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Conversion of dietary *trans*-vaccenic acid to *trans*11,*cis*13-conjugated linoleic acid in the rat lactating mammary gland by Fatty Acid Desaturase 3-catalyzed methyl-end Δ13-desaturation

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ABSTRACT

In vitro, the rat Fatty Acid Desaturase 3 (FADS3) gene was shown to code for an enzyme able to catalyze the unexpected Δ13-desaturation of *trans*-vaccenic acid, producing the *trans*11,*cis*13-conjugated linoleic acid (CLA) isomer. FADS3 may therefore be the first methyl-end *trans*-vaccenate Δ13-desaturase functionally characterized in mammals, but the proof of this concept is so far lacking *in vivo*. The present study therefore aimed at investigating further the putative *in vivo* synthesis of *trans*11,*cis*13-CLA from dietary *trans*-vaccenic acid in rodents. During one week of pregnancy and two weeks post-partum, Sprague-Dawley female rats were fed two diets either high (10.0% of fatty acids and 3.8% of energy intake) or low (0.4% of fatty acids and 0.2% of energy intake) in *trans*-vaccenic acid. The *trans*11,*cis*13-CLA was specifically detected, formally identified and reproducibly quantified (0.06% of total fatty acids) in the mammary gland phospholipids of lactating female rats fed the high *trans*-vaccenic acid-enriched diet. This result was consistent with *FADS3* mRNA expression being significantly higher in the lactating mammary gland than in the liver. Although the apparent metabolic conversion is low, this physiological evidence demonstrates the existence of this new pathway described in the lactating mammary gland and establishes the FADS3 enzyme as a reliable mammalian *trans*-vaccenate Δ13-desaturase *in vivo*.

1. Introduction

In mammalian species, the Fatty acid desaturase (*Fads*) gene cluster includes *Fads1* (Δ5-desaturase), *Fads2* (Δ6-desaturase) and a third gene member, named *Fads3* [1–6]. According to its high degree of nucleotide sequence homology with both *Fads1*/*Fads2*, *Fads3* was promptly suspected to code for a new mammalian membrane-bound fatty acid (FA) desaturase. However, no catalytic activity was attributed to the FADS3 protein for a long time, although alternative mRNA transcripts of *Fads3* were described [3,7] and *Fads3*-knockout mice were generated [8,9].

Recently, we showed *in vitro* the specific ability of both recombinant and native rat FADS3 to catalyze the Δ13-desaturation of *trans*-vaccenic acid (*trans*11-18:1; TVA) [10], producing a conjugated linoleic acid (CLA) isomer with *trans*11,*cis*13 double bonds (*trans*11,*cis*13-18:2). This FA was already described as a minor component of ruminant milk [11]. As initially suspected, this *in vitro*-elucidated pathway demonstrated that FADS3 enzyme has a FA desaturase catalytic activity, but with quite unexpected features. The main unexpected feature was the Δ13-position of the inserted double bond, suggesting that FADS3 was consequently the first methyl-end desaturase described in mammals [12] and not a front-end desaturase as previously hypothesized from its amino-acid sequence [13]. Another surprising feature was that, inside a large panel of FAs, TVA was the single substrate to be identified [10].

However, these *in vitro* data have for now not been replicated *in vivo* [9] and their physiological reliability is reasonably questioned, although we recently provided several lines of evidence that the *trans*11,*cis*13-CLA detected in ruminant milk [11] may partly originate from direct FADS3-catalyzed Δ13-desaturation of TVA in ruminant mammary tissue [6].

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In order to address the questions raised by this *in vitro* unexpected catalytic desaturase activity, the present study aimed at investigating further the putative *in vivo* synthesis of *trans*11,*cis*13-CLA by FADS3-catalyzed Δ13-desaturation of dietary TVA in rodents. Considering the previous results showing the presence of this specific FA in milk fat and suggesting its synthesis in the ruminant mammary gland [6], the study was mainly focused on the lactating mammary tissue in non-ruminant animals. To achieve this, Sprague-Dawley female rats were fed two diets either high (10.0% of fatty acids) or low (0.4% of fatty acids) in TVA during 1 week of pregnancy and 2-weeks post-partum. The *trans*11,*cis*13-CLA was specifically detected, formally identified and reproducibly quantified (0.06% of FAs) in the mammary gland phospholipids of lactating female rats fed the high-TVA diet.

2. Materials and methods

2.1. Chemical synthesis of ethyl trans-vaccenate added to the diets

Large-scale synthesis (about 100 g) of TVA ethyl ester (purity over 95%) was based on optimized literature procedures [14,15], as recently described [16].

2.2. Diets and animal feeding

Two lipid mixtures, either high or low in TVA (Table 1), were prepared by adding the purified TVA ethyl ester fraction to a common lipid base made of commercial oils (olive, rapeseed, palm and corn oils). In the high-TVA diet, TVA represented 10.08% of fatty acids and 3.8% of energy. In the low-TVA diet, TVA represented only 0.44% of fatty acids (0.2% of energy) and compensation was obtained by adding ethyl *cis*-vaccenate to keep the diets isocaloric and isolipidic. These two lipid mixtures were then similarly combined at 20% (g/100 g of final diets) with 37.5% of starch, 18.8% of sucrose, 17.7% of casein, 1.6% of cellulose, 3.6% of mineral mix and 0.8% of vitamin mix (Unité de Production d'Aliments Expérimentaux, INRA, Jouy en Josas, France). Both diets were free of any conjugated linoleic acid (Table 1).

