14(*R*,*S*)-[^{18}F]Fluoro-6-thia-heptadecanoic acid as a tracer of free fatty acid uptake and oxidation in myocardium and skeletal muscle

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Abstract. 14(*R*,*S*)-[^{18}F]Fluoro-6-thia-heptadecanoic acid ([^{18}F]FTHA) is a long-chain fatty acid substrate for fatty acid metabolism. [^{18}F]FTHA has been used to study fatty acid metabolism in human heart and skeletal muscle. It has been suggested that the rate of radioactivity accumulation in the myocardium reflects the beta-oxidation rate of free fatty acids (FFAs). However, the net accumulation of FFAs in tissue always represents the sum of FFA oxidation and incorporation into triglycerides. The fraction of [^{18}F]FTHA entering directly into mitochondria for oxidation has not been previously measured. Eight anaesthetized pigs were studied with [^{18}F]FTHA and positron emission tomography (PET). Immediately after each PET experiment, tissue samples from myocardium and skeletal muscle were taken for the isolation of mitochondria and measurements of radioactivity accumulation, and for intracellular [^{18}F]FTHA metabolite analysis. Fractional [^{18}F]FTHA uptake rates were calculated both by graphical analysis of PET data and by measuring ^{18}F in the tissue samples. Fractional [^{18}F]FTHA uptake rates based on the analysis of tissue samples were 0.56±0.17 ml g^{-1} min^{-1} and 0.037±0.007 ml g^{-1} min^{-1} for myocardium and skeletal muscle (mean ± SD), respectively. The myocardial results obtained from the PET data (0.50±0.11 ml g^{-1} min^{-1}) were similar to the values obtained from the tissue samples (r=0.94, P=0.002). We also found that 89%±23% (mean±SD, n=7) of the ^{18}F entered mitochondria in myocardium, as compared with only 36%±15% (mean±SD, n=7) in skeletal muscle. Intracellular [^{18}F]FTHA metabolite analysis showed that a major part of [^{18}F]FTHA is metabolized in the mitochondria in the heart. Our data suggest that ~89% of [^{18}F]FTHA taken up by the heart enters mitochondria. This supports the hypothesis that [^{18}F]FTHA traces FFA beta-oxidation in the heart. In contrast to this, only ~36% of [^{18}F]FTHA accumulated in skeletal muscle appears to directly enter mitochondria; the majority is taken up by the other cell fractions, suggesting that in skeletal muscle [^{18}F]FTHA traces FFA uptake but not specifically FFA beta-oxidation.

Keywords: Myocardial metabolism – Skeletal muscle metabolism – Fatty acids – Positron emission tomography

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Introduction

Free fatty acids (FFAs) are the main fuel of heart and skeletal muscle. After uptake by the tissues, FFAs enter mitochondrial beta-oxidation or are incorporated into the triglyceride pool. In the myocardium, 80%–84% of FFA uptake is directly oxidized and only a small fraction enters the intracellular lipid pool (triglycerides) in the fasting state [1, 2, 3]. In contrast, in skeletal muscle the oxidized fraction appears to be smaller, and the larger fraction is stored as triglycerides [4].

14(*R*,*S*)-[^{18}F]Fluoro-6-thia-heptadecanoic acid ([^{18}F]FTHA) has recently been used to study fatty acid metabolism in humans [5, 6, 7, 8, 9, 10]. [^{18}F]FTHA has been used in

determining skeletal muscle and myocardial fatty acid uptake rates [8, 10], and these studies yielded consistent results with earlier FFA uptake measurements with various methods.

[^{18}F]FTHA is a long-chain fatty acid substrate for fatty acid metabolism [11]. [^{18}F]FTHA has a high first-pass uptake. After transport into the mitochondria it enters beta-oxidation and after formation of two acetyl-CoA molecules, the rest of the chain is trapped in the mitochondria. According to the previous studies, inhibition of FFA oxidation by use of POCA (carnitine palmitoyltransferase I inhibitor 2[5(4-chlorophenyl)pentyl]oxirane-2-carboxylate) resulted in a 81%–87% decrease in [^{18}F]FTHA uptake in rat heart [11]. Thus, accumulation of [^{18}F]FTHA has been suggested to trace FFA beta-oxidation in the heart [11, 12]. However, there are no direct measurements of which fraction of [^{18}F]FTHA enters mitochondria for beta-oxidation and which goes to triglyceride synthesis.

