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Experimental Studies on the Role of Fructose in the Development of Diabetic Complications

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We examined the role of fructose in the development of diabetic complications. Compared with glucose, fructose increased the fluorescence intensity and the cross-linking of glycated collagen, and promoted the polymerization of proteins. Therefore fructose accelerated the production of advanced glycation end-products more than glucose. In addition, fructose enhanced the reactive oxygen or oxygen radical generation and the associated degeneration of proteins and lipids. These actions of fructose appeared to be due to the formation of dicarbonyl compounds such as 3-deoxyglucosone, a highly reactive intermediate product formed in the advanced glycation stage. These results suggest that fructose is closely involved not only in glycation but also in the polyol pathway and peroxidation reactions through free radical formation. Thus, fructose is considered to be a more critical reducing sugar associated with the progression of diabetic complications than it has been thought until now.

Glycation (Maillard reaction), a non-enzymatic binding reaction between protein and sugar, is considered to be a pathogenic factor of diabetic complications, aging, and arteriosclerosis. This reaction has recently attracted attention because of its association with oxidation reactions accompanied by reactive oxygen generation and cleavage reactions of intermediate metabolites (1,2,3). In diabetes mellitus, the polyol pathway is accelerated. Noting fructose is produced in this metabolic pathway, we evaluated the effects of fructose on glycation and associated reactive oxygen generation. In addition, to clarify the role of fructose in the development of diabetic complications, an aldose reductase inhibitor was administered to diabetic rats, and the association between glycation and polyol metabolism was evaluated.

MATERIALS AND METHODS

(1) Experiment examining the effects of fructose on glycation

Type IV collagen (20 mg/ml: Sigma Chemical Co., St. Louis, MO) as a model protein for glycation was incubated with 200 mM glucose or fructose at 37°C in a 200 mM phosphate buffer (pH,7.4) for 4 weeks. As a parameter of advanced glycation end-products(AGEs), the fluorescence intensity of the soluble fraction of glycated collagen was measured. Glycated collagen was digested with 100 units collagenase (Sigma Chemical Co., St. Louis, MO) at 37°C in a 0.02 mM Hepes buffer (pH,7.5) for 48 hours. The supernatant soluble collagen fraction was obtained, and the fluorescence intensity was measured using a
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fluorospectrophotometer at an excitation wavelength of 370 nm and a fluorescence wavelength of 440 nm. In addition, the amount of insoluble collagen fraction was measured, and the percentage (%) of the amount of soluble collagen to total collagen subjected to glycation (sum of soluble and insoluble collagen) was calculated as the digestibility, and used as a parameter of cross-linking by AGEs. The amount of collagen was measured by a hydroxyproline assay (4). 20 mg/ml lysozyme (6× crystallized, from egg white; Seikagaku Kogyo Co., Tokyo, Japan) was also dissolved in a 200 mM phosphate buffer and incubated with 100 mM glucose or fructose at 37°C for 4 weeks, and the degree of protein polymerization was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was conducted on a gradient gel (PAA 4/30; Pharmacia Co., Uppsala, Sweden) according to the method of Weber and Osborn (5). In each experiment with glucose or fructose, 10 mM or 20 mM aminoguanidine (hemisulfate; Sigma Chemical Co., St. Louis, MO) was added, and the degree of glycation inhibition was observed.

(2) Experiment examining oxidation reactions in glycation

Autoxidation of reducing sugar and the formation of reactive oxygen and dicarbonyl compounds

Glucose (100 mM) or fructose (100 mM) was incubated in a 500 mM phosphate buffer under physiological aerobic conditions by a method similar to the above for 7 days, and the amounts of reactive oxygen and dicarbonyl compounds produced by the autoxidation of reducing sugar were observed through the course of time. In addition, 10 mM L-lysine was incubated with 100 mM glucose or fructose by a similar method for 7 days, and the amount of reactive oxygen produced by glycation was observed time after time. The effect of addition of 5 mM aminoguanidine was also observed in the process of similar experiments. Dicarbonyl compounds were measured by the Girard-T reaction (6). The absorbance of the disubstituted compound, which is formed by binding of the two Girard-T reagents to dicarbonyl groups, was measured using glyoxal with a known concentration as a standard at a pH of 9.2 and a maximum absorption wavelength of 325 nm. As reactive oxygen, hydroxyl radicals were measured using benzoic acid as a radical scavenger (7,8). Each sample was incubated with 1 mM benzoic acid, and hydroxylated benzoic acid was measured at an excitation wavelength of 305 nm and a fluorescence wavelength of 410 nm and expressed as the equivalence of salicylic acid.

