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Arachidonic acid synthetic pathways of the oyster protozoan parasite, *Perkinsus marinus*: evidence for usage of a delta-8 pathway

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Abstract

The meront stage of the oyster protozoan parasite, *Perkinsus marinus*, is capable of synthesizing saturated and unsaturated fatty acids including the essential fatty acid, arachidonic acid [20:4($n-6$)]. Eukaryotes employ either delta-6 (Δ-6) or delta-8 (Δ-8) desaturase pathway or both to synthesize arachidonic acid. To elucidate the arachidonic acid synthetic pathways in *P. marinus*, meronts were incubated with deuterium-labeled precursors [18:1($n-9$)-d6, 18:2($n-6$)-d4, 18:3($n-3$)-d4, and 20:3($n-3$)-d8]. The lipids were extracted, converted to fatty acid methyl esters, and analyzed using gas chromatography/mass spectrometry and gas chromatography/flame ionization detection. Deuterium-labeled 18:2($n-6$), 20:2($n-6$), 20:3($n-6$), and 20:4($n-6$) were detected in meront lipids after 1-, 3-, 5-, and 10-day incubation with 18:1($n-9$)-d6. Deuterium-labeled 20:2($n-6$), 20:3($n-6$) and 20:4($n-6$) were found in lipids from meronts after incubation with 18:2($n-6$)-d4 methyl ester. No labeled 18:3($n-6$) was detected in either incubation. Apparently, when incubated with 18:1($n-9$)-d6, the parasite first desaturated 18:1($n-9$)-d6 to 18:2($n-6$)-d6 by Δ-12 desaturase, then to 20:2($n-6$)-d6 by elongation, and ultimately desaturated to 20:3($n-6$)-d6 and 20:4($n-6$)-d6 using the sequential Δ-8 and Δ-5 desaturation. Similarly, when incubated with 18:2($n-6$)-d4, *P. marinus* converted the 18:2($n-6$)-d4 to 20:2($n-6$)-d4 by elongation and 20:2($n-6$)-d4 to 20:3($n-6$)-d4 by Δ-8 desaturase then by Δ-5 desaturase to 20:4($n-6$)-d4. These results provide evidence that *P. marinus* employed the Δ-8 rather Δ-6 pathway for arachidonic acid synthesis. Additional support for the presence of a Δ-8 pathway was the demonstrated ability of the parasite to metabolize 18:3($n-3$)-d4 to 20:3($n-3$)-d4 and 20:4($n-3$)-d4, and 20:3($n-3$)-d8 to 20:4($n-3$)-d6 and 20:5($n-3$)-d6 using the sequential position-specific Δ-8 and Δ-5 desaturases.

Keywords: Arachidonic acid; Desaturases; Fatty acid synthetic pathways; Oyster; Oyster parasite; *Perkinsus marinus*

1. Introduction

Although parasitic protozoans effectively incorporate lipids from culture media and/or host and convert them to constitutive lipids [1–4], their ability for chain elongation and desaturation of fatty acids are limited [1,5–8]. Only three parasitic protozoans, *Trypanosoma cruzi*, *Plasmodium falciparum*, and *Leishmaina donovani*, have been shown to synthesize fatty acids from ^{14}C-acetate [9–11]. In these studies, no saturated fatty acid with chain length more than 18 carbons and unsaturated fatty acids other than 18:1 and 18:2 were found to incorporate ^{14}C from labeled acetate. However, results of our recent study [12] revealed that the fatty acid synthetic capability of the oyster protozoan parasite, *Perkinsus marinus*, is far beyond those described for *T. cruzi*, *P. falciparum*, and *L. donovani*. The in vitro cultured meronts of *P. marinus* synthesized a wide range of saturated and unsaturated fatty acids including the essential fatty acid, arachidonic acid [20:4($n-6$), AA], employing ^{13}C-acetate [12].

