Fructose Ingestion Enhances Atherosclerosis and Deposition of Advanced Glycated End-products in Cholesterol-fed Rabbits

Yoshihisa Tokita, Yoshitake Hirayama, Akihiro Sekikawa, Hidetoshi Kotake, Takayoshi Toyota, Teruo Miyazawa, Takashi Sawai, and Shinichi Oikawa

1 Division of Molecular Metabolism and Diabetes, Tohoku University Graduate School of Medicine, Sendai, Japan. 2 Laboratory of Biodynamic Chemistry, Graduate School of Life Science and Agriculture, Tohoku University, Sendai, Japan. 3 Department of Pathology, School of Medicine, Iwate Medical University, Morioka, Japan. 4 Division of Endocrinology and Metabolism, Department of Medicine, Nippon Medical School, Tokyo, Japan.

This study was performed to investigate whether the plasma concentration of phosphatidylcholine hydroperoxide (PCOOH), which is a marker of oxidized stress in the blood, increased in cholesterol-fed rabbits, and fructose ingestion promoted this process and aggravated atherosclerosis. Male Japanese white rabbits (age: 12 weeks, and body weight: around 2.0 kg, n = 15) were divided into three groups, (1) a NN group as a normal control fed a standard diet (n = 5), (2) a CN group fed 1.0% cholesterol, and (3) a CF group given both 1.0% cholesterol and 10% fructose-containing tap water. During 8 weeks, plasma PCOOH levels increased significantly in the CN and CF groups compared to the NN group and fructose further raised the PCOOH level. The atherosclerosis was significantly promoted and the deposition of advanced glycation end products (AGEs) was marked in the CF group compared to the CN group. Fructose worsened the atheromatous lesions caused by cholesterol feeding. The mechanism is most likely through lipid peroxidation, which was increased by cholesterol feeding-induced hyperlipidemia, and the formation of AGEs. J Atheroscler Thromb, 2005; 12: 260–267.

Key words: Fructose, AGE, PCOOH, Oxidative stress

Introduction

It has been suggested that oxidized low-density lipoprotein (oxLDL) plays a pivotal role in foam cell formation (1–3). The detection of oxLDL in atheromatous lesions implicates oxLDL in atherogenicity (4). Such a role for oxLDL is supported by studies in vivo using probucol, a strong anti-oxidant, which protected the aorta from atherosclerosis by reducing oxLDL formation (5, 6). Moreover, oxLDL can be detected in blood serum as a result of oxidative stress in vivo (7, 8). Oxidative stress may be augmented in some metabolic disorders such as hyperlipidemia and diabetes mellitus. Recently, chemiluminescence-high performance liquid chromatography (CL-HPLC) has been used to assess the level of oxidative stress by measuring phosphatidylcholine hydroperoxide (PCOOH) (9–11). We have previously reported the presence of high PCOOH levels in venous blood of aged subjects and patients with primary hyperlipidemia (12). Phospholipids coat the surface of lipoproteins and may be the initial target of oxidative stress reactions causing lipid peroxidation and subsequent hyperlipidemia. High plasma PCOOH levels were found in diabetic patients even in those without hyperlipidemia and vascular complications (13). It is thought that hyperglycemia is related to oxidative stress (14) via glycation (15, 16).

As mentioned above, lipid peroxidation would occur during hyperlipidemia and glycation. Glycation is induced
when glucose concentrations are high. Furthermore, another reduced sugar known to induce strong glycation is fructose (17–20). Therefore, it is expected that lipid peroxidation as a result of oxidative stress is increased in vivo by cholesterol feeding and ingestion of fructose.

In the present study, we assessed atherosclerosis in the aorta and measured plasma POOH levels in rabbits fed a high cholesterol diet. The aim of the study was to determine whether plasma PCOOH levels and severity of atherosclerotic lesions are modified or exaggerated by fructose ingestion.

Materials and Methods

Materials

Fructose was purchased from Wako Pure Chemical Industries (Tokyo, Japan). L-α-phosphatidylcholine, β-oleyl-γ-palmitoyl (C18:1, cis-9/C16:0) was purchased from Sigma (St. Louis, MO, USA). For immunostaining, we obtained commercially available monoclonal antibodies against pyrraline, pentosidine and carboxymethyl lysine (CML) from Kumamoto Immunochemical Laboratory Co., (Kumamoto, Japan). A Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) was purchased from Wako Pure Chemical Industries. All other chemicals were of the best grade available from commercial sources.

