Trapping and Metabolism of Radioiodinated PHIPA 3-10 in the Rat Myocardium

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PHIPA 3-10 [13-(4'-iodophenyl)-3-(p-phenylene)tridecanoic acid] is a p-phenylene-bridged, radioiodinated ω-phenyl fatty acid that has recently been developed to study coronary artery disease or cardiomyopathies. Here, we demonstrate that PHIPA 3-10 exhibits the characteristics of a long-chain fatty acid, including its ability to be efficiently taken up by myocytes and to function as a substrate for beta-oxidation before it is trapped. **Methods:** Myocardial metabolism of carrier-added and carrier-free ^{131}I-PHIPA 3-10 preparations were investigated in rats in vivo and in isolated Langendorff rat hearts. Heart extracts were analyzed by high-performance liquid chromatography, negative-ion electrospray mass spectrometry and investigation of intracellular distribution using density-gradient centrifugation. **Results:** A single, rapidly formed metabolite was found in the heart extract and also, surprisingly, in the hydrolyzed lipids. The total amount of metabolite increased from 43% to 51% between 15 and 60 min postinjection. By high-performance liquid chromatography comparison with synthetic potential catabolites, the metabolite was assigned the name PHIPA 1-10 [11-(4'-iodophenyl)-1-(p-phenylene)undecanoic acid] and was the product of one beta-oxidation cycle. Additional proof was obtained from the mass spectrometric analysis of the metabolite formed in vivo. The formation of this metabolite could be suppressed by Etomoxir, a carnitine palmitoyl transferase I inhibitor, indicating beta-oxidation of ^{131}I-PHIPA 3-10 in mitochondria. Final evidence for the involvement of mitochondria in the degradation of ^{131}I-PHIPA 3-10 was obtained by density-gradient centrifugation of homogenized rat heart tissue. The position of the labeled free PHIPA 3-10 and free metabolite peaked within the fraction containing mainly mitochondria. **Conclusion:** In spite of its bulky structure, ^{131}I-PHIPA 3-10 is extracted by the myocardium in a manner similar to the extraction of the unmodified fatty acid analog, IPPA. The retention of PHIPA 3-10 in heart muscle results from the presence of the p-phenylene group, which prevents more than one beta-oxidation cycle. Intracellular free PHIPA 3-10 and free PHIPA 1-10 are present in the mitochondria, whereas most of the esterified metabolite was found in the cytosolic lipid pool. Hence, the rapid appearance of PHIPA 1-10 in the lipid pool must be accounted for by mitochondrial leakage or by an unknown in–out transport system.

Key Words: radioiodinated fatty acids; PHIPA 3-10; metabolism; trapping mechanism

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Various radiolabeled fatty acid analogs have been used as probes for the assessment of altered myocardial perfusion and metabolic function resulting from coronary artery disease or cardiomyopathies of various origins. The rapid metabolic degradation of radioiodinated aliphatic and ω-phenyl-substituted long-chain fatty acids proceeds through several biochemical pathways with complicated kinetics (*1–3*). As a result of the biochemical degradation processes, a series of radioactive metabolites is produced (*4–9*) that may obscure the assessment of regional catabolism in the heart muscle. Myocardial scintigrams obtained with these probes, therefore, depict a sum of all radioactive components, including those which are not relevant to the tissue function of interest, namely, its capacity for beta-oxidation. Additionally, the time-consuming transaxial heart imaging by SPECT becomes difficult due to the rapid disappearance of radioactivity from the myocardium.

To circumvent these problems, fatty acids were chemically modified to permit only partial beta-oxidation and subsequent trapping of the resulting metabolites. Thus, as depicted in Figure 1, the intrachain replacement of a methylene group by a tellurium (C_{17}-9-Te) or sulfur atom (fluoro-6-thiaheptadecanoic acid or FTHA) and the substitution of β-methylene hydrogens by one [3-(R,S)-BMIPP] or two methyl groups (DMIPP) resulted in prolonged myocardial retention (*10–13*). Especially radioiodinated 3-(R,S)-BMIPP, which showed a significantly delayed myocardial tracer washout, was thoroughly investigated in clinical studies and has been reviewed recently (*14*). One interesting result obtained with this agent is the mismatch between perfusion tracer distribution and the regional [^{123}I]−3-(R,S)-BMIPP distribution in jeopardized but viable myocardium (*15*).

Another approach that has been used to enhance the retention of radiolabeled fatty acids is the incorporation of p-phenylene, as a beta-oxidation-inhibiting group, into the aliphatic chain. This choice was made because a phenyl group proved to be tolerated by myocytes, as shown for IPPA (*16*). From the 12-p-*ph*enylene-bridged radio*i*odinated ω-*p*henyl fatty *a*cids (PHIPAs) tested in rats, those with 13 methylene groups displayed the highest myocardial uptake (*17*). The retention of radioactivity in the myocardium strongly depended on the position of the p-phenylene group in the aliphatic chain. Thus, the p-phenylene group located close to the carboxyl function (Fig. 1), as in [^{123}I]-PHIPA 3-10 {13-(4'-[^{123}I]iodophenyl)-3-(p-phenylene)tridecanoic acid}, enhanced the retention of the radioactivity, whereas more remote p-phenylene groups induced the opposite effect. The increase in retention was attributed to a hypothetical mitochondrial trapping mechanism (*17,18*).

