Coupling of Mitochondrial Fatty Acid Uptake to Oxidative Flux in the Intact Heart

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ABSTRACT The coordination of long chain fatty acid (LCFA) transport across the mitochondrial membrane (V_{PAL}) with subsequent oxidation rate through β-oxidation and the tricarboxylic acid (TCA) cycle (V_{tca}) has been difficult to characterize in the intact heart. Kinetic analysis of dynamic ^{13}C-NMR distinguished these flux rates in isolated rabbit hearts. Hearts were perfused in a 9.4 T magnet with either 0.5 mM [2,4,6,8,10,12,14,16-$^{13}C_8$] palmitate ($n = 4$), or 0.5 mM ^{13}C-labeled palmitate plus 0.08 mM unlabeled butyrate ($n = 4$). Butyrate is a short chain fatty acid (SCFA) that bypasses the LCFA transporters of mitochondria. In hearts oxidizing palmitate alone, the ratio of V_{TCA} to V_{PAL} was 8:1. This is consistent with one molecule of palmitate yielding eight molecules of acetyl-CoA for the subsequent oxidation through the TCA cycle. Addition of butyrate elevated this ratio; $V_{TCA}/V_{PAL} = 12{:}1$ due to an SCFA-induced increase in V_{TCA} of 43% ($p < 0.05$). However, SCFA oxidation did not significantly reduce palmitate transport into the mitochondria: $V_{PAL} = 1.0 \pm 0.2$ μmol/min/g dw with palmitate alone versus 0.9 ± 0.1 with palmitate plus butyrate. Thus, the products of β-oxidation are preferentially channeled to the TCA cycle, away from mitochondrial efflux via carnitine acetyltransferase.

INTRODUCTION

This work is the first dynamic measurement of long chain fatty acid (LCFA) transport and oxidation in the mitochondria of intact, beating hearts via ^{13}C- NMR spectroscopy. Fatty acids are an important source of energy in the heart, skeletal muscle, and liver. In heart alone, >60% of the energy requirements are met by the oxidation of fatty acids (Belke et al., 1999; Goodwin and Taegtmeyer, 1999), and this demand can increase under conditions of fasting and diabetes (McGarry et al., 1989). Significant changes or defects in fatty acid oxidation have been reported for conditions of hypertrophy, ischemic heart disease, diabetic cardiomyopathies, and congenital defects (Barger and Kelly, 1999; Brivet et al., 1999; Kantor et al., 1999; Lopaschuk et al., 1994); yet little information exists from intact hearts regarding the coordination of relative fluxes for fatty acid transport into the cell and mitochondria, and oxidation via β-oxidation and the tricarboxylic acid (TCA) cycle. This study addresses the problem using a novel method that provides on-line monitoring of the LCFA metabolism using a previously unavailable ^{13}C-enriched fatty acid, [2,4,6,8,10,12,14,16-$^{13}C_8$] palmitate.

The metabolism of LCFA begins with transport across the cell membrane by the fatty acid transporters. Although a number of fatty acid transporters at the sarcolemma have been identified, it has yet to be determined what kinetic effects these transporters have on LCFA metabolism in the cardiomyocyte (Lopaschuk et al., 1994). Once in the cytosol, LCFA binds to the fatty acid binding protein (FABP), or is converted to an acyl-CoA ester, transported across the mitochondrial membrane by the carnitine shuttle, and catabolism through β-oxidation to produce acetyl-CoA. Acetyl-Co is then oxidized through the TCA cycle. Although LCFA metabolism could arguably be regulated by any of the enzymes involved in the pathway, it is generally presumed that the rate-determining step of LCFA metabolism is the transport of the fatty acid across the mitochondria by the carnitine shuttle (Lopaschuk et al., 1994; McGarry and Brown, 1997; Van Der Vusse et al., 1992). In this study we quantify this rate-determining flux and distinguish it from the rate of TCA cycle activity.

Earlier studies have provided information on fatty acid oxidation in healthy and diseased intact heart (Belke et al., 1999; Crass et al., 1969; Goodwin and Taegtmeyer, 1999; Neely at al., 1969; Vanoverschelde et al., 1993). That work was based on either oxygen consumption rates from arterial and venous sampling, PET detection of net label accumulation, or $^{14}CO_2$ release into the extracellular space. Although the methods provided valuable insight into fatty acid metabolism, they could not distinguish specific metabolic pathways in the beating heart. ^{13}C-NMR provides a means to monitor specific pathways in intact functioning tissue by directly observing the dynamic labeling of intermediate metabolite pools (Lewandowski, 1992; Malloy et al., 1989; Robitaille et al., 1993; Weiss et al., 1992).

Until recently, dynamic NMR studies of the whole heart had been limited by the use of labeled substrates, which were both non-physiological and excluded LCFA metabolism. The use of ^{13}C-labeled palmitate alleviates this previous limitation. In this study, the link between LCFA entry

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into the mitochondria and TCA cycle flux was elucidated in the presence of palmitate and an alternative short chain fatty acid (SCFA) to provide the first glimpse of fatty acid uptake and transport kinetics in relation to oxidative flux rates in functioning heart.

