Effect of Statil (ICI 128436) on Erythrocyte Viscosity In Vitro

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The hypothesis that sorbitol accumulation could contribute to a reduced erythrocyte deformability in diabetes was investigated. Erythrocyte sorbitol and erythrocyte viscosity at high and low shear rates were studied in 20 insulin-dependent diabetic (IDDM) and 20 matched control subjects. An increased erythrocyte sorbitol and an increased low-shear erythrocyte viscosity were found in the IDDM patients, but there was no significant correlation (r = .11, NS) between the parameters. Incubation (3 h, 37°C) in a Krebs buffer containing 33.3 mM glucose resulted in a significant sorbitol accumulation, but erythrocyte viscosity was not affected. Despite this fact, addition of 1 mM statil (ICI 128436) in the 5.5- and 33.3-mM glucose media not only prevented erythrocyte sorbitol accumulation but also improved erythrocyte viscosity in diabetic and control subjects. The effect was more pronounced at the low (~16%) than at the high (~2%) shear rate. The effect on erythrocyte viscosity disappeared by washing the erythrocytes after incubation, although erythrocyte sorbitol remained different. Our results suggest that sorbitol accumulation does not contribute to an increased erythrocyte viscosity in diabetes, and statil shows a positive effect on erythrocyte viscosity independent of its aldose reductase–inhibiting property. *Diabetes* 37:471–75, 1988

An increased flux through the polyol pathway (Fig. 1) as a consequence of hyperglycemia has been implicated in the pathogenesis of neuropathy, nephropathy, retinopathy, and cataracts in diabetes (1). Support for a role of aldose reductase in diabetic complications has been derived from the fact that some structural abnormalities could be prevented with different aldose reductase inhibitors in diabetic and galactosemic animals (2–4). The presence of the aldose reductase enzyme within microvessels is consistent with a possible role of the polyol pathway in the etiology of diabetic microvascular disease. However, it is not clear whether the primary defect leading to vascular disease in diabetes resides in the vasculature itself (5). Rheological abnormalities have indeed been postulated to play a role in the pathogenesis of diabetic microangiopathy (6). A possibly important factor in this respect is erythrocyte deformability, which is the ability of the erythrocyte to pass through capillaries smaller than its own diameter. Normal erythrocyte deformability is necessary for adequate oxygen supply in the microcirculation. Erythrocyte deformability has been reported to be decreased in diabetes and has been hypothesized to contribute to the development of diabetic microangiopathy (7,8).

Aldose reductase is present in erythrocytes, and an enhanced sorbitol synthesis in erythrocytes from diabetic subjects is well known (9,10). An increased aldose reductase activity leads to secondary biochemical alterations in different cell types. Osmotic swelling, reduced Na^+-K^+-ATPase activity, and a change in the redox status of the pyridine nucleotides and glutathione are reported to be associated with enhanced polyol-pathway activity (3,4,11,12). Such secondary alterations could lead to reduced erythrocyte deformability in diabetes. Therefore, we investigated a possible relationship between erythrocyte sorbitol accumulation and erythrocyte deformability in diabetic patients by use of in vitro incubation experiments.

MATERIALS AND METHODS

Subjects. Erythrocyte viscosity and sorbitol were investigated in 20 ambulatory insulin-dependent diabetic (IDDM) subjects (6 men, 14 women aged 19–40 yr; mean ± SD 29 ± 6 yr) with a mean duration of diabetes of 9 ± 5 yr (range 1–23 yr). All patients were treated with one or more subcutaneous insulin injections per day. They showed normal renal and hepatic function tests and did not suffer from hematological or cardiovascular disease. IDDM patients with

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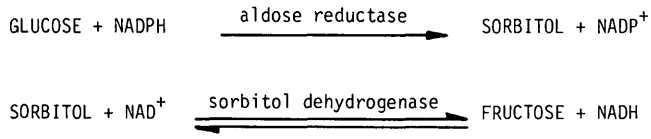

FIG. 1. Schema of polyol pathway.

proliferative retinopathy were excluded. The patients were compared with 20 age- and sex-matched healthy control subjects (6 men, 14 women; aged 19–37 yr, mean 30 ± 5 yr). However, for incubation experiments, erythrocytes were obtained from IDDM and control subjects without regard to age and sex matching.