In order to measure the accumulation of [^{18}F]FTHA in mitochondria and other cell fractions, myocardial and skeletal muscle tissue samples from eight pigs were analysed after tracer injection. The mitochondrial fraction was isolated and the total radioactivity and metabolite fractions were measured.

Materials and methods

Study protocol. Eight pigs (weight 25–30 kg) were studied. The animal preparation protocol was reviewed and approved by the Ethical Committee for Animal Experiments of the University of Turku. The pigs had fasted more than 24 h before starting anaesthesia. Anaesthesia was induced with ketamine (1 g) intramuscularly and diazepam (10 mg) intravenously and maintained with intravenous infusion of ketamine (350 mg/h) and pancuron (15 mg/h) (Fig. 1). Animals were ventilated mechanically through tracheal intubation with normal room air (~8 l/min, Respiration Pump Mod 607, Harward Apparatus Co., Inc. Millis, Mass., USA). Medial sternotomy was performed and the pericardium was opened. Left carotid artery (samples for determination of ^{18}F and microsphere radioactivity and FFA, lactate, glucose and insulin concentrations), left jugular vein (infusion of [^{18}F]FTHA), coronary sinus (samples for plasma lactate) and left atrium (infusion of microspheres) were cannulated. Left ventricular dimensions and wall thicknesses were measured with echocardiography. The open chest was closed and myocardial blood flow was measured with radioactive microspheres infused into the left atrium. [^{18}F]FTHA (677±166 MBq) was infused over 90 s into the jugular vein, and dynamic PET scanning for 32 min (12×15 s, 4×30 s, 2×120 s, 1×180 s, 4×300 s) was started. Twenty to 23 blood samples for measurements of arterial plasma radioactivity were taken during the PET scanning (11–13 of these samples were taken during the first 5 min after injection). Thereafter the pigs were euthanized with intracardiac injection of potassium and tissue samples were obtained from myocardium and skeletal muscle.

Production of [^{18}F]FTHA and analysis of blood samples. [^{18}F]FTHA was synthesized by nucleophilic labelling of benzyl 14(*R*,*S*)-tosyloxy-6-thia-heptadecanoate with [^{18}F]fluoride [7, 8, 13]. The reactivity of the [^{18}F]fluoride was enhanced by using aminopolyether and potassium carbonate. After formation of the ^{18}F-labelled fatty acid complex, the protecting groups were removed by basic hydrolysis with 2 *M* potassium hydroxide. [^{18}F]FTHA was separated by preparative high-performance liquid chromatography (HPLC); the HPLC eluent was subsequently evaporated and the final product formulated in a 4% albumin solution, which was then sterile filtered. The product identity has been verified by an authentic reference of unlabelled FTHA. The radiochemical purity exceeded 98% as analysed by HPLC and radio thin-layer chromatography (radio-TLC). The HPLC was performed using a Waters Radial Pak C-18 column and a solution of methanol/water/acetic acid (85:15:0.4) as eluent. The radio-TLC was performed on reverse phase plates (Merck, DC-alufolien RP-18 F_{254}) using methanol/water/acetic acid (100:5:0.4) as mobile phase.

