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Restoration of Microsieve Filterability of Human Red Cells After Exposure to Hyperosmolarity and Lactacidosis: Effect of Vinpocetine

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ABSTRACT

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A new technique has been utilized to assess quantitatively the filterability (i.e., the ability to pass restricted pores) of leukocyte-free red blood cell (RBC) suspensions by means of computer-assisted *conductometry* of novel precision metal microsieves with uniform pore diameters of 4.2 μm (MyNiPore®). RBCs were exposed to adverse conditions of chemical "stress" (400 mOsmol/liter, pH 6.8), thereafter, the effects of vinpocetine (Methyl-Apo-Vipamin, Thiemann-Arzneimittel GmbH, Waltrop, FRG) were tested. When added in concentrations between 10^{-5} and 10^{-2} mol/liter to the "stressed" RBCs, vinpocetine restored the red cell microrheological performance (or behavior) when tested under low shear stresses. The present results suggest that in interpreting vinpocetine's effects in low flow situations, a possible protective microrheological action should be taken into consideration.

Key words: RBC filter ability, rheology, vinpocetine, MyNiPore®

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INTRODUCTION

Disturbances in microcirculation may explain the pathogenesis of cerebral hypoperfusion, resulting in a deficit due to the obliteration of large supplying arteries. A reduction of the blood's fluidity and/or an abnormal microrheological behavior of blood elements is thought to be based either on generalized abnormalities (i.e., a kind of "hyperviscosity syndrome") or on local rheological events. Rigidification of red cells owing to abnormal biochemical composition of the blood in the areas of local ischaemic anoxia is an example of the latter.

There have been a number of attempts to protect red cells pharmacologically; this idea is based on in vitro data demonstrating that the pivotal "fluidity" of the erythrocyte is disturbed easily when it is exposed to abnormal biochemical and biophysical condition, e.g., hyperosmolarity, lactacidosis, hypercapnia, or—more specifically—high K^+ and PO_4^- concentrations.

The interesting possibility arises that metabolic alterations in sessile parenchymal cells might act on mobile blood cells, altering them in their hemorheological behavior, but only when they enter a hypoperfused, ischaemic area.

The conventional technology used to test the ability of red cells to pass restricted microchannels has recently been severely criticized. The present experiments, which aim to test the protective hemorhelogical effects of vinpocetine, were therefore performed using a newly developed system of high-precision metal microsieves, a filtrometer employing low and decreasing driving pressures, and a computer-assisted evaluation of filter conductance during a perfusion experiment.

MATERIALS AND METHODS

Filtrometry

The Filtrometer MF4 (Fig. 1a; Myrenne GmbH, Steffensgasse 9, D-5160 Roetgen/Aachen) consists of a U-shaped measuring tube in which the microsieve is mounted so that a red-cell suspension (8% washed RBC in an isotonic-buffered saline solution containing 5 mMol/liter D-glucose) is passed through the filter from below (Fig. 1c) in order to avoid sedimentation artifacts. As the fluid rises, the height of the blood column increases, and this volumn change is registered photometrically. Provided the high precison U-tube is filled properly (volumn = 2.00 ml), the height of the suspension (decaying from 30 to zero mm) in the tube of a precisely defined diameter (and thus a known cross-sectional area) is proportional to the driving force (30 mm $H_2O \rightarrow 0$, 300 $\rightarrow$ 0 Pa) and to the already-filtered volume at any given time. The rate of change of the volume is proportional to the flow rate. Using an optoelectronic level detection device and appropriate data processing, the entire flow experiment is recorded and stored by monitoring (once per sec) the volume passed through the microsieve as a function of time.

MyNiPore® Microsieves

In this study, new, reusable MyNiPore® microsieves, manufactured from nickel by galvanic procedures, were used exclusively. As can be seen in Fig. 1b, these novel microsieves have round pores made in a regular hexagonal array. In the present study, microsieves with a pore diameter of 4.2 $\pm$ 0.2 μm were used.

Filtrometric Data Processing

The filtrometric data are presented in a normalized form; the flow rates are calculated from the change in filtered volume per time unit (μl/sec), and the corresponding hydraulic conductance is calculated by dividing the flow rate by the pressure.