Perkinsus marinus is an alveolate in the class Perkinsasida [13]. It is one of the two important pathogenic protozoans causing severe mortality in American (eastern) oysters (*Crassostrea virginica*) from the mid-Atlantic to the Gulf coasts since the 1950s. The disease caused by *P. marinus* is infectious (see review by Chu [14]) and four

Abbreviations: GC/MS, gas chromatography/mass spectrometry; GC/FID, gas chromatography/flame ionization detection; CI, positive chemical inoization; FAME, fatty acid methyl ester; PUFAs, polyunsaturated fatty acids; EPA, eicosapentaenoic acid, 20:5($n-3$); DHA, docosahexaenoic acid, 22:6($n-3$); AA, arachidonic acid, 20:4($n-6$); PG, prostaglandin

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life stages: meront (trophozoite), prezoosporangia (hypnospores), zoosporangia, and biflagellated zoospores have been identified and described [15,16]. The meront stage is believed to be the primary agent for disease transmission [14,16].

The finding of de novo synthesis of AA in the meront stage of *P. marinus* is novel. Thus far, only free-living protozoans such as amoeba and ciliates have been reported to have the ability to synthesize AA de novo [5,17–20]. No parasitic protozoan has been reported to be capable of synthesizing long chain essential fatty acids, such as AA. To synthesize ($n-6$) 20-carbon polyenoic acids such as AA, usually one of two pathways is employed by organisms, either the common Δ-6 pathway or the alternative Δ-8 pathway. Usage of Δ-6 pathway for 20:4($n-6$) synthesis has been described in ciliates, trypanosomatids [5] and some phytoplankton species [21]. Conversely, employing the Δ-8 pathway to synthesize 20:4($n-6$) has been described in the ciliated protozoan *Tetrahymena pyriformis* [18]. The objective of the present study was to elucidate the 20:4($n-6$) synthetic pathways (Δ-6 or Δ-8) in *P. marinus* meronts employing deuterium-labeled 18:1 ($n-9$), 18:2($n-6$), 18:3($n-3$), and 20:3($n-3$).

2. Materials and methods

2.1. Axenic P. marinus meront cultures

Perkinsus marinus meronts were cultivated at 28 °C as previous described [12,22]. The medium was prepared as described by La Peyre et al. [23] and contained amino acids, nucleotides, carbohydrates, and vitamins, but no fetal bovine serum. The lipid concentration of this medium was estimated to be 14.5 μg lipid/ml, based on the amount of lipid added to the medium. Meronts were inoculated (approximately $1 \times 10^6\,\mathrm{ml}^{-1}$) and cultivated in 10-ml aliquots of medium in T-10 tissue culture flasks at 28 °C. Meronts at exponential growth phase (7 or 9 days old) were harvested and used for all stable isotope precursor incubation or growth experiments.

2.2. Deuterium-labeled fatty acids and fatty acid methyl esters

Deuterium-labeled fatty acid and fatty acid methyl esters were synthesized by Richard Adlof and his associates at USDA (ARS, NCAUR, 1815 N. University, Peoria, IL) using previously published methods [24]. Chemical purities of the synthesized fatty acids (as fatty acid methyl esters; FAME) were determined on a Varian 3400 GC (Varian Instruments, Palo Alto, CA) equipped with a 30 m × 0.32 mm SP2380 (Supelco, Inc., Bellefonte, PA) capillary column and flame ionization detector (FID). Helium was utilized as carrier gas. Methyl ester peaks were identified by comparison with standard FAME mixtures of known composition.

Gas chromatography/mass spectrometry (GC/MS) was used to determine the isotopic purities of the deuterium-labeled fatty acids. Analyses (as FAME) were made on a Hewlett-Packard Model 5889 GC/MS (quadrapole; positive chemical ionization mode; isobutane as ionizing gas) equipped with a 30 m × 0.25 mm Supelcowax 10 fused silica capillary column (Supelco, Inc., Bellefonte, PA). Data collection and manipulation have been described previously [25]. The isotope purities of the labeled fatty acids used in the present study were confirmed to be: 18:1($n-9$)-d6 = 90.4%, 18:2($n-6$)-d4 = 84%, 18:3($n-3$)-d4 = 97.1%, 20:3($n-3$)-d8 = 92.4%.

2.3. Experiments

A series of experiments were conducted to elucidate the arachidonic acid (AA) synthetic pathways in *P. marinus* meronts. To investigate whether *P. marinus* meronts use delta-8 (Δ-8), and/or delta-6 (Δ-6) pathways to synthesize AA, the deuterium-labeled fatty acids, 18:1($n-9$)-d6 [18:1($n-9$)-14,14,15,15,17,18-d6], 18:3($n-3$)-d4 [18:3($n-3$)-6,6,7,7-d4], and 20:3($n-3$)-d8 [20:3($n-3$)-3,3,4,4,8,8,9,9-d8], and 18:2($n-6$)-d4 [18:2($n-6$)-15,15,16,16-d4] methyl ester were used.

