Amino Acids (2011) 41:969–979
DOI 10.1007/s00726-010-0797-7

ORIGINAL ARTICLE

A role for PPARα in the regulation of arginine metabolism and nitric oxide synthesis

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Received: 10 September 2010 / Accepted: 22 October 2010 / Published online: 10 November 2010

Abstract The pleiotropic effects of PPARα may include the regulation of amino acid metabolism. Nitric oxide (NO) is a key player in vascular homeostasis. NO synthesis may be jeopardized by a differential channeling of arginine toward urea (via arginase) versus NO (via NO synthase, NOS). This was studied in wild-type (WT) and PPARα-null (KO) mice fed diets containing either saturated fatty acids (COCO diet) or 18:3 n-3 (LIN diet). Metabolic markers of arginine metabolism were assayed in urine and plasma. mRNA levels of arginases and NOS were determined in liver. Whole-body NO synthesis and the conversion of systemic arginine into urea were assessed by using $^{15}N_2$-guanido-arginine and measuring urinary $^{15}NO_3$ and [^{15}N]-urea. PPARα deficiency resulted in a markedly lower whole-body NO synthesis, whereas the conversion of systemic arginine into urea remained unaffected. PPARα deficiency also increased plasma arginine and decreased citrulline concentration in plasma. These changes could not be ascribed to a direct effect on hepatic target genes, since NOS mRNA levels were unaffected, and arginase mRNA levels decreased in KO mice. Despite the low level in the diet, the nature of the fatty acids modulated some effects of PPARα deficiency, including plasma arginine and urea, which increased more in KO mice fed the LIN diet than in those fed the COCO diet. In conclusion, PPARα is largely involved in normal whole-body NO synthesis. This warrants further study on the potential of PPARα activation to maintain NO synthesis in the initiation of the metabolic syndrome.

Keywords Metabolic syndrome · Amino acids · α-Linolenic acid · n-3 PUFA · Urea

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Introduction

The metabolic syndrome, the prevalence of which is increasing worldwide, is a cluster of abnormalities including visceral obesity, dyslipidemia, insulin resistance, and elevation in blood pressure that results in a higher risk of cardiovascular diseases and type-2 diabetes (Cornier et al. 2008). Intense and sustained research efforts aiming to the prevention and care of metabolic syndrome have enlightened the importance of a group of nuclear receptors known as peroxisome activated receptors (PPAR) as efficient targets for nutritional and pharmaceutical interventions (Kersten et al. 2000; Robinson and Grieve 2009). Among PPARs, PPARα has been identified as a major modulator of various aspects of lipid metabolism, such as fatty acid (FA) catabolism (uptake, trafficking, oxidation, and ketogenesis), polyunsaturated FA synthesis, and lipoprotein metabolism (assembly, transport, and catabolism). PPARα is activated by pharmacological (fibrates) and nutritional (n-3 polyunsaturated FA, PUFA) agonists, which are both widely used as regulators of lipid metabolism, especially as hypolipidemic agents (Hachem and Mooradian 2006). Following its activation, the transcriptional regulation of target genes by PPARα is achieved by

its direct binding to specific, yet ubiquitous, nucleotidic sequences known as peroxisome proliferators response elements (PPRE). In consequence, beyond lipid metabolism, the effects of PPARα extend to a number of target genes involved in the metabolism of glucose, glycerol and glycogen, and of bile acids, as well as in inflammation, detoxification, and hepatocarcinogenesis (Mandard et al. 2004, 2007; Patsouris et al. 2004). In line with these pleiotropic effects, PPARα has also been suggested to play a role in the regulation of amino acid metabolism, but the current literature remains limited. The use of PPARα-null (KO) mice, or fibrate-treated rodents, has evidenced that PPARα is directly implicated in the regulation of a number of hepatic target genes involved in transamination, deamination and urea synthesis (Edgar et al. 1998; Kersten et al. 2001; Sheikh et al. 2007).

In the context of the metabolic syndrome, interest in amino acids has focused on L-arginine, because it is the precursor of nitric oxide (NO), a key player in vascular homeostasis. Indeed, the impairment in NO synthesis/bioavailability is closely associated with vascular endothelial dysfunction, which is the hallmark of atherosclerosis and is closely associated with insulin resistance (Brunner et al. 2005; Forstermann 2010; Kim et al. 2006). However, arginine is implicated in many metabolic pathways, and is largely used for urea synthesis, quantitatively much more than for NO synthesis. Accordingly, the literature suggests that NO synthesis could depend on the differential arginine channeling toward either urea (via arginase) versus NO (via NO synthase, NOS) (Morris 2009). Interestingly, PPARα could be involved in this differential channeling of arginine. Indeed, PPARα-null mice exhibit a higher systolic blood pressure and a lower renal NOS activity (Newaz et al. 2005), together with higher fasting levels of hepatic arginase mRNA and plasma urea (Kersten et al. 2001). In parallel, treatment by pharmacological PPARα agonists improves endothelial function both in vitro and in vivo, including in the human (Touyz and Schiffrin 2006). Exact in vivo mechanisms for these beneficial actions remain unclear, but would probably involve NO synthesis. Indeed, pharmacological PPARα activation was suggested to increase NO synthesis in the rat (Linz et al. 2009; Newaz et al. 2004, 2005), renal NOS activity in the WT mouse (Newaz et al. 2005), and eNOS protein expression and phosphorylation in the rat (Bulhak et al. 2009; Linz et al. 2009), whereas plasma arginine concentration was decreased (Linz et al. 2009). Thus, PPARα activation appears to play a major role in the regulation of arginine metabolism.