Erythrocyte sorbitol. Erythrocyte sorbitol was determined according to Malone et al. (9). The plasma from heparinized samples was separated, and the packed cells were washed three times with isotonic saline at 4°C. The hemoglobin content of the washed cells was determined with the cyanohemoglobin method according to D.L. Drabkin (hemoglobin reagent 3060, Baker Chemical, Phillipsburg, NJ).

Two milliliters of the packed cells were mixed with 2 ml of 4 M cold $HClO_4$. The proteins were precipitated after centrifugation. Two milliliters of the protein-free filtrate were neutralized with 3.2 M KOH. The neutralized solution was centrifuged again at 4°C to remove insoluble $KClO_4$. The remaining clear supernatant was used for the sorbitol assay; 0.9 ml of the supernatant was pipetted in 0.6 ml of 0.05 M glycine buffer (pH 9.5) containing 0.8 mg NAD (127329, Boehringer Mannheim, Mannheim, FRG). The reaction was started by adding 20 μl of a sorbitol dehydrogenase solution containing 4 mg enzyme protein/ml (no. 109399, Boehringer Mannheim). All samples were measured in duplicate against their own blank. After a 1-h incubation at room temperature, the relative fluorescence due to NADH was measured on a spectrofluorometer (model 430, Turner, Palo Alto, CA). Sorbitol standards run simultaneously were linear up to 10 g/ml. All results were expressed in terms of nanomoles sorbitol per gram hemoglobin.

Erythrocyte viscosity. Erythrocyte suspension viscosity measurements can be used as an indirect measure of erythrocyte deformability (13,14). In this method, suspending-fluid viscosity and cell concentrations must be controlled. It is claimed that erythrocyte viscosity at high shear rates is mainly influenced by intracellular viscosity, but at low shear rates the influence is more by membrane properties and cell geometry (13). To improve sensitivity, erythrocyte suspensions with a high hematocrit (>60%) were used (15).

After an overnight fast, antecubital vein blood anticoagulated with K_3 EDTA was taken. Plasma and buffy coat were removed by gentle aspiration after centrifugation. The erythrocytes were washed twice in phosphate-buffered saline (PBS). After washing, the cells were resuspended in Krebs buffer (see below) to which bovine serum albumin (10 g/L) and glucose (5.5 or 33.3 mM) were added. For viscosity measurements, hematocrit was adjusted in this buffer to 70%. The hematocrit was determined via microhematocrit centrifugation.

Viscosity measurements were performed in duplicate at 37°C with a Contraves low-shear 30 viscosimeter (Zurich, Switzerland) at high (128.5-s^{-1}) and low (0.945-s^{-1}) shear rates. This viscosimeter was reported to be suitable for blood viscosity measurements at the low shear rate (16).

Incubation of erythrocytes. To evaluate a possible consequence of an increased polyol pathway activity on erythrocyte deformability, erythrocytes were incubated under normo- and hyperglycemic conditions with and without an aldose reductase inhibitor. Three milliliters of washed, packed erythrocytes were suspended in 8 ml Krebs bicarbonate medium (pH 7.4, g/L as follows: 5.54 NaCl, 0.35 KCl, 0.28 $CaCl_2$, 0.16 KH_2PO_4, 0.29 $MgSO_4 \cdot 7\ H_2O$, and 2.10 $NaHCO_3$) with glucose (5.5 or 33.3 mM) and 10 g/L bovine serum albumin (fraction V; Sigma, Poole, UK). In duplicate samples the aldose reductase inhibitor statil (17) (ICI 128436), dissolved in 1% sodium bicarbonate, was added to the flask to a final concentration of 10^{-3} or 10^{-4} M.

The osmolality of the medium containing 5.5 or 33.3 mM glucose was 290 or 320 mosmol/kg, respectively. The erythrocytes were incubated for 3 h with gentle agitation at 37°C in an atmosphere of 95% O_2/5% CO_2. After incubation, viscosity and sorbitol were measured as described above.

In a separate series of experiments the erythrocytes were first washed twice with PBS after incubation and then resuspended in Krebs buffer containing 5.5 mM glucose and 10 g/L bovine serum albumin for viscosity measurements.

RESULTS

We found an increased low-shear viscosity and an increased sorbitol level in the erythrocytes of the diabetic group (Table 1). There was no significant correlation between the low-shear viscosity and sorbitol levels in the diabetic patients (r = .11, NS). To determine whether an enhanced aldose reductase activity could lead to a decreased erythrocyte deformability, the cells were incubated for 3 h at 37°C in a Krebs buffer containing varying amounts of glucose (5.5 or 33.3 mM). To achieve complete inhibition of the sorbitol accumulation, the aldose reductase inhibitor statil was added at a final concentration of 1 mM (Tables 2 and 3).

