Arch. Pharm. Res. 11(2), 149-154(1988)

Effects of Brazilin on Erythrocyte Deformability and Its Related Biochemical Factors in Streptozotocin Induced Diabetic Rats

Chang-Kiu Moon, Jin-Ho Chung, You-Mie Lee, Soo-Hwan Lee Gwi-Seo Hwang, Kwang-Sik Park, Myung-Soo Mock, Seong-Gon Kim Young-Soo Ahn and Jeong-Hee Ann

College of Pharmacy, Seoul National University, 151-742 Seoul, Korea
(Received June 1, 1988)

Abstract □ Impaired erythrocyte deformability was considered to play an important role in microcirculatory disturbances. We recently confirmed that brazilin, the main active principle of *Caesalpinia sappan,* enhanced activity of erythrocyte deformability and reduced blood viscosity. In this study, we examined the effects of brazilin on three biochemical parameters (ATP, 2,3-diphosphoglycerate, and calcium) which influenced erythrocyte deformability. Treatment with brazilin increased erythrocyte deformability and ATP concentrations in streptozotocin-induced diabetic rats. Concentrations of 2,3-diphosphoglycerate and calcium in diabetic rats following brazilin administration were decreased significantly compared to those of diabetic control rats. The results suggest that brazilin have a potential effect to improve rheological abnormalities in diabetes.

Keywords □ brazilin, erythrocyte deformability, streptozotocin-induced diabetes, ATP, 2,3-diphosphoglycerate, calcium

An erythrocyte can pass through blood vessels smaller than its own diameter because of its ability to alter its dicoid shape. Although the size of erythrocyte is usually 8 m in diameter, it is capable of passing through glass tubes whose diameter is only 3 m[1,2]. Following passages, the erythrocyte returns quickly to its dicoid shape. This erythrocyte property is usually refered to deformability. The deformability of erythrocyte plays a prominant physiological role in oxygen transport capacity. It was well known that erythrocytes from diabetic patients had a significantly lower deformability activity[3-5]. Therefore, the understanding of the biochemical mechanisms which give use to deformability in diabetic patients would serve an important contribution to diabetic-related research. There are several evidences that mechanical behavior of the erythrocyte membrane is strongly related with its metabolic state[6]. Weed *et al.* reported that ATP depletion induces an increase in erythrocyte rigidity[7]. In 1969, Weed group also demonstrated that decreased deformability of the aging erythrocyte is associated with the levels of ATP and 2,3-diphosphoglycerate (2,3-DPG)[8]. Furthermore, there was another report that increasing Ca^{++} content with a ionopore reduces deformability[9]. It was suggested that this might be due to alterations of cross-linking of cytoskeleton protein, spectrin, through activation of a transglutaminase[10].

The heart wood of *Caesalpinia sappan* has been commonly used as an emmenagogue, and analgesic, a cure for contusion and sprain as well as a remedy for thrombosis in oriental medicine. The main constituent of *Caesalpinia sappan* is brazilin which has a flavonoid structure. Our laboratory published a series of papers related to effects of brazilin on diabetic complications[11,12]. It has been previously shown that erythrocyte deformability in alloxan-induced diabetic rats was significantly improved by treatment of brazilin. As a continuing research, the effects of brazilin on erythrocyte deformability in streptozotocin-induced diabetic rats were investigated. In addition, the alteration of biochemical parameters which are involved in erythrocyte deformability was studied.

EXPERIMENTAL METHODS

Reagents

Streptozotocin, phosphoglyceric acid buffered solution, NADPH, glyceraldehyde-3-phosphate dehydrogenase/3-phosphoglycerate phosphokinase enzyme mixture, adenosine 5'-triphosphate, phosphoglycolic acid, phosphoglycerate mutase and triethanolamine buffer solution were purchased from Sigma Chemical Co., USA and (+)-brazilin monohydrate were obtained from Aldrich Chemical Co., USA. Pentoxifylline was kindly gifted from Hoechst Pharm. Co., Korea. All other chemicals used were reagent grade.

Experimental animals

Male Spraque-Dawley rats (180-250 gm) supplied from the Experimental Animal Breeding Center of Seoul National University were used. Experimental rats were adapted for 2 weeks with alternating 12 hr periods of light/darkness cycle and temperature, humidity were controlled with automatic thermo-humidistat as 22 ± 1 °C and 60 ± 50%, respectively. Rats were fed on normal chow diet (Samyang Feed Production Co., Korea), which is composed of crude protein 22.1%, crude fat 3.5%, calcium 0.6%, phosphorus 0.4%, crude cellulose 5.0% and crude ash 8.0%.

