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Bencyclane as an anti-sickling agent

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Summary. A vasodilating Ca^{2+} channel blocker, bencyclane, was used in 18 patients with homozygous sickle cell anaemia (SCD) to test the possible anti-sickling effect. With bencylane intervention the Na^{+}-K^{+} ATPase activity increased from 256 ± 29 to 331 ± 37 nmol Pi/mg protein/h ($P < 0{\cdot}0001$) and the Ca^{2+}-Mg^{2+} ATPase level increased from 172 ± 12 to 222 ± 44 nmol Pi/mg protein/h ($P < 0{\cdot}0001$). The intracytoplasmic Ca^{2+} concentration reduced from $3{\cdot}5 \pm 0{\cdot}6$ to $2{\cdot}7 \pm 0{\cdot}25$ μmol/l ($P < 0{\cdot}0001$). The patient's blood contained fewer irreversibly sickled cells (ISCs) (a reduction from 21·4% to 14·4%) ($P < 0{\cdot}05$). At the same time MCHC of the erythrocytes decreased from 34·5 to 33·0 g/dl ($P < 0{\cdot}05$). Bencyclane appears to be a promising anti-sickling agent that can be used orally in SCD.

Keywords: sickle cell, anti-sickling agent, ISC, MCHC, bencyclane.

The polymerization of Hb S in sickle cell anaemia (SCD) is closely associated with reduction in cell ion and water content (cell dehydration) (Hofrichter *et al*, 1974); dehydrated sickle erythrocytes have increased haemoglobin concentration (MCHC) and density. The dense cells, on the other hand, with irreversible (ISC) or reversible deformed shapes contribute to both the haemolytic (Serjeant *et al*, 1969; Hebbel, 1991) and vaso-occlusive features of SCD (Kaul *et al*, 1986). The prevention of sickle cell dehydration and reducing the MCHC is one of the therapeutic strategies in SCD.

It has previously been demonstrated that intracellular K^{+} and water is low in sickle erythrocytes as a consequence of a defective Na^{+}-K^{+} pump in cell membrane (Clark *et al*, 1978; Glader & Nathan, 1978). Sickle erythrocytes were also shown to have elevated calcium content (Eaton *et al*, 1973; Palek, 1973).

Therefore some pharmacological agents that act on red cell membrane and transport systems might have anti-sickling properties by changing the cell cation and water content and so decreasing the intracellular concentration. Some examples of these investigated drugs are cetiedil (Asakura *et al*, 1980), bepridil (Reilly *et al*, 1993), phenothiazines (Thompson *et al*, 1993) and clotrimazole (De Franceschi *et al*, 1994). We have examined the anti-sickling properties of bencyclane, a vasodilating Ca^{2+} channel blocker, which is classified in the same group as bepridil.

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MATERIALS AND METHODS

The follow-up homozygous sickle cell (SS) patients of the haematology clinic included in the study had signed the informed consent approved by the ethical committee of the faculty of medicine. They all were in a steady state and none of them had been transfused during the study period. The diagnosis of SCD was made by the haemoglobin electrophoresis on cellulose acetate method at pH 9·2. Bencyclane was given in an oral dose of 100 ng three times daily. Blood specimens were collected for the following measurements prior to starting treatment and after 1 month: (a) a routine blood count by Coulter Counter S Plus; (b) a reticulocyte count done manually by a technician unaware of the study protocol; (c) a count of ISCs expressed as a percentage of erythrocytes counted from a peripheral smear by a physician included in the study.

For the measurements of ATPase the erythrocyte membranes were prepared by the procedure of Beutler *et al* (1983). The ATPase activity was based on the measurements of inorganic phosphate released each hour for each mg protein in the presence of 3 mM disodium adenosine 5′-triphophatase according to the method derived by Atkinson *et al* (1973). For the measurement of Na^{+}-K^{+} ATPase Na^{+} and for the measurements of Ca^{2+}-Mg^{2+} ATPase Ca^{2+} was

Table I. The mean values for ATPases, Ca^{2+}, MCHC and percent ISCs in 18 SCD before and after bencyclane treatment.