Provided leukocytes are removed to less than 50 per μl in the suspension, the effects of

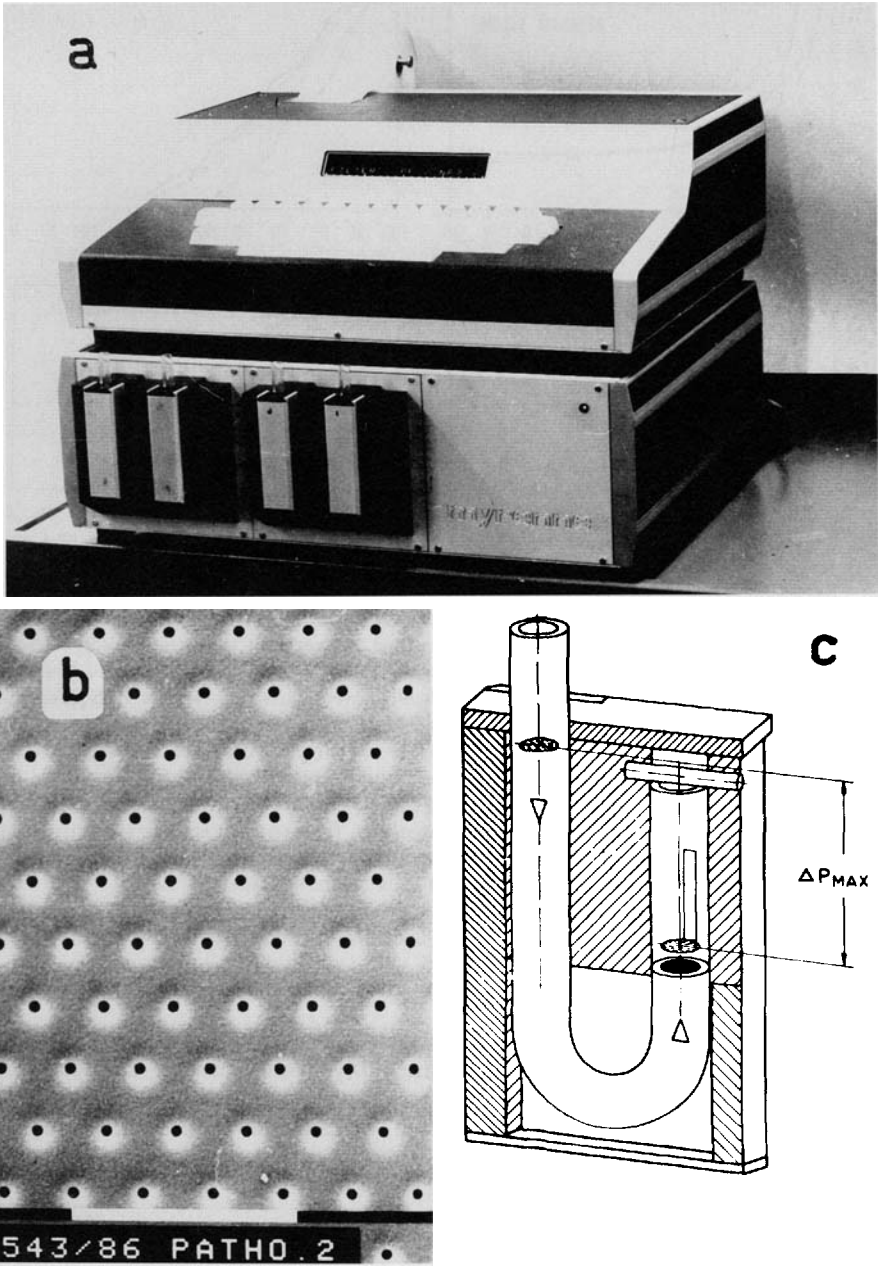

Fig. 1.**a:** Photograph of the Filtrometer MF4. **b:** Scanning electron microscopic picture of a MyNiPore microsieve (courtesy of Dr. Hollweg, Department of Pathology, RWTH Aachen). Note uniform diameter (small holes), regular arrangement, and absence of doublets. **c:** Schematic drawing of the U-shaped measuring chamber in the Filtrometer MF4. A precision glass tube is bent and cut so that the microsieve (plus two tightening gaskets, and rubber washers) can be mounted between the two parts of the U-shaped sample holder. A precise amount of a RBC suspension (2000 μl) is pipetted into the left part of the sample holder. A pressure differential (DP max) is created, driving the suspension through the microsieve. The height of the rising column of the suspension is monitored continuously by an optoelectronic sensor and recorded as a function of time.

