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Asymmetric dimethylarginine reduced erythrocyte deformability in streptozotocin-induced diabetic rats

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Abstract

To investigate the effect of asymmetric dimethylarginine on erythrocyte deformability in streptozotocin-induced diabetic rats, a single intraperitoneal injection of streptozotocin (STZ, 65 mg/kg) in male Sprague–Dawley rats was carried out to induce diabetes and normal erythrocytes were incubated with asymmetric dimethylarginine or aortic rings from diabetic rats in the presence of L-arginine or vitamin E. We found that erythrocyte deformability was significantly decreased in diabetic rats. The levels of asymmetric dimethylarginine in plasma and erythrocytes of diabetic rats were elevated significantly from 2-week diabetic duration to 8-week diabetic duration. Nitric oxide in erythrocytes was decreased at 8-week diabetic duration while plasma nitric oxide remained unchanged all along. The content of malondialdehyde in erythrocytes of diabetic rats was increased. After incubation of erythrocytes with asymmetric dimethylarginine (10^{-6} M) for 30 min, erythrocyte deformability and nitric oxide level in erythrocytes were decreased markedly. Reactive oxygen species and malondialdehyde production in erythrocytes were promoted by asymmetric dimethylarginine. Both L-arginine and vitamin E reversed the effects of asymmetric dimethylarginine. After incubation of erythrocytes with aortic rings from diabetic rats, erythrocyte deformability was decreased, which was attenuated by L-arginine. These results indicated that reduction of erythrocyte deformability in diabetic rats was associated with promoted oxidant stress as well as impaired nitric oxide synthesis by elevation of asymmetric dimethylarginine.

Keywords: Nitric oxide synthase; Nitric oxide; Microcirculatory disturbances; Oxidant stress

Introduction

Decreased erythrocyte deformability is an important initiator contributing to microcirculatory disturbances under many pathological conditions, such as hypertension and diabetes mellitus (Juhan et al., 1982). It has been documented that nitric oxide (NO) participated in modulation of erythrocyte deformability (Kazushi et al., 2000; Bor-Kucukatay et al., 2003). Evidence shows that decrease of bioavailability of NO might be related to elevation of endogenous nitric oxide synthase inhibitors including asymmetric dimethylarginine (ADMA) in diabetes (Lin et al., 2002). Recent studies showed that erythrocyte deformability was decreased concomitantly with increased plasma level of ADMA in patients with hypertension (Kazushi and Ichiro, 2005). Since the plasma levels of ADMA are increased in diabetic animals and patients (Yan et al., 2005), we postulate that the increased level of ADMA might be an important contributor to reduction of erythrocyte deformability in diabetes.

It was reported that lipid peroxides could impair erythrocyte membrane and decrease erythrocyte deformability (Bekyarova et al., 1996; Chung et al., 1998). Recently, it has been found that ADMA, besides inhibiting NO synthesis, directly induces oxidant stress (Böger et al., 2000; Teerlink, 2005; Scalera et al., 2005). In the present study, therefore, we also tested

Abbreviations: ADMA, asymmetric dimethylarginine; NOS, nitric oxide synthase; STZ, streptozotocin; NO, nitric oxide; MDA, malondialdehyde; ROS, reactive oxygen species; Vit E, vitamin E; Hb, hemoglobin; SNP, sodium nitroprusside; L-NAME, *N* (G)-nitro-L-arginine methyl ester; DCFH-DA, 6-Carboxy-2′, 7′-dichlorodihydrofluorescein diacetate.

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whether decreased erythrocyte deformability induced by ADMA involves oxidant stress.

Materials and methods

Major reagents

L-arginine, ADMA and streptozotocin (STZ) were purchased from Sigma Chemical Co (St. Louis, MO, USA). Vitamin E was obtained from Shuang-He Medical Corporation (Beijing, China). NO and malondialdehyde (MDA) assay kits were purchased from Ju-Li Biological Medical Engineering Institute (Nanjing, China). Reactive oxygen species (ROS) assay kit was purchased from Beyotime Institute of Biotechnology (Jiangsu, China).

Animals

Male Sprague–Dawley rats (200±10 g) were obtained from Central South University Animal Services (Changsha, China). All experimental procedures were adhered to the ILAR *Guide for Care and Use of Laboratory Animals* and were approved by the Animal Care and Use Committee of Central South University on animal experiment.