A growing body of evidence suggests that not only pharmacological, but also nutritional PPARα agonists favor NO synthesis. This is the case in endothelial cells or in isolated arteries not only in response to one of the long-chain PUFA found in fish oil (EPA, 20:5 n-3) (Omura et al. 2001; Singh et al. 2010), but also in response to its direct precursor α-linolenic acid (ALA, 18:3 n-3) (Zhang et al. 2007). More interestingly, in vivo studies suggested that dietary n-3 PUFA intake enhanced whole-body NO synthesis, as indirectly assessed by determination of nitrate and nitrite levels in hypertensive rats (in response to ALA) (Sekine et al. 2007), or urinary nitrate in healthy volunteers (in response to fish oil) (Harris et al. 1997), or by measurement of NOS activity and eNOS phosphorylation in brain and muscle of piglets (in response to another long-chain PUFA found in fish oil, DHA, 22:6 n-3) (Li et al. 2008). However, it remains to be determined to what extent these beneficial effects of dietary n-3 PUFA on NO synthesis involve PPARα, on the one hand, and regulation of amino acid metabolism, and especially of arginine, on the other hand. In this respect, studies on the liver are a special interest, since this organ plays a central role in the metabolism of amino acids and is a key target of PPARα activation.

The aim of the present study was, therefore, to evaluate the role of PPARα in the regulation of arginine metabolism, with special emphasis on the impact on NO synthesis, using wild-type (WT) and PPARα-null (KO) mice. We used a state-of-the-art tracer-based method to measure whole-body NO synthesis, using [$^{15}N_2$-(guanido)]-arginine. Because we also made the hypothesis that a change in NO synthesis could originate from a competition between NO synthesis and urea production, we also evaluated the conversion of systemic arginine into urea, using the same tracer method. Lastly, because the liver is a key target of PPARα activation, we evaluated the gene regulation of some hepatic enzymes involved in arginine metabolism.

We have shown previously in the mouse that a number of hepatic genes known to be regulated essentially via PPARα were up-regulated by ALA, as they are by its long-chain derivatives. This was observed not only in WT mice fed rather high-fat diets (Morise et al. 2009) but also in WT mice fed low-fat diets, albeit to a lesser extent (Martin et al. 2007). For these reasons, as a secondary objective, we aimed to assess the contribution of dietary n-3 PUFA to PPARα activation, by exposing mice to contrasted diets, containing mostly either saturated FA or ALA.

Materials and methods

Experimental procedures

Animals

Male PPARα-deficient mice (Lee et al. 1995) were bred locally in INRA's facility in Toulouse, in which several additional rounds of backcrossing have been performed

initially to increase the C57BL/6J genetic background and to generate the animals used (Costet et al. 1998). Age-matched male C57BL/6J mice were obtained from Charles River (L'Arbresle, France) and were acclimated to local animal facility conditions for 4 weeks. In vivo studies were conducted under European Union guidelines for the use and care of laboratory animals and were approved by the institutional ethical committee. The same protocol was conducted on two successive series of 30 mice.

Animals were housed collectively on wood litter, at 22 ± 2°C under 12-h light/dark cycles (light on at 06:00 am). They were fed ad libitum a standard pelleted diet for 4 weeks (Teklad 20-18S, Harlan, Gannat, France). At 10–11 weeks of age, mice were fed one of the experimental diets during 8 weeks. They had free access to food and tap water. Food consumption (as assessed per collective cage, and expressed relatively to the mean body weight of mice in each cage) and individual body weight were recorded weekly.

Diets

The two experimental diets were provided as pellets by UPAE-INRA (Jouy-en-Josas, France) as described previously (Martin et al. 2007). The calculated composition (in weight) of the two diets was 21.0% protein, 69.2% carbohydrate, 4.8% lipid, 4.0% vitamins and 4.0% minerals. The experimental diets were isoenergetic, with lipids providing 11.3% of total energy intake. The choice of a low-fat diet was determined by a previous nutrigenomic study of some of the present authors, showing significant effects of PPARα deficiency on lipid and xenobiotic metabolism in mice on the same diet as in the present study (Martin et al. 2007). Oils used for experimental diet preparation were hydrogenated coconut oil for a saturated FA diet (SFA, 99%, COCO diet), and linseed oil for an ALA-rich oil (58%, LIN diet). Their fatty acid composition was (in weight %): 99.6 SFA, 0.3 monounsaturated FA (MUFA) and 0.1% PUFA for the COCO diet; 9.0 SFA, 18.0 MUFA and 72.9 PUFA (58 n-3 PUFA) for the LIN diet (Martin et al. 2007).