2.3.1. Uptake and metabolic kinetics of the deuterium-labeled oleic acid, 18:1($n-9$)-d6, in P. marinus meronts

This experiment was conducted to determine the time course of AA biosynthesis from deuterated fatty acid precursors and to identify intermediates. Medium containing 18:1($n-9$)-d6 was prepared by adding the deuterated fatty acid directly to lipid concentrate solution (GibcoBRL #21900-030), which was then used in preparing the media (10 ml lipid concentrate/l). The final nominal concentration of 18:1($n-9$)-d6 in the media was 20 μM (6 μg/ml). Medium was also prepared with a similar concentration of non-deuterated 18:1($n-9$). *P. marinus* meronts from a 7-day-old culture were used to innoculate 24 flasks containing 10 ml of one of the two media (deuterated or non-deuterated) with 10^7 meronts (10^6 meronts/ml). A sample of the innoculum was saved for fatty acid analysis. At days 1, 3, 5, and 10 three flasks from each of the two treatments were centrifuged and the cell pellets were harvested for fatty acid analysis.

2.3.2. Metabolism of the deuterium-labeled linoleic acid methyl ester, 18:2($n-6$)-d4, and deuterium-labeled fatty acids 18:3($n-3$)-d4 and 20:3($n-3$)-d8 in P. marinus meronts

Incubations with 18:2($n-6$)-d4 methyl ester and fatty acids, 18:3($n-3$)-d4 and 20:3($n-3$)-d8, and their non-deuterated counterparts were conducted in a similar manner to the 18:1($n-9$)-d6 incubations, except that the substrates were added directly to the media from 10 mg/ml ethanol stock solutions to final nominal concentrations of 10 μg/ml media. For each substrate tested, three replicate 10 ml flasks of cells were harvested after 3 days to analyze for the uptake and metabolism of the substrates by *P. marinus* meronts.

2.4. Lipid and fatty acid analyses

Detailed methodologies for lipid extraction, and analysis of deuterium-labeled and non-labeled fatty acids have been described previously by Chu et al. [12]. Thus, only a brief description is provided here. Total lipids were extracted from meronts according to the procedure described by Bligh and Dyer [26]. Fatty acid composition and contents of meronts were first analyzed using GC/FID (Varian 3300, equipped with a FID; Varian Analytical Instruments, Sunnydale, CA) using a DB-WAX capillary column (25 m × 0.32 mm; 0.2 μm film thickness; J&W Scientific, Folsom, CA), after trans-methylation [12,27]. Identification of FAMEs was based on the comparison of their retention times with those of authentic standards and confirmed by GC/MS. Peaks containing deuterated fatty acids were tentatively identified by comparison to chromatograms of cultures incubated with the same non-deuterated substrate using GC/FID and then confirmed by chemical ionization GC/MS (Varian 3400 gas chromatograph equipped with a Varian Saturn 4D GC/MS/MS detector) using the same column. Values are mean ± S.D. for total micrograms of deuterated fatty acids recovered in the cell pellets of the cultures.

2.5. Statistical analyses

Data from the 18:1($n-9$)-d6 time series incubations were subjected to analysis of variance. When appropriate, comparisons between sampling dates were conducted using Tukey's test.

