Original Article

Green tea catechin improves microsomal phospholipase A_2 activity and the arachidonic acid cascade system in the kidney of diabetic rats

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The purpose of this study was to investigate the effects of green tea catechin on the microsomal phospholipase A_2 activity and arachidonic acid cascade in the kidneys of streptozotocin-induced diabetic rats. Sprague-Dawley male rats weighing 100 ± 10 g were assigned randomly to one normal and three streptozotocin-induced diabetic groups. The diabetic groups were the DM-0C group (n = 10), fed a catechin-free diet, the DM-0.25C group (n = 10), fed a 0.25 g catechin per 100 g diet, and the DM-0.5C group (n = 10), fed a 0.5 g catechin per 100 g diet. The kidney microsomal phospholipase A_2 activity was higher in the diabetic groups than in the normal group, while it was lower in the DM-0.25C and DM-0.5C groups than in the DM-0C group. The percentage of phosphatidylcholine hydrolysed in the kidney microsomes was not significantly different between any of the four groups. The percentage of phosphatidylethanolamine hydrolysed in the kidney microsomes was progressively higher in the DM-0.5C, DM-0.25C and DM-0C groups, respectively, compared to the normal group. The formation of thromboxane A_2 was significantly higher while the formation of prostacyclin was lower in kidney microsomes of the streptozotocin-induced diabetic groups compared with the normal group, but this condition was improved by catechin supplementation. Kidney microsomal vitamin E concentrations were progressively lower in the DM-0.5C, DM-0.25C, and DM-0C groups, respectively, compared to the normal group. The kidney thiobarbituric acid reactive substance (TBARS) contents became higher in the DM-0C and DM-0.25C groups as compared with the normal group, whereas the DM-0.5C group did not differ from the normal group. Kidney function appears to be improved by green tea catechin supplementation due to its antithrombus action, which in turn controls the arachidonic acid cascade system.

Key words: antithrombogenic, diabetes, green tea catechin, lipid peroxidation, phospholipase A_2.

Introduction

The occurrence of diabetes has rapidly increased, especially in developed and developing countries, due to ageing populations, obesity and unsound dietary habits. In most cases, diabetes is accompanied by triopathy including retinopathy, neuropathy and nephropathy, and it is the cause of several other metabolic diseases. In particular, kidney disease, including kidney failure, has a high incidence rate and, once it occurs, there is currently no effective method of interrupting its progress. This complication has become a serious medical, social and economic problem; for example, it has been reported that approximately 40–50% of diabetics in Korea die of kidney failure.[1] Accordingly, the study of the mechanisms of microvascular dysfunction or impairment that cause kidney failure is very important. However, until now, few studies have focused on the nutritional aspects of the pathological mechanisms of diabetic kidney failure.

In more recent studies on the functional damage of glomeruli and microvascular vessels such as renal tubules, it has been reported that the damage is related to eicosanoids, which are metabolites of arachidonic acid synthesized in the kidney cells.[2] Eicosanoids synthesized in the kidney cells have an effect on renal haemokinesis, glomerular filtration rate, sodium excretion, renin secretion and urinary concentration. In a normal kidney, the production of thromboxane A_2 (TXA_2) and prostacyclin (PGI_2) is controlled and the balance between them is important in maintaining homeostasis *in vivo*; yet for patients with chronic renal diseases, this balance is destroyed.[3–5] The production of TXA_2, PGI_2, and prostaglandin E_2 (PGE_2) depends on the activity of phospholipase A_2 (PLA_2), a rate-limiting enzyme of the arachidonic acid (AA) cascade, plus the composition of the fatty acid that is a product of phospholipase A_2.[6] Linos *et al.*[7] reported that glomeruli isolated from rats with nephritis, induced by injecting an antiglomerular basement antibody, had a TXA_2 synthesis rate that was 10 times higher than in glomeruli from normal rats, and that the excretion of urinary

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protein was increased in the nephrotic rats. Therefore, the destruction of the balance of the PGI_2/TXA_2 ratio accelerates thrombogenesis in the renal tubules, arteriosclerosis and ageing. Accordingly, rectifying this balance will result in an attenuation of renal arteriosclerosis, the proper control of renal haemokinesic and glomerular filtration rates, and an improvement in renal function, which will ultimately contribute to preventing diabetic kidney failure.

