Effects of Green Tea Catechin on Hepatic Microsomal Phospholipase A\textsubscript{2} Activities and Changes of Hepatic Phospholipid Species in Streptozotocin-Induced Diabetic Rats

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Summary In this study, male Sprague-Dawley rats weighing 70±5 g were divided into a control (normal) group and three different diabetes mellitus (DM) groups that were supplemented with catechin-free (DM-0C), 0.5% catechin dietary (DM-0.5C), and 1% catechin (DM-1.0C). The animals were maintained on an experimental diet for four weeks. At this point, they were injected with streptozotocin (STZ) to induce diabetes, and they were sacrificed on the 6th day to determine the activities of phospholipase A\textsubscript{2} (PLA\textsubscript{2}) and the changes of phospholipid species by catechin supplementation. In liver tissues, no significant differences were found between the PC hydrolysis of a normal group and a diabetic group; however, PE hydrolysis of the DM-0C, DM-0.5C, and DM-1.0C groups increased by 68.9%, 34.01%, and 26.9%, respectively, compared with the normal group. Although the PLA\textsubscript{2} activity of the DM-0C group in the liver tissues increased 91% compared with the normal group, the PLA\textsubscript{2} activity of DM-0.5C and DM-1.0C, which were fed catechin, increased 50% and 56%, respectively, compared with the normal group. In the liver tissues, peroxide values of the DM-0C, the DM-0.5C, and the DM-1.0C groups were increased 109%, 32.8%, and 46.7%, respectively, compared with the normal group. Based on these results for STZ-induced diabetic rats, lipid peroxidation seems to be accelerated specifically with the increased PLA\textsubscript{2} activities. Thus if antioxidants like catechin were supplementation, the activity of PLA\textsubscript{2} and PE hydrolysis can be altered and the accumulation of lipid peroxide would be decreased. Therefore we concluded that the antioxidant catechin for diabetic animals can significantly reduce PLA\textsubscript{2} activities and lipid peroxide formation.

Key Words diabetes, catechin, phospholipase A\textsubscript{2}, phospholipid species, lipid peroxidation

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It is known that phospholipase A2 (PLA2) releases arachidonic acid from the phospholipid of the biological membrane because of many factors. Free arachidonic acid is metabolized to the eicosanoids, such as prostaglandins, thromboxanes, and leukotrienes, that are related to various metabolisms and diseases via two pathways: lipoxygenase and cyclooxygenase. In this metabolic process, lipid peroxidation is induced by free radicals (1–4).

PLA2 hydrolyzes the mixture of fatty acid ester in the sn-2 position of glycerol-3-phospholipid and generates free fatty acids and lysophospholipids (5, 6). Of these products, isolated fatty acid plays a role in increasing blood vessel permeability and prolonging blood vessels in inflamed regions (7), and the lysophospholipids change physiological activity, such as the action of hemolysis and the aggregation of platelets (8). Furthermore, it was reported by Dennis (9) that isolated arachidonic acid from the phospholipid by PLA2 offers either a precursor to forming eicosanoid or a precursor to forming factors of platelet activity providing acyl ether linkage included in position one of phospholipid.

It has been reported that PLA2, known for the rate-limiting enzyme of arachidonic acid cascade, is activated depending either on various external stresses or on internal pathological conditions to enhance lipid peroxidation, but its activity is inhibited by an antioxidant (11). In the report by Takahashi et al (10), the isolated arachidonic acid and PLA2 activities in platelets of diabetic rats increased remarkably in comparison with those of normal rats.

But when brazilin, used as a natural pigment, is injected into the platelets of diabetic rats, the isolation of arachidonic acid and the PLA2 activities were both inhibited (11). Borowitz and Montgomery (12) reported that PLA2 activity is inhibited by PLA2 inhibitors like chloropromazine, megacrine, and P-bromphenacyl bromide, and the accumulation of lipid peroxide diminishes depending on the PLA2 inhibition. It has been reported by Panganamala and Cornwell that a proper injection of antioxidant like vitamin E can inhibit the increase of PLA2 activity (1). Therefore it could be possible that a proper supplementation of antioxidant inhibits the accumulation of lipid peroxide, since lipid peroxidation in tissues is stimulated by the increase of PLA2 activity. Thus we have investigated the antioxidative effects of natural materials that can protect the damages by lipid peroxidation in tissues by inhibiting PLA2 activity. Recently the studies on green tea as a natural antioxidant have been under progress (13, 14). The preliminary study showed that the reaction of lipid peroxidation is accelerated in streptozotocin (STZ) induced diabetic rats compared with normal rats, but the peroxidation lesion was relieved when the catechin, a component of green tea, was supplementally offered as an antioxidant during an experimental period (15).