Parameter	Before bencyclane	After bencyclane 100 mg t.i.d.	*P*
Na^+-K^+ ATPase (nmol Pi/mg protein/h)	256 ± 29	331 ± 37	<0·0001
Ca^{2+}-Mg^{2+} ATPase (nmol Pi/mg protein/h)	172 ± 12	222 ± 44	<0·0001
Ca^{2+} (μmol/l)	3·5 ± 0·59	2·7 ± 0·25	<0·001
MCHC (g/dl)	34·5 ± 2·2	33 ± 2·2	<0·05
ISC (%)	21·4 ± 7·0	14·4 ± 7·5	<0·05

added to the medium. The results were given in nmol Pi/mg protein/h. The intracytoplasmic determination of calcium was carried out after dilution by lanthanum chloride (1%) at a ratio of 1/50. Readings were performed with a Perkin-Elmer 2380 atomic absorption spectrophotometer at a wavelength of 422·7 nm.

The paired *t*-test has been used for statistical analyses.

RESULTS

This report is based on the data obtained from the first 18 consecutive patients who were treated with bencyclane for a period of 1 month. None of the patients stopped taking the drug before the end of the month. There were seven male and 11 female SS patients. The mean age was 24·5 ± 7·0 (range 15–47).

The mean values for ATPases, Ca^{2+} concentration, ISCs, MCHC at the start of trial (baseline) and after taking bencyclane 100 mg t.i.d. p.o. for 1 month are shown in Table I.

The mean Na^+-K^+ ATPase in 18 sickle cell patients was found to be 256 ± 29 nmol Pi/mg protein/h at baseline. After a month of bencyclane use the ATPase level was found to be 331 ± 37 nmol Pi/mg protein/h. The difference between these two values was significant ($P < 0·0001$). The normal value for Na^+-K^+ ATPase in our laboratory is 343 ± 25 nmol Pi/mg protein/h ($n = 10$). Thus the Na^+-K^+ ATPase in SS patients were significantly lower than normal ($P < 0·0001$) before drug intervention, and increased to near normal values with the drug (Fig 1). The Ca^{2+}-Mg^{2+} ATPase in 10 normal controls in our laboratory was found to be 259 ± 22 nmol. In SS patients this value was found to be 172 ± 12 nmol, which was significantly lower ($P < 0·0001$). With bencyclane therapy it rose to 222 ± 44 nmol. The difference between the values before and after therapy was significant ($P < 0·0001$) (Fig 1).

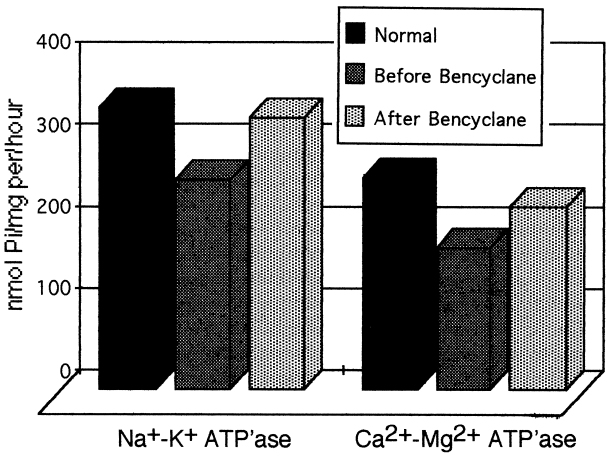

Fig 1. The Na^+-K^+ and Ca^{2+}-Mg^{2+} ATPase levels in normal blood and in SCD before and after bencyclane treatment.