MATERIALS AND METHODS

Experimental protocol

Animal use conformed to the "Guide for the Care and Use of Laboratory Animals" (National Research Council, revised 1996). Hearts from Dutch Belted rabbits were isolated and perfused in retrograde fashion as previously described (Johnston and Lewandowski, 1991; Lewandowski, 1992; Neely et al., 1967; O'Donnell et al., 1998). Left ventricular developed pressure (LVDP), heart rate (HR), and myocardial oxygen consumption (MVO_2) were monitored throughout experiments. All hearts were initially perfused with buffer containing 5 mM unlabeled glucose, and coronary effluent was discarded. At the start of each protocol, a ^{31}P-NMR spectrum was acquired to confirm the energetic stability of the preparation. The buffer supply was then switched from media containing glucose to that containing either 0.5 mM unlabeled palmitate ($n = 4$) or 0.5 mM unlabeled palmitate plus 0.08 mM unlabeled butyrate ($n = 4$). Butyrate is a four-carbon SCFA that enters β-oxidation, but bypasses the transport of LCFA into the mitochondria. The potassium salt of palmitic acid was complexed to 3% albumin and dialyzed before use (Johnston and Lewandowski, 1991). Hearts were perfused with unlabeled media for 10 min, and background ^{13}C-NMR signals of naturally abundant (1.1%) ^{13}C were acquired for digital subtraction of background signal.

At the start of the isotope enrichment protocol the buffer supply was then switched to recirculated media containing 0.5 mM [2,4,6,8,10,12,14,16-$^{13}C_8$] palmitate alone, or with 0.08 mM unlabeled butyrate. Sequential ^{13}C-NMR spectra (2.6-min blocks) were acquired for 40 min. The hearts were than removed from the magnet, freeze-clamped, and prepared for in vitro analysis of metabolite content and isotopic enrichment. ^{31}P- and ^{13}C-NMR measurements were acquired at 9.4 T by methods described extensively elsewhere (O'Donnell et al., 1998; Yu et al., 1995, 1997).

Tissue chemistry

Perchloric acid extracts were obtained from ventricle as previously described (Lewandowski, 1992). Glutamate, α-ketoglutarate, citrate, and aspartate concentrations were determined from spectrophotometric and fluorometric techniques (Bergmeyer, 1974). The remainder of the extract was lyophilized and resuspended in D_2O for high-resolution NMR analysis (O'Donnell et al., 1998). From frozen tissue samples, lipids were extracted and separated as described by Bowyer and King (1977). Thin-layer chromatography (TLC) was used to isolated triglycerides for GC-MS analysis. Total tissue tri, di, and monoglyceride content was determined by measuring glycerol release following saponification of the isolated lipids (Lopaschuk et al., 1986).

GC-MS analyses of extracted fatty acid methyl esters were performed on a Hewlett-Packard 1 GC/MS. For GC-MS quantitation, on-line computer-controlled electro-ionization detection was used.

Isotope-labeling scheme

^{13}C-labeled palmitate is transported across the mitochondrial membrane by the carnitine shuttle (CPT1, CPT2, and CAT), is oxidized through β-oxidation, and contributes to the formation of acetyl-CoA enriched at the second carbon position. Acetyl-CoA enters the TCA cycle, and subsequently enriches the C4 carbon position of α-ketoglutarate (Fig. 1). Chemical exchange between α-ketoglutarate and glutamate pools results in labeling at the 4-carbon of glutamate. Oxidation of α-ketoglutarate in the mitochondrial matrix produces succinate, shifting the label to the 2- or 3-carbon position with equal probability. As the carbon label recycles to the first span of the TCA cycle, the recycled label then becomes incorporated at the 2- or 3-carbon of α-ketoglutarate, and ultimately glutamate (Chance et al., 1983). The rate of isotope exchange between these metabolic compartments has been previously reported (LaNoue et al., 1970; Yu et al., 1995).

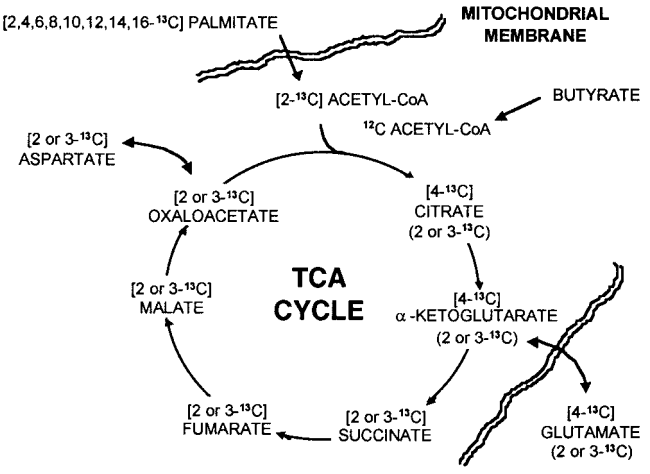

FIGURE 1 Schematic representation of palmitate and butyrate uptake and incorporation into the TCA cycle. ^{13}C from labeled palmitate enters the first span of the TCA cycle as acetyl-CoA, then labels α-ketoglutarate, and exchanges with the glutamate pool to produce [4-^{13}C] glutamate, as shown. Recycling of the label through the second span of the TCA cycle then enriches both the 2- and 3-carbon positions with equal probability. Oxidation of unlabeled butyrate reduces the ^{13}C enrichment of glutamate from labeled palmitate.

