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Anti-atherogenic property of ferulic acid in apolipoprotein E-deficient mice fed Western diet: Comparison with clofibrate

E.Y. Kwon [a], G.M. Do [a], Y.Y. Cho [a], Y.B. Park [b], S.M. Jeon [a], M.S. Choi [a,*]

[a] Department of Food Science and Nutrition, Center for Food and Nutritional Genomics, Kyungpook National University, Daegu 702-701, South Korea
[b] School of Life Science and Biotechnology, Kyungpook National University, Daegu 702-701, South Korea

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ABSTRACT

Anti-atherogenic effect of ferulic acid (0.02%, w/w) was investigated in comparison with the clofibrate (0.02%, w/w) in apolipoprotein E-deficient (apo $E^{-/-}$) mice fed Western diet. Concentrations of total cholesterol (total-C), apolipoprotein B (apo B) in the plasma and epididymal adipose tissue weight were significantly lower in the ferulic acid and clofibrate supplemented groups compared to the control group. The ratio of apo B to apo A-I was also significantly lower in those groups than in the control group. Activities of hepatic ACAT and HMG-CoA reductase were only significantly lower in the ferulic acid and clofibrate groups, respectively than in the control group. The numbers of mice that exhibited aortic fatty plaque were 8/10 in control groups vs. 0/10 in the ferulic acid or clofibrate group. The activities of anti-oxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and paraoxonase) in the hepatocyte and erythrocyte were significantly higher in the ferulic acid group than in the control group. In contrast, hepatic TBARS level was only markedly lower in the ferulic acid group. These results provide a new insight into the anti-atherogenic property of ferulic acid in the apo $E^{-/-}$ mice fed a Western diet.

1. Introduction

Oxidative stress plays a crucial role in the development of atherosclerosis by oxidation of low-density lipoproteins (LDL) that subsequently lead to formation of foam cells (Carew, 1989). Conversely, high-density lipoproteins (HDL) are a well-known anti-oxidant that prevents atherosclerosis (Barter and Rye, 1996). Paraoxonase (PON) is a calcium-dependent esterase closely associated with HDL containing apolipoprotein A-I (apo A-I) that has been reported to confer anti-oxidant properties on HDL by decreasing the accumulation of lipid peroxidation products (Mackness et al., 1991). The PON hydrolyzes paraoxon, the toxic metabolite of organophosphate anticholinesterases, whose presence has also been suggested in the pathogenesis of atherosclerosis (Mackness et al., 1993a). A decreased PON activity has been documented in patients with myocardial infarction (McElveen et al., 1986) and consumption of anti-oxidants also enhances serum PON activity (Hayek et al., 1997).

Phenolics are widely distributed in the plant kingdom and are therefore an integral part of the diet, with significant amounts being reported in vegetables, fruits and beverages (Luximon-Ramma et al., 1996). Although the dietary intake of phenolics varies considerably among geographic regions, it is estimated that daily intake ranges from about 20 mg to 1 g, which is higher than that for vitamin E (Hollman and Katan, 1998).

Belonging to the phenolics, ferulic acid is a ubiquitous plant constituent that arises from the metabolism of phenylalanine and tyrosine. Due to its phenolic nucleus and an extended side chain conjugation, ferulic acid readily forms a phenoxy radical which accounts for its potent anti-oxidant potential. Ferulic acid has been shown to potentially exert several beneficial effects on health. For example, it acted as a peroxyl radical scavenger and increased the resistance of LDL to oxidation (Castelluccio et al., 1995) and protected against some chronic diseases such as diabetes (Balasubashini et al., 2004), Alzheimer's (Yan et al., 2001), colon and breast cancers (Hudson et al., 2000) and atherosclerosis (Hiramatsu et al., 1990).

One of cardiovascular drugs, fibrates, exhibits antilipidemic activity by reducing production of very low-density lipoproteins (VLDL), and by increasing VLDL clearance due to their stimulation of lipoprotein lipase activity. These drugs often reduce the plasma concentration of triglycerides (TG), whilst raising the HDL-cholesterol concentration (Zimetbaum et al., 1991). For these reasons, fibrate compounds can be used as one of positive control for lipid-lowering agents in human and animal trials.

Accordingly, the aim of the present study was to investigate how ferulic acid affects the cholesterol and anti-oxidant

* Corresponding author. Tel.: +82 53 950 6232; fax: +82 53 958 1230.
E-mail address: mschoi@knu.ac.kr (M.S. Choi).