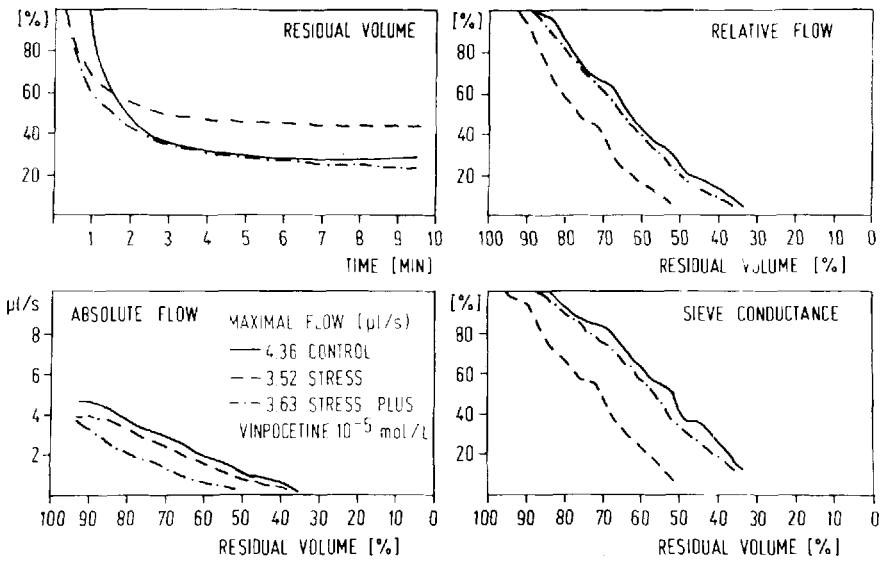

Fig. 2. Representative example of direct and computed filtrometric data obtained from the Filtrometer MF4 (using MyNiPore microsieves with pore diameter of 4.2 µm). **Upper left:** residual volume (not yet filtered suspension) plotted as a function of filtration time. **Lower left:** flow rate (dV/dt) plotted as a function of residual volume (and thus of filtration pressure), 100% residual volume being equivalent to a driving pressure of 30 mm H_2O or 300 Pa. Control cells have a maximum flow of 4.36 µl/sec, which decays almost linearly with pressure (solid line). The initial flow rate of the "stressed" cells is 3.52 µl/sec (hatched line) and decays with about the same slope as the control cells, reaching zero flow at 50% residual volume or 150 Pa. The "stressed" cells have a slightly higher maximal flow rate (3.63 µl/sec) after the addition of vinpocetine (10 µM, hatch-dot curve), the flow decays much slower than in the "stressed" cells before treatment. **Upper right:** suspension flow (normalized for maximum flow = 100%), plotted as function of residual volume. **Lower right:** Comparison of microsieve conductance during filtration of normal red cells, "stressed" red cells before and after addition of vinpocetine (10 µM). Note the steep drop of microsieve conductance due to blockade of pores with "stressed" cells, whereas microsieve conductance remains high in both control cells and in "stressed" cells after vinpocetine treatment.

rigid subpopulations of red cells can be monitored conveniently. When present, such subpopulations give rise to a progressive decrease in MyNiPore® microsieve conductance. In an attempt to express in a single numerical value the continuous process by which the pores in the microsieves are excluded from the perfusion, we calculated the pressure corresponding to half-maximum microsieve conductance (PC/50%). A representative example is given in Figure 2.

Preparation of Red Cell Suspensions

The precision methodology for filtering red cells, as previously described, requires great scrutiny in removing white cells. For this purpose, we developed a special technique for sequential centrifugation and cotton wool prefiltration, not detailed here however.

Biological Stress Model: exposure of erythrocytes to lactacidosis and hyperosmolarity

To expose the erythrocytes to a combination of lactacidosis and hyperosmolarity, a 1-ml volume of RBCs is stirred gradually and carefully into 100 ml of an electrolyte solution (400

± 5 mOsmol/Kg), to which lactic acid previously was added to reduce the pH to 6.5. Subsequently, the cells are centrifuged, and most of the supernatant electrolyte solution is discarded and 8% hematocrit suspensions are prepared.

