Appearance of a Cathodic Band in the Electrophoretogram of Blood Creatine Kinase Isoenzyme-MM Fraction during Hypoxia in Rats

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In electrophoretograms of creatine kinase (CK; EC 2.7.3.2) in patients' blood, a band, presumably of mitochondrial origin, is occasionally observed on the cathodic side of the CK-MM fraction. We studied the implications of this phenomenon in rats exposed to hypoxic conditions. In the hypoxic cardiac muscle, the proportions of CK-MB and CK-MM were not significantly different from controls, but that of the mitochondrial CK was lower. In the corresponding blood, the cathodic mitochondrial CK band appeared, but disappeared as the animals recovered from hypoxia. The CK-MB isoenzyme was increased in the blood of the control rats, as obtained by heart puncture, but no mitochondrial fraction was detected. We believe that changes in myocardial mitochondria during hypoxia are related to the appearance of the cathodic band. Cytoplasmic CK-MB, unlike mitochondrial CK, markedly increased in the rats' blood during the recovery stage rather than during the hypoxia.

Additional Keyphrases: mitochondrial and cytoplasmic creatine kinase · cardiac muscle · myocardial infarction

Since the appearance of an atypical band in the electrophoretic analysis of macro-creatine kinases (CK; EC 2.7.3.2) was reported (1–3), there have been several investigations as to its clinical implications. The diagnostic importance of CK isoenzymes has generally been discussed in relation to their association with myocardial infarction. The myocardium is maintained by aerobic metabolism, and the disturbance in its oxygen demand–supply balance constitutes the direct cause of ischemic heart disease. We studied the changes in various enzyme activities and in the proportion of CK isoenzymes under hypoxia in the cardiac muscle and blood of rats. An atypical band observed in the analysis of hypoxic blood for CK isoenzymes, and its implications, were investigated by observing the short-term changes in the isoenzymes associated with the rapid increase in the partial pressure of blood oxygen during recovery from hypoxia.

Materials and Methods

Male Wistar rats weighing 200 to 350 g were used. Eight rats were placed in an air-tight glass dome (capacity about 4000 mL) connected to a spirometer, into which nitrogen gas was gradually introduced, to achieve an oxygen content of about 10%. The animals gradually exhibited dyspnea, after tachypnea, and became apneic about 30 min later, after several deep mandibular respirations shortly before death. Most of the rats in this stage died after the sampling. A different group of eight rats were returned from hypoxia without having the sample taken during the apneic stage, and were used in the recovery-stage study. Blood was sampled by heart puncture into a heparinized syringe. Blood gas analysis for blood collected in the apneic stage indicated severe hypoxemia, with low oxygen tension and extremely acidosis (Table 1). Blood was also sampled from control animals that had been rendered unconscious by a blow on the head. In some hypoxic rats the blood samples were collected during apnea, and in other hypoxic rats during the recovery stage, about 5 min after they resumed respiration.

The hearts from the rats that became apneic and died were removed and homogenized soon afterwards. Myocardial homogenates were prepared by the method of Solaro et al. (4), and paired with those of control rats. Each heart was washed with ice-cooled isotonic saline, and weighed after the atrium, connective tissues, and fatty masses were removed. Slices of the ventricles were homogenized in a fivefold (by weight) amount of Tris HCl buffer (20 mmol/L, pH 6.8) containing, per liter, 0.25 mol of sucrose, 2 mmol of MgCl₂, 2 mmol of ethylene glycol bis(β-aminoethyl ether) tetraacetic acid (EGTA), and 2 mmol of Na₃P₂O₇, in an Ultrax homogenizer. The homogenate was diluted 10-fold by weight with the same Tris buffer mixture, in this case also containing 16 mmol of Triton X-100 detergent per liter, homogenized again in a Potter-type homogenizer, and centrifuged at 17 000 × g for 15 min. The supernate was examined. All of the above procedures were performed at 0–4°C.