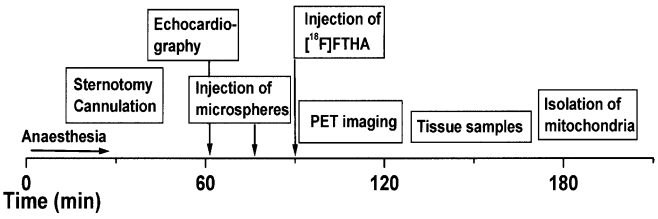

Fig. 1. Study protocol

Fractions of [^{18}F]FTHA and radioactive metabolites were determined from arterial plasma samples taken at 2, 5, 10, 15, 20, 25 and 30 min after [^{18}F]FTHA injection. To an aliquot of 250 µl from the samples was added 350 µl of a methanol/acetic acid solution (100:0.4). The samples were centrifuged and the supernatant analysed by HPLC equipped with a radioactivity detector. The column (Waters µBondapac C-18, 7.8 mm×300 mm) was eluted with a solution consisting of methanol/water/acetic acid (85:10:0.4) [7, 8]. Standards were prepared by adding [^{18}F]FTHA to non-radioactive plasma, followed by similar plasma sample handling.

Image acquisition, processing and corrections. The pigs were positioned in a 15-slice ECAT 931/08–12 tomograph (Siemens/CTI Inc., Knoxville, Tenn., USA) with a measured technical axial resolution of 6.7 mm and 6.5 mm in plane. To correct for photon attenuation, transmission scanning was performed for 15 min for thoracic regions prior to the emission scan. All data were corrected for deadtime, decay and photon attenuation and reconstructed in a 128×128 matrix. The final in-plane resolution in reconstructed and Hann-filtered (0.3 cycles/pixel) images was 9.5 mm full-width at half-maximum. The myocardial time-activity curves were corrected for spill-over from cavity and partial volume using information from echocardiographic measurements of wall thickness and left ventricular diameter as explained previously [14]. Transaxial PET slices were visually aligned and 15–20 elliptical regions of interest (ROIs) were placed on the left ventricular myocardium [14].

Heart and skeletal muscle sample preparation. Six myocardial samples from the left ventricular anterior and posterior walls and four samples from skeletal muscle (m. quadriceps femoris, vastus medialis) were obtained (Fig. 2). The first samples (A) from myocardium and skeletal muscle were used for isolation of mitochondria (described in detail below). The second samples (B) were directly homogenized and used in metabolite analysis by radio-TLC. Samples C and D were used for determination of ^{18}F (5–7 h after injection of [^{18}F]FTHA) and cerium-141 (>24 h after injection of [^{18}F]FTHA). The radioactivity measurements were performed using an automatic gamma counter (Wizard 1480 3"; Wallac, Turku, Finland). Radiolabelled ^{141}Ce microspheres (15 µm, NEN-TRAC

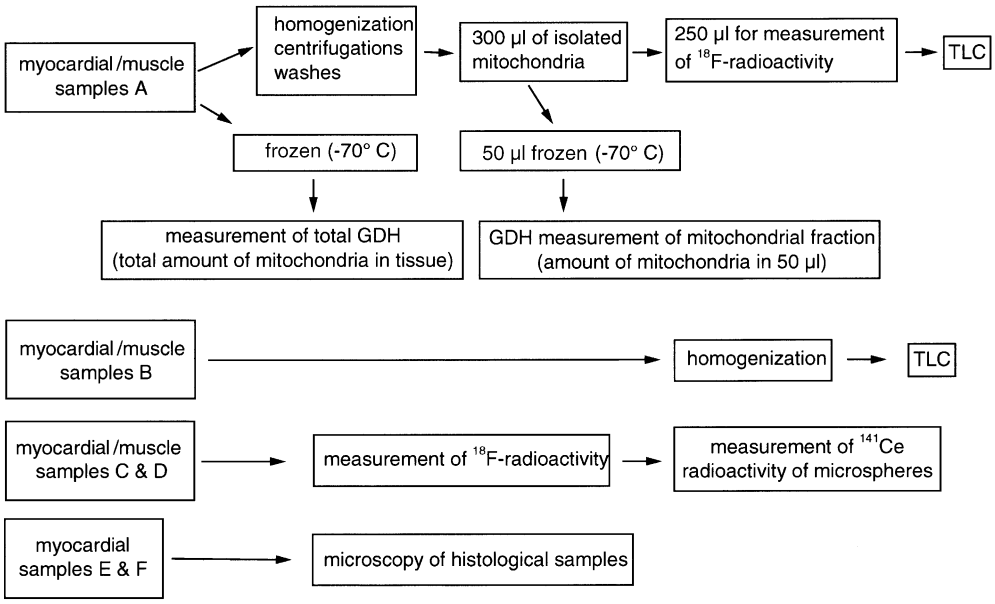

Fig. 2. Schematic presentation of myocardial and skeletal muscle sample preparation. *GDH*, Glutamate dehydrogenase; *TLC*, thin-layer chromatography

