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Influence of calcium-induced workload transitions and fatty acid supply on myocardial substrate selection

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Abstract

Because of differences in energy yield and oxygen demand, the selection of oxidative fuels is important in the hypoxic or ischemic heart muscle. The aim of the present study was to clarify the contradictions observed in the effects of workload and fatty acid supply on myocardial fuel preference in isolated perfused rat hearts. Nuclear magnetic resonance spectroscopy combined with the administration of substrates labeled with the stable isotope carbon 13 and isotopomer analysis of glutamate labeling offers an opportunity to simultaneously measure metabolic fluxes in pathways feeding into the tricarboxylic acid (TCA) cycle. The work output was modulated by changes in extracellular calcium. In the presence of 5 mmol/L glucose, 0.5 mmol/L octanoate in the perfusate dominated the oxidative metabolism, and workload had little effect on the ratio of glucose to fatty acid utilization. This was the case even when the octanoate concentration was lowered to 50 μmol/L. The relative rate of replenishment of the TCA cycle intermediates was higher at a low workload. The redox state of flavoproteins in the intact heart was monitored fluorometrically to obtain an estimate of the mitochondrial reduced/oxidized nicotinamide-adenine dinucleotide ratio (NADH/NAD ratio) for assessment of the dominant level of regulation of cell respiration, and the myoglobin spectrum was simultaneously monitored to evaluate the oxygenation status of the myocardium. Commencement of octanoate infusion (50 μmol/L or 0.5 mmol/L) caused a large but transient reduction of mitochondrial NAD and, conversely, its cessation elicited NADH oxidation and rebound reduction. During glucose oxidation, an increase in workload led to oxidation of the mitochondrial NADH, but this effect was much smaller in the presence of 50 μmol/L octanoate and absent in the presence of 0.5 mmol/L. This indicates that strong control of oxygen consumption during glucose oxidation is exerted in the mitochondrial respiratory chain, whereas equal control during fatty acid oxidation is excerted within the metabolic pathway upstream from the respiratory chain. It is concluded that when a medium-chain fatty acid is available, myocardial workload and energy consumption have little influence on fuel preference and glucose oxidation remains suppressed.

1. Introduction

Oxygen consumption in the heart muscle is geared toward energy expenditure, an indication of mitochondrial respiratory control [1,2]. Under physiological conditions, fatty acids are the main oxidizable substrate of the myocardium [3], but because these have a tendency to increase oxygen consumption, an important question, especially in hypoxic or ischemic tissue volumes, is the selection of a fuel substrate. Fatty acids may increase oxygen consumption by several mechanisms: First, the yield of adenosine triphosphate (ATP) molecules per atom of oxygen consumed (ATP/O ratio) of fatty acid oxidation is lower than that of glucose oxidation because some of the reducing equivalents bypass complex I, the first energy conserving site in the respiratory chain. Second, fatty acids may cause mitochondrial uncoupling or decoupling, although they do not act as classical uncouplers because no deenergization has been observed [4]. Third, fatty acids are efficient reducers of mitochondrial NAD and, by increasing the concentration of this substrate of the respiratory chain, they are able to raise oxygen consumption [5].

Means of influencing cardiac fuel preference could be advantageous. Early in vivo catheterization data suggest substrate switching between fatty acid and glucose oxidation upon changes in energy expenditure in the human heart

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[6]. Some studies using adrenergic stimulation to increase the workload have supported this view [7,8], but in contrast, the contribution of substrates has been shown to remain unaltered upon primary changes in work output in the absence of hormones [9,10].

In the present case, we set out to study the substrate preference of the heart muscle by means of carbon 13 nuclear magnetic resonance and isotopomer analysis. The questions asked were: First, does the fatty acid supply determine the rate of fatty acid oxidation? Second, do changes in myocardial energy consumption influence the relative contributions of various substrates as well as the gross rates of oxidative metabolism? Third, is replenishment (anaplerosis) of the tricarboxylic acid (TCA) cycle intermediates affected by the energy consumption during fatty acid oxidation? To reduce the system under study and to focus on glucose and fatty acid oxidation proper, only glucose and octanoate, a medium-chain fatty acid, were used as exogenous substrates. Because the contractile function of the heart muscle expresses an absolute requirement for extracellular calcium, adjustments of the latter were used to elicit changes in the mechanical work output. It is shown that octanoate dominates the oxidative metabolism both at low and high concentrations, independently of energy expenditure, when the competing exogenous substrate is glucose.

2. Materials and methods

2.1. Stable isotopes

Isotopes [1-^{13}C]glucose, [1,6-^{13}C]glucose, [1,2,3,4-^{13}C] octanoic acid, and [U^{13}-C]octanoic acid (all 99% enriched) were purchased from Cambridge Isotopic Laboratories, Inc, Andover, Mass.

2.2. Animals and perfusion methods

The experiments were conducted according to the guiding principles regarding the care and use of laboratory animals and with prior approval from the Laboratory Animals Committee of the University of Oulu. Male Sprague-Dawley rats in the stocks of the Laboratory Animal Center of the University of Oulu were kept under a 12-hour light/12-hour dark cycle, the dark period being from 7 PM to 7 AM. The experiments were performed between 12 noon and 3 PM and without a preceding fasting period. The rats were anesthetized with intraperitoneal sodium pentobarbital, and 500 IU heparin was injected into the inferior vena cava. The heart was excised 1 minute later, immediately cooled in an ice-cold Krebs-Henseleit bicarbonate buffer, and tied onto an aortic cannula. Retrograde perfusion was begun with a modified Krebs-Henseleit bicarbonate buffer containing 121 mmol/L NaCl, 4.7 mmol/L KCl, 1.5 mmol/L $CaCl_2$, 0.5 mmol/L EDTA, 1.28 mmol/L $MgSO_4$, and 24.8 mmol/L $NaHCO_3$ and supplemented with exogenous substrates as described in the protocol below. The perfusion system was maintained at 37°C by means of water mantles and the medium was gassed with O_2/CO_2 (19:1). A constant perfusion pressure of 100 cm H_2O was maintained by means of a hydrostatic overflow.