Kinetic model and analysis

An existing set of differential equations that describes the concentration history of the ^{13}C in each metabolite and developed in our laboratory was modified to include the additional, rate-determining components of LCFA uptake into the mitochondria. The original model has been used extensively to examine both TCA cycle flux and metabolite transport between the mitochondria and cytosol (O'Donnell et al., 1998; Yu et al., 1995, 1997) in heart.

The inclusion of LCFA metabolism requires the addition of a 10th compartment that reflects the rate-determining transport of LCFA across the mitochondrial membrane and the subsequent accumulation of labeled fatty acid intermediates in the mitochondria. Fig. 2 illustrates the modified compartment model. The sum of intermediary fatty acyl pools within the mitochondria is represented as the 10th compartment (PAL). The rate of LCFA transport across the mitochondrial membrane and into the fatty acid compartment pool is defined as V_{PAL}. At steady state, the rate of metabolic flux from the fatty acid pool into the TCA cycle at citrate is equal to the TCA cycle flux, V_{TCA}, when palmitate is the sole substrate.

The revised model was formulated for a simultaneous, three-parameter fit to the ^{13}C enrichment curves of the 2- and 4-carbon positions of glutamate as detected by NMR. Ten differential equations were used to describe the compartment model, as shown below. Other compartments are handled similarly, as previously described (Yu et al., 1995, 1997). Nonlinear least-square fitting of the model to ^{13}C-NMR data of glutamate 2- and 4-carbon enrichment using the Levenberg-Marquardt method (MATLAB, The MathWorks Inc., Natick, MA) provides TCA cycle flux (V_{TCA}),

the interconversion rate between cytosolic glutamate and mitochondrial α-ketoglutarate (F_1), and the transport rate of LCFA into the mitochondria before oxidation (V_{pal}).

Differential equations describing the pre-steady-state ^{13}C labeling of each metabolite pool of the TCA cycle are listed here for the metabolism of palmitate. PAL is the fractional enrichment level of the mitochondrial fatty acid pool. (i.e., PAL = [^{13}C-fatty acid pool of the mitochondria]/[total fatty acid pool of mitochondria]. F_p is the fractional enrichment of the labeled palmitate substrate (100%). CIT4 is the fractional enrichment level of ^{13}C at the 4-carbon position of citrate (i.e., CIT4 = [(4-^{13}C)CIT]/[CIT]) see Yu et al., 1995, 1997 for additional detail). The equation describing malate enrichment includes anaplerotic and cataplerotic effects (Yu et al., 1995, 1997).

$$\frac{d}{dt}\mathrm{PAL} = \frac{F_p \times V_{PAL}}{[\mathrm{PAL}]} - \frac{V_{TCA}}{[\mathrm{PAL}]}\mathrm{PAL}$$

$$\frac{d}{dt}\mathrm{CIT4} = \frac{V_{TCA}}{[\mathrm{CIT}]}\mathrm{PAL} - \frac{V_{TCA}}{[\mathrm{CIT}]}\mathrm{CIT4}$$

$$\frac{d}{dt}\alpha\mathrm{KG4} = \frac{V_{TCA}}{[\alpha\mathrm{KG}]}\mathrm{CIT4} - \frac{V_{TCA}+F_1}{[\alpha\mathrm{KG}]}\alpha\mathrm{KG4} + \frac{F_1}{[\alpha\mathrm{KG}]}\mathrm{GLU4}$$

$$\frac{d}{dt}\mathrm{GLU4} = \frac{F_1}{[\mathrm{GLU}]}(\alpha\mathrm{KG4} - \mathrm{GLU4})$$

$$\frac{d}{dt}\mathrm{CIT2} = \frac{V_{TCA}}{[\mathrm{CIT}]}(\mathrm{OAA2} - \mathrm{CIT2})$$

$$\frac{d}{dt}\alpha\mathrm{KG2} = \frac{V_{TCA}}{[\alpha\mathrm{KG}]}\mathrm{CIT2} - \frac{V_{TCA}+F_1}{[\alpha\mathrm{KG}]}\alpha\mathrm{KG2} + \frac{F_1}{[\alpha\mathrm{KG}]}\mathrm{GLU2}$$

$$\frac{d}{dt}\mathrm{GLU2} = \frac{F_1}{[\mathrm{GLU}]}(\alpha\mathrm{KG2} - \mathrm{GLU2})$$

$$\frac{d}{dt}\mathrm{MAL2} = \frac{V_{TCA}}{[\mathrm{MAL}]}\left[\frac{1}{2}1\alpha\mathrm{KG2} + \frac{1}{2}\alpha\mathrm{KG4} - (1+y)\mathrm{MAL2}\right]$$

$$\frac{d}{dt}\mathrm{OAA2} = \frac{V_{TCA}}{[\mathrm{OAA}]}\mathrm{MAL2} - \frac{V_{TCA}+F_2}{[\mathrm{OAA}]}\mathrm{OAA2} + \frac{F_2}{[\mathrm{OAA}]}\mathrm{ASP2}$$

$$\frac{d}{dt}\mathrm{ASP2} = \frac{F_2}{[\mathrm{ASP}]}(\mathrm{OAA2} - \mathrm{ASP2})$$

Statistical analysis

Data set comparisons were performed with Student's unpaired, two-tailed *t*-test. Differences in mean values were considered statistically significant at a probability level of <5% ($p < 0.05$).