3. Results

3.1. Uptake and metabolic kinetics of the deuterium-labeled oleic acid, 18:1($n-9$)-d6, in P. marinus meronts

Perkinsus marinus meronts effectively incorporated and utilized 18:1($n-9$)-d6. Deuterium-labeled 18:2($n-6$), 20:2($n-6$), 20:3($n-6$), and 20:4($n-6$) were detected in *P. marinus* meronts lipids after 1-, 3-, 5-, and 10-day incubation with the substrate, 18:1($n-9$)-d6 (Fig. 1; Table 1). The chromatograms generated from GC/FID analysis demonstrated that 18:1($n-9$)-d6 and its metabolites resolved with baseline, or near baseline separation from their non-deuterated counterparts (Fig. 1). Approximately 8.7% of the 18:1($n-9$)-d6 added to the medium was recovered in the *P. marinus* lipids and 3.0, 1.3, 0.2, and 5.3% of the added 18:1($n-9$) were converted to 18:2($n-6$)-d6, 20:2($n-6$), 20:3($n-6$)-d6, and 20:4($n-6$)-d6, respectively, after 72-h incubation (Table 2). The deuterium-labeled arachidonic acid (AA) constituted 11% of the total AA (deuterated AA plus non-deuterated AA) in the *P. marinus* lipids. No 18:3($n-6$)-d6 was detected. These results indicate that in *P. marinus* meronts, 18:1($n-9$)-d6 was first desaturated to 18:2($n-6$)-d6 by Δ-12 desaturase, then elongated to 20:2($n-6$)-d6, and ultimately desaturated to 20:3($n-6$)-d6 and 20:4($n-6$)-d6 using, presumably, the sequential Δ-8 and Δ-5 desaturases (Table 2). The AA synthetic activity was relatively slow in the first 24 h (day 1). But, the quantity of AA increased almost three-fold by day 3 compared to day 1. No significant additional

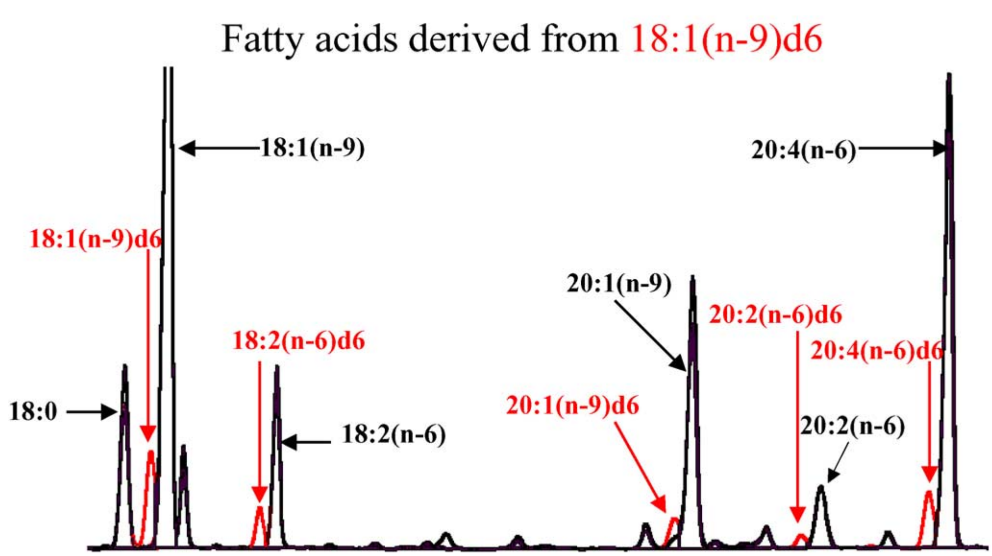

Fig. 1. Evidence of arachidonic acid, 20:4($n-6$), synthesis by *P. marinus* using the deuterated fatty acid substrate 18:1($n-9$)-d6. Red chromatogram from a culture with added 18:1($n-9$)-d6 overlaid on a black chromatogram from a culture with added 18:1($n-9$).

Table 1
Uptake and metabolism of 18:1($n-9$)-d6 by *P. marinus*

	Total FA (μg)			
	Day 1	Day 3	Day 5	Day 10
18:1($n-9$)-d6	9.7 ± 0.8[a]	5.1 ± 0.1[b]	5.0 ± 0.7[b]	4.5 ± 0.4[b]
18:2($n-6$)-d6	1.5 ± 0.1	1.8 ± 0.1	1.7 ± 0.3	1.6 ± 0.2
20:2($n-6$)-d6	0.3 ± 0.0[a]	0.8 ± 0.1[b]	0.9 ± 0.2[b]	0.7 ± 0.1[b]
20:3($n-6$)-d6	Trace	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:4($n-6$)-d6	1.3 ± 0.1[a]	3.5 ± 0.2[b]	3.7 ± 0.8[b]	3.8 ± 0.6[b]
	Total deuterated FAs (μg)			
	12.8 ± 1.0	11.2 ± 0.3	11.3 ± 1.9	10.6 ± 1.3
	Total deuterated FAs (mg/g DW)			
	1.4 ± 0.3	1.3 ± 0.0	0.9 ± 0.0	1.2 ± 0.2
	Cell mass (mg DW)			
	9.5 ± 1.9	8.8 ± 0.4	12.9 ± 2.4	9.1 ± 0.9

