Decreased Turnovers of Glutathione and Ascorbic Acid in Watanabe Heritable Hyperlipidemic Rabbits

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Summary Oxidative stress has been postulated to play important roles in the pathogenesis of various diseases such as atherosclerosis in hyperlipidemic subjects. Although the possible role of oxidation of low-density lipoprotein (LDL) in the etiology of atherosclerosis has been studied extensively, the turnover of endogenous antioxidants, which is an important protection system against oxidative stress, remains to be elucidated. The aim of our study was to determine the change of the turnover of endogenous antioxidants such as glutathione and ascorbic acid in case of hyperlipidemia, using Japanese white rabbits (JW) and Watanabe heritable hyperlipidemic rabbits (WHHL). The levels of total glutathione and low molecular weight thiols in the liver, kidney, and other organs in both strains of rabbits were similar. However, a kinetic analysis using L-buthionine-(S,R)-sulfoximine revealed that the rate of glutathione turnover in the liver and kidney of WHHL was about 50% lower than that of JW. Furthermore, intravenously administered ascorbic acid disappeared more slowly in WHHL than in JW. These results indicate that the turnovers of both glutathione and ascorbic acid in WHHL are depressed in comparison with that in JW. These changes would be closely related to the increased oxidizability of lipids in the circulation of hyperlipidemic subjects.

Key Words antioxidant, oxidative stress, hyperlipidemia, glutathione, ascorbic acid

Oxidative stress has been postulated to play important roles in the pathogenesis of atherosclerosis in patients with hyperlipidemia (1, 2). Natural antioxidants, such as reduced glutathione (GSH), ascorbic acid (AA), and α-tocopherol play important roles in the protection of cells and tissues from oxidative injury (3). Several synthetic antioxidants, such as probucol, have been reported to suppress the oxidation of LDL and to retard the progression of atherosclerosis in hyperlipidemic animals (2). The results of the clinical trials of antioxidant therapy, however, remain unclear (2, 4), and the efficacy of antioxidant therapy for atherosclerosis is still controversial.

It has been well documented that metabolisms of natural antioxidants such as GSH occur via intra- and interorgan cycles (5). The rate of its turnover is changed significantly in the pathologic subjects, even if the apparent levels in tissue and plasma are not changed (5). Moreover, this change would affect the redox status in the body. Therefore it is important to know the rate of the turnover of endogenous antioxidants in hyperlipidemic subjects as well as their apparent levels in tissue and plasma.

GSH and related tripeptides are secreted from the liver and other tissues into the systemic circulation, degraded to their constituent amino acids by tissues that have γ-glutamyltransferase (γ-GTP) and peptidases that hydrolyze cysteinylglycine bond. The constituent amino acids thus formed are transported into cells for GSH regeneration and protein synthesis. AA and its oxidized metabolites are also translocated across plasma membranes of various cells by either Na+-dependent active transport system(s) (6, 7) or facilitated transport systems, such as glucose transporters (GLUT 1 and 4) (6–8). The oxidation of AA occurs both inside and outside cells and tissues, and the enzymatic reduction of monodehydroascorbic acid and dehydroascorbic acid, which are the oxidized forms of AA, occurs predominantly inside cells at the expense of either NADPH or GSH, respectively (9–11). Therefore AA metabolism may also occur via intra- and interorgan cooperation.

Although the metabolism of various antioxidants in normal and pathologic subjects have been studied extensively (1,2), information about rates of the metabolic cycle of GSH and AA in hyperlipidemic subjects is lacking. The present work shows the decreased turnovers of GSH and AA metabolisms in Watanabe heritable hyperlipidemic rabbits (WHHL), an animal model for familial hyperlipidemia (13, 14).

MATERIALS AND METHODS

Chemicals. L-Buthionine-(S,R)-sulfoximine (BSO) and glutathione reductase were obtained from Sigma (St.

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Louis, MO). GSH, AA, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and other reagents were purchased from Wako Chemicals (Osaka, Japan). All the reagents used were of the highest commercial grade available.

