CHAPTER IV

DISCUSSIONS

Molecular oxygen (O_2) is essential for aerobic organisms. Exposure of tissues or cells to hypoxia induces a variety of adaptive or pathologic responses. An increase in erythrocyte 2,3-BPG in hypoxic condition has been interpreted to mean a greater availability of oxygen to tissues. The compensatory nature of 2,3-BPG depends on its ability to cause the oxygen-hemoglobin binding curve to shift to the right, thereby facilitating the unloading of oxygen from oxyhemoglobin to tissues. Its concentration is elevated above normal values in certain clinical and environmental conditions such as in anemia, congenital heart disease, and at an altitude. In the same way, exercise requires an adequate supply of oxygen to maintain aerobic energy metabolism, and it probably causes an increase in erythrocyte 2,3-BPG level. However, the literatures on the response of the erythrocyte 2,3-BPG to exercise at sea level are repeated with inconsistency. Lignen and associates (Lignen et al., 1986) have shown an increase in erythrocyte 2,3-BPG level after 50 minutes cross-country run in athletes. Later, Lignen and associates (Lignen et al., 1988) also found an increase in erythrocyte 2,3-BPG immediately after a marathon race and up to 12 hours later. In accordance with Austin and coworkers (Austin *et al.*, 1973) in which the level of erythrocyte 2,3-BPG increased after walking until exhaustion on a treadmill at 3.5 mile per hour. Hespel *et al* (1988) also observed an increase in erythrocyte 2,3-BPG level immediately after training. In contrast, Bonner *et al* (1975) reported that erythrocyte 2,3-BPG level was not changed in women after walking until exhaustion on a tredmill. Odje *et al* (1995) also reported that the level of erythrocyte 2,3-BPG was not changed immediately after untrained men exercised on a bicycle ergometer at 122.5 W for 10 minutes and 2,3-BPG level decreased significantly after 50 minutes of rest. These may be due to the effects of several factors that regulate the metabolism of erythrocyte 2,3-BPG during exercise. In addition, different kinds of anticoagulant and blood storage are attributed to the conflicting reports. The propose of the present study was to investigate the change in erythrocyte 2,3-BPG levels and the factors influencing erythrocyte 2,3-BPG levels during exercise in male Wistar rats. The study was designed under the assumption that anticoagulant and blood storage, exercise programs may help

in clarifying the ambiguity in the literature concerning the response of 2,3-BPG to exercise. Furthermore, the effects of a decrease in pH, an elevated temperature, Ca^{2+} and pO_2 that occurred during exercise on 2,3-BPG metabolism were studied *in vitro*.