Deuterated substrates were added to fresh *P. marinus* media to a concentration of 10 μg/ml (100 μg/culture flask) using 10 mg/ml EtOH stock. *P. marinus* cells from a 7-day-old culture were pelleted and resuspended in the media at a concentration of 10^6 cells/ml. Ten milliliter cultures ($n = 3$) were incubated at 28 °C for 1, 3, 5, and 10 days, then collected and processed for fatty acid analysis. Peaks containing deuterated fatty acids were tentatively identified by comparison to chromatograms of cultures incubated with the same non-deuterated substrate and confirmed by chemical ionization GC/MS. Values are mean ± S.D. for total micrograms of deuterated fatty acids recovered in the cell pellets of the cultures. FA: fatty acid; DW: dry weight. Different letters in superscript (a and b) denote significant differences at the $P < 0.05$ level.

AA synthetic activity was noted from day 3 to day 10 (Table 1).

3.2. Metabolism of the deuterium-labeled linoleic acid [18:2($n-6$)-d4] methyl ester, in P. marinus meronts

Deuterium-labeled 20:2($n-6$), 20:3($n-6$), and 20:4($n-6$) were found in lipids extracted from *P. marinus* meronts after incubation/cultivation with 18:2($n-6$)-d4 methyl ester for 3 days (72 h) (Fig. 1; Table 2). Twelve percent of the added 18:2($n-6$)-d4 methyl ester was incorporated in *P. marinus* lipids and about 4.2, 0.2, and 1.1% were metabolized to 20:2($n-6$)-d4, 20:3($n-6$)-d4, and 20:4($n-6$)-d4, respectively. These results further provide evidence that at the meront stage, the parasite employed the Δ-8 pathway for AA synthesis: elongation of 18:2($n-6$) to 20:2($n-6$) and desaturation of 20:2($n-6$) to 20:3($n-6$) by Δ-8 desaturase then Δ-5 desaturase to make 20:4($n-6$). The deuterium-labeled 20:4($n-6$)-d4 accounted for 21% of the total AA pool.

3.3. Metabolism of deuterium-labeled $n-3$ fatty acids, 18:3($n-3$)-d4 and 20:3($n-3$)-d8 in P. mainus meronts

Deuterium-labeled 20:3($n-3$) and 20:4($n-3$) were detected in lipids of *P. marinus* meronts after incubation/cultivation with 18:3($n-3$)-d4 for 72 h and 20:4($n-3$)-d6 and 20:5($n-3$)-d6 were found after incubation with 20:3($n-3$)-d8 (Table 3). The incorporation of 18:3($n-3$)-d4

Table 2
Metabolism of deuterated $n-6$ and $n-9$ fatty acids by *P. marinus*

Presumptive enzymes	Substrates and products			
	18:1($n-9$)-d6		**18:2($n-6$)-d4 methyl ester**	
	μg/g DW	% recovery	μg/g DW	% recovery
	18:1($n-9$)-d6		–	
	594 ± 4	8.7 ± 0.1		
Δ-12 Desaturase	18:2($n-6$)-d6		**18:2($n-6$)-d4**	
	206 ± 1	3.0 ± 0.1	1146 ± 25	12.1 ± 1.8
Elongase	20:2($n-6$)-d6		20:2($n-6$)-d4	
	88 ± 7	1.3 ± 0.1	399 ± 119	4.2 ± 1
Δ-8 Desaturase	20:3($n-6$)-d6		20:3($n-6$)-d4	
	16 ± 4	0.2 ± 0.1	14 ± 1	0.2 ± 0
Δ-5 Desaturase	20:4($n-6$)-d6		20:4($n-6$)-d4	
	366 ± 15	5.4 ± 0.4	107 ± 34	1.1 ± 0.3
Total recovered	1270 ± 36	18.6 ± 0.6	1666 ± 405	17.6 ± 3.0