Induction of diabetes mellitus

Diabetes was induced by a single intraperitoneal injection of 65 mg/kg STZ (dissolved in 0.05 M citrate buffer, pH 4.5) after fasting for 24 h. Onset of diabetes mellitus normally occurred 3 days after STZ injection and was confirmed by rapid weight loss, glycosuria and hyperglycemia. Animals in the diabetic group were excluded from the study if plasma glucose was less than 16.7 mM determined by an Accu-Chk Active Blood Glucose Instrument (Accu-Chk Co, Germany).

Measurement of erythrocyte deformability

Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (30 mg/kg), and blood samples (anticoagulated with heparin 15 IU/ml) collected from carotid artery were placed in tubes containing 15% polyvinylpyrrolidone (PVP-K30) solution (PVP 150 g/l, Na_2HPO_4 2.84 g/l, KH_2PO_4 0.68 g/l, NaCl 3.8 g/l, pH 7.4). Erythrocyte deformability was determined by laser diffraction analysis using an ektacytometer (LBY-BX3, Pu-Lisheng Corporation, Beijing, China) (Bor-Kucukatay et al., 2003). In brief, a low hematocrit (2.5%) suspension of erythrocytes in 15% polyvinylpyrrolidone solution was added to the sample cup, then laser beam was directed through and erythrocytes were sheared at a series of shear stresses. During the passage of the laser beam, the laser light was diffracted forming an image correlating to the shape of all erythrocytes that pass the laser beam. Thus, the diffraction image represented the mean deformability of all these erythrocytes and was analyzed by a microcomputer. On the basis of the geometry of diffraction pattern, an elongation index (EI) was calculated: $EI=(L-W)/(L+W)$,where L and W were the length and width of the diffraction pattern, respectively. Integrated elongation index (IEI) is an overall measurement of erythrocyte deformability when shearing erythrocytes at a series of shearing stresses. Increased EI or IEI indicates greater erythrocyte deformability. All measurements were carried out at 37°C. Each sample was examined three times.

Determination of ADMA

After resuspension of erythrocytes in double distilled water for 2 h, hemolysis supernatant was prepared by centrifugation (13,000×*g*, 30 min) at 4°C. After deproteinating of plasma or hemolysis supernatant by 5-sulfosalicylic acid, the content of ADMA was measured by high-performance liquid chromatography (Shimadzu LC-6A, Shimadzu Corporation Kyoto, Japan) using *o*-Phthaldialdehyde for fluorescence determination according to methods described (Jiang et al., 2002). ADMA contents were expressed as μmol/l for plasma and nmol/g Hb for erythrocytes, respectively.

Determination of nitrite/nitrate, MDA, ROS and hemoglobin

The contents of nitrite and nitrate measured by Griess method with a spectrophotometer were used to reflect NO levels (Yan et al., 2005; Jiang et al., 2002; Tedesco et al., 2001). MDA content in erythrocytes was measured by thiobarbituric acid method (Jain, 1985). Hemoglobin (Hb) was measured with a hemocyte analysator (CD1700, ABBOTT Co, USA) based on the cyanomethemoglobin method. NO levels in plasma and erythrocytes were expressed as μmol/l and μmol/g Hb, respectively.

Reactive oxygen species (ROS) was measured as previously described with some modification (Tedesco et al., 2001). In brief, erythrocytes were incubated with 6-Carboxy-2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA) at a final concentration of 10 mM for 20 min and washed 3 times with HEPES buffer. ROS production in erythrocytes was measured fluorometrically with excitation and emission settings at 488 and 525 nm, respectively (F-4000, HITACHI, Japan) and expressed as arbitrary units.

Preparation of aortic rings

At 8-week duration after diabetes induction, rats were anesthetized (sodium pentobarbital, 30 mg/kg, ip). The thoracic aortas were carefully obtained and cut into rings of 1 cm long. Rings were suspended in ice-cold Krebs' solution (mM: 119.0 NaCl; 25.0 $NaHCO_3$; 4.7 KCl; 1.2 KH_2PO_4; 1.2 $MgSO_4{\cdot}7H_2O$; 2.5 $CaCl_2$; 11.0 glucose, pH 7.4) implied with 95% O_2 and 5% CO_2 (Jiang et al., 2002).