Induction of diabetes mellitus

Streptozotocin was dissolved in citrate buffer (0.2 M, pH 4.0) and kept in ice bath. Streptozotocin solution was prepared 15 minutes prior to experiment. Previously adapted Sprague-Dawley rats were fasted overnight and injected streptozotocin 50 mg/kg body weight through the tail vein to a total volume of about 0.4 ml. A drop of blood was drawn from tail vein 48 hours after without fasting streptozotocin injection and blood glucose level was determined by Reflomat Glucometer (Boeringer Manheim, West Germany). Rats which has blood glucose level more than 300 mg% were used in this experiment. All rats had free access to fresh water and solid laboratory chow throughout this experiment.

Treatments of animal

Rats were divided into 6 groups; normal rats, brazilin treated normal rats, pentoxifyllin treated normal rats, diabetic rats, brazilin treated diabetic rats and pentoxifyllin treated diabetic rats. Brazilin (100 mg/kg) and pentoxifyllin (91.4 mg/kg, equimolar to brazilin) were dissolved in saline(5 ml/kg), and then were administered i.p. once a day for 2 weeks. Control rats were administered saline only as a vehicle. Due to its poor solubility in water and high sensitivity to light, brazilin solution was suspended by 3-hour sonication and kept in brown vaccum bottle.

In the case of *in vitro* study, rats were divided into 2 groups; normal and diabetic groups. The brazilin or pentoxifylline was added to the blood at different doses (10^{-6} to 10^{-3} M), same volume of physiological saline was added to the control. These were incubated for 5 minutes at 37 °C.

Determination of erythrocyte deformability

Each blood sample was obtained by cardiac puncture with heparinized syringe. The number of erythrocyte was counted in a hemacytometer of known dimensions. Whole blood was centrifuged at 3,000 rpm for 10 minutes and buffy coat was carefully removed. The red cells were washed two times with tris-buffered NaCl (tris-NaCl), and 1 volume of cells were suspended with 4 volume of tris-NaCl buffer (pH 7.4 containing 0.25% albumin) to make 20% red blood cell suspension. Erythrocyte deformability was determined by means of the filtration equipment of Reid *et al.*[13] using method of Schmid-Schonbein *et al.*[4]. All measurements were carried out at 37 °C and results were expressed as the volume of red blood cells filtered per minute (VRBC ml/min.).

Assay of ATP in erythrocyte

Each blood sample was collected by cardiac puncture with the acid citrate dextrose solution containing syringe. The ATP assay was carried out based on the reaction described by Bucher[14] as modified by Adams.

Assay of 2,3-diphosphoglycerate

Heparinized blood was centrifuged to obtain the erythrocytes. Buffy coat was carefully removed and the cells were washed with physiological saline. 1ml of erythrocytes was added to 3 ml of 8% cold trichloroacetic acid (TCA) and vortexed vigorously for deproteinization. Deproteinization mixtures were frozen to -20 °C for further assays. It has been reported that 2,3-DPG in TCA mixture is stable at least two weeks at −20 °C. On the day of analysis, frozen samples were thawed and centrifuged to obtain supernatant for use in the assay. 2,3-DPG level of erythrocyte was measured according to Lowry's method[15].

Determination of calcium in erythrocyte

Erythrocyte calcium levels were determined according to the method of Weed *et al.*[6]. Heparinized blood was centrifuged to get the erythrocyte pellet. Buffy coat was carefully removed and the cells were washed two times with tris-NaCl buffer (pH 7.4, 0.25% albumin) and 1 volume of cells lysed with 2 volume of distilled, deionized water followed by an initial extraction with 1 m*l* of 20% TCA containing 0.5% lanthanium chloride. After centrifugation, the supernatant was transferred to a 10 ml volumetric flask and the precipitate reextracted one time with 2 ml of 20% TCA containing 0.5% lanthanium chloride, to bring the volume to 10 ml and red cell calcium measured by Inductively Coupled Plasma.

