ANTISICKLING EFFECTS OF ZINC

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SUMMARY

Low levels of zinc incorporated into sickle cells consistently improves their filterability at intermediate oxygen tensions. Zinc also appears to antagonize calcium binding by red cell membrane-hemoglobin complexes. Since calcium may be involved in the formation of irreversibly sickled cells, zinc may improve filterability of sickle cells by a calcium antagonizing mechanism. Zinc appears to have important therapeutic potential in sickle cell disease.

INTRODUCTION

Increasingly it is apparent that zinc administration can play a therapeutic role in sickle cell anemia, first because many patients are zinc deficient and need replacement (1-3), and second because of two, possibly independent, pharmacological effects of zinc.

The first potential pharmacological effect comes from the observation that zinc binds to hemoglobin, and increases hemoglobin oxygen affinity (4). This may be of benefit in inhibiting sickling provided high enough levels of red cell zinc can be obtained. In addition, zinc may be a new molecular probe for hemoglobin. Zinc does not bind to any of the usual sites affecting oxygen affinity, including the Bohr sites, the DPG sites, and β cysteine-93 (5). The effect of zinc on hemoglobin (increased affinity, unchanged Bohr effect, and decreased Hill coefficient) is reminiscent of certain mutations affecting the dimer contacts, and we have postulated that zinc binds in a dimer contact region (5). Most exciting from the standpoint of therapeutic potential is the second pharmacological effect--an improvement in sickle cell filterability--which is the subject of this communication.

METHODS AND RESULTS

Filterability. Fresh whole venous blood, drawn in heparin from adult

patients with homozygous sickle cell anemia, was used. Four parts of whole blood from a patient were diluted with one part of 0.145 M saline containing a sufficient amount of zinc sulfate to produce the final concentrations indicated in Table 1, and incubated for two hours at 4°C. The control sample was diluted with saline and handled in a manner similar to the zinc samples. All samples within an individual experiment were aliquots of the same blood sample. After incubation, the samples were further diluted with 0.01 M Tris-0.145 M saline buffer at pH 7.4 (containing 10 mg% glucose) to a final dilution as indicated in Table 1, and equilibrated for 10 minutes with oxygen-nitrogen mixtures to a final oxygen pressure as indicated in Table 1. Filtration was carried out at 37°C by techniques similar to those of Lacelle (6), with 10 cm of water negative pressure using high density Nuclepore* filters with a pore size of 3.0 μ and a diameter of 25 mm. During filtration the gas phase above the cell suspension was identical to that used in the equilibration. The measurement of filterability used was the length of time for 2.0 ml of the cell suspensions to pass through the filter.

From Table 1 it is apparent that zinc in a concentration as low as 0.3 mM, but not at lower concentrations, markedly improved filterability of sickle cells at oxygen pressures varying from 30 mm Hg to 15 mm Hg. Assay of zinc in the red cells by atomic absorption spectroscopy revealed that the amount of additional zinc incorporated in the cells after incubation with 0.3 mM zinc in these experiments equaled a zinc/hemoglobin tetramer ratio of less than 0.05. The effect on sickle cell filterability at such a low zinc concentration suggested an effect of zinc on the sickle cell membrane.

Studies on the Competitive Effect of Zinc on Calcium Incorporation into Cell Membranes. Recent studies have implicated calcium incorporation as a pathogenic event in the formation of irreversibly sickled cells (7). It is well established that calcium accumulation in red cells greatly decreases

*Nuclepore Corp., Pleasanton, California 94566.

