Impaired reductive regeneration of ascorbic acid in the Goto–Kakizaki diabetic rat

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Ascorbic acid (AA) is a naturally occurring major antioxidant that is essential for the scavenging of toxic free radicals in both plasma and tissues. AA levels in plasma and tissues have been reported to be significantly lower than normal in diabetic animals and humans, and might contribute to the complications found at the late stages of diabetes. In this study, plasma and hepatic AA levels and AA regeneration were studied in the Goto–Kakizaki diabetic rat (GK rat) to elucidate the mechanism of decreasing plasma and hepatic AA levels in diabetes. AA concentrations in the plasma and liver were significantly lower in GK than in control rats. AA levels in primary cultured hepatocytes derived from GK rats were lower than those derived from control Wistar

rats with or without dehydroascorbic acid (DHA) in the medium. Among various enzyme activities that reduce DHA to AA, the NADPH-dependent regeneration of AA in the liver was significantly suppressed in GK rats. Northern blot analysis revealed that only the expression of 3- α -hydroxysteroid dehydrogenase (AKR) was significantly suppressed in these rats. These results suggest that decreased AA-regenerating activity, probably through decreased expression of AKR, contributes to the decreased AA levels and increased oxidative stress in GK rats.

Key words: 3- α -hydroxysteroid dehydrogenase, dehydroascorbic acid, diabetic complications, hepatocytes, RNA species.

INTRODUCTION

There is evidence to suggest that oxidative stress is increased in humans with diabetes and in animal models of diabetes [1–3]. L-Ascorbic acid (AA) is a naturally occurring major antioxidant that is essential to the scavenging of toxic free radicals in both plasma and tissues [4,5]. AA is oxidized to monodehydroascorbic acid and dehydroascorbic acid (DHA). AA levels in plasma and tissues have been reported to be significantly lower than normal in diabetic animals and humans (reviewed in [6]). Because the supplementation of AA decreases sorbitol levels in various tissues and increases the stability of blood vessels in patients with diabetes, a decrease in AA levels might contribute to the diabetic complications found at the late stages of the disease [7–10]. Levels of DHA in the liver and other tissues in diabetic subjects are reportedly increased [11–13]. This metabolite has also been postulated to be involved in diabetic complications [14,15].

AA levels in plasma and tissues are maintained predominantly by the intracellular reduction of an oxidized form of this vitamin. It has been known that reductive regeneration of AA is catalysed by NADPH-dependent regenerating enzymes such as 3- α -hydroxysteroid dehydrogenase (AKR) [16] and thioredoxin reductase (TR) [17]. AA is also regenerated from DHA by GSH-dependent enzymes such as glutaredoxin (GRX) [18], protein disulphide-isomerase (PDI) [18] and GSH-dependent DHA re-

ductase (DAR) [19,20]. These enzymes rapidly reduce DHA to maintain the steady-state levels of AA in cells and plasma. However, only limited information is available on the properties of AA regeneration in diabetic subjects [21].

The Goto–Kakizaki diabetic rat (GK rat) is an animal model for Type II diabetes mellitus without obesity [22]. This model was produced by repeated selective breeding of rats with glucose intolerance starting from a non-diabetic Wistar rat colony. The GK rats exhibit mild hyperglycaemia at first and an impaired glucose tolerance on glucose loading. As the disease progresses, this diabetic model develops characteristic complication-like tissue damage in the peripheral nerves as well as in the kidney, recapitulating systemic manifestations encountered in human Type II diabetes [22–24]. The present study investigated the plasma level and metabolism of AA in GK rats.

EXPERIMENTAL

Animals

GK and Wistar rats (16 weeks old) were obtained from Japan Clea (Tokyo, Japan). They were maintained in individual cages on a rotating 12 h/12 h light/dark cycle at an ambient temperature of 24 ± 1 °C and were provided with free access to both standard rat chow (Oriental Food, Tokyo, Japan) and water.

Abbreviations used: AA, ascorbic acid; AGE, advanced glycation end product; AKR, 3- α -hydroxysteroid dehydrogenase (AKR1C9); DAR, GSH-dependent dehydroascorbate reductase; DHA, dehydroascorbic acid; ECD, electrochemical detection; GK rat, Goto–Kakizaki diabetic rat; GRX, glutaredoxin; PDI, protein disulphide-isomerase; TR, thioredoxin reductase.