Experiment examining protein fragmentation in glycation

10 mg/ml crystallized dry bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO) was incubated with 200 mM glucose or fructose under conditions similar to the above in the presence of 100 μM copper ions as a catalyst for 7 days. Protein fragmentation was observed by SDS-PAGE. Similar experiments were performed with the addition of 10 mM aminoguanidine.

Experiment on lipid peroxidation in glycation

Human low density lipoprotein (LDL: 2 mg/ml) was incubated with 100 mM glucose or fructose under conditions similar to the above in the presence of 50 μM iron ions for 32 hours, and LDL peroxidation was observed from time to time. LDL peroxidation reactions were measured using a hemoglobin-methylene blue derivative (9). Human LDL was prepared from nondiabetic subjects as previously described (8).

(3) Experiment examining the association between glycation and the polyol pathway

An aldose reductase inhibitor (ARI: epalrestat; Ono Pharmaceutical Co., Osaka, Japan) was administered to rats, and the erythrocyte fructose concentration and fluorescence intensity of renal cortex collagen as a parameter of AGEs were determined. Male Wistar rats weighing about 300 g were allocated to a normal group (Group 1, n=4), a diabetic group

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(Group 2, n=4), and an ARI-treated diabetic group (Group 3, n=4). In the diabetic group, diabetes mellitus was induced by intravenous injection of 30 mg/kg streptozotocin (STZ; Sigma Chemical Co., St, Louis, MO). In the ARI-treated diabetic group, 50 mg/kg ARI was orally administered daily for 8 weeks to rats with STZ-induced diabetes mellitus. Body weight and blood glucose were measured, and the erythrocyte fructose concentration was determined by the enzyme method. The fluorescence intensity of renal cortex collagen was measured by Monnier's method (10). The renal cortexes of rats were collected, homogenized, washed, degreased, purified, and digested with 200 IU collagenase for 48 hours as described above. The Fluorescence intensity of the supernatant soluble fraction of collagen was measured.

(4) Statistical analysis

Statistical analysis was carried out using Student's t-test.

RESULTS

(1) Effects of fructose on glycation

The fluorescence intensity of the soluble fraction of glycated collagen was significantly higher after incubation with fructose than after incubation with glucose. Addition of aminoguanidine to each sugar inhibited the fluorescence intensity. The digestibility of glycated collagen was almost 100 % without addition of sugar, 86.0 % with glucose, and 15.9 % with fructose. Addition of aminoguanidine to each sugar resulted in recovery of the decreased digestibility (Table I). Concerning lysozyme polymerization evaluated by SDS-PAGE, a dimer was formed in the glucose system and a trimer was formed in the fructose system, showing accelerated polymerization. Addition of aminoguanidine inhibited dimer or trimer formation (Fig. 1). Compared with glucose, fructose enhanced fluorescence intensity, protein cross-linking, and protein polymerization more than glucose. Aminoguanidine inhibited these actions.

TABLE I. Fluorescence intensity and digestibility of glycated collagen after incubation with or without aminoguanidine for 4 weeks.

	C	C+AG	C+G	C+G+AG	C+F	C+F+AG
(n)	(5)	(5)	(5)	(5)	(5)	(5)
Fluorescence intensity (A.U./mg protein)	2.48±0.24	2.47±0.22	6.72±0.48	2.45±0.34	42.76±1.81	3.68±0.82
	└── NS ──┘		└── P<0.01 ──┘		└── P<0.01 ──┘	
	└── P<0.01 ──┘		└── P<0.01 ──┘			
Digestibility (%)	97.9±2.3	98.8±2.4	86.0±3.9	97.2±2.6	15.9±3.1	88.2±5.1
	└── NS ──┘		└── P<0.01 ──┘		└── P<0.01 ──┘	
	└── P<0.01 ──┘		└── P<0.01 ──┘			

C: 20 mg/ml collagen AG: 20 mM aminoguanidine G: 200 mM glucose F: 200 mM fructose

Data are means±S.D.