Experimental protocols

Diabetic rats were randomly divided into three groups of 2-, 4- and 8-week feeding duration after diabetes induction. Non-diabetic control animals matched for age and weight were injected with equal volumes of vehicle. All animals were kept under the same conditions.

To further test the effect of ADMA on erythrocyte deformability, erythrocytes were treated with ADMA in HEPES buffer (mM: 123 NaCl, 5 KCl, 1 $MgCl_2{\cdot}6H_2O$, 1.3 $CaCl_2$, 10 glucose and 25 HEPES; pH 7.4).

To examine the effect of the endothelium on erythrocyte deformability, erythrocytes were incubated with aortic rings from diabetic or non-diabetic rats in HEPES.

Statistics

Results were expressed as means±SEM. Statistical comparisons among groups were carried out by ANOVA followed by either Newman–Keuls posttest or nonparametric tests according to the results of homogeneity of variance test. Significance was accepted at $p<0.05$.

Results

Plasma glucose concentration

As shown in Table 1, blood glucose level was elevated significantly in diabetic rats, but there were no significant differences in blood glucose levels among different diabetic durations.

Erythrocyte deformability

At 4-week and 8-week diabetic duration, EI or IEI, reflecting erythrocyte deformability, was decreased in diabetic rats (Table 2). Incubation of erythrocytes with ADMA at a concentration of 10^{-6} M or 10^{-5} M for 30 or 45 min markedly decreased erythrocyte deformability (Fig. 1), which could be reversed by L-arginine (Fig. 2A) or vitamin E (Fig. 2B).

Table 1
Plasma concentrations of glucose, ADMA and NO in diabetic rats (DM)

	Glucose (mM)		ADMA (μM)		NO (μM)	
	Con	DM	Con	DM	Con	DM
2 weeks	6.2±0.5	30.6±1.1[b]	0.86±0.11	2.42±0.15[a]	32.5±1.8	36.7±1.7
4 weeks	5.9±0.4	29.9±0.8[b]	0.96±0.10	2.79±0.14[b]	25.1±1.9	28.2±1.6
8 weeks	6.8±0.2	31.3±1.3[b]	1.12±0.06	2.38±0.12[b]	24.3±0.5	24.6±0.7

Data are expressed as means±SEM, $n=8$. [a]$p<0.05$, [b]$p<0.01$ *vs* Con.

ADMA in plasma and erythrocytes

Plasma levels of ADMA in STZ-induced diabetic rats were increased markedly at 2-week duration and retained at high levels at both 4- and 8-week duration (Table 1). Similarly, the level of asymmetric dimethylarginine in erythrocytes was also significantly increased (Table 3).

NO and MDA level

Plasma levels of NO remained unchanged throughout the experiment (Table 1), while at 8-week diabetic duration, the level of nitric oxide in erythrocytes was decreased (Table 3). The content of MDA in erythrocytes was elevated in a duration-dependent manner in diabetic rats (Table 3).

Effects of ADMA on NO, MDA and ROS production

Incubation of erythrocytes with ADMA (10^{-6} M) for 30 min significantly decreased NO content, an effect which was attenuated by L-arginine (Fig. 3). Exogenous ADMA also increased MDA content and ROS production, which could be attenuated by vitamin E, respectively (Figs. 4 and 5).

Influence of the endothelium on erythrocyte deformability

After incubation of erythrocytes with aortic rings, erythrocyte deformability in diabetic group was lower than non-diabetic control, which was reversed by L-arginine (Figs. 6 and 7).

Discussion

It is well known that erythrocyte deformability is of crucial importance for maintaining normal circulation. Decreased erythrocyte deformability impairs microcirculatory perfusion, resulting in hypoxia, endothelial dysfunction and platelet aggregation. These pathological changes are closely associated with serious complications of some diseases such as atherosclerosis, hypertension and diabetes mellitus.