The goals of this article were to investigate myocardial metabolism of carrier- and non-carrier-added ^{131}I-PHIPA 3-10 preparations in intact rats and in isolated Langendorff rat hearts. The metabolism was studied by high-performance liquid chromatography (HPLC), negative-ion electrospray mass spectrometry (neg-ESI-MS) and investigation of intracellular distribution using density-gradient centrifugation. With the following results, we demonstrate that radioiodinated PHIPA 3-10 exhibits the characteristics of a long-chain fatty acid. This includes its ability to be taken up efficiently by the myocardium and to function as a substrate for beta-oxidation before it is trapped.

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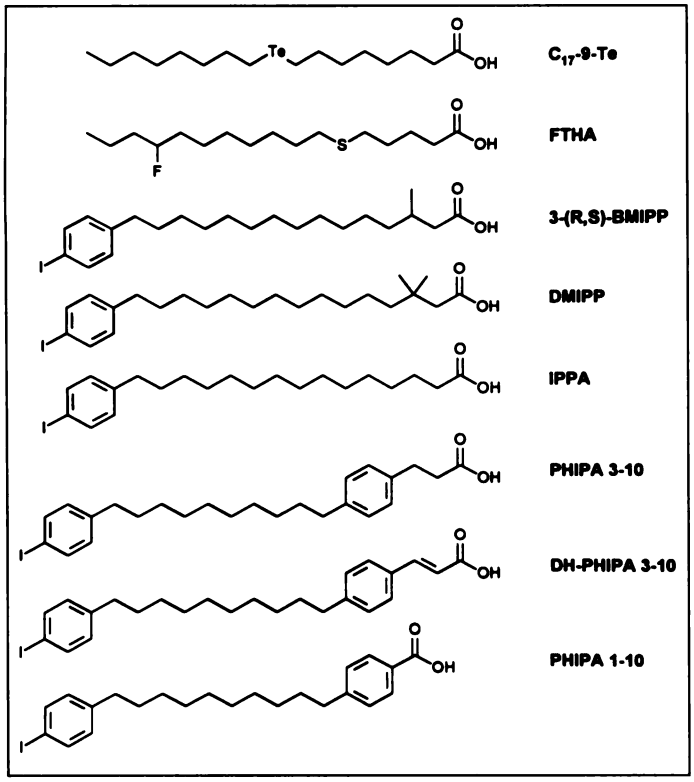

FIGURE 1. Chemical structures of modified fatty acids. The order of compounds is the same order in which they appear in the text.

MATERIALS AND METHODS

Materials

All commercially available chemicals were of analytical grade and were used without further purification. Na[^{131}I] was obtained in 0.02 *M* NaOH solution from Amersham International (Amersham, England). The specific activity was 90 GBq/μmol at the time of delivery. Racemic sodium salt of Etomoxir was purchased from ASAT AG (Zug, Switzerland). HPLC was performed on Latek systems equipped with variable UV (Latek, Heidelberg, Germany) and gamma-radiation detectors (Berthold, Wildbad, Germany). The reverse-phase HPLC columns (270 × 4 mm) used were Nucleosil 5-μm C_8 and C_{18} columns (Macherey & Nagel, Düren, Germany). The solvent mixtures used throughout the experiments consisted of MeOH and 0.2% aqueous formic acid. The detectors were equipped with a dual-channel integrator C-R5A (Shimadzu Europe, Duisburg, Germany). All HPLC data were processed with CLASS-UniPac software (Shimadzu) run under Windows 3.1 (Microsoft). ^{1}H-NMR spectra were obtained at 11.7 T (^{1}H frequency = 500 MHz) with an AM-500 spectrometer (Bruker, Karlsruhe) using conventional FT-NMR techniques. Detailed proton assignments were made by selective decoupling experiments and detection of ^{4}J couplings between aromatic protons and phenyl-group substituents (—CH_2—). The chemical shifts are reported in ppm downfield from Me_4Si, and the coupling constants are given in Hz. All mass spectrometric analyses were performed using a TSQ 7000 triple-quadrupole model instrument (Finnigan, Bremen, Germany) equipped with a Finnigan ESI source. The electron multiplier was operated at 1.4 keV, and the transfer capillary was maintained at 200°C. Argon was used as collision gas at a nominal pressure of 2 milliTorr. The samples were dissolved in methanol infused at a rate of 3 μl/min.

Synthesis of 13-(4′-Iodophenyl)-3-(p-Phenylene) Tridecanoic Acid (PHIPA 3-10)

The unlabeled precursor, PHIPA 3-10, was synthesized essentially according to a procedure described previously (*17*). Iodination was performed in two steps:

1. An equimolar amount of $Tl(TFA)_3$ was added to 0.05 *M* PHIPA 3-10 in trifluoroacetic acid (TFA) and left for 15 min at ambient temperature.
2. After the addition of equimolar NaI (1 *M*, in H_2O), the crude product precipitated from the reaction mixture. Preparative HPLC separation and recrystallization from petroleum ether/Et_2O afforded pure PHIPA 3-10 (66%); melting point, 98–99°C.