Substrates are shown in bold. Deuterated substrates were added to fresh *P. marinus* media to a concentration of 10 μg/ml using 10 mg/ml EtOH stock. *P. marinus* cells from a 7-day-old culture were pelleted and resuspended in the media at a concentration of 10^6 cells/ml. Ten milliliter cultures ($n = 3$) were incubated at 28 °C for 72 h, then collected, and processed for fatty acid analysis. Peaks containing deuterated fatty acids were tentatively identified by comparison to chromatograms of cultures incubated with the same non-deuterated substrate and confirmed by chemical ionization GC/MS. Values presented are μg/g DW ($n = 3$). DW: dry weight.

in *P. marinus* lipids was about 16%. Approximately 13.8 % of the 18:3($n-3$)-d4 added to the medium was elongated to 20:3($n-3$)-d4 and 0.5% was transformed to 20:4($n-3$). The recovery of 20:3($n-3$)-d8 was highest, 30.5%, compared to other deuterated substrates. While no deuterium-labeled 20:5($n-3$) was detected in the lipid when *P. marinus* was incubated with 18:3($n-3$)-d4, about

Table 3
Metabolism of deuterated $n-3$ fatty acids by *P. marinus*

Presumptive enzyme	Substrates and products			
	18:3($n-3$)-d4		**20:3($n-3$)-d8**	
	μg/g DW	% recovery	μg/g DW	% recovery
	18:3($n-3$)-d4		–	
	1.6 ± 0.1	15.5 ± 0.6		
Elongase	20:3($n-3$)-d4		**20:3($n-3$)-d8**	
	1.4±0.1	13.8 ± 0.6	3.2 ± 0.1	30.5 ± 0.7
Δ-8 Desaturase	20:4($n-3$)-d4		20:4($n-3$)-d6	
	0.1±0.0	0.5 ± 0.1	0.1 ± 0.0	0.6 ± 0.0
Δ-5 Desaturase	–		20:5($n-3$)-d6	
			0.3 ± 0.01	2.8 ± 0.1
Total recovered	3.0 ± 0.2	29.9 ± 1.1	3.6 ± 0.1	33.8 ± 0.7

Substrates are shown in bold. Deuterated substrates were added to fresh *P. marinus* media to a concentration of 10 μg/ml using 10 mg/ml EtOH stock. *P. marinus* cells from a 7-day-old culture were pelleted and resuspended in the media at a concentration of 10^6 cells/ml. Ten milliliter cultures ($n = 3$) were incubated at 28 °C for 72 h, then collected and processed for fatty acid analysis. Peaks containing deuterated fatty acids were tentatively identified by comparison to chromatograms of cultures incubated with the same non-deuterated substrate and confirmed by chemical ionization GC/MS. Values presented are mg/g DW ($n = 3$). DW: dry weight.

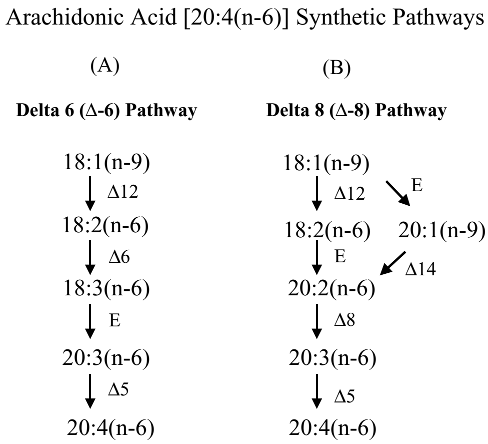

Fig. 2. Arachidonic acid [20:4($n-6$)] synthetic pathways. (A) Delta-6 (Δ-6) pathway. (B) Delta-8 (Δ-8) pathway. E: elongation; Δ6, Δ-6 desaturase; Δ8, Δ-8 desaturase; Δ12, Δ-12 desaturase; Δ14, Δ-14 desaturase; Δ5, Δ-5 desaturase.

0.6% of the 20:3($n-3$)-d8 added into the medium was found converted to 20:5($n-3$)-d6. Results of this experiment further confirm the existence of a Δ-8 pathway in *P. marinus* meronts.