The intracytoplasmic total calcium concentration in 10 normals was found to be 1·99 ± 0·28 μmol/l, whereas in the 18 patients with SCD it was 3·5 ± 0·59 μmol ($P < 0·0001$). With bencyclane therapy the Ca^{2+} decreased to 2·75 ± 0·25 μmol. The difference of the mean level before and after the drug was significant ($P < 0·0001$).

The mean number of ISCs before and after bencyclane were 21·4% and 14·4%, respectively. The difference was significant ($P < 0·05$).

The mean MCHC was 34·5 g/dl before the drug treatment and 33 g/dl after. The lowering effect of bencyclane in MCHC was significant ($P < 0·05$).

The WBC and platelet counts have not been affected by the drug.

DISCUSSION

We have tested the anti-sickling effects of bencyclane, a vasodilator drug, which has been used for the treatment of peripheral arterial occlusive diseases (Trubestein *et al*, 1989).

The effect of bencyclane in sickled red blood cells has not previously been studied. The mechanism of action on the reduction of cell density might be similar to the effect of other anti-sickling agents. For clotrimazole and cetiedil it was suggested that they inhibit the K^+ efflux pathways, like the Gardos channel, to decrease the potassium and water loss (Stuart *et al*, 1987; De Franceschi *et al*, 1994). Bencyclane, with a formula of N-(-(1-benzylcycloheptyloxy)propyl)-N,N-dimethylammonium hydrogen fumarate, is a non-selective calcium entry blocker which acts on calcium and fast sodium channels. It is classified in the same group as bepridil (Godfraind *et al*, 1986). We chose to study the effects of bencyclane in SCD because we had already observed that it raised the Na^+-K^+ pump activity in SS blood cells *in vitro*

(unpublished observation). To manipulate the cell membrane permeability is one of the ways to change the concentration of intracellular haemoglobin. Cetiedil was the first drug to be reported to inhibit erythrocyte sickling by acting directly on the cell membrane (Asakura *et al*, 1980). The data that we obtained with bencyclane clearly shows increased Na^+-K^+ and Ca^{2+}-Mg^{2+} pump activities (Fig 1). At the same time the Ca^{2+} inside the cell decreased. A possible explanation for the observed effect of bencyclane on cation transport might be that it accumulates Ca^{2+} around the cell while decreasing intracellular Ca^{2+}. Calcium accumulation in turn might inhibit the K^+ loss from the cell. A similar mechanism has been suggested by Schmidt *et al* (1982) to explain cetiedil's efficacy in inhibiting K^+ loss when Ca^{2+} is added to the cell medium.

With bencyclane treatment Na^+-K^+ ATPase activity increased to near normal levels (Fig 1). Although we could not measure the intracytoplasmic K^+ concentration, with the normalization of Na^+-K^+ pump system and increasing the Ca^{2+} extracellularly, a mechanism as suggested above might operate to reverse the defect causing K^+ and water loss.

Our data confirms the significantly high Ca^{2+} content of the SS cell. As well as its reduction with bencyclane intervention, Ca^{2+} stimulated ATPase which requires Mg^{2+}, ATP is also involved in the efflux of Ca^{2+} from the cell (Caroni & Carofoli, 1980). The activity of this pump system in SCD has not been reported previously. We have demonstrated that the Ca^{2+}-Mg^{2+} pump was lower than normal in SS (Fig 1). With bencyclane intervention the activity of this pump was found to increase. This could be another reason for the reduction of intracellular calcium with bencyclane treatment.

Previous studies on sickle erythrocytes indicate that calcium accumulation, potassium loss and dehydration are involved with membrane injury and sickling. Bencyclane appears to change this cation composition towards normal by a yet undetermined mechanism. Data from this study should serve as a rationale to continue investigation of bencyclane as a promising anti-sickling agent.

REFERENCES

Asakura, T., Ohnishi, S.T., Adachi, K., Ozguc, K., Hashimoto, K., Singer, M., Russel, O. & Schwartz, E. (1980) Effect of cetiedil on erythrocyte sickling: new type of antisickling agent that may affect erythrocyte membranes. *Proceedings of the National Academy of Sciences of the United States of America*, 77, 2955–2959.

