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A SYMBIOTIC RELATIONSHIP OF ENERGY METABOLISM BETWEEN A 'NON-GLYCOLYTIC' MAMMALIAN RED CELL AND THE LIVER

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Summary

The red cell of newborn pig loses the ability to carry out glycolysis within a month after birth. The metabolic energy source for this 'non-glycolytic' mammalian red cell is unknown. Hepatectomy of an adult pig results in the loss of red cell ATP with a characteristic half-time of 7—8 h which is identical to the rate with which ATP disappears in the pig cells under in vitro substrate-free incubation. Exposure of pig red cells with either normal or depleted levels of ATP to isolated hepatocytes causes a net synthesis of red cell ATP during a 12 h incubation. These findings suggest that a symbiotic relationship of energy metabolism may exist between the red cell and the liver of the pig.

Introduction

Metabolic energy source of the pig red blood cell is unknown. Almost a half century ago, Engelhardt and Ljubimova [1] discovered that domestic pig red cells, unlike other mammalian red cells, were unable to consume glucose. Based on measurement of plasma level glucose which did not change in a prolonged incubation with the pig cells, Kolotilova and Engelhardt [2] reasoned that the cell membrane must not be permeable to glucose, thereby depriving the cells from the benefit of glycolysis. This prediction was recently supported by the work of Kim and McManus [4] who demonstrated the presence of latent glycolytic capacity of cells when the normal membrane barrier to glucose was broken by treatment with an antifungal drug, amphotericin B. Apart from this unusual membrane impermeability to glucose, membrane transport characteristics in general are not unlike other mammalian

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Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

cells. For example, the pig cells maintain rather steep chemical potential gradients with respect to Na^+ and K^+ across the cell membranes by means of ATP-dependent membrane ATPase [5—8].

While the adult pig red cell is non-glycolytic, the fetal pig red cells show normal glucose permeability [9—11] and metabolism [12]. The membrane permeability to glucose which plays the limiting role in glycolysis is entirely lost within a month after birth. The findings by Kim and Luthra [11] suggest that this transition from glucose-permeable fetal state to the glucose-impermeable adult state is brought about essentially by two mechanisms: (1) a rapid elimination of fetal cells from the circulation, and (2) an extensive dilution of fetal cells by glucose-impervious cells produced shortly after birth.

It is not clear why pig red cells in the early postnatal stage discard their glucose permeability, the prerequisite for the sole metabolic pathways utilized by all other mammalian cells, thus far tested. In this communication, evidence will be presented in support of a view that a metabolically symbiotic relationship exists between the red cells and the liver of the pig.

Methods

ATP measurements. ATP was measured using a modified firefly luciferin-luciferase system [13]. In this assay, 100 mg of lyophilized firefly tails (Sigma) were ground and dissolved in 75 ml of 0.04 M $MgCl_2$/0.1 M Na_2HAsO_4 (1 : 1, v/v). The particulate matter was removed by centrifugation before use. Red cell samples were prepared by washing cells in isotonic NaCl three times and diluted to 50% hematocrit. 0.1 ml of this suspension was extracted with 1 ml of cold 0.56 M perchloric acid and neutralized with K_2CO_3. An aliquot of diluted extract was added to a glass scintillation vial and placed in a Beckman LS-250 scintillation counter at room temperature. Then 0.6 ml of firefly extract was forcefully introduced from a syringe into the vial and immediately counted for 0.2 min using all channels. The assay was sensitive from 20 to 250 pmol of ATP.

Porcine hepatectomy. In vivo red blood cell membrane studies in hepatic pigs posed several special problems related to the technique of hepatectomy. Swift interruption of both blood inflow (hepatic arterial and portal venous) and outflow (vena cava) was necessary to prevent generation of toxic products of liver cell decomposition. Portal venous occlusion was restricted to only a few minutes to prevent accumulation of potassium and other toxins in the congested intestinal venous blood. Since red blood cell transfusions were prohibited by the experimental design, a technique of hepatectomy with minimal blood loss was required. The following technique of porcine hepatectomy was designed to fulfill these objectives.

Healthy 40 k pigs were fasted overnight, sedated with intravenous surital, 12 mg/kg body weight, and anesthetized with 1% penthrane and 40% oxygen delivered through an endotracheal tube by a Bird Respirator.

Using clean, but not sterile technique, a long midline incision was used to enter the peritoneal cavity. An iliac artery and vein were cannulated for continuous monitoring of arterial blood pressure, serial sampling of arterial blood, and continuous infusion of 5% glucose solution as required to maintain serum glucose over 80 mg/100 ml.