Firstly, the study began with the assumption that different kinds of anticoagulant (heparin, sodium fluoride and potassium oxalate) and blood storage are important in evaluating the effects of exercise on erythrocyte 2,3-BPG levels. The results demonstrated that heparin, sodium fluoride and potassium oxalate gave similar results in the levels of erythrocyte 2,3-BPG when immediately determined. The more powerful preservation of erythrocyte 2,3-BPG levels occurred in potassium oxalate than in sodium fluoride and heparin. Potassium oxalate could preserve 2,3-BPG levels of red blood cells when incubated in their own plasma at $4^{\circ}C$ until 5 days of storage. A number of investigation have stated that potassium oxalate is an inhibitor of plasma lactate dehydrogenase (LDH) (Emerson *et al.,* 1965), L type (red cell) pyruvate kinase (PK) (Buc *et al.,* 1978) and red cell phosphoglycerate mutase (PGM) (Beulter *et al.,* 1987). On the basis of these finding, it is reasonable to propose that the effects of potassium oxalate on the maintenance of erythrocyte 2,3-BPG levels is to affect the change in glycolysis and 2,3-BPG metabolism in red blood cell. Therefore, the mechanism of action of potassium oxalate can be explained that it affects on inhibiton of PK reaction, causing the accumulation of substrates proximal to the PK reaction, ie, PEP, 2-PG, and 3-PG. The latter compound inhibits BPGP and stimulates BPGM. LDH reaction is also inhibited by potassium oxalate and resulting in the accumulation of pyruvate, 2-PG and 3-PG. These may affect the erythrocyte 2,3-BPG levels. In addition to its effect on the PK and LDH reaction, the mechanism of action is possible at the PGM reaction, causing the accumulation of 3-PG and thereby increasing in erythrocyte 2,3-BPG level. In the same way, sodium fluoride, an inhibitor of enolase in glycolysis pathway is the most widely used antiglycolytic agent for glucose preservation (Chan *et al.,* 1992). The mechanism of action of sodium fluoride in preserving the levels of erythrocyte 2,3-BPG is similar to potassium oxalate, but its effect is less than potassium oxalate. Sodium fluoride can affect only the enolase step of glycolysis pathway, producing the accumulation of substrates proximal to the enolase reaction, ie, 2-PGA, 3-PGA and 1,3-BPG. The later compound is the substrate for the synthesis of 2,3-BPG. Interestingly, potassium oxalate is a normal plasma constituent, circulating at a level of approximately 4 μmole/L. As shown in this study that potassium oxalate can preserves the erythrocyte 2,3-BPG, it is possible that this compound may exert *in vivo* regulatory effect.

The second purpose of the present study was to evaluate the effects of different exercise programs on erythrocyte 2,3-BPG level. The studies were designed under the assumption that the intensity of exercise and the training states might help to clarify a controversy in the conflicting reports concerning the response of erythrocyte 2,3-BPG to exercise. The exercise programs used in this study were different in both intensity and training state. Male Wistar rats subject to run by these programs. The changes in erythrocyte 2,3-BPG levels were investigated along with the level of blood lactate, triglycerides and glucose. The effects of different exercise programs and the alteration of both glucose and triglycerides metabolism had been studied in order to understand the metabolic adaptation of muscle tissues from anaerobic to aerobic metabolism after exercises. The results from these studies showed that the metabolism of glucose and triglycerides were not changed in sedentary control. Both endurance and exhaustion exercise caused a high anaerobic energy metabolism. However, after a period of training, exercised rats were adapted to a greater extent of aerobic energy metabolism and more triglycerides were used as energy source. However, exercise programs used in this study did not cause the change in blood glucose levels.

At the beginning of exercise, muscle glycogen is the principal energy source. Subsequently, uptake of blood-borne glucose and free fatty acids (FFA) increases. As muscle glycogen supply is depleted during the beginning of exercise, blood-borne glucose as supplying from liver glycogen becomes the major source of carbohydrate (Appenzellern *et al.,* 1983). It is corresponding to our findings that the level of plasma glucose did not change after both acute and training exercise. With continued exercise, glucose utilization declines while FFA utilization increases and becomes the predominant energy source (Appenzellern *et al.,* 1983 and Bove *et al.,* 1983). In accordance with previous studies (Brooks *et al.,* 1994, Phillips *et al.,* 1996), prolong moderate exercise intensity has been shown to result in a time-dependent increase in fat oxidation and a decrease in carbohydrate oxidation. On the basis of this finding, it has been proposed that apparently occuring with training is a reduction in circulating FFA due to the increase of its oxidation by muscle during exercise (Phillips *et al.,* 1996). Correspondingly, our data showed that training status of exercise caused a decrease in plasma triglycerides higher than acute exercise. Moreover, our findings showed that a decrease in plasma triglycerides by exhaustion training exercise was higher than endurance training exercise. A number of investigations have reported that malonyl-CoA decreases in liver during exercise in an exercise