^{1}H NMR ($CDCl_3$, 30°C) δ = 1.26 ppm (broad s, 4H, IPh$(CH_2)_4$ CH_2CH_2); 1.28 (m, 8H, IPh$(CH_2)_2CH_2CH_2$ and $CH_2CH_2(CH_2)_2$ $PhCH_2$); 1.56 (pentet, 2H, J = 7.6, IPhCH_2CH_2); 1.58 (pentet, 2H, J = 7.4, $CH_2CH_2PhCH_2$); 2.53 (pseudo t, AA′ part of AA′XX′ system, 2H, $^3J_{ave}$ = 7.6, IPhCH_2); 2.56 (pseudo t, AA′ part of AA′XX′ system, 2H, $^3J_{ave}$ = 7.7, $CH_2CH_2PhCH_2$); 2.67 (MM′ part of AA′MM′ system, 2H, $^3J_{trans}$ = 9.5, $^3J_{gauche}$ = 6.2, CH_2PhCH_2 CH_2COOH); 2.93 (AA′ part of AA′MM′ system, 2H, $^3J_{trans}$ = 9.5, $^3J_{gauche}$ = 6.2, CH_2CH_2COOH); 6.92 (XX′ part of AA′XX′ system, 2H, $J_{ortho}+J_{para}$ = 8.4, ^{4}J to CH_2 = 0.6, H_{ortho} relative to CH_2 in IPhCH_2); 7.10 and 7.11 (AA′BB′ system, 4H, CH_2PhCH_2); 7.58 (AA′ part of AA′XX′ system, 2H, J_{ortho} + J_{para} = 8.4, H_{meta} relative to CH_2 in IPhCH_2). neg-ESI-MS: *m/z* 491 ([M-H^+]$^-$, 100%). Elemental analysis: theoretical (%) C 60.98, H 6.75; found (%) C 60.66, H 6.92.

Synthesis of 11-(4′-Iodophenyl)-1-(p-Phenylene) Undecanoic Acid (PHIPA 1-10)

An Et_2O solution of 1-phenyl-10-(4′-iodophenyl)decane (0.26 *M*), synthesized according to a procedure described previously (*17*), was added to activated Mg. The Grignard reaction was started with 1,2-dibromoethane and gentle heating. Thereafter, gaseous CO_2 was bubbled through this solution. Addition of aqueous HCl, extraction with Et_2O and recrystallization from petroleum ether/Et_2O yielded pure PHIPA 1-10 (38%); melting point, 91°C. Iodination of PHIPA 1-10 was achieved as described above. Preparative HPLC separation and recrystallization from petroleum ether/Et_2O afforded pure PHIPA 1-10 (33%); melting point, 152°C.

^{1}H NMR ($CDCl_3$, 30°C) δ = 1.26 ppm (broad s, 4H, IPh$(CH_2)_4CH_2CH_2$); 1.34–1.27 (m, 8H, IPh$(CH_2)_2CH_2CH_2$ and $CH_2CH_2(CH_2)_2PhCH_2$); 1.57 (pentet, 2H, J = 7.5, IPhCH_2CH_2); 1.63 (pentet, 2H, J = 7.5, $CH_2CH_2PhCH_2$); 2.53 (t, 2H, J = 7.8, IPhCH_2); 2.67 (t, 2H, J = 7.8, $CH_2CH_2PhCO_2H$); 6.92 (d, 2H, J = 8.4, H_{ortho} relative to CH_2 in IPhCH_2); 7.27 (d, 2H, J = 8.6, CH_2PhCO_2H); 7.57 (d, 2H, J = 8.6, H_{meta} relative to CH_2 in IPhCH_2); 8.02 (d, 2H, J = 8.6, CH_2PhCO_2H). neg-ESI-MS: *m/z* 463 ([M-H^+]$^-$, 100%). Elemental analysis: theoretical (%) C 59.49, H 6.29; found (%) C 59.03, H 6.48.

Synthesis of Trans-2-[13-(4′-Iodophenyl)-3-(p-Phenylene)] Tridecenoic Acid (DH-PHIPA 3-10)

1-Phenyl-10-(4′-iodophenyl)decane (0.13 *M* in Et_2O) was reacted at ambient temperature with an equimolar amount of $Tl(TFA)_3$ (0.2 *M* in TFA). After solvent evaporation, a 1.2-fold molar excess of acrylic acid methyl ester and an equimolar amount of Li_2PdCl_4 (0.025 *M* in MeOH) were added, and the mixture was heated for 3 hr at 55°C (*19*). Evaporation, extraction with Et_2O/1 *N* HCl and hydrolysis yielded a crude cis and trans product mixture. Separation of the trans product by preparative HPLC and recrystallization from petroleum ether/Et_2O yielded pure DH-PHIPA 3-10 (15%); melting point, 159°C.

^{1}H NMR ($CDCl_3$, 30°C) δ 1.26 (broad s, 4H, $IPh(CH_2)_4CH_2CH_2$); 1.27–1.34 (m, 8H, $IPh(CH_2)_2CH_2CH_2$ and $CH_2CH_2(CH_2)_2PhCH_2$); 1.57 (pentet, 2H, J = 7.5, $IPhCH_2CH_2$); 1.610 (pentet, 2H, J = 7.4, CH_2CH_2PhCH = CH); 2.53 (t, 2H, J = 7.7, $IPhCH_2$); 2.62 (t, 2H, J = 7.6, CH_2PhCH = CH); 6.41 (d, 1H, J = 15.9, trans-CH = $CHCO_2H$); 6.92 (d, 2H, J = 8.5, H_{ortho} relative to CH_2 in $IPhCH_2$); 7.21 (d, 2H, J = 8.4, CH_2PhCH = $CHCO_2H$); 7.46 (d, 2H, J = 8.4, CH_2PhCH = CHCOOH); 7.58 (d, 2H, J = 8.5, H_{meta} relative to CH_2 in $IPhCH_2$); 7.76 (d, 1H, J = 15.9, trans-CH=$CHCO_2H$). neg-ESI-MS: *m/z* 489 ($[M-H^+]^-$, 58%). Elemental analysis: theoretical (%) C 61.23, H 6.37; found (%) C 61.13, H 6.45.