It has been found that erythrocyte deformability is regulated by multiple factors. As mentioned above, NO is one of the endogenous active substances responsible for maintaining normal erythrocyte deformability. There is evidence that *N* (G)-nitro-L-arginine methyl ester (L-NAME), a kind of NOS inhibitor, decreased erythrocyte deformability at stress levels of 5 Pa or less, and incubation of erythrocytes with NO donor

Table 2
Erythrocyte deformability at different shear stresses in diabetic rats (DM) and non-diabetic controls (Con)

		EI (%)			IEI (%)
		1 Pa	5 Pa	100 Pa	
2 weeks	Con	13.5±0.9	11.5±0.7	56.4±0.7	22.1±0.6
	DM	12.5±0.8	9.9±0.4	54.7±0.7	21.1±0.4
4 weeks	Con	15.1±0.9	11.5±0.5	55.6±0.4	22.9±0.4
	DM	11.0±0.6[b]	10.0±0.5[a]	54.4±0.3[a]	21.4±0.4[b]
8 weeks	Con	14.7±1.2	13.0±1.1	54.5±0.4	22.8±0.5
	DM	9.8±1.0[b]	10.2±0.3[a]	51.8±0.9[a]	19.9±0.4[b]

Data are expressed as means±SEM, $n=8$. [a]$p<0.05$, [b]$p<0.01$ *vs* Con.

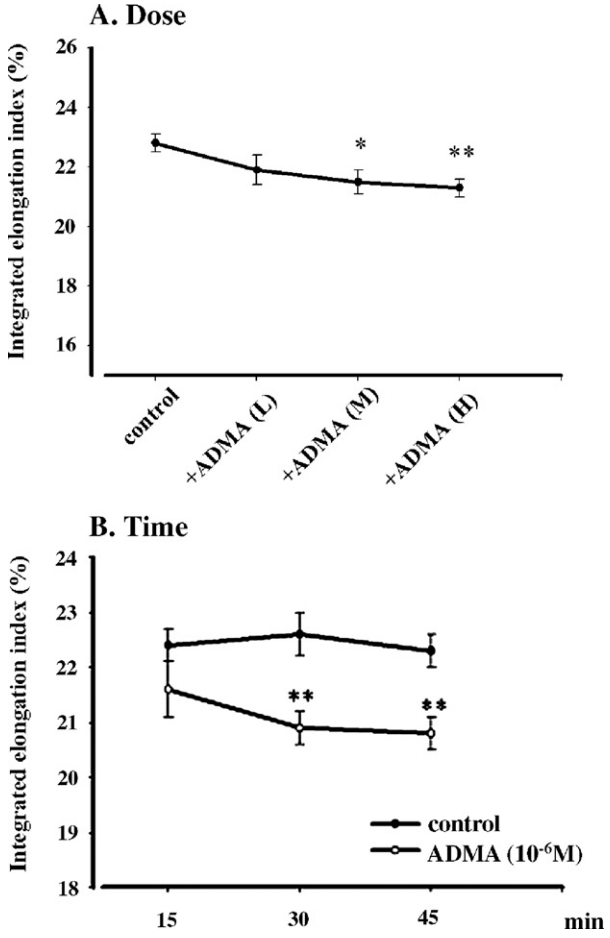

Fig. 1. Effect of ADMA on erythrocyte deformability. The dose–effect (A) and the time–effect (B) relationship of ADMA. ADMA (L, M, H): ADMA at a concentration of 10^{-7}, 10^{-6} or 10^{-5} M, respectively. Data are expressed as means±SEM, $n=8$. *$p<0.05$, **$p<0.01$ *vs* control.

