Volume 275, number 1,2, 117-120 FEBS 09127 November 1990

Stable rightward shifts of the oxyhemoglobin dissocation curve induced by encapsulation of inositol hexaphosphate in red blood cells using electroporation

Youssef Mouneimne[1], Roula Barhoumi[1], Tim Myers[2], Stephen Slogoff[2] and Claude Nicolau[1]

[1]*Cell Biology Section, Institute of Biosciences and Technology, Texas A&M University, College Station, TX 77843-2401, USA* and [2]*Texas Heart Institute, The Texas Medical Center, Houston, TX 77225, USA*

Received 18 September 1990

Rightward shifts of 50–100% of the P_{50} values in the oxygen dissociation curve of intracellular hemoglobin are obtained after encapsulation of inositol hexaphosphate in mouse and dog red blood cells (RBC) by electroporation. Life spans of mouse RBC-myo-inositol hexaphosphate in circulation are unchanged from the normal RBC values.

Allosteric effector of hemoglobin; Red blood cell life span; Oxygen delivery; Red blood cell mean volume; Mean hemoglobin content

1. INTRODUCTION

Inositol hexaphosphate (IHP), a strong allosteric effector of hemoglobin induces highly significant rightward shifts of the Hb-O_2 dissociation curve when it is located in hemoglobin's allosteric pocket [1]. This phenomenon, observed first in Hb solutions, might serve as a basis for the molecular therapy of different afflictions which result in the impairment of O_2 supply to organs and tissues [2]. IHP at physiological pH is a dissociated polyanion unable to cross the red blood cell plasma membrane.

Different techniques have been used to introduce IHP in RBC [3–6], all of them producing strong rightward shifts of the Hb-O_2 dissociation curves, i.e. increasing the P_{50} value of the intracellular hemoglobin (P_{50} = O_2 partial pressure at which hemoglobin is 50% saturated with O_2).

When transfused to piglets, the rightward shift of the ODC was concomitant with an increase of the arterial P_{O_2} and of the arteriovenous O_2 content difference. The cardiac output was shown to be inversely related to the P_{50} value. Despite the O_2-transport reduction (37%), consumption was maintained due to enhanced O_2 extraction [6,7].

In long-term physiological experiments with RBC-IHP transfused piglets, the effects of IHP-loaded erythrocytes were increased O_2 release and reduced cardiac output [8]. The reduced O_2 affinity of the IHP-loaded erythrocytes was still effective 20 days after transfusion in awake piglets. The electrolyte concentration appeared stable over the 5-day observation period except for a transient, but significant hyperkalemia immediately after transfusion. Introduction of IHP into viable erythrocytes improves tissue oxygenation when, for any reason, normal blood flow is impaired [8].

The life span of IHP-loaded erythrocytes equalled that of control erythrocytes [9].

The potential therapeutic applications of IHP-loaded red blood cells led us to search for simpler methods of encapsulation of this allosteric effector of Hb in intact erythrocytes; method applicable to RBC derived from different species. We report here IHP encapsulation in dog and mouse RBC using electroporation, a simple and efficient alternative to the techniques which we already mentioned [3–6]. Electroporation has been used for encapsulation of foreign molecules in different cell types [10] including red blood cells [11]. However, no allosteric effector has been thus encapsulated. Kinosita and Tsong have shown that electroporated red blood cells in which sucrose had been encapsulated retained their normal life span in circulation [12].

2. MATERIALS AND METHODS

2.1. *Erythrocytes*

Mouse erythrocytes were separated from heparinized fresh whole blood obtained from BALB/c strain mice by retroorbital sinus puncture. Dog erythrocytes were separated from fresh whole blood obtained on citrate buffer (CPD) from mongrel dogs by jugular vein collection. After centrifugation (1000 × *g* for 10 min), the plasma was collected and the erythrocytes were washed three times with 0.15 M NaCl solution at 4°C. The supernatant and buffy coat were discarded.