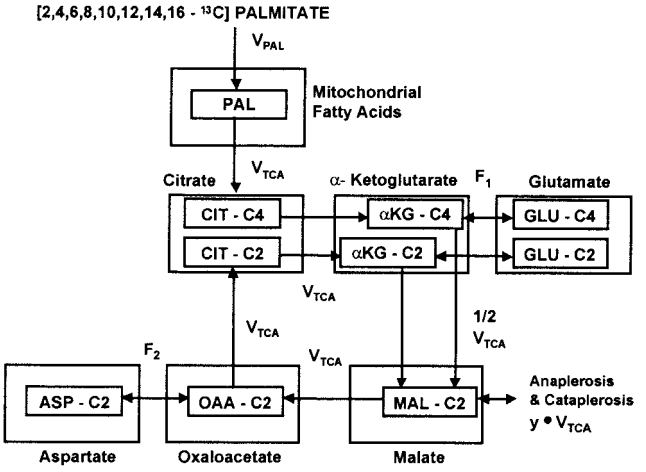

FIGURE 2 Compartment model of metabolic intermediates used in TCA flux analysis of palmitate oxidation. The PAL compartment represents the sum of the fatty acid intermediates within the mitochondria. In addition: CIT, citrate; αKG, α-ketoglutarate; GLU, glutamate; MAL, malate; OAA, oxaloacetate; ASP, aspartate. V_{PAL} is the flux of long chain fatty acid transport into the mitochondria, V_{TCA} is the TCA cycle flux, and F_1 is the interconversion rate between α-ketoglutarate and glutamate. F_2 is set equal to F_1 (Yu et al., 1995, 1997).

RESULTS

Physiological function

Contractile function was similar in both groups of hearts. Before the start of each protocol, hearts initially developed a rate-pressure-product (RPP) of 20,000 ± 4200 mm Hg beats/min with glucose as the sole substrate. Substrate was then switch as outlined above. For hearts perfused with buffer containing 0.5 mM palmitate ($n = 4$), RPP was 16,000 ± 3500 (mm Hg beat/min), and hearts perfused with buffer containing 0.5 mM palmitate plus 0.08 mM butyrate ($n = 4$) developed an RPP of 14,300 ± 1800 (NS). Oxygen consumption with palmitate alone was 21.07 ± 7.42 μmol/min/g dw, and 18.7 ± 9.8 (NS) with palmitate plus butyrate. The ratio of wet to dry heart mass was the same for either group; wet/dry = 9:1.

Dynamic ^{13}C-NMR data

Proton-decoupled ^{13}C spectra of an isolated heart perfused with [2,4,6,8,10,12,14,16-$^{13}C_8$] palmitate are shown in Fig. 3. Peak assignments include glutamate carbons C2, C4, and C3 at 56, 34, and 28 ppm, respectively. Fig. 4 shows the averaged time course of glutamate carbons C2 and C4 enrichment (mean ± SD) for both experimental groups. The fit of the kinetic model to the enrichment data is shown as the solid curves in Fig. 4.

The incorporation of ^{13}C-labeled palmitate into the endogenous triglyceride pool is also apparent in the ^{13}C-NMR spectra of Fig. 3. The broad (5 ppm) resonance centered at 31 ppm corresponds to five of the eight labeled carbons of

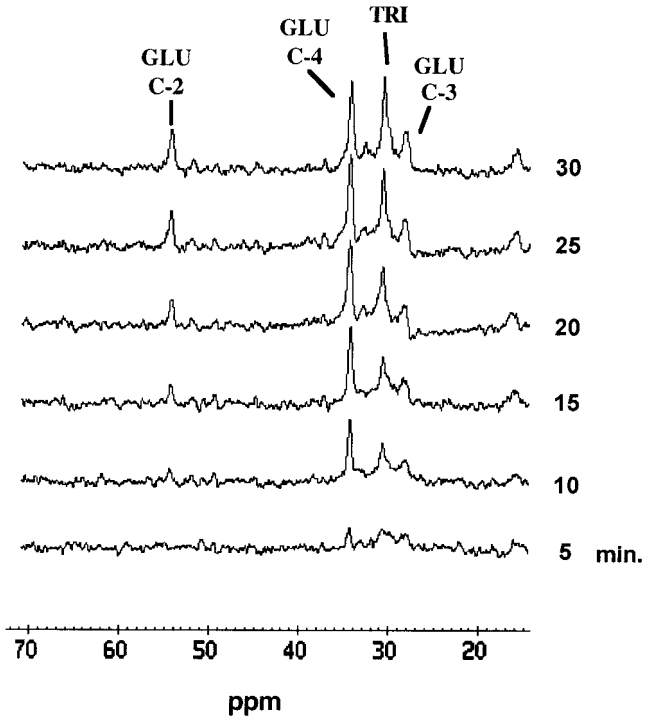

FIGURE 3 Selected ^{13}C spectra from a dynamic data set obtained from an isolated rabbit heart perfused with 0.5 mM [2,4,6,8,10,12,14,16-$^{13}C_8$] palmitate. Peak assignments are glutamate carbon-2 (GLU-C2), glutamate carbon-3 (GLU-C3), and glutamate carbon-4 (GLU-C4). Resonances detected at 30 and 15 ppm correspond to enrichment of triglycerides.