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Materials

AA was obtained from Wako (Osaka, Japan). DHA was synthesized as reported previously [25]. In brief, 50 μ l of aqueous bromine was added to 1 ml of standard 200 mM AA solution and mixed for 30 s. Excess bromine was removed by bubbling with nitrogen gas for 10 min. Other chemicals were obtained from Sigma (St Louis, MO, U.S.A.).

Measurement of AA

For the analysis of AA in the plasma, blood samples were collected in heparin-containing tubes. The blood was then immediately centrifuged at 15000 *g* for 3 min; the plasma fraction was vigorously mixed with an equal volume of 10% (w/v) metaphosphoric acid solution for 3 min. After centrifugation of the samples at 15000 *g* for 15 min, the acid-soluble fractions were measured for AA and DHA. Frozen tissue samples were homogenized in 5 vol. of 5% (w/v) metaphosphoric acid. The samples were centrifuged at 15000 *g* for 15 min and acid-soluble fractions were measured for AA and DHA. Cultured hepatocytes (5×10^5 per dish) were washed with PBS and lysed in 5% (w/v) metaphosphoric acid solution. Lysates were mixed vigorously and centrifuged at 15000 *g* for 15 min; the acid-soluble fractions were measured for AA. AA was measured by HPLC with an electrochemical detection system (HPLC-ECD, Irica, Kyoto, Japan) [26]. AA + DHA was also measured by HPLC-ECD after reduction with dithiothreitol. In brief, 100 μ l of the acid-soluble fraction described above was mixed with 50 μ l of 10 mM dithiothreitol in 1 M potassium phosphate buffer. After 5 min at room temperature the solution was mixed with 50 μ l of 25% (w/v) metaphosphoric acid and injected into the HPLC-ECD system.

Isolation and maintenance of hepatocytes

The liver was perfused with 200 ml of Ca^{2+} -free Gey's balanced salts solution, and then with 150 ml of Gey's balanced salts solution containing Ca^{2+} and 0.05% (w/v) collagenase [27]. The liver was removed and minced gently. The resulting suspension was filtered through several layers of mesh and sedimented by centrifugation at 50 *g* for 1 min. The cell pellet was washed three times. Cell viability was typically 70–90% as determined with Trypan Blue. Cells were seeded on 35 mm plastic dishes at a density of 5×10^5 cells per plate. Before use, cells were maintained for 1 day in Williams E medium with L-glutamine, containing 5% (v/v) fetal calf serum and supplemented with insulin (1%), dexamethasone (1 μ M), penicillin (100 units/ml) and streptomycin (100 mg/ml).

Preparation of liver homogenate

Frozen liver (100 mg) was homogenized in 5 ml of ice-cold 0.1 M Tris/HCl buffer, pH 7.4, containing 1 mM EDTA. The homogenates were centrifuged at 100000 *g* for 1 h at 4 °C. The supernatants were dialysed against 3 litres of 0.1 M Tris/EDTA buffer, pH 7.4, at 4 °C with three changes. The dialysed samples had no detectable AA and GSH. The pellet was resuspended in 0.1 M Tris/EDTA buffer, pH 7.4, to obtain microsomal fractions.

Assay of NADPH-dependent and GSH-dependent DHA reductase activity

The conversion of DHA into AA was measured by incubating DHA and the dialysates in the presence of GSH or NADPH, as reported previously [17]. The reaction mixture contained, in a

final volume of 1 ml, 10 mM DHA and 50 μ l of the cytosolic or microsomal fraction and 0.4 mM NADPH or 1 mM GSH. The reaction was started by the addition of DHA. After incubation at 37 °C in air for various periods as indicated in the Figure legends, the reaction was stopped by adding 0.5 ml of 25% (v/v) metaphosphoric acid. The mixture was centrifuged at 15000 *g* for 15 min and aliquots of the supernatants were assayed for AA by HPLC-ECD. It was confirmed that NADPH alone did not reduce DHA under the conditions used. GSH reduces DHA slightly and the formation of AA by this reaction was subtracted from the total.

