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Effects of Brazilin on Lipid and Phosphatidyl Fatty Acid Composition of Erythrocyte Membrane in Streptozotocin-induced Diabetic Rats

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In diabetes, the abnormal increase of the membrane cholesterol/phospholipid ratio (C/PL) is considered to be the main reason for the decreased membrane fluidity, which then results in impaired erythrocyte deformability and subsequent microcirculatory disturbances. In this study, we examined the effects of brazilin on lipid and phosphatidyl fatty acid composition of erythrocyte membranes in streptozotocin induced diabetic rats. Treatment of brazilin (10 mg/kg or 100 mg/kg for 2 weeks, i.p) altered phospholipid and cholesterol contents in diabetic erythrocyte membranes. The C/PL ratio of brazilin treated groups decreased compared with that of diabetic control group while no change was observed in normal erythrocytes. In streptozotocin induced diabetic rats, alterations in phosphatidyl fatty acid composition of erythrocyte membranes were observed and brazilin could reverse these alterations. Arachidonic acid level returned to a normal level while linoleic acid level remained unchanged by the treatment of brazilin. The results suggest that brazilin might increase erythrocyte membrane fluidity which plays a key role in regulating erythrocyte deformability, thereby it could exert positive effects on microcirculatory disturbances.

Key words: Brazilin, Erythrocyte, Phospholipid, Cholesterol, Diabetes, Fluidity, Membrane lipid, Phosphatidyl fatty acid composition

INTRODUCTION

One of the diabetic complications, microangiopathy is known to result from abnormal function of erythrocytes, *i.e.*, reduced erythrocyte deformability along with increased blood viscosity. The increase of erythrocyte membrane microviscosity implies the changes and disturbances of membrane structure in diabetic conditions (Chandramouly and Carter, 1975; Baba *et al.*, 1979; Schmid-Schoenbein and Volger, 1976; William *et al.*, 1985). Insulin dependent diabetic patients showed the changes of lipid composition in plasma and erythrocyte membrane. The fluidity or deformability of erythrocyte depends on the composition of erythrocyte membrane which consists of phospholipid and cholesterol. For that reason, the cholesterol/phospholipid ratio (C/PL) is important in evaluating the fluidity of erythrocyte membrane and it was reported that increase of C/PL ratio is one of the major causes of reduced fluidity of diabetic erythrocyte membrane (Bryszewska *et al.*, 1986).

Also, the fluidity depends on the fatty acid composition of membrane. It is reported that the contents of polyunsaturated fatty acid, particularly arachidonic acid, in adipocyte membrane phospholipid have been decreased (Boivin, 1988). Arachidonic acid deficiency and elevated linoleic acid levels have been a consistent finding in a variety of tissues in experimental diabetes including the red blood cell (Holman *et al.*, 1983; Huang *et al.*, 1984). Changes of arachidonic acid levels have been of particular current interest because changes in platelet aggregation, thromboxane- and prostacyclin-levels may relate to the enhanced vascular disease in diabetes (Dang *et al.*, 1988).

Brazilin, the main constituent of *Caesalpinia sappan*, has been found to have various biological activities including the reduction of blood glucose and viscosity (Moon *et al.*, 1988). Based on these findings, we studied the effects of brazilin on lipid and the phosphatidyl fatty acid composition of erythrocyte membrane in streptozotocin-induced diabetic rats.

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MATERIALS AND METHODS

Animals

Male Spraque-Dawley (SD) rats (200-250 g) were supplied from the Experimental Animal Breeding Center of Seoul National University. They were adapted for more than 14 days before experiment.

All rats were housed in wire cages, in groups of 4-5 rats, fed water *ad libitum* and usual laboratory chow (Samyang Feed Production Co.) and kept with alternating 12 hrs period of light/dark cycle throughout the experiments. Temperature and humidity were controlled with automatic thermo-humidistat at 22± 1℃ and 60± 5%, respectively.

Induction and treatment of experimental diabetes

Male SD rats weighing between 200 and 250 g were fasted overnight and injected intravenously with streptozotocin (Sigma Chem. Co.) 45 mg/kg body weight. Streptozotocin was dissolved in citrate buffer (pH 4.0), kept on ice bath and administered within 15 minutes. Control animals were treated with same volume of citrate buffer.