Experimental design

At 18–19 weeks of age, after 8 weeks of experimental diets, mice were fasted for 5 h, then weighed and anesthetized. Blood was taken by cardiac puncture, and mice were then killed by exsanguination. Plasma was separated by centrifugation (1,700*g*, 20 min, 4°C) and aliquots were stored at −80°C.

The abdominal cavity was then opened and the liver was carefully removed and weighed. Several liver samples were immediately frozen in liquid nitrogen and stored at −80°C. Epididymal adipose tissue (EpAT, visceral localization) and inguinal adipose tissue (IngAT, subcutaneous localization) were removed and weighed.

Before killing, mice were used for the evaluation of in vivo whole-body NO synthesis as described previously in rats (Magne et al. 2009). Briefly, mice were fasted for 3 h, administered IP 90 mg/kg body wt of [$^{15}N_2$-(guanido)]-arginine:HCl, then placed immediately in a metabolic chamber and fed ad libitum. Urine was collected over 23 h in glass tubes containing antibiotics for the determination of whole-body NO synthesis, as described below.

Analyses

Plasma and urine metabolites

Blood glucose concentration was determined with an Accu-Chek® glucometer (Roche Diagnostics, Meylan, France). Plasma cholesterol, triglycerides, and urea were determined by colorimetric enzymatic methods using the kits Cholesterol RTU, Triglycerides PAP 150 and Urea-Kit S 1000 (Bio-Merieux, Craponne, France) (Fawcett and Scott 1960; Fossati and Prencipe 1982; Richmond 1973).

Plasma amino acids were determined by ion-exchange chromatography with postcolumn ninhydrine detection (Aminotac JLC-500/V; Jeol, Tokyo, Japan). To calculate the total excretion of urea, nitrate and creatinine during the tracer study (see below), urinary urea concentration was determined as in plasma after the samples were diluted 1/2,000, and urinary creatinine and nitrate concentrations were measured using colorimetric kinetic (Biomerieux) and fluorimetric methods, respectively (Marzinzig et al. 1997; Vassault et al. 1992).

Assessment of whole-body NO synthesis and conversion of systemic arginine into urea

Whole-body NO synthesis was measured as the relative recovery of ^{15}N as urinary nitrate following the IP administration of [$^{15}N_2$-(guanido)]-arginine (Cambridge Isotope Lab), based on a method described by Forte et al. (1997), and as reported in detail previously (Blouet et al. 2007; Magne et al. 2009). This method was preferred to the measure of urinary nitrate excretion, which is highly confounded by other sources, in particular the dietary nitrate (Forte et al. 1997). In contrast, this tracer-based method has the advantage of being quantitative and specific for the L-arginine/NO pathway, and is considered as a state-of-the-art technique to assess whole-body NO synthesis in vivo. Briefly, urinary nitrate were extracted by ion-exchange chromatography and the microdiffusion technique, and the isotopic enrichment was determined by Elemental analyzer coupled to Isotope Ratio Mass Spectrometry (EA-IRMS).

The conversion of systemic arginine into urea was quantified as the relative recovery of ^{15}N in urinary urea following the same IP administration of [$^{15}N_2$-(guanido)]-arginine. Urea was isolated from urine as previously described by Mariotti et al. (2000). Briefly, urine ammonia was first extracted using a cation exchange resin (Dowex AG-50X8, Mesh 100-200, BioRad, France). Then, urea was extracted from the ammonia-free urine by conversion into ammonium through hydrolysis with urease (Sigma, Saint-Quentin-Fallavier, France) for 2 h at 30°C, pH 7, on cation exchange resin. Urea-derived ammonia were eluted from the resins by the addition of 2.5 mol/L KH_2SO_4, and ~50 µL eluate samples were combusted in the EA-IRMS to determine isotopic enrichment.

To calculate whole-body NO synthesis and systemic conversion of arginine into urea, the amounts of ^{15}N recovered as nitrate and urea in the 23-h urine collection were corrected for the excretion of creatinine, so as to correct for possible differences in urinary excretion rate between animals and treatments and for possible incomplete urinary collection (Forte et al. 1997). Thus, values were expressed as % of the amount of ^{15}N administered as [$^{15}N_2$]-arginine as follows: QE/dC, where Q are the total amounts of nitrate (or urea) and C the total amounts of creatinine excreted in urine, E the ^{15}N enrichment (as atom percent excess) in urinary nitrate (or urea, respectively) and d the amount of ^{15}N injected as [$^{15}N_2$-(guanido)]-arginine.