Radioiodination of PHIPA 3-10

Labeling of PHIPA 3-10 with ^{131}I was performed using the $Tl(TFA)_3/^{131}I^-$ method as described previously (*20*). Iodine-131-PHIPA 3-10 purified by HPLC was dissolved in 3 ml 0.1 *M* phosphate buffer (pH 7.4) containing 50 mg of bovine serum albumin (BSA). Experiments with carrier-added PHIPA 3-10 required equimolar BSA for complete dissolution.

Metabolism in Rat Hearts

The animal experiments described here were performed according to the German animal protection laws. Overnight-fasted male Sprague–Dawley rats, weighing 180–250 g, were given an intravenous injection (tail vein) of ^{131}I-labeled PHIPA 3-10/BSA solution (20–30 MBq). The injected volume was determined by weighing the syringe before and after injection. At specific times postinjection, the animals were weighed, killed by cervical dislocation and dissected. The hearts were weighed, immediately cooled on ice and counted along with standards of the injected dose in a gamma counter. The heart uptake was calculated as %ID/g.

The ice-cold hearts were cut within seconds into small pieces and homogenized in 40 ml of acetone:chloroform:ethanol (2:1:1, v/v/v). The clear supernatant was collected, and the extraction was repeated. On the basis of the recovery of ^{131}I, the extraction efficiency was >90%. Evaporation of the solvents and redissolving in 1 ml of ethanol proceeded with almost complete recovery of the radioactivity. One-half of this solution was hydrolyzed by the addition of 0.5 ml of 10 *M* aqueous KOH and heating at 60°C for 60 min. After adjusting to pH <1 by the addition of HCl, the liberated fatty acids were extracted twice with Et_2O, evaporated to dryness and redissolved in 0.5 ml of EtOH.

The analyses of the heart extracts were performed with RP-C_8 columns using methanol/water and a linear gradient, starting at 60% methanol and increasing to 100% over 90 min. RP-C_8 columns were used because they do not retain lipids. Free fatty acids of hydrolyzed heart extracts were analyzed with higher resolution using RP-C_{18}, columns starting at 80% and ending at 100% MeOH after 90 min.

For the mass spectrometric analysis of metabolites formed by rat hearts, unlabeled PHIPA 3-10 was also injected. Therefore, animals received 24 MBq of ^{131}I-PHIPA 3-10 and 5.7 mg of cold PHIPA 3-10 dissolved in 3.7 ml BSA (20%). One hour later, the heart was excised, extracted as described above and hydrolyzed. To reduce the amount of unlabeled free fatty acids from the hydrolyzed rat heart extract, the radioactive compounds were purified by preparative RP-C_{18} HPLC using 93% MeOH. The combined HPLC eluates were concentrated to 100 μl and analyzed by neg-ESI-MS/MS.

Metabolism in Langendorff Rat Hearts

Male Sprague–Dawley rats weighing 180–250 g were fasted overnight, anesthetized with 30 mg thiopental sodium salt and treated with 2.5 mg of heparin sodium salt by intraperitoneal injection 10 min before the heart preparation commenced. The preparation was performed according to the Langendorff technique as described previously (*21*). Excised hearts were chilled for 2 sec in ice-cold normal saline and promptly perfused at 37°C with nonrecirculating, modified Krebs–Henseleit buffer containing 5 m*M* glucose at a flow rate of 7 ml/min. Before and during perfusion, the buffer was saturated with O_2 and adjusted with carbogen (95% O_2/5% CO_2) to pH 7.40. The heart rate was stable at 180–200 beats/min, and the effluent O_2 tension was 200–230 mm Hg. The isolated hearts were functionally viable, as demonstrated by a stable heart rate. At the end of the experiments, the myocardial tissue was soft and of light color, indicating good perfusion and intact heart cell function. ATP and other substrates characterizing the energy situation of the heart muscle proved to be normal.

An initial 15 min of perfusion was performed for heart stabilization, followed by coinjection of ^{131}I-PHIPA 3-10/BSA solutions within the indicated time periods using a syringe pump (Perfusor V; Braun, Melsungen, Germany). At the end of the experiments, the hearts were rapidly cooled on ice and analyzed as described above.

The perfusion solution was acidified with HCl to pH <1 and extracted using a C-18 Sep-Pak cartridge (Waters). The extracted radioactivity was eluted from the cartridge and concentrated to 1 ml in ethanol. The analysis of this solution was performed with RP-C_8 columns and a methanol gradient as described above.

Langendorff rat hearts pretreated with Etomoxir were stabilized for 15 min after mounting on the perfusion apparatus and perfused for 15 min with 1.55 ml of 20 m*M* racemic Etomoxir/BSA solution. Two minutes of normal perfusion, 5 min of perfusion with ^{131}I-PHIPA 3-10/BSA and finally 30 min of normal perfusion completed this experiment. Work-up and analyses were performed as described above.