Four weeks after glycation, the fluorescence intensity of the soluble collagen fraction was significantly higher after incubation with fructose (42.76±1.81 A.U./mg protein) than after incubation with glucose (6.72±0.48 A.U./mg protein). Addition of aminoguanidine to each sugar inhibited the fluorescence intensity after incubation. The digestibility of glycated collagen was almost 100 % without addition of sugar, 86.0 % with glucose, and 15.9 % with fructose. Addition of aminoguanidine to each sugar resulted in recovery of the decreased digestibility.

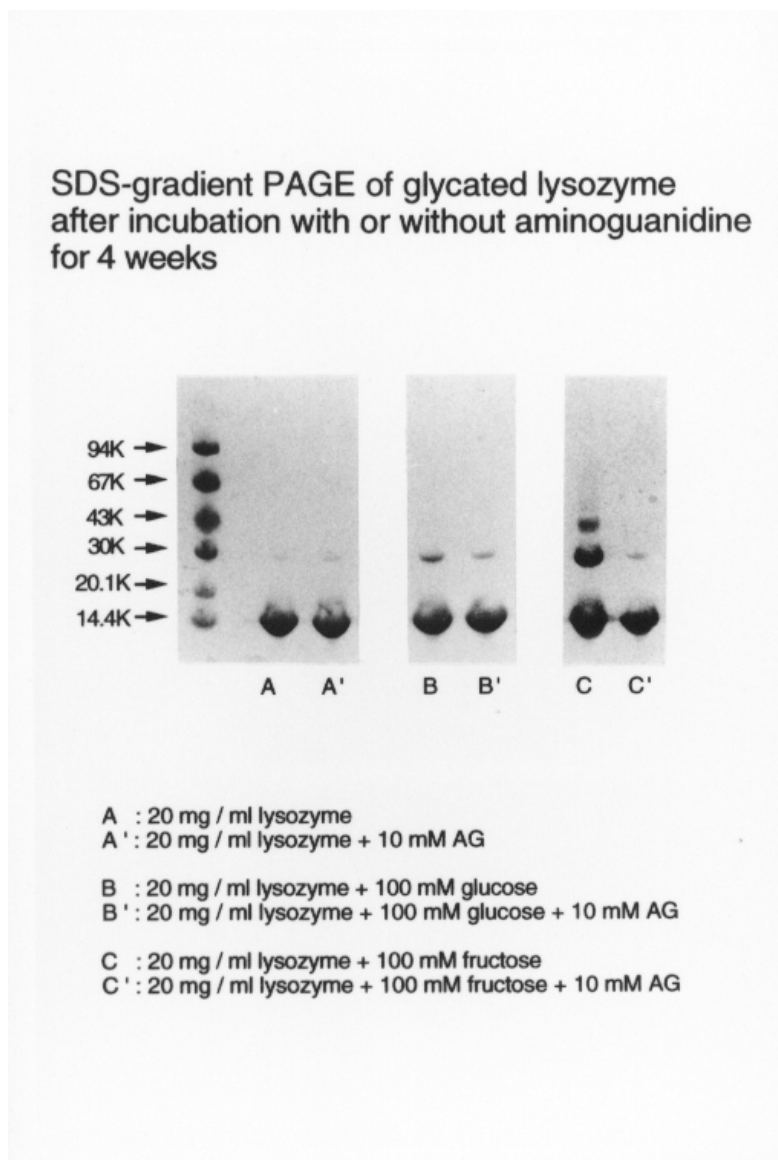


FIG. 1. A dimer was formed after lysozyme incubation with glucose (B-lane), and a trimer was also formed after incubation with fructose, showing accelerated polymerization (C-lane). Addition of aminoguanidine inhibited the formation of the dimer or trimer (B'-lane, C'-lane).