2.3. Protocol

The hearts were perfused with 12 IU/L insulin, 5 mmol/L glucose, and 50 μmol/L or 0.5 mmol/L octanoate. After a balancing period of 20 minutes with nonlabeled substrates in the presence of 1.5 mmol/L Ca^{2+}, the once-through perfusion was switched to recirculation with the same concentrations of ^{13}C-labeled substrates ([1,6-^{13}C]glucose and [U-^{13}C]octanoate). The Ca^{2+} concentration was lowered to 0.5 mmol/L after 20 minutes in the low workload group, whereas in the high workload group it was increased to 2.5 mmol/L. After 30 minutes of recirculating perfusion with ^{13}C substrates, the hearts were quickly frozen and stored in liquid nitrogen for the preparation of nuclear magnetic resonance (NMR) samples. An identical protocol was observed with 50 μmol/L octanoate, but recirculation of perfusate was not used, as the labeled [1,2,3,4-^{13}C]octanoate was introduced through a side infusion, and the 5 mmol/L glucose was unlabeled. This was done because during a 30-minute recirculation, the uptake of octanoate would have significantly lowered its concentration in the perfusate and the sensitivity of the detection of ^{13}C enrichment in the glutamate from octanoate had to be maximized.

An additional set of experiments was carried out to study the effect of octanoate on oxygen consumption. The hearts were initially perfused with a medium containing 5 mmol/L glucose, 12 IU/L insulin, and 1.5 mmol/L $CaCl_2$. After a 15-minute balancing period, the $CaCl_2$ concentration was raised to 2.5 mmol/L, and after 10 more minutes, 50 μmol/L or 0.5 mmol/L octanoate was added to the perfusate for 10 minutes. Five minutes later, the calcium concentration was returned to 1.5 mmol/L for a further 20-minute balancing period. Thereafter, a similar sequence of 50 μmol/L or 0.5 mmol/L octanoate perfusion was performed in the presence of 0.5 mmol/L Ca^{2+} with the same heart. Finally, the calcium concentration was returned to 1.5 mmol/L to test maintenance of the metabolic and hemodynamic parameters. The protocol is presented graphically in the lower panel of Fig. 5.

2.4. Left ventricular pressure, heart rate, and oxygen consumption

Left ventricular pressure was monitored by inserting a water-filled latex balloon into the left ventricle and connecting it to an Isotec pressure transducer (Hugo Sachs Elektronik-Harvard Apparatus GmbH, March-Hugstetten, Germany) and Statham SP1400 pressure monitor (Statham Instruments, Oxnard, Calif). The heart rate was measured from the pressure curve with a digital frequency counter or was calculated online from the pressure curve by means of the data logger software. Mechanical work output was expressed as the pressure-rate product (PRP; ie, peak systolic pressure multiplied by heart rate). Coronary flow was monitored throughout the experiments using a laboratory-made optical drop counter with analogue output. The oxygen

concentration in the effluent perfusate was monitored continuously with an oxygen electrode. Oxygen consumption was calculated by multiplying the arteriovenous concentration difference by the coronary flow. The outputs of the monitoring devices were fed to a Lab-PC data acquisition board (National Instruments, Austin, Tex) and a personal computer for data storage and analysis.

2.5. Surface fluorometry and reflectance photometry

Fluorescence of flavoproteins and myoglobin absorption spectrum changes were monitored on the epicardial surface using a custom-made triple wavelength spectrometer-fluorometer [11] that was optically connected to the surface of the heart by means of a 2-branch quartz fiber light guide. Fluorescence and reflectance were measured simultaneously using 465-nm excitation selected by an interference filter and by detecting the fluorescence above 515 nm through a long-pass filter. The myoglobin oxygenation state was monitored in terms of the reflectance difference at wavelengths of 582 and 630 nm, as selected from grating monochromators.

2.6. Nuclear magnetic resonance spectroscopy

After storage in liquid nitrogen, the hearts were powdered and homogenized in 6% $HClO_4$. After centrifugation, the supernatant was neutralized to pH 7 and $KClO_4$ precipitated with 3.0 mol/L K_2CO_3/0.5 mol/L KOH, centrifuged, and the supernatant freeze-dried. The powder was redissolved in 0.6 mL of D_2O/H_2O (1:1) and placed in a 5-mm NMR tube.

The ^{13}C NMR spectra of the heart extracts were obtained in a Bruker AM-200 spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany) operating at 50.32 MHz. Protons were decoupled with the composite pulse decoupling sequence WALTZ [12]. The samples were spun at 20 Hz to reduce field inhomogeneity. The spectra were collected using an excitation pulse resulting in a 45° tilt angle, an acquisition time of 0.69 second, and an interpulse delay of 4.40 seconds. A spectral width of 12 kHz and 8-k data points was used to collect the spectra. Typically, 9000 free induction decays were accumulated. These were zero-filled to 32-k data points and multiplied by an exponential function equivalent to a 1- to 2-Hz line broadening before Fourier transformation to improve the signal-to-noise ratio.

2.7. ^{13}C isotopomer analysis and calculation of metabolic fluxes

The hearts were perfused to a steady state of ^{13}C enrichment in intracellular glutamate. An approach for a general case, modified from that of Malloy et al [13,14], was used for analysis of the ^{13}C NMR spectra. The set of simultaneous equations was the same as that used by Malloy et al [13,14]. Glutamate is a good indicator metabolite of 2-oxoglutarate labeling in the TCA cycle because of its high concentration and preferentially mitochondrial location [15]. This kinetic model omits the glutamine pool. Formation of glutamine can be detected, although glutamate formation from glutamine in the rat heart remains undetectable [16]. Thus, the glutamine cycling rate is low enough to justify its omission from the model. It has been shown previously that the isotopic steady state presupposed by the method is achieved in 20 minutes in rat and rabbit hearts [17,18]. The method was modified by taking into account the long-range spin-spin couplings of C2 and C4 of glutamate to C5 and C1 by 2.0 ± 0.5 and 1.5 ± 0.5 Hz, respectively. Therefore, each of the 4 multiplets of C2 and C4 may be split once more by the long-range spin-spin couplings, resulting in a total of 8 possible multiplets for each carbon. These couplings provide additional information on the relative concentrations of C1 and C5 in ^{13}C-enriched glutamate isotopomers, and taking them into the calculations enhances the fit of the experimental values to the simulated spectra. The other long-range spin-spin couplings of glutamate were less than 1 Hz and were not used in the analysis as in the study of Carvalho et al [19] because of the higher line broadening values of the spectra in the current measurements.

