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ACUTE AND CHRONIC ADMINISTRATION OF INSULIN TO RATS- IMPLICATIONS ON RED BLOOD CELL FUNCTION.

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ABSTRACT

The effect of insulin administration on functional and physico-chemical parameters of red blood cell (RBC) of male albino rats is reported. The parameters studied included the haemoglobin P_{50} for oxygen determined from the oxygen haemoglobin dissociation curve; activities of red cell enzymes of carbohydrate metabolism including those of hexokinase (HK), phosphofructokinase (PFK), glucose-6-phosphate dehydrogenase (GPD), bisphosphoglycerate mutase (BPG-mutase), pyruvate kinase (PK) and lactate dehydrogenase (LDH). Glucose, 2,3-bisphophoglycetate (2,3-BPG), inorganic phosphate, pyruvate, and lactic acid concentrations were also determined in the RBC. Erythrocyte resistance to hemolysis was assessed using the erythrogramme method with 0.001 HCl serving as hemolytic agent. The hormone in acute and chronic doses elicited increase in the haemoglobin oxygen affinity via reduction in rate of 2,3-BPG synthesis. The activities of HK, PFK, GPD, PK and LDH were increased with accumulation of acidic metabolites, pyruvate and lactate. The erythrocyte resistance to hemolysis increased in both instances of hormone action.

KEY WORDS: Insulin, Phospofructokinase, erythrocyte, erythrogramme, haemoglobin.

INTRODUCTION

The oxygen regimen of an organism including the supply and maintenance of optimal tension of the gas in tissue cells, is a coordinated and intricately regulated process. At the molecular level, the process hinges on the reversible oxygenation-deoygenation of the haemoglobin, a complex tetrameric protein found in the red blood cells of most animals. As the haemoglobin performs its gas transport function, the red blood cell does not simply serve as an inert vessel, but as one, which actively provides the right functional and metabolic environment for the respiratory protein.

The integrated system, which regulates the composition of the environment surrounding the haemoglobin in the red blood cell, is far from elucidated. One aspect of it concerns the regulatory role of insulin on the erythrocyte carbohydrate metabolism. While some workers declined to acknowledge the direct involvement of insulin in the red blood cell processes (Dormandy and Larday; 1969), others have reported the presence of insulin receptors on the red cell membrane (Czech and Masague, 1982). The red blood cell has also been reported to possess insulin-degrading proteolytic activity (Lamer et al, 1982; Kendish 1983), which implies that the hormone may have direct bearing on erythrocyte metabolism. Given this background, very little is known about how the haemoglobin function is regulated to ensure optimal tension of oxyger. in the tissues following insulin action. This work links physico-chemical and metabolic changes to functions of the RBC system in response to insulin.

MATERIALS AND METHODS

Male albino rats of body weights between 160-200g were used in this experiment. The animals were divided into three groups, the first group serving as the control. Animals of the second group were subjected to acute insulin administration. This was done by injecting 0.20 I.U of the hormone per 100g body weight into animals and the effect analysed after 3 hours of administration. The third group of animals was subjected to chronic administration of the hormone. For the purpose, animals were injected with the hormone in the dose of 0.10 I.U/100g once every 12 hours for three days, and the effect studied thereafter.

All operations were performed in the cold ($0^{\circ}C$-$4^{\circ}C$). Fresh blood samples from animals were obtained by decapitation with heparin serving as anticoagulant. To obtain plasma free red blood cell (RBC) suspensions, samples were washed three times in physiological brine (0.85% NaCl) with centrifugation each time at 2,500 rev./min for 5 minutes. RBC hemolysates were obtained through freez-thaw, conducted by immersing test tubes with cell suspensions in liquid nitrogen and then centrifuging at 18,000 rev/min. under refrigeration for 15 minutes to remove cell debris. The hemolysates so obtained, which contained all the intra red cell ingredients, were used for analysis.