(2) Oxidation reactions in glycation

The autoxidation of each reducing sugar increased the dicarbonyl compounds and hydroxyl radicals with time during the 7-days' incubation. The amounts of dicarbonyl compounds and hydroxyl radicals from fructose were significantly greater than those from glucose (Table II). During 7-days' incubation of L-lysine with glucose or fructose, the amounts of hydroxyl radical formation from fructose were significantly greater than those from glucose. Addition of aminoguanidine to each sugar inhibited hydroxyl radical formation

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(Table III). Analysis of protein cleavage by SDS-PAGE showed more marked BSA fragmentation after incubation with fructose in the presence of copper ions, compared with the case of glucose. Addition of aminoguanidine inhibited this fragmentation (Fig. 2). LDL peroxidation was accelerated by its incubation with glucose or fructose. In particular, fructose more rapidly and markedly accelerated LDL peroxidation than glucose (Fig. 3). These results suggest that fructose produces greater amounts of dicarbonyl compounds and reactive oxygen in both autoxidation and glycation, and more markedly accelerates protein fragmentation and lipid peroxidation than glucose.

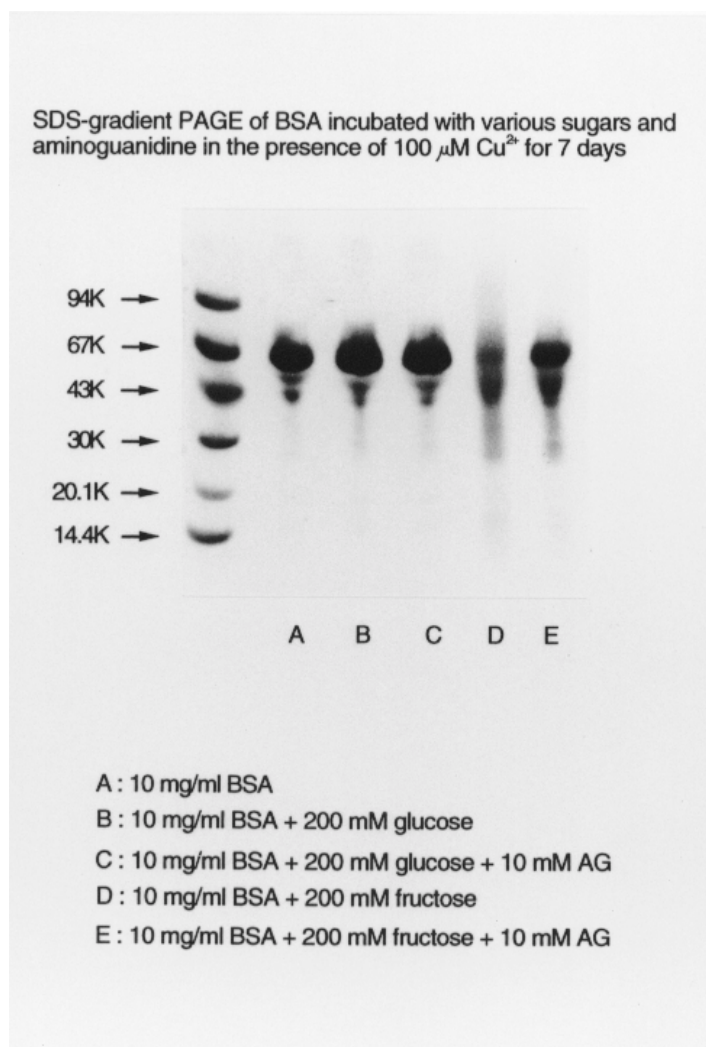


FIG. 2. The degree of protein fragmentation in the presence of copper ions was more marked after incubation of BSA with fructose (D-lane) than after incubation of BSA with glucose (B-lane). Addition of aminoguanidine inhibited fragmentation (E-lane).