The purpose of this study is to investigate the effects of green tea catechin on the change of phospholipid species and the formation of lipid peroxides, which are caused by the change of PLA2 activity in diabetic rats.
MATERIALS AND METHODS

Experimental animals and diet. Male Sprague-Dawley rats (2 weeks old) were obtained from KRICT Korea (Taegjon, Korea). The Taegu-Hyousung Catholic University’s guide for the care and use of laboratory animals was followed in this study. Rats weighing $70 \pm 5$ g were adjusted for one week after arrival; they were divided into a normal group and three different diabetic groups by a completely randomized block design. Each of the four groups with 10 rats were fed for four weeks with experimental diets. The diabetic groups were classified as DM-0C (catechin-free diet), DM-0.5C (0.5% catechin of diet), and DM-1.0C (1% catechin of diet), according to the level of dietary catechin supplementation.

The rats were fed the respective diets for four weeks ad libitum. They were housed individually in stainless cages placed in an air-conditioned room maintained at 22–24°C with 50% humidity under controlled lighting conditions (12h/12h light-dark cycle). Throughout the experimental period, the body weight and food intake were monitored every day.

The induction of diabetes, measurements of body weight, and the efficiency of diet. Diabetes was induced by intravenous injection of 55 mg/kg body weight of STZ in sodium citrate buffer (pH 4.3) after 4 weeks of feeding three experimental diets. The rats were sacrificed on the 6th day of the diabetic states. The animals whose blood glucose concentration exceeded 300 mg/dL after six days were selected for the further experiments. The body weight was measured regularly at the same time every other day throughout the experimental period. The efficiency of diet was calculated by dividing the body weight by the dietary intake in the same period.

Collecting samples and sample preparations. Streptozotocin (STZ)-induced diabetic rats were fasted for 12 h and sacrificed on the sixth day. The livers were excised, washed in 0.9% of NaCl, frozen rapidly in liquid nitrogen, stored at $-80$ °C, then prepared for the experiment. The microsomes were isolated from the liver tissues by using a previously reported method (16).

Separation and measurement of phospholipid species.

1) The method of lipid extraction and determination of phosphorus: The lipids were extracted according to the Bligh and Dyer method (17). Chloroform: methanol:d-H$_2$O = 1:1:1 was added to liver microsome, stirred vigorously, and centrifuged. The chloroform fraction was taken, vaporized with N$_2$ gas, and formed into lipid film. The determination of phosphorus was carried out by the Marinetti method (18).

2) The separation of phospholipid species: A two-dimensional TLC was applied to the separation of phospholipid; chloroform:methanol:acetic acid = 65:25:10 was used as a first developing solvent, and chloroform:methanol:formic acid (88%) = 65:25:10 as a second developing solvent. Separated lipids on TLC plates (No. 5721, Merek Co., 20 cm × 20 cm, silicagel 60, without fluorescent indicator) were detected with I$_2$ vapor, then redetected with ninhydrin (Fig. 1).

The hydrolyses of phosphatidylcholine and phosphatidylethanolamine were
Fig. 1. Thin-layer chromatogram of various kinds of phospholipids. 1: lysophosphatidylcholine, 2: sphingomyelines, 3: phosphatidylcholine, 4: lysophosphatidylethanolamine, 5: phosphatidylinositol, 6: phosphatidylserine, 7: phosphatidylethanolamine, 8: phosphatidic acid, 9: cardiolipin, 10: unknown.

monitored by a determination of phosphorus of phospholipid species, such as phosphatidylcholine (PC), lysophosphatidylcholine (LysoPC), phosphatidylethanolamine (PE), and lysophosphatidylethanolamine (LysoPE), according to the above procedures (18).

3) The measurement of phospholipase A$_2$ activity: As substrate, 1-pal-2-[1-$^{14}$C] linoleoyl PE was used. Isolated linoleic acid was measured by the Dole and Meinertz method (19). Liver microsome was used as an enzyme source, and 1-pal-2-[1-$^{14}$C] linoleoyl PE was used as a substrate. Added to distilled water and incubated at 37°C was 0.05 M Tris-HCl (pH 7.0), 40 mM CaCl$_2$. Furthermore, a supernatant centrifuged in Dole reagent and distilled water was put into heptane. After it was added to the cocktail solution of the toluene system, isolated linoleic acid was measured from the liquid scintillation counter.

The determination of lipid peroxide. Lipid peroxides were determined by the measurement of thiobarbituric acid reactive substances (TBARS) (20).

The measurement of protein. The protein concentration of hepatic microsome was measured by using bovine serum albumin as a standard solution by the Lowry method (21).