The fitting of the spectra was improved, as the simultaneous equations for the isotopomer analysis were not solved on the basis of measured peak areas of the spectrum but by fitting to the experimental spectrum a simulated one calculated from the metabolic variables used as seed values for the optimization procedure.

The program was written in the MATLAB environment (MATLAB Optimization Toolbox, The Mathworks, Natick, Mass). A simulated spectrum was calculated using the first-order spectrum theory, which is sufficient for a ^{13}C spectrum with large separation between the resonances compared with the spin-spin couplings. A Lorentzian line shape was assumed to model the simulated spectrum. The 3 resonances C2, C3, and C4 were fitted simultaneously, which allowed the use of a minimum number of fitted parameters. The same spin-spin coupling between carbons C2 and C3, for example, was used to determine multiplets in both C2 and C3 resonances. For the fitting itself, the constrained nonlinear least squares optimization algorithm available in the MATLAB Optimization Toolbox was used to solve a set of simultaneous equations in which the minimized parameter was the difference between the experimental and calculated spectra.

The use of constraints allows a priori information to be used for both the metabolic parameters and the spectral ones. The values for the latter are well known in advance so that these parameters can be fixed or can have strict constraints. For the metabolic parameters, the choice of substrates and the original enrichment of the source compound determine the maximal fractional enrichments obtainable for acetyl-coenzyme A (CoA) and oxaloacetate. For example, the highest possible enrichment of acetyl-CoA and oxaloacetate obtainable using [1-^{13}C]glucose and [1,2,3,4-^{13}C]octanoate as exogenous sources is 50%, which was included as a constraint on the fit.

The metabolic network under study is shown in Fig. 1. Also, some of the anaplerotic or metabolite disposal routes

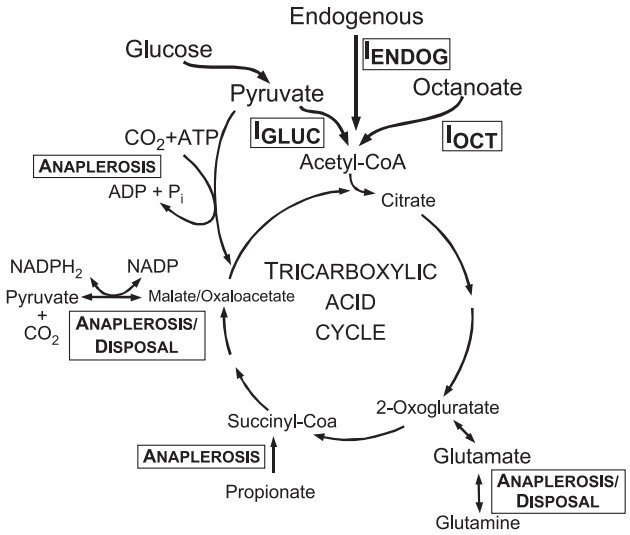

Fig. 1. The metabolic network. The equilibrium of the aminotransferase reactions and the high intracellular concentration of glutamate allow its use for measuring the intramolecular distribution of a ^{13}C label in 2-oxoglutarate, which is dependent on the fractional contributions of the oxidation of glucose and fatty acid labeled in definite positions and on the rate of anaplerosis. Some of the main routes of anaplerosis and metabolite disposal are indicated.

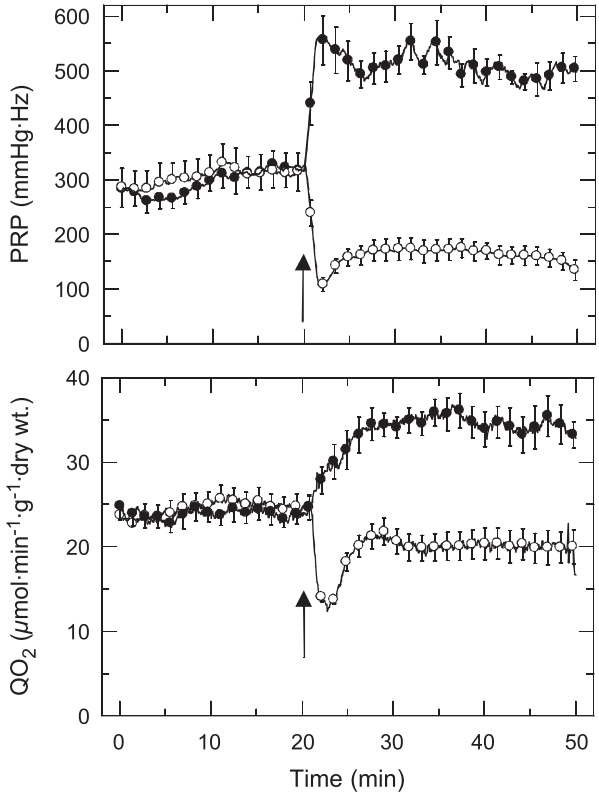

Fig. 2. Effects of perfusate calcium concentration on work output and oxygen consumption in isolated perfused rat hearts oxidizing 5 mmol/L glucose and 50 μmol/L octanoate. Upper panel: PRP. Lower panel: oxygen consumption. The initial perfusate [Ca^{2+}] was 1.5 mmol/L, after which (at the time point marked by the arrow) the Ca^{2+} concentration was altered to 0.5 mmol/L (lines with open symbols) or 2.5 mmol/L (lines with solid symbols). The lines show means at 4-second intervals and the vertical bars ± SE for every 40 data points in 7 independent experiments.

are indicated in Fig. 1, although only their combined flux can be estimated by ^{13}C NMR isotopomer analysis. The absolute acetyl-CoA fluxes were calculated using the oxygen consumption values and other information gained from the isotopomer analysis, as described previously [20]. The average oxygen consumption during 30 minutes of perfusion with ^{13}C-labeled substrates was used (expressed as μmol/(min g) dry wt). The acetyl-CoA input into the TCA cycle originates from labeled exogenous sources or unlabeled endogenous sources. As the identity of the endogenous source could not be identified by the experimental setup used here, it was assumed that this was derived from tripalmitoyl glycerol, largely because the ^{13}C NMR data showed that octanoate suppressed the utilization of glucose. When assuming that glycogen was the only source of endogenous acetyl-CoA production, the difference in the results was less than 1%, which is smaller than the experimental uncertainty of the method.