Correspondence address: C. Nicolau, Cell Biology Section, Institute of Biosciences and Technology, Texas A&M University, College Station, TX 77843-2401, USA

Abbreviations: IHP, myo-inositol hexaphosphate; RBC, red blood cells; ODC, oxygen dissociation curve; P_{50}, oxygen partial pressure at which Hb is 50% saturated with O_2

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

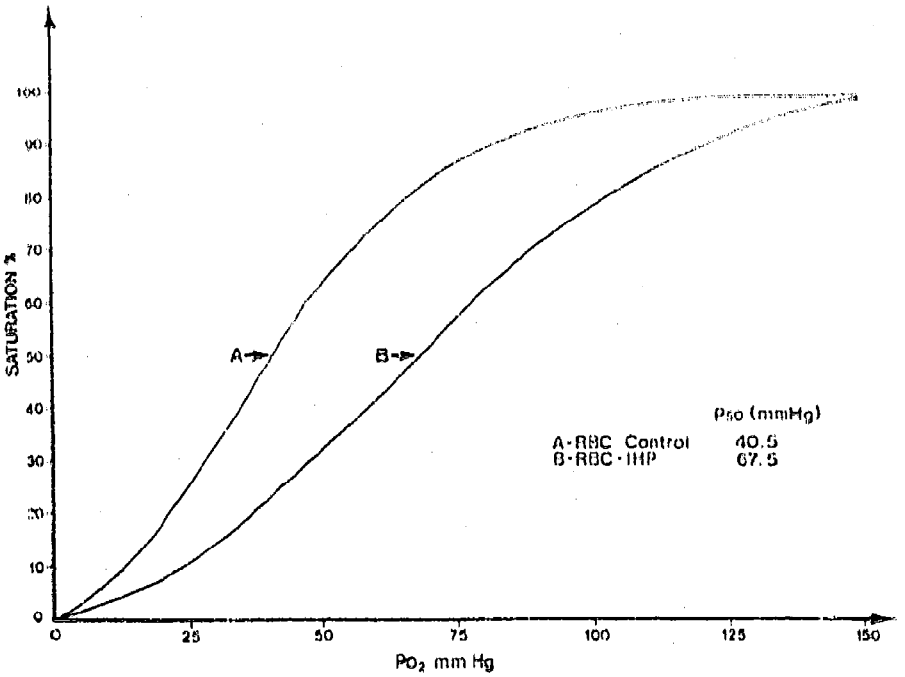

Fig. 1. Oxygen dissociation curves of mouse RBC drawn at pH 7.4 and 37°C. (A) Control RBC. (B) RBC loaded with inositol hexaphosphate (IHP). Field strength: 3.5 kV/cm; pulse duration: 3.0 ms. The P_{50} of RBC electroporated in the absence of IHP was 39.5±0.5 mm Hg.

2.2. *Electroporation*

The pulse generator used was a BTX T100 device delivering an exponential decay pulse. The voltage and duration of the pulse were monitored by a pulse checker (BTX). The electroporation chamber used for the encapsulation of IHP was a cuvette with parallel stainless steel electrodes with a 1.9 mm gap.

2.3. *IHP encapsulation*

The erythrocytes were suspended at 50% hematocrit in an isoosmolar solution of the sodium salt of inositol hexaphosphate (IHP-12Na Sigma, St. Louis, MO, USA). This solution had been neutralized to pH 7.4 using 1 M hydrochloric acid and the concentration of inositol hexaphosphate was 23 mM. The suspension in the electroporation cuvette was subjected to pulses of 3.5 kV/cm and 3 ms at 4°C. After 5 min, incubation at 4°C, resealing of the cells was performed by incubation at 37°C for one hour. The erythrocytes were then washed twice with 0.15 M NaCl.

Oxyhemoglobin dissociation curves were obtained with a dissociation curve analyzer (TCS Huntington Valley, PA, USA) for RBC-IHP and for the control cells, i.e. fresh erythrocytes not subjected to electroporation.

2.4. *Erythrocyte life span measurements*

Biotinylation of erythrocytes was performed according to the method of Suzuki and Dale [13]. Briefly, 1 mg of biotin (6-(+)-biotinylamido-hexanoic acid *N*-hydroxysulfosuccinimide ester Na-salt, Serva, Heidelberg, NY, USA) was added per 100 μl of pellet of cells. After 20 min incubation at 4°C, the cells were washed twice with a 0.15 M NaCl solution. The erythrocytes were then suspended in a 0.15 M NaCl solution at 50% hematocrit and injected into the mouse through the tail vein. After injection, a sample was collected at the specified time points and labelled with phycoerythrin-Avidin (Molecular Probes Inc., Eugene, OR, USA) - by incubating the washed RBC with Avidin-PE for 30 min at room temperature and subsequent washing three times with phosphate buffered saline PBS at 7.4. Using flow cytometry (EPICS Profile Instrument, Coulter, Hialeah, FL, USA), with the phycoerythrin fluorescence emission measured by a 575 nm band pass filter, the percentage of the RBC-IHP in circulation was determined.