Activities of CK, lactate dehydrogenase (LDH; EC 1.1.1.27), aspartate aminotransferase (AST; EC 2.6.1.1), and alanine aminotransferase (ALT; EC 2.6.1.2) in the cardiac muscle and plasma were measured by ultraviolet-absorbance methods by centrifugal analysis (Centrifichem; Union Carbide) (5) and continuous-flow analysis (AutoAnalyzer; Technicon) (6). The protein content of the homogenates was determined according to the method of Lowry et al. (7), and the activity of each enzyme per gram of protein was expressed in international units (U). CK isoenzymes were separated electrophoretically on cellulose acetate by the procedure marketed by Helena Laboratories (6).

| Table 1. Blood Gas Analyses of Seven Rats in an Apneic State at the Peak of Hypoxia |
|---------------------------------|-----------------|-----------------|-----------------|
|                                 | Mean (and SD)   |                 |                 |
|                                 | pH              | 6.699 (0.168)   |                 |
|                                 | Pco₂ (mmHg)     | 95.3 (16.4)     |                 |
|                                 | Hco₃⁻ (mmol/L)  | 8.5 (10.7)      |                 |
|                                 | Hb (g/L)        | 10.6 (2.1)      |                 |
|                                 | BE (mmol/L)     | –24.7 (5.7)     |                 |
|                                 | O₂ CT (ml/dL)   | 1.4 (2.3)       |                 |
|                                 | O₂ SAT (%)      | 6.1 (9.9)       |                 |
|                                 | Total CO₂ (%)   | 12.4 (2.0)      |                 |
|                                 | Hb (g/dL)       | 150 (24)        |                 |

BE, base excess; CT, content; SAT, saturation.
Results

Changes in Enzyme Activities and CK Isoenzymes in the Hypoxic Cardiac Muscle

Enzyme activities (Table 2). High activities of CK, LDH and AST were observed in myocardial homogenates. The changes in these activities under hypoxia were not significant, probably because the duration of the hypoxic state was short.

CK isoenzymes (Table 2). CK isoenzymes in rat myocardial homogenate consisted of four fractions, namely, CK-BB, CK-MB, CK-MM, and mitochondrial CK (mit. CK). Under hypoxia, CK-MB increased and mit. CK decreased, as compared with the values in the controls. The former was not significant, but the latter was (p < 0.05).

Changes in Plasma CK Isoenzymes after Hypoxia

We compared CK isoenzyme patterns in plasma with those in cardiac muscle at the peak of hypoxia. Figure 1 shows a representative densitometric pattern of each. In plasma, the proportion of the CK-BB isoenzyme was high, and the CK-MB fraction was clearly detectable, but particularly interesting was the appearance, on the cathodic side of the CK-MM band, of a minor fraction that corresponded in migration with the mit. CK fraction in the myocardial homogenate. In short, the CK electrophoretogram of hypoxic plasma resembled that for cardiac muscle except for the CK-BB.

Changes in Plasma CK Isoenzymes during Recovery from Hypoxia

Serial changes in a rat. Blood from the one surviving animal was sampled 4 and 8 min after its removal from the hypoxic condition. The plasma was examined for serial changes during the recovery phase. During peak hypoxia CK-BB and CK-MM activities were high, and CK-MB activity was detectable but low (Figure 2). Although we did not see the mit. CK fraction in this rat, the CK-MM fraction in the electrophoretogram was broad and atypical. We noted the following serial changes: the CK-BB activity decreased, the CK-MB activity became more distinct and increased, and the CK-MM profile returned to its original sharp contour.

Differences between the hypoxic state and the recovery stage. Plasma activities of CK isoenzymes and total CK during peak hypoxia were compared with those 3 to 5 min after termination of the hypoxic state. Table 3 shows the results for each group of eight animals. Like the serial changes discussed above, a decrease in CK-BB and an increase in CK-MB were notable during recovery. Although decreases in mit. CK were not significant, mit. CK could be seen in the patterns for six of the eight rats during peak hypoxia, but in only two of the eight during recovery. The atypical CK-MM profile during peak hypoxia was not observed during recovery.

Changes in plasma enzyme activities during recovery. The activities of various enzymes in plasma rapidly and significantly increased after the hypoxic state was terminated (Table 3). Changes in enzyme activities released into blood, indicating the impairment of organs, were more notable during the recovery than during peak hypoxia.

Comparison of plasma CK isoenzyme activities in hypoxic and control rats. Activities of CK isoenzymes in plasma during peak hypoxia were compared with those in the control rats (Figure 3). CK-BB and CK-MM activities were not significantly different between the two groups, but the proportion of CK-MB was significantly (p < 0.05) higher in the controls than in the hypoxic rats. The mit. CK fraction,
seen on the cathodic side of the CK-MM fraction in the hypoxic rats, was completely absent from the pattern for the controls.

