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Respiratory control in the glucose perfused heart

A ^{31}P NMR and NADH fluorescence study

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The phosphate metabolites, adenosine diphosphate (ADP), inorganic phosphate (P_i), and adenosine triphosphate (ATP), are potentially important regulators of mitochondrial respiration in vivo. However, previous studies on the heart in vivo and in vitro have not consistently demonstrated an appropriate correlation between the concentration of these phosphate metabolites and moderate changes in work and respiration. Recently, mitochondrial NAD(P)H levels have been proposed as a potential regulator of cardiac respiration during alterations in work output. In order to understand better the mechanism of respiratory control under these conditions, we investigated the relationship between the phosphate metabolites, the NAD(P)H levels, and oxygen consumption (Q_{0_2}) in the isovolumic perfused rat heart during alterations in work output with pacing. ATP, creatine phosphate (CrP), P_i and intracellular pH were measured using ^{31}P NMR. Mitochondrial NAD(P)H levels were monitored using spectrofluorometric techniques. Utilizing glucose as the sole substrate, an increase in paced heart rate led to an increase in Q_{0_2} from 1.73 ± 0.09 to 2.29 ± 0.12 mmol Q_2/h per g dry wt. No significant changes in the levels of P_i, PCr, ATP, or the calculated ADP levels were detected. Under identical conditions, an increase in heart rate was associated with a 23 ± 3% increase in NAD(P)H fluorescence. Thus, under the conditions of these studies, an increase in Q_{0_2} was not associated with an increase in ADP or P_i. In contrast, increases in Q_{0_2} were associated with an increase in NAD(P)H. These data are consistent with the notion that increases in the mitochondrial NADH redox state regulate steady-state levels of respiration when myocardial work is increased.

ADP; ATP; Energy metabolism; P_i; Creatine phosphate; Mitochondria; (Rat heart)

1. INTRODUCTION

The healthy beating heart has the ability to match closely its cellular energy requirements with the production of energy [1]. When work is performed by the heart it responds metabolically by increasing mitochondrial oxygen consumption (Q_{O_2}) and producing adenosine triphosphate (ATP) at a faster rate. The intracellular signals that control this conversion remain an area of active investigation. Early studies on isolated mitochondria led to the notion that alterations in cytoplasmic ADP or P_i levels [2], due to changes in ATP hydrolysis by the work-producing ATPases, are responsible for the coordination of metabolism and work. Later studies on isolated mitochondria and cells suggested that the absolute concentrations of ADP and P_i may not be critical in this process, but rather the ratio of ATP/ADP [3] or the

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total phosphorylation potential [4]. The most recent evidence from heart mitochondria supports the theory that it is the level of ADP which is of primary importance [5]. All of these models, independent of the actual mechanism, predict that alterations in work output should result in changes in the adenylate phosphates and P_i.

Many studies in the perfused heart have failed to detect appropriate changes in high-energy phosphates when the heart is stimulated to do more work [6–9]. Most recently, ^{31}P NMR studies in intact dog heart were not able to detect changes in phosphate levels with maneuvers which stimulated work and oxygen consumption [10,11]. These studies leave doubt as to whether the high-energy phosphates are the key intracellular control of oxidative metabolism in the heart and suggest that some other parameter may be controlling this process.

The NADH redox state has been entertained as a potential regulator of respiration in numerous systems [4]. In isolated mitochondria [12], kidney tubules [13], liver [14,15] and heart [16] increases in NADH due to various alterations in mitochondrial substrate metabolism are associated with increased Q_{O_2} at constant or even decreased levels of ADP. Therefore, the mitochondrial NADH redox state is a possible control step for the coordination of work and energy metabolism. The mitochondrial NAD(P)H level can be followed noninvasively utilizing spectrofluorometric techniques [17,18]. Here, we utilized ^{31}P NMR and optical fluorescence to follow the metabolism of high-energy phosphates and NADH levels in the isolated perfused rat heart. Both ^{31}P NMR and optical fluorescence are non-destructive and therefore each heart can be used as its own control while Q_{O_2} is monitored during changes in work output induced by pacing.