Microspheres, NEN Life Science Products, Boston, Mass., USA) were used to measure myocardial and skeletal muscle blood flow according to the method of Heymann et al. [15]. In addition, samples E and F from myocardium were forwarded to histological analysis in order to confirm normal cellular structure.

Calculation of the [^{18}F]FTHA fractional uptake and FFA uptake indices. The uptake indices for the heart were calculated both from PET data and from samples C and D. The values for skeletal muscle were based on tissue samples only. Metabolite-corrected plasma time-activity curves and myocardial time-activity curves obtained from PET data were analysed graphically [8, 16]. The slope of the plot in the graphical analysis is equal to the fractional uptake constant of [^{18}F]FTHA, K_i (ml g^{-1} min^{-1}). Tissue sample fractional uptake rates (K_i) were calculated by dividing the decay-corrected ^{18}F of the tissue sample by the area under the metabolite-corrected plasma activity curve. The FFA uptake indices (µmol g^{-1} min^{-1}) were calculated by multiplying K_i by the mean serum FFA concentration during the [^{18}F]FTHA study.

Isolation of mitochondria. The isolation of mitochondria was performed on both myocardial and skeletal muscle samples (samples A, Fig. 2) weighing on average 90±21 mg and 105±42 mg, respectively. The samples were homogenized using a glass homogenizer in a solution containing 100 m*M* KCl, 50 m*M* KH_2PO_4, 50 m*M* Tri3, 5 m*M* $MgCl_2 \cdot 6H_2O$ and 1 m*M* EDTA. The homogenate was first centrifuged for 3 min at 3,000 rpm, and then the supernatant was centrifuged for 3 min at 14,000 rpm. The supernatant was removed and 1 ml of sucrose buffer solution (180 m*M* sucrose) was added to the pellet, which was recentrifuged for 3 min at 14,000 rpm. The supernatant was removed and sucrose buffer solution was added to the pellet containing mitochondria, so that the total volume of the mitochondrial fraction was 300 µl. Two hundred and fifty microlitres of the mitochondrial fraction was forwarded first to measurement of ^{18}F and thereafter to radio-TLC metabolite analysis (described below). Glutamate dehydrogenase (GDH) is a mitochondrial enzyme which has been used in determining the recovery of mitochondria from heart and skeletal muscle tissue [17, 18]. GDH activity of the mitochondrial fraction was measured from the remaining 50 µl of the mitochondrial fraction as previously described [18]. Analysis of total GDH activity was also done on whole myocardial and skeletal muscle samples as previously described [18]. The GDH activity of the mitochondrial fraction was compared with the total GDH activity in order to measure the yield of mitochondria after isolation.

Analysis of intracellular metabolites. Radio-TLC was used to analyse the ^{18}F-labelled metabolites from tissues (myocardium and skeletal muscle) and isolated mitochondria. Radio-TLC was used instead of HPLC owing to its high sensitivity in detection of radioactivity. The radio-TLC method was cross-calibrated with the analytical HPLC method.

Samples were precipitated in methanol/acetic acid (100:0.4) and centrifuged. The supernatant was applied onto TLC plates (Merck, DC-alufolien RP-18 F_{254}) and eluted in a methanol/water/acetic acid (100:5:0.13) solution. Standards were prepared as described in the section "Production of [^{18}F]FTHA and analysis of blood samples". Distribution of radioactive compounds on the plate was measured by digital autoradiography (Bio-Rad Model GS-250 and Bio-Rad Imaging Screen-HS, Richmond, Calif., USA).