**Blood gas analysis during hypoxia and recovery.** Although severe acidosis and hypoxemia were observed at the peak of hypoxia, the pH was unchanged between the hypoxic and recovery stages, indicating intense acidosis (Figure 4). Oxygen partial pressure \( (P_02) \), oxygen content, and oxygen saturation increased rapidly and significantly in the recovery stage (Figure 4).

**Discussion**

The anoxemia test [exposure to 10% oxygen (9)] is currently used in the diagnosis and evaluation of ischemic heart diseases. One of us previously studied myocardial metabolism in clinical cases by the hypoxia test, using coronary vein catheterization (10). In the present study we noted severe acidosis and hypoxemia in the rats during hypoxia, but no changes in myocardial enzyme activities, probably because of the short duration of the hypoxic state. The CK-MB and CK-MM fractions showed no significant changes, but mit. CK, migrating to the cathodic side of the MM fraction, tended to decrease. Mit. CK is considered to be bound to the outer surface of the inner mitochondrial membrane (11, 12). Electron microscopy revealed immediate changes in these myocardial mitochondria under hypoxia (13, 14), and their morphological as well as functional return to normal after the termination of hypoxia has also been reported (15, 16).

Electrophoresis of plasma obtained at the peak of hypoxia revealed a fraction with the same migration as mit. CK, which decreased in the cardiac muscle. Bohner et al. (17) categorized macro-CK isoenzymes in plasma into two types. Type 1, immunoglobulin-bound macro CK-BB with an electrophoretic migration between CK-BB and CK-MM, is considered to be present in blood over a long period of time and not correlated to any specific disease. Type 2, which migrates on the cathodic side of CK-MM in electrophoresis, has been reported in severely ill patients with advanced malignant tumors (17–19), shock (20), or myocardial infarction (21), and is considered to be of mitochondrial origin on the basis of clinical as well as biochemical observations (17, 21).

The proportions of CK isoenzymes in plasma changed rapidly after the hypoxic condition was ended. Mit. CK was observed in six of the eight rats during peak hypoxia, but in only two during the recovery stage. However, in the animals in which no mit. CK was identified, the CK-MM band was broad and atypical during peak hypoxia, returning to the normal pattern with the recovery. Wevers et al. (22) electrophoretically examined CK isoenzymes in human myocardial mitochondria and found two to be present. One, with a zymographic position identical to that of the CK-MM subfraction, is not electrophoretically differentiated, but the other reportedly appears on the cathodic side of CK-MM. If this can also be demonstrated in rats, CK isoenzymes from myocardial mitochondria may be involved in the appearance of the atypical MM-band in hypoxia.

CK-BB is the dominant CK isoenzyme in rat plasma (23), and we found no significant difference in this fraction between the hypoxic and control animals. The proportion of CK-MB, on the other hand, was significantly higher in the control plasma than in the hypoxic samples. In humans, the activities of total CK and CK-MM in blood are increased by muscular exercise (24) or intramuscular injections (25). At blood sampling by cardiac puncture, the hypoxic rats were apneic and their pulse rates were less than 100/min; the pulse rate in the controls remained about 400/min. Therefore, a greater amount of the CK-MB fraction of the myocardial cytoplasmic isoenzymes was apparently released in the controls. At any rate, we found it particularly interesting that mit. CK was absent in the control plasma despite the increase in CK-MB, but was present in the hypoxic plasma.

The proportion of CK-BB activity rapidly decreased after the hypoxic conditions were ended. Rather than resulting from the recovery from the brain injury, however, this decrease may be more related to the increases in CK-MB and CK-MM exceeding the increase in CK-BB released from the brain. The increases in CK-MB and CK-MM suggest that cardiac muscle is impaired by hypoxia. The short-term changes in the activities of various enzymes and CK isoenzymes in plasma during the recovery stage rather than the hypoxic state may be explained by the rapid changes in the \( P_02 \). A decrease in arterial \( P_02 \) induces hypoxia in tissues, and affects cytosolic and mitochondrial enzymes according to their intracellular distribution or form. Changes in enzyme activities in blood are further affected by alterations in blood flow to the organs, which may be responsible for the appearance of mit. CK during intense hypoxia and for the increase in CK-MB from cytosol during the recovery stage. Skeletal muscles may also be involved. However, because the circulatory system responds to hypoxia by dilating the vessels and increasing the blood flow through vital organs such as the brain and heart, and constricting those through skeletal muscles and digestive organs (26), studies of
changes in enzyme activities during hypoxia should emphasize the cardiac muscle.

References