RNA analysis

Preparation of probe for mRNA measurement

The cloning of a cDNA for rat DAR has been reported previously [20]. A partial rat GRX cDNA was cloned from a rat liver cDNA library (Clontech, Palo Alto, CA, U.S.A.) by using a fragment of human GRX cDNA (GenBank[®] accession no. D21238); it exhibited 88% identity with the human GRX amino acid sequence from residue 9 to the C-terminus. cDNA samples for AKR, TR1, TR2 and PDI were obtained from a rat liver cDNA library by a PCR amplification. Forward and reverse primer sequences for AKR, TR1, TR2 and PDI were: AKR forward primer, 5'-GTG CCT GAG AAG GTT GCT AAG G-3'; AKR reverse primer, 5'-TCA TGT CCT CTG AAG CCA ACT G-3'; TR1 forward primer, 5'-CAC CAA CTC CTC TCG GAA CG-3'; TR1 reverse primer, 5'-TGG ACC GCA CCA TTA CAG TG-3'; TR2 forward primer, 5'-GAA CTG GGG TCA TCG TGT CC-3'; TR2 reverse primer, 5'-TCT GGT CTC TGG AAC TCG CC-3'; PDI forward primer, 5'-AGA ACG GTC ATT GAT TAC AAC GG-3'; PDI reverse primer, 5'-AGG GGT CTC AGG GAG GTC TG-3'. A PCR cocktail containing the library, gene-specific primer and *Taq* DNA polymerase (Perkin Elmer) were denatured (6 min, 95 °C). The mixture was then overlaid at 95 °C and annealed at 55 °C for 1 min. The cDNA was extended at 72 °C for 2 min; 30 cycles of amplification were performed, followed by a 10 min final extension at 72 °C. The amplified products were cloned into TA vector (Invitrogen, Carlsbad, CA, U.S.A.) and then sequenced.

Northern blots

Total RNA was prepared from the tissue of each rat with TRIzol reagent (Gibco BRL, Gaithersburg, MD, U.S.A.). The concentration of RNA was determined spectrophotometrically. Total RNA (15 μ g per lane) was loaded on to a 1% agarose/formaldehyde gel and transferred on a nylon membrane (Hybond N⁺; Amersham, Arlington, IL, U.S.A.). The blots were pre-hybridized for 4 h in hybridization fluid [0.75 M NaCl/0.05 M NaH_2PO_4 (pH 7.4)/5 $\times$ Denhart's solution/10 mg/ml salmon sperm DNA/0.5% SDS] at 42 °C. Blots were hybridized overnight with [α -³²P]dCTP-labelled cDNA species in the hybridization fluid at 42 °C. Hybridized blots were washed in 1% SDS at 42 °C for 10 min (three times) and at 65 °C for 30 min, then exposed to X-ray film (Fujifilm, Tokyo, Japan) at –80 °C with the use of an intensifying screen. Signals were quantified with Fujix Bio-Imaging Analyzer BAS2000 (Fujifilm). Equivalent loading was verified by staining the rRNA bands with Methylene Blue after transfer.

Other assays

Protein was measured by the method of Bradford (Pierce, Rockford, IL, U.S.A.). Plasma glucose levels were determined by the glucose oxidase method (glucose B-test; Wako, Osaka,

Japan). Immunoreactive insulin concentrations were determined with a radioimmunoassay kit (catalogue no. RI-13K; Linco Research, St. Charles, MO, U.S.A.) and standardized against rat insulin.

RESULTS

Group characteristics

Table 1 shows characteristic features of control and GK rats. No significant difference was found in body weight and food intake between control and GK rats. The plasma concentration of glucose increased significantly in GK rats ($P < 0.05$). Plasma insulin levels in GK rats were also increased significantly ($P < 0.05$).

Table 1 Characteristic features of control and GK rats

Results are means $\pm$ S.D. * $P < 0.05$ compared with control.

Rat type	Body weight (g)	Food intake (g/day)	Plasma glucose (mM)	Plasma insulin (ng/ml)
Control	347 $\pm$ 9	12.6 $\pm$ 1.2	10.5 $\pm$ 1.0	2.93 $\pm$ 0.80
GK	361 $\pm$ 26	15.0 $\pm$ 1.6	30.2 $\pm$ 8.8*	4.41 $\pm$ 0.88*

Figure 1 Concentration of AA in control and GK rats

Concentration of AA (open bars) and total AA (AA + DHA, hatched bars) in the plasma (a), liver (b) and brain (c). ** $P < 0.01$ compared with control; *** $P < 0.01$ compared with control and $P < 0.05$ compared with AA in GK.