Other measurements. Blood pressure was measured every 15 min from the carotid artery using a Uniflow 43-600 (Baxter Healthcare Corporation, Uden, Holland) and a Patient Data Monitor 565A (Kone Corporation, Espoo, Finland). M-mode and two-dimensional echocardiography were performed with Acuson 128XP/5 (Acuson Inc., Calif., USA) immediately after catheterization (~60 min after induction of anaesthesia) for measurement of left ventricular cavity dimensions and wall thickness. Serum FFAs were determined by an enzymatic method (ACS-ACOD Method, Wako Chemicals GmbH, Neuss, Germany). Serum-free insulin concentrations were measured using a double antibody radioimmunoassay (Pharmacia Insulin RIA kit, Pharmacia, Uppsala, Sweden) after precipitation with polyethylene glycol. Plasma lactate and serum glucose were measured by enzymatic methods using a Hitachi 917 Automatic Analyzer (Boehringer Mannheim, Mannheim, Germany).

Statistics. Comparisons were done with paired Student's *t* tests or the Wilcoxon matched pairs test where appropriate. Pearson's correlations were calculated. The statistical calculations were performed

with the Statistica (StatSoft, Tulsa, Oklahoma, USA) statistical program. The results are expressed as mean ± standard deviation (SD).

Results

The characteristics of the study animals, blood substrate concentrations, left ventricular dimensions and haemodynamic parameters are shown in Table 1. The arterial–sinus coronarius difference in plasma lactate was 0.64±0.58 m*M*, indicating net lactate uptake in the heart (P=0.02). In the histological analysis, the myocardium of each pig was normal.

FFA uptake

Fractional [^{18}F]FTHA uptake rates calculated using tissue samples from myocardium and skeletal muscle were 0.56±0.17 and 0.037±0.007 ml g^{-1} min^{-1}, respectively. Thus, fractional [^{18}F]FTHA uptake was ~15-fold higher in the myocardium than in the skeletal muscle (P<0.001). Calculated FFA uptake indices were 12.1±5.3 and 0.84±0.48 µmol 100 g^{-1} min^{-1} for myocardium and skeletal muscle tissue samples, respectively. The fractional myocardial [^{18}F]FTHA uptake rate calculated from PET data was 0.50±0.11 ml g^{-1} min^{-1} and it correlated well with the fractional [^{18}F]FTHA uptake obtained from myocardial tissue samples (r=0.94, P=0.002, n=7, Fig. 3). There was no association between blood flow and fractional [^{18}F]FTHA uptake.

Accumulation and intracellular metabolism of [^{18}F]FTHA

Based on the measurements of the isolated mitochondria, 89%±23% of total ^{18}F was detected in this fraction in the myocardium (Table 2). Radio-TLC analyses of isolated heart mitochondria were successful on only five pigs, but showed that only 7.0%±2.5% of ^{18}F was unmetabolized [^{18}F]FTHA in mitochondria. In contrast, radio-TLC analysis of homogenized heart muscle samples (which contained all cell fractions, including mitochondria) showed that 32%±12% of ^{18}F was unmetabolized [^{18}F]FTHA. Taking into account that about 89% of the total radioac-

Table 1. Characteristics of the study animals (mean±SD)

No.	8
Weight (kg)	27±3
Serum FFAs (µ*M*)	239±147
Serum glucose (m*M*)	8.0±0.6
Plasma lactate (m*M*)	4.9±2.5
Serum insulin (p*M*)	41±13
Blood pressure (mmHg)	90±6/70±7/58±7
Heart rate (beats min^{-1})	130±23
Rate-pressure product (mmHg beats min^{-1})	11,750±2,630
LV posterior wall, diastole (mm)	7±0.2
Interventricular septum, diastole (mm)	8±0.2
LV diameter, diastole (mm)	34±0.5
LV diameter, systole (mm)	20±0.8
Ejection fraction (%)	72±2
Myocardial blood flow (ml 100 g^{-1} min^{-1})	148±34
Skeletal muscle blood flow (ml 100 g^{-1} min^{-1})	7.8±4.2