2. MATERIALS AND METHODS

In these studies 300–400 g male Sprague Dawley rats were anesthetized with an intraperitoneal injection of 0.5 g sodium pentobarbitol. 1000 U heparin were also injected intraperitoneally to prevent blood coagulation. After the onset of surgical anesthesia, the hearts were rapidly excised and perfused retrograde in a Langendorff fashion [19]. A latex balloon was inserted into the left ventricle through an incision in the left atrium, resulting in an isovolumic contraction pattern. The hearts developed from 60 to 80 mmHg peak pressures. The hearts were perfused at a pressure of 90 mmHg with media containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM $NaHPO_4$, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 11 mM glucose and 25 mM $NaHCO_3$. The perfusate equilibration with 95% O_2/5% CO_2 at 37°C was confirmed using a blood gas analyzer (Radiometer). The perfusate pH was 7.4. The perfused heart was placed in an air-tight glass chamber and oxygen consumption (Q_{O_2}) was monitored using a flow meter to determine coronary flow, and sampling the difference between the afferent and efferent perfusate oxygen tensions using a blood gas analyzer. Work by the heart was altered by pacing the tissue at varying rates using electrical stimulation via agar bridges.

^{31}P NMR spectra were obtained on a Nicolet WB-360 magnet operating at a phosphorus frequency of 146.2 MHz. The detection coil was constructed in a Helmholtz configuration (2 × 2 cm) from 14-gauge magnet wire to minimize losses associated with the conductive sample [20]. Spectra were obtained in 5-min blocks using a 60° pulse angle (20 μs with 100 W) and a 1.1 s recycle time. This short delay time between each radiofrequency pulse was used to maximize the signal-to-noise ratio over a reasonable experimental time frame [21]. The signal-to-noise ratio obtained allowed changes of $< 5\%$ in the phosphate resonance intensities of ATP to be observed. The signals observed under these conditions are differentially saturated and do not represent the total amounts of metabolites present. However, these signals can be corrected using the spin lattice relaxation times (T_1) of the phosphate compounds [22,23] and the nutation angles used [21]. With these values the fully relaxed PCr to ATP ratio is calculated to be 1:22, in good agreement with previous preparations [8,9,22].

NAD(P)H fluorescence was measured as described [24] from the heart using a chamber simulating that used for NMR. Briefly, a rapid-scanning video fluorometer utilizing laser excitation at 337 nm was used to detect the NAD(P)H fluorescence. The fluorescence signal was obtained from a 1 cm^2 area of the left ventricle. The depth of NAD(P)H signal detection has been shown to be of the order of 0.5 mm under similar conditions

[25]. To correct for artifacts associated with motion or changes in myoglobin absorption a fluorescent standard 5(and 6)-carboxy-2′,7′-dichlorofluorescein (Molecular Probes) was added to the heart. This internal inert fluorescent standard permitted the correction of the surface fluorescence for motion and myoglobin absorption artifacts which are difficult to compensate for in a freely beating heart preparation. The NAD(P)H fluorescence from the heart was determined using the 426/592 nm wavelength pair from data obtained by averaging 25 fluorescence spectra acquisitions. Each averaged fluorescence spectrum (from 380 to 605 nm) took 0.8 s to collect and was acquired once every minute.

3. RESULTS

The protocol consisted of two periods each lasting 40 min preceded by a period of stabilization (10–15 min) to ensure a steady state before data acquisiton. The first period represented the control where the heart was paced near its basal rate of 250–300 bpm, followed by the second period of increased work where the rate was increased to 590 bpm. A separate group of hearts served as time controls and were evaluated for the length of the entire protocol (two 40 min periods) without a pacing increase. Oxygen consumption was stable throughout the time course controls at 1.57 mmol O_2/h per g dry wt (fig.1). The ^{31}P NMR data are summarized in table 1 where the integrated NMR signals are normalized to the control PCr signal. There was no change in ATP or P_i over the two 40 min periods as judged from the absolute intensities (table 1) and difference spectrum (not shown). A small decrease in the level of PCr was observed over the course of the control experiment. Note that the high P_i levels are due to the combined integration of intra- and extracellular (1.2 mM in the perfusate) P_i signals. These two areas were combined since in a number of preparations the intracellular P_i peak was only partially resolved from the extracellular peak preventing accurate individual integrations. It should be pointed out that if significant changes in intracellular P_i were occurring with these perturbations a significant intracellular P_i peak should have been observed in the difference spectra. This would occur since the perfusate P_i signal does not change and completely subtracts out in the difference spectrum leaving only the changes in the intracellular P_i. Even with the limited resolution, the chemical shift of the intracellular and extracellular P_i signals could be measured in spectra when only 5 Hz line broadening was applied. This permitted the estimation of intracellular pH [26]. Based on this direct determination of the intracellular pH and the absence of a frequency shift in the difference spectrum we conclude that no change in intracellular pH occurred over the time course of these experiments.