Density-Gradient Ultracentrifugation of Rat Heart Homogenate

A Langendorff rat heart, prepared as described above, was perfused with ^{131}I-PHIPA 3-10/BSA solution for 5 min in addition to the Krebs–Henseleit solution, followed by 15 min of normal perfusion. To soften the heart tissue, the following perfusion sequence was applied: 5 min of Ca^{2+}-free Krebs–Henseleit solution, 10 min of Krebs–Henseleit solution containing 15,000 units of collagenase (Sigma, München, Germany) and finally a purging phase with normal Krebs–Henseleit solution for 5 min. One-half of the soft and swollen heart, which released single cells upon pulling apart, was dissected and briefly homogenized at 4°C using a Potters homogenizer rotating at 800 min^{-1}. The remaining tissue was extracted, hydrolyzed and analyzed by HPLC as described above.

Subcellular fractionation of the rat heart homogenate was performed essentially as described previously for the separation of a rat liver light mitochondrial fraction (*22*). Briefly, the homogenate was layered on top of a density gradient made up of 14.5%–45% (w/v) Nycodenz in 10 m*M* glycylglycine (pH 7.4), 1 m*M* EDTA and 0.1% ethanol. To correct the density gradient for proper osmolarity, the Nycodenz gradient contained a reversed sucrose gradient of 8%–0% (w/v). Centrifugation was performed in a vertical rotor at 45,000 × g for 45 min at 4°C. Fractions were recovered from bottom to top.

The density-gradient fractions were counted and calculated as % of total heart activity. Lipids and free fatty acids present in these fractions were separated on aluminum-baked silica gel thin-layer chromatography (TLC) plates (Merck, Darmstadt, Germany) using petroleum ether:Et_2O:acetic acid (70:30:1). Quantification was performed using a TLC linear analyzer (Berthold).

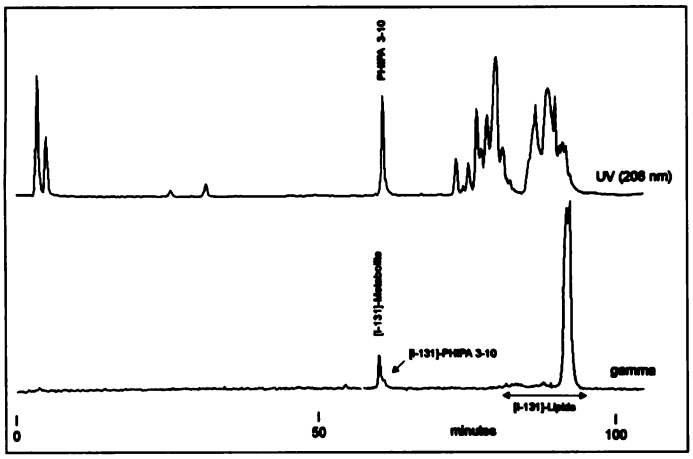

FIGURE 2. RP-C_8 HPLC of a rat heart extract 60 min after the injection of ^{131}I-PHIPA 3-10.

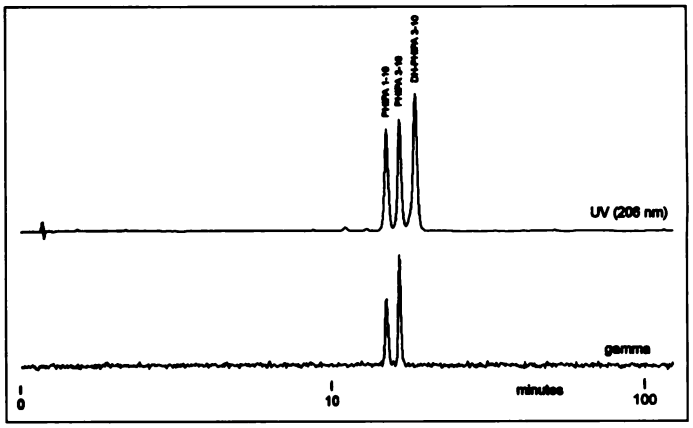

FIGURE 3. RP-C_{18} HPLC of hydrolyzed rat heart lipids 15 min after injection of ^{131}I-PHIPA 3-10.

RESULTS

Metabolism of [^{123}I]-PHIPA 3-10 in Rat Hearts

Fifteen, 30 and 60 min after intravenous injection of 20–30 MBq non-carrier-added ^{131}I-PHIPA 3-10/BSA, the animals showed heart uptake values that slowly decreased from 4.6% to 3.9%ID/g. RP-8 HPLC of the heart extracts showed small variations in the radioactivity distributed between esterified and free radioactive fatty acids. As an example, the UV- and gamma-HPLC readouts of a 60-min heart extract are shown in Figure 2. Free ^{131}I-PHIPA 3-10 eluting at 60 min was identified by spiking with cold PHIPA 3-10. A metabolite could also be detected eluting somewhat earlier. Table 1 summarizes the results evaluated from the gamma-HPLC chromatograms. The labeled free fatty acids decreased with time from 14.8% at 15 min to 10.3% at 60 min after injection, whereas the relative amount of free metabolite increased from 60.1% to 85.4%.