It is known that the catechin found in green tea exerts a variety of medical actions, including the reduction of blood cholesterol levels,[8] anti-oxidation[9] and suppression of platelet agglutination function.[10] The suppression of platelet agglutination by green tea extract has been reported[11] and *in vitro* studies have been carried out on the action of green tea on platelet agglutination and thrombus suppression. However, studies carried out *in vivo* are insufficient.

Although the anti-oxidant and antithrombogenic actions of green tea catechin are well known, there have been no reports detailing the mechanisms of these actions. Basic studies are therefore required to study the mechanism by which green tea catechin acts to improve fine vessel malfunction in renal tissues.[10] The present study was carried out by feeding diets varying in catechin to white rats before inducing the onset of diabetes, so as to analyse the effect of green tea catechin on the AA cascade and PLA_2 activity in renal tissue.

Materials and methods

Experimental animals and diets

Male Sprague-Dawley rats weighing between 70 and 80 g were purchased from KRITC (Daejon, Korea). Rats were housed individually in stainless steel cages in a room with controlled temperature (20–23°C) and lighting (alternating 12-h periods of light and dark) and fed a pelleted commercial non-purified diet (Samyang, Seoul, Korea) for 6 days after arrival. They were divided randomly into one normal group and three diabetic groups, and the diabetic groups were further classified into DM-0C (catechin free), DM-0.25C (catechin = 0.25 g/100 g diet) and DM-0.5C (catechin = 0.5 g/100 g diet), according to the level of catechin supplemented. The four groups (n = 10 for each group) were fed the experimental diets for a period of 4 weeks. This experimental design was approved by the committee of the Catholic University of Daegu for the care and use of laboratory animals.

Experimental diabetes. Diabetes was induced by an intravenous injection of streptozotocin (STZ; 55 mg/kg bodyweight) in a citrate buffer (pH 4.3) via the tail vein. Rats with a blood glucose level above 16.7 mmol/L after 6 days were used for the experiment described herein.

Sample collection and preparation. Streptozotocin-induced diabetic rats were deprived of food for 12 h and killed on day 6. Their kidneys were excised, washed in 9 g/L NaCl, frozen rapidly in liquid nitrogen and stored at –80°C until use. They were then homogenized in a buffer (10 mmol/L N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, 10 mmol/L KCl and 280 mmol/L sucrose, pH 7.4), centrifuged at 9000 *g* for 15 min, recentrifuged at 12 000 *g* for 20 min to remove any mitochondrial contamination of mitochondria, and then centrifuged at 105 000 *g* for 60 min to yield the microsomal fraction.

Measurement of vitamin E content

The vitamin E contents of kidney microsomes were determined according to the method of Kayden *et al.*[12] Kidney tissue microsomes were prepared as described previously. Briefly, 5 mL of 20 g/L pyrogallol was added to the microsomes on heating. After being mixed, incubated at 70°C for 2 min and mixed with 0.3 mL of KOH, the microsomes were then incubated at 70°C for another 30 min. The incubated microsomes were cooled in an ice bath (approximately 4°C), mixed with 4 mL of distilled H_2O and 10 mL of hexane, and vortexed vigorously for 2 min. After centrifugation at 1500 *g* for 10 min, 7 mL of the resultant mixture was then used for the vitamin E extraction. The extract was dried under N_2 gas and mixed with 0.8 mL of 5 g/L ferric chloride and 0.8 mL of 5 g/L dipyridyl. After vortexing, 2 mL of ethanol and ferric chloride was added to the mixture and the optical density of the colour formed was measured at 520 nm.