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metabolism compared to the clofibrate in an apolipoprotein E-deficient (apo $E^{-/-}$) mice fed Western diet. These particular mice were shown to be an excellent atherogenic model since they become hyperlipidemic even with a low fat diet and develop complex atherosclerotic lesions (Palinski et al., 1994).

2. Materials and methods

2.1. Animals and diets

Thirty-four-weeks-old male apo E-deficient mice (C57BL/6J background) that were homozygous for the disrupted apo E gene were purchased from Jackson Laboratories (Bar Harbor, ME, USA). The mice were all treated in strict accordance with Kyungpook National University guidelines for the care and use of laboratory animals. The animals were individually housed in stainless steel cages in a room at 22 ± 2 °C on a 12 h light–dark cycle and fed a pelletized commercial chow diet for 2 weeks after arrival. Then, the animals were randomly divided into three groups and fed supplemented powder type of Western diet containing 21% milk fat, 0.15% cholesterol for 15 weeks with two of the groups receiving 0.02% (w/w) ferulic acid (Sigma Chemical Co.) or 0.02% (w/w) clofibrate (Sigma Chemical Co.). The pellet type of Western diet was purchased from DYETS Inc. (Pennsylvania, USA), then grounded and mixed with the ferulic acid or clofibrate. The diets were prepared every week and stored in dark at −4 °C. All mice were given free access to food and distilled water and the food consumption and body weight gain were measured every day and once a week, respectively. At the end of the experimental period, the mice were anesthetized with Ketamine after a 12 h fast and blood samples taken from the inferior vena cava into the heparin-coated tube. The plasma and erythrocyte were obtained by centrifuging the blood at 1000*g* for 15 min at 4 °C. The livers were removed, rinsed, and immediately stored at −70 °C.

2.2. Preparation of samples

After centrifugation of blood, the plasma and buffy coat were carefully removed. The separated cells were then washed three times by resuspension in a 0.9% NaCl solution followed by centrifugation. The washed cells were lysed in an equal volume of water and then mixed thoroughly. The hemoglobin concentration was estimated in an aliquot of the hemolysate, using a commercial assay kit (No. 525-A, Sigma, Chemical Co.). An appropriate dilution of the hemolysate was then made from the erythrocytes suspension by the addition of distilled water prior to estimation of the catalase (CAT), glutathione peroxidase (GSH-Px) and glutathione reductase (GR) activities. In order to remove the hemoglobin by precipitation with chloroform:ethanol (McCord and Fridovich, 1969), 0.2 ml of an ethanol:chloroform (3:5, v/v) mixture was added to an aliquot (0.5 ml) of the hemolysate cooled in ice. This mixture was stirred constantly for 15 min and then diluted with 0.1 ml of water. After centrifugation for 10 min at 1600*g*, the pale yellow supernatant was separated from the protein precipitate and used to assay the superoxide dismutase (SOD).

Fraction for enzyme source in the liver was prepared according to the method developed by Hulcher and Oleson (1973) with slight modifications. A 20% (w/v) homogenate was prepared in a buffer containing 0.1 mol/L triethanolamine. 0.02 mol/L EDTA and 2 mmol/L DTT (pH 7.0), then centrifuged at 600*g* for 10 min to discard any cell debris, and the supernatant centrifuged at 10,000*g* followed by 12,000*g* for 20 min at 4 °C to remove mitochondrial pellets. Thereafter, the supernatant was ultracentrifuged twice at 100,000*g* for 60 min at 4 °C to obtain the cytosolic supernatant. The mitochondrial and microsomal pellets were then redissolved in 800 μL of a homogenization buffer and the protein content was determined by the Bradford method (1976) using BSA as the standard.

2.3. Plasma lipids

Plasma total cholesterol (total-C), HDL-cholesterol (HDL-C) and triglyceride (TG) were determined using an enzymatic method (Sigma Diagnostics, St Louis, MO, USA). Plasma apolipoprotein A-I and B concentrations were also measured using an immunoassay method (Nitto Boseki Co. Ltd, Japan).

2.4. Hepatic 3-hydroxy-3-methylglutary CoA (HMG-CoA) reductase and acyl coenzyme A: cholesterol acyltransferase (ACAT) activities

The microsomal HMGR activities were measured with [^{14}C]-HMG-CoA as the substrate, based on a modification of the method of Shapiro et al. (1974). The micromic ACAT activities were determined using [^{14}C]-oleoyl CoA according to the method of Gillies et al. (1986).