In the experimental hearts the protocol was repeated but the heart rate was increased from 255 to 590 bpm after 40 min. Following all the same parameters the Q_{O_2} increased 32% from 1.73 ± 0.09 to 2.29 ± 0.12 mmol O_2/h per g dry wt (fig.1). The NMR spectra obtained (fig.2) demon-

Table 1

Time controls for the perfused rat heart

	1st period	2nd period	%Δ
Heart rate (bpm)	250	250	NC
O_2x	1.57 ± 0.08	1.58 ± 0.17	NC
PCr	100	88.0 ± 3.1	-12[a]
ATP	121.0 ± 5.6	111.0 ± 2.1	NC
P_i	145.3 ± 8.0	155.6 ± 2.3	NC
PCr/ATP	0.83 ± 0.04[b]	0.79 ± 0.02	NC

[a] $p < 0.05$
[b] Not corrected for partial saturation (see text)
$n = 3$; x mmol/h per g dry wt

Table 2

Effect of pacing increase on perfused rat heart

	1st period	2nd period	%Δ
Heart Rate (bpm)	255	590	+131[a]
$Q_{O_2}x$	1.73 ± 0.09	2.29 ± 0.12	+32[a]
PCr	100	103.5 ± 2.5	NC
ATP	121.3 ± 5.2	109.8 ± 5.4	NC
P_i	166.0 ± 3.6	159.5 ± 5.9	NC
PCr/ATP	0.83 ± 0.04[b]	0.95 ± 0.03	+14[a]

[a] $p < 0.05$
[b] Not corrected for partial saturation (see text)
$n = 4$; x mmol/h per g dry wt

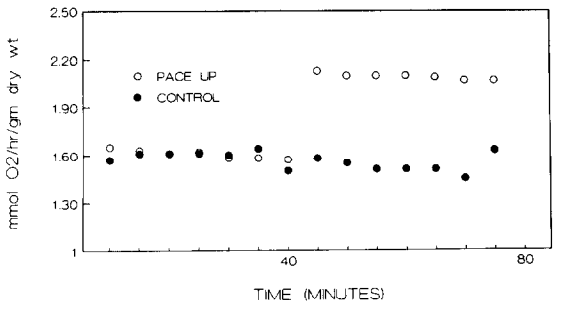

Fig.1. Effects of increased heart rate on oxygen consumption. Oxygen consumption of a glucose-perfused heart beating at 250 bpm (●, control) and one which was beating at 250 bpm and then paced up to 590 bpm (○, pace up).

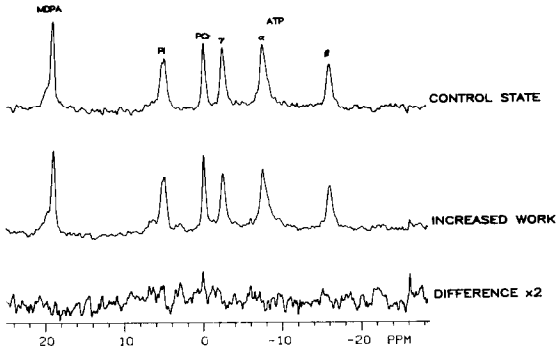

Fig.2. Effects of increased work on ^{31}P NMR detected metabolites in the perfused heart. ^{31}P NMR spectra obtained from a glucose-perfused heart in the control state (250 bpm) and after increasing work by pacing to 590 bpm. Spectra were acquired using a 60° pulse and a 1.1 s recycle time [20]. Peaks: MDPA, methylenediphosphonic acid placed in the left ventricular balloon; P_i, P_i with additional 1.2 mM NaH_2PO_4 from perfusate, representing extra- and intracellular P_i; PCr, partially saturated phosphocreatine; and the α-, β- and γ-phosphates of ATP. The difference spectrum shows that no significant change occurred in any of the phosphates with increased work.