Separation and measurement of phospholipid species

Lipid extraction and determination of phosphorus. Lipids were extracted according to the method of Bligh and Dyer.[13] A mixture of chloroform, methanol and dH_2O (1 : 1 : 1, v/v) was added to the kidney microsomes, which were then stirred vigorously and centrifuged. The chloroform fractions obtained were evaporated under N_2 gas to form a lipid film. Determination of the phosphorus included in the extracted lipids was carried out using the Marinetti method.[14]

Separation of phospholipid species. Two-dimensional thin layer chromatography (TLC) was used for separating the phospholipid species. A mixture of chloroform, methanol and acetic acid (65 : 25 : 10, v/v) was used as the first developing solvent, and a mixture of chloroform, methanol and 88% formic acid (65 : 25 : 10, v/v) was used as the second. The lipids separated on the TLC plate (No. 5721, 20 cm × 20 cm, silica gel 60, no fluorescent indicator; Merck, Darmstadt, Germany) were detected with I_2 vapour and redetected with ninhydrin. To determine the phosphorus present in the phospholipid species, such as phosphatidylcholine (PC), lysophosphatdylcholine (LysoPC), phosphatidylethanolamine (PE) or lysophosphatidylethanolamine (LysoPE), we monitored the hydrolysis of phosphatidylcholine and phosphatidylethanolamine, according to the method previously described.[15] Briefly, the percentage PC and PE hydrolysis were calculated using the following equations:

$$\text{PC hydrolysis} = \frac{\text{LysoPC}}{\text{LysoPC} + \text{PC}} \times 100$$

$$\text{PE hydrolysis} = \frac{\text{LysoPE}}{\text{LysoPE} + \text{PE}} \times 100$$

Measurement of phospholipase A_2 activity

Kidney microsomes (20 μg) were used as the enzyme source, and 1-pal-2-[1-^{14}C] linoleoyl PE was used as the substrate. Briefly, the 1-acyl-2-[1-^{14}C] linoleoyl-sn-glycero-3-phosphatidylethanolamine (16.8 Bq/nmol) in ethanol/toluene (1:1) was evaporated under N_2 gas, forming a lipid film. A small amount of distilled water was added to the lipid film and sonicated with an ultrasonic cleaner (Branson 2200; Branson, Danbury, CT, USA) for 3 min. The standard reaction mixture (200 μL) contained 0.05 mol/L Tris-HCl (pH 7.0), 40 mmol/L $CaCl_2$ and 20 nmol/L substrate (16.8 Bq/nmol). The reaction was carried out at 37°C for 20 min and stopped by adding 1.25 mL of Dole's reagent.[16] The radioactive linoleic acid released was extracted using a method described previously.[17]

Measurement of thromboxane A_2 and 6-keto prostaglandin F_{1a}

Isolated microsomes (2.0 mg protein/tube) were incubated in a 280 mmol/L Tris-KCl buffer (pH 7.4) at 37°C with continuous agitation (100 orbits/min, 1.6 Hz) and centrifuged at 10 000 *g* for 5 min. The supernatant was adjusted with the Tris-KCl buffer to a final volume of 1.0 mL and used as the source of TXA_2 and 6-keto prostaglandin F_{1a}. The 6-keto prostaglandin F_{1a} and TXA_2 contents were measured using a commercially available radioimmunoassay kit (RPA 515 and 516; Amersham, Cleveland, OH, USA). The radioactivity of the iodinated final products was measured using a Packard liquid scintillation counter (Packard Tricarb 1600TR; Packard, Meriden, CT, USA).

Determination of lipid peroxide

Lipid peroxide in the kidney tissues was determined by measuring the thiobarbituric acid reactive substances (TBARS) contents, an index of lipid peroxidation.[15]

Protein determination

Protein in the kidney tissues was determined using the method of Lowry *et al.*,[18] with bovine serum albumin as the standard.

Statistical analysis

Results were assessed by ANOVA and Tukey's Honestly Significant Difference test. Differences were considered significant at $P < 0.05$.

Results

Phospholipase A_2 activity in kidney microsomes

Kidney microsomal PLA_2 activity in the DM-0C group was 63% greater than in the normal group (Fig. 1). However, the PLA_2 activity in the DM-0.25C and DM-0.5C groups were not significantly different from that of the normal group.

Phosphatidylcholine and phosphatidylethanolamine hydrolysed in kidney microsomes

The percentage of PC hydrolysed in kidney microsomes was not significantly different between any of the four groups. Compared with the normal group, the percentage of PE hydrolysed in the kidney microsomes was 115% greater in the DM-0C group, 41% greater in the DM-0.25C group and 55% greater in the DM-0.5C group (Table 1).