4. Discussion

Our previous studies demonstrated the ability of in vitro cultivated *P. marinus* meronts to synthesize arachidonic acid (AA) de novo using a 2-carbon substrate, ^{13}C-labeled acetate [12]. Only free-living protists have previously been reported to have such a capability. Results of the present study further confirm this synthetic capability in *P. marinus* meronts, which synthesized AA utilizing the provided deuterated substrates, 18:1($n-9$)-d6 and 18:2($n-6$)-d4. Deuterium-labeled AA represented 11–21% of the total AA pool in the parasite.

Two pathways have been described for AA synthesis, the common delta-6 (Δ-6) pathway and the alternate delta-8 (Δ-8) pathway (Fig. 2). The substrate for both pathways is 18:2($n-6$). The common pathway for synthesis of ($n-6$) 20-carbon polyenoic acids begins with Δ-6 desaturation of 18:2($n-6$) to 18:3($n-6$) followed by 2-carbon elongation to 20:3($n-6$) and then further desaturation to 20:4($n-6$) by a Δ-5 desaturase. The alternate Δ-8 pathway begins with an elongation of 18:2($n-6$) to 20:2($n-6$) followed by Δ-8 desaturation to 20:3($n-6$) and a second desaturation at the Δ-5 position to form 20:4($n-6$). Thus, when the initial substrate is 18:2($n-6$), the obligatory intermediates would be 18:3($n-6$) and 20:3($n-6$) for AA synthesis, if the Δ-6 desaturase pathway were employed. On the other hand, if the synthesis of AA were Δ-8 desaturation dependent, the obligatory intermediates would be 20:2($n-6$) and 20:3($n-6$), respectively. In the present study, the inability to detect the intermediate, 18:3($n-6$), but the presence of 20:2($n-6$) and 20:3($n-6$) when *P. marinus* meronts were incubated/cultivated with substrates 18:1($n-9$) or 18:2($n-6$) indicated that the meront stage of this parasite synthesized 20:4($n-6$) employing the Δ-8 pathway and Δ-6 desaturation was absent. In our previous study, incorporation of ^{13}C-acetate into 18:3($n-6$) was also not detected, but found in 20:2($n-6$), 20:3($n-6$), and 20:4($n-6$) [12]. Additionally, the fatty acid 18:3($n-6$) was not present in *P. marinus* cultivated in two different media [22]. The lower level of deuterium-labeled 20:3($n-6$) relative to 20:2($n-6$) and 20:4($n-6$) detected in meront lipids suggests that once 20:3($n-6$) was produced, it was readily desaturated to 20:4($n-6$) by a Δ-5 desaturase. Although further study is needed to define the processes of Δ-8 synthetic pathway enzyme systems and associated activities, the evidence of precursor–intermediate–product relation between 18:2($n-6$), 20:2($n-6$)/20:3($n-6$), and 20:4($n-6$) from the present study proves the presence and expression of Δ-8 desaturase in *P. marinus*. Apparently, *P. marinus* can first convert 18:1($n-9$) to 18:2($n-6$), from which to synthesize 20:4($n-6$) and/or use directly the 18:2($n-6$) methyl ester provided in the media for the same purpose. Additional support for a Δ-8 pathway was provided by metabolism of the deuterium-labeled substrates, 18:3($n-3$) to 20:3($n-3$) and 20:4($n-3$), and 20:3($n-3$) to 20:4($n-3$) and 20:5($n-3$) using the sequential position-specific Δ-8 and Δ-5 desaturases. However, when 18:3($n-3$) was used as the initial substrate, no 18:4($n-3$) or 20:5($n-3$) were detected. This further suggests that Δ-6 desaturase was not available to desaturate 18:3($n-3$) to 18:4($n-3$). The existence of Δ-14 desaturase in a eukaryotic system (the Asian corn borer, *Ostrinia furnacalis*) was first reported by Zhao et al. [28]. While we cannot rule out the conversion of 20:1($n-9$) to 20:2($n-6$) via a Δ-14 desaturase, the conversion of 18:1($n-9$)-d6 to 18:2($n-6$)-d6 via Δ-12 desaturase and the elongation of 18:2($n-6$)-d4 to 20:2($n-6$)-d4 suggest it is unlikely that a Δ-14 desaturase was used for arachidonic acid synthesis in *P. marinus*.