In experiments studying the effects of vinpocetine, the compound was added in concentrations of 1.0, 0.1, 0.01, and 0.001 mM (i.e., 10^{-3} to 10^{-6} M) to the prepared suspension, which was then incubated 10 min at 37°C before the actual filtration experiments or 10 min. The drug was added from a stock solution of 3 mMol/liter.

RESULTS

Filtration of Normal and Red Cells After Exposure to Stress

The filtrometric measurement in the Filtrometer MF4 apparatus depends on the spontaneous flow of a red-cell suspension through the microsieves under the influence of gravity. Figure 2 shows a typical curve from data obtained with this instrument, and residual volume (300 μl = 100%) is plotted as a function of time. From the raw data, the flow rate (dV/dt, μl/sec) is calculated and plotted as a function of the residual volume, which, in turn, is proportional to the driving pressure (3 cm H_2O = 100% Residual Volume = 300 Pa driving pressure). Thus, the absolute (Fig. 2b) and relative (Fig. 2c) flow rates (100% = maximum flow rate) of different suspensions, plotted as a function of driving pressure, can be compared. When comparing control RBCs suspended in a isotonic, phophate-buffered, albuminated (0.1% HSA) solution with cells in a hypertonic media (400 mOsmol/liter, made acidotic by lactic acid pH 6.8), the flow rate of the latter decays more rapidly during the experiment. The addition of vinpocetine (10 μMol/liter) shifted the flow curve back toward the control value; i.e., the maximum flow rate is higher, and the flow rate decays less rapidly.

In filtration experiments using altered red cells, the flow rate drops faster than does the driving pressure. There are two reasons for this: the cells are deformed less easily as the pressure falls, and the small subpopulation of especially stiff cells present in any blood sample begins to clog an increasing number of microsieve pores. The rate of microsieve pore occlusion can be observed by comparing the slopes of the curves in Figure 2c and best in the curve in Figure 2d, in which the microsieve conductance (ratio of instantaneous flow rate to corresponding instantaneous driving pressure) is calculated. When RBCs are sieved under ideally filterable conditions, i.e., freshly drawn, not previously incubated cells, the microsieve conductance drops only slightly during filtration. In the present series, control cells were treated exactly as the "stressed" cells and the vinpocetine-treated "stressed" cells; this includes a 30-min incubation period at 37°C and a total time of 120–180 min between blood

TABLE 1. Dose Dependency of Vinpocetine on Microsieve Filtration of 8% RBC (stressed by exposure to 400 mOsmol/liter, pH 6.8) x ± S.D., n = 5

	V_{max} (μl/sec at 270 Pa)	$^{P}C_{50}$ (Pa)
Isotonic control cells pH 7.4, 290 mOsm/L	4.22 ± 0.74	153 ± 28
Stressed cells	3.60 ± 1.1	218 ± 23
Stressed cells + Vinpocetine		
10^{-6} Mol/L	3.74 ± 0.9	187 ± 54
10^{-5} Mol/L[a]	3.95 ± 1.8	168 ± 29
10^{-4} Mol/L	3.99 ± 2.4	158 ± 27
10$s-3$ Mol/L	2.04 ± 1.5	216 ± 33
10^{-2} Mol/L[6]	1.65 ± 0.8	240 ± 34