The hepatic artery, portal vein, inferior vena cava just above the renal veins, and the inferior vena cava above the diaphragm within the pericardium were encircled with non-occlusive tapes. Portal systemtic anastomosis and vena caval bypass were accomplished with a Y-tube constructed of polyvinyl chloride plastic and designed specifically for this purpose. The long limb of the Y-tube was designed to pass through the liver within the vena cava such that the lower end rested just above the renal veins and the upper end rested just below the right atrium. Tributary veins from the diaphragm to the vena cava were ligated in continuity. The short end of the Y-tube was designed to fit into the portal vein to permit immediate portal systemic bypass.

Devascularization of the liver was accomplished in the following manner. The long limb of the tube was inserted into the vena cava just above the renal veins using vascular clamps to minimize blood loss. The short end was then quickly inserted into the portal vein which had been mobilized and transected with only brief occlusion. Cannulas were secured with ligatures. Blood within the liver was recovered by infusing 500 ml of normal saline through the hepatic artery; then the hepatic artery was ligated. The ligatures around the vena cava within the pericardim were tied, thus effecting complete devascularization of the liver with vena caval bypass and portal-systemic shunt. Following this, bloodless hepatectomy was performed to insure thal all viable liver tissue was removed.

Light general anesthesia was maintained, with continuous monitoring of blood pressure and electrocardiogram, and serial monitoring of arterial pH, pCO_2 and pO_2, serum glucose, hematocrit, and serum electrolytes. Animals were killed at the end of procedure.

Hepatocyte preparation. A 7-day-old pig was anesthetized with diethyl ether and a midline incision made with lateral extensions. The portal vein was cannulated and the vena cava opened at the site of the left kidney. Perfusion was started at this time with a Ca^{2+}-free buffer containing 140 mM NaCl, 10 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.3 mM $MgSO_4 \cdot 7\ H_2O$ and 60 μg/ml penicillin adjusted to pH 7.35. The animal was terminated by cutting through the diaphragm and the liver dissected carefully while it was being flushed with perfusate. The cannulated liver was then placed into a larger apparatus. The apparatus included the following features: (a) bubble trap and cannula, attached to a flexible tube permitting in situ cannulation and non-recirculatory flush out as well as recirculatory perfusion; (b) perfusion vessel and silastic tubing oxygenator, jacketed and warmed by a circulating water thermostat (37°C); (c) a low pulsatility Harvard perfusion pump and in line 10 μm perfusate filters. The initial non-circulatory flush-out period of approximately 3 min was followed by 12 min recirculatory perfusion in the apparatus with 300 ml Ca^{2+}-free buffer at a rate of 100 ml/min and with an inflow-outflow differential oxygen partial pressure of 300 mmHg. After this period, 100 mg Worthington collagenase CLS II and 2.5 mmol Ca^{2+} were added and perfusion continued for 10—15 min until stroking of the surface of the perfused organ with a glass rod indicated tissue disintegration. At this time the system was switched to a non-circulatory mode again and extensively flushed free of collagenase with the original Ca^{2+}-free buffer.

The partially digested organ was placed on a 1000 μm pore nylon sieve and the capsule removed by gentle opening of scissors. Cell clumps were washed through the sieve and then agitated and washed through a second sieve of 100 μm pore using the Ca^{2+}-free buffer. The resulting cell suspension was centrifuged at 50 × *g*, resuspended and recentrifuged three times. The final pellet was suspended in a tissue culture medium and adjusted to 30 mg of wet weight cells/ml (approximately $5 \cdot 10^6$ cells/ml). The culture medium was basically Waymouth's medium (MG 752/1 Gibco) buffered with 20 mM Hepes instead of the customary $NaHCO_3$ to pH 7.35 and fortified with 60 μg/ml penicillin and 1 μg/ml gentamycin as well as 20% homologous pig serum (from the adult pig which donated the red cells). Adult pig red cells were suspended in the same culture medium to a hemocrit of 50% and 1 ml aliquots were tied into small dialysis bags. Six bags were placed into 250 ml of the hepatocyte suspension and agitated in a gyratory shaker (100 rev./min) at 25° C. At predetermined times, sample bags were removed and analyzed for ATP. The ratio of hepatocytes to red cells was chosen arbitrarily to give approximately 30 red cells per hepatocyte.

Results

Fig. 1 summarized salient features of energy metabolism of red cells derived from fully grown pigs. ATP levels vital for the cellular functions fall with a characteristic half-time of approximately 8 h (Fig. 1A) regardless of whether glucose is present or not as reported previously [4]. It is also evident that the cells suspended in their own plasma did not fare any better. These findings make it clear that plasma-born glucose is not the normal substrate for the pig red cells.