3. RESULTS

Fig. 1 shows a significant rightshift of the Hb-O_2 dissolution curve in mouse erythrocytes having incorporated IHP by electroporation (curve B). Curve A represents the ODC (oxygen dissociation curve) of intact mouse erythrocytes. The Hill coefficients are just slightly reduced, as previously observed with RBC-IHP [3-6] but the shape of the curve is sigmoid and the P_{50}

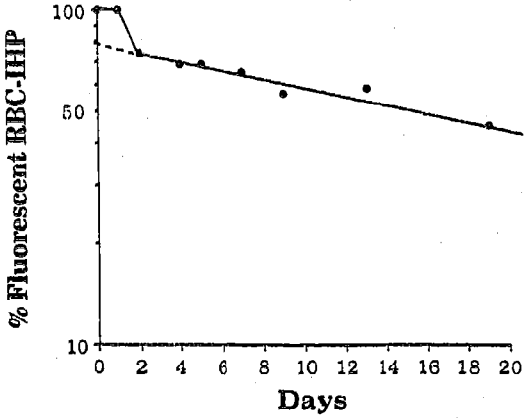

Fig. 2. In vivo life span of mouse RBC loaded with IHP by electroporation. RBC-IHP were biotinylated and detected by Avidin-PE using flow cytometry analysis (see section 2).

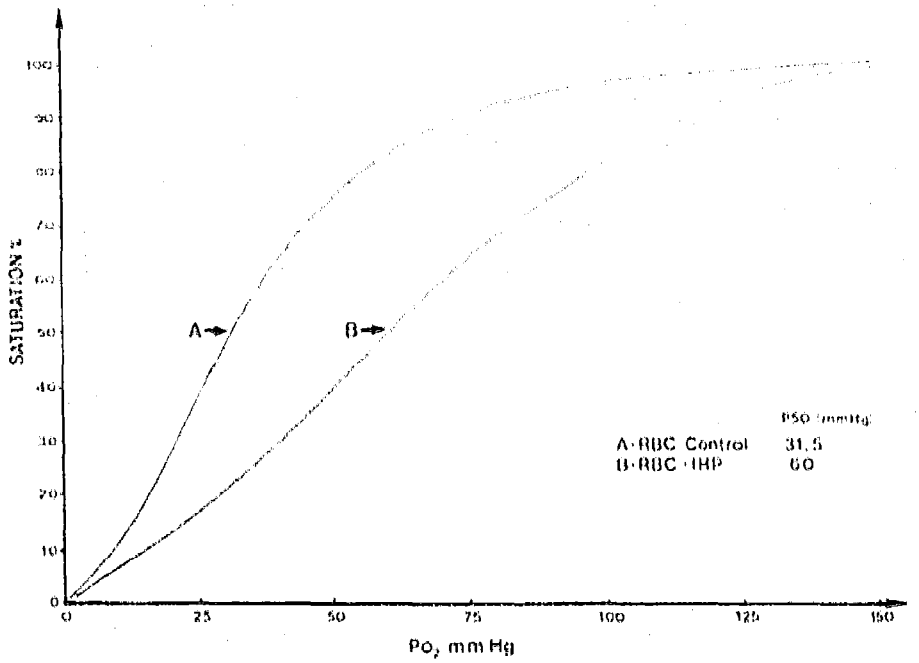

Fig. 3. Oxygen dissociation curves of dog RBC drawn at pH 7.4 and 37°C. (A) Control RBC. (B) RBC loaded with inositol hexaphosphate (IHP). Field strength: 3.5 kV/cm; pulse duration: 3.0 ms. The P_{50} of RBC electroporated in the absence of IHP was 30 ± 0.5 mm Hg. The Hill number was calculated from the slope of a straight line between 30% and 70% saturation on a log Sat/(100-Sat) vs log P_{O_2} plot.

is markedly increased (67.5 mm Hg as compared to 40.5 mm Hg in the control, at 37°C).

Measurement at 24 h and 48 h after electroporation showed a stable value of P_{50}, indicating that resealing was permanent. The important parameter to follow was the life span of the RBC-IHP in vivo.