Figure 2 Accumulation of AA in hepatocytes derived from control and GK rats

Accumulation of AA in hepatocytes derived from control (○) and GK (●) rats incubated with DHA. At 30 min after the addition of DHA to the medium, cells were washed with PBS and the concentration of AA was measured. ** $P < 0.01$. uM = μ M.

Level of AA in the plasma, liver and brain

Figure 1 shows the AA concentrations in the plasma, liver and brain of control and GK rats. AA levels were decreased signifi-

Figure 3 Regeneration of AA from DHA by liver cytosol and microsome fraction in control and GK rats

Reaction mixtures contained, in a final volume of 1 ml, 10 mM DHA and cytosol (A, B) or microsome fraction (C, D) in Tris/EDTA buffer in the presence of 1 mM GSH (A, C) or 0.4 mM NADPH (B, D). The reaction was started by the addition of DHA. After incubation at 37 °C, the reaction was stopped by the addition of 0.5 ml of 25% (v/v) metaphosphoric acid. The mixture was centrifuged at 15 000 g for 15 min and aliquots of the supernatant were measured for AA. Symbols: ○, control; ●, GK. ** $P < 0.01$.

Figure 4 Northern blot analysis and quantification of the relative levels of AA-regenerating enzymes in the livers of control and GK rats

Upper panels: autoradiographs of Northern blot analyses of various enzymes, as indicated. Lower panels: mRNA levels of various enzymes, as indicated. The mRNA levels were normalized to total RNA. The y-axis indicates the relative levels of mRNA (levels of mRNA in control animals were set arbitrarily at 1.0). Results are means $\pm$ S.D. ** $P < 0.01$.

cantly in both the plasma and the liver of GK rats. The amount of total reduced and oxidized forms of AA + DHA also decreased both in the plasma and liver in GK rats. However, the difference in AA + DHA was less than that in AA. Plasma levels of DHA (calculated by subtracting AA from AA + DHA) were increased in GK rats. Hepatic levels of DHA were also increased in GK rats. Levels of AA and DHA in the brain were similar in GK and in control rats.

Accumulation of AA in hepatocytes derived from control and diabetic rats

Figure 2 shows the accumulation of AA in primary cultured hepatocytes when DHA was added to the medium. AA was accumulated in the hepatocytes, as reported with other cell lines [20,28]. AA levels in the hepatocytes derived from GK rats were lower than those derived from control Wistar rats, both with and without DHA in the medium.

Figure 5 Regeneration of AA from DHA by the brain cytosol and microsomal fraction in control and GK rats

Reaction mixtures contained, in a final volume of 1 ml, 10 mM DHA and cytosol (A, B) or microsomal fraction (C, D) in Tris/EDTA buffer in the presence of 1 mM GSH (A, C) or 0.4 mM NADPH (B, D). The reaction was started by the addition of DHA. After incubation at 37 °C, the reaction was stopped by the addition of 0.5 ml of 25% (v/v) metaphosphoric acid. The mixture was centrifuged at 15 000 *g* for 15 min and aliquots of the supernatant were measured for AA. Symbols: ○, control; ●, GK.

Activity and mRNA levels of AA-regenerating enzymes in the liver

Figure 3 shows the GSH- and NADPH-dependent regeneration of AA from DHA in the liver. In the presence of GSH, the rate of AA regeneration in the liver cytosolic fraction of GK rats was similar to that in control animals (0.6 unit/mg of protein). However, NADPH-dependent regenerating activity was lower in GK (0.38 unit/mg protein) than control (0.56 unit/mg of protein) animals. Although microsomal fractions of the liver also reduced DHA to AA in the presence of GSH, their activities were similar in the two animal groups (0.31 unit/mg of protein). Liver microsome did not regenerate AA from DHA even in the presence of NADPH. Figure 4 shows the hepatic mRNA levels for six enzymes involved in the regeneration of AA from DHA. All these enzymes were expressed in the liver of control and GK rats. The expression of mRNA species for AA-regenerating enzymes (the GSH-dependent regenerating enzymes GRX, DAR and PDI, and the NADPH-dependent regenerating enzymes TR1 and TR2) was similar in the two groups. However, the expression of AKR was suppressed significantly in GK rats.