Usage of Δ-6 pathway or Δ-8 pathway or both for 20:4($n-6$) synthesis is known to occur in higher eukayotes and protists [5]. Utilization of Δ-6 pathway for 20:4($n-6$) synthesis has been described in ciliates, trypanosomatis [5], and some phytoplankton species, such as *Porphyridium cruentum* (Rhodophyceae), *Ochromonas danica* (Chrysophyceae), and *Spirula platensis* (Cyanophyceae) [21]. Conversely, employing the Δ-8 pathway to synthesize 20:4($n-6$) has been described in the ciliated protozoan *T. pyriformis* [18] and in the soil amoebae, *Acanthamoeba* sp. [29,30]. In higher animals including mammals, AA is more commonly synthesized by the Δ-6 pathway. However, use of the Δ-8 pathway for 20:4($n-6$) synthesis had been reported in rat liver and testis [31,32], in mouse liver [33], and in mammals including humans [34,35]. The significance of using Δ-8 pathway for 20:4($n-6$) synthesis in higher animals is unclear, because of the presence of the competing Δ-6 desaturase pathway. However, the recent advancement of molecular technology in identifying and cloning Δ-8 and Δ-6 desaturases isolated from organisms should allow for the investigation of their expression separately in the yeast, *Saccharomyces cerevisiae*. A Δ-8 desaturase has recently been isolated from the protist, *Euglena gracilis* and its expression has been tested in *S. cerevisiae* [36].

The fatty acid composition of *P. marinus* is similar to *Acanthamoeba* sp. [22,37]. Evidence of usage of Δ-8 pathway in AA synthesis, suggests that *P. marinus* shares a common ancestor with the free-living protists, *T. pyriformis* and *Acanthamoeba* sp. *P. marinus* disease transmission is via dispersal of infective cells in the water column. In nature, meronts are released from the infected host. The water born meronts are considered free-living prior to their entrance to a new host oyster as it feeds and filters water. The phylogenetic position of *P. marinus* has not been completely resolved. It had been placed in the Phylum Apicomplexa based on morphological similarity [13,38]. However, results of recent morphological and genetic analyses suggest that the phylogenetic position of *P. marinus* is closer to the dinoflagellates than to apicomplexans [39–41].

Polyunsaturated fatty acids (PUFAs), both $(n-3)$ and $(n-6)$, are not only essential membrane components, but also precursors of important molecules that play vital roles in most eukaryotes [42,43]. Mammals can synthesize $(n-3)$ and $(n-6)$ PUFAs from 18:2$(n-6)$ and 18:3$(n-3)$ derived from dietary sources [44]. The ability of *P. marinus* to synthesize PUFAs was not discovered until recently [12,22]. De novo AA synthesis may contribute to the virulence of *P. marinus*. Excess AA may produce harmful effects on the immune system due to a surplus of AA-derived eicosanoids [45–47]. There is increasing evidence that *T. brucei* and *P. falciparum* are capable of producing prostaglandins (PGs) from AA and its metabolites acquired from the host and that these AA-derived PGs are suppressive of host immune response [45–50]. Parasite-derived PGs are believed to be at least in part, responsible for the clinical symptoms, including fever and immunosuppression in patients with chronic African trypanosomiasis [51]. Unlike *P. falciparum* and *T. brucei* whose mammalian hosts are capable of de novo synthesis of AA, the oyster host of *P. marinus* (*C. virginica*) does not have such an ability and must acquire AA from dietary sources [44,52,53]. Additionally, the PUFA profile of oysters is dominanted by $(n-3)$ fatty acids such as 20:5$(n-3)$ (eicosapentaenoic acid, EPA) and 22:6$(n-3)$ (docosahexaenoic acid, DHA) [22]. EPA and DHA accounted for 17 and 9%, respectively, of the total fatty acids and AA accounted for only about 5% of the total fatty acids. However, the purpose of synthesizing 20:4$(n-6)$ de novo in *P. marinus* meronts is unknown and whether or not *P. marinus* meronts produce eicosanids remains to be investigated.

In summary, the present study demonstrated that *P. marinus* meronts synthesized arachidonic acid, 20:4$(n-6)$, from deuterium-labeled substrates 18:1$(n-9)$ and 18:2$(n-6)$ employing the Δ-8 synthetic pathway. After 3-day incubation/cultivation, the deuterium-labeled 20:4$(n-6)$ represented 11–21% of the total 20:4$(n-6)$ in the parasite.

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