Effect of pH and halothane on muscle and liver mitochondria

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Malignant hyperpyrexia is a genetic disorder characterized by a marked rise in temperature in response to certain anesthetics. It is known in both man (7) and pigs (2) and is often accompanied by rigidity of skeletal muscle.

Inhibition of mitochondrial respiration and uncoupling of oxidative phosphorylation by anesthetics such as halothane have been observed at the concentrations occurring in tissues during clinical anesthesia (6, 9, 13). The site of the respiratory inhibition was located in the region of NADH-coenzyme Q reductase and uncoupling of oxidative phosphorylation was found to be slight in comparison with 2,4-dinitrophenol (13). Miller and Hunter (14) showed that other processes such as calcium uptake and phosphate-induced swelling of mitochondria were inhibited in the presence of halothane rather than uncoupled. However, the similarity of the malignant hyperpyrexic response to anesthetics with the high temperature and skeletal muscle rigidity present in animals poisoned with 2,4-dinitrophenol (13) has led to the suggestion that fundamental relationships might exist and thus be worthy of further investigation (22).

The function of mitochondria in skeletal muscle is not fully understood but an obvious function is that they act as a source of ATP for both muscle contraction and the uptake of calcium by the sarcotubular system. It may be of significance that in both red and white skeletal muscle they are located in the regions where calcium is accumulated. As both glycogenolysis and muscle contraction require the release of calcium from the sarcotubular system and are correspondingly inhibited by its re-uptake by this system, an inhibition of mitochondrial ATP production may have special significance. Further, as muscle contraction is sustained, continued glycolysis will lead to a fall in the pH of the myoplasm and this in turn may affect mitochondrial function.

The present investigation examines the separate and combined effects of halothane and increasing hydrogen ion concentration on rates of respiration and oxidative phosphorylation by mitochondria from the longissimus dorsi muscle of pigs. Comparative studies on mitochondria from the liver of pigs and skeletal muscle from rats were also made.

Materials and Methods

EDTA, 1,2-bis(dicarboxymethylaminoethoxy)ethane (EGTA), and ADP were obtained from Sigma Chemical Company and halothane from Imperial Chemical Industries, Macclesfield, England. Bovine serum albumin (BSA) (Cohn fraction V) was obtained from the Commonwealth Serum Laboratories, Melbourne, Australia. Other chemicals were A. R. grade.

Pig muscle and liver tissues were obtained from a local slaughterhouse from animals which had been killed up to 20 min previously. Longissimus dorsi muscle was used because it tends to develop a pale, soft, exudative condition, a disease related to malignant hyperpyrexia (3, 16). Strips of longissimus dorsi muscle were stored in ice-cold 0.15 M KCl and pieces of liver were kept in ice-cold 0.3 M sucrose until homogenization after return to the laboratory. Rats (an inbred Buffalo strain) were killed by stunning, bled, and the muscles of the hindlegs were removed and chilled in ice-cold 0.15 M KCl.

Preparation of Mitochondria

Muscle. The isolation medium was based on that used by Lindenmayer et al. (12) and consisted of 0.18 M KCl, 10 mM EDTA, and 0.5% BSA, pH 7.4. Muscle was dissected to remove connective tissue and fat, blotted, weighed, and then minced finely with scissors. The minced muscle (10 g) was then homogenized with 3 volumes of ice-cold isolation medium in a Sorvall Omnimixer at 8,000 rpm for 7 sec. After homogenization 2 volumes of medium were added to
the homogenate which was then centrifuged at 600 × g for 10 min at 2–5 C. Connective tissue which packed at the surface was removed and the supernatant suspension was filtered through fine muslin and collected. This suspension was centrifuged at 14,000 × g for 10 min. The supernatant liquid was discarded, the pellet allowed to drain, and the surface washed with 0.25 M sucrose. The pellet was resuspended in a total of 5 ml of 0.25 M sucrose, 0.5 % BSA, and again centrifuged at 14,000 × g for 10 min. The pellet was finally suspended in 0.25 M sucrose, 0.5 % BSA, to give a concentration of 5–15 mg mitochondrial protein per milliliter.

Liver. The liver was dissected to remove obvious connective tissue and the mitochondria prepared using the method described previously (11). The final mitochondrial pellet was suspended in 0.3 M sucrose, 0.5 % BSA, with a concentration of 10–25 mg mitochondrial protein per milliliter.