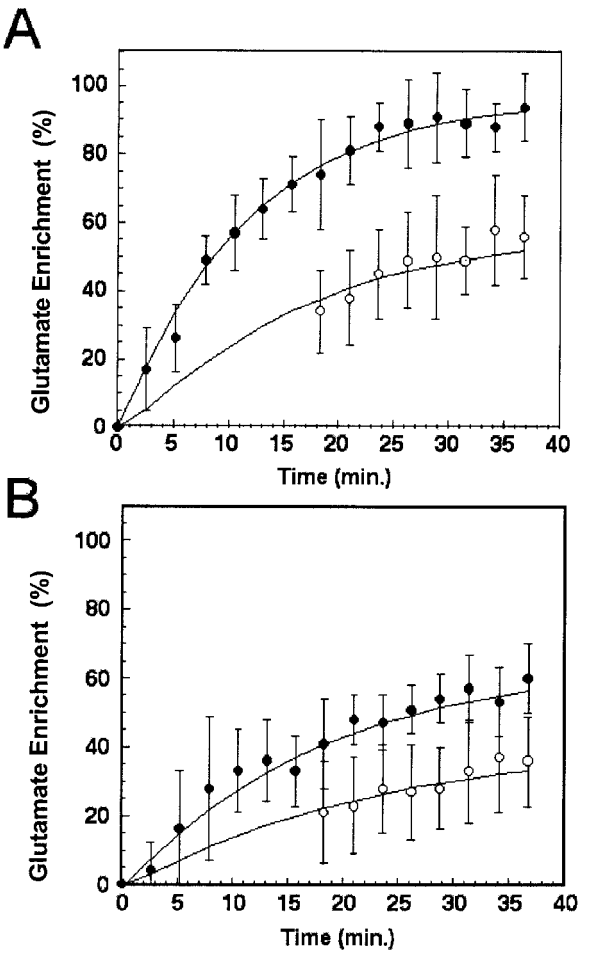

FIGURE 4 Time course of glutamate ^{13}C enrichment and kinetic analysis. The first 15 min of C2 enrichment are not shown due to low enrichment levels below detectable concentrations that do not contribute significantly to the kinetic fit. *Solid circles*, glutamate C4 enrichment; *open circles*, glutamate C2 enrichment; *solid lines*, least-squares fitting of the model to enrichment curves. (*A*) Palmitate group; (*B*) palmitate plus butyrate group.

palmitate. Assignment of labeled triglyceride to the resonance at 31 ppm is based on a high-resolution ^{13}C-NMR spectrum of the labeled palmitate (not shown) and TLC-MS analysis of lipid extracts prepared from the heart samples.

Fatty acid transport and oxidation rates

Fig. 5 illustrates the relationship between LCFA entry into mitochondria and TCA cycle rates from kinetic analysis of the isotope enrichment curves. For hearts oxidizing palmitate alone, TCA cycle flux was eight times the rate of palmitate transport: $V_{TCA} = 7.8 \pm 1.0$ μmol/min/g dw; and $V_{PAL} = 1.0 \pm 0.2$ μmol/min/g dw. The rate of palmitate oxidation can be calculated from the TCA cycle flux and the fraction of acetyl-CoA enriched from labeled palmitate ($F_c = 92\%$) (palmitate oxidation = $V_{TCA} \times F_c/8$). The denominator, eight, accounts for one molecule of palmitate providing eight molecules of acetyl-CoA that enter the TCA cycle. In this protocol, the rate of palmitate oxidation is 0.9 μmol/min/g dw. While palmitate oxidation can be measured by conventional $^{14}C/^{3}H$ methods in intact beating hearts, palmitate uptake (V_{PAL}) and TCA cycle flux (V_{TCA}) cannot. This is the first study to report all three rates for the same intact functioning heart.

In the second experimental group, fatty acid oxidation was perturbed by the presence of an SCFA, butyrate, to uncouple LCFA transport from the oxidation rate in the mitochondria (V_{TCA}). As shown in Fig. 5, the ratio of TCA cycle flux to LCFA transport was perturbed, increasing significantly (12:1) relative to hearts oxidizing palmitate as the sole substrate (8:1). V_{TCA} was 11.2 ± 0.5 μmol/min/g dw, and V_{PAL} was 0.9 ± 0.1 μmol/min/g dw. The increase in V_{TCA} ($p < 0.05$) is consistent with the regulated oxidation of SCFA. The calculated rate of palmitate oxidation is 0.8 μmol/min/g dw.

The third parameter, exchange between α-ketoglutarate and glutamate (F_1), was not affected by the change in substrate availability. In hearts oxidizing palmitate, F_1 was 2.6 ± 0.5 μmol/min/g dw, and in hearts oxidizing palmitate plus butyrate F_1 was 2.9 ± 1.5 ($p > 0.05$).