In addition, the heart extracts were hydrolyzed and analyzed by HPLC. Figure 3 shows the RP-18 gamma-HPLC of a hydrolyzed heart extract, which was obtained 15 min after the injection of ^{131}I-PHIPA 3-10. The UV trace demonstrates the retention behavior of the two potential metabolites PHIPA 1-10 and DH-PHIPA 3-10 (Fig. 1). Accordingly, the more lipophilic gamma-HPLC peak was identified to be PHIPA 3-10, and the less lipophilic component was assigned to PHIPA 1-10. The relative amount of the metabolite as compared to the parent compound increased with time from 42.5% at 15 min to 51.0% at 60 min after injection. The data are summarized in Table 1.

To obtain additional proof of the identity of PHIPA 1-10, mass spectrometric analysis was performed with the HPLC-purified hydrolyzed heart extract from an animal that received unlabeled PHIPA 3-10 in addition to ^{131}I-PHIPA 3-10/BSA solution. One hour after application, the heart showed 1.9%ID/g heart tissue. The heart uptake was generally found to be diminished if unlabeled PHIPA 3-10 was added, indicating a saturable transport mechanism. The labeled fatty acids extracted from the heart amounted to 0.1 mg, which contained 28.7% of the metabolite after hydrolysis of the lipids. The purified and concentrated radiolabeled fatty acids were analyzed by neg-ESI-MS/MS. Using the [iodide]$^-$-fragment at *m/z* 126.9 and the parent scan mode, the specific signal of the metabolite could be selectively detected without contribution of other contaminants in the sample. Figure 4 illustrates this feature of ESI-MS/MS, showing the parent-ion peaks of PHIPA 3-10 (*m/z* 491) and PHIPA 1-10 (*m/z* 463).

TABLE 1
Summary of Results Obtained with Iodine-131-PHIPA 3-10 Injected into Rats

Time (min)	Heart uptake* (% ID/g)	Free fatty acids (% heart activity)	Free metabolite† (% free fatty acids)	Total metabolite (% heart activity)
15	4.6 ± 0.6	14.8 ± 1.5	60.1 ± 7.0	42.5 ± 4.1
30	4.5 ± 0.5	10.7 ± 1.6	83.2 ± 8.9	45.1 ± 4.8
60	3.9 ± 1.0	10.3 ± 1.1	85.4 ± 9.3	51.0 ± 4.9

*Mean of three animals ± s.d.
†^{131}I-PHIPA 1-10.

Metabolism of ^{131}I-PHIPA 3-10 in Langendorff Rat Hearts

Stable beating Langendorff rat hearts were perfused for 5 min with ^{131}I-PHIPA 3-10/BSA solutions before a 30-min normal perfusion period. Table 2 summarizes heart uptake data and labeled fatty acid and metabolite concentrations. At the end of perfusion, the heart uptake amounted to 35.7%, and the radioactivity released during the second phase ranged between 0.5% and 1.0%. No metabolites were detected in the perfusion solutions. Additionally, rat hearts with depleted mitochondrial activity, induced by preperfusing racemic Etomoxir, a carnitine palmitoyl transferase I (CPT-I) inhibitor, were investigated under comparable conditions. The heart showed reduced uptake (6.8%), and more labeled free fatty acids were present (22.8%) as compared to untreated rat hearts (9.6%). While the amount of free metabolite was reduced in Etomoxir-treated hearts, the difference in total metabolite content was rather small.

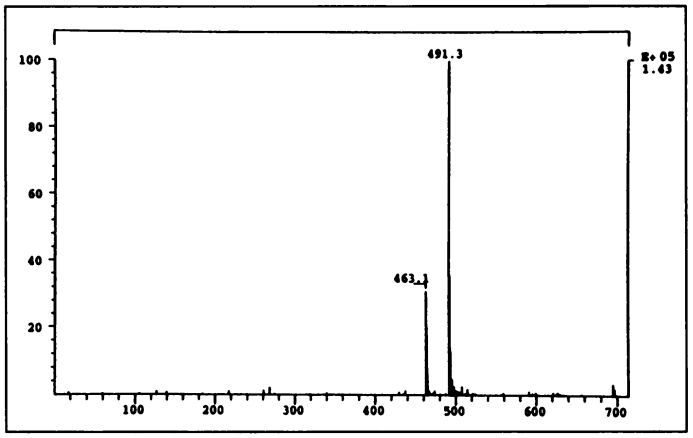

FIGURE 4. Negative-ion ESI-MS/MS spectrum of hydrolyzed and HPLC-purified rat heart lipids.

TABLE 2
Summary of Results Obtained with Iodine-131-PHIPA 3-10 Perfused through Langendorff Rat Hearts

Heart uptake* (% ID/g)	Free fatty acids (% heart activity)	Free metabolite† (% free fatty acids)	Total metabolite (% heart activity)	Intervention
35.7 ± 4.1	9.6 ± 1.3	72.9 ± 6.5	62.7 ± 5.2	—
6.8 ± 0.9	22.8 ± 1.5	52.1 ± 7.2	59.6 ± 4.9	Etomoxir
13.5	17.7	88.8	63.0	Collagenase‡

*Mean of three animals ± s.d.
†^{131}I-PHIPA 1-10.
‡One animal.