The haemoglobin gas transport function was studied by spectrophotometric determination of the oxygen-haemoglobin dissociation curve according to Ivanov (1975). The activity of bisphosglycerate mutase was determined according to the Rapoport-Luebering optical method (Rapport and Luebering, 1952), while those of the other enzymes of carbohydrate metabolism in the RBC, namely, hexokinase (HK), glucose -6-phosphate dehydrogenase (GPD), phosphofructokinase (PFK), pyruvate kinase (PK) and lactate dehydrogenase (LDH), were assayed spectrophotometrically at 340nm based on the oxidation-reduction of nicotinamide cosubstrates (Chapman et al. 1962). Glucose, pyruvate and lactase levels in the hemolysates were determined as described (Chmelovsky 1985), while those of 2,3 bisphosphoglycerate (2,3-BPG) and inorganic phosphate (Pi) were determined by nonenzymatic methods as described by Dyce and Bessman (1973). The RBC resistance to hemolysis was studied using Gitelzon's erythrogramme method (Gitelzon and Terskov, 1969) consisting of time monitoring of the kinetics of red cell hemolysis with 0.001N HCl as haemolytic agent. To achieve the aim, cell suspensions were preliminarily diluted with physiological solution to 0.700 extinction at 600nm. To 2.50 ml of diluted suspensions in a cuvette, 0.50 ml of the haemolytic agent was added and cell demolition monitored from extinction readings at $25^{\circ}C$ and at 30 second intervals. The percentage of cells haemolysed was calculated from extinction values and then plotted against time to give a haemolytic curve or erythrogramme. Differences between the control and experimental groups were compared and level of significance checked using the student's t test.

RESULTS

Information on changes in the gas transport function of the haemoglobin following acute and chronic administration of insulin into rats is presented in Table 1 and Fig. 1. While

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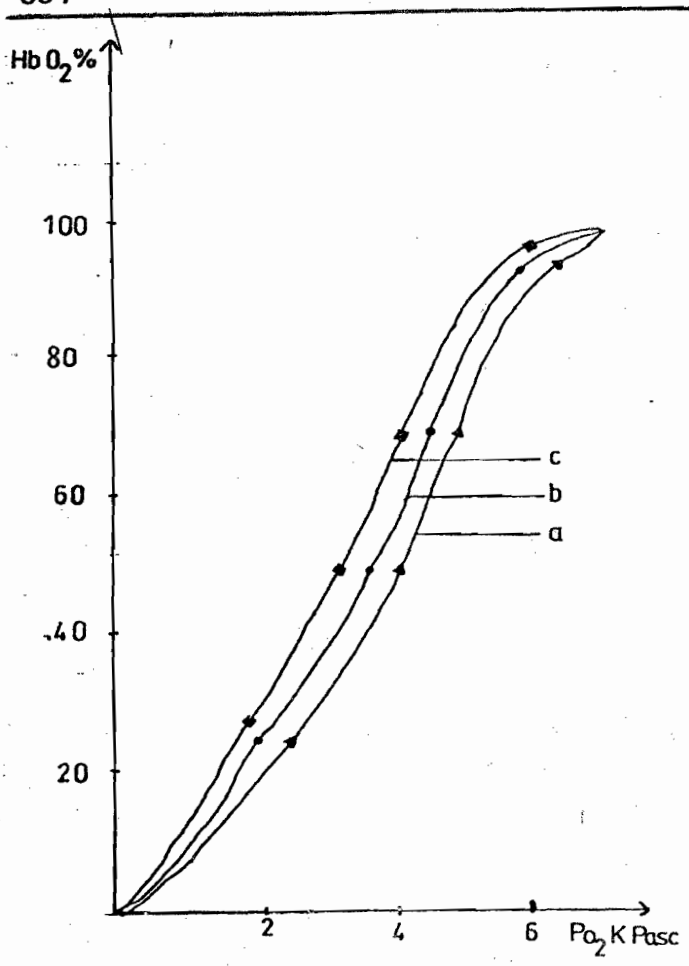

Fig 1: oxygen – Haemoglobin dissociation curves following insulin administration into rats: a-control; b- acute dose; c-chronic dose.