72 hours later, blood glucose level was determined by Reflomat Glucometer (Boehringer Mannheim). and animals with blood glucose level higher than 300 mg/dl were used as diabetic rats.

Rats were divided into seven groups and each group consisted of five experimental animals; normal control, normal brazilin treated (10 mg/kg, 100 mg/kg respectively), diabetic control, diabetic brazilin treated (10 mg/kg, 100 mg/kg respectively), diabetic insulin treated. Normal and diabetic brazilin treated rats were administered 10 mg/kg or 100 mg/kg of brazilin through i.p. injection for 14 days and diabetic insulin treated rats were injected with 8 units of protamine zinc insulin (Green Cross Co.) subcutaneously daily for 5 days prior to killing. Normal control rats were administered with the same volume of saline.

Blood sampling and Extraction of RBC membrane lipid

Blood was collected from abdominal aorta with heparinized syringe. Whole blood was centrifuged at 3, 000 rpm for 10 min at 4℃. After centrifugation plasma and buffy coat were removed and the packed red blood cell (RBC) was washed twice with cold phosphate-buffered saline.

One part of packed RBC was used in extraction of intact RBC lipid and the other part of packed RBC was used in the ghost preparation.

In the ghost preparation, intact RBC was lysed with 40 volumes of 10 mM Tris (pH 7.4) at 4℃ for 1 hr and centrifuged at 3,000 rpm for 10 min and the remained intact RBC was removed followed by centrifugation at 15,000 rpm for 40 min at 4℃. Precipitated pellet was taken and washed thoroughly with hemolyzing solution (10mM Tris buffer) in order to remove the hemoglobin.

RBC membrane lipids were extracted with chloroform/methanol as described by Folch (Folch *et al.*, 1957).

Determination of phosphorus content

Phosphorus contents of intact RBC and RBC membrane lipids were determined as described by Fiske and Subbarow (Cooper, 1977). Lipids in $CHCl_3$ were evaporated to dryness under nitrogen stream, and 70% perchloric acid (Aldrich Chemical Co.) was added. The samples were then heated for 30 min to 1 hour at 200℃ in furnace until the color turned to pale yellow or white and cooled to room temperature.

After adjusting to equal total volume, ammonium molybdate and ascorbic acid were added. After vortexing, samples were heated for 5 min and cooled at room temperature for 5 min and then the absorbance at 820 nm was determined.

Determination of cholesterol content

Cholesterol contents were determined according to the method of Findaly and Evans (1987). Glacial acetic acid and 2.5% $FeCl_3 \cdot 6H_2O$ in 85% orthophosphoric acid were added to the sample and vortexed vigorously. After standing at room temperature for 10 min, the absorbance at 550 nm was determined and cholesterol content was calculated from standard calibration curve.

Analysis of phosphatidyl fatty acid composition by Gas Chromatography

Glycolipids were separated by thin-layer chromatography using silica gel G7739 plates (Merck) (Broekhuyyse, 1969; Skipski *et al.*, 1964). Spots corresponding to phospholipid (which remained at the origin) were scraped and put into a silica gel column to be eluted with methanol. The collected eluent was evaporated and transmethylated (Holman *et al.*, 1983; Dang *et al.*, 1988). Fatty acid composition was determined using a Hewlett-Packard Gas Chromatograph (Model 5890A).

Identification of fatty acids was based on retention times of authentic standards. Fatty acid composition was expressed as mean percent± SE of each individual fatty acid vs total fatty acids.

Statistics

The statistical analysis was performed by student t-test. The significant differences between groups were evaluated with the level set at 0.05.