Density-Gradient Distribution of ^{131}I-PHIPA 3-10 in Rat Heart Homogenates

The evaluation of the intracellular distribution of the radiolabeled fatty acids and lipids in a ^{131}I-PHIPA 3-10-perfused rat heart was performed using density-gradient centrifugation. Before homogenization, the connecting tissue of the Langendorff rat heart had to be softened. Therefore, collagenase was added for a short period to the Krebs–Henseleit solution. During this intervention, the heart released some of its radioactivity (13.5% compared to 35.7%ID/g), which was either due to the leakage of degrading cells or to the loss of single cells into the effluent. The labeled free fatty acids were enhanced (17.7% compared to 9.6% heart activity), indicating an unknown effect during enzyme catalyzed lysis of connecting heart tissue. The amount of metabolite found after hydrolysis was comparable to that found for untreated hearts (Table 2).

Figure 5 illustrates the distribution of radioactivity after ultracentrifugation, measured in 32 gradient fractions. The increasing fraction numbers correlate with decreasing density of the gradient. Two prominent peaks were found that coincided with maximum enzyme activity of catalase and cytochrome c oxidase, marker enzymes of peroxisomes and mitochondria, respectively. Beyond fraction 22, radioactivity belonged to ^{131}I-labeled compounds associated with endoplasmic reticulum, lysosomes and cytosol.

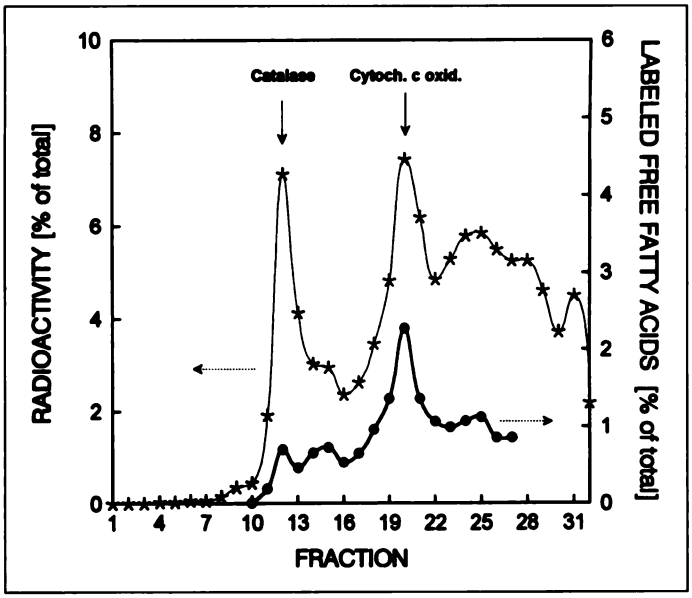

FIGURE 5. Distribution of total radioactivity and free-labeled fatty acids after density-gradient separation of a homogenized rat heart perfused with ^{131}I-PHIPA 3-10.

To locate the position of the free-labeled fatty acids within the gradient, all fractions were analyzed by TLC. The result is shown in Figure 5, indicating that the labeled free PHIPA 3-10/PHIPA 1-10 peaked with the fraction containing mainly mitochondria.

DISCUSSION

In several cases, structural modifications of the fatty-acid chain have resulted in increased retention of the corresponding radiolabeled fatty acids and/or their metabolites in the myocardium. PHIPA 3-10 belongs to this group of modified long-chain fatty acids, showing an effective retention of radioactivity in the rat heart [$T_{1/2(biol)}$ = >6 hr] (*17*). Myocardial trapping was initially attributed to mitochondrial uptake and storage of PHIPA 3-10 and potential lipophilic metabolites because long-chain fatty acids are known not to be transported back into the cytosol.

Preliminary information about the metabolic fate of PHIPA 3-10 in myocytes was received from the HPLC of rat heart extracts. The radioactive components were almost completely extracted from the heart tissue, indicating no deposition of catabolites into proteins, as was observed with 14(R,S)-[^{18}F]fluoro-6-thiaheptadecanoic acid (*12*). The heart extracts were found to comprise free and esterified fatty acids, analogous to results obtained with [^{123}I]IPPA (*6,9*), an unmodified ω-iodophenyl fatty acid. A metabolite of PHIPA 3-10 was detected by HPLC with a slowly increasing methanol gradient. Figure 2 illustrates the HPLC of a rat heart extract that was obtained 60 min after the injection of ^{131}I-PHIPA 3-10. The major part of the free fatty acids was found as a somewhat less lipophilic metabolite. The amount of this metabolite increased with time, whereas the fraction of free fatty acids was reduced (Table 1).

Because most of the radioactivity was present in esterified form, we investigated the nature of labeled fatty acids hidden in the lipid pool. Therefore, the lipids of the heart extracts were hydrolyzed and analyzed by HPLC. As an example, the gamma-HPLCs of hydrolyzed heart lipids are shown in Figure 3. The two peaks indicate ^{131}I-PHIPA 3-10 and a single metabolite, which was identical to that shown in Figure 2. Initial evidence for the structure of this metabolite was obtained by comparing its retention time on HPLC to those of DH-PHIPA 3-10 and PHIPA 1-10, two potential degradation products of PHIPA 3-10, depicted in Figure 1. The agreement of retention times pointed to PHIPA 1-10 as the metabolite. Additionally, mass amounts of this metabolite were generated in vivo by adding unlabeled PHIPA 3-10 to the ^{131}I-PHIPA 3-10/BSA solution before being injected into the animal. From the mass spectrometric analysis of the hydrolyzed heart extract, the formation of PHIPA 1-10 could be confirmed. The neg-ESI-MS spectrum of this solution shows the parent ion $[M\text{-}H^+]^-$ peaks of PHIPA 3-10 (*m/z* 491) and the metabolite PHIPA 1-10 (*m/z* 463) (Fig. 4).