strated no significant changes in any of the phosphate peak areas. There was no significant change in intracellular pH, nor the individual levels of PCr nor ATP (table 2). However, the PCr/ATP ratio, which has been used to reflect changes using the creatine kinase equilibrium reaction (see section 4),

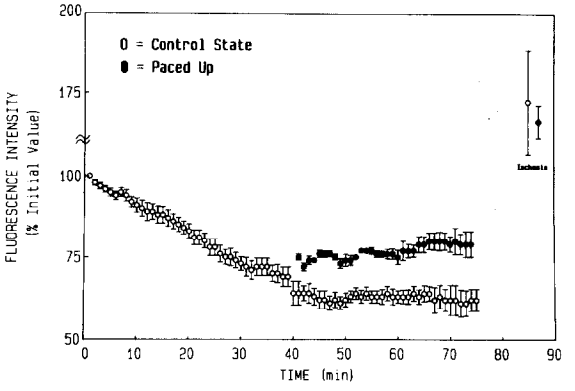

Fig.3. Effects of increased heart rate on NADH fluorescence. Time course of fluorescence changes (NADH/ClCF) for hearts perfused with glucose. For the first 40 min hearts were paced at 250 bpm and then either pacing was continued at this rate (○, controls) or paced up to 590 bpm (●, paced up).

increased 14% ($p<0.05$) indicating that the ADP level may have actually dropped slightly even though the respiratory rate increased.

Using the identical protocol, fluorescence spectra were obtained from the perfused heart preparation. The NAD(P)H fluorescence signal from the heart was followed for the two 40 min periods. A decrease in fluorescence during the first 40 min was seen in control hearts which leveled out after approx. 30 min (fig.3) and may be due to depletion of endogenous substrates [27]. This conclusion is supported by the observation that a much smaller drop in fluorescence occurs with pyruvate (11 mM) as a substrate (unpublished). Due to this lack of a steady-state NAD(P)H fluorescence, the time courses of fluorescence changes were compared between the control hearts, paced at basal rates for 80 min and the experimental hearts that were paced up to 590 bpm for the second 40 min period (fig.3). When pacing was increased in the experimental hearts, the NAD(P)H fluorescence increased or a significant inhibition of the control periods' slow decrease in fluorescence was observed (fig.3). On average, in the second 40 min period, the experimental hearts, which are paced at 590 bpm, had a 32% increase in Q_{O_2} and a 23% higher level of NAD(P)H than did the hearts beating at 300 bpm (fig.3).

4. DISCUSSION

These data demonstrate that when the glucose-perfused rat heart responds to increased work via electrical pacing it respires at a higher Q_{O_2} with an increase in NAD(P)H levels and no significant changes in the NMR-detected phosphates. This increase in NAD(P)H represents an effect on mitochondrial redox state opposite to that expected if respiration were controlled only by ADP and P_i levels, and implies that it is the NAD(P)H redox state which controls respiration under these conditions.

The relative free ADP concentration was determined in these studies using the creatine kinase equilibrium method [28]. The creatine kinase reaction has been shown by both classical chemical extraction techniques and ^{31}P NMR to be near equilibrium in heart tissue [9,22,28]. The equilibrium equation for the creatine kinase reaction can be expressed in terms of ADP as:

$$[ADP] = \frac{K_{eq}[ATP][Cr]}{[CrP][H^+]} \quad (1)$$

If the intracellular pH, [ATP] and total PCr + Cr pool are assumed to remain constant, then the value of CrP/ATP should reflect alterations in [ADP]. In these studies the pH and ATP concentration were contant over our experimental periods. Additionally, the total creatine pool has been found to be very stable (approx. 11.1 μmol/g wet wt) in the glucose-perfused heart at different work loads [16]. Therefore, it is reasonable to assume that under these conditions the CrP/ATP ratio is a good indication of the relative free ADP concentration within a given heart. Studies of isolated mitochondria have revealed a saturable Michaelis-Menton type relationship between extramitochondrial ADP and respiration. The apparent affinity (K_m) of ADP is approx. 20 μM which is close to the calculated free cytosolic [ADP] in heart muscle [28] and other tissues [29]. Of interest here is that the slope of this relation between [ADP] and respiration is never greater than 1:1. That is, a 30% increase in ADP results in a 30% increase in respiration near the K_m for [ADP]. Therefore, if this simple mechanism is responsible for respiratory control in the intact cell, a 30% increase in work or respiration should be accompanied by a 30% increase in cytosolic [ADP]. In our experiments the respiration was increased approx. 32% which should result in a 20% drop in the [CrP] according to eqn 1 using published values for the total creatine pool in the glucose-perfused heart [8,16]. However, there was no decrease in CrP detected in these studies. In contrast, the CrP/ATP ratio increased significantly when compared to the control period or the time control hearts. These latter data suggest that ADP levels actually decreased with the increase in respiration induced in these studies. Clearly, these data indicate that neither cytosolic [ADP] nor [P_i] increased with a work-induced increase in Q_{O_2} suggesting, based on studies of isolated mitochondria, that these compounds alone cannot be responsible for stimulating Q_{O_2}.