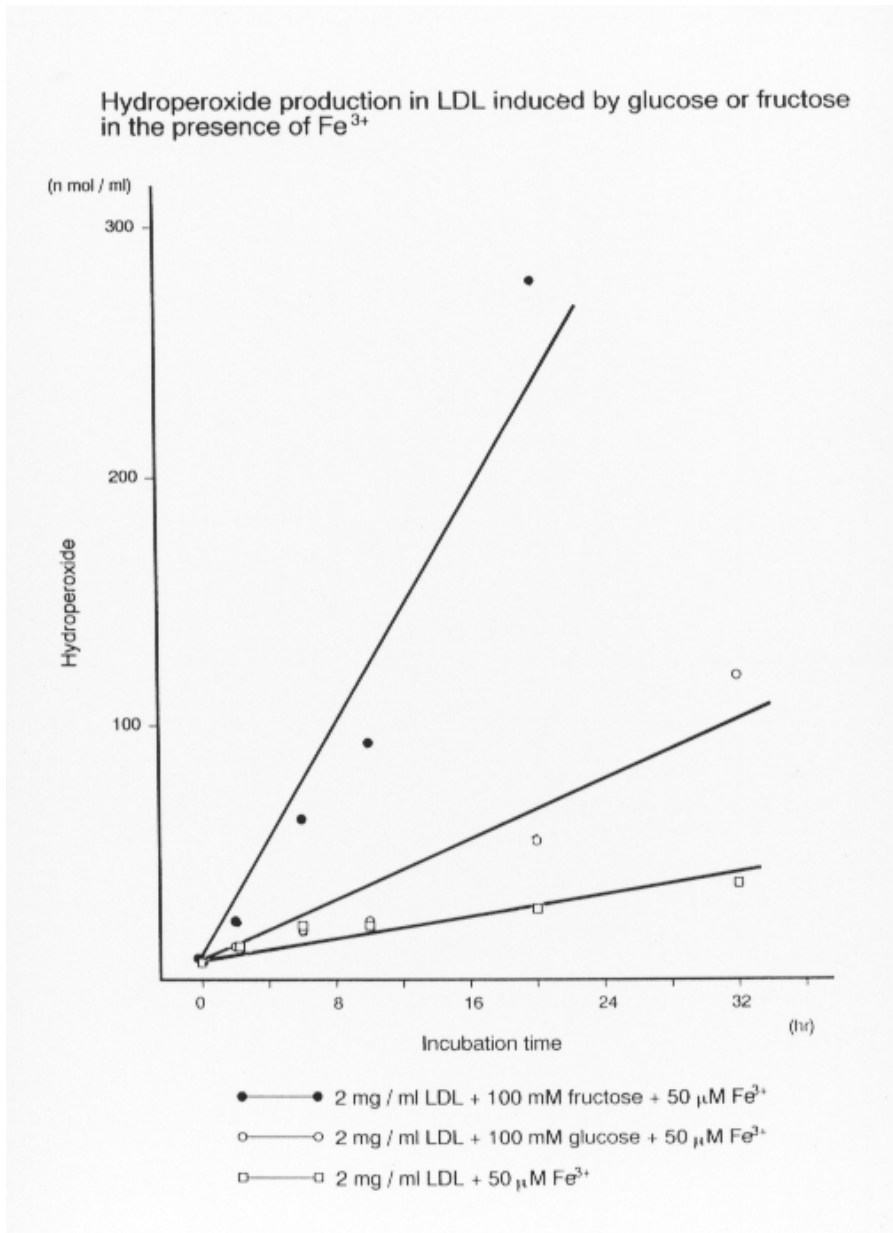


FIG. 3. LDL peroxidation was accelerated by incubation of LDL with glucose or fructose. In particular, more rapid and marked LDL peroxidation was observed in case of fructose.

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TABLE II. Dicarbonyl compounds and hydroxyl radicals generated by autoxidation of glucose or fructose in 100 mM sodium phosphate buffer for 7 days.