Activity and mRNA levels of AA-regenerating enzymes in the brain

Figure 5 shows the AA-regenerating activity from DHA in brain cytosolic and microsomal fractions from control and GK rats. In the presence of GSH, the cytosolic fraction from GK rats exhibited similar levels of AA-regenerating activity to those from control animals (1.1 units/mg protein). Although both cytosolic fractions regenerated AA in the presence of NADPH, their activities were significantly lower (0.14 unit/mg protein) than in the presence of GSH.

Figure 6 Northern blot analysis and quantification of the relative levels of AA-regenerating enzymes in the brains of control and GK rats

Upper panels: autoradiographs of various enzymes, as indicated. Lower panels: mRNA levels of various enzymes, as indicated. The mRNA levels were normalized to total RNA. The y-axis indicates the relative levels of mRNA (levels of mRNA in control animals were set arbitrarily at 1.0). Results are means $\pm$ S.D.

Figure 6 shows mRNA levels for the enzymes regenerating AA from DHA in the brain. mRNA levels for GSH-dependent enzymes (GRX, DAR and PDI) in GK rats were similar to those in control animals; mRNA levels for TR1 and TR2 were also the same as in controls. However, no mRNA for AKR was found in GK or control rats.

DISCUSSION

The plasma and hepatic AA levels in the GK rat are significantly low compared with control values, as reported for other animal models of diabetes [3]. The present study revealed that hepatic AA-regenerating activity was decreased in GK rats and also that NADPH-dependent regenerating activity in the liver was significantly suppressed in GK rats. Northern blot analysis indicated that, of the various enzymes that reduce DHA to AA, only AKR in GK rats was significantly suppressed in its expression. It is noteworthy that, because AA is a water-soluble compound, cytosolic enzymes such as DAR, GRX, TR1 and AKR rather

than microsomal enzymes might be important for maintaining its level. It has been reported that the level of GSH is lower in diabetic animals [29,30]. Because GRX and DAR use GSH as a cofactor and their K_m values for GSH are relatively high (1.6–8.7 and 2.8 mM respectively) [18,19], NADPH-dependent cytosolic enzymes such as TR1 and AKR might be responsible for the regeneration of AA from DHA, particularly when tissue GSH levels were decreased. In the present study, only the gene expression of AKR was decreased.

Although AA levels in plasma and in the liver of GK rats decreased significantly, those in the brain were unchanged. This is understandable because the NADPH-dependent regeneration of AA and the expression of AKR mRNA decreased in the liver of GK rats but not in the brain. Sun et al. [31] reported that the hepatic AA level decreased markedly 4 weeks after the administration of streptozotocin, whereas the level in the brain remained unchanged; this is consistent with the present findings.

A decrease in the activity of AA-regenerating enzymes might result in lower AA levels and higher DHA levels in tissues. One or more degradation products of DHA have been postulated to enhance the formation of advanced glycation end products (AGEs) [32]. In patients with diabetes mellitus, AGEs accumulate in the extracellular matrix, which is one of the main causes of diabetic complications. Therefore AA-regenerating enzymes should be important not only in maintaining adequate levels of AA but also in preventing the generation of AGEs. It has been reported that the accumulation of sorbitol, another potential cause of diabetic complications, was restored by AA supplementation [7–10].

Besides having AA-regenerating activity, AKR catalyses the NAD(P)-linked oxidoreduction of a wide variety of substrates such as quinone [33], aromatic alcohol [33], dihydrodiol [33,34] and hydroxyprostaglandin [35]. The pathophysiological roles of this abnormality of AKR expression in GK rats need further investigation. It is also necessary to investigate the role of human AKR in maintaining plasma and tissue AA levels and its expression in humans, especially in those with diabetes.

In summary, low AA levels in plasma and tissue resulting from a decrease in AA regeneration, probably caused by a decrease in AKR, might increase oxidative stress and thereby contribute to the manifestation of complications in GK diabetic rats, synergistically or additionally with other contributing factors.

We thank Dr A. Matsumoto and Dr K. Umegaki for helpful suggestions, and Ms N. Kumagai and Mr K. Ohwada for technical support.

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Received 13 April 2000/28 June 2000; accepted 27 July 2000