Amperometric Estimation of Oxidative Phosphorylation

Oxidative phosphorylation was estimated amperometrically at 30 C using an oscillating platinum electrode and 0.1 ml of mitochondrial suspension. Mitochondria from muscle were studied in 1.5 ml of a medium which contained 23.3 mM potassium phosphate, 67 mM KCl, and 0.5 % BSA. To produce conditions of maximum respiratory control, mitochondria from the longissimus dorsi muscle were incubated in the presence of 8 mM MgCl₂, and rat muscle mitochondria were incubated with 2 mM MgCl₂ and 8 mM EDTA. Substrates used were either 10 mM succinate, or 6.7 mM pyruvate plus 0.7 mM malate. The pH conditions were as indicated in the text. L-Lactic acid (150 mM) was used to produce the desired changes of pH.

Addition of Halothane to Mitochondria

Glass-distilled water was saturated with halothane at 30 C and was calculated to give a concentration of 21 mM (20).

<table>
<thead>
<tr>
<th>Source of Mitochondria</th>
<th>Substrates</th>
<th>State 3 Respiration, μg-atom O/min per mg protein</th>
<th>ADP/O Ratios</th>
<th>Respiratory Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig longissimus dorsi muscle</td>
<td>Pyruvate + Malate</td>
<td>0.41 ± 0.04 (16)</td>
<td>9.73 ± 0.04</td>
<td>10 ± 0.6</td>
</tr>
<tr>
<td>Rat hindleg</td>
<td>Pyruvate + Malate</td>
<td>0.39 ± 0.02 (6)</td>
<td>2.85 ± 0.04</td>
<td>7.0 ± 0.9</td>
</tr>
<tr>
<td>Pig liver</td>
<td>Pyruvate + Malate</td>
<td>0.11 ± 0.01 (6)</td>
<td>2.42 ± 0.08</td>
<td>3.1 ± 0.2</td>
</tr>
</tbody>
</table>

Results for mitochondria from pig and rat muscles were obtained at pH 6.9 and from pig liver at pH 7.2. Other conditions were as described in MATERIALS AND METHODS. Results indicate mean values ± se, and figures in parentheses the number of determinations. Respiratory control expressed as the ratio of State 3 and State 4 respiration.

Halothane was introduced into the mitochondrial incubations from a closed 2-μl Chance Repette syringe immediately before the beginning of an experiment. Each experiment was completed within several minutes.

**TABLE 1. Rates of oxygen uptake, respiratory control, and ADP/O ratios of mitochondria at normal pH values**

**FIG. 1. Effect of pH on mitochondrial respiration and ADP/O ratios. Substrate was pyruvate plus malate, other conditions were as described in MATERIALS AND METHODS. A: State 3 respiration is shown as the percentage of State 3 respiration at pH 6.9 and pH 7.2 for muscle and liver mitochondria, respectively. State 2 (○) and State 4 (■) respiration of pig longissimus dorsi muscle mitochondria are shown as percentage of State 3 respiration at pH 6.9. B: ADP/O ratios are shown as percentage of ADP/O ratios at pH 6.9 and at pH 7.2 for muscle and liver respectively. Values are means and vertical lines represent ± se. Number of experiments is in parentheses.**
Mitochondrial Protein Estimations

Protein estimations were made using the biuret method (8) after mitochondrial lipid was extracted with the use of a suitable solvent (1).

RESULTS

Effects of pH on Mitochondrial Respiration and ADP/O Ratios

Table 1 shows that the isolation procedure gave mitochondria with acceptable rates of respiration, ADP/O ratios, and respiratory control.

Figure 1A shows that the State 3 respiration (5) of mitochondria from both rat and pig muscles was only slightly inhibited when the pH was lowered from 6.9 to about 6.5. Similarly liver mitochondria were inhibited little when the pH was lowered from 7.2 to 6.6. Below pH 6.5 respiration was inhibited to an increasing extent with further drop of pH. State 2 and State 4 respiration of mitochondria from all the tissues were only slightly raised by lowering the pH of the medium.

The ADP/O ratios were not markedly affected until the pH fell to 6.0. Below this value it fell rapidly (Fig. 1B). Thus the rate of ATP formation and the efficiency of its

FIG. 2. Effect of halothane on respiration of mitochondria prepared from pig longissimus dorsi muscle. pH was 6.9 and other conditions were as described in MATERIALS AND METHODS. Substrate was A: succinate; B: pyruvate plus malate. Values are means and vertical lines represent ± se. Number of experiments is in parentheses.

FIG. 3. Effect of halothane on mitochondrial ADP/O ratios. Mitochondria were prepared from pig longissimus dorsi muscle. Conditions were as described in Fig. 2. Values are means and vertical lines represent ± se. Number of experiments is in parentheses.