		Dicarbonyl compounds (μM)							
(Days)	before		2		4		7		
100 mM glucose (n=5)	4.1 \pm 0.6	⌈	5.1 \pm 1.3	⌈	9.2 \pm 1.5	⌈	27.4 \pm 8.1	⌈	
100 mM fructose (n=5)	4.1 \pm 0.7	⌋	27.1 \pm 5.2	⌋	85.1 \pm 10.8	⌋	197.0 \pm 11.3	⌋	
		NS		P<0.01		P<0.01		P<0.01	

		Hydroxyl radical (μM salicylate)							
(Days)	before		2		4		7		
100 mM glucose (n=5)	0.27 \pm 0.01	⌈	0.35 \pm 0.03	⌈	0.56 \pm 0.06	⌈	0.72 \pm 0.06	⌈	
100 mM fructose (n=5)	0.27 \pm 0.02	⌋	2.07 \pm 0.09	⌋	2.64 \pm 0.11	⌋	6.29 \pm 0.92	⌋	
		NS		P<0.01		P<0.01		P<0.01	

Data are means \pm S.D.

Dicarbonyl compounds and hydroxyl radicals continuously formed by the autoxidation of reducing sugars during 7-days' incubation. The amount of dicarbonyl compounds formation was significantly higher in the case of fructose (197.0 \pm 11.3 μM) than in the case of glucose (27.4 \pm 8.1 μM), and that of hydroxyl radicals was also significantly higher in the case of fructose (6.29 \pm 0.92 μM salicylate) than in the case of glucose (0.72 \pm 0.06 μM salicylate).

TABLE III. Hydroxyl radical generation after incubation of L-lysine with glucose or fructose and aminoguanidine for 7 days.

	L	L+AG	L+G	L+G+AG	L+F	L+F+AG
(n)	(5)	(5)	(5)	(5)	(5)	(5)
Hydroxyl radical (μM salicylate)	5.8 \pm 1.9	6.2 \pm 2.4	45.0 \pm 2.8	32.3 \pm 3.9	125.0 \pm 6.5	32.3 \pm 3.9
	⌈ NS ⌋		⌈ P<0.01 ⌋		⌈ P<0.01 ⌋	
	⌈ P<0.01 ⌋			⌈ P<0.01 ⌋		

L: 10 mM L-lysine AG: 5 mM aminoguanidine G: 100 mM glucose F: 100 mM fructose

Data are means \pm S.D.

The amount of hydroxyl radicals formed by incubation of L-lysine with glucose or fructose over 7-days was significantly higher with fructose (125.0 \pm 6.5 μM salicylate) than with glucose (45.0 \pm 2.8 μM salicylate). Addition of aminoguanidine to each sugar inhibited hydroxyl radical formation.

(3) Association between glycation and the polyol pathway

The blood glucose level was significantly higher in the diabetic group and the ARI-treated diabetic group than in the normal group, but it did not differ between the former 2 groups. The erythrocyte fructose level was significantly higher in the diabetic group than in the normal group, while it was significantly lower in the ARI-treated diabetic group than in the diabetic group. The fluorescence intensity of renal cortex collagen was significantly higher in the diabetic group than in the normal group, while it was significantly lower in the ARI-treated diabetic group than in the diabetic group (Table IV). Thus, compared with the diabetic group, the ARI-treated diabetic group showed a similar glucose level, but decreases in the erythrocyte fructose level and the fluorescence intensity of renal cortex collagen.

TABLE IV. Blood glucose, red cell fructose and fluorescence intensity of renal cortex collagen in normal group, diabetic group and diabetic group treated with ARI for 8 weeks.

	Blood glucose (mg/dl)		Red cell fructose (nmol/g·Hb)		Fluorescence intensity (A.U./mg protein)
Normal group (n=4)	181.1±16.3	P<0.01	164.3±35.9	P<0.05	1.10±0.12
Diabetic group (n=4)	538.3±57.2		576.1±128.5		1.93±0.17
		P<0.01		NS	
Diabetic group + ARI (n=4)	493.6±37.2		223.8±56.3		1.27±0.19
					NS
					P<0.05

Data are means ±S.D.