intensity-dependent manner and the change induces fatty acid oxidation (Hutber *et al.,* 1997 and Rasmussen *et al.,* 1997). Malonyl CoA is synthesized by acetyl CoA carboxylase (ACC), and is an inhibitor of fatty acid oxidation in liver and cardiac muscle (Awan *et al.,* 1993 and Saddik *et al.,* 1993). Inactivation of ACC leads to a decrease in the concentration of malonyl CoA, a potent inhibitor of carnitine palmitoyltransferase I (CPT-I). During exercise, the active muscle is subject to stress, causing ATP depletion and leading to an elevation of the AMP/ATP ratios. Since adenylate kinase 2ADP $\rightleftharpoons$ ATP + AMP is close to equilibrium at all times. The rise in AMP activates AMP-activated protein kinase (AMPK) cascade (as shown in Figure 4-1). AMPK is a metabolic stress sensing enzyme that plays a role in the regulation of energy homeostasis (Stein *et al.,* 2000). AMPK is allosterically activated by AMP and also promotes the phosphorylation of an upstream kinase, termed AMPK kinase (AMPKK). A number of investigators have cited that the decrease in malonyl CoA is accompanied by increase in AMPK activity and by decrease in ACC activity (Hutber *et al.,* 1997, Vavvas *et al.,* 1997 and Winder and Hadie, 1996). The mechanism has been postulated to couple the muscle contraction with an increase in the rate of fatty acid oxidation in muscle during exercise (Winder *et al.,* 1998, Winder and Hardie, 1996): 1) Muscle contraction increases AMP concentration in the muscle. 2) The increase in AMP activates AMPK allosterically and also activates AMPK kinase. 3) The AMPK kinase phosphorylates AMPK, resulting in further activation. 4) AMPK phosphorylates ACC, resulting in a decrease activity. 5) The decrease in ACC results in a decrease in malonyl CoA, which decrease an inhibition of CPT-I, allowing increased fatty acid oxidation in the mitochondria. It has been suggested that malonyl CoA is an important regulator of fat metabolism in skeletal muscle (Hutber *et al.,* 1997, Rasmussen and Wolfe, 1999).

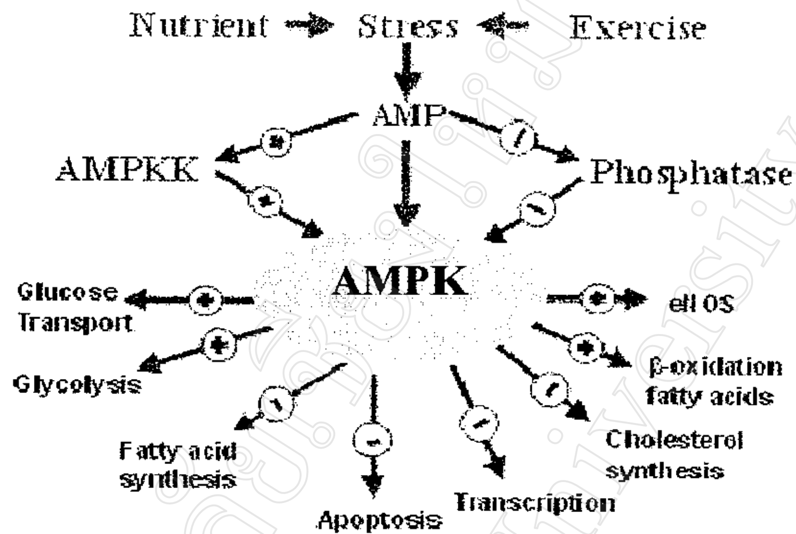

Figure 4-1: Schematic illustration of AMPK cascade. AMP-activated protein kinase (AMPK) is a metabolic stress sensing enzyme that is responsible for regulating meet of energy demands and match energy supply. During exercise, ATP supplies are reduced (caloric restriction) and leaded to increase in the ratio of AMP to ATP. AMPK is allosterically activated by the rise in cellular AMP and by phosphorylation by AMPK kinase (AMPKK). An activated AMPK is involved in regulating several metabolic pathways in response to cellular stress.