Table 1 contains data on the effect of insulin on the controlling parameters of the haemoglobin oxygenation-deoxygenation process, Fig. 1 shows the effect of the hormone on the position of the oxygen -haemoglobin dissociation curve. Insulin, in acute and chronic doses elicited backward displacement of the oxygen -haemoglobin equilibrium curve, with the effect being more pronounced during chronic than during acute administration of the hormone. Significant decreases in the value of the haemoglobin P_{50} for oxygen were correspondingly obtained in both cases of action of the hormone (Table 1). The activity of bisphosphoglycerate mutase (BPG-mutase) as well as the concentration of 2,3-bisphossphoglycerate significantly decreased in both acute and chronic actions of insulin (Table 1).

Table 2 contains information on the effect of insulin on the metabolic activities of enzymes of carbohydrate metabolism in the RBC. The activities of hexakinase, pyruvate kinase and lactate dehydrogenase were increased in both acute and chronic action of the hormone. Although the phosphofructokinase activity remained unchanged in acute dose, the activity of the enzyme increased slightly but significantly ($P<0.05$) during chronic action of insulin. The activity of glucose –6-phosphate dehydrogenase of the pentose phosphate shunt increased significantly ($P<0.05$) in both cases of hormone action.

The effect of insulin on the levels of metabolites in the RBC of rats was recorded in Table 3. While the concentration of glucose decreased, that of lactate remained significantly high ($P<0.05$) in both cases of hormone administration. The level of pyruvate was elevated with the effect being more pronounced in chronic than in acute doses of insulin. The concentration of inorganic phosphate decreased significantly ($P<0.05$) in chronic dose but remained virtually at the control level during acute hormone administration.

Fig. 2 shows the effect of insulin on the erythrocyte haemolytic process. Acute administration of the hormone elicited increase in the height of the maximum of the haemolytic curve, however without shifting its position from the 3.5 minutes of the control. In chronic dose, the erythrogramme maximum shifted forward, relative to control, to a new, 4.0-minute position.

DISCUSSION

The gas transport function of the haemoglobin in the red blood cell (RBC) is dependent on the intracellular pH as well as on the concentration of 2,3-bisphosphoglycerate, among other factors. As the pH within the erythrocyte decreases, the haemoglobin oxygen affinity also decreases. This phenomenon is called the Bohr's effects (Astrup et al 1966; Freidman 1992; Delvin 2002).2.3- Bisphosphoglycerate influences the haemoglobin gas transport function in two capacities. Firstly, and as an acid, the compound adds to the Bohr's effect. Secondly, and as allosteric modulator of oxygneation, it binds to the beta-chains of the haemoglobin molecule, stabilising the deoxy-form of the protein and forcing oxygen from oxyhaemoglobin (Busa, Nuccitelli 1984; Gavrilov et al 1985). That is, allosterically and via the Bohr's effect, 2,3-BPG decreases the haemoglobin oxygen affinity, thus promoting the offloading of the gas from oxyhaemoglobin to metabolising tissue cells of the organism.

Table 1: RBC gas transport parameters following acute and chronic administration of insulin into rats. N = 5; P<0.05

	Parameters		
Expt. Condition	HbP_{50} (Pasc.x10^3)	BPG-MUTASE (Micromole/min/mlRBC	2,3-BPG Concentration (micromole/mlRBC)
Control	4.31± 0.19	1.52 ± 0.07	3.83 ± 0.24
Acute Insulin Action	3.58 ± 0.2*	1.11± 0.05 *	2.42 ± 0.14*
Chronic Insulin Action	3.27 ±0.16 *	1.02 ± 0.02 *	2.84 ± 0.13 *

Significant differences compared to control.

Table 2: Influence of Insulin on activities of rat RBC enzymes of carbohydrate metabolism: n=5;P<0.05

ENZYMES ACTIVITIES (micro M/min./ml RBC					
Expt Condition	HK	PFK	PK	LDH	G.6PD
Control	0.14±0.10	4.45±0.23	1.83±0.11	4.85±0.14	1.28±0.06
Acute Insulin Action	0.21± 0.01 *	4.53±0.31	2.41±0.06*	6.51±0.17*	1.98±0.03*
Chronic Insulin Action	*0.26±0.02	*5.32±0.19	2.53±0.12*	6.63±0.15*	2.07±0.06*

- Difference significant compared to control.