The blood glucose level was significantly higher in the diabetic group (538.3±57.2 mg/dl) and the ARI-treated diabetic group (493.6±37.2 mg/dl) than in the normal group (181.1±16.3 mg/dl), but did not significantly differ between the former 2 groups. The erythrocyte fructose level was significantly higher in the diabetic group (576.1±128.5 nmol/g·Hb) than in the normal group (164.3±35.9 nmol/g·Hb), but was significantly lower in the ARI-treated group (223.8±56.3 nmol/g·Hb) than in the diabetic group. The fluorescence intensity of renal cortex collagen was significantly higher in the diabetic group (1.93±0.17 A.U./mg protein) than in the normal group (1.10±0.12 A.U./mg protein), but was significantly lower in the ARI treated diabetic group (1.27±0.19 A.U./mg protein) than in the diabetic group.

DISCUSSION

Glycation (Maillard reaction) is a non-enzymatic reaction between reducing sugars and protein amino acid residues. This reaction has been studied as a browning phenomenon in the field of food chemistry. This reaction is a universal reaction also occurring in various proteins *in vivo* and induces changes in protein structure and function, and therefore, has been considered to play an important role in the pathogenesis of diabetic complications, aging, and arteriosclerosis. In glycation, reducing sugars such as glucose bind to amino acid residues of protein, forming reversible Schiff bases, which are converted to relatively stable Amadori compounds by Amadori rearrangement. The process prior to this conversion is the early glycation stage. Amadori compounds repeat complicated dehydration and rearrangement, forming intermediate active substances such as dicarbonyl compounds represented by 3-deoxyglucosone (3DG), which promote progression to the advanced glycation stage. AGEs includes pentosidine (11), crossline (12), pyrraline (13) and carboxy methyl lysine (CML) (14). Pentosidine and CML are produced by oxidation reactions in the advanced stage, while pyrraline is produced by non-oxidation reactions from α -oxoaldehydes such as 3DG (15). Not all AGEs have been identified. This is because most AGEs from inter or intra molecular cross-links, undergo complicated changes such as polymerization and insolubilization, begin to emit fluorescence and finally become brown pigments called melanoidin. In this study, AGEs were measured using the intensity of fluorescence, which is one of their characteristics, as a parameter. In addition, protein polymerization based on cross-linking and changes in enzyme digestibility were also measured.

We studied on the role of fructose in the development of diabetic complications. In the pathogenesis of diabetic complications, the polyol pathway is important. Noting fructose produced in the polyol pathway, we experimentally observed the effects of fructose on glycation and oxidation reactions and evaluated the role of fructose in the pathogenesis of diabetic complications. In case of comparing fructose with glucose in the effects on glycation,

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fructose more markedly enhanced the fluorescence of glycated collagen, and reduced the digestibility of collagen by collagenase from 86.0 % to 15.9 % which suggested closer involvement of fructose rather than glucose in cross-linking. SDS-PAGE showed greater promotion of lysozyme polymerization by fructose compared with glucose, suggesting the marked effects of fructose on the production of AGEs. These findings can be theoretically explained as follows. As a reducing sugar, fructose has 300 times as many highly reactive chain structures as glucose (16). In addition, fructose differs from glucose in the pathway for the formation of dicarbonyl compounds such as highly reactive 3-deoxyglucosone (3DG), which are intermediates in the advanced glycation stage. The primary pathway for 3DG formation from glucose is a reaction between glucose and amino acid residues, resulting in the formation of Amadori compounds, from which 3DG is produced. Fructose is converted to fructose- 3-phosphate by phosphorylation, from which 3DG is formed (17). In addition, Shin et al. showed another pathway that proceeds even in the absence of protein, i.e., the direct production of 3DG by the autoxidation of fructose without the formation of Amadori compounds (18). It has also been suggested that dicarbonyl compounds such as 3DG and methylglyoxal are directly produced from fructose by cleavage from fructose to triose or catabolism of ketone bodies (19). These may be associated with high reactivity of fructose in glycation.