Table I

Effect of Zinc on Filterability of Sickle Cells

Experi-	Final Blood Dilution (During Filtration)	Oxygen Pressure (mm Hg)	Zinc* Concentration During Incubation	Time for Filtration of 2.0 mls (Seconds) Trial 1	Trial 2	Mean % Improvement Over Control
1	1:100	15	Control	318	426	
	1:100	15	1.5	168	225	48
2	1:100	15	Control	450	304	
	1:100	15	0.3	220	252	37
	1:100	15	1.5	192	260	44
3	1:100	15	Control	220	264	
	1:100	15	0.03	261	---	0
	1:100	15	0.15	270	145	8
	1:100	15	0.3	132	114	50
4	1:100	18	Control	754	744	
	1:100	18	0.3	515	560	28
5	1:50	30	Control	432	440	
	1:50	30	0.3	322	331	25

*Zinc sulfate was used, but in preliminary experiments, zinc chloride was found to have a comparable effect on filterability.

their deformability (8). To evaluate the possibility that zinc might be acting on cell deformability through an effect on calcium, we have studied the effect of zinc on calcium incorporation into red cell membranes (ghosts).

In a preliminary experiment, ghosts were prepared by the technique of Hoffman (9) with minor modifications (10) and aliquots of the ghost pellet mixed with appropriate cation solutions (1.5 mM zinc sulfate, 1.0 mM calcium chloride, and a combination of the two) and incubated at 23°C for 20 minutes. The ghosts were then resealed with 0.01 M Tris buffer, pH 7.4 in 0.145 M saline (henceforth called "resealing solution"), washed three times with 10 volumes of resealing solution and an aliquot assayed for zinc and calcium by atomic absorption spectroscopy. Only one-sixth as much calcium was found in the membranes in the presence of zinc as in its absence.

In another preliminary experiment ghosts were prepared as above, resealed, and then dialyzed against the above cation-containing solutions for two days at 4°C. Some reduction (about 20%) in calcium levels was observed in the presence of zinc, but the effect was less marked than above. Of course, in this experiment in order to bind inside the membrane the cations were required to diffuse through the membrane.

Subsequent work involved radioactive techniques for measuring cation incorporation. Ghosts were prepared in two ways. In the first, single stage red cell ghosts were prepared as above (9, 10). Calcium to a final concentration of 1.0 mM and containing a tracer amount of ^{45}Ca, and zinc to a final concentration of 1.5 mM and containing a tracer amount of ^{65}Zn, were added to the appropriate aliquots during hemolysis. A comparable concentration of saline was added to control aliquots. Subsequently the membranes were resealed and washed twice with 10 volumes of resealing solution. The membranes were then packed by centrifugation, counted for radioactivity (see footnote to Table 2 for methods), and the concentration of ghost particles in the preparation determined with a Model ZBI Coulter Counter. Results were expressed as atoms of calcium incorporated on a per ghost basis. Table 2A presents the results

Table 2

Atoms of Zinc and Calcium Incorporated into Red Cell Ghost Particles as Measured by Radioactive Tracer Techniques

Experiment	Additions NaCl mM	Additions $ZnSO_4$ mM	Additions $CaCl_2$ mM	Hemoglobin gm% in Final pellet	Zinc* Atoms Incorporated per Ghost x 10^7	Calcium* Atoms Incorporated per Ghost x 10^7
A						
1	3.0			0.81		
(Normal	1.5	1.5		0.45	4.25	
Ghosts)	1.5		1.0	16.3		3.1
		1.5	1.0	0.34	3.70	0.89
2	3.0			1.12		
(Normal	1.5	1.5		0.78	7.78	
Ghosts)	1.5		1.0	29.7		1.36
		1.5	1.0	0.68	5.50	0.675
3	3.0			1.39		
(Sickle	1.5	1.5		1.22	6.55	
Ghosts)	1.5		1.0	38.8		2.80
		1.5	1.0	1.37	6.65	0.520
B					Zinc Atoms Incorporated per mg. dry wt. x 10^{14}	Calcium Atoms Incorporated per mg. dry wt. x 10^{14}
4	.03	.03			28.0	
(Normal	.03		.02	Negligible		9.59
Ghosts)		.03	.02	Negligible	27.3	3.33
5	.03	.03			23.0	
(Normal	.03		.02	Negligible		13.0
Ghosts)		.03	.02	Negligible	22.0	7.3
6	.03	.03			59.1	
(Normal	.03		.02	Negligible		39.0
Ghosts)		.03	.02	Negligible	73.0	26.0