After 3 h of incubation in 33.3 mM glucose, erythrocyte sorbitol significantly increased compared with the preincubation value and the value after incubation in the presence of 5.5 mM glucose, but there was no significant effect on erythrocyte viscosity. Incubation in 5.5 mM glucose resulted in a small but significant decrease in erythrocyte sorbitol in

TABLE 1
Erythrocyte viscosity and erythrocyte sorbitol in IDDM and control subjects

	IDDM patients	Control subjects	P*
Fasting glycemia (mM)	11.7 ± 1.2	4.4 ± 0.2	<.001
Erythrocyte sorbitol (nmol/g Hb)	59.8 ± 7.7	39.5 ± 3.3	<.01
Erythrocyte viscosity			
High shear† (mPa · s)	8.6 ± 0.1	8.6 ± 0.1	NS
Low shear‡ (mPa · s)	39.9 ± 0.7	37.3 ± 0.5	<.01

All results are expressed as means ± SE. n = 20 for IDDM and control group.
*Calculated by a one-tailed Mann-Whitney U test.
†128.5 s^{-1}.
‡0.945 s^{-1}.

TABLE 2
Effect of glucose and aldose reductase inhibition on erythrocyte sorbitol and erythrocyte viscosity

Condition	Erythrocyte sorbitol (nmol/g Hb)	Erythrocyte viscosity (mPa · s)	
		High shear	Low shear
Erythrocytes from IDDM patients			
Before incubation	61.5 ± 9.3	8.6 ± 0.2	39.9 ± 1.3
After incubation			
In 5.5 mM glucose	56.0 ± 7.7*	8.5 ± 0.2	39.1 ± 2.2
In 33.3 mM glucose	261.8 ± 38.4†	8.5 ± 0.2	39.3 ± 2.5
In 33.3 mM glucose + 1 mM statil	42.8 ± 8.2*‡	8.3 ± 0.1*‡	33.1 ± 1.1*‡
Erythrocytes from control subjects			
Before incubation	35.7 ± 4.9	8.6 ± 0.2	37.5 ± 0.7
After incubation			
In 5.5 mM glucose	44.4 ± 4.9§	8.5 ± 0.2	37.1 ± 1.3
In 33.3 mM glucose	177.8 ± 24.1†	8.5 ± 0.2	37.5 ± 1.2
In 33.3 mM glucose + 1 mM statil	31.8 ± 4.4‡	8.3 ± 0.2*‡	31.0 ± 0.9*‡

All results are expressed as means ± SE. $n = 6$ for IDDM and control groups.
*$P < .025$ significantly lower than before incubation.
†$P < .025$, significantly higher than before incubation in 5.5 mM glucose with statil (one-tailed Wilcoxon test).
‡$P < .025$, significantly lower than when incubated in 33.3 or 5.5 mM glucose.
§$P < .025$, significantly higher than before incubation.

the diabetic patients, in contrast with a small increase in the control subjects. This difference is due to the fact that before incubation, the blood glucose level in the diabetic patients was >5.5 mM, whereas in the control subjects the blood glucose level was <5.5 mM (IDDM, 11.2 ± 2.2 mM glucose; control, 4.3 ± 2.2 mM glucose). No such different effect on erythrocyte viscosity was observed.

The addition of statil abolished the sorbitol accumulation, as indicated by the erythrocyte sorbitol values. In the presence of the inhibitor, the sorbitol values fell even below the preincubation values, although statistical significance was reached only in the diabetic group. At the same time, the presence of statil resulted in a significant improvement of erythrocyte viscosity in diabetic and control subjects. Considering the control group, the decrease in erythrocyte viscosity was not caused by a decrease in erythrocyte sorbitol, because there was no significant difference in sorbitol values before incubation and after incubation in the presence of statil. A small but significant decrease was found for the high-shear erythrocyte viscosity (~2%), and a more pronounced decrease of the low-shear erythrocyte viscosity was observed (~16%). There was no detectable change in mean cell volume or mean cell hemoglobin concentration after incubation. Additional experiments, with 1 mM statil in the 3-h incubation at 5.5 mM glucose, confirmed the improvement of erythrocyte viscosity by statil at physiologic glucose concentration (Table 3). The effect on erythrocyte viscosity was similar for erythrocytes from diabetic or control subjects. Further erythrocyte experiments were done only on the IDDM patients.