Data of blood lactate levels showed that exercise caused an increase in the lactate levels. This agrees with numerous studies in human subjects and laboratory animals (Issekutz *et al.*, 1976) which show an increase in blood lactate after exercise. The current results demonstrated that an exhaustion training exercise caused an increase of lactate level higher than endurance training exercise. This agrees with the previous studies (Diprampero,, 1981, Fabio *et al.*, 1989) that an increase in lactate production increases with the intensity of exercise. It has been suggested that an increase in lactate level during exercise is due to an insufficient amount of oxygen to maintain aerobic energy metabolism. This leads to an accumulation of cytosolic NADH, thereby increasing the amount of pyruvate that is reduced to lactate and results in an increase in an anaerobic metabolism (Fabio *et al.*, 1989 and Turner *et al.*, 1995). In addition, the results of this study showed that the level of blood lactate obtained after training exercise was lower than that obtained after acute exercise. It is supported by previous study (MacRae *et al.*, 1992) that training attenuates blood lactate accumulation during exercise. One explanation for training induced a decrease in lactate production is partly due to an improving fatty acid oxidation. The improvement in fat oxidation as a result of training reduces the requirement for ATP formation via glycolysis and thus reduces the rate of lactate production (Rennie *et al.*, 1976, MacRae *et al.*, 1992). It is corresponding to our finding that training exercise causes a decrease in plasma triglycerides levels. Moreover, it has been shown that trained rats achieve higher rates of lactate removal at blood concentrations above 1 mM than in untrained rats (Donuvan and Pagliasssotti *et al.*, 1990).

The results from the effects of different exercise programs on the change in erythrocyte 2,3-BPG levels showed that exercise increases the level of erythrocyte 2,3-BPG. As summarized in Table 3-7, all programs of exercise caused an increase in erythrocyte 2,3-BPG levels by different magnitude. These findings agree with previous studies (Lijnen *et al.*, 1986, Hespel *et al.*, 1988, Shappell *et al.*, 1971) which showed an increase in erythrocyte 2,3-BPG after exercise. It is noteworthy to notice that 8 weeks of exhaustion training, exercise caused an increase in erythrocyte 2,3-BPG level higher than endurance training exercise. This result can be related to the change in intensity of training exercise. It has been reported that the decrease in intensity of training might have provided a small stimulates to increase erythrocyte 2,3-BPG levels (Resina *et al.*, 1994). Furthermore, Taunton and Klein (Taunton *et al.*, 1974, Klein, 1980) concluded that only exhaustion exercise provided change in erythrocyte necessary to stimulate 2,3-BPG

production. This type of exercise is known to cause greater changes than submaximal exercise in deoxygenated hemoglobin, circulating plasma catecholamine (Banister *et al.,* 1972) and plasma phosphate concentration (Klein et al., 1980), and each of them may stimulate glycolysis, thus promoting elevation 2,3-BPG. In addition, the current results demonstrated that those of training exercise which compose of endurance training, exhaustion training and endurance exhaustion exercise caused the rise in erythrocyte 2,3-BPG level higher than acute exercise. The explanation for the training exercise-related rise in erythrocyte 2,3-BPG could be associated with the circulating mean red blood cell population. Seaman and associates (1980) reported that the glycolytic activity was higher in younger erythrocytes than the older. Mairbaur and associates (1983) also revealed that physical training rejuvenates the circulating red cell population. In addition, Ricci and associates (1988) have been demonstrated that erythropoietin production was percistently increased, whereas hematocrit values were not changed during the regular exercise of basal training. Thus, it is possible that the training induced a rise in erythrocyte 2,3-BPG level has been attributed to a rejuvenation of the circulating mean red blood cell population due to repeated and preferential elimination of older red cells during exercise. The higher glycolytic activity of younger erythrocytes comparing to the older then results in an enhanced formation of erythrocyte 2,3-BPG via the Rapoport-Luebering shunt, a side path of the glycolysis. Thus, it can be inferred that from this result both intensity and training exercise causes an increase in erythrocyte 2,3-BPG level. After exercise, the rise in erythrocyte 2,3-BPG level is partly due to an adaptation of skeletal muscle from anaerobic to aerobic energy metabolism.