Experimental design

The experiments were carried out on male Japanese white rabbits (age: 12 weeks, and body weight: around 2.0 kg, n = 15). Rabbits were housed in individual cages in an air-conditioned room under a 12/12 h light/dark cycle. They were randomly divided into three groups. The first group represented the normal control and no-cholesterol (NN) group fed a standard diet (n = 5). The other two groups of rabbits were fed a standard diet supplemented with 1.0% cholesterol for 8 weeks. In one of these cholesterol groups, tap water containing 10% fructose was provided instead of plain drinking water for 8 weeks. Rabbits on the cholesterol diet alone were designated the CN (cholesterol, non-fructose) group (n = 5), and the other group drinking 10% fructose water was designated the CF (cholesterol-fructose feeding) group (n = 5). All rabbits were given the experimental diet at 100 g/day for 8 weeks, and had free access to water.

Blood samples were obtained from the ear vein every 4 weeks after overnight fasting and collected into tubes containing 2 mM EDTA and kept on ice. Plasma was separated from whole blood by centrifugation at 3,000 rpm for 15 min at 4°C and stored at 4°C for lipid analysis. For the measurement of lipid peroxide, plasma was stored at –80°C and used within 2 weeks. At the end of the feeding period, rabbits were sacrificed by intravenous injection of pentobarbital sodium (50 mg/kg body weight). The aorta was excised from the aortic arch for histological and lipid analyses. Aorta samples were immediately frozen in dry ice and stored at –80°C prior to use.

Lipid analysis

Plasma total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and triglyceride (TG) levels were analyzed by enzymatic assay, using kits purchased from Wako Pure Chemical Industries and the phospholipid (PL) concentration was determined by the colorimetric method of Bartlett (21). In the case of the aorta, within 24 h after its preparation, the aorta was homogenized with four volumes (w/v) of 0.15 M NaCl. The total lipids were extracted from tissue homogenates with a mixture of chloroform/ methanol (2:1, v/v) containing 0.002% (w/v) butylated hydroxytoluene (BHT) as an antioxidant according to Folch’s procedure (22). As with plasma, TC, TG and PL levels in the aorta were determined by enzymatic assay.

Measurement of phospholipid hydroperoxides

Plasma and aorta concentrations of phosphatidylcholine hydroperoxide (PCOOH) were measured by the method of CL-HPLC as described previously (9–11). The phosphatidylethanolamine hydroperoxide (PEOOH) level in the aorta was studied. PEOOH levels were not measured in the present study, because PEOOH is present in plasma membrane as oxidized PE and detected in the tissue or cells, but not in the blood stream. Briefly, an aliquot of the total lipid sample from plasma and aorta was subjected to CL-HPLC. The HPLC column was a JASCO Finepak SIL-NH2-5 [250 × 4.6 mm (i.d.), 5 µm particle size; JASCO] with a mixture of 2-propanol-methanol-water (26:9:5, v/v/v), and the flow rate was maintained at 1.0 ml/min by a JASCO 880-PU pump. The hydroperoxide-specific post-column chemiluminescence reagent was a mixture of 2.5 µg cytochrome c and 0.2 µg luminol in 1 ml of 50 mM borate buffer delivered at a flow rate of 1.8 ml/min by a JASCO 880-PU pump. The chemiluminescence detector CLD-100 (Tohoku Electronic Industries) was used for post-column detection. Phospholipid hydroperoxide concentrations were quantified by comparison with standards. L-α-phosphatidylcholine, β-oleyl-γ-palmitoyl (C18:1, cis-9/C16:0, Sigma) was used as a standard for phospholipids. PCOOH and PEOOH levels in aorta were expressed as mmol/mol PL of the aorta.

Analysis of thiobarbituric acid reactive substances (TBARS)

TBARS were analyzed by the method of Yagi, using 1,1,3,3-tetramethoxypropane as a standard (23). After precipitation of the lipids from 10 µl of plasma using 0.5 ml of 10% phosphotungstate, the precipitate was mixed with 2 ml of N/12 H2SO4 and 0.3 ml of 10% phosphotungstate, and the mixture was centrifuged at 3,000 rpm.
for 10 min. The precipitate was suspended in 4.0 ml of distilled water and 1.0 ml of 0.67% sodium thiobarbiturate. The reaction mixture was heated at 95°C for 60 min in a water bath. The reaction product was extracted from the solution with 5 ml of n-butanol. After centrifugation at 3,000 rpm for 10 min, the fluorescence of the extracted solution was analyzed by spectrofluorometry (Ex: 515 nm, Em: 553 nm). In the case of aorta, TBARS were analyzed by the methods of Ohkawa et al. (24). To 0.1 ml of 20% tissue homogenate (w/v), we added 0.2 ml of 8.1% sodium dodecylsulfate, 1.5 ml of 20% acetate buffer (pH 3.5), 1.5 ml of 0.8% sodium thiobarbiturate and 0.7 ml of distilled water. The mixture was incubated in a water bath at 100°C for 60 min. After cooling, TBARS were extracted with a mixture of n-butanol and pyridine (15:1, v/v), and the absorbance of the extracted solution was analyzed at 532 nm.