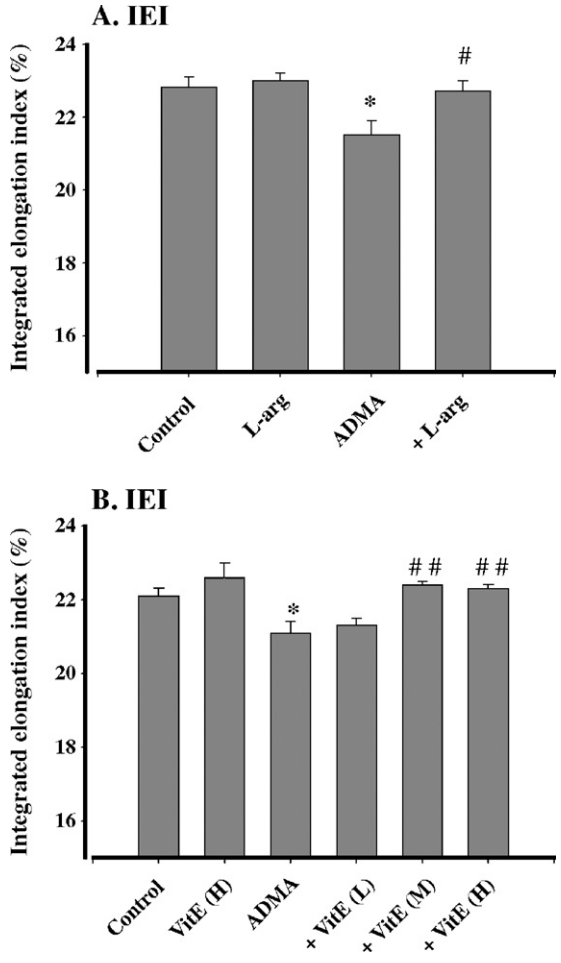

Fig. 2. The effect of ADMA on erythrocyte deformability was reversed by L-arginine (A) and vitamin E (B). Control: HEPES; ADMA: erythrocytes were incubated with ADMA (10^{-6} M); +L-arg: erythrocytes were incubated with ADMA (10^{-6} M) for 30 min in the presence of L-arginine (10^{-5} M); +Vit E (L, M, H): erythrocytes were incubated with ADMA (10^{-6} M) in the presence of vitamin E at a concentration of 10^{-5}, 10^{-4} or 10^{-3} M, respectively. Data are expressed as means±SEM, $n=8$. *$p<0.05$, **$p<0.01$ *vs* control; $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs* ADMA group.

sodium nitroprusside (SNP) increased erythrocyte deformability (Bor-Kucukatay et al., 2003).

Nitric oxide released from the vascular endothelium is one of the main sources in circulation system. It has been shown that endothelium-dependent relaxation (a sign of endothelial NO bioavailability) is decreased in diabetic animals (Yan et al., 2005). In the present study, we tested the effect of the endothelium on erythrocyte deformability. The results showed

Table 3
Contents of ADMA, NO and MDA in erythrocytes of diabetic rats

	ADMA (nmol/g Hb)		NO (μmol/g Hb)		MDA (nmol/g Hb)	
	Con	DM	Con	DM	Con	DM
2 weeks	20.2±1.2	28.4±1.3^{b}	23.3±1.1	22.9±0.7	10.4±0.6	11.8±0.9
4 weeks	17.9±1.2	30.8±3.1^{a}	19.5±1.4	18.4±1.0	9.9±1.1	21.2±0.9^{b}
8 weeks	23.6±2.3	43.5±4.9^{b}	20.8±1.0	11.7±0.7^{b}	13.1±0.7	24.2±0.8^{b}

Data are expressed as means±SEM, $n=8$. $^{a}p<0.05$, $^{b}p<0.01$ *vs* Con.

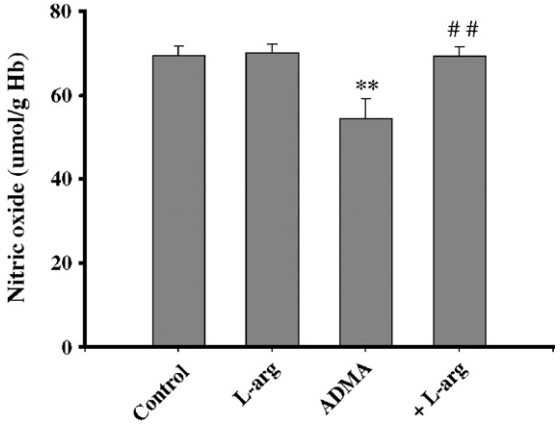

Fig. 3. Effect of ADMA on nitrite/nitrate level in erythrocytes. Control: HEPES; ADMA: erythrocytes were incubated with ADMA (10^{-6} M); +L-arg: erythrocytes were incubated with ADMA (10^{-6} M) for 30 min in the presence of L-arginine (10^{-5} M). Data are expressed as means±SEM, $n=8$. *$p<0.05$, **$p<0.01$ *vs* control; $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs* ADMA group.

that, after incubation of erythrocytes with aortic rings from diabetic rats, erythrocyte deformability was significantly decreased, which was reversed by L-arginine, in support of the hypothesis that NO from endothelium might play an important role in regulation of erythrocyte deformability.