All protocols complied with the European Union Guideline for animal care and use (2010/63/CEE). The experimental procedure (#3059-2015-120718477744) was approved by the French Animal Care Committee (Rennes). Height pregnant Sprague-Dawley female rats (day 14) were purchased from Envigo (Gannat, France) and randomly assigned to one of the two experimental diets (n = 4/group). Dams were housed individually with 12 h-12 h light-dark cycles. The rats were allowed free access to food and water. The fresh diets were given for three weeks to the maternal rats daily. At postnatal day 14, dams were kept for overnight fasting and euthanized by intraperitoneal injection of pentobarbital (Euthasol Vet, 140 mg/kg). Tissues were collected and frozen in liquid N_2 before storage at −80 °C.

Table 1
Fatty acid composition (% of total fatty acids) of the experimental diets either low or high in *trans*-vaccenic acid (TVA). SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; ND: not detected.

Fatty acid (%)	low-TVA diet	high-TVA diet
C14:0	0.07	0.07
C16:0	9.90	9.95
C17:0	0.02	0.02
C18:0	2.02	2.04
C20:0	0.39	0.39
ΣSFA	**12.40**	**12.47**
C16:1 n-9 *cis*	0.06	0.06
C16:1 n-7 *cis*	0.41	0.41
C18:1 n-9	55.71	56.09
C18:1 n-7 *cis* (CVA)	13.14	3.02
C18:1 n-7 *trans* (TVA)	0.44	10.08
C20:1 n-9	0.62	0.62
ΣMUFA	**70.38**	**70.28**
C18:2 n-6	13.57	13.60
Σ n-6 PUFA	**13.57**	**13.60**
C18:3 n-3	3.65	3.65
Σ n-3 PUFA	**3.65**	**3.65**
C18:2 *cis*-9,*trans*-11	ND	ND
C18:2 *trans*-11,*cis*-13	ND	ND
Σ CLA	**ND**	**ND**

2.3. Lipid extraction, isolation of lipid species and phospholipid classes

Lipids from collected mammary tissue and liver were extracted using a mixture of dimetoxymethane/methanol (4:1, v/v) [17]. Lipid species were separated by thin layer chromatography as described [18] and phospholipid classes were subsequently separated by a second thin layer chromatography using silicagel H plates and a mixture of methanol/ethanol/water/trimethylamine (20:50:7:30 v/v/v/v) for development [19].

Isolated lipid species and phospholipid classes were saponified for 30 min at 70 °C with 1 mL of 0.5 mol/L NaOH in methanol and methylated with 1 mL BF_3 (12% in methanol) for 15 min at 70 °C [20]. The fatty acid methyl esters (FAMEs) were extracted with pentane.

2.4. FA analysis, identification and quantification

FAMEs were analyzed by GC-MS using an Agilent 7890N (Agilent Technologies, Santa Clara, CA, USA), as recently described [16].

Identification of the FAMEs was based upon retention times (Rt) obtained for methyl ester of authentic standards, when available. The commercial FAs used as standards were rumenic acid (*cis*9,*trans*11-CLA), a mixture of *cis*11,*trans*13-CLA, *cis*11,*cis*13-CLA and *trans*11,*trans*13-CLA (Matreya, Pleasant Gap, PA, USA) and pure *trans*11,*trans*13-CLA (Larodan, Malmö, Sweden). Since there is no commercially available standard for the *trans*11,*cis*13-CLA [10,21], we also designed for this study our own FAME standard reference mixture containing this FA. To achieve this, pooled FA samples extracted from COS-7 cells expressing recombinant rat FADS3 and producing the *trans*11,*cis*13-CLA when incubated with TVA [10] were converted to FAMEs and partially purified by separation on preparative silver ion thin layer chromatography (silicagel H impregnated with 3% w/w $AgNO_3$) using a mixture of diethylether/hexane (10/90 v/v) for development [22]. This standard reference mixture contained therefore the *trans*11,*cis*13-CLA but also rumenic acid synthesized from TVA by the native Δ9-desaturase present in COS-7 cells, and several C20:1 isomers, including the *trans*13-C20:1 isomer produced by TVA elongation [10].

2.5. Identification of CLA after their reaction with 4-methyl-1,2,4-triazoline-3,5-dione

To confirm the identity of CLA isomers, FAMEs from phospholipid samples of the lactating mammary glands were pooled and partially purified by separation on preparative silver ion thin layer chromatography as described above. Collected CLAs were converted to Diels-Alder adducts [23,24] by reaction with 4-methyl-1,2,4-triazoline-3,5-dione (MTAD). CLAs in dichloromethane were mixed with MTAD (1 mg/mL, Santa Cruz Biotechnology) at 0 °C for 10 s. The reaction was stopped immediately by addition of a fivefold excess of 1,3-hexadiene (Santa Cruz Biotechnology). MTAD adducts

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