In a later series of experiments, the erythrocytes were washed twice in PBS after the incubation in 33.3 mM glucose and resuspended in Krebs buffer containing 5.5 mM glucose for viscosity measurements (Table 4). After washing and resuspending, there was no difference in erythrocyte viscosity between the cells incubated with or without statil, although erythrocyte sorbitol remained significantly different.

These experiments suggested a direct effect of statil on erythrocyte viscosity, independent of sorbitol accumulation. Therefore, we further investigated the effect of a 10-fold decrease in concentration of statil (0.1 mM) on erythrocyte viscosity. From this concentration the influence of statil on erythrocyte viscosity was clearly diminished; there was no more difference at high shear rate, and there was only a small (~3%), although significant, decrease at low shear rate (Table 5). However, erythrocyte sorbitol accumulation was still prevented, to a level comparable with the higher dose of statil (Table 2).

TABLE 3
Effect of aldose reductase inhibition on erythrocyte sorbitol and erythrocyte viscosity at physiologic glucose concentration

Condition	Erythrocyte sorbitol (nmol/g Hb)	Erythrocyte viscosity (mPa · s)	
		High shear	Low shear
Erythrocytes from IDDM patients			
After incubation in 5.5 mM glucose	55.4 ± 6.9	8.4 ± 0.1	38.8 ± 0.9
After incubation in 5.5 mM glucose + 1 mM statil	21.2 ± 7.3*	8.2 ± 0.1*	32.3 ± 1.1*
Erythrocytes from control subjects			
After incubation in 5.5 mM glucose	45.6 ± 5.0	8.4 ± 0.1	37.9 ± 0.7
After incubation in 5.5 mM glucose + 1 mM statil	17.6 ± 3.8*	8.2 ± 0.1*	32.1 ± 0.8*

All results are expressed as means ± SE. $n = 6$ for both groups.
*$P < .025$, one-tailed Wilcoxon test.

TABLE 4
Effect of aldose reductase inhibition on diabetic erythrocyte viscosity after washing and resuspending the cells in 5.5 mM glucose buffer

Condition	Erythrocyte sorbitol (nmol/g Hb)	Erythrocyte viscosity (mPa · s)	
		High shear	Low shear
After incubation in 33.3 mM glucose	227.2 ± 20.3	8.4 ± 0.1	41.4 ± 1.5
After incubation in 33.3 mM glucose + 1 mM statil	46.1 ± 7.7*	8.3 ± 0.1	41.8 ± 1.6

All results are expressed as means ± SE. $n = 6$.
*$P < .025$, one-tailed Wilcoxon test.

DISCUSSION

We found an increased erythrocyte sorbitol and an increased low-shear erythrocyte viscosity in a group of IDDM patients. An increased erythrocyte sorbitol level in diabetes, due to hyperglycemia, is well known (9,10). Erythrocyte viscosity measurements provide an indirect measure of erythrocyte deformability (13). A normal high-shear but an increased low-shear erythrocyte viscosity has been demonstrated by McMillan and Utterback (18), and in a previous study we confirmed this finding (19). McMillan suggested that the viscosity results were caused by an increased "resistance to bending" of the diabetic erythrocyte membranes. These results seem to parallel prior micropipette and filtration studies showing a reduced erythrocyte deformability in diabetes (8,20,21).

In this study, there was no correlation between the sorbitol level and the low-shear erythrocyte viscosity in the diabetic group. Sorbitol accumulation per se, through an eventual osmotic effect, did not contribute to an increased low-shear erythrocyte viscosity in IDDM subjects.

However, because of a different sorbitol dehydrogenase activity, a high polyol pathway activity was not necessarily associated with a high sorbitol accumulation (12). A causal relationship between polyol pathway activity and erythrocyte deformability remained possible even in the absence of a correlation between erythrocyte sorbitol and the low-shear erythrocyte viscosity. Therefore, erythrocytes were incubated in buffer under normo- and hyperglycemic conditions. To prevent aldose reductase activity, the inhibitor statil was added to the buffer. In the presence of statil, an increase in polyol-pathway activity was abolished as indicated by the erythrocyte sorbitol values. Without statil, there was neither an effect of the 3-h incubation on erythrocyte viscosity nor was there any difference in erythrocyte viscosity after incubation in 5.5 or 33.3 mM glucose. Therefore, the hypothesis that an increased polyol-pathway activity should lead to a reduced erythrocyte deformability in diabetes could not be confirmed by these experiments.