2.5. Anti-oxidant enzyme activities

Superoxide dismutase activity was spectrophotometrically measured using a modified version of the method developed by Marklund and Marklund (1974). One unit was determined as the amount of enzyme that inhibits the oxidation of pyrogallol by 50%. Catalase activity was measured using Aebi's method, (1974) with slight modifications, in which the disappearance of hydrogen peroxide was monitored spectrophotometrically for 5 min at 240 nm. A molar extinction coefficient of 0.041 mM^{-1} cm^{-1} was used to determine CAT activity. GSH-Px activity was measured using Paglia and Valentine method (1967) with a slight modification. The reaction mixture contained 1 mM of glutathione, 0.2 mM of NADPH, and 0.24 units of glutathione reductase in 0.1 M Tris–HCl (pH 7.2) buffer. The reaction was initiated by adding 0.25 mM of H_2O_2 and the absorbance was measured for 5 min at 340 nm. GR activity was determined using the method of Pinto and Bartley (1969) by monitoring the oxidation of NADPH at 340 nm. The reaction mixture contained 1 mM EDTA and 1 mM GSSG in a 0.1 M potassium phosphate buffer (pH 7.4).

2.6. Lipid peroxidation levels and paraoxonase activity

Erythrocyte samples were mixed with 5% trichloroacetic acid (TCA) and 60 mmol/L thiobarbituric acid (TBA). After incubation at 80 °C for 90 min, the supernatants were centrifuged at 1000*g* for 15 min at 4 °C, and the absorbance recorded at 535 nm by using tetramethoxypropane (sigma chemical Co.) as the standard. Hepatic homogenates containing 8.1% sodium dodecylsufate (SDS) and distilled water mixed with 20% acetic acid (pH 3.5) and 0.8% aqueous TBA solution, and subsequently heated at 95 °C for 60 min. After cooling, n-butanol and pyridine (15:1, v/v) solutions were added and the samples were centrifuged. The absorbance of the upper layer was measured at 535 nm. Paraoxonase (PON) activities were spectrophotometrically assayed using plasma and hepatic microsomes. The assay mixture consisted of 1 mM paraoxon in 0.1 M Tris–HCl buffer (pH 8.0) containing 2 mM $CaCl_2$. The increase in absorbance was monitored photometrically for 90 s at 405 nm and 25 °C.

2.7. Histological analyses of atherosclerotic lesions

Each aortic arch was removed and wrapped with saline-soaked gauze after removing the connective tissues. All were fixed in 10% paraformaldehyde/PBS, embedded in paraffin, and then stained with hematoxylin and eosin (H&E). Another section of the aortic arch was cryosectioned and stained with Oil-Red O solution.

2.8. Statistical analysis

All data are presented as means ± SE. The data were evaluated by a one-way ANOVA using the SPSS program, and by the differences between the means assessed using a Duncan's multiple-range test. Statistical significance was considered at $p < 0.05$.

3. Results

3.1. Body weight gain, food intake, FER and weights of adipose tissue

There were no significant differences in body weight gain, food intake and FER (Table 1). The epididymal fat weight was significantly lower in the ferulic acid and clofibrate groups than in the control group. In particular, the visceral fat weight was only significantly lower in the ferulic acid group than in the control group. The structure of ferulic acid is shown in Fig. 1.

Table 1
Effect of ferulic acid and clofibrate supplementation for 15 weeks on body weight gain, food intake, FER and weights of adipose tissue in Western diet fed apo $E^{-/-}$ mice.

	Control	Ferulic acid	Clofibrate
Body weight gain (g/day)	0.15 ± 0.01	0.14 ± 0.01	0.14 ± 0.01
Food intake (g/day)	4.18 ± 0.04	4.18 ± 0.04	4.19 ± 0.05
FER	0.034 ± 0.002	0.034 ± 0.002	0.034 ± 0.002
Adipose tissue weights (g/100 g body weight)			
Interscapular brown fat	0.54 ± 0.03	0.55 ± 0.02	0.51 ± 0.03
Epididymal fat	3.73 ± 0.10[a]	3.14 ± 0.24[a]	3.19 ± 0.15[a]
Perirenal fat	0.96 ± 0.07	0.88 ± 0.1	0.98 ± 0.06
Visceral fat	4.64 ± 0.13[a]	3.97 ± 0.33[a]	4.16 ± 0.17[a]

Mean ± SE, $n = 10$.
FER: food efficiency ratio = body weight gain/food intakes per day, visceral fat: epididymal fat plus perirenal fat.
[a] Means in the row not sharing a common letter are significantly different between groups at $p < 0.05$ as determined by a one-way ANOVA test.