**Histological analysis of aortic atherosclerotic lesions**

The aortic arch (just 1 cm from the heart) was removed, and was washed with 0.15 M NaCl. Each specimen was fixed overnight in a 50% formalin aqueous solution, and embedded in paraffin. The embedded samples were cut 5 μm thick with a microtome. Slices were made at 5 μm intervals. Each section of aortic arch was stained with eosin and the nuclei were stained with hematoxylin by standard methods. Elastica Masson staining was performed using standard methods in preparation for a morphometric analysis. As shown in Fig. 1, each area (A, B, and C) was measured on the computer using NIH image analysis software. The intima-to-media ratio (B/C) and intima-to-lumen ratio (B/A) were calculated to determine the intimal thickening. The mean value obtained form five sections was calculated for one rabbit.

**Fig. 1. Morphometry of atherosclerotic lesions.**

Each area (A, B, and C) was measured on a computer using NIH image analysis software. Intima-to-media ratio (B/C) and intima-to-lumen ratio (B/A) were calculated to determine intimal thickening. A: area of vessel lumen, B: area of intimal layer, C: area of medial layer.

For immunohistological analysis, aortic arch specimens were fixed in O.C.T. compound, snap-frozen in liquid nitrogen, and stored at −80°C prior to use. For the detection of pyrraline, pentosidine, and CML, the avidin-biotin complex method was used. Specimens were rinsed with phosphate-buffered saline (PBS) and then normal horse serum diluted with PBS (1:10) was added for 30 minutes. After this serum had been removed, specimens were incubated with 50 µl of each antibody solution (diluted to 1:400) including monoclonal antibodies against pentosidine, pyrraline, and CML for 12 hours at 4°C. The sections were rinsed with PBS three times and incubated with H2O2-methanol (1:100) for 20 minutes. Anti-mouse antibody solution (50 µl, diluted to 1:50) was added for 30 minutes, and ABC reagent was added for 30 minutes. After being rinsed with PBS, sections were incubated with DAB and nuclei were stained with methyl green. The sections were visualized with DAB and nuclei were counterstained with methyl green.

**Statistical analysis**

Data are presented as the mean ± SEM. Differences between groups were examined for statistical significance using the ANOVA test (StatView® v4.5). A p value less than 0.05 denoted the presence of a statistically significant difference.

**Results**

**Changes in plasma lipids and PCOOH concentration**

There were no significant differences in body weight among the three groups over the 8-week period (mean body weight was 2.0 kg at the start and 2.6 – 2.8 kg at the end of the experiment). Changes in plasma lipids are
Fructose Ingestion and Atherosclerosis

The concentration in plasma of TC, TG, PL and HDL-C significantly increased after 1.0% cholesterol feeding. TG levels were significantly higher in the CF group than the CN group.

Time-dependent increases in PCOOH levels were also observed, which were different from the changes in TBARS levels. At 8 weeks, PCOOH levels were significantly greater in the CF group than in the CN group (Fig. 3).
Immunohistochemical study of aortas

Immunostaining for AGEs including pentosidine, pyrraline, and CML was performed in the aortas of each group (Fig. 9). Cholesterol feeding exaggerated the atheromatous lesions and induced the deposition of AGEs in the lesions. Pentosidine, pyrraline, and CML were detected around foam cells. The area and density of AGE staining was increased in the CF group. Further analysis showed that the severity of atherosclerotic lesions paralleled the level of AGE deposition.

Histological findings

Cross-sections of aortic arch are shown in Fig. 4. No changes were found in the NN group, whereas marked atherosclerotic lesions were observed in the CN and CF groups, with the degree of atherosclerosis being greater in the CF group. Morphometric analysis was also applied to clarify the significance of intima thickening. The ratios of the areas of intima/media and intima/lumen were calculated and presented in Fig. 5. The intima/media (CN: 0.170 ± 0.013, CF: 0.268 ± 0.017) and intima/lumen (CN: 0.114 ± 0.007, CF: 0.156 ± 0.007) ratios were significantly higher in fructose-fed rabbits than the CN group.