RESULTS AND DISCUSSION

It has been reported that, in the diabetic patients, haematological abnormalities could occur in the pathological conditions such as increases of platelet aggregation, platelet adhesion to vessel, platelet turnover rate, viscosity of plasma and decreases of the deformability of erythrocyte[4,5,16,17]. These abnormalities may result in the pathogenesis of small vessel disease[16,17].

In the search of active principles to exhibit the preventive effect on haematological abnormalities, brazilin, a plant pigment, was extensively studied in our laboratory for its effects on the platelet aggregation, and malondialdehyde-formation in platelet (unpublished data). In addition, it has been demonstrated that brazilin treatment leads to the increase in erythrocyte deformability and the decrease in blood viscosity in diabetic Sprague-Dawley rats (unpublished data). In the present study, we further characterized the effects of brazilin on three biochemical parameters related to erythrocyte deformability.

Fig. 1 represents the effect of brazilin on the erythrocyte deformability in normal and diabetic rats. Erythrocyte deformability was investigated by the measurement of erythrocyte-filtration rate. Pentoxifyllin, which promotes blood flow and increases peripheral circulation was used as a positive control in this experiments. As expected, erythrocyte from diabetic animal significantly decreased the filtration rate compared to normal control. A treatment with brazilin significantly increased filtration rate of erythrocyte in normal control(ca. 15%) as well as in diabetic animal(ca. 28%). Therefore, it was suggested that brazilin treatment significantly enhanced the erythrocyte deformability in normal and diabetic animals.

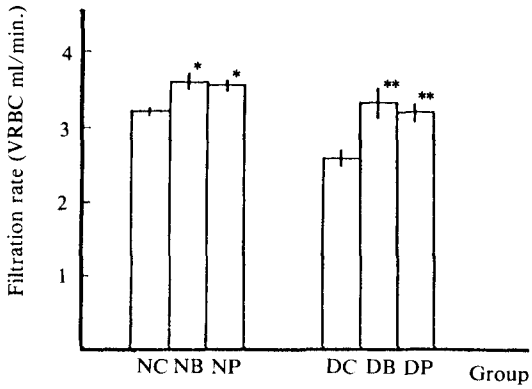

Fig. 1. Effects of brazilin and pentoxifylline on filtration rates of erythrocytes in normal and diabetic rats.

Brazilin (100mg/kg b.w./day) or pentoxifylline (91.4 mg/kg b.w./day) or saline was injected i.p. to normal and diabetic rats for 2 weeks. Filtration was measured with 20% erythrocyte suspension in tris-NaCl buffer. Filtration rates were expressed as VRBC(volume of erythrocyte). Experimental groups are; NC (normal control), NB (normal animals with brazilin-treated), NP(normal animals with pentoxifylline-treated), DC (diabetic control), DB(diabetic animals with brazilin-treated), and DP(diabetic animals with pentoxifylline-treated).

*represent significant differences from normal control ($p < 0.05$); **represent significant differences from diabetic control ($p < 0.05$).

The effect of brazilin on erythrocyte deformability was tested at its various concentrations *in* vitro. As shown in Fig. 2, significant increases of erythrocyte deformability was caused by the treatment of brazilin in a dose dependent manner. Normal animals, however, did not show any significant difference among groups at the various concentrations of brazilin *in vitro* (data are not shown). Therefore it was concluded that treatment with brazilin significantly enhanced erythrocyte deformability *in vivo* as well as *in vitro*.

Nakao *et al.*[18] and Weed *et al.*[6] previously demonstrated that deformability of erythrocyte was closely related to the content of ATP. Thus the substances which improve erythrocyte deformability could increase ATP level in erythrocyte. Since our previous results showed that brazilin enhanced erythrocyte deformability *in vivo* and *in vitro*, it was of our interest to study the change of ATP level in erythrocyte followed by brazilin treatment. As seen in Fig. 3, ATP contents were significantly

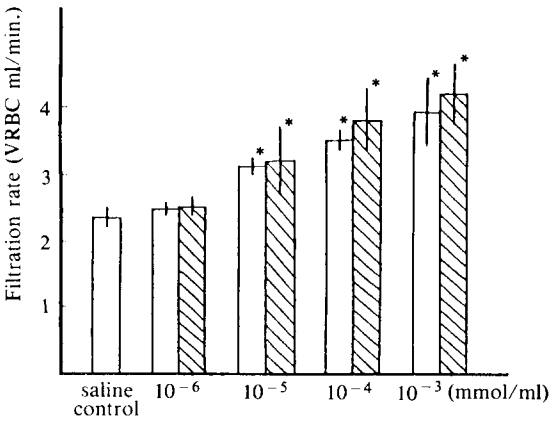

Fig. 2. Filtration rates of erythrocyte from diabetic rats treated with various concentrations of brazilin and pentoxifylline *in vitro*.
Filtration rate was measured with 20% erythrocyte suspension in tris-NaCl buffer. Samples were incubated at 37 °C and tested after 5 min, □ represents brazilin-treated and ▨ represents pentoxifylline-treated. *represent significant differences from diabetic control ($p < 0.05$).