Animals. The experimental protocol was approved by the Animal Care and Use Committee of Osaka City University. Five-mo-old male Japanese white rabbits (JW, 3.3–3.7 kg) and WHHL (3.2–3.5 kg) were purchased from Kitayama Labes Co. (Nagano, Japan). All animals were permitted free access to a standard laboratory chow (LRC4: Oriental Yeast, Tokyo, Japan) and water and housed in a room controlled at 24°C with a 12-h light/dark cycle for 1 wk before use. Venous blood and tissue samples were obtained from animals under pentobarbital anesthesia without fasting.

Measurements of lipids and lipid peroxidation products in plasma. Plasma levels of total cholesterol, triglycerides, and phospholipids were measured by enzymatic methods using Cholestezyme-V555 “Eiken,” Triglyzyme-V “Eiken” (Eiken Kagaku Co., Osaka), and Phospholipid C-test Wako (Wako Chemicals), respectively. The extent of lipid peroxidation in plasma was assessed by measuring thiobarbituric acid-reactive substances (TBARS) (15). The amount of TBARS was calculated as the nmol equivalent of malondialdehyde.

Chemical analysis of endogenous antioxidants. Levels of total glutathione (GSH+2 glutathione disulfide (GSSG)) and other free thiols in plasma and tissues were determined by the method of Tietze (16) and Ellman (17), respectively. Fresh plasma samples obtained from heparinized blood were mixed with 0.5 volume of ice-cold 10% trichloroacetic acid containing 1 mM diethylenetriamine-pentaacetic acid (DTPA). The excised tissues were homogenized in 3 volumes of ice-cold 10% trichloroacetic acid containing 1 mM DTPA. After centrifugation at 12,000 g for 20 min, concentrations of low molecular weight thiols and total glutathione in the acid-soluble fractions were determined.

Because tissue glutathione levels are determined by the dynamic equilibrium between the biosynthesis and utilization of GSH, their turnover was determined in animals that were intravenously administered BSO, a specific inhibitor of γ-glutamylcysteine synthetase, as described previously (18). Before and after the administration of 1 mmol/kg of BSO (0, 0.5, 3, and 6 h), glutathione levels in liver and kidney were measured as described above. From our preliminary experiment, the inhibition of GSH biosynthesis continued at least 6 h from just after the BSO administration. Its administration inhibited the decreased turnover of GSH, their levels versus the time after BSO administration, the half-lives of glutathione in liver and kidney were calculated. Based on these half-lives and the initial levels of glutathione, the amounts of its turnover were calculated as described previously (5). The half-lives of glutathione corresponded to those of low molecular weight thiol, suggesting that the decrease of glutathione reflects the decrease of GSH.

Fig. 1. Plasma levels of various lipids in JW and WHHL. The plasma levels of total cholesterol, triglycerides, and phospholipids in JW (open columns) and WHHL (closed columns) were determined as described in the text. Data are expressed as the mean±SE (n=12). *p=0.05.

AA levels in plasma and tissues were determined by the method using high-performance liquid chromatography (HPLC) equipped with an electrochemical detection system (19). Acid-soluble fractions of plasma and tissues were subjected to the HPLC analysis. Time-dependent changes in plasma AA levels were also determined after an intravenous administration of 50 μmol/kg of AA. From the semilogarithmic plots of plasma AA levels versus time after its administration, its half-life in the circulation was calculated.

Statistics. Data are expressed as the means±SE derived from 4 or 12 animals. A statistical analysis of the data was performed, using Student’s t-test, and the level of significance was put at p<0.05.

RESULTS

Lipids and lipid peroxidation products in plasma

Figure 1 shows the profiles of lipids in plasma of JW and WHHL. The plasma levels of total cholesterol, triglycerides, and phospholipids were significantly higher in WHHL than those in JW. These profiles of high plasma lipid levels are characteristic of WHHL (14, 20).

The plasma level of TBARS, one of the oxidative stress markers, was also measured. It was about 4 times higher in WHHL than in JW, suggesting that the lipid peroxidation proceeds significantly higher in WHHL than in JW (JW: 0.22±0.03, WHHL: 0.87±0.07 nmol/mL).