LV, Left ventricular

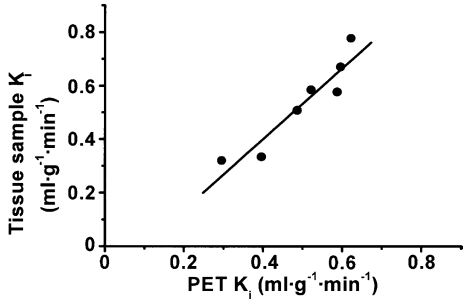

Fig. 3. Association between fractional myocardial [^{18}F]FTHA uptake (K_i) calculated from PET data and fractional [^{18}F]FTHA uptake obtained from myocardial tissue samples. The slope of the regression line is 1.32±0.21 and the intercept is –0.13±0.11 (r=0.94, P=0.002, n=7)

Table 2. Accumulation of ^{18}F in mitochondria

	Yield (%)	Radioactivity (%)	Accumulation of ^{18}F in mitochondria (%)
Heart			
Pig 1	40	25	63
Pig 2	46	30	65
Pig 3	42	41	97
Pig 4	35	28	80
Pig 5	[a]	29	
Pig 6	40	42	105
Pig 7	43	36	82
Pig 8	31	40	128
Mean	39	34	89
SD	5	7	23
Skeletal muscle			
Pig 1	16	3	16
Pig 2	70	34	49
Pig 3	29	10	36
Pig 4	39	15	38
Pig 5	[a]	6	
Pig 6	12	7	60
Pig 7	28	7	24
Pig 8	39	12	31
Mean	33	12	36
SD	19	10	15

Yield = yield of mitochondria in isolation; Radioactivity = ratio of mitochondrial fraction to total radioactivity. Accumulation of ^{18}F in mitochondria (%) is obtained by dividing the radioactivity by the yield

[a] Not determined for technical reasons

tivity was located in the mitochondrial fraction, it can be estimated that in the extramitochondrial spaces the metabolite fraction was negligible. This was also supported by the finding that there was an inverse correlation between accumulation of ^{18}F in mitochondria and the fraction of unmetabolized [^{18}F]FTHA in homogenized heart muscle samples (r=–0.92, P=0.004).

In skeletal muscle 36%±15% of total ^{18}F activity was found in the mitochondria (Table 2). Analyses of mitochondrial metabolite fractions were not successful owing to the small amount of radioactivity. TLC analysis of homogenized muscle samples showed that 17%±7% (n=6, P=0.005 vs 32%±12% in myocardium) of ^{18}F was unmetabolized [^{18}F]FTHA.

Discussion

Fluorine-18 labelled 6-thia-heptadecanoic acid ([^{18}F]FTHA) has recently been used to study myocardial and skeletal muscle FFA metabolism in humans. Based on the chemical structure of [^{18}F]FTHA, it is assumed to enter mitochondrial beta-oxidation, but after formation of two acetyl-CoA molecules, the rest of the chain is trapped in mitochondria. Therefore, it has been suggested that [^{18}F]FTHA specifically traces FFA beta-oxidation [11, 12]. In the present study we were able to directly measure the accumulation of radioactivity in the mitochondria and estimate the intracellular metabolites of [^{18}F]FTHA in the pig heart and skeletal muscle. We found that 89% of [^{18}F]FTHA uptake in the heart enters mitochondria. However, in skeletal muscle this fraction was only 36%.

Only two previous studies have investigated the association between [^{18}F]FTHA uptake and oxidative FFA metabolism. In the study by DeGrado et al. [11], inhibition of beta-oxidation by blocking FFA transport into mitochondria resulted in an 81%–87% decrease in [^{18}F]FTHA uptake in mice hearts. In the study by Stone at al. [12], myocardial [^{18}F]FTHA uptake and the fatty acid beta-oxidation rate (as determined with tritiated palmitate) were closely associated in extracorporeally perfused pig hearts. Both of these studies [11, 12] are in agreement with the findings of the present study. The vast majority of [^{18}F]FTHA appears to be accumulated in the mitochondria in the heart, and this fraction can be suggested to trace beta-oxidation.