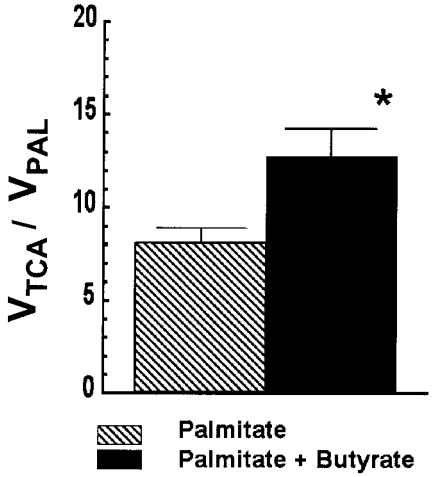

FIGURE 5 Palmitate transport, V_{PAL}, and TCA cycle flux, V_{TCA}, for hearts oxidizing either palmitate (*cross-hatched bar*) or palmitate + butyrate (*solid bar*). V_{TCA} is eight times greater than V_{PAL} in hearts oxidizing palmitate. In hearts oxidizing both palmitate and butyrate, V_{TCA} is significantly increased. This is consistent with increased substrate availability (butyrate) which bypasses the regulatory proteins of palmitate oxidation. ($* p < 0.05$.)

Acetyl-CoA enrichment

The fraction of ^{13}C-enriched acetyl-CoA entering the TCA cycle (F_c) was determined from high-resolution ^{13}C-NMR spectra (Malloy et al., 1989). As expected, F_c values from hearts oxidizing palmitate plus butyrate were significantly lower (54% enrichment) compared to hearts oxidizing palmitate (92%). This is consistent with unlabeled butyrate being incorporated into the acetyl-CoA pool. The anaplerosis-to-citrate synthase ratio (y) was 0.34 ± 0.06 in the palmitate group and 0.29 ± 0.05 in the palmitate plus butyrate group ($p > 0.05$).

Metabolite content

Steady-state metabolite contents are listed in Table 1. α-Ketoglutarate, aspartate, and glutamate were not statistically different between experimental groups ($p > 0.05$). Citrate was significantly higher in hearts oxidizing both palmitate and butyrate.

Triglyceride content and enrichment

Triglyceride content was not statistically different between experimental groups: 29.6 ± 2.6 μmol/g dw in hearts oxidizing palmitate alone, and 23.6 ± 5.7 in hearts oxidizing both palmitate and butyrate ($p > 0.05$). Table 2 provides a list of the major fatty acyl groups composing the triglyceride pool. Hearts perfused with buffer containing labeled palmitate incorporated the LCFA into the endogenous triglyceride pool. From MS analysis 8% of the triglyceride pool (or 22 ± 4% of the C16:0 palmitate isomer pool) contained acyl chains from the exogenous [2,4,6,8,10,12,14,16-$^{13}C_8$] palmitate. In hearts oxidizing both labeled palmitate and unlabeled butyrate, there was no greater accumulation of palmitate in the triglyceride pool. Nine percent of the acyl groups in the triglyceride pool (or 24 ± 7% of the C16:0 palmitate isomer pool) was from the labeled palmitate.

TABLE 2 Fraction of long chain fatty acid isomers making up the triglyceride pool as determined by GC-MS analysis

	Perfusate Condition	
Long Chain Fatty Acid Isomer	Palmitate	Palmitate + Butyrate
C12:0 Laurate	0.00 ± 0.01	0.00 ± 0.01
C14:0 Myristate	0.03 ± 0.01	0.03 ± 0.01
C15:0 Pentadecanate	0.01 ± 0.00	0.01 ± 0.01
C16:0 Palmitate	0.35 ± 0.03*	0.38 ± 0.02†
C16:1 Palmitoleate	0.03 ± 0.02	0.02 ± 0.02
C17:0 Heptadecanoate	0.01 ± 0.01	0.01 ± 0.01
C18:0 Stearate	0.13 ± 0.01	0.17 ± 0.02
C18:1 Oleate	0.22 ± 0.01	0.24 ± 0.02
C18:2 Linoleate	0.19 ± 0.01	0.13 ± 0.04
C18:3 Linolenate	0.01 ± 0.01	0.01 ± 0.01

*22 ± 4% of the C16:0 fatty acid pool was labeled palmitate.
†24 ± 7% of the C16:0 fatty acid pool was labeled palmitate.

DISCUSSION

This study is the first to elucidate the rate-determining component of LCFA entry into mitochondria in the intact beating heart, and to distinguish this transport process from the oxidative activity of the TCA cycle. We also determine substrate oxidation rate and assess the competitive oxidation of long and short chain fatty acid substrates. Although others have examined substrate oxidation and competition by conventional ^{14}C/^{3}H radiotracer techniques (Belke et al., 1999; Crass et al., 1969; Lopaschuk et al., 1986, 1997; Oram et al., 1973), such measurements do not distinguish between LCFA transport and TCA cycle activity as distinct components of overall oxidation in the beating heart. Additionally, we demonstrate the progressive incorporation of

TABLE 1 Steady-state metabolite and triglyceride content in intact hearts

Perfusate	Glutamate	α-Ketoglutarate	Aspartate	Citrate	Triglyceride
Palmitate	20.0 ± 2.9	0.16 ± 0.09	5.13 ± 0.93	0.49 ± 0.35	29.6 ± 2.6
Palmitate + Butyrate	22.3 ± 6.1	0.42 ± 0.34	5.79 ± 1.44	1.84 ± 1.05*	23.6 ± 5.7

All values are means ± SD. Metabolite concentrations are measured by enzymatic assays and listed as μmol/g dry tissue wt.
*$p < 0.05$.

the labeled substrate into the triglyceride pool. As the fate of free fatty acids in the myocyte continues to be an important aspect of energy metabolism in both normal and diseased hearts, this work provides the basis for future studies on the highly regulated processes of LCFA transport.