The calculation takes advantage of the fact that the amount of [^{15}N-^{14}N]-arginine is negligible as compared to the amount of [^{15}N-^{15}N]-arginine, since the former originate from the recycling of ^{15}N-citrulline that originates from a very minor pathway (<1%) of utilization of [^{15}N-^{15}N]-arginine (Luiking and Deutz 2003). Accordingly, the $^{15}NO3$ formed from [^{15}N-^{14}N]-arginine is also negligible compared to that formed from [^{15}N-^{15}N]-arginine, and, likewise, the amount of [^{15}N-^{14}N]-urea is negligible as compared to the amount of [^{15}N-^{15}N]-urea.

Real-time quantitative RT-PCR

Total RNA was extracted from a liver sample homogenized with an UltraTurrax in the presence of Trizol reagent (Invitrogen, Carlsbad, USA). Total RNA concentrations were determined by measuring absorbance at 260 nm with a Nanodrop spectrophotometer (Labtek, Paris, France). A 260 nm/280 nm ratio of ~2 indicated that samples were essentially free from contaminants such as protein and phenol. The quality and integrity of total RNA were assessed on 1% agarose.

Synthesis of cDNA was performed on 400 ng of total RNA using a high capacity cDNA reverse transcription kit, based on the use of both oligodT and hexamers (Applied Biosystems, Foster City, USA). Primers were designed using Primer Express software (Applied Biosystems, Foster City, CA) with the following sequences: Cyp4a14: forward primer, TCAGTCTATTTCTGGTGCTGTTC; reverse primer, GAGCTCCTTGTCCTTCAGATGGT; ARG1 (Arginase 1): forward primer, GCAGAGGTCCAGAAGAA TGGAA; reverse primer, GCGTGGCCAGAGATGCTT; ARG2 (Arginase 2): forward primer, CTTGGGATCC AGAAGGTGATG; reverse primer, GCCTCTGCCTT TTGCCAAT; NOS3 (endothelial nitric oxide synthase, eNOS): forward primer, GCACAAGAGCTACAAAAT CCGATT; reverse primer, GCCGCCAAGAGGATACCA; NOS2 (inducible nitric oxide synthase, iNOS): forward primer, GCAAACCCAAGGTCTACGTTCA; reverse primer, GAGCACGCTGAGTACCTCATTG; ALAT (Alanine aminotransferase): forward primer, AAGGCACT TACCACTTCAGAATGAC; reverse primer, ACCTTG TGGAGCACGGTCTT; cASAT (aspartate aminotransferase, cytosolic precursor): forward primer, AGCTGTGC TTCTCGCCTAGTTC C; reverse primer, GCCTGTCCC TCCCAAAGACT; mASAT (aspartate aminotransferase, mitochondrial precursor): forward primer, TGCCAAT CGTATGCCAAGAAC; reverse primer, CCTTTTGGCT TCTTCTGCATCT. To normalize the mRNA abundance of each gene, 18S rRNA was used as a housekeeping gene, with the exception of Cyp4a14 gene, for which TBP (tata-box binding protein) was used. Sequences of the corresponding primers were: 18S: forward primer, ACGGA AGGGCACCACCAGGAG; reverse primer, GCACCAC CACCCACGGAAACG; TBP: forward primer, ACTTCG TGCAAGAAATGCTGAA; reverse primer, GCAGT TGTCCGTGGCTCTCT. In order to not amplify possible residual genomic DNA, only primers for which at least one sequence of the pair overlaps an exon/exon junction on the mRNA sequence, or primers located on different exons, were chosen. PCR reactions were performed using a 7300 real-time PCR system (Applied Biosystems), as described previously (Chotechuang et al. 2009). Each cDNA was amplified in a 20 µL volume containing 15 µL of 2× SYBR Green master mix (Applied Biosystems) and 500 nM concentrations of the gene-specific primer. Thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min and 40 amplification cycles at 95°C for 15 s and 60°C for 1 min. For each run, data were analyzed as previously described for residual genomic DNA amplification and for primer–dimers formation (Chotechuang et al. 2009). Gene expression was determined using the $2^{-\Delta Ct}$ formula where ΔCt = (Ct target gene − Ct housekeeping gene).

Statistical analyses

Data are presented as mean ± standard errors. They were analyzed using the SAS program (SAS Institute, Cary,

USA). The effects of the genotype (KO vs. WT), the diet (LIN vs. COCO), and the genotype–diet interaction were tested using a two-way ANOVA with genotype, diet, and series as factors, using the GLM procedure. When the effect of genotype and/or diet factor was significant, differences between means were tested for significance using the post hoc Tukey–Kramer procedure. Significance level was set at $P < 0.05$. When a series effect was significant, data were normalized as % of the WT group fed the COCO diet.