Production of thromboxane A_2 and prostacyclin in kidney microsome

The formation of TXA_2 in the kidney microsomes was 159% greater in the DM-0C group and 143% greater in the DM-0.25C group, compared with that of the normal group; however, the TXA_2 levels in the DM-0.5C and normal groups did not differ (Fig. 2a). The formation of PGI_2 in the kidney microsomes was 44% lower in the DM-0C group compared with the normal group (Fig. 2b). However, the levels of PGI_2 formation in the DM-0.25C and DM-0.5C groups were not significantly different from that of the normal group (Fig. 2b). The ratio of PGI_2/TXA_2 (Fig. 2c) was 67% and 44% lower in the DM-0C and DM-0.25C groups, respectively, compared to that in the normal group, while the ratio in the DM-0.5C group did not differ from that in the normal group (Fig. 2c).

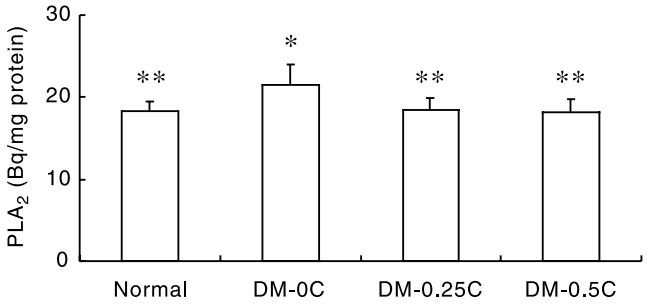

Figure 1. Effect of 0 (DM-0C), 0.25 (DM-0.25C) or 0.5 (DM-0.5C) g catechin/100 g diet on kidney microsomal phospholipase A_2 (PLA_2) activity in streptozotocin-induced diabetic rats. All values are mean ± SE, $n = 10$. *, ** and *** are significantly different at $P < 0.05$. Microsomal phospholipase A_2 activity was measured using radioactive released linoleic acid.

Table 1. Effect of 0 (DM-0C), 0.25 (DM-0.25C) or 0.5 (DM-0.5C) g catechin/100 g diet on hydrolysed of kidney microsomal PC and PE in streptozotocin-induced diabetic rats

Groups	PC hydrolysed (%)†	PE hydrolysed (%)‡
Normal	6.878 ± 0.844¶	7.761 ± 0.830[b]
DM-0C	8.178 ± 0.904	15.168 ± 0.681[a]
DM-0.25C	8.142 ± 1.026	11.909 ± 2.265[ab]
DM-0.5C	8.296 ± 0.845	12.042 ± 1.625[ab]

PC, phosphatidylcholine; PE, phosphatidylethanolamine. †(LysoPC/PC + LysoPC) × 100. ‡(Lyso PE/PE + Lyso PE) × 100. ¶ Not significant. [a,b]Values within a column with different superscripts are significantly different at $P < 0.05$.

Vitamin E concentration in Kidney tissues

Kidney microsomal vitamin E concentrations were 49, 42 and 37% lower in the DM-0C, DM-0.25C, and DM-0.5C groups, respectively, compared to the normal group (Table 2).

Level of thiobarbituric acid reactive substances in kidney tissues

The kidney TBARS contents, of the DM-0C and DM-0.25C groups, were 162% and 128% greater, respectively, compared to the normal group. The TBARS content of the DM-0.5C group did not differ significantly from that of the normal group (Fig. 3).

Discussion

The purpose of this study was to investigate the effects of green tea catechin on microsomal phospholipase A_2 activity and the arachidonic acid cascade in the kidneys of STZ-induced diabetic rats. The activity of PLA_2, a rate-limiting enzyme of the AA cascade, was measured in the kidney microsomes of STZ-induced diabetic rats supplemented with different levels of catechin. When compared with the normal group, the PLA_2 activity of the DM-0C group, which received no catechin supplementation, showed a 64% increase. In contrast, the PLA_2 activity of the DM-0.25C and DM-0.5C groups were not significantly different from that of the normal group.