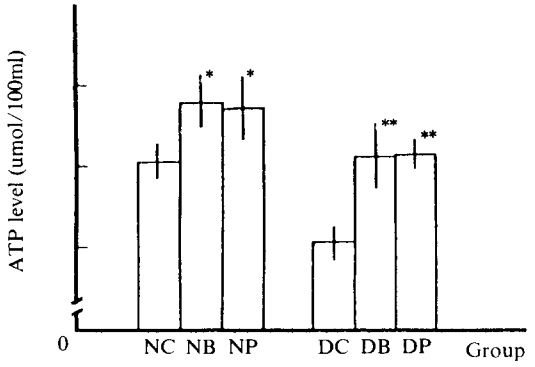

Fig. 3. Effects of brazilin on erythrocyte ATP concentration of normal and diabetic rats.
Experimental methods and abbreviations are the same as described in figure 1.
*represent significant differences from notmal control ($p < 0.05$); **represent significant differences from diabetic control ($0 < 0.01$).

reduced in erythrocytes from diabetic control animals compared to those in normal control animals. Brazilin treatment of normal and diabetic animals increased ATP levels by ca. 15% and ca. 28% respectively. These data is consistent with the previous experiment on erythrocyte deformability measured by filtration rate. Administration of the equimolar dose of pentoxifylline to normal and diabetic rats increased ATP levels to the same extent as treatments of brazilin. Consistent with these findings, Stefanovich[19] reported that ATP levels in erythrocyte were increased in normal and hypoxic rats following oral administration of pentoxifylline. The increases in filtration rates of erythrocyte by brazilin treatment are nicely correlated with the increases in levels of ATP in normal and diabetic animals. Thus it was concluded that the change of ATP levels by brazilin treatment might have a important role for improvement of erythrocyte deformability.

The changes of erythrocyte ATP level were also investigated *in vitro* at its various concentrations (Fig. 4). ATP level of erythrocytes were increased in dose-dependent manner in diabetic animals by the treatment of brazilin. It was confirmed in the *in vitro* study that changes of ATP level in erythrocytes due to different concentration of brazilin (Fig. 4) were correlated with increase in erythrocyte deformability (Fig. 2).

The contribution of ATP levels to the deformability and life-span of erythrocytes has been suggested in earlier study[6]. According to Weed *et al.*, the ATP in erythrocytes was chelated with intracellular calcium under normal circumstances. However, when the calcium level attains supranormal levels, the flexibility of erythrocyte is greatly reduced due to a reduction in ATP levels through binding of excess calcium. In order to clarify further the mechanism of erythrocyte deformability, the levels

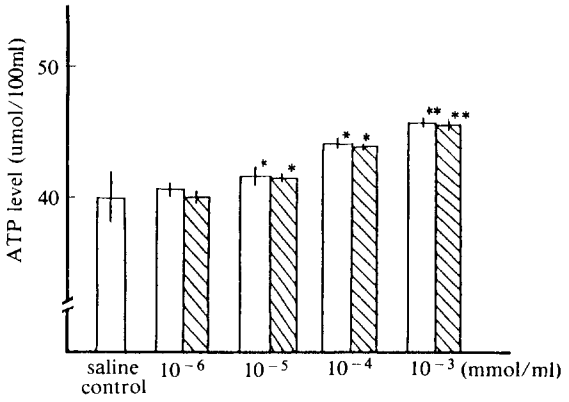

Fig 4. Effect of brazilin on erythrocyte ATP levels at its various concentrations in diabetic animals *in vitro*.
*represent significant differences from diabetic control ($p < 0.05$); **represent significant differences from diabetic control ($p < 0.01$).
Brazilin treated and pentoxifylline treated groups and shown as (□) and (▨), respectively.