Results

Markers of PPARα deficiency (Table 1)

Throughout the 8 weeks of the experimental protocol, daily food intake was similar in all groups (±120 mg/g body weight per day) whatever the genotype or the diet (data not shown). In WT mice, the dietary treatment did not influence the body weight and composition significantly. PPARα deficiency resulted in a higher body weight than in WT mice, but between-group differences were significant only in those fed the COCO diet. Similarly, when compared to their WT counterparts, KO mice fed the COCO diet exhibited higher proportions of liver and of epididymal (visceral) and inguinal (subcutaneous) adipose tissue, but not those fed the LIN diet.

PPARα deficiency resulted in lower glycemia and higher triglyceridemia, the degree of which was irrespective of the diet. In contrast, hypercholesterolemia found in the KO mice was more pronounced in mice fed the COCO diet than in those fed the LIN diet.

Plasma amino acids (Table 2)

As concerns the amino acids involved in the urea cycle, their plasma concentration in the WT group fed the COCO diet was: arginine, 53.1 μmol/L; ornithine, 44.0 μmol/L; citrulline, 53.0 μmol/L. Whatever the genotype, the dietary treatment did not influence their plasma concentration (Table 2). When compared to the WT mice (COCO and LIN diet together), PPARα deficiency resulted in a higher arginine concentration and a lower citrulline concentration, whereas ornithine concentration tended to decrease, but was not significantly affected. Besides, post hoc analysis revealed that, in KO mice, the increase in arginine concentration was more pronounced in those fed the LIN diet, whereas the decrease in ornithine and citrulline concentration was accounted for by mice fed the COCO diet mainly. Accordingly, ornithine/arginine ratio and citrulline/arginine ratio were significantly lower in KO mice than in WT ones, whereas ornithine/citrulline ratio was not affected by the genotype (Table 2).

Some other amino acids were also sensitive to the genotype. PPARα deficiency resulted in increased plasma concentrations of serine, valine, methionine, histidine and lysine, and in decreased plasma concentrations of taurine, glycine and α-amino-*N*-butyric acid. A few amino acids were sensitive to the diet, their plasma concentration being lower in the LIN group than in the COCO diet. This was the case for histidine, lysine, and α-amino-*N*-butyric acid, with no interaction with the genotype.

Hepatic expression of target genes (Fig. 1)

Relative mRNA levels were expressed as the % of the WT group fed on the COCO diet. Level of mRNA for Cyp4a14, a typical PPARα target gene, was measured to check whether the genotype and/or the diet were associated with changes in PPARα activity in the liver. Cyp4a14 was almost null in PPARα-null mice (±2% of the WT COCO group), but significantly increased in WT mice fed the LIN diet, as compared to those fed the COCO diet. As concerns the target genes of arginine metabolism, level of mRNA for arginase 1

Table 1 Markers of PPARα deficiency in WT and PPARα-null (KO) mice fed diets rich in either saturated FA (COCO diet) or ALA (LIN diet) for 8 weeks

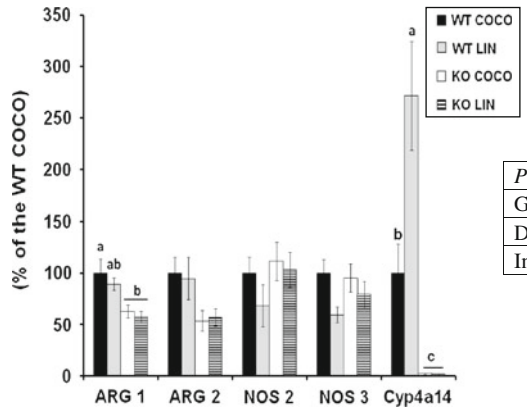
	WT		KO		*P* values		
	COCO	LIN	COCO	LIN	Genotype (*G*)	Diet (*D*)	Interaction $G \times D$
Body weight (g)	29.5 ± 0.5^{b}	29.6 ± 0.5^{b}	36.7 ± 1.0^{a}	32.7 ± 1.5^{b}	<0.0001	0.0379	0.0261
EpAT weight (%)	2.31 ± 0.16^{b}	2.58 ± 0.19^{ab}	3.47 ± 0.28^{a}	2.91 ± 0.40^{ab}	0.0038	0.5062	0.0720
IngAT weight (%)	1.46 ± 0.19^{b}	1.78 ± 0.12^{ab}	2.50 ± 0.23^{a}	1.79 ± 0.22^{ab}	0.0090	0.3293	0.0102
Liver weight (%)	3.88 ± 0.10^{b}	3.88 ± 0.25^{b}	4.50 ± 0.12^{a}	3.71 ± 0.12^{b}	0.1403	0.0112	0.0123
Blood glucose (g/L)	1.94 ± 0.18^{b}	1.83 ± 0.21^{b}	1.48 ± 0.17^{ab}	1.17 ± 0.12^{a}	<0.0001	0.1892	0.4690
Plasma TG (mg/dL)	34.5 ± 3.0^{ab}	35.3 ± 3.1^{b}	45.7 ± 3.0^{a}	50.3 ± 5.1^{a}	<0.0001	0.2957	0.4611
Plasma CT (mg/dL)	83.4 ± 3.7^{bc}	67.5 ± 5.7^{c}	120.7 ± 4.4^{a}	95.5 ± 6.3^{b}	<0.0001	<0.0001	0.3136

