EFFECTS OF EXOGENOUS CYTOCHROME C ON RESPIRATORY CAPACITY OF HEART AND SKELETAL MUSCLE

Peter J. Van Handel, William R. Sandel, and Paul A. Mole
Department of Physiology
LSU Medical Center
New Orleans, LA 70119

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Cytochrome c is easily extracted from mitochondria by salt buffers. Yet studies of mitochondrial respiratory capacity, which routinely use KCl solutions for preparation of muscle, have failed to include the enzyme in the assay medium. The present study demonstrated that exogenous cytochrome c stimulated the oxidation of pyruvate + malate by 120, 66, 65, and 35% for the mitochondrial fraction of heart, fast-twitch red, mixed and fast-twitch white rodent muscle. Lesser increases were obtained for whole homogenate preparations. These data suggest that previous studies may have underestimated the true respiratory capacity of these tissues.

Cytochrome c is easily extracted from muscle treated with salt solutions (3, 5, 13, 14, 27). Perhaps on this basis, a KCl buffer has been used to remove the enzyme for subsequent analysis (22). Paradoxically, similar KCl solutions are routinely used in the preparation of muscle for determination of respiratory capacity (1, 2, 3, 4, 8, 9, 10, 11, 12, 18, 21). With one exception, these studies have not included cytochrome c in the assay medium used to measure oxygen consumption. Since the cytochromes of the respiratory assembly appear to occur in simple molar ratios (14), the loss of cytochrome c may alter activity of the whole unit, resulting in an underestimation of the true respiratory capacity of the muscle. Moreover, exogenous cytochrome c may have variable effects on oxidation of heart and muscle fiber types since it has been shown that these tissues have different cytochrome c contents which are proportional to their differences in oxidative capacity (1, 10, 26). Based on these considerations, a study was undertaken to determine the effects of cytochrome c on the respiratory capacity of heart, mixed gastrocnemius, and the red and white portions of the quadriceps.

METHODS: Twelve adult female rats of a Wistar strain (specific pathogen-free CFN) with a mean body weight of 297.3 ± S.E. 7.4 g were sacrificed by decapitation over a period of 3 weeks. The heart (H), gastrocnemius (G), and red (RQ) and white (WQ) portions of the quadriceps were rapidly excised, rinsed in

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cold saline, and placed on ice. Each skeletal muscle was cleaned of connective tissue and fat, minced and thoroughly mixed. The valves and atria were removed from the heart and the ventricles minced on ice. Each muscle mince was weighed and a 1:10 (w/v) homogenate prepared with cold 175 mM KCl + 2 mM EDTA, pH 7.30. Homogenization was accomplished with two complete passes of the glass pestle. The homogenates had a pH of 6.30 to 6.50 which was adjusted to 7.00 with 100 mM KOH. An aliquot of each was diluted with the KCl solution so that muscle concentration was 0.025 g/ml (1:40 w/v) for heart and 0.05 g/ml (1:20 w/v) for the G, RQ and WQ. These whole homogenates were immediately used for the determination of respiratory capacity. Mitochondria were prepared from the remainder of the KCl homogenates as follows. Each sample was centrifuged at 700 X G for 15 mins at 2°C and the supernatant fluid decanted and stored on ice. The pellet was resuspended in the KCl solution, the pH adjusted to 7.00 if necessary, and again centrifuged as indicated above. The supernatant fractions of the two 700 X G spins were combined and recentrifuged at 8000 X G for 15 mins at 2°C. The resulting mitochondrial pellet was suspended in 250 mM sucrose + 2 mM EDTA, pH 7.20. The pH was checked and adjusted to 7.20.

Oxidative capacity was measured in at least duplicate with a Gilson Polarograph at 30°C. Assay conditions for state 3 respiration (Qo_2 in the presence of nonlimiting amounts of ADP, Pi and pyruvate + malate) were established such that doubling the amount of homogenate or mitochondria resulted in a proportional increase in O_2 consumption. The final concentrations of the reagents in the 2 ml reaction mixture were: Na pyruvate, 10 mM; Na malate, 1 mM; ADP, 2 mM; EDTA, 1.4 mM; KPO_4 buffer, 20 mM; $MgCl_2$, 5 mM; sucrose, 125 mM for mitochondria and 100 mM for whole homogenates; and KCl, 20 mM for mitochondria and 37.5 mM for whole homogenates. Muscle equal to 0.005 - 0.02 g (whole homogenate) or mitochondria equivalent to 0.04 - 0.16 g of muscle was added to the reaction vessel. The respiratory control index (RCI) for mitochondrial fractions was determined as the ratio of Qo_2 in state 3 to Qo_2 in state 4 (5,6). Cytochrome c (0.1 ml, 1.55 mM Sigma Type IIA) was subsequently added to determine its effect on state 3 Qo_2. Respiration with cytochrome c was not evident in the absence of tissue. Mitochondrial protein was determined by the method of Mokrasch (16, 17). A paired t-test was used to determine whether cytochrome c altered respiratory capacity. Statistical significance for mean differences was established at the 0.05 level. Least square regression analysis was used to construct the linear curves showing the relationship between the increase in respiration due to cytochrome c and Qo_2.