Our conclusion agrees with McMillan et al. (8) and Juhan et al. (21), who also did not find any difference in erythrocyte deformability between the cells after incubation in normo- or hyperglycemic conditions. However, Bareford et al. (22) and Robey et al. (23) found that increased glucose concentration affected erythrocyte deformability. The use of different rheological techniques may explain the discrepancy.

Our addition of 1 mM statil resulted in not only an inhibition of aldose reductase activity but also a significant decrease of erythrocyte viscosity at physiologic and increased glucose concentrations in diabetic and control subjects. Because sorbitol production had no effect on erythrocyte viscosity, this effect of statil on erythrocyte viscosity seemed to be independent of aldose reductase inhibition. Further experiments, with a lower concentration of statil, and viscosity measurements after washing the incubated erythrocytes confirmed this conclusion. Bareford et al. (22) showed a reduction in erythrocyte filterability through 3-μm pores for erythrocytes after incubation in 50 mM glucose, but there was no effect of incubation in 25 mM glucose. This loss of erythrocyte filterability was prevented by the addition of the aldose reductase inhibitor sorbinil. Probably an extremely high sorbitol accumulation led to a rheological effect that could be detected with the 3-μm–pore filtration technique, which is very sensitive to changes in cell geometry (24). Robey et al. (25) found a positive effect of sorbinil on erythrocyte deformability, assessed by whole-blood filtration through 5-μm pores, in diabetic rats. In a later in vitro study, they concluded that this effect might be independent of its aldose reductase–inhibiting property (23). Our data seem to confirm their conclusion.

Washing the erythrocytes after incubation abolished the effect of the aldose reductase inhibitor on erythrocyte viscosity, although erythrocyte sorbitol remained significantly different because sorbitol is relatively impermeable to cell membranes. This result also indicated that the effect of the inhibitor on erythrocyte viscosity is not acquired but seems to depend on the presence of the inhibitor in the medium.

TABLE 5
Effect of 0.1 mM statil on diabetic erythrocyte sorbitol and erythrocyte viscosity

Condition	Erythrocyte sorbitol (nmol/g Hb)	Erythrocyte viscosity (mPa · s)	
		High shear	Low shear
After incubation in 33.3 mM glucose	252.5 ± 29.6	8.5 ± 0.1	39.0 ± 1.1
After incubation in 33.3 mM glucose + 0.1 mM statil	46.1 ± 6.0*	8.5 ± 0.1	37.9 ± 1.1*

All results are expressed as means ± SE. $n = 6$.
*$P < .025$, one-tailed Wilcoxon test.

The effect on erythrocyte viscosity almost disappeared with a 10-fold lower concentration of statil (0.1 mM), although the concentration was sufficient to prevent sorbitol accumulation.

The effect of statil on erythrocyte viscosity was small, although significant, at the high shear rate but was more pronounced at the low shear rate for diabetic as well as control subjects. Low-shear erythrocyte viscosity measurements are influenced merely by cell geometry and membrane flexibility (13). The fact that no effect of statil on mean cell volume could be found may suggest an effect on membrane flexibility. Because aldose reductase inhibitor is needed in the medium, a direct interaction with the erythrocyte membrane seems to occur. Cohen (4) showed that sorbinil binds to membranes of glomeruli. The same drug showed an improvement of erythrocyte filterability in diabetic rats (25). Virtually all aldose reductase inhibitors have common molecular features. Generally they consist of a planar aromatic ring system with a specifically located electrophilic group (26). Further investigation is needed to compare the rheological impact of different aldose reductase inhibitors and to elucidate the molecular mechanism involved.

Some caveats are necessary before extrapolations from these in vitro experiments can be applied to an in vivo situation. First, a high dose of statil is needed to show the effect on erythrocyte deformability in vitro. Second, long-term metabolic effects of aldose reductase inhibition, and so on erythrocyte deformability, cannot be investigated in vitro. Further in vivo research of aldose reductase inhibitors is of interest not only because of their effect on the polyol pathway but also because their improvement of erythrocyte deformability might be beneficial to oxygen supply in peripheral tissues.

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