While previous efforts to monitor fatty acid metabolism in the heart have relied on extracellular measurements, this study provides new information on the regulatory role of LCFA transport on oxidative metabolism via the first direct, on-line observation of dynamic metabolic events inside the myocytes of functioning hearts. This study also demonstrates, for the first time, the practical application of stable isotope kinetics in the elucidation of metabolite transport rates that regulate oxidative flux in the intact, beating heart. The administration of the novel, ^{13}C-enriched long chain fatty acid, [2,4,6,8,10,12,14,16-$^{13}C_8$] palmitate, enabled sequential acquisition of ^{13}C-NMR spectra from the whole heart. The result of this approach facilitated a simple kinetic analysis based on the evolution of the detected enrichment pattern that has been previously described for fuels with shorter carbon chain length, but of less physiological significance than that of palmitate (Lewandowski, 1992; Robitaille et al., 1993; Weiss et al., 1992).

TCA cycle flux and fatty acid transport in hearts oxidizing palmitate

The TCA cycle flux was $V_{TCA} = 7.8 \pm 1.0$ μmol/min/g dw, and the transport rate of palmitate into the mitochondria was $V_{PAL} = 1.0 \pm 0.2$. The ratio (V_{TCA}/V_{PAL}) nearly matches the stoichiometric ratio predicted for palmitate conversion to acetyl groups. That is, palmitate passes through β-oxidation seven times to produce eight molecules of acetyl-CoA. Each molecule of acetyl-CoA is metabolized in one turn of the TCA cycle. Thus, the TCA cycle rate should be eight times the rate of palmitate transport when palmitate is the sole substrate. The model accurately determined this ratio from the fit to the NMR data.

As a separate entity, we find the rate of palmitate oxidation is 0.9 μmol/min/g dw. This value compares well with the oxidative rate measured by conventional ^{14}C/^{3}H techniques (0.2–1.3 μmol/min/g dw) (Belke et al., 1999; Crass et al., 1969; Lopaschuk and Barr, 1997; Lopaschuk et al., 1986; Oram et al., 1973; Saddik et al., 1993). The rate matches the rate of palmitate transport into the mitochondria (V_{PAL}). This match is expected under these steady-state metabolic conditions. As previously stated, while palmitate oxidation can be measured by ^{14}C/^{3}H methods, palmitate transport (V_{PAL}) and TCA cycle activity (V_{TCA}) cannot.

TCA cycle flux and fatty acid transport in hearts oxidizing palmitate and butyrate

Palmitate and butyrate, the long and short chain saturated fatty acids used in these experiments, enter the mitochondria through different processes. Unlike palmitate, butyrate does not depend upon transport across the cell membrane by the fatty acid transporter, activation in the extra-mitochondrial space, or transport into the matrix by the carnitine shuttle (CPT1, CPT2, and CAT). This SCFA readily crosses both the sarcolemmal and mitochondrial membranes, and is activated to the CoA ester in the matrix. Consequently, while LCFA utilization may be tightly regulated by the activity of the carnitine shuttle, SCFA bypass this control network. We wanted to determine how bypassing this highly regulated and rate-determining step would affect metabolic rates.

In hearts oxidizing both palmitate and butyrate, TCA cycle flux was $V_{TCA} = 11.2 \pm 0.5$ μmol/min/g dw and palmitate transport was $V_{PAL} = 0.9 \pm 0.1$. The ratio ($V_{TCA}/V_{PAL} = 12$) is greater compared to hearts oxidizing palmitate alone ($V_{TCA}/V_{PAL} = 8$). The increase is consistent with the oxidation of the second substrate, butyrate. A 41% drop in the fractional enrichment of acetyl-CoA (F_c) also follows oxidation of butyrate. Importantly, adding the second substrate did not reduce the transport rate of palmitate into the mitochondria, V_{PAL}, despite constant oxygen consumption and workload. This results provides important insight into the transport processes regulating fatty acid oxidation.

There are a number of proposed mechanisms regulating palmitate transport into the mitochondria. Oram et al. (1973) have shown that when palmitate is provided as a sole substrate and the concentration is raised from 0 to 0.4 mM, uptake increases proportionately. As the level of exogenous palmitate is raised from 0.6 to 1.2 mM, no further increase in uptake is observed. It is proposed that the oxidation of the fatty acid may be limited at high concentrations by either the activity of the TCA cycle or the availability of cytosolic CoA and carnitine (Oram et al., 1973). In our experiment, butyrate does not require cytosolic carnitine or CoA. Therefore, this regulatory mechanism is not expected to limit palmitate transport. When we bypass this regulatory mechanism, we find TCA cycle activity increases. Thus, TCA cycle flux may not limit palmitate oxidation at these concentrations.