Atkinson, A., Gotenby, A.D. & Lowe, A.D. (1973) The determination of inorganic orthophosphate in biological systems. *Biochimica et Biophysica Acta*, 320, 195–204.

Beutler, E., Kuhl, W. & Sacks, P. (1983) Sodium–potassium ATPase activity is influenced by ethnic origin and not by obesity. *New England Journal of Medicine*, 309, 756–760.

Caroni, P. & Carofoli, E. (1980) An ATP-dependent Ca^{2+} pumping system in dog heart sarcolemma. *Nature*, 283, 765–767.

Clark, M.R., Unger, R.C. & Shohet, S.B. (1978) Monovalent cation composition and ATP and lipid content of irreversibly sickled cells. *Blood*, 51, 1169–1178.

De Franceschi, L., Saadane, N., Trudel, M., Alper, S.L., Brugnara, C. & Beuzard, Y. (1994) Treatment with oral clotrimazole blocks Ca^{2+}-activated K^+ transport and reverses erythrocyte dehydration in transgenic SAD mice: a model for therapy of sickle cell disease. *Journal of Clinical Investigation*, 93, 1670–1676.

Eaton, J.W., Skelton, T.D., Swafford, H.S., Koplin, C.E. & Jacop, H.S. (1973) Elevated erythrocyte calcium in sickle cell disease. *Nature*, 246, 105–106.

Glader, B.E. & Nathan, D.G. (1978) Cation permeability alteration during sickling: relationships to cation composition and cellular hydration of irreversibly sickled cells. *Blood*, 51, 983–989.

Godfraind, T., Miller, R. & Wibo, M. (1986) Calcium antagonism and calcium entry blockade. *Pharmacological Reviews*, 38, 321–416.

Hebbel, R.P. (1991) Beyond haemoglobin polimerization: the red blood cell membrane and sickle cell disease pathophysiology. *Blood*, 77, 214–218.

Hofrichter, J., Ross, P.D. & Eaton, W.A. (1974) Kinetics and mechanism of deoxyhemoglobin S gelation. *Proceedings of the National Academy of Sciences of the United States of America*, 71, 4864–4868.

Kaul, D.K., Fabry, M.E. & Nagel, R.L. (1986) Vaso-occlusion by sickle cells: evidence for selective trapping of dense cells. *Blood*, 68, 1162–1166.

Palek, J. (1973) Calcium accumulation during sickling of hemoglobin S red cells. *Blood*, 42, 988–1000.

Reilly, M., Horiuchi, K. & Asakura, T. (1993) Dose-dependent red cell volume increase induced by bepridil. *General Pharmacology*, 24, 1323–1329.

Schmidt, W.F., Asakura, T. & Schwartz, E. (1982) Effect of cetiedil on cation and water movements in erythrocytes. *Journal of Clinical Investigation*. 69, 589–594.

Serjeant, G.R., Serjeant, B.E. & Milner, P.F. (1969) The irreversibly sickled cell: a determinant of haemolysis in sickle cell anaemia. *British Journal of Haemotology*, 17, 527–533.

Stuart, J., Stone, P.C.W., Biltom, Y.Y. & Keidan, A.J. (1987) Oxpentifylline and cetiedil citrate improve deformability of the dehydrated sickle cell. *Journal of Clinical Pathology*, 40, 1182–1186.

Thomson, A.A., Cornelius, A.S., Asakura, T. & Horiuchi, K. (1993) Comparative studies of phenothiazine derivatives for their effects on swelling of normal and sickle erythrocytes. *General Pharmacology*, 24, 999–1006.

Trubestein, G., Trubestein, R. & Ludwig, M. (1989) Bencyclane in stage II arterial occlusive disease: results of a controlled study. *Fortschritte der Medizin*, 107, 781–784.