Values are mean ± standard errors for 11–13 mice per group. Mean values within a row sharing a same superscript letter, or without superscript letter, were not significantly different at $P < 0.05$

Table 2 Plasma amino acids in WT and PPARα-null (KO) mice fed diets rich in either saturated FA (COCO diet) or ALA (LIN diet) for 8 weeks

	WT		KO		*P* values		
	COCO	LIN	COCO	LIN	Genotype (*G*)	Diet (*D*)	Interaction $G \times D$
Serine	100 ± 3	95 ± 6	112 ± 5	116 ± 7	0.0044	0.9040	0.3943
Valine	100 ± 5	96 ± 3	106 ± 5	111 ± 7	0.0287	0.9187	0.2969
Methionine	100 ± 4	100 ± 6	118 ± 7	122 ± 10	0.0083	0.8054	0.8084
Histidine	100 ± 5	86 ± 3	109 ± 5	105 ± 5	0.0039	0.0495	0.2772
Lysine	100 ± 8^{ab}	75 ± 5^{b}	108 ± 8^{a}	99 ± 9^{ab}	0.0366	0.0484	0.2448
Taurine	100 ± 9	109 ± 8	90 ± 7	81 ± 6	0.0268	0.9932	0.2680
Glycine	100 ± 5^{a}	97 ± 5^{ab}	81 ± 4^{b}	95 ± 4^{ab}	0.0144	0.2232	0.0827
α-Amino-*N*-butyric acid	100 ± 8^{a}	70 ± 4^{ab}	74 ± 9^{ab}	66 ± 4^{b}	0.0340	0.0139	0.1321
Threonine	100 ± 3^{ab}	95 ± 6^{ab}	87 ± 5^{b}	113 ± 7^{a}	0.7852	0.0754	0.0061
Arginine	100 ± 5^{b}	93 ± 6^{b}	111 ± 5^{ab}	129 ± 7^{a}	0.0008	0.3789	0.0483
Ornithine	100 ± 5^{a}	81 ± 6^{ab}	80 ± 6^{b}	87 ± 6^{ab}	0.1053	0.2477	0.0197
Citrulline	100 ± 5^{a}	92 ± 6^{ab}	79 ± 4^{b}	88 ± 5^{ab}	0.0104	0.9679	0.1096
Ornithine/arginine	100 ± 7^{a}	88 ± 7^{ab}	70 ± 5^{b}	67 ± 5^{b}	0.0002	0.2197	0.5362
Citrulline/arginine	100 ± 5^{a}	100 ± 6^{a}	71 ± 4^{b}	69 ± 4^{b}	<0.0001	0.7827	0.7675
Ornithine/citrulline	100 ± 7	87 ± 4	100 ± 6	99 ± 9	0.4346	0.3208	0.3828

Due to some statistically significant inter-series effects, values are expressed as % of the WT group fed the COCO diet. Only amino acids of which plasma concentration was influenced by the genotype and/or the diet are reported, with the exception of amino acids of the urea cycle, of which concentrations are reported in Fig. 1. Values are mean ± standard errors for 11–13 mice per group. Mean values within a row sharing a same superscript letter, or without superscript letter, were not significantly different at $P < 0.05$



P values	ARG1	ARG2	NOS2	NOS3	Cyp4a14
Genotype (G)	0.0005	0.0052	0.1974	0.5390	<.0001
Diet (D)	0.3792	0.9441	0.2640	0.0241	0.0023
Interaction (G*D)	0.7793	0.7742	0.5196	0.3209	0.0021

Fig. 1 Hepatic mRNA levels of CYP4a14 and arginine metabolism key genes in WT and PPARα-null (KO) mice fed diets rich in either saturated FA (COCO diet) or ALA (LIN diet) for 8 weeks. Values (after correction relative to mRNA TBP or 18S levels) are expressed as % of the WT group fed the COCO diet (mean ± standard errors for 11–13 mice per group). For each parameter, *columns* sharing a *same superscript letter*, or *without superscript letter*, were not significantly different at $P < 0.05$

(ARG1), which is cytosolic and mainly ascribed to the urea cycle in the liver, was not affected by the dietary treatment. In contrast, it was significantly decreased by PPARα deficiency. Arginase 2 is mitochondrial, more ubiquitous, and competes with NOS3 (eNOS) for NO synthesis. Similar to arginase 1, arginase 2 (ARG2), mRNA level was not diet-responsive, but was significantly lower in KO mice. The expression of (constitutive) NOS3 (eNOS) was not affected by the genotype, but was significantly lower in mice fed the LIN diet. In contrast, the expression of (inductible) NOS2 (iNOS) was affected neither by genotype nor by the diet. Lastly, expression of the three transaminase genes (ALAT, cASAT, mASAT) was not affected by either PPARα deficiency or the diet (data not shown).