Lipid composition of aorta

The lipid contents of the aorta in each group are shown in Fig. 6. Cholesterol feeding significantly increased tissue cholesterol and triglyceride concentrations, but did not alter phospholipid levels. These changes were enhanced by ingestion of fructose. To assess lipid peroxidation, we measured phospholipid hydroperoxide levels. As shown in Fig. 7, cholesterol increased PCOOH and PEOOH levels in the aorta and fructose further increased the levels of these hydroperoxides. Similar to PCOOH levels, levels of TBARS in the aorta were significantly increased in CF rabbits compared with the CN group (Fig. 8).

Fig. 4. Atherosclerotic lesions in the rabbit aorta.
Elastica Masson staining (∗10). Calibration bar = 300 µm.

Fig. 5. Intimal thickness of atherosclerotic lesions.
Data are the mean ± SEM (n = 5). The ratios of intima/media and intima/lumen were significantly (p < 0.01 and p < 0.02, respectively) higher in the CF group than CN group.

Fig. 6. Lipid content of the aorta.
Data are the mean ± SEM (n=5). *p < 0.05 vs. NN, **p < 0.05 vs. CN.
The major findings of the present study were (i) cholesterol feeding in rabbits increased PCOOH levels in both plasma and aorta, (ii) ingestion of fructose with cholesterol feeding (CF group) increased PCOOH levels in plasma and aorta more than cholesterol feeding alone (CN group), and (iii) fructose worsened the atherosclerotic lesions and induced marked deposition of AGEs in the lesions caused by cholesterol feeding. Chajara et al. (25) reported that fructose administration induced an insulin-resistant condition and was associated with increased levels of hyaluronan, hyaluronidase production and hyaluronan degradation in the damaged rat aorta. In
the present study, our histological findings clearly indicate a worsening of lesions and marked deposition of AGEs in the atheroma of the CF group compared to the CN group.

We have previously shown high levels of PCOOH in plasma in primary hyperlipidemia (12). The increase in PCOOH levels did not differ among the various phenotypes of hyperlipidemia. Dietary hyperlipidemia as shown in the present study also increased plasma PCOOH levels. These results indicate that the increases in lipoprotein particle number and lipid mass relate to increases in PCOOH levels.

Fructose ingestion increased PCOOH levels. It has been shown that fructose administration increased glycation and produced AGEs (17–20), and the process could trigger lipid peroxidation (26, 27). In our previous study, serum PCOOH levels increased in diabetic patients and this increase correlated with the level of HbA1c, a marker of glycation (13). The increase in PCOOH levels in cholesterol-fed rabbits (CN group) would be exaggerated by glycation induced by fructose ingestion as shown in the CF group. The difference in PCOOH levels between the CN and CF groups was not substantial. These results indicate that plasma PCOOH levels are strongly dependent on lipid mass or lipoprotein particle number. Therefore, patients with hyperlipidemia might be exposed to oxidative stress via lipid peroxidation as shown by the relationship between hyperlipidemia and oxidation products (28), and be in the process of developing atherosclerotic diseases through changes to the vascular antioxidant system (29).

Piotrowski et al. (30) have shown that human atherosclerotic lesions contain peroxidized phosphatidylcholine as a major lipid peroxide. Our study demonstrated similar results in that the atherosclerotic aorta contained large amounts of PCOOH, and the contents correlated with the extent of the lesions (Fig. 7). Fructose ingestion increased the extent of atheromatous lesions and induced the deposition of AGEs in the atheromatous area in the cholesterol-fed rabbits (CF group) compared to the rabbits fed cholesterol alone (CN group). The deposition of AGEs in human atherosclerotic lesions has been reported previously (31, 32), and is expected to be enhanced by fructose-induced glycation. Fructose ingestion alone did not influence plasma PCOOH levels or result in any histological changes of the aorta (data not shown). Therefore fructose may act as a modifie of atherogenic lipoproteins and atheroma lesions. But the ability of fructose to worsen the lesions and to induce deposition of AGEs might be dependent on hypercholesterolemia.

In conclusion, we have demonstrated that the ingestion of fructose induced an exaggeration of atheromas in the aortas of cholesterol-fed rabbits. The mechanism is most likely through lipid peroxidation, which was increased by hyperlipidemia and AGE formation. At present, it is not clear whether the deposition of AGEs was the cause or the result of lipid peroxidation.

Acknowledgements: This work was supported by Health Sciences Research Grants from the Ministry of Health, Labor and Welfare, and grants from the Japanese Ministry of Science, Education and Culture (13671202).

References