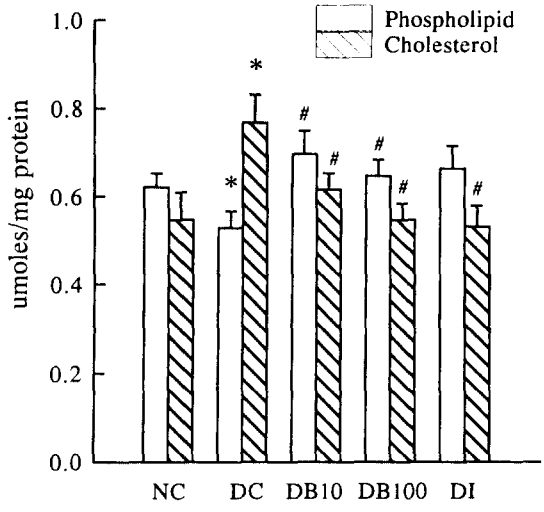

Fig. 1. Effects of brazilin on phospholipid and cholesterol level in diabetic erythrocyte membrane.
NC: Normal control
DC: Diabetic control
DB 10: Diabetic brazilin treated (10 mg/kg)
DB 100: Diabetic brazilin treated (100 mg/kg)
DI: Diabetic insulin treated
Each group consisted of five experimental animals.
Values are mean± SD
*: $p<0.05$ vs NC
#:$p<0.05$ vs DC

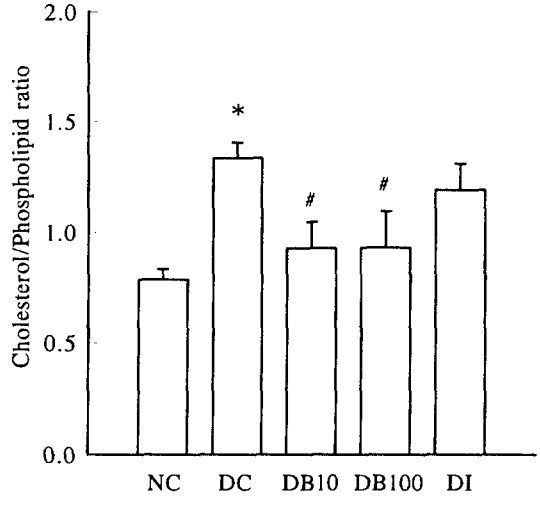

Fig. 2. Effects of brazilin on cholesterol/phospholipid ratio in diabetic erythrocyte membrane.
NC: Normal control
DC: Diabetic control
DB 10: Diabetic brazilin treated (10 mg/kg)
DB 100: Diabetic brazilin treated (100 mg/kg)
DI: Diabetic insulin treated
Each group consisted of five experimental animals.
Values are mean± SD
*: $p<0.05$ vs NC
#: $p<0.05$ vs DC

RESULTS AND DISCUSSION

As shown in Fig. 1, in streptozotocin-induced diabetic rats, there was a significant decrease in the phospholipid content (0.52± 0.03 μmoles/mg protein in diabetic rats versus 0.62± 0.02 μmoles/mg protein in control rats) and a significant increase in the level of cholesterol (0.76± 0.06 μmoles/mg protein in diabetic rats versus 0.54± 0.05 μmoles/mg protein in control rats). Insulin treatment increased phospholipid content (23%) and decreased cholesterol level (30.3%) in diabetic rats. Brazilin showed similar effects on these parameters. In diabetic brazilin treated group, phospholipid content increased by 32.7% and 23.1% respectively in reponse to 10 mg/kg and 100 mg/kg brazilin administration. And brazilin treatment (10 mg/kg, 100 mg /kg) decreased the cholesterol level by 19.7% and 28.9 % respectively. Thus, brazilin and insulin appears to have similar effects in terms of normalization of phospholipid and cholesterol contents in diabetic rats.

It is well known that there is a significant increase of cholecterol/ phospholipid (C/PL) ratio in diabetes (Bryszewska *et al.*, 1986) and our data also showed same pattern. Brazilin (10 mg/kg, 100 mg/kg) and insulin reduced the C/PL ratio to the nearly same level of normal control (Fig. 2). The increased rigidity of erythrocyte membranes in diabetes is thought to be resulted from the increase of cholesterol and the decrease of phospholipid contents (William *et al.*, 1985; Bryszewska *et al.*, 1986). Brazlin, like insulin, decreased cholesterol content and increased phospholipid level (thus, C/PL ratio decreased). In contrast to the diabetic animals, brazilin did not have any effect on phospholipid content in erythrocytes from normal animals (data not shown).