Because it is known that β-oxidation of a fatty acid molecule proceeds to completion, 1 mol of octanoate must require 11 mol of oxygen, 23 mol of palmitate, and 6 mol of glucose. One mole of octanoate produces 4 mol of acetyl-CoA in β-oxidation, whereas glucose produces 2 mol and palmitate 8 mol. The relation between oxygen consumption (QO_2) and the absolute acetyl-CoA fluxes can be expressed by Eq. (1):

$$QO_2 = 11\ I_{OCT}/4 + 6\ I_{GLUK}/2 + 23\ I_{ENDOG}/8 \qquad (1)$$

where I_{OCT}, I_{GLUK}, and I_{ENDOG} denote the absolute rates of acetyl-CoA input into the TCA cycle from octanoate,

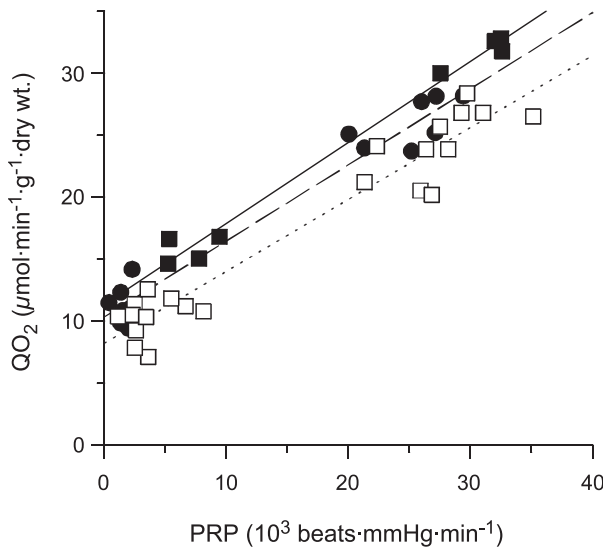

Fig. 3. Correlation between oxygen consumption and work output in isolated perfused rat hearts oxidizing glucose and various concentrations of octanoate. Open squares and dotted regression line: (slope, 0.58 ± 0.035; intercept, 8.22 ± 0.69) 5 mmol/L glucose. Solid circles and dashed regression line: (slope, 0.61 ± 0.036; intercept, 10.33 ± 0.65) 5 mmol/L glucose plus 50 μmol/L octanoate. Solid squares and solid regression line: (slope, 0.65 ± 0.031; intercept, 11.35 ± 0.71) 5 mmol/L glucose plus 0.5 mmol/L octanoate.

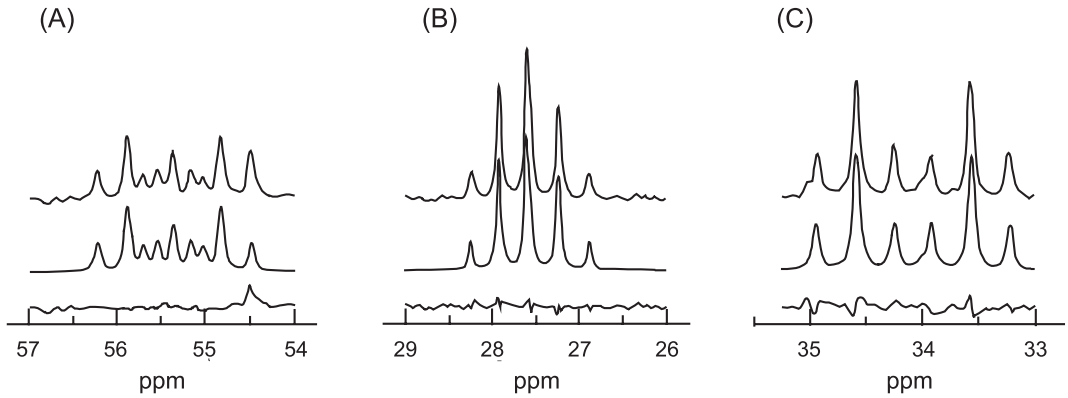

Fig. 4. Fitting of simulated to observed ^{13}C NMR spectra for the 2, 3, and 4 carbons of glutamate in a perchloric acid extract from a rat heart perfused with 5 mmol/L [1-^{13}C]glucose and 0.5 mmol/L [1,2,3,4-^{13}C]octanoate. Upper curves, observed spectra; middle curves, fitted spectra; lower curves, difference. A, Carbon-2. B, Carbon-3. C, Carbon-4.

glucose, and endogenous sources, respectively, when the endogenous substrate is palmitate. Using the nomenclature of Malloy et al [13] for fractional fluxes and the position of label enrichment, F_{c0} denotes a nonlabeled flux; F_{c1}, a flux labeled in C1 of acetyl-CoA; F_{c2}, a flux labeled in C2; and F_{c3}, a flux labeled in both C1 and C2. As the ratio of the absolute fluxes is the same as the ratio of the respective F_c values, the absolute flux of acetyl-CoA derived from octanoate, for example, can be calculated from Eq. (2):

$$I_{OCT} = QO_2/(11/4 + 3\ F_{c2}/F_{c3} + 23\ F_{c0}/8\ F_{c3}) \quad (2)$$

Acetyl-CoA fluxes originating from other substrates can be calculated in a similar manner.

2.8. Statistical analysis

The data were analyzed by one-way analysis of variance followed by a modified t test using the Bonferroni modification for multiple comparisons when appropriate. The results are presented as means ± SE.

