeks after starting the fish diet and hydralazine treatment. The residual activity in the supernatant after the antigen-antibody reaction was higher in the crude enzyme extracts prepared from SHRSP after administering the fish diet and hydralazine treatment as compared with that obtained before either of the treatments (Fig. 5). Accordingly, an immunochemical titration was conducted by using these crude enzyme extracts. As shown in Fig. 6, the relationship between the residual GSH-Px activity in the supernatant and the activity prior to the addition of the anti-GSH-Px antibody was the same before and after each of these treatments. Therefore, the recovery of GSH-Px activity in erythrocytes after the fish diet and hydralazine treatments was due to the increased biosynthesis of GSH-Px protein.

**Discussion**

SHRSP obtained by line separation from Wistar-Kyoto rats (WKY) with normal blood pressure by Okamoto et al. are sufferers from spontaneous hypertension and cerebrovascular diseases, and can be profitably used as a animal model for studying essential hypertension and cerebral stroke. The onset mechanism and process of stroke lesions have been widely studied in experiment on SHRSPs and they have proved to be an excellent model for elucidating...
the incipient mechanism and incipient process of cerebral strokes in humans. It is important to accurately determine the onset time of a stroke to elucidate the incipient process of cerebrovascular disease. Akiyuki et al.\cite{19} have used such hyperkinesia promoting offensive or hypersensitive activity as an index of cerebral stroke incidence. Tomita et al.\cite{9} have studied the incidence of cerebral strokes and the change in lipid peroxide level in blood of SHRSP, and suggested that a sudden elevation of the lipid peroxide level in blood just before the incidence of a stroke was a factor for inducing the stroke. Takahashi et al.\cite{17} have recently studied magnetic resonance imaging (MRI) in relation to pathological findings in SHRSP during cerebral strokes, and reported that the time-course change due to the incidence of a cerebral stroke in SHRSP could be detected and identified noninvasively by MRI. The present authors\cite{9,10} have found in previous studies on the relationship between improving proteinous nutrients from the progressive stage of hypertension and preventing cerebrovascular lesions that the GSH-Px activity in erythrocytes dropped slightly prior to the elevation of serum lipid peroxide level. The present studies were conducted with the expectation that measuring the GSH-Px activity in erythrocytes may be useful as a biochemical indicator for predicting the incidence of a cerebral stroke in SHRSP. The GSH-Px activity was slightly elevated with age, but shortly after the blood pressure had reached more than 250 mmHg, a drop in the activity was observed. Once this fall in GSH-Px activity in erythrocytes had begun, it fell rapidly, dropping markedly after the activity was close to 22 units/ml of blood. After the GSH-Px activity began to drop, the rats were sacrificed, and the presence or absence of a cerebral stroke was examined, and the level of GSH-Px activity was measured. Stroke lesions were not apparent in SHRSP just after the activity started to drop, but as the activity dropped more, the incidence of cerebral strokes increased, and a high correlation was noted between the fall in GSH-Px activity level and the incidence of cerebral strokes. The drop in GSH-Px activity observed at the incidence of a stroke was found to be caused by decrease in enzyme protein, and not by any inactivation of the enzyme. These results are in good agreement with the those reported by Tomita et al.\cite{9,19} in which the serum lipid peroxide level was shown to increase suddenly just before incidence of a cerebral stroke, and the activities of SOD and GSH-Px that participated in the formation and degradation of lipid peroxide were greatly lowered. When a nutritional improvement or hypotensive treatment was applied at the initial stage of the stroke, recovery of erythrocyte GSH-Px activity was observed, and prolonged survival was apparent, although these effects are considered to be due to suppressing the progress of stroke lesions, and not to its cure. In this respect, Okamoto et al.\cite{19} had already pathologically studied with malignant SHRSP (M-SHRSP) in the onset period of a stroke the effects of a fish meal diet or of the administration of a hypotensive drug on cerebrovascular diseases. They reported that the site of a lesion in the brain remained in a vacuolated state in treated M-SHRSP, showing that the progress of the lesion had been stopped. Therefore, by accurately knowing the change in erythrocyte GSH-Px activity, it is through possible to biochemically predict the progress of stroke lesions in SHRSP or the state of the cure.

References