Plasma and urinary arginine metabolism (Table 3)

Plasma urea concentration was not affected by genotype. In contrast, it was higher in the LIN group than in the COCO one, this effect being significant in KO mice only.

Table 3 Urinary 23-h excretion of creatinine, urea, and nitrate, and plasma urea concentration in WT and KO mice fed on diets rich in either saturated FA (COCO diet) or ALA (LIN diet) for 8 weeks

	WT		KO		*P* values		
	COCO	LIN	COCO	LIN	Genotype (*G*)	Diet (*D*)	Interaction $G \times D$
Plasma urea (mmol/L)	6.99 ± 0.56^{ab}	7.33 ± 0.44^{ab}	6.56 ± 0.38^{a}	8.57 ± 0.43^{b}	0.4803	0.0069	0.0809
Urinary creatinine (μmol)	4.17 ± 0.60	2.59 ± 0.25	3.02 ± 0.23	3.09 ± 0.43	0.3512	0.0896	0.0569
Urinary urea (mmol)	2.19 ± 0.29^{a}	1.60 ± 0.20^{ab}	1.56 ± 0.15^{ab}	1.45 ± 0.13^{b}	0.0600	0.0660	0.1916
Urinary nitrate (μmol)	4.37 ± 0.59	3.59 ± 0.60	3.12 ± 0.37	2.91 ± 0.48	0.0528	0.3220	0.5624
Urinary urea (mmol/μMol creatinine)	0.53 ± 0.03	0.59 ± 0.08	0.52 ± 0.03	0.53 ± 0.04	0.4680	0.4657	0.3840
Urinary nitrate (μmol/μmol creatinine)	1.10 ± 0.13	1.22 ± 0.13	1.04 ± 0.10	0.99 ± 0.19	0.3010	0.8372	0.5106

Mean values ± standard errors for 11–13 mice per group. Urinary excretions of urea and nitrate are expressed in absolute values and relative to creatinine excretion. Mean values within a row sharing a same superscript letter, or without superscript letter, were not significantly different at $P < 0.05$

Urinary excretions of creatinine, urea, and nitrate were not affected by either dietary treatment or genotype. However, when compared to WT mice, absolute values of urea and nitrate excretions in KO mice tended to be lower ($P = 0.0600$ and 0.0528, for urea and nitrate, respectively), but this trend disappeared when urinary excretions of urea and nitrate were expressed relatively to the creatinine excretion.

NO synthesis and the competition with systemic arginine conversion into urea (Fig. 2)

The conversion of systemic arginine into urea did not differ with either the genotype or the diet (Fig. 2a). In contrast, the conversion of systemic arginine into nitrate (a measure of whole-body NO synthesis) was significantly lower in KO mice, but remained not affected by the diet (Fig. 2b). When expressed as relative to the conversion of systemic arginine into urea, the effect of PPARα on whole-body NO synthesis was diet-sensitive (diet × genotype interaction) and the effect of PPARα inactivation was significant in mice fed the LIN diet but not in those fed COCO diet (Fig. 2c).