*Zinc and calcium incorporation into the ghosts was determined with ^{65}Zn and ^{45}Ca tracers. ^{65}Zn was counted with a Packard gamma scintillation counter with a window setting from 1.0 to 1.2 Mev. ^{45}Ca was counted by drying 100λ of sample on a piece of filter paper, which was placed in a vial with 7.0 mls of toluene base scintillation fluid, and counted in a Packard-Tri carb liquid scintillation spectrometer with a window setting chosen to minimize error due to counts from ^{65}Zn (less than 3.0% of the calcium counts were due to ^{65}Zn). Amounts of radioactivity in the ghost preparations were approximately 2500 cpm/ml for ^{65}Zn, and 20,000 cpm/ml for ^{45}Ca.

and shows that calcium incorporation was reduced by 50 to 80% in the presence of zinc. Results were comparable whether sickle cell ghosts (experiment 3) or normal red cell ghosts (experiments 1 and 2) were used.

Because hemoglobin retention in the ghosts differed in the various preparations (it has been previously established that calcium causes hemoglobin retention [11]) and this could effect relative calcium retention, ghosts were also prepared in which the hemoglobin content was reduced to a minimum in all preparations. In this method packed red cells were lysed in 10 volumes of water, and the resulting, unsealed, membranes then washed twice with 10 volumes of water (buffered to pH 7.4 with 0.01 M Tris) to get rid of most of the hemoglobin. After the second wash aliquots of unsealed packed ghosts were mixed with the appropriate radioisotope-containing cation solutions and allowed to stand for 20 minutes at room temperature. These preparations were then washed twice with 10 volumes of water. It proved difficult to count the membrane particles from these preparations with the Coulter Counter, so they were dried and the radioactivity of the samples related to mg of dry weight.

Results are shown in Table 2B. Hemoglobin retention was negligible in all preparations. Table 2B shows that calcium incorporation into the membranes in these experiments was reduced by zinc to almost the same extent as under conditions of hemoglobin retention.

DISCUSSION

The filterability studies demonstrate that zinc consistently improves deformability of sickle cells at intermediate oxygen tensions. This effect appears to take place at low red cell zinc concentrations. During a two hour incubation with whole blood, most zinc is bound to plasma proteins and the amount incorporated into red cells is less than 0.05 atoms (probably less than 0.03) of zinc per hemoglobin tetramer. This effect at a low concentration suggests that zinc is not acting on hemoglobin, at least on hemoglobin alone, to improve deformability. Consequently we turned to membrane studies.

We investigated the effect of zinc on calcium incorporation into red cell

membranes since zinc and calcium have a number of antagonistic effects, and since recent work suggests that calcium incorporation into red cells or red cell membranes may be involved in producing irreversibly sickled cells. Under all circumstances studied, zinc had an antagonistic effect on calcium incorporation or calcium levels in red cell membranes. This effect was perhaps most marked in the presence of considerable amounts of hemoglobin. We have previously shown that zinc antagonizes the hemoglobin-retaining effect of calcium on red cell ghosts (9). The effect of zinc on calcium binding in the red cell membrane is a possible mechanism for the improvement in sickle cell filterability brought about by zinc. The mechanism of the harmful effect of calcium on sickle cells is not clear at present. A sol-gel transformation in the membrane has been suggested (8) and we have suggested that calcium may cross-link hemoglobin to the membrane and in that manner decrease deformability (10). Whatever the mechanism of harmful calcium action on sickle cells, it appears that it may be opposed by zinc, and this may lead to therapeutic benefit.

Preliminary studies of zinc administration to a limited number of patients with sickle cell anemia complicated by chronic pain has resulted in considerable relief from that pain (12). Of course, until double-blind, controlled, studies are done we must be cautious in interpreting results with a subjective response such as pain. However, if pain relief does occur with zinc administration, then the studies reported here in which zinc improves filterability provide a possible mechanism. Our concept is that the improved deformability resulting from zinc results in less obstruction of blood vessels, and therefore less pain.

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