RESULTS: Figure 1 illustrates state 3 Qo_2 of whole homogenates (panel A) and mitochondrial fractions (panel B)of rodent heart (H), gastocnemius (G), and the red (RQ) and white (WQ) portions of the quadriceps muscles in the presence or absence of exogenous cytochrome c. The magnitude of Qo_2, with or without cytochrome c, was found to follow the order: H > RQ = G > WG for both whole homogenate and mitochondrial fractions. These differences in Qo_2 reflect the amount of mitochondria contained in each tissue, as indicated by mitochondrial protein concentration (Table 1). The high Qo_2 and RCI values, as well as the mitochondrial yields, indicates excellent structural and functional integrity of the preparations (9).

Exogenous cytochrome c affected the Qo_2 of the muscle preparations

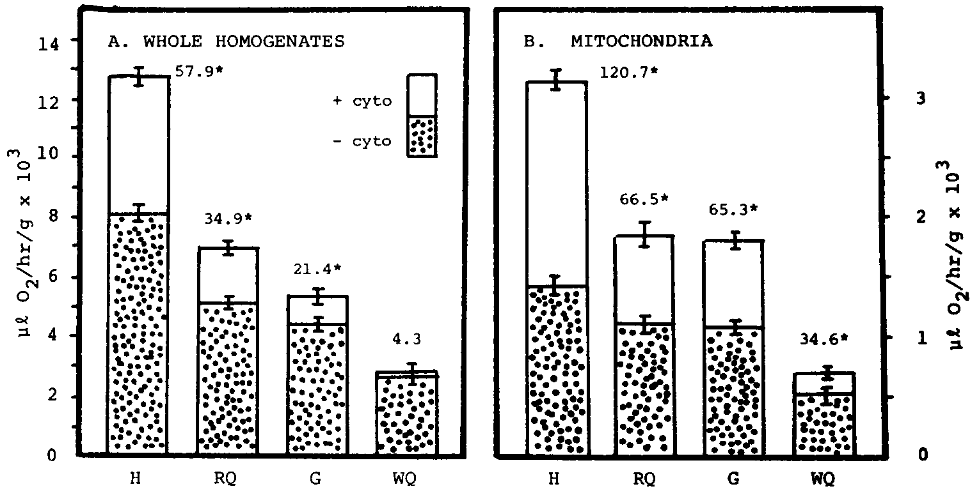

FIGURE 1. The Effects of Exogenous Cytochrome c (C) on the Respiratory Capacity of Heart (H), Red Quadriceps (RQ), White Quadriceps (WQ) and Mixed Gastrocnemius (G) Muscles. Values represented by the bars are means ± SEM for whole homogenates (Panel A) and mitochondrial fractions (Panel B). The % increase in respiration due to cytochrome c is noted above each bar. Asterick (*) = $Qo_2 + C$ versus $Qo_2 - C$, $p < 0.001$, $n = 12$.

TABLE 1. The Respiratory Control Index (RCI) and Protein Concentration of Mitochondrial Fractions from Rat Heart, Red Quadriceps, White Quadriceps and Gastrocnemius Muscles[a]

Variable	Heart	RQ	WQ	Gastroc
RCI	13.2 ± 4.0	20.9 ± 8.3	15.2 ± 6.0	19.8 ± 6.3
Protein mg/g	18.2 ± 1.9	5.9 ± 0.5	3.7 ± 0.4	5.0 ± 0.3

[a]Values are the means ± SEM of 12 female rats.

differently (Fig. 1 and 2), i.e. mitochondrial fractions >> whole homogenates, as evidenced by the percent increases shown above the bars in Figure 1. The relative increase in Qo_2 was also different for the four tissues; heart preparations showing the greatest effect, WQ the least. This differential effect of cytochrome c appears to be related to the inherent respiratory capacity of

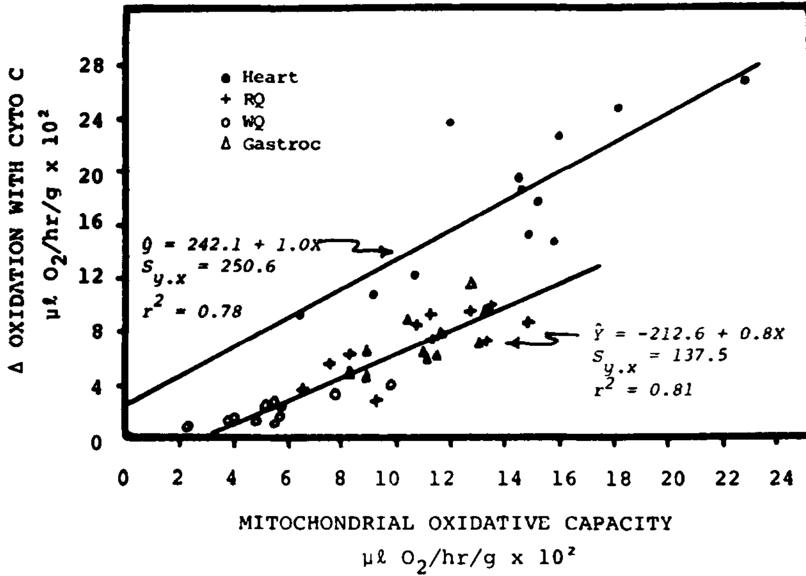

FIGURE 2. Cytochrome c Induced Pyruvate Oxidation as Related to Mitochondrial Oxidative Capacity of Heart and Skeletal Muscles.