Glutathione and low molecular weight thiols in various tissues

Figure 2 shows the levels of glutathione and low molecular weight thiols in various organs. Although the levels of glutathione differed from one tissue to another, profiles of their steady-state levels were similar between
two animal groups except for less than 20% statistically significant decreases in the kidney, lung, spleen, and cerebrum of WHHL. Levels of low molecular weight thiols were also identical in the two animal groups, except for a 10% statistically significant decrease in the kidney of WHHL. Plasma levels of glutathione were 2.9±0.3 μM in JW and 2.8±0.1 μM in WHHL. Plasma levels of low molecular weight thiols were 27±2 μM in JW and 28±3 μM in WHHL. That is, the level of plasma glutathione was about 10%, compared with that of low molecular weight thiols in both strains of rabbits.

Effect of BSO on hepato-renal glutathione levels

To investigate the rates of the metabolic cycle of GSH in JW and WHHL, animals were intravenously injected with BSO, and time-dependent changes in tissue glutathione levels were determined. BSO rapidly reduced glutathione levels in the liver and kidney. From semilogarithmic plots of tissue glutathione levels, the half-lives of hepatic glutathione were calculated to be 10 and 15 h in JW and WHHL, respectively (Table 1). Renal glutathione levels in JW and WHHL decreased with half-lives of 30 and 50 min, respectively. The half-lives of glutathione in adrenal gland and ileum were 3 and 6 h in JW and 5 and 13 h in WHHL, respectively. No significant changes in glutathione levels were found in other tissues within 6 h after the administration of BSO. The BSO-induced decrease in glutathione levels in liver and kidney reflects the rate of its utilization and excretion by these tissues (5). Based on the initial levels and half-lives of glutathione in liver and kidney, the amounts of glutathione mobilized from these tissues were calculated (Table 1). The total amounts of glutathione mobilized from liver and kidney in WHHL were about 50% lower than those in JW.

<table>
<thead>
<tr>
<th>Initial level (μmol/g)</th>
<th>Half-life (h)</th>
<th>Turnover (μmol/g/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JW-liver 8.08±0.37</td>
<td>10 h</td>
<td>0.40±0.02</td>
</tr>
<tr>
<td>WHHL-liver 7.74±0.05</td>
<td>15 h</td>
<td>0.26±0.00</td>
</tr>
<tr>
<td>JW-kidney 2.68±0.06</td>
<td>30 min</td>
<td>2.68±0.06</td>
</tr>
<tr>
<td>WHHL-kidney 2.14±0.12</td>
<td>50 min</td>
<td>1.28±0.07</td>
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Before and after intravenous administration of BSO (1 mmol/kg), glutathione levels (GSH+2GSSG) in liver and kidney were determined. The half-lives of glutathione were calculated from the semilogarithmic plots of the changes in its tissue levels after BSO treatment (n=4).

AA status in JW and WHHL

Figure 3 shows the levels of AA in plasma, liver, kidney, and adrenal gland of JW and WHHL. Although AA levels in these tissues of WHHL were similar to those of JW, the plasma level was 2-fold higher in the former than in the latter. To investigate the rate of AA metabolism in the circulation, time-dependent changes in its plasma levels were determined after its administration.
As shown in Fig. 4, intravenously injected AA rapidly disappeared from the circulation of both strains of rabbits in a biphasic manner. The rate of AA disappearance was lower in WHHL than in JW. From the semilogarithmic plots of the changes in plasma AA levels, its half-lives at the rapid and slow phases in JW and WHHL were calculated. The half-life of AA at slow phase in WHHL was about 1.6 times significantly longer than in JW.

**DISCUSSION**

The present results indicate that the turnovers of both GSH and AA are significantly lower in WHHL than in JW. The decreased turnover of these antioxidants would closely correlate with the increased oxidizability of lipids in plasma, an important factor for the deterioration of atherosclerosis in hyperlipidemic subjects.