FFAs are the main fuel of heart and skeletal muscle. After being taken up by the tissues, they enter mitochondrial beta-oxidation or are incorporated into the triglyceride pool. It has been found that 80%–84% of FFA uptake in the heart undergoes rapid oxidation [1, 2, 3] and only a small fraction enters the intracellular lipid pool in the fasting state. Measurements of skeletal muscle FFA uptake and beta-oxidation are more controversial [4, 19, 20, 21, 22]. In experimental studies the fraction of FFAs undergoing direct oxidation has ranged from 10% [19] to 90% [20]. In humans the fraction directed to beta-oxidation has been found to be 34%–58% in forearm or leg muscles [4, 21, 22]. Thus, it appears that the behaviour of [^{18}F]FTHA in the heart and skeletal muscle is similar to that of FFAs in general. Due to the physiological differences in FFA metabolism between the heart and skeletal muscle, [^{18}F]FTHA traces mainly FFA beta-oxidation in the heart but FFA uptake in the skeletal muscle.

The metabolite analysis of myocardial tissue samples suggests that >90% of radioactivity found in the mitochondria was accounted for by [^{18}F]FTHA metabolites. Furthermore, the distribution of radioactivity and metabolites between mitochondria and other cell fractions suggested that the majority of radioactivity in the cell fractions other than mitochondria was unmetabolized [^{18}F]FTHA. However, we were not able to directly measure [^{18}F]FTHA and its metabolites in extramitochondrial fractions. These were estimated by subtracting mitochondrial measurements from the whole tissue results. For this reason, and because TLC analysis was successful only in five pigs, we could calculate only rough estimates of [^{18}F]FTHA and its metabolites in the heart.

The fraction of unmetabolized [^{18}F]FTHA was smaller in homogenized skeletal muscle than in myocardial sample, which probably reflects both the faster extramitochondrial metabolism of [^{18}F]FTHA and the decreased accumulation of [^{18}F]FTHA in mitochondria in skeletal muscle. We assume that mitochondrial [^{18}F]FTHA metabolites are formed locally in the cell, because [^{18}F]FTHA has been shown to be trapped in mitochondria [11]. However, we could not directly measure from where the metabolites originate, which leaves open questions for further studies.

In this study the fractional myocardial [^{18}F]FTHA uptake rates were calculated from both tissue samples and PET data, and the results were in accordance. This provides additional confirmation that PET is a reliable method in measuring myocardial [^{18}F]FTHA uptake. Furthermore, the estimated myocardial FFA uptake rates were concordant with the values reported from previous studies in animals (3–22 μmol 100 g^{-1} min^{-1}) [1, 23, 24, 25, 26].

The results of this study are dependent on the accuracy of mitochondrial isolation. We estimated the total error in measurement of mitochondrial ^{18}F to be at maximum 15% for the myocardium and 25% in the skeletal muscle (data not shown). Based on these estimations, the range of ^{18}F accumulation in mitochondria would be 75%–102% in myocardium and 27%–45% in skeletal muscle. Therefore, the problems in the reproducibility of mitochondrial isolation are unlikely to have biassed the main results of this study. Our results provide information on the intracellular metabolism of [^{18}F]FTHA under aerobic and fasting conditions. We do not know whether the results can be extrapolated to postprandial or ischaemic tissue.

In conclusion, the results of this study suggest that in the heart ~89% of [^{18}F]FTHA accumulation enters myo-

cardial mitochondria and only 11% is found in the other cell fractions. In contrast to this, only ~36% of [^{18}F]FTHA accumulation in skeletal muscle appears to directly enter mitochondria, and the majority of [^{18}F]FTHA is taken up by the other cell fractions. These findings are in close agreement with the data known about FFA metabolism in general. The results support the hypothesis that in the heart, [^{18}F]FTHA traces mainly FFA beta-oxidation, while in skeletal muscle it traces FFA uptake but not specifically beta-oxidation.

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