The third purpose of the present study was to investigate the effect of several factors, such as acidic pH, an elevated temperature, Ca^{2+}, and pO_2 on the response of erythrocyte 2,3-BPG levels. As mentioned above, exercise caused increases in erythrocyte 2,3-BPG levels and lactate production. At the site of active muscle tissues, there are adaptive mechanisms that may leads to change in erythrocyte 2,3-BPG metabolism and results in 2,3-BPG accumulation. The effects of each of several factors on the change in erythrocyte 2,3-BPG levels were studies *in vitro*. At first, the present study was to investigate the effects of acidic pH ranging from 6.4 to 7.2 on erythrocyte 2,3-BPG levels. It was observed in this study (Table 3-8 and Figure 3-3) that acidic pH affected the change in erythrocyte 2,3-BPG metabolism. The current results showed that high acidic pH ranging from 6.4-6.6 caused a reduction of 2,3-BPG levels. This outcome is inaccordance with the previous report that at subphysiologic pH values, a reducing

intraerythrocytic pH stimulates 2,3-BPG degradation by activating bisphosphoglycerate phosphatase (BPGP), the enzyme that degrades 2,3-BPG (Roseand and Liebowitz, 1970, Rapoport *et al.,* 1977, Momsen and Vestergaard-Bogind, 1978). It has been shown that BPGP has a pH optimum at about 6.2 (Bunn *et al.,* 1977). It is strongly activated by 2-phosphoglycolate and Pi, probably the most important factors *in vivo* and can be inhibited by 2-phosphoglycerate and 3-phosphoglycerate (Rose and Liebowitz, 1970). Also, the mutase activity (BPGM) can be inhibited by its product (2,3-BPG), inorganic phosphate and decreasing pH values (Momsem and Vestergaard-Bogind, 1978, Mulquiney and Kuchel, 1999); this inhibition may be due to a decrease supply of 1,3-BPG or pH-dependent inhibition of the BPGM (Rapoport *et al.,* 1977). It is known that the rate of red cell glycolysis varies directly with pH. At high acidic pH, the red cell glycolysis rate trends to decline and results in 2,3-BPG depletion (Bunn et al., 1977). Furthermore, the importance of pyruvate kinase (PK) in regulating 2,3-BPG levels has long been recognized. For example, an abnormally high activity of pyruvate kinase causes 2,3-BPG levels to fall (Tanaka and Paglia, 1971). It was possible, then, that a marked increase in the activity of pyruvate kinase caused the decline in 2,3-BPG rather than the co-operative H^+ inhibition of BPGM. Two possible allosteric effectors that were ignored in the model of pyruvate kinase were Pi and 2,3-BPG. Pi is an activator, while 2,3-BPG is an inhibitor of pyruvate kinase. Thus, the combination of the accumulation of Pi and the decline of 2,3-BPG may increase the activity of pyruvate kinase (Mulquiney and Kuchel, 1999). All of these reasons, it is possible to infer that the main cause of the net breakdown of 2,3-BPG at high acidic pH ranging from 6.4 to 6.6 may be an activation in BPGP reaction, an inhibition in the BPGM reaction, a decrease in red cell glycolysis rate and an increase in the activity of pyruvate kinase.