3. Results

3.1. Work output and oxygen consumption

The changes in mechanical work output and the concomitant changes in oxygen consumption are presented in Fig. 2. Raising of the calcium concentration from the

Table 1
Effects of workload transitions evoked by perfusate [Ca^{2+}] changes on the relative and absolute metabolic fluxes of the main pathways feeding into the TCA cycle

Substrate and label	Workload		n	Pyruvate oxidation	Fatty acid oxidation	Nondefined	Total TCA cycle flux
5 mmol/L glucose + 50 μmol/L [U-^{13}C]-octanoate							
	Low[a]	Fractional input into TCA cycle (%)	3	–	97 ± 2	3 ± 2	
		Absolute input rate [μmol/(min g) dry wt]	3	–	4.37 ± 0.16	0.14 ± 0.11	4.51 ± 0.07
	High[b]	Fractional input into TCA cycle (%)	3	–	89 ± 6	11 ± 6	
		Absolute input rate [μmol/(min g) dry wt]	3	–	10.11 ± 1.51	1.13 ± 0.55	11.24 ± 1.00*
5 mmol/L [1-^{13}C]-glucose + 0.5 mmol/L [1,2,3,4-^{13}C] octanoate							
	Low	fractional input into TCA cycle (%)	4	8 ± 5	88 ± 4	4 ± 2	
		absolute input rate [μmol/(min g) dry wt]	4	0.6 ± 0.4	7.4 ± 0.4	0.4 ± 0.2	8.4 ± 0.1
	High	fractional input into TCA cycle (%)	4	3 ± 1	95 ± 4	2 ± 1	
		absolute input rate [μmol/(min g) dry wt]	4	0.4 ± 0.1	12.5 ± 0.9*	0.3 ± 0.2	13.0 ± 1.0*

The values are means ± SE for n independent experiments.
[a] [Ca^{2+}] = 0.5 mmol/L.
[b] [Ca^{2+}] = 2.5 mmol/L.
* $P < .005$, compared with low-work heart.

basal 1.5 to 2.5 mmol/L increased PRP and oxygen consumption concomitantly, whereas a decrease in calcium concentration 1.5 to 0.5 mmol/L caused a sharp fall in PRP and oxygen consumption.

Because our unpublished data with octanoate as the sole exogenous substrate indicated that octanoate lowers the PRP/Q_{O_2} ratio, an additional set of experiments was performed in which a heart was exposed to different Ca^{2+} concentrations in the presence of 5 mmol/L glucose plus either 0.05 or 0.5 mmol/L octanoate or in the absence of octanoate to avoid the confounding effect of perfusion time. The results are shown in Fig. 3. The ordinate intercept of Q_{O_2}, representing zero work, was 8.2 ± 0.69 (SE) in the glucose group, 10.3 ± 0.65 μmol/(min g) dry wt in the glucose plus 0.05 mmol/L octanoate group ($P < .05$ compared with the glucose group), and 11.4 ± 0.71 in the glucose plus 0.5 mmol/L octanoate group ($P < .01$ compared with the glucose group. These values are in accord with those observed in K^+-arrested rat hearts [21]. The reciprocal of the slope gives the PRP/Q_{O_2} ratio, and as work represents ATP consumption, the PRP/Q_{O_2} ratio reflects the ATP/O ratio of oxidative phosphorylation. The differences in slope between the glucose and glucose plus octanoate groups were not statistically significant.

3.2. Preference for oxidizable substrate at different workloads

The simulated and experimental glutamate ^{13}C NMR spectra of perchloric acid extracts from a rat heart perfused with 5 mmol/L [1-^{13}C]glucose or 0.5 mmol/L [1,2,3,4-^{13}C] octanoate are fitted in Fig. 4, which also demonstrates the performance of the present method. The fractional and absolute metabolic fluxes complying with the glutamate isotopomer pattern are shown in Table 1. The exogenous octanoate almost entirely accounts for the acetyl-CoA input into the TCA cycle at both 50 μmol/L and 0.5 mmol/L octanoate concentrations. The workload alterations have no significant effect on the contribution of fatty acid oxidation. Because the contribution of pyruvate oxidation could not be specified, it is included in substrate oxidation from non-defined sources in the low octanoate group because labeled glucose was not used. The values given in Table 1 were calculated on the assumption that the endogenous acetyl-CoA input is derived from palmitate, but the results would only differ by about 1% if glycogen were the nonlabeled endogenous source.

3.3. Replenishment of citric acid cycle intermediates (anaplerosis)

The ^{13}C NMR methodology also gives the fractional contribution of the combined anaplerotic fluxes to carbon entry into the TCA cycle. The relative rate of anaplerosis was 29% ± 5% of the acetyl-CoA flux in the low workload group, a value significantly higher than that of 13% ± 3% in the high workload group when 0.5 mmol/L octanoate was used. This gives absolute anaplerotic fluxes of 2.42 ± 0.55 and 1.63 ± 0.48 μmol/(min g) dry wt in the low and high workload groups, respectively. The anaplerotic flux could not be reliably determined in the 50 μmol/L octanoate group due to low isotope enrichment.

3.4. Redox state of flavoproteins and oxygenation state of myoglobin

The results of the surface-optical experiments are presented in Fig. 5. An increase in calcium concentration from 1.5 to 2.5 mmol/L caused a small but clear amount of flavin oxidation, with an initial overshoot, and when [Ca^{2+}] was 2.5 mmol/L, an addition of 50 μmol/L octanoate caused flavin reduction, again with an initial overshoot. Termination of octanoate infusion resulted in an extensive flavin oxidation overshoot before the redox state returned to its basal value. The behavior of the flux was different when [Ca^{2+}] was kept at 0.5 mmol/L, namely, infusion of 50 μmol/L octanoate caused more extensive flavin reduction, with an initial reduction spike, but no oxidation