Studies by Prichard *et al.*,[19] with vitamin E supplementation, and Choi *et al.*,[20] with catechin supplementation, have reported the effects of anti-oxidants on platelets and hepatic tissue of white rats with STZ-induced diabetes. As with the present study, these indicated that there was a tendency toward the reduction of lipid peroxide and the suppression of PLA_2 activity. In addition, large quantities of dietary vitamin E supplementation have been shown to produce a similar suppression of PLA_2 activity in renal tissue.[21] This indicates that while a higher activity of PLA_2 in epicytes with more oxidative stress leads to a higher level of arachidonic acid in diabetic rats, the activity of PLA_2 is decreased by suppressing the elaboration of free radical material and lipid peroxide of tissues, resulting from the anti-oxidant action of catechin or vitamin E. Also, Borowitz and Montgomery[22] reported that when a biomembrane is exposed to a free radical production system, the level of endogenous lysophospholipid increases. In the present study, a change in the phospholipid

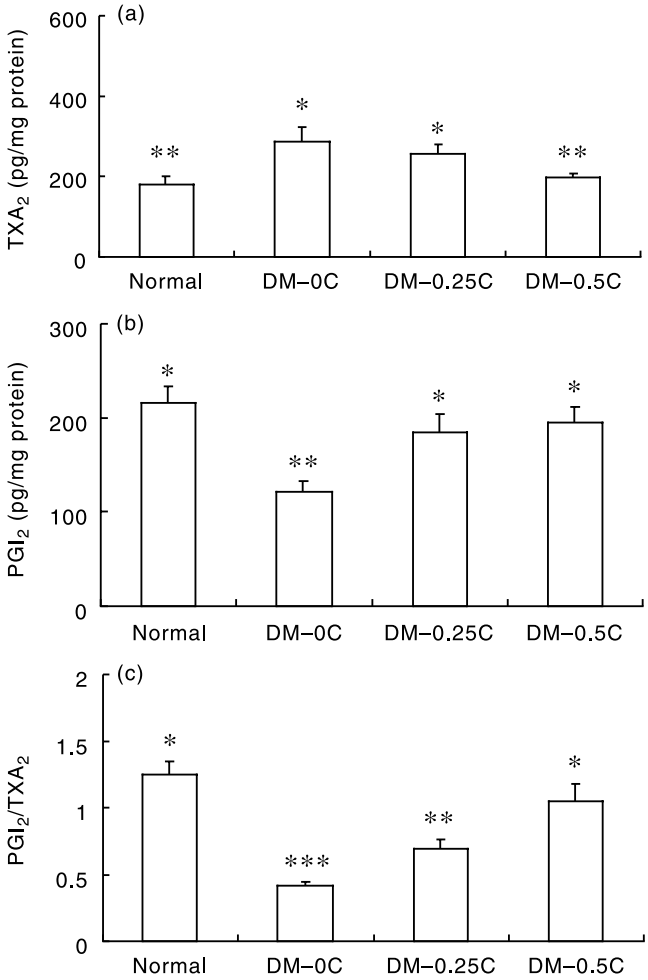

Figure 2. Effect of 0 (DM-0C), 0.25 (DM-0.25C) or 0.5 (DM-0.5C) g catechin/100 g diet on kidney microsomal (a) thromboxane A_2 (TXA_2), (b) prostacyclin (PGI_2) synthesis and (c) PGI_2/TXA_2 ratio in streptozotocin-induced diabetic rats. All values are mean ± SE, $n = 10$. *, ** and *** are significantly different at $P < 0.05$.

Table 2. Effect of 0 (DM-0C), 0.25 (DM-0.25C) or 0.5 (DM-0.5C) g catechin/100 g on kidney weight and microsomal vitamin E concentration in streptozotocin-induced diabetic rats

Groups	Vitamin E (μg/mg protein)
Normal	0.320 ± 0.028^a
DM-0C	0.163 ± 0.034^b
DM-0.25C	0.184 ± 0.014^b
DM-0.5C	0.201 ± 0.025^b

All values are given as mean ± SE, $n = 10$. Values within a column with different superscripts are significantly different, $P < 0.05$.