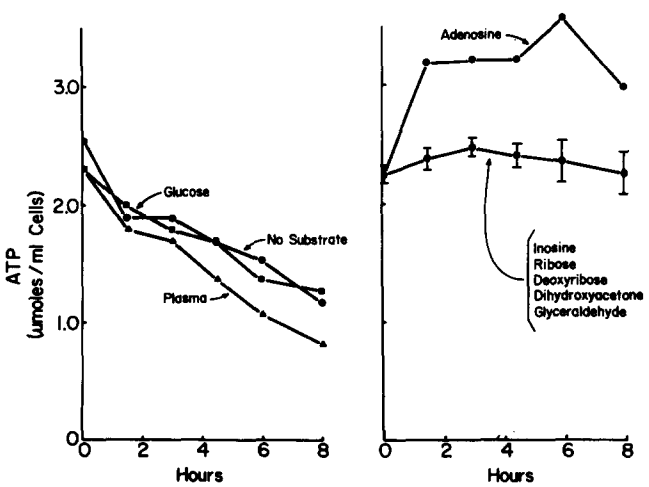

Fig. 1. ATP maintenance of pig red cells by a variety of metabolic substrates. Red cells were suspended to give a hematocryt of 20% in a balance salt solution of the following compositions (mM): 90 NaCl; 5 KCl; 20 phosphate buffer, pH 7.4; 30 glycylglycine/$MgCO_3$ buffer, pH 7.4. All substrate concentration: 5 mM, except ribose, 3 mM. Temperature 37° C.

Even though the pig red cell is non-glycolytic, the metabolic machinery is by no means simple. The cell is amply endowed with 'alternate metabolic pathways'. The results depicted in Fig. 1B show that ATP can be maintained by a host of substrates including adenosine [6,8], inosine [14,15], ribose [3,4], deoxyribose [3,4], dihydroxyacetone and glyceraldehyde [16]. With adenosine, a net synthesis of ATP takes place presumably catalyzed by a series of enzymes, including adenosine kinase, adenosine deaminase and nucleoside phosphorylase.

As expected, chemical analysis for the presence of these compounds in plasma revealed none of them to be present in a significant amount except for glucose (3 mmol/l plasma). Nevertheless, some energy source must exist with which to sustain cellular activity. These findings led us a few years ago to suggest that some localized region in the circulation such as the lungs, spleen, kidney and the liver supply a low amount of substrate to the cells as they pass through [3,4,16]. Indeed, Spinner and Faulhaber [17] have found that perfusion of the pig red cells through intact rat livers resulted in an acceleration of methemoglobin reduction. Following this line of reasoning, experiments were designed to ascertain whether or not a metabolic interaction takes place between the red cells and organs of the body, since the putative compound(s) must not be present in a sufficient quantity to be detected in blood samples derived from the peripheral circulation.

The liver was selected as the target organ. It was of interest to see what would happen to the energy metabolism of cells once the liver is surgically removed from the animal. Fig. 2 shows the effect of hepatectomy on the red cell ATP levels. Upon complete removal of the liver from the animal, the ATP content in red cells steadily falls with a half-time of approximately 7.5 h. It should be recalled that the rate of ATP loss shown in Fig. 1 occurred with the half-time of 8 h. It would seem as though the liver is the sole energy source. However, another interpretation is also possible, especially since it has not been possible to keep the animals alive for more than 4—5 h. In most cases, the animal died within an hour after hepatectomy. Without the liver, much

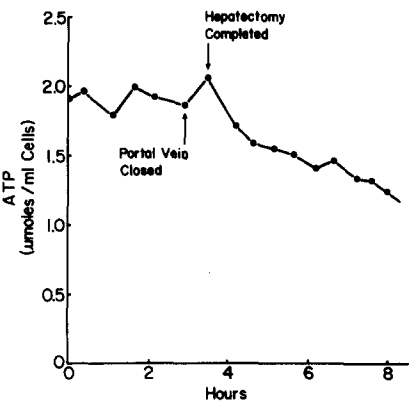

Fig. 2. Effect of hepatectomy on pig red cell ATP levels.

of the physiological regulations of bodily functions has been irreversibly perturbed. It would seem conceivable that the depletion of ATP seen in red cells subsequent to hepatectomy could be due to the inhibition of red cell metabolism by toxic metabolites no longer removed by the liver.

In an effort to gain more definitive evidence, the isolated pig hepatocytes were employed as an alternate route to determine whether the liver plays a role in supporting red cell metabolism. Because of the technical difficulties associated with perfusing a large size liver of adult pig with collagenase, a step necessary in the course of hepatocyte isolation, the livers of 7-day-old pigs were employed.

Prior to hepatocyte exposure, red cells with differing ATP contents were prepared by starving cells for 6 and 12 h, respectively. The amount of hepatocytes relative to red cells was arbituarily decided to give four times more hepatocytes than a unit mass of red cells.