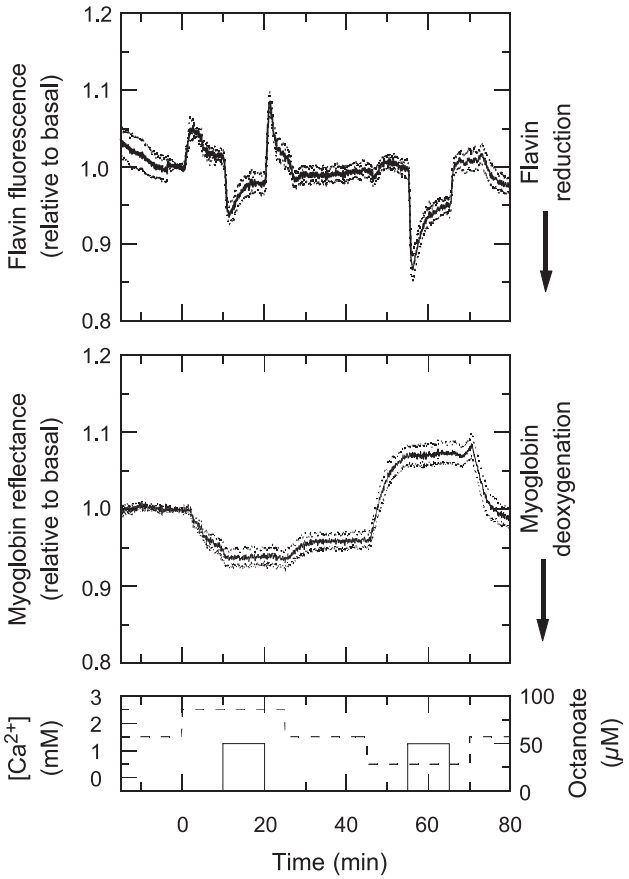

Fig. 5. Effects of calcium concentration changes and 50 μmol/L octanoate on the redox state of flavins and oxygenation of myoglobin in isolated perfused rat hearts. Upper panel: epicardial flavin fluorescence. Downward deflection indicates flavin reduction. Middle panel: the epicardial reflectance difference at 582 and 630 nm. Downward deflection of the trace indicates myoglobin deoxygenation. Flavin and myoglobin panels: the thick line gives the mean for 6 independent experiments; the thin dotted lines, ±SE. Lower panel: calcium (dashed line) and octanoate (solid line) concentrations in the perfusate.

overshoot was observed on the discontinuation of octanoate infusion. The temporal pattern of the redox changes with 0.5 mmol/L octanoate under the same conditions was similar but more intensive.

Raising the calcium concentration caused a slight deoxygenation of myoglobin, whereas its lowering elicited myoglobin oxygenation. Octanoate infusion had no effects on the oxygenation state of myoglobin (Fig. 5), although it did increase oxygen consumption.

4. Discussion

4.1. Substrate selection

The present results show that exogenous octanoate is the main oxidizable substrate in the heart within the concentration range of 50 to 500 μmol/L when the only competing exogenous substrate is 5 mmol/L glucose in the presence of 12 IU/L insulin. Availability has been considered to be one of the main factors controlling the rate of fatty acid oxidation in the heart [22,23]. In the present case, however, even 50 μmol/L octanoate almost completely saturated the myocardial substrate oxidation at both a low and a high workload. The absolute acetyl-CoA input from octanoate into the TCA cycle increased when the octanoate supply was increased to 500 μmol/L, but this was obviously due to the higher work output in the 5 mmol/L glucose plus 500 μmol/L octanoate group. When the work output (Fig. 3) was taken into account, there were no differences in the rate of octanoate oxidation as a function of exogenous octanoate supply in the range of 50 to 500 μmol/L at either a low or a high workload. The current results differ from those of previous work using ^{14}C and hydrogen 3 tracer methods in the isolated rat heart, where an increase in octanoate or palmitate concentration in the range of 0.4 to 2.4 mmol/L increased the rate of fatty acid oxidation, reducing the contribution of glucose to substrate oxidation from 16% to 50% to 4% to 28% [23,24]. The previous ^{13}C study by Jeffrey et al [25] points to a minor role for glucose oxidation, although the substrate supply was more complicated in their experiments: 0.35 mmol/L of a mixture of fatty acids, 5.5 mmol/L glucose, 0.17 mmol/L acetoacetate, and 1.2 mmol/L lactate. This mixture resulted in a 49% to 52% contribution of fatty acids to acetyl-CoA input at both low and high workloads, with no glucose oxidation at all. Similarly, a noticeable contribution of lactate and pyruvate to the TCA cycle flux has been observed using the glutamine isotopomer methodology [26]. To reduce the present system to a case of competition between glucose and a fatty acid, a supraphysiological concentration of insulin was used and no other competing substrates such as lactate, pyruvate, or ketone bodies were present.

It should be pointed out that once-through perfusion had to be used in the experiments with 50 μmol/L octanoate (Table 1) because fatty acid uptake exhausted the octanoate pool during a prolonged closed setup. In a recirculating perfusion, a glucose contribution of up to 30% to the TCA flux was observed, but this high proportion was due to octanoate exhaustion (data not shown).

Our results are in accordance with the "glucose-fatty acid cycle" of Randle et al [27], where fatty acid oxidation suppresses glucose oxidation. This has been thought to be mediated by decreases in the uptake and phosphorylation of glucose and inhibition of phosphofructokinase [28] and pyruvate dehydrogenase [29,30]. The pyruvate dehydrogenase complex (PDH) is inhibited by increases in the reduced/oxidized nicotinamide-adenine dinucleotide ratio (NADH/NAD ratio), ATP/ADP, and acetyl-CoA/CoA ratios. We show here that rapid flavin reduction occurs during octanoate infusion, reflecting an increased mitochondrial NADH/NAD ratio. Monitoring of the oxygenation state of myoglobin, an intracellular oxygen probe, shows that the concomitant redox changes cannot be secondary to oxygen limitation in a hemoglobin-free perfused heart. The initial overshoot in flavin reduction or oxidation at the onset or cessation of octanoate infusion is intriguing. It may be an indication of the regulation of β-oxidation proper, the respiratory chain, or complementary NADH-producing pathways. It has been shown that octanoate infusion into a perfused rat heart causes conversion of the PDH complex to its inactive form [29]. Although octanoate elicits an increase in oxygen consumption, the partial recovery of NAD reduction cannot be due to decoupling, which, according to the PRP/QO_2 plots (Fig. 3), must be negligible. The present observations rather support the interpretation that the fatty acid-induced increase in oxygen consumption is due to an increase in the mitochondrial concentration of the reduced forms of NAD and flavins [5]. Another possibility is autoregulation of β-oxidation, although the increase in oxygen consumption indicates that this can only be marginal relative to the flux. A third, and most plausible, explanation would be that the rebound in reduction upon the commencement of fatty acid infusion is due to the onset of PDH inhibition and that the corresponding overshoot in oxidation at the cessation of infusion is due to a lag in the reactivation of PDH and NADH generation. The critical role of PDH in substrate selection is also supported by a previous observation that an addition of 1 mmol/L hexanoic acid completely inhibited the acetyl-CoA input from glucose but that the conversion of pyruvate to lactate and alanine continued [31]. It has also been observed that an addition of exogenous fatty acids to the perfusate when glucose is the sole external substrate induces a 2.5-fold increase in lactate production, which also points to an inactivation of PDH [32].