Using a flow cytometer, the number of fluorescent RBC-IHP were followed in the mouse. RBC-IHP were biotinylated (see section 2) and upon drawing blood they were stained with PE-Avidin [13].

Fig. 2 shows that in mice the RBC-IHP obtained by electroporation have their normal halflife of 11 days. Just as in the case of IHP encapsulation by lysis and resealing ~20% of the retransfused RBC-IHP are lost during the first 24 h after transfusion. A similar result was obtained also when IHP was encapsulated in RBC using a DMSO-shock technique [6].

It seems that independent of the method of IHP-encapsulation used, a fraction of the RBC-IHP, fragilized by the procedure would lyse soon after transfusion. These may be the oldest RBC population in circulation. Using the same procedure of IHP-encapsulation with dog erythrocytes, large, stable rightward shifts were obtained as well (Fig. 3).

Just as in the case of mouse erythrocytes, the main hematological parameters of the IHP-RBC are practically unchanged as compared to normal dog erythrocytes (Table I). Encapsulation of IHP by electroporation does not alter the mean corpuscular hemoglobin content with dog and mouse blood (Table I). Electroporation does not seem to affect the shape of the RBC either (data not shown). The simplicity of the method and the fact that it can be used with red blood cells which reseal with great difficulty (e.g. dog), suggest that electroporation may be a valuable method in introducing non-permeant allosteric effectors of hemoglobin into red blood cells without affecting their physiology.

Physiological investigations on dogs with electroporated RBC-IHP will be reported elsewhere.

Table I

Hematological parameters of mouse and dog erythrocytes loaded with IHP by electroporation

	Dog erythrocytes		Mouse erythrocytes	
	Control	IHP	Control	IHP
Hill coefficient	2.45	2.24	2.94	2.72
Mean corpuscular volume (μm^3)	61.1 ± 1.7	64.4 ± 1.7	48.5 ± 3.1	50.7 ± 6.3
Mean corpuscular hemoglobin content (g/100 ml)	32.8 ± 2	32.3 ± 2	31.3 ± 2	30.5 ± 2
P_{50} (mmHg)	31.5 ± 0.5	60 ± 0.5	40.5 ± 0.5	67.5 ± 0.5

Acknowledgements: The excellent assistance of Dr Ellen M. Kasari and Mr. Jeffrey H. Greene, Laboratory Animal Care, Texas A&M University College of Veterinary Medicine, is acknowledged.

REFERENCES

[1] Benesch, R.E., Edalji, R. and Benesch, R. (1977) Biochemistry 16, 2594–2599.
[2] Nicolau, C., Ropars, C. and Teisseire, B. (1986) in: Phytic Acid, (Cirad, E. ed.) pp. 265–290, Pilatus Press, Minneapolis, MI.
[3] Giersonde, K. and Nicolau, C. (1979) Blut 39, 1–7.
[4] Nicolau, C. and Giersonde, K. (1979) Naturwiss. 66, 567–570.
[5] Ropars, C., Chassaigne, M., Villereal, M.C., Avenard, G., Hural, C. and Nicolau, C. (1986) Bibl. Haematol. 51, 82–91.
[6] Franco, R.S., Weiner, M., Wagner, K., Mantelo, O.J., Rietto, G., Pivacek, L.E. and Valeri, R.C. (1985) Blood 66 (Suppl. 1), 272a.
[7] Teisseire, B.P., Ropars, C., Vallez, M.O., Hérigault, R.A. and Nicolau, C. (1985) J. Appl. Physiol. 58, 1810–1817.
[8] Teisseire, B., Ropars, C., Villeréal, M.-C. and Nicolau, C. (1987) Proc. Natl. Acad. Sci. USA 84, 6894–6898.
[9] Ropars, C., Teisseire, B., Avenard, G., Chassaigne, M., Hurel, C., Cinot, R. and Nicolau, C. (1985) Ann. NY Acad. Sci. 445, 477–491.
[10] Neumann, E. and Rosenheck, K. (1972) J. Membr. Biol. 10, 279–290.
[11] Zimmermann, U., Riemann, R. and Pilwat, G. (1976) Biochim. Biophys. Acta. 436, 460–474.
[12] Kinoshita, K. and Tsong, T.Y. (1978) Nature 272, 258–260.
[13] Suzuki, K. and Dale, G.L. (1987) Blood 70, 791–795.