The retrograde perfusion of isolated rat hearts with ^{131}I-PHIPA 3-10 (Langendorff model) revealed a ~10-fold higher uptake of radioactivity as compared to the heart uptake in whole animals (Table 2). The high myocardial uptake was, on one hand, due to an enhanced fraction of ^{131}I-PHIPA 3-10 perfused through the coronary arteries and, on the other hand, to the molecular characteristics of PHIPA 3-10, corresponding to a long-chain fatty acid. Aside from this effect, the amounts of metabolites formed in the Langendorff rat hearts were comparable in order of magnitude to those found for whole-animal experiments (Table 1).

Pretreatment of animals with the CPT-I inhibitor 2-[5-(4-

chlorophenyl)pentyl]oxirane-2-carboxylate has been used to investigate whether radiolabeled fatty acids are substrates of mitochondrial beta-oxidation (*1,12,23*). Similarly, Etomoxir (2-[6-(4-chlorophenoxy)hexyl]oxirane-2-carboxylate), a more recently developed CPT-I inhibitor, was perfused through the Langendorff rat heart before the administration of ^{131}I-PHIPA 3-10 and was found to significantly reduce heart uptake. This effect has also been observed with other radiolabeled fatty acids (*12,23*). The impairment of myocardial PHIPA 3-10 extraction may be associated with Etomoxir mediated inhibition on fatty acid transport into mitochondria, which signals saturation to the cellular uptake process. In addition, the elevated free-labeled fatty acids content in the heart extract indicate mitochondrial dysfunction. In respect to the specific inhibitory effect of Etomoxir on fatty-acid transport into mitochondria and substrate depletion for beta-oxidation, the fraction of free metabolite was found to be highly reduced (Table 2).

The interpretation of these results may appear straightforward, but it also leaves an important biochemical question unanswered. The rapid and extensive accumulation of this metabolite in the lipid fraction of the myocytes is not consistent with the mitochondrial trapping hypothesis because fatty acid esters are not located in this cell compartment. In addition, these results are not in agreement with the general understanding that long-chain fatty acids are transported out of the mitochondria. The rapid formation of PHIPA 1-10 and its appearance in the ester fraction must, however, imply transport through mitochondrial membranes. This result appears surprising because PHIPA 1-10 is a long-chain fatty acid with about the same molecular dimension as palmitic acid, whereas IPPA and PHIPA 3-10 are structurally related to stearic acid.

Evidence for the catabolic degradation of PHIPA 3-10 in mitochondria instead of in other cell compartments, like peroxisomes, was obtained by the density-gradient separation of a rat heart cell homogenate. The vast portion of free ^{131}I-PHIPA 3-10 and free ^{131}I-PHIPA 1-10 was indeed found to be associated with the mitochondrial fraction (Fig. 5). The esterified fatty acids, on the other hand, were mainly located within the cytosol, endoplasmatic reticulum and peroxisomes.

CONCLUSION

From these observations the following conclusions can be drawn. Like the long-chain ω-iodophenyl fatty acid, IPPA, and in spite of its bulky structure, PHIPA 3-10 is efficiently extracted by the myocardium. The retention of ^{131}I-PHIPA 3-10 in heart muscle results from the presence of the p-phenylene group, which prevents more than one beta-oxidation cycle. Intracellular free ^{131}I-PHIPA 1-10 and free unused ^{131}I-PHIPA 3-10 are present in the mitochondria, the location of catabolic fatty acid degradation. Most of ^{131}I-PHIPA 1-10, however, was found in the cytosolic lipid pool. The rapid appearance of ^{131}I-PHIPA 1-10 in the lipid pool must, therefore, be accounted for by mitochondrial leakage or an unknown in–out transport system. Finally, with respect to its effective myocardial uptake, retention and biochemical activity in the rat heart, [^{123}I]-PHIPA 3-10 may be considered a useful radiopharmaceutical for the study of fatty acid utilization in coronary artery disease or cardiomyopathies. In humans, [^{123}I]-PHIPA 3-10 showed high myocardial uptake and retention (*24,25*). The clinical evaluation of patients with chronic coronary artery disease revealed decreased ^{123}I-PHIPA 3-10 uptake, at rest and during stress, in the majority of myocardial areas with stress-induced perfusion abnormalities (*24*). Additional findings showing reversible ^{201}Tl defects with deficient PHIPA 3-10 uptake resembles the "mismatching" pattern between regional [^{123}I]−3-(R,S)-BMIPP and ^{99m}Tc-sestamibi flow tracer distribution in infarcted human hearts, indicating jeopardized but viable myocardium (*14,15*). Support regarding the potential of this agent to differentiate between residual viable myocardium and scar has recently been demonstrated in comparison to ^{99m}Tc-sestamibi by a double-tracer study of an infarcted explanted human heart (*26*). The diagnostic benefit of PHIPA 3-10 against BMIPP now remains to be elucidated by a comparative clinical study, currently being performed.

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