It has been well documented that GSH metabolism occurs via inter- and intraorgan cycles in which liver and kidney play important roles (5). In rodents, the circulating GSH is derived predominantly from the liver and degraded preferentially by the kidney and other tissues enriched with γ-GTP and peptidases. Therefore GSH is predominantly responsible for the major fraction of low molecular weight thiols in plasma (5). It should be noted that the plasma level of glutathione was significantly lower than that of low molecular weight thiols in both strains of rabbits. Because the activity of γ-GTP in the livers of humans and rabbits is as high as that in their kidneys, significant fractions of GSH coming out from hepatocytes would be degraded within the liver sinusoid before entering into the systemic circulation (21). This is one reason for the lower levels of plasma glutathione in these species than in those in rodents (22).

Although glutathione levels in various tissues were similar with the two animal groups, the increased half-lives of glutathione in liver and kidney of WHHL indicated that its turnover was significantly depressed in these animals. Furthermore, the half-life of injected AA from the circulation at slow phase was significantly longer in WHHL than in JW. Many factors regulate the clearance of AA from the circulation. Among these, a rapid uptake by organs and a metabolic change in organs would be two of the important factors. The former mainly affects the rate of rapid phase clearance, and the latter mainly affects the rate of slow phase clearance. Considering that the half-life of AA at slow phase was significantly longer in WHHL than in JW, AA metabolism might also be depressed in WHHL. It is well known that vitamins having antioxidant activity cooperate with one another for protecting cells and tissues from oxidative stress. For example, AA interacts with tocopherol radicals in cell membrane lipid/bilayers, thereby forming monodehydroascorbic acid (23, 24). Monodehydroascorbic acid undergoes spontaneous dismutation to form dehydroascorbic acid. Monodehydro-ascorbic acid and dehydroascorbic acid are reduced to AA at the expense of NAD(P)H and GSH, respectively (9–11). This sequence of reactions plays an important role in protecting cell membranes from the hazardous chain reaction of free radicals. Therefore a decreased turnover of GSH in WHHL would metabolically correlate, at least in part, with the decreased turnover of AA.

The mechanism by which the metabolisms of both GSH and AA were depressed in WHHL is not now clear. It has been generally accepted that the turnovers of GSH and related antioxidants are accelerated by increasing the oxidative stress (25). A depressed antioxidant system, however, is closely related to the increased lipid peroxidation and oxidative tissue injury.

The functions of liver and kidney of WHHL alter with time as the animals become severely atherosclerotic. Such changes could affect the metabolism and transport of endogenous molecules including AA and GSH. The present experiments were carried out by using 5-mo-old rabbits. Although WHHL showed a marked hyperlipidemia and mild atherosclerosis, no appreciable sign for hepato-renal injury was obtained. Therefore the depression of the turnovers of GSH and AA in WHHL would occur independently from the decrease in hepato-renal functions caused by atherosclerotic changes.

An important point remains unexplained: whether decreased turnover of the antioxidants observed in WHHL is applicable to other general hyperlipidemic subjects. In other words, whether this change is caused by a unique genetic nature of WHHL or not. To the best of our knowledge, this is the first report to describe the change in turnover of antioxidants in hyperlipidemic subjects. Therefore it is now difficult to compare the phenomenon in WHHL with that in other hyperlipidemic animal models. We suppose, however, that the hyperlipidemic state itself could generally lead to the reduction of the turnovers of GSH and other related antioxidants. There are many kinds of enzymes and functional proteins that regulate the metabolism of antioxidants such as glutathione peroxidase and glutathione-S-transferase for GSH. Although the exact mechanism is uncertain, it is possible that the hyperlipidemic state
affects the activities of these proteins that regulate the antioxidant metabolism. Therefore precise molecular mechanisms for the depression of the turnover of these antioxidants in WHHL and the turnover of these antioxidants in other animal models should be studied further.

Because of the presence of oxidized LDL in atherosclerotic lesions of the aorta (26), oxidative modification of LDL has been postulated to be an important factor for the progression of atherosclerosis in hyperlipidemic subjects (1, 2). The increased oxidizability of LDL has also been reported in patients with hyperlipidemia (27, 28). The endogenous antioxidant system is a critical factor for determining the oxidation resistance of the body. Thus examining the dynamic aspect of natural antioxidants in patients with hyperlipidemia and/or atherosclerosis would also be important for the treatment of this disease.

REFERENCES