Substrate selection in the heart upon an increase in workload remains a contradictory matter. Some studies suggest that increased workload is selective for carbohydrates, with little or no increase in fatty acid oxidation [6-8,33], but there are studies showing that fatty acid and glucose oxidation increase in parallel upon increasing work output [9,10]. The administering of a "physiological" mixture

of substrates, as in the study of Jeffrey et al [25], leads to complete suppression of glucose oxidation, and the workload increase has no effect on the relative contributions of fatty acids and glucose to substrate oxidation. Using ^{13}C isotopomer analysis by gas chromatography–mass spectometry, Comte et al [34] have demonstrated that in the presence of lactate and pyruvate, 0.2 mmol/L octanoate suppresses pyruvate decarboxylation rate to 4.1% of the TCA flux in the isolated perfused rat heart. In the presence of octanoate, an increase in the work output elicited by the of addition of 1 μmol/L norepinephrine tended to slightly increase the pyruvate decarboxylation rate (to 6.6% of the TCA cycle flux). However, norepinephrine activates second-mediator cascades affecting several metabolic pathways so that the principal regulator remains undetermined.

Our results support the view that an increase in cardiac workload does not significantly alter the balance between oxidation of exogenous glucose and external medium-chain fatty acid. This seems to be the case even when the workload is increased by calcium, which is a known activator of phosphoprotein phosphatase, leading to PDH activation and increased usage of glucose [35,36]. Calcium is also known to increase glycogenolysis in muscles through a calmodulin-dependent protein kinase [37]. It appears that inhibitory signals from fatty acid oxidation overrun the PDH stimulation by calcium even in the presence of a moderate fatty acid supply. Glycogenolysis accounts for most of the flux from endogenous nonlabeled sources in the present experiments. As this nonlabeled flux remains unchanged upon an increase in work performance (Table 1), calcium activation of glycogenolysis seems to be of less significance under the conditions used.

Fatty acid import into the mitochondrion is regulated by the malonyl-CoA–sensitive carnitine acyltransferase I located in the outer mitochondrial membrane [38]. Aerobic glycolysis and pyruvate oxidation produce acetyl-CoA in mitochondria, and it has been suggested that this could lead to the export of acetyl-CoA into the cytosol, where its conversion to malonyl-CoA, even in the heart, would inhibit carnitine acyltransferase I and thereby the β-oxidation of long-chain fatty acids [39]. It has been shown, however, that this mechanism does not affect fatty acid oxidation in nonstressful conditions or in the absence of high fatty acid concentrations [23]. In the present case, we used a C_8 fatty acid, which is independent of the carnitine system as far as penetrating the inner mitochondrial membrane is concerned [23,40]. Octanoate was chosen because it is readily available labeled with ^{13}C in multiple positions and because it can be solubilized in protein-free media, where its free concentration is accurately known, and oxygenation of the perfusate does not result in foaming problems. Oleate as an albumin complex lowered the contractile activity of the heart [41], as recently confirmed by Vincent et al [42], such that in the present experiments, long-chain fatty acid was not used because effects of energy consumption were under study.

4.2. Regulation of oxygen consumption

The regulation of oxygen consumption was evaluated here on the basis of flavin redox data. It is known that mitochondrial and whole tissue flavin fluorescence originates mainly from a component of the mitochondrial 2-oxoacid dehydrogenases, namely, lipoamide dehydrogenase, which is in rapid equilibrium with the free NADH/NAD pool of the mitochondrial matrix and serves as a compartment-specific indicator of the redox state of NADH/NAD [43-45].

Extracellular calcium adjustments were used here to elicit changes in mechanical function. This approach was selected to link the present observations to previous results obtained at this laboratory with ^{31}P NMR using isolated rat hearts [41]. Electric pacing was not practicable in the latter experimental setup because electric conductors cannot be placed near the sensitive volume of the NMR probe coil without degrading the signal. It is known that contraction in the heart muscle expresses dependence on extracellular calcium and that, during electric pacing of cardiac myocytes, the contraction frequency is reflected in even mitochondrial calcium transients and diastolic $[Ca^{2+}]_f$ (for a review, see Ref. [46]). The mechanics are linked to calcium in the heart muscle so that it is not possible to alter mechanical performance without affecting the time-averaged intracellular calcium.

It is well established that calcium regulates carbohydrate metabolism and oxidation. Glycogenolysis in muscles is subject to regulation by a calmodulin-dependent protein kinase [37], and the mitochondrial pyruvate dehydrogenase complex and isocitrate and 2-oxoglutarate dehydrogenases are also calcium-regulated [35]. It is evident, however, that work rather than calcium is the main signal during glucose oxidation, as can be seen from Fig. 5, which shows that a calcium increase causes oxidation of mitochondrial flavoproteins (ie, oxidation of matrix NADH). If calcium were the main trigger of the metabolic effect, an NAD reduction could be expected because the abovementioned NADH producers are calcium-activated.

Although the work-related changes in the redox state of NADH/NAD in the mitochondrial matrix were smaller when the hearts were perfused with 5 mmol/L glucose and 50 μmol/L octanoate than in the presence of glucose only (data not shown), minor flavin oxidation occurs upon an increase in Ca^{2+}, and, correspondingly, a decrease in Ca^{2+} results in flavin reduction. This indicates that metabolic control of the NADH consumer (the respiratory chain) during work output transitions is tighter than that of the NADH producer (glycolysis, β-oxidation, and the TCA cycle).