Others have found that when palmitate is provided in combination with pyruvate or glucose, there is a dramatic increase in carnitine acetyltransferase activity and malonyl CoA content (Lysiak et al., 1986; Saddik et al., 1993). A subsequent drop in fatty acid oxidation is consistent with the inhibitory effect of malonyl-CoA on CPT1. It is proposed that the acetyl-CoA generated from pyruvate may be readily accessible to this inhibitory pathway. On the contrary, Lysiak (1986) and Abdel-aleem et al. (1996) have shown that the acetyl-CoA generated from the β-oxidation of fatty acids may not have the same access and may be preferentially shuttled to the TCA cycle. Our data support their results described for isolated mitochondria and myocytes. We observe an increase in TCA cycle activity without an inhibition of palmitate transport in hearts oxidizing only fatty acids.

Our results were observed at tightly controlled exogenous fatty acid concentrations. At higher concentrations of butyrate (0.10 mM), we observed complete inhibition of palmitate oxidation ($V_{PAL} = 0$, $F_c = 0$). We hypothesize that inhibition was not due to inhibition at CPT1, but rather preferential utilization of mitochondrial CoA by butyrate to the exclusion of palmitate.

Importantly, the purpose of our study was not to further elucidate the feedback mechanisms acting on CPT1. The intent was to examine the dynamic interactions of flux coupling between LCFA transport and TCA rate. This study is the first to report these rates for intact functioning heart. Indeed, this study is an important prelude to in vivo MRS studies because the in vivo heart oxidizes a menu of available fuels. The oxidative flux supported by alternate fuels must be distinguished from the transport and entry rates of the predominant fatty acid fuels for oxidative metabolism.

$^{14}C/^{3}H$ radiotracer versus ^{13}C-NMR measurements of oxidative flux

With NMR we can detect and distinguish among TCA cycle flux, LCFA transport, substrate oxidation, and metabolite exchange between the mitochondria and cytosol. In contrast, with conventional $^{14}C/^{3}H$ radiotracer measurements, only substrate oxidation and glycolysis rates are measure in intact tissue (Lopaschuk and Barr, 1997). These measurements are made by providing the heart with ^{14}C or ^{3}H radio-labeled substrates. The rate of $^{14}CO_2/^{3}H_2O$ release provides a measure of the oxidation of a particular radiolabeled carbon source. The experimental system must be tightly sealed because measurements are made external to the heart and CO_2 could be lost as it passes from the mitochondria to the CO_2 trapping solution. This approach does not lend itself well to in vivo studies. With ^{13}C-NMR, metabolic rates are assessed based on direct detection of metabolite enrichment inside the cardiomyocytes of the intact tissue. ^{13}C-NMR can be done in vivo, and could be advanced to give even more quantitative data on specific enzymatic reactions of metabolism.

Long chain fatty acid oxidation and the kinetic model

In formulating the kinetic model, the oxidation of LCFAs requires the addition of a compartment that reflects the rate-determining transport of LCFA across the mitochondrial membrane and the subsequent accumulation of fatty acid intermediates in the mitochondria. Consideration was given as to whether the intermediary fatty acid pools of the mitochondria would affect the ^{13}C enrichment rate of glutamate. In the compartment model, the FA pool represents the sum of the fatty acid intermediates between uptake at the mitochondrial membrane and entry into the TCA cycle at citrate. The concentration of the larger pools of metabolites were taken from literature on hearts perfused under comparable conditions; fatty acyl-CoA 0.10 μmol/g dw (Lopaschuk et al., 1986; Whitmer et al., 1978), fatty acyl-carnitine 0.6 μmol/g dw (Idell-Wenger et al., 1978; Lopaschuk et al., 1990; Whitmer et al., 1978); and acetyl-CoA 0.2 μmol/g dw (Randle et al., 1970; Saddik et al., 1993). The effect of possible variations or errors in these pool sizes, plus contributions from the smaller intermediate metabolite pools of β-oxidation, were evaluated by sensitivity analysis with 500% changes in pool sizes. Results from kinetic simulations showed that even the most dramatic changes in these relatively small pool sizes will have minimal impact on the labeling kinetics of glutamate.

CONCLUSIONS

In conclusion, sequential ^{13}C-NMR spectra were obtained from intact hearts supplied perfusate containing the long chain fatty acid, palmitate, or palmitate co-infused with the short chain fatty acid, butyrate. The use of [2,4,6,8,10,12,14,16-$^{13}C_8$] palmitate, uniquely labeled at every other carbon, provided a means to monitor the rate-determining and competitive oxidation of long chain versus short chain fatty acids. A kinetic model was developed for the analysis of ^{13}C enrichment data to evaluate the transport rate of palmitate relative to the TCA cycle flux. As expected, TCA cycle flux followed fatty acid transport when palmitate was provided as the sole substrate. When the transport of the LCFA was bypassed by the availability of a short chain fatty acid, TCA cycle activity increased and LCFA transport remained constant. This result supports the hypothesis that the products of β-oxidation are preferentially channeled to the TCA cycle, and away from mitochondrial efflux via carnitine acetyltransferase. These ^{13}C-NMR measurements are not only sensitive to TCA activity, as previously reported, but also to the highly regulated transport processes that ultimately determine rates of long chain fatty acid oxidation.

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