At mild acidic pH in the range of 6.8 to7.2, the current results showed that the erythrocyte 2,3-BPG levels were increased when compared to the physiologic pH (7.4). The pH 7.2 caused the highest increase of erythrocyte 2,3-BPG levels. Findings from the present study are at variance with the previous study in which subphysiologic pH values caused a reduction in erythrocyte 2,3-BPG (Momsen *et al.,* 1978). Moreover, Mulquiney and Kuchel (1999) studied an *in vivo* kinetic characterization of bisphosphoglycerate mutase/phosphatase (BPGM/BPGP). This work states that BPGM shows a significant pH-dependance which results largely from a strong co-operative inhibition of the BPGM activity by proton. Whereas, the data presented in their report are corresponded to our findings in which exercise causes an increase in erythrocyte

2,3-BPG levels. It has been known that lactic acid cause a decrease in venous blood pH during exercise. The previous finding (Douglas, 1974) reveals that an increase in 2,3-BPG levels could be due to: (1) an increase in overall glycolytic rate (increase flux); (2) an increased conversion of 1,3-BPG to 2,3-BPG; (3) a decreased degradation of 2,3-BPG to 3-PG ; or (4) combinations of certain of the above. From the first mechanism, the intracellular pH is known to be an important factor in red cell glycolysis control. As the pH increases, glycolysis increases. The major effect of pH seems to be on the PFK step, which has a pH optimum around pH 8.0. As pH increases, the level of fructose-1,6-bisphosphate (FBP) increase, results in 1,3-BPG accumulation. In addition, PFK is activated by inorganic phosphate (Douglas., 1974). It has also been reported that at very high phosphate concentration (up to 40 mM) the rate of glycolysis is increased by a factor more than 2. It is probable that phosphate reduces the inhibition of PFK by ATP (Mulquiney and Kuchel, 1999). It is supported by a number of research groups showing that phosphate loading increases erythrocyte 2,3-BPG levels. A decrease in serum Pi, particularly if prolonged inhibits glycolysis primary by limiting the GA-3-PD reactions (Douglas, 1974). Furthermore, the current results showed that pH 7.2 caused the optimal increase in erythrocyte 2,3-BPG levels. An increase in pH value has shown to activate BPGM reaction. On the basis of these findings, it can be proposed that pH 7.2 is the optimal. Furthermore, some unknown agents that can block the pathway below 3-PG and cause 2,3-BPG accumulation may be presented. Based on these reasons, it is possible to propose that mild acidic condition (pH 6.8-7.2) causes an increase of erythrocyte 2,3-BPG levels.

Since body temperature is increased during exercise, the effects of temperature on erythrocyte 2,3-BPG level were examined *in vitro*. In the present study, the data of the change in erythrocyte 2,3-BPG level at different temperature demonstrated that an elevated temperature affected an increase in the erythrocyte 2,3-BPG levels (Table 3-10 and Figure 3-6). The temperature of 43°C was optimal in causing the highest increases in erythrocyte 2,3-BPG levels. A number of investigators have showed that an increase in temperature moves the dissociation curve to the right. The physiological advantage of such a shift is that during exercise the accompanying rise in temperature permits unloading of additional oxygen to the tissues (Grimes, 1980). It has been reported that the maintenance of the stationary level of 2,3-BPG must result from a balance between the rates of two reactions constituting the pathway (Figure 1-9). The synthesis of 2,3-BPG from 1,3-BPG catalyzed by BPGM thus must equal the breakdown of

2,3-BPG to 3-PG and Pi catalyzed by BPGP (Momsen and Vestergaard-Bogind, 1978). In addition, we also investigated the effect of temperature ranging from 37°C to 45°C on deoxyhemoglobin in rat erythrocytes (as shown in Figure 3-8). This outcome reveals that the absorption spectrum of rat erythrocyte were similar among the incubations at different temperature (37°C to 45°C). The similar absorption spectrum included two bands of oxyhemoglobin, whereas the single band of deoxyhemoglobin was not appeared. Thus, it is possible to propose that elevated temperature causes the rise in erythrocyte 2,3-BPG level but not relates to deoxyhemoglobin. On the basis of these findings, the present study showed that temperature higher than physiological temperature directly affected the change in erythrocyte 2,3-BPG metabolism.