Statistical analysis. All the results were assessed by variance analysis (ANOVA) to investigate the standard differences among groups. If significance was found by variance analysis, the level of significance among the groups was analyzed by Tukey’s HSD test.
Table 1. Body weight gains, food intakes, and food efficiency ratios of rats.

| Groups      | Body weight gains (g/4 wk) | Food intakes (g/4 wk) | FER         |
|-------------|---------------------------|-----------------------|-------------|
| Normal      | 200 ± 26<sup>NS</sup>     | 351 ± 51<sup>NS</sup> | 0.58 ± 0.02<sup>NS</sup> |
| STZ-diabetes |                           |                       |             |
| DM-0C       | 245 ± 12                  | 443 ± 18              | 0.55 ± 0.02 |
| DM-0.5C     | 208 ± 4.4                 | 451 ± 27              | 0.47 ± 0.02 |
| DM-1.0C     | 244 ± 13                  | 414 ± 23              | 0.60 ± 0.02 |

After STZ injection (g/6 d)

| Groups      | Body weight gains (g/6 d) | Food intakes (g/6 d) | FER         |
|-------------|---------------------------|-----------------------|-------------|
| Normal      | 17.5 ± 3.2<sup>a</sup>    | 73.1 ± 14.7<sup>NS</sup> | 0.25 ± 0.04<sup>a</sup> |
| STZ-diabetes |                           |                       |             |
| DM-0C       | −25.0 ± 7.6<sup>b</sup>   | 105.6 ± 26.8          | −0.47 ± 0.24<sup>b</sup> |
| DM-0.5C     | −38.3 ± 13.6<sup>b</sup>  | 91.7 ± 6.7            | −0.43 ± 0.17<sup>b</sup> |
| DM-1.0C     | −26.0 ± 4.9<sup>b</sup>   | 81.0 ± 12.6           | −0.36 ± 0.09<sup>b</sup> |

All values are mean ± SE (n = 10).
Values within a column with different superscripts are significantly different at p < 0.05 by Tukey’s test.
NS: not significant.

RESULTS

Body weight, food intake, and feed efficiency

The results of body weight gain, food intake, and feed efficiency are shown in Table 1. During the period of the experiment, no significant differences were found between the normal group and the DM groups in body weight change before the injection of streptozotocin (STZ), but all of the DM groups lost body weights after an injection of STZ in comparison with the normal group. No effects of catechin on weight grains were found.

The normal group and the DM groups showed no differences in food intakes throughout animal feeding. No significant differences were noted between the normal group and the experimental group in the efficiency of diet before the injection of STZ. But after the injection, the efficiency of diet decreased in all the experimental groups.

The change of phospholipid species

Phosphatidylcholine (PC) hydrolysis and phosphatidylethanolamine (PE) hydrolysis. To quantify the degree of PC hydrolysis, spots of PC and lysoPC were scraped off to determine phosphate after a separation of two-dimensional TLC.
Table 2. The effects of green tea catechin on the microsomal phosphatidylecholine and phosphatidylethanolamine hydrolysis in liver microsome of streptozotocin-induced diabetic rats.

| Groups   | PC hydrolysis (%) | PE hydrolysis (%) |
|----------|-------------------|-------------------|
| Normal   | 3.10±0.22<sup>NS</sup> | 7.85±0.63<sup>a</sup> |
| DM-0C    | 3.90±0.25         | 13.27±1.53<sup>b</sup> |
| DM-0.5C  | 3.25±0.21         | 10.52±0.83<sup>c</sup> |
| DM-1.0C  | 2.82±0.15         | 9.96±0.87<sup>c</sup> |

All values are mean±SE (n=10). Values within a column with different superscripts are significantly different at p<0.05 by Tukey’s test.

There was no significant difference in the value of PC hydrolysis between the normal group and experimental groups.

The results of PE hydrolysis, carried out by the quantification of phosphate by scraping the PE and lysoPE spots after separation by a two-dimensional TLC, are shown in Table 2. PE hydrolysis of the DM-0C group was increased 68.9%, that of the DM-0.5C group 34.01%, and that of the DM-1.0C group 26.9%, compared with the normal group. And the DM-0.5C group and the DM-1.0C group were lower by 20.7% and 24.5%, respectively, compared with the DM-0C group.

The changes in phospholipase A<sub>2</sub> activities

It was shown that hepatic PLA<sub>2</sub> activities of the DM-0C group was higher by 91.0% and the DM-0.5C group and DM-1.0C group by 50.4% and 56.4%, respectively, compared with those of the normal group. The DM-0.5C group and the DM-1.0C group were decreased 21.3% and 18.2% in comparison with the DM-0C group (Fig. 2). It suggests that PLA<sub>2</sub> plays a specific role in PE hydrolysis by presenting a similar phase with the value of hydrolysis in this PLA<sub>2</sub> activity change. Furthermore, it shows that no correlation exists between PLA<sub>2</sub> activity and PC hydrolysis, whereas PLA<sub>2</sub> and PE hydrolysis shows a positive correlation at the level of p<0.001 (Fig. 3).