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the tissue (Fig. 2). The stimulation of Qo_2 for the mitochondrial fraction of the heart however, was exaggerated. Its regression curve was found to be different from that of skeletal muscle ($p < 0.001$).

DISCUSSION: Early studies indicated that exogenous cytochrome c may stimulate the respiratory activity of mixed skeletal muscle (5, 9) and heart (19) mitochondria. For unknown reasons, more recent studies of heart and skeletal muscle fiber types (2, 4, 8, 10, 11, 12, 18, 21) have not included the enzyme in the assay medium. The present data show however, that exogenous cytochrome c is required to determine maximum pyruvate oxidation for both whole homogenate and mitochondrial fractions of heart and skeletal muscle fiber types. A similar requirement has been previously reported for long-chain fatty acid oxidation of mixed gastrocnemius and quadriceps muscles (15). Moreover, the data are comparable to those obtained for similar preparation and assay conditions (1). In contrast, studies using similar preparation techniques, but excluding cytochrome c, have obtained pyruvate oxidations approximately 2 to 4 times

lower than the present values (2, 4, 8, 10, 11, 12, 18, 21). These data clearly indicate that respiratory capacity may be significantly underestimated if cytochrome c is excluded from the assay medium.

The extent of this underestimation appears to be related to the nature of the preparation (mitochondria >> whole homogenates; Figure 1) and the muscle fiber type (heart >> RQ >> G >> WQ; Figures 1 and 2). The basis for these differences cannot be elucidated from the present data. It is likely however, that (1) the KCl solution used in this study extracted at least some of the endogenous cytochrome (3, 9, 13, 27) (as opposed to inactivating it), and (2) that this extraction is, in part, related to the initial cytochrome c content of the muscle. If KCl-EDTA mediated inactivation occurred, the addition of exogenous cytochrome to the same KCl whole homogenate preparation (Figure 1A) presumably would not have stimulated oxidation. In addition, the normal structure of KCl extracted cytochrome c, as determined by spectral analysis, is unchanged (23).

Veeger (25) has indicated that muscle mince must be washed for an extended period of time if a cytochrome c deficient mitochondrial sample is desired. This may explain the large differences in percent increase in oxidation found between the whole homogenate and mitochondrial preparations. The muscle (see METHODS) remains in contact with the KCl solution approximately 4 times longer during preparation of the mitochondria as compared to the whole homogenate fraction, perhaps extracting more enzyme. Figure 2 illustrates that the stimulating effect of cytochrome c is proportional to the respiratory capacity of the muscle. This suggests that the extent to which the enzyme is extracted may be dependent upon its initial concentration, since respiratory capacity of the muscle is generally proportional to its cytochrome c concentration (1, 10, 26).

It should be noted that alterations in homogenate pH may also have been a contributing factor in the present study. Even though the muscle was kept on ice, the time required to excise, clean, mince and weigh each tissue was such

that large amounts of lactate would be produced. Since the KCl-EDTA solution (pH 7.3) used does not have buffering capacity, the pH of the homogenates was actually measured at 6.30 - 6.50 and therefore adjusted to and maintained at 7.0 (3, 20, 24, 27). In preliminary studies we found that extremes of pH (6.30 < pH > 8.00) resulted in an irreversible decrease in respiratory activity of both whole homogenate and mitochondrial fractions. Apparently such pH alterations damage mitochondria (20) and may impair energy-linked functions by extracting cytochrome c (3). That we adjusted the homogenate pH to neutral levels, may also account for the fact that the present oxidations, RCI, and mitochondrial yields are higher than any previously reported. We are unable to determine if the oxidation studies cited above have considered this pH problem. The drop in pH as the homogenates were prepared may also have contributed to the magnitude cytochrome c stimulated respiration of heart vs skeletal muscle. For example, WQ muscle (low oxidative, high glycolytic) may be able to tolerate pH changes to a much greater degree than does heart (high oxidative). Such tolerance may include resistance to alterations in protein charge and migration and membrane permeability. Zak (27) has also suggested that the response of cardiac muscle to fractionation techniques may be different than that of skeletal muscle while others have indicated that inhibition of Mg^{2+} activated myofibrilar ATPase by KCl is different for the two muscle types (7). On this basis, it may be that heart is affected by KCl or pH to a greater degree than skeletal muscle resulting in a proportionally greater extraction of cytochrome c (Fig. 2). In summary, it appears that there may be differences in susceptibility of muscle fiber types to alterations induced as the tissue is prepared for studies of oxidative capacity. Further studies are needed to examine the relationships between homogenate pH, enzyme loss from mitochondria, muscle fiber type and oxidative capacity. The extent to which exercise training alters these relationships may also be of interest.

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