of calcium followed by treatment of brazilin were investigated (Fig. 5). The calcium concentrations in erythrocyte of diabetic animals was higher than in those of normal animals. Administration of brazilin significantly decreased calcium level in diabetic erythrocyte but had no effect in erythrocytes of normal animals. These data support the premises that lower level of calcium might improve erythrocyte deformability. On the other hand, treatment of pentoxifylline did not have any effect both in diabetic and in normal animals. Unlike brazilin, pentoxifylline improve deformability of erythrocyte without changes of calcium concentration. It is suggested that the mechanism which is involved in erythrocyte deformability by these two agents could be different. This study is currently undergoing in our laboratory.

It has been reported that 2,3-diphosphoglycerate (2,3-DPG) level in erythrocytes from the diabetic animal increase significantly with concomitant decrease of erythrocyte deformability and ATP concentration[20]. Therefore, levels of 2,3-DPG in erythrocytes of normal and diabetic animals was investigated following administration of brazilin (Fig. 6). Contradictory results from previous report[20] were obtained that 2,3-DPG did not increased significantly in diabetic animals compared to normal animals. Although brazilin did not alter 2,3-DPG level in normal rats, treatment of diabetic animal with brazilin significantly decrease 2,3-DPG in its erythrocytes. It was suggested that brazilin could improve erythrocytes deformability in diabetic animal by decreasing 2,3-DPG. It has been known that rigidity of the erythrocyte is dependent upon conformational changes of cytoskeleton protein.

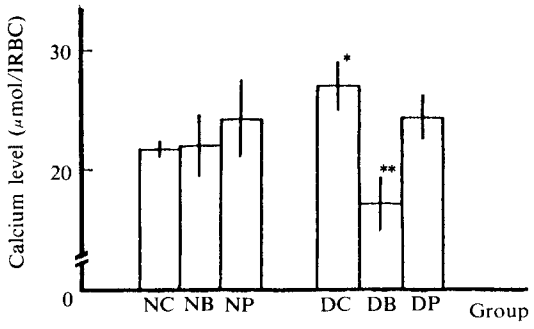

Fig. 5. Effects of brazilin on erythrocyte calcium concentrations of normal and diabetic rats.
Experimental method and abbreviations are the same as described in Figure 1. *represent significant differences from normal control ($p < 0.05$); **represent significant differences from diabetic control ($p < 0.05$).

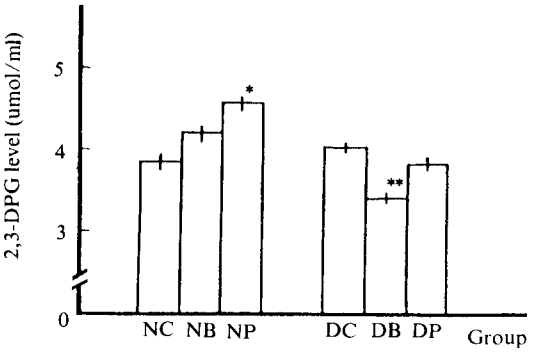

Fig. 6. Effect of brazilin on erythrocyte 2,3-diphosphoglycerate concentrations of normal and diabetic rats.
Experimental methods and abbreviations are the same as described in Figure 1. *represent significant differences from normal control ($p < 0.05$); **represent significant differences from diabetic control ($p < 0.05$).

One of the important structural protein, spectrin, usually bind to actin. However, spectrin's affinity to actin might alter erythrocyte deformability depending on the levels of 2,3-DPG and thus affect the changes of erythrocyte deformability.

In summary, erythrocyte deformability in diabetic animals decreased significantly compared to those of normal animals. Treatment of diabetic rats with brazilin resulted in significant increase of erythrocyte deformability in both normal and diabetic animals. To characterize the mechanisms of erythrocytes deformability, three possible biochemical parameters were investigated following administration of brazilin. In diabetic animals, increase of ATP levels and decreases of Ca^{++} and 2,3-DPG levels were occurred by treatment with brazilin. These data suggest that brazilin protect against rigidity of erythrocytes in diabetic animals through alterations of three biochemical parameters. However, the interrelationships of three biochemical parameters which give rise to erythrocyte deformability needs further investigations.

ACKNOWLEDGEMENT

This work was supported by the research grant from the Korea Research Foundation, Republic of Korea.

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