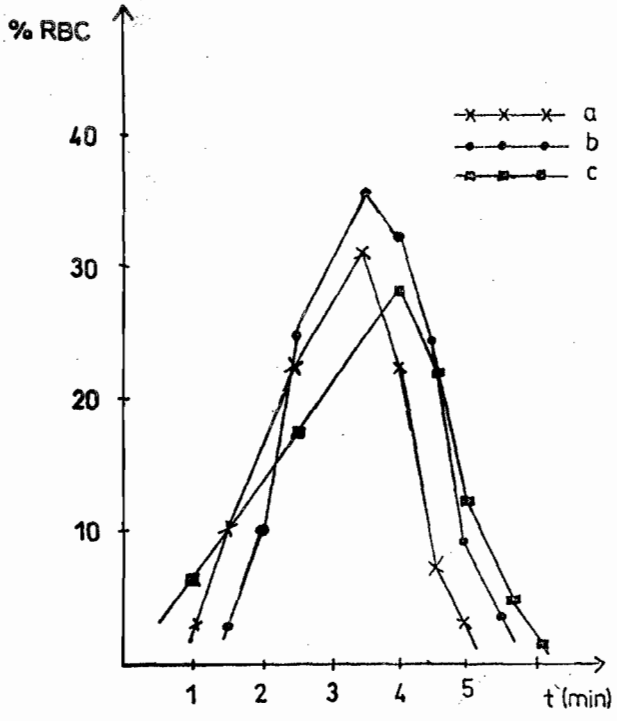

Fig.2: The effect of insulin on the RBC hemolytic process:
a – control: b- acute dose; c-chronic dose.

The metabolic activity of bisphophoglycerate mutase is the critical determinant of the concentration of 2,3-BPG in the RBC. The activity of this enzyme decreased significantly during acute and chronic administration of insulin. The concentration of 2,3-BPG also follows suit in both instances of hormone action (Table 1). This situation amounted to increase in the haemoglobin oxygen affinity. As recorded in Fig. 1, the oxygen–haemoglobin dissociation curve was displaced to the left during both acute and chronic action of insulin, which implies increased affinity of the respiratory protein to oxygen. Although it remained virtually unchanged during acute action of insulin, the concentration of organic phosphate dropped significantly under chronic dosage of the hormone (Table 4). The Bohr's effect, therefore, is more reduced during chronic dose, and consequently, the oxygen-haemoglobin dissociation curve was more shifted to the left in the chronic than in acute action of insulin.

The inhibitory effect of insulin on the levels of 2,3-BPG and inorganic phosphate recorded in this work may account for the opposite effect as observed in the RBC of diabetic patients. Lekakin et al (1981) reported that the concentrations of 2,3-BPG and inorganic phosphate are increased in RBC in diabetes. In the same vein, Dormandy and Larday (1965), Dormmandy (1965) reported decreased incorporation of labelled phosphorus (^{32}P) into 2,3-BPG during in vitro incubation of RBC in media with insulin. The decrease in inorganic phosphate concentration as observed in our experiment under chronic action of insulin (Table 3) may be the consequence of increased diversion of phosphate in favour of phospholipid synthesis for the RBC membrane. The decrease may also be associated with increased phosphorylation of the RBC membrane protein, spectrin, following activation of appropriate kinases by insulin (Conway and Tao 1981). Such increased phosphorylation may lead to redistribution of electrical charges on the spectrin molecule resulting in decreased affinity of the protein to 2,3-BPG. Free from complex with spectrin, the unbound 2,3-BPG then interacts with BPG – mutase, inhibiting the enzyme. This may explain why the activity of bisphophoglycerate mutase fell and why the concentration of the enzyme product, 2,3-BPG, also fell under the effect of insulin.

The decreased synthesis of 2,3-BPG as explained above may lead to increased haemoglobin oxygen affinity as evident in the backward movement of the oxygen – haemoglobin dissociation curve. While the situation is beneficial to the organism at first glance, in that it promotes effective saturation of the haemoglobin with oxygen at the lungs, subsequent dissociation of oxyhaemoglobin into the deoxy-form and oxygen for tissue cells, is made difficult in normoxic conditions on account of the tight binding of the ligand to the protein (Van Leer and Stikney 1962). Oxygen starvation or hypoxia thus threatens the tissues cells. The need, therefore, arises for appropriate adjustment reactions to be initiated in the organism in order to bring about decrease in the haemoglobin oxygen affinity and thus, enhance the offloading of the gas to tissue cells.