As the erythrocytes pass through the capillaries in active muscle, the cells are deformed and threatened. This phenomenon causes the erythrocyte membrane to alter its permeability to Ca^{2+} ion. Thus, the effect of Ca^{2+} ion on the erythrocyte 2,3-BPG level was examined in this study using calcium ionophore A23187. The result of the effects of A23187 on erythrocyte 2,3-BPG level was showed in Table 3-10 and Figure 3-6. The presence of A23187 in erythrocyte caused an increase in 2,3-BPG levels. It is implied that Ca^{2+} caused an increase in erythrocyte 2,3-BPG levels. This result can be related to red blood cell metabolism. A number of investigations have stated that in erythrocyte, the cell shape and deformability are regulated by calcium (Sarkadi, 1980). The physiological Ca^{2+} concentration in human erythrocyte is between 10 and 20 μM and most of Ca^{2+} is attached to the cell membrane. Cytoplasmic Ca^{2+} concentration is in the range of 10^{-6} to 10^{-7} M, about three order of magnitude smaller than that in the plasma. The red cells are virtually impermeable to Ca^{2+}. Ca^{2+} is bound to the membrane but also diffuses rather slowly into the cells, it is extruded rapidly by ATP-dependent mechanism. The calcium pump appears to be a vital component in red cell which needs to preserve a very low level of this cation for their continuing function. Failure to expel excess Ca^{2+} leads to an increased leakage of potassium (K^+) via Gardos effect and the red cells subsequently become shrunken, spherical and rigid (Douglas, 1974, Grimes, 1980,Clark et al., 1981). An excess of Ca^{2+} causes K^+ and water loss from red cells. The preservation of K^+ and water *status quo* in red cells requires the expenditure of energy and the likeliest source is ATP. Based on these reasons, it is possible to propose that an excess Ca^{2+} causes the increase of ATP utilization for Ca^{2+} extrusion and to maintain of sodium (Na^+) and potassium (K^+) homeostasis and results in the rise of ADP to ATP ratio. Also, it has been

known that the synthesis of 2,3-BPG in the red cell is directly dependent on the rate of glycolysis (Bunn *et al.,* 1977). In the red cell, phosphofructokinase (PFK) is a particularly sensitive step. This reaction is unusual in that it is activated by ADP and inhibited by ATP (Bunn *et al.,* 1977). From this reaction, 1,3-BPG trends to increase and results in the rises of 2,3-BPG synthesis. In addition, Assouline-Cohen and Beitner (1999) suggested that the Ca^{2+} induced activation of membrane skeleton-bound (PFK), and thereby glycolysis, the sole source of energy in erythrocytes, may be a defense mechanism to surmount the damage induced by high Ca^{2+} levels. At this point, it seems likely to propose that an excess Ca^{2+} in erythrocyte causes an increase in erythrocyte 2,3-BPG levels by directly dependent upon the rate of red cell glycolysis.

It is noteworthy to notice that a marked decrease in pO_2 in active muscle tissue capillaries is another factor that causes the rise in erythrocyte 2,3-BPG levels, as shown in Table 3-11 and Figure 3-6. It is possible that, at the site of active muscle, pO_2 in the capillaries is low due to more O_2-unloading and more deoxyhemoglobin is present in the erythrocytes. Deoxyhemoglobin binds 2,3-BPG with high affinity. Binding of 2,3-BPG to deoxyhemoglobin may prevent 2,3-BPG from degradation by BPGP. Thus, more deoxyhemoglobin existing in erythrocytic may cause an elevation of 2,3-BPG level. However, this study showed that incubation of rat erythrocytes at 37°C for 15 minutes under different values of reduced pO_2 did not cause an increase in the level of deoxyhemoglobin (Figure 3-7) as determined by light absorption spectra. The mechanism of reduced pO_2 on elevation of erythrocyte 2,3-BPG level is waiting for further investigation.