Recently, carbonyl stress in diabetic complications such as renal failure has been considered to be important. Fructose enhances carbonyl stress (20). Speculating that the formation of highly reactive dicarbonyl compounds is a major factor associated with acceleration of AGEs production, we evaluated the autoxidation of reducing sugars and associated reactive oxygen generation. AGEs accumulate in long-lived proteins such as collagen. These end stage products, therefore may be more closely related to the degradation of tissue proteins than the early stage products of glycation. On the other hand, the rate of sugar autoxidation is short in the presence of transition metals ions, and the amounts of α -ketoaldehydes and oxidizing agents such as hydroxyl radicals formed over the typical time courses of in vitro glycation studies (days to weeks) (1). In the autoxidation of reducing sugars under an aerobic conditions, fructose produced greater amounts of dicarbonyl compounds and hydroxyl radicals than glucose. Glycation of L-lysine by glucose resulted in greater amounts of hydroxyl radicals than autoxidation of glucose. Fructose produced greater amounts of dicarbonyl compounds and reactive oxygen than glucose in both glycation and autoxidation. Wolff et al. reported that glucose is auto-oxidized in the presence of transition metal ions and oxygen, producing dicarbonyl compounds via endiol, and generating superoxides in this process (1). Fructoselysine, which is an Amadori compound formed by glycation of lysine residues by glucose, undergoes advanced glycation and produces AGEs. In oxidation reactions in this process, fructoselysine produces endiol as in the autoxidation of reducing sugars, forming dicarbonyl compounds such as 3DG. In this process, superoxides are also generated, converted to H_2O_2 due to superoxide dismutase activity, and produce highly reactive hydroxyl radicals in the presence of metal ions. These results suggest that there is a common basis for glycation and oxidation. In particular, the production of dicarbonyl compounds such as highly reactive 3DG and the associated production of reactive oxygen may degenerate proteins, lipids and nucleic acids, resulting in damaging proteins, enzymes, cells, and tissues in the body (21,22). In this study, the glycation of BSA by glucose or fructose in the presence of metal ions resulted in protein fragmentation. Protein fragmentation was more marked after incubation with fructose rather than glucose. LDL peroxidation was observed in the presence of both glucose and fructose, but earlier and more marked peroxidation occurred in the presence of fructose. Malondialdehyde formed by lipid

peroxidation may increase collagen cross-linking (23). Fructose produces a great amount of reactive oxygen in the glycation process and caused marked lipid peroxidation.

Furthermore we studied the relationship of glycation and the polyol pathway. After ARI administration to diabetic rats, the association between polyol metabolism via fructose and the formation of AGEs was evaluated. Diabetic rats showed the increases in the erythrocyte fructose level and the fluorescence intensity of renal cortex collagen, which were inhibited by ARI administration. These results suggest that fructose increases in the tissue level in the polyol pathway, and produce more AGEs. Therefore, when the activity of the polyol pathway in tissue is enhanced, the reaction between fructose and tissues protein may readily occur. Therefore, fructose produced in the polyol pathway appears to be a critical reducing sugar involved in the production of AGEs.

Aminoguanidine has attracted attention as an inhibitor of glycation (24,25). In this study, the effects of aminoguanidine on glycation and oxidation were also experimentally evaluated. Aminoguanidine inhibited the production of AGEs and reactive oxygen in the glycation. Furthermore this drug inhibited the oxidative cleavage of protein in the presence of metal ions. We previously reported that highly reactive intermediate metabolites, such as 3DG, in the advanced glycation stage are important as the site of aminoguanidine action in glycation (26). Aminoguanidine may also affect oxidation reactions occurring in the production process of these intermediate metabolites, inhibiting reactive oxygen generation.

Our experiments mainly on glycation suggest that fructose more markedly accelerates the advanced glycation stage and enhances oxidative stress than glucose. In the pathogenesis of diabetic complications, not only glycation but also the polyol pathway and oxidation reactions due to free radical formation may closely involved. Fructose may be a critical reducing sugar that determines the relationships among these factors. Aminoguanidine inhibited not only glycation but also oxidation reactions, therefore this drug could play an important role in the prevention of diabetic complications.

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