Discussion

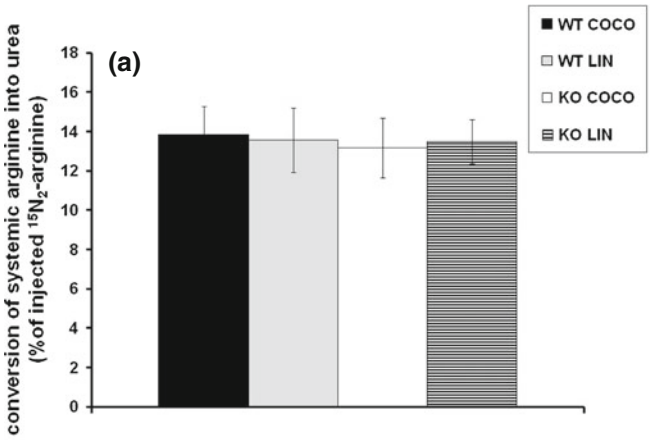
Validation of the model

In the present study, PPARα-null mice exhibited characteristic phenotypic alterations such as obesity, hepatic hypertrophy, hypertriglyceridemia, hypercholesterolemia, and glycemic dysregulation (Table 1). These alterations are consistent with those initially described for lipid metabolism (Costet et al. 1998) and glucose homeostasis (Kersten et al. 1999; Neschen et al. 2007) and resemble those clustered in the metabolic syndrome (Cornier et al. 2008). Besides, in the present study, the degree of some alterations was sensitive to the nature of dietary fatty acids. Indeed, in KO mice, the LIN diet limited obesity, hepatic hypertrophy, and hypercholesterolemia, and aggravated the decrease in glycemia. These data are in line with our previous reports that an ALA-rich diet (as compared to saturated FA) alleviates some of the risk factors associated with the metabolic syndrome resulting from PPARα deficiency, and that even with a very low intake (Martin et al. 2007; Morise et al. 2009). Besides, in accordance with our previous study (Martin et al. 2007), they confirm that even a very low fatty acid intake (<5% in weight) can modulate markedly the phenotypic consequences of PPARα deficiency. In parallel, mRNA level of Cyp4a14, a typical PPARα target gene, was nearly abolished in KO mice, which indicated the absence of PPARα activity (Fig. 1). It was increased in WT mice fed the LIN diet (vs. the WT COCO group), showing that PPARα activity was sensitive to the diet.

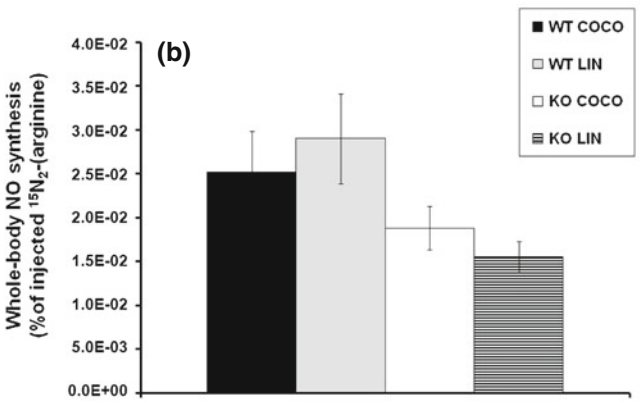
Thus, our experimental conditions reproduced the classical phenotype associated with PPARα deficiency, as well as the previously reported influence of dietary fatty acids on the degree of these alterations. This allowed us to consider our model as valid to investigate the effects of PPARα deficiency and dietary fatty acids on another metabolic pathway involved in the metabolic syndrome, i.e. NO synthesis from arginine.

PPARα deficiency alters arginine metabolism and NO synthesis

A few animal studies have investigated the role of PPARα in the regulation of amino acid metabolism, and especially of arginine metabolism. Using PPARα-null mice or PPARα pharmacological agonists, they clearly showed that PPARα regulates the mRNA level of key enzymes of arginine metabolism, such as arginase, deaminases, or transaminases (Edgar et al. 1998; Kersten et al. 2001; Sheikh et al.



P values	Urea	NO	NO/Urea
Genotype (G)	0.8015	0.0093	0.0325
Diet (D)	0.9786	0.9389	0.9661
Interaction (G*D)	0.8403	0.3314	0.0653



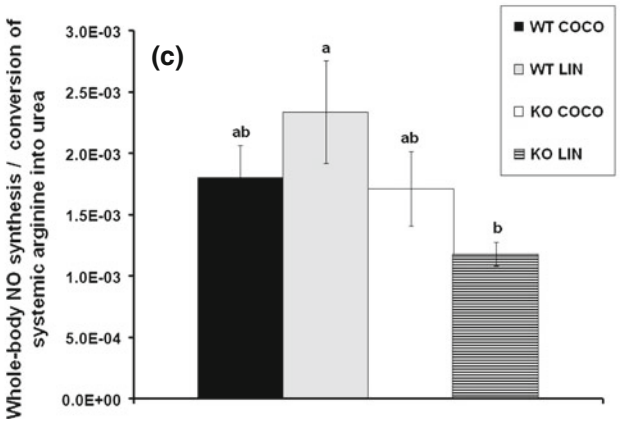


Fig. 2 Whole-body nitric oxide (NO), synthesis and conversion of systemic arginine into urea in WT and PPARα-null (KO) mice fed diets rich in either saturated FA (COCO diet) or ALA (LIN diet) for 8 weeks. Values are mean ± standard errors for 11–13 mice per group. For each parameter, *columns* sharing a *same superscript letter*, or *without superscript letter*, were not significantly different at $P < 0.05$. **a** Tracer-based measurement of the conversion of systemic arginine into urea. Values are presented as the percentage of the injected $^{15}N_2$-(guanido)-arginine dose that has been converted into ^{15}N-urea and recovered in the 23-h urine collection, adjusted for urinary creatinine excretion (in μmol). **b** Whole-body synthesis of nitric oxide (NO) after a systemic dose of $^{15}N_2$-(guanido)-arginine. Values are presented as the percentage of injected $^{15}N_2$-(guanido)-arginine converted into ^{15}N-nitrate, after adjustment of ^{15}N-