The fact that the redox state of NADH/NAD remained constant during the work output transitions when 0.5 mmol/L octanoate was infused with 5 mmol/L glucose (data not shown) could be interpreted as implying that the regulatory power within the metabolic path upstream the respiratory chain is appreciable. However, because the (free)

NADH/NAD ratio is high and can be estimated to reach a value of 3 to 4 during octanoate oxidation [5,47,48], it is difficult to detect minor changes in this by monitoring NADH, the changes in which are small compared with those in the ratio.

Evaluation of metabolic control by means of the "cross-over theorem" of Chance et al [49] is useful. In brief, a control site can be identified by observing directions of changes in the sizes of pools of metabolic intermediates or amounts reduced and oxidized electron carriers (as in the present case) upon a change in metabolic flux. An oxidation of mitochondrial NADH (decrease in the NADH/NAD ratio) during glucose oxidation then indicates that control (activation) is exerted downstream of NADH (ie, in the respiratory chain). Nonresponsiveness of the redox state of the mitochondrial NADH/NAD pool to a change in metabolic flux during octanoate oxidation indicates that the control is more equally distributed upstream and downstream of mitochondrial NADH/NAD in the metabolic pathway.

Pre–steady state ^{13}C NMR has shown that the oxygen consumption of a working heart has a better correlation with the initial labeling rate of the glutamate pool (via the TCA cycle) than with the phosphocreatinine/ATP ratio [18]. This finding has been interpreted as showing that respiratory activity is regulated by the generation of reducing equivalents in substrate-level dehydrogenations and not by the respiratory chain being adjusted by the ATP/ADP system. In the present case, the redox state of the NADH/NAD couple in the mitochondrial matrix was monitored using flavin as a compartment-specific probe. Flavin oxidation was observed upon an increase in work when either 5 mmol/L glucose or 5 mmol/L glucose plus 50 μmol/L octanoate was used as the substrate. The effect vanished in the presence of a high fatty acid concentration, however, which may indicate a shift toward substrate-level regulation when excessive amounts of reducing equivalents are fed into the mitochondrial respiratory chain. Observations that calcium stimulation of β-oxidation in the heart is negligible [39,50] further support a role for a thermodynamic driving force in increased substrate level regulation during fatty acid oxidation.

Fatty acids intrinsically increase oxygen consumption, and they also cause a prominent reduction of matrix NAD and ubiquinone in the mitochondrial respiratory chain in spite of an increase in the $[ATP]/[ADP]\cdot[P_i]$ ratio [5]. The effect of fatty acids in increasing oxygen consumption cannot be due to their lower P/O ratio, as can be deduced from the PRP/QO_2 plots in Fig. 3.

4.3. Anaplerosis

Our laboratory has previously demonstrated by ^{14}C isotope labeling analysis that an anaplerotic flux of carbon into the TCA cycle is an intrinsic part of metabolism in the heart [51-53]. Lack of anaplerosis leads to a decline in myocardial contractile function, which is prevented or rapidly reversed by the addition of anaplerotic substrates [54]. Peuhkurinen [55] and Peuhkurinen et al [52] pointed out that some cycle intermediates serve as metabolic signals so that their concentrations are regulated by replenishment and disposal mechanisms that then become involved in the regulation of other pathways. This was verified in subsequent ^{13}C studies in which citrate efflux accounted for 17% to 21% of the rate of anaplerotic pyruvate carboxylation, depending on the blood concentration of pyruvate and the presence of octanoate [42,56]. Thus, anaplerosis is essential for the normal functioning of the heart, and it also plays a role in metabolic signal transmission between mitochondria and the cytosol.

In the present case, the anaplerotic rate in the presence of 0.5 mmol/L octanoate was found to be 13% and 29% at high and low workloads, respectively. This is in good agreement with previous results derived from ^{14}C tracer data, which gave a unidirectional anaplerotic flux of 12% in the beating rat heart and 47% in the KCl-arrested heart [52]. Similar results have been acquired by ^{13}C NMR technology in beating or arrested rabbit hearts, which show anaplerosis values of 9% and 32%, respectively [18], although Malloy et al [14] report low values, below 5% of the TCA cycle flux. The anaplerotic fluxes seem in fact to be fairly high, but one should bear in mind that the ^{13}C methodology gives unidirectional fluxes. Thus, simultaneous disposal of the TCA cycle intermediates [55] must occur in TCA in a metabolic steady state, even in a tissue that lacks the capacity for gluconeogenesis or fatty acid synthesis.

Pyruvate carboxylation by pyruvate carboxylase or the reversal of the decarboxylating malate dehydrogenase (malic enzyme), propionyl-CoA carboxylation, the pyridine nucleotide cycle, and glutamine deamidation are potential anaplerotic reactions, but in-myocardium pyruvate carboxylation is a central mechanism of anaplerosis [16,52,53,57]. The present experiments cannot distinguish between these mechanisms, but previous work suggests that carboxylation of pyruvate is important in anaplerosis. In-myocardium pyruvate carboxylation is resistant to biotin deficiency so that the malic enzyme may be responsible, as also suggested by its equilibrium poise in rat heart [58,59]. Although the reason for the increased rate of anaplerosis at a decreased workload is unclear, it could result from an increase in the acetyl-CoA/CoA ratio, as observed upon arrest of the heart [21]. This may also be the case in the present experiments, where the work output is reduced by a decrease in extracellular calcium. The CoA/CoA ratio is a known activator of pyruvate carboxylase.

4.4. Summary

Our results show that even a moderate concentration of medium-chain fatty acids is sufficient to dominate substrate oxidation in the heart muscle, subduing the use of external glucose and endogenous substrates irrespective of the workload. The unresponsiveness of the fractional contribution of octanoate oxidation to calcium-induced workload alterations indicates that glucose oxidation–inhibiting

signals from fatty acid oxidation overrun the PDH stimulation brought about by calcium. Flavoprotein redox data indicate that respiratory control is mainly exerted at the level of the mitochondrial respiratory chain when glucose is the sole external substrate. An addition of fatty acid shifts the respiratory control toward the substrate level. The rate of anaplerosis in the heart muscle is significant during fatty acid oxidation and increases significantly upon a lowering of the work output.

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