At frequent intervals, the dialysis bags containing red cells were removed and extracted for ATP assays as shown in Fig. 3. Fresh pig cells undepleted of ATP displayed ATP content of 2.4 μmol/ml red blood cells. In the presence of hepatocytes, a net synthesis of ATP takes place during a 12 h incubation period as opposed to control cells which were treated identically except lacking hepatocytes lose ATP with a half-time of roughly 10 h. This somewhat slower rate of ATP depletion is attributable to a lower incubation temperature of 25° C employed in order to minimize thermal denaturation of hepatocytes. The same batch of red cells, not shown in Fig. 3, displayed ATP depletion with a half-time of 6 h when incubated in the tissue culture medium at 38° C.

Pig cells whose ATP was depleted to 1.2 μmol/ml red blood cells exhibit a steady recovery of ATP reaching a level slightly above the initial value of 2.4 when incubated with hepatocytes for 12 h. Again, in the absence of hepatocytes, cellular ATP continues to decline. The metabolic contribution of hepatocytes to energy metabolism of the red cell is also evident in cells whose ATP was depleted as low as 0.7 μmol/ml cells.

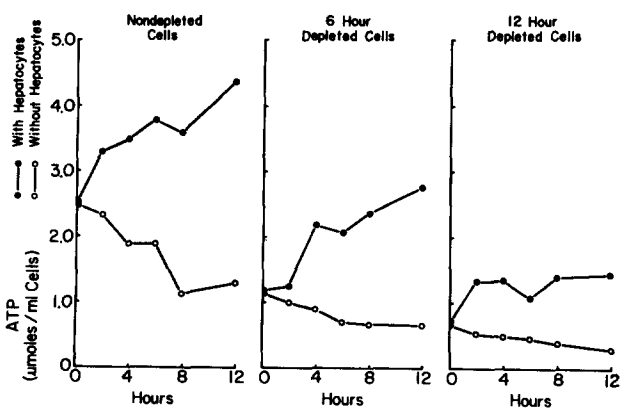

Fig. 3. Effect of co-incubation of isolated hepatocytes and pig red cells on red cell ATP content. Temperature 25° C.

Discussion

The findings reported herein lend support to the notion that the red cells receive a supply of metabolic substrate(s) during their journey through the sinusoidal microcirculatory beds of the liver which, in turn, drives oxygen from the red cells.

Pertaining to this postulate, it would be instructive to consider the following feature of the pig red cells. First, it would be useful to ascertain the metabolic cost of the minimum free energy necessary to power the cell. An estimate may be made from ribose utilization which exhibited a substrate inhibition-type kinetics at high ribose concentrations [3]. By plotting the extent to which ATP was maintained vs. lactate produced in the presence of varying concentrations of ribose, it was possible to deduce the metabolic requirement of substrate input necessary for keeping ATP at the physiological level. It was found that a steady supply of substrate, yielding lactate production at least a minimum rate of 1 μmol/ml cells per h will have to be made available to the cells in order to maintain ATP. Presumably the liver would be capable of producing enough substrate to meet the metabolic needs of the cells. Second, the slower the rate of ATP depletion, the easier the maintenance of ATP will be in the face of an intermittent supply of energy source. Indeed, the conservative handling of ATP in substrate-free conditions is another distinguishing feature of the pig red cells. Much shorter half-times of ATP depletion, 1.0, 2.5 and 4 h, respectively, have been reported for the cow, rabbit and human cells [18—20].

Pertaining to the question of the true nature of in vivo metabolic substrate, increased ATP seen with hepatocytes may serendipitously turn out to be the clue. As shown in Fig. 1, the only compound capable of yielding a net synthesis of ATP under in vitro conditions is adenosine.

In point of fact, adenosine has already been cast for a number of possible physiological roles such as a direct precursor of erythrocyte adenine nucleotides [21—24] and as a modulator serving to hence coronary blood flow [25]. Adenosine is primarily formed from AMP in the liver [26—28] and other organs [29,30] by a 5′-nucleotidase. It seems conceivable that red cells endowed with a facilitated diffusion-type mechanism with an exceptionally high affinity for adenosine [31] could readily pick up adenosine synthesized within and released from the liver as they pass through.

Clearly, chemical analysis of hepatocyte extrudate or the perfusion fluid of the liver would seem to hold much promise for our attempt to identify the metabolic substrate of this non-glycolytic mammalian red cell.

Note added in proof (Received November 5th, 1979)

Since the submission of this paper for publication, we identified inosine as the major nucleoside present in the perfusate of pig hepatocytes or the liver. The preliminary result has been presented elsewhere [32].

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