Insulin exerts stimulatory effect on glycolysis in many tissues (Beitner and Kalant, 1971; Rozen 1984). In our experiment, this effect is realized through increases in the metabolic activities of key and regulatory enzymes of the pathway in the RBC. Thus, the activities of hexokinase, phophofructokinase and pyruvate kinase, are increased (Table 2). The concentration of glucose decreased (Table 3) showing increased utilisation of the energy substrate. The activity of glucose –6-phosphate dehydrogenase was increased (Table 2) which means increased metabolisation of glucose –6-phosphate via the pentose phosphate shunt. Thus activated, the pentose phosphate pathway then shunts metabolites, namely, fructose-6-phosphate and 3-phophoglyceraldehyde, generated in the transaldolase and transketolase reactions, into the main glycolytic sequence. Consequently, phosphofructokinase and the enzymes situated towards the end of the pathway, (pyruvate kinase and lactate dehydrogenase) are additionally activated. While all these

Table 3. Effect of Insulin on concentration of metabolites in rat RBC. n=5;(micro mole/ml RBC)

	Metabolites			
Expt Condition	Glucose	Pyruvate	Lactate	Pi
Control	5.13±0.21	0.18±0.02	2.63±0.13	0.21±0.02
Acute Insulin Action	3.03± 0.11*	0.21±0.03	4.19±0.21*	0.22±0.02
Chronic Insulin Action	*3.41±0.17	*0.27±0.02	4.63±0.21*	0.13±0.02*

* Difference significant compared to control. P<0.05.

occur, the activity of bisphosphoglycerate mutase falls (Table 1). That is, insulin causes decrease in the activity of the Rapoport –Luebering bypass but stimulates metabolic flux through the main glycolytic sequence. The activation of pyruvate kinase and lactate dehydrogenase results in accumulation of acidic metabolites (pyruvate and lactate) in the RBC (Table 3). These products, by increasing the Bohr's effect in the erythrocyte, decreased the affinity of haemoglobin to oxygen, thus facilitating the offloading of the gas to metabolising tissue cells of the organism. Thus, the observed activation of the main glycolytic sequence in the red blood cell by insulin constitutes a biochemical strategy aimed at combating the threat of tissue hypoxia.

Our data on the effect of insulin on the kinetics of RBC hemolysis are presented in Fig. 2. The erythrogramme maximum is increased in height during acute action of insulin, however, without shift from the 3.5-minute position of the control. In chronic action of the hormone, the erythrogramme maximum is shifted rightward to a new, 4.0-minute position. All these imply increase in the RBC resistance to hemolysis. The increased activity of glucose –6-phosphate dehydrogenase recorded in both instances of insulin action may lead to increased formation of NADPH required by the glutathione – dependent reductase system that maintains the integrity of the red cell membrane (Feodorov 1976). Decrease in metabolic activity of glucose –6-phosphate dehydrogenase is usually associated with hemolytic conditions of the RBC (Van Bennet 1985; Delvin 2002). As mentioned earlier, the observed decrease in organic phosphate concentration (Table 3) may mean increased phosphorylation of membrane spectrin, or increased synthesis of phospholipids for red cell membranogenesis. All these may be contributory to the observed reduction in fragility of the RBC to hemolysis. Thus and as observed, the red blood cell is made more resistant and less prone to osmotic shock and hemolysis in condition of increase in levels of acidic metabolites following insulin action.

CONCLUSION

Insulin, during acute and chronic action, elicited functional and metabolic changes in the red blood cell. At the onset, the hormone triggered increase in the haemoglobin oxygen affinity. This was realized through inhibition of 2,3-BPG synthesis. Although this promotes haemoglobin saturation with oxygen at the lungs, subsequent offloading of the gas to tissue cells is defacilitated. Hypoxia threatens the tissue cells of the organism. Following this, the main glycolytic sequence was stimulated resulting in accumulation of acidic products. The later, via the Bohr's effect, facilitated oxygen delivery by haemoglobin to the tissues. This way, the oxygen regimen in the tissues was adjusted and optimised to the needs of the organism during insulin action.

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