Recently, it has been documented that there is an endogenous mechanism to regulate NO synthesis. ADMA can competitively inhibit NOS activity and decrease NO synthesis (Cooke, 2000). Previous investigations have shown separately that erythrocyte deformability was decreased (Juhan et al., 1982) and the plasma level of ADMA was elevated in diabetes mellitus (Yan et al., 2005). In the present study, we examined the relationship between erythrocyte deformability and ADMA in STZ-induced diabetic rats. The results revealed that erythrocyte deformability was significantly decreased concomitantly with increased

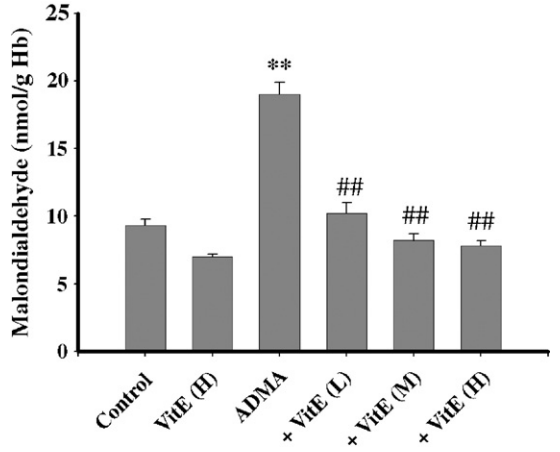

Fig. 4. Effect of ADMA on malondialdehyde (MDA) production in erythrocytes. Control: HEPES; ADMA: erythrocytes were incubated with ADMA (10^{-6} M); +Vit E (L, M, H): erythrocytes were incubated with ADMA (10^{-6} M) in the presence of vitamin E at a concentration of 10^{-5}, 10^{-4} or 10^{-3} M, respectively. Data are expressed as means±SEM, $n=8$. *$p<0.05$, **$p<0.01$ *vs* Control; $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs* ADMA group.

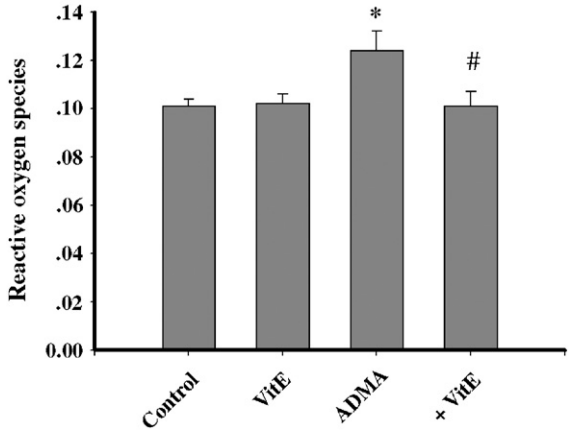

Fig. 5. Effect of ADMA on reactive oxygen species (ROS) production in erythrocytes. Control: HEPES; ADMA: erythrocytes were incubated with ADMA (10^{-6} M); +Vit E: erythrocytes were incubated with ADMA (10^{-6} M) in the presence of vitamin E at a concentration of 10^{-4} M. Data are expressed as means±SEM, $n=6$. *$p<0.05$, **$p<0.01$ *vs* Control; $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs* DM.

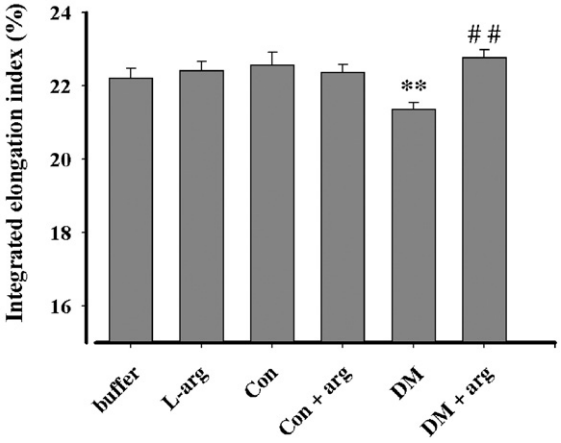

Fig. 7. L-arginine reversed the effect of diabetic endothelium on erythrocytes. Buffer: HEPES; +L-arg: erythrocytes were incubated with L-arginine (10^{-5} M); Con: erythrocytes were incubated with aortic rings from control rats; Con+L-arg: erythrocytes were incubated with aortic rings from control rats in the presence of L-arginine (10^{-5} M); DM: erythrocytes were incubated with aortic rings of diabetic rats; DM+L-arg: erythrocytes were incubated with aortic rings of diabetic rats in the presence of L-arginine (10^{-5} M). The incubation was carried out at 37°C and lasted for 30 minutes Data are expressed as means±SEM, $n=8$. *$p<0.05$, **$p<0.01$ *vs* Con; $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs* DM.