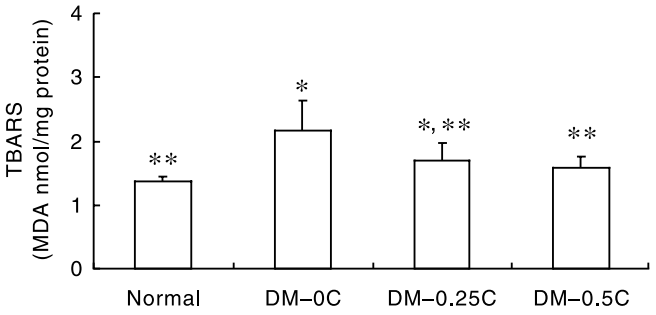

Figure 3. Effect of 0 (DM-0C), 0.25 (DM-0.25C) or 0.5 (DM-0.5C) g catechin/100 g diet on kidney thiobarbituric acid reactive substance (TBARS) contents in streptozotocin-induced diabetic rats. All values are mean ± SE, $n = 10$. *, ** and *** are significantly different at $P < 0.05$.

species, such as PC, lysoPC, PE and lysoPE, was observed in the kidney microsomes of diabetic rats supplemented with different levels of green tea catechin.

The percentage of PC hydrolysed in the kidney microsomes was not significantly different between any of the four groups. The amount of PE hydrolysed in the kidney microsomes was 115% greater in the DM-0C group, 41% greater in the DM-0.25C group and 55% greater in the DM-0.5C group, compared with the normal group.

The results obtained in the present study support those recorded by Choi *et al.*[20] Phospholipid hydrolysis increased in the hepatic tissue of rats with diabetes and then decreased to the level of the normal group after dietary supplementation with green tea catechin. In addition, the results seem to bear some relation to the reduction of PLA_2 activity, resulting from the depression of PLA_2 activity by catechin.

Thromboxane A_2, PGI_2 and PGE_2 are produced from arachidonic acid via cyclooxygenase. Klahr and colleagues[23] have previously reported that thromboxane and PGI_2, which functions as a vasoconstrictor and a vasodilator, respectively, are synthesized in the kidney and have an effect on kidney function by controlling renal haemokinesis and the glomerular filtration rate. In particular, TXA_2 functions as a strong accelerator of vasoconstriction and thrombogenesis, and exacerbates kidney diseases by constricting mesangial cells and reducing the glomerular filtration rate.

The formation of TXA_2 in kidney microsomes was 159% and 143% greater in the DM-0C and DM-0.25C groups, respectively, compared to that in the normal group; however, TXA_2 formation in the DM-0.5C and normal groups did not differ. Compared with the normal group, the formation of PGI_2 in the kidney microsomes was 44% lower in the DM-0C group. However, the level of PGI_2 formation in the DM-0.25C and DM-0.5C groups was not significantly different from that of the normal group.

The ratio of PGI_2/TXA_2 was 67% lower in the DM-0C group and 44% lower in the DM-0.25C group, compared to that of the normal group, while the ratio in the DM-0.5C group did not differ from that of the normal group. The PGI_2/TXA_2 ratio, which is an important element in determining the thrombogenesis index and vasoconstriction function, was reduced in the diabetic groups; however, the ratio increased in proportion to the level of dietary vitamin E supplementation. This result was supported not only by Yang *et al.*'s report,[10] which showed that the unbalanced ratio of PGI_2/TXA_2 in platelets was improved by the catechin found in green tea, but also Gwag *et al.*'s report,[21] which showed that an unbalanced PGI_2/TXA_2 ratio in renal tissue was improved by dietary supplementation with vitamin E.

The large increase in lipid peroxide observed in the present study seems to be caused by the increased release of arachidonic acid and increased activity of PLA_2 in biomembrane lipids. This leads to the accentuation of the lipoxygenase and cyclooxygenase system, an increase in free radicals in the metabolic process and a reduction in anti-oxidants such as vitamin E. This result indicates that dietary green tea catechin improved the PGI_2/TXA_2 balance in the kidney of diabetic rats by decreasing PLA_2 activity and TXA_2 synthesis, and increasing PGI_2 synthesis.

Conclusion

It would appear that kidney function is improved by green tea catechin supplementation due to its antithrombogenic action, which in turn controls the arachidonic acid cascade system.

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