The levels of thiobarbituric acid reactive substances in liver tissues

The results of measurement of thiobarbituric acid reactive substances, known for the index of peroxidational damage in biological membrane, are shown in Fig. 2. The level of lipid peroxide in the DM-0C group is approximately twofold higher, in the DM-0.5C group 1.3-fold higher, and in the DM-1.0C group 1.5-fold higher than that of normal. In the levels of lipid peroxides, the DM-0.5C group and the DM-1.0C group were decreased 37% and 30%, respectively, compared with the
Effects of green tea catechin on microsomal phospholipase A₂ activity (A) and thiobarbituric acid reactive substances (B) in the liver of streptozotocin-induced diabetic rats. Mean ± SE. Bars with different letters are significantly different at p<0.05 by Tukey’s test.

DM-0C group. As the results show in Fig. 2, the changes of lipid peroxide levels are similar to the changes of PLA₂ activities. PLA₂ and TBARS show a positive correlation at the level of p<0.001 (Fig. 3). Thus the catechin supplementation may relieve the peroxidative damage in tissues in STZ-induced diabetic rats.

DISCUSSION

This study was designed to investigate the changes of PLA₂ activities and phospholipid species in the livers of STZ-induced diabetic groups and the degree of accumulation of lipid peroxides by the supplementation of green tea catechin.

In this experiment, the significant weight loss after the injection of STZ is compared with reports by Park and Cho (22) and by Lau and Failla (23). No
Fig. 3. Correlation coefficients between PLA₂ activity and major parameters (PC hydrolysis (A), PE hydrolysis (B), and TBARS (C)).
differences were found in food intakes between groups before or after diabetes was induced by STZ. It is assumed that the feed efficiency ratio became lower in diabetes because of severe weight loss (24–26).

PLA₂ among phospholipases can hydrolyze ester conjugation of phospholipids, which forms lysophospholipids and free fatty acids (5, 6). The free fatty acids tend to be peroxidized, resulting in the formation of lipid peroxides. In this study, PC hydrolysis was not significantly different between the control group and experimental groups, but PE hydrolysis diabetic groups, which were not fed catechin, increased 68.9%, and the DM-0.5C group and the DM-1.0C group, which were fed catechin, increased 34.01% and 26.9%, respectively, compared with the normal group. It was thought that the increase of PE hydrolysis resulted from the specific activity of phospholipase A₂ on PE.

PLA₂ activity is known to function as a rate-limiting enzyme in the cascade of arachidonic acid, which was not isolated in cells, but which had ester conjugation in position two of the phospholipid of biological membrane, whereas arachidonic acid was metabolized to enhance the lipid oxidation. In this study, the activity of PLA₂ increased in the DM-0C group, which was on no-catechin supplement, and in the DM-0.5C and DM-1.0C groups which were supplemented with catechin by 96%, 50.4%, and 56.4%, respectively, compared with the normal group. For these results, we were led to conclude that among phospholipases, PLA₂ is the enzyme that induced PE hydrolysis.

As determined by the peroxide values, which indicated the degree of peroxidative damage of the interior of the body, the peroxide values of the DM-0C group, DM-0.5C group, and DM-1.0C group were increased by 109%, 32.8%, and 46.7%, respectively.

In lipids of biological membrane, the increment of free arachidonic acid resulted from the increased activity of PLA₂, thus two pathways such as lipoxygenase and cyclooxygenase were enhanced and the production of free radical increased, which was like Panganamala’s report (1). This was caused by increased peroxide values in the induced-diabetic groups.

Peroxide values remarkably were decreased with catechin supplement because catechin inhibited the activity of PLA₂, which may inhibit the metabolism of the free arachidonic acid so that the activity of lipoxygenase and cyclo-oxygenase diminished, and the production of free radical was also decreased. Moreover, the catechin played a role as a chain-breaking antioxidant in the oxidation of unsaturated fatty acid of cell membrane (27), which is in accord with the Park (25) and the Prichard et al (3) reports that state the antioxidant (Vit E) treated diabetic rats decreased in the accumulation of peroxidized lipids in the liver. Throughout this result, in the liver tissues of diabetic rats were high PE hydrolyses and an accumulation of peroxidized lipids with the increase of PLA₂ activity, but as catechin from green tea restrained the activity of PLA₂, PE hydrolysis and metabolic products of free arachidonic acid were decreased and lipid peroxidation was inhibited.

In the future, it will be necessary to clearly reveal the interrelation between
the role of PLA₂ and the metabolism of peroxidized lipid in the experiment by using a PLA₂ inhibitor. Through the experiments interrelated with antioxidants and PLA₂, we need to know about how much the antioxidants affect the activity of PLA₂ and lipid peroxidation.

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