Alterations of fatty acid composition in the membrane phospholipid are well known observations in streptozotocin induced dibetes (Faas *et al.*, 1988). As expected, contents of linoleic acid (18 : 2n-6) were significantly increased and those of arachidonic acid (20 : 4n-6) were decreased (Table 1). Other alterations included the increases in palmitic acid (16 : 0) and eicosatetraenoic acid (20 : 4n-3) and the decrease in stearic acid (18 : 0). The major cause of these changes in contents of linoleic and arachidonic acid are thought to be diminished fatty acid desaturation in diabetic animals (Faas and Carter, 1983; Faas *et al.*, 1988). And also, fatty acid composition changes in diabetic state could be due to changes of free fatty acid (FFA) pool and/or incorporation pattern from FFA pool (Dang *et al.*, 1988). Brazilin and insulin returned the contents of palmitic acid and arachidonic acid to normal levels in diabetic animals. However, linoleic acid (18 : 2n-6) and stearic acid (18 : 0) remained unchanged by the treatment of

Table I. Effects of brazilin on phospholipid fatty acid composition in diabetic erythrocyte membrane

Fatty acid	Normal Control	Diabetic			
		Control	+Brazilin (10 mg/kg)	+Brazilin (100 mg/kg)	+Insulin
16 : 0	48.54± 1.65	54.28± 0.90[a]	49.87± 1.00[b]	51.51± 1.64	46.62± 0.90[b]
16 : 1n-7	ND	1.54± 0.22	ND	0.42± 0.17	1.27± 0.59
18 : 0	21.07± 0.20	14.48± 0.35[c]	14.42± 0.51	14.66± 0.71	13.20± 0.86
18 : 1n-9(t)	2.70± 0.20	3.65± 0.14[a]	3.79± 0.14	3.19± 0.29	4.59± 0.20[b]
18 : 1n-9(c)	1.76± 0.58	1.88± 0.16	2.02± 0.03	2.37± 0.40	2.21± 0.07
18 : 2n-6	6.06± 0.25	12.74± 0.67[c]	12.90± 0.50	12.23± 0.54	14.15± 0.88
20 : 1n-9	5.16± 0.64	ND	ND	ND	ND
20 : 4n-6	10.43± 0.58	7.71± 0.56[a]	10.92± 0.76[b]	10.28± 0.53[b]	12.09± 1.38[b]
20 : 4n-3	5.72± 0.23	2.00± 0.19[c]	2.76± 0.26	3.02± 0.33[b]	2.33± 0.21
20 : 5n-3	0.77± 0.05	ND	1.37± 0.08	1.13± 0.14	1.03± 0.11
20 : 6n-3	1.47± 0.14	1.54± 0.12	3.13± 0.20[d]	2.20± 0.22	2.35± 0.70

Brazilin was administered i.p. for 14 days.
Insulin was administered s.c. for 5 days prior to sacrifice.
Each group consisted of five experimental animals.
Values are expressed as mean± SE of percentages of each fatty acid over total fatty acids identifiable.
a: $p<0.05$ vs normal control
b: $p<0.05$ vs diabetic control
c: $p<0.01$ vs normal control
d: $p<0.01$ vs diabetic control
t: trans c: cis ND: not detected

brazilin or insulin. Insulin is known to normalize fatty acid composition of red blood cell by improving metabolic disturbances in the diabetic patient (Tilvis 1986, Tilvis 1985). However, it is not yet determined how brazilin could ameliorate the alterations of fatty acid composition in the diabetic animals. This could be due to its direct effects on desaturase system or secondary effects following improving diabetic control (Faas, 1988). In fact, brazilin significantly reduced blood glucose level of diabetic animal under this experimental condition (368± 28 vs 197± 68; diabetic control and diabetic, brazilin (100 mg/kg) treated group, respectively, $P<0.05$) The precise mechanism how brazilin affects fatty acid composition remains to be elucidated.

In summary, brazilin could normalize C/PL ratio and phosphatidyl fatty acid composition of erythrocyte membrane from streptozotocin induced diebetic rats. These results suggest that brazilin could improve erythrocyte membrane fluidity, which plays a key role in regulating erythrocyte deformability, thereby it could exert beneficial effects on microcirculatory disturbances.

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