These NMR data confirmed earlier studies demonstrating a lack of correlation between [ADP], [P_i] and Q_{O_2} in the heart. The fluorescence studies were then conducted to establish what was occurring to the mitochondrial NADH redox state during these perturbations. The surface fluorescence signal from pyridine nucleotides of the heart could contain components from NADPH as well as NADH. In addition, both cytoplasmic and mitochondrial (NAD(P)H could contribute to this fluorescence signal. However, it has recently been shown that the cytoplasmic NADH contribution in the heart is negligible compared to the mitochondrial NADH signal [18]. In our studies, we have found that maximally uncoupling the tissue with 1799 results in a greater than 90% decrease in fluorescence (unpublished). The 10% residual fluorescence is similar to that found in isolated mitochondria after treatment with 1799. This low fluorescence background with uncoupler exists in the intact heart even when the cytoplasmic NADH level should be increased by the elevated tissue lactate content due to the stimulation of glycolysis. This result confirms earlier studies indicating that the cytoplasmic component of the NADH fluorescence signal is negligible in this preparation. With regard to NADPH contributions to the fluorescence signal, there is much more NADH than NADPH in heart tissue with a ratio of NADH/NADPH of 4:1 [30]. Therefore, when isolated heart mitochondria undergo stimulation of respiration by the addition of ADP there is a 6 μM change in NADH vs only 0.3 μM change in NADPH [30]. These findings, combined with the

estimates that NADH fluorescence is 2-4-fold more intense than NADPH [31], lead us to interpret the (NAD(P)H) fluorescence signal from the heart to be due to mitochondrial NADH.

In these protocols the control NAD(P)H signal was observed to decrease with time consistent with the utilization and depletion of endogenous substrates [26]. Interestingly, the CrP concentration decreased during this period suggesting that ADP may have increased. With a higher pacing rate an immediate increase in NADH fluorescence or an inhibition of the control periods NADH oxidation was observed. This increase in NADH reduction level is unlikely to be due to hypoxia, as a result of the increase in Q_{O_2}, since no change in the high-energy phosphates was observed. In addition, our previous studies have demonstrated a large excess of oxygen delivery in this preparation using the NAD(P)H fluorescence technique [24]. A possible mechanism to explain the increase in NAD(P)H in response to an increase in work output can be found in the literature. Denton and McCormick [32] have proposed that an increase in mitochondrial calcium will activate mitochondrial dehydrogenases which in turn will result in an increase in reduced NAD. Hansford [33] has shown in isolated blowfly mitochondria that this type of activation can result in a net reduction of mitochondrial NAD when the mitochondria are subjected to high concentrations of ADP. This latter result is consistent with the data presented here in the perfused heart. That is, an increase in work output causes an increase in cytosolic calcium which then activates mitochondrial dehydrogenases and increases NADH without requiring a significant change in phosphates. Other possibilities are that the stimulation of oxygen consumption by ADP is much more sensitive than detected in isolated mitochondria. That is, in vivo a transient change in ADP or P_i could produce a large change in respiration due to other cooperativity effects. It is also possible that with increased work there is a change in the metabolic pathways used to produce NADH. For example, the activation of fatty acid or glycolytic metabolism in association with increases in work may result in a net increase in NADH.

In summary, the present data add an important dimension to understanding the control of respiration in the heart when work is stimulated. The data support the notion that the NADH redox state is a primary regulator of mitochondrial respiration during work transitions when the high-energy phosphates do not change significantly.

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