ADMA levels both in plasma and erythrocytes. Exogenous ADMA caused a decrease of erythrocyte deformability in a concentration-dependant manner, and the effect of ADMA was reversed by L-arginine. These findings suggest that ADMA might be an important contributor to reduction of erythrocyte deformability.

It is worthy being noted that, in the present study, plasma NO in diabetic rats showed no changes until 8-week duration though ADMA was elevated obviously. Reasons for NO and ADMA separation are not clear. Similar phenomenon has also been seen in patients with hypertension (Kazushi and Ichiro, 2005).

It has been reported that oxidant stress plays an important role in modulating erythrocyte deformability and it enhances in diabetes mellitus (Bekyarova I et al., 1996; Chung et al., 1998; Ahmet et al., 2001). It is known that oxygen radicals formed in excess of the detoxifying capacity of erythrocytes will attack the polyunsaturated fatty acids of membrane lipids, which results in MDA production. The content of MDA is an intermediate product of polyunsaturated fatty acid peroxidation. It can cross-link with membrane constituents of erythrocytes, but may also cross-link hemoglobin to cytoskeletal proteins (especially spectrin). It has been reported that peroxidative damage of erythrocyte membranes can cause progressive echinocyte transformation and increase membrane rigidity. This renders the erythrocytes less flexible, leading to microcirculatory disorders. The results of our study confirmed previous observations that erythrocyte deformability was decreased concomitantly with an elevation of MDA level and antioxidant agent vitamin E was able to reverse the decrease in erythrocyte deformability by suppressing MDA accumulation in diabetic rats (Bekyarova I et al., 1996; Chung et al., 1998).

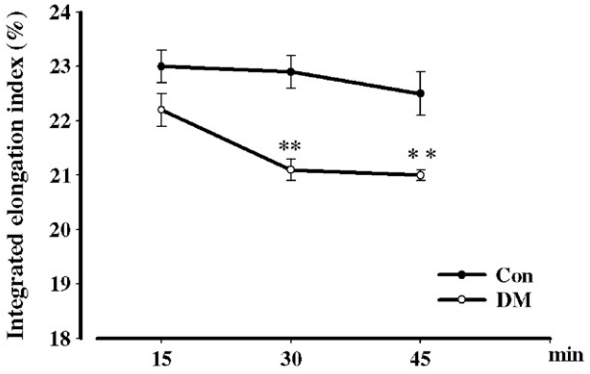

Fig. 6. Effect of the endothelium on erythrocyte deformability. Con: erythrocytes were incubated with aortic rings from control rats; DM: erythrocytes were incubated with aortic rings from diabetic rats. Data are expressed as means± SEM, $n=6$. *$p<0.05$, **$p<0.01$ *vs* Con.

More recently, ADMA, besides regulating synthesis of NO, was documented to induce oxidant stress. For example, incubation with ADMA increased endothelial superoxide radical elaboration in a concentration-dependent manner, which was reversed by L-arginine (Böger et al., 2000). For these reasons, we hypothesized that reduction of erythrocyte deformability induced by ADMA might be due to stimulation of oxygen radical generation. The present results showed that ADMA decreased erythrocyte deformability concomitantly with increases in ROS and MDA production, and the effects of ADMA were attenuated by vitamin E. Recently, it has been documented that decreased membrane fluidity of erythrocytes is related to elevation of ADMA level in patients with hypertension. These results suggest that ADMA plays important role in modulation of erythrocyte deformability.

In summary, the present results suggest that the reduction of erythrocyte deformability in STZ-induced diabetic rats is associated with the stimulation of ADMA production. Besides the classical L-arginine/NO pathway, oxidant stress is also involved in the mechanism of ADMA.

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