FIG. 4. Combined effects of halothane and pH on mitochondrial respiration conditions were as described in Fig. 1. Pig liver mitochondria and mitochondria from longissimus dorsi muscle were treated with 1.2 mM halothane, and rat muscle mitochondria with 1.4 mM halothane. ○, State 3 respiration of halothane-treated mitochondria as percentage of control respiration at each pH value. ●, State 3 respiration minus State 2 respiration of halothane-treated mitochondria as a percentage of control respiration at each pH value. Values are means and vertical lines represent ± se. Number of experiments is in parentheses.
FIG. 5. Combined effects of halothane and pH and ADP/O ratios. Conditions were as described in Fig. 4. ADP/O ratios of halothane-treated mitochondria are shown as percentage of control ADP/O ratios at each pH value. Values are means and vertical lines represent ± SE. Number of experiments is in parentheses.

Ejfects of Halothane on Mitochondrial Respiration and ADP/O Ratios

Increasing concentrations of halothane up to 3 mM had little effect on the oxidation of succinate by mitochondria from pig muscle but above this concentration inhibition became more pronounced (Fig. 2A). This inhibition of State 3 respiration was accompanied by an increase in the State 2 and State 4 respiration rates. Thus the mitochondria were both inhibited and uncoupled by halothane. Figure 2B shows that with pyruvate + malate as the substrate the inhibition of State 3 respiration was very responsive to the concentration of halothane.

Figure 3 shows that with both succinate and pyruvate + malate as a substrate the ADP/O ratio was lowered by increasing the concentration of halothane. Above 3 mM the inhibition became marked and at about 4–5 mM it was complete.

Combined Effect of Halothane and pH on Mitochondrial Respiration and ADP/O Ratios

Figure 4, A–C, shows the percentage response of the State 3 respiration by mitochondria from the three tissues to a fall in the pH of the medium in the presence of a low concentration of halothane. Since respiration in the presence of substrate and absence of ADP was stimulated by halothane, for comparative purposes this rate has been subtracted from the apparent State 3 respiration rate to give a better idea of the effect of this anesthetic on the respiratory activity during the State 3 phase of respiration. Figure 4, A–C, shows that halothane at 1.2–1.4 mM inhibits respiration over the whole pH range tested and that in pig longissimus dorsi the effect of halothane on the corrected rate of respiration was much greater below about pH 6.0.

Mitochondria from the three sources behaved similarly with respect to the effect of halothane on ADP/O ratios.

FIG. 6. Effect of halothane on respiration and ADP/O ratios at various pH values. Mitochondria from pig longissimus dorsi muscle were used under conditions described in Fig. 1. A: State 3 respiration as percentage of State 3 respiration at pH 6.9, without halothane. B: ADP/O ratios as percentage of ADP/O ratios at pH 6.9, without halothane. Values are means and vertical lines represent ± SE. Number of experiments is in parentheses.
Halothane (1.2–1.4 mm) lowered the ADP/O ratio by 15–30% but below pH 6.0 the combined effects of halothane and hydrogen ion concentration caused a dramatic fall in the ratio (Fig. 5). Thus vigorously contracting muscle (leading to a low intracellular pH value) exposed to halothane would have impaired mitochondrial function.

Figure 6A shows the effect of varying the concentration of halothane at various pH values on the State 3 respiration of mitochondria from pig longissimus dorsi muscle. The effect of halothane is shown to be additional to the effect of lowering the pH value of the medium. Figure 6B shows a similar overall effect on the ADP/O ratios. It is clear, however, that at lower pH values a lower concentration of halothane will produce the same fall in the ADP/O ratio.

**DISCUSSION**

The intracellular pH of skeletal muscle ranges from pH 6.9 at rest to approximately pH 6.4 after exercise to exhaustion (10). Our results indicate that respiration of muscle mitochondria was only slightly inhibited down to pH 6.4, but that below this level pH inhibition of State 3 respiration progressively increased. Uncoupling was slight until the pH value reached pH 6.0, but below this value ADP/O ratios were markedly lowered.

Similar effects of pH on respiration were shown by rat heart mitochondria (4, 19). ADP/O ratios of heart mitochondria were shown to fall off (4). Liver mitochondria were inhibited and uncoupled at essentially the same pH values as skeletal muscle mitochondria. Unless other factors were important, the effect of normal fluctuations of blood pH on liver mitochondria would be slight since even after exhaustive exercise the blood pH is above 6.9 (10). Tobin et al. (23) obtained a similar inhibition and uncoupling of liver mitochondria at essentially the same pH values. It is clear, however, that below this pH the ADP/O ratios are markedly lowered.

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