[a]Donors not identical to those listed in Fig. 3 and 2
[b]Marked hemolysis

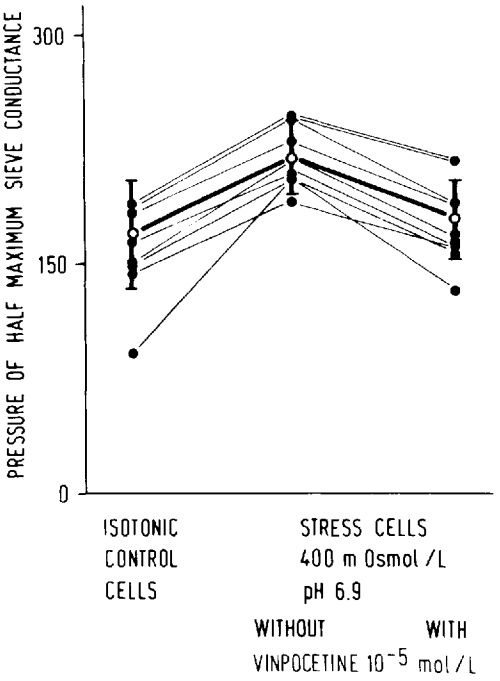

Fig. 3. Pressure of half-maximum microsieve conductance (PC/50%, ordinate) of MyNiPore microsieves with pores of 4.2-μm diameter during the perfusion with 8% RBC suspensions. Comparison of cells under isotonic conditions (= 100%) with those suspended under hyperosmolar and lactacidotic conditions before and after addition of vinpocetine 10 micromolar.

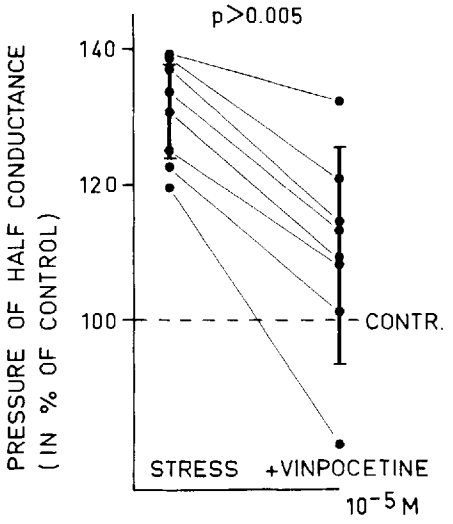

Fig. 4. Same data as in Figure 3, presented as normalized values.

withdrawal and measurement. This is the reason that control samples had a more rapid drop in microsieve conductance than with red cells freshly drawn. This is indicative of a an overall slight rigidification of all cells and an increase in the proportion of altered cells unable to pass the microsieve following from the mere incubation (Figures 3 and 4.)

DISCUSSION

It is especially interesting that vinpocetine, a drug thought previously to have primarily metabolic effects on the brain, exerts rather dramatic hemorheological effects in situations modeling biochemical and biophysical conditions likely to occur in tissues under chronic hypoperfusion. In these cases, the hemodynamic situation is characterized by a low driving pressure and sluggish perfusion, a condition known to favour the manifestation of abnormal RBC rheological behavior. The changes likely to occur include a shift to anaerobic glycolysis, with a concomitant alteration in the electrolyte state (hyperosmolarity), in the pH of the interstitium, and of the blood perfusing the affected capillaries.

In other words, in situations where one can assume changes in the biochemical composition of blood in the microvessels followed by a local alteration of the fluidity of creeping blood, a membrane-active drug like vinpocetine can protect the RBC against adverse local biochemical alterations. The exact mechanism of action remains to be elucidated.

The results of this study have shown that vinpocetine, in clinically useful doses (1 to 10 μm), improves the ability of "stressed" human red cells to pass restricted pores in vitro.

The effects of combined hyperosmolarity and acidosis on RBCs are very dramatic as produced in the present experiments when measuring the perfusion of a natural vascular bed. When perfusing an isolated mesentery preparation of the rat under hemodynamically controlled conditions with suspensions of human red cells before and after exposure to the combination of hyperosmolarity and lactacidosis (400 mOsmol/liter, pH 6.8), a dramatic reduction in perfusion and frequent capillary flow stasis has been monitored. Unpublished observations of high-resolution intravital microscopy has shown that the altered cells tend to clog capillaries at natural narrowings and by crowding at bifurcations points, a finding similar to those reported after infusion of irreversibly sickled red cells and cells rigidified by other chemical means.

SUMMARY

Using newly developed microrheological tests, we demonstrated a clearcut effect of vinpocetine on the microrheological performance (narrow pore filterability) of red cells exposed to a combination of lactacidosis (pH 6.8) and hyperosmolarity (400 mOsmol/liter). The latter alteration, which is known to occur in the blood under conditions of hypoxic metabolism (i.e., during physical exercise of muscles during ischemic anoxia) reduces the in vitro "flexibility" of erythrocytes. Therefore, the possibility of a pharmacological prevention of erythrocyte stiffening, such as suggested from the present experiment by use of vinpocetine in the 1 to 10 μm range, opens new posibilities for treatment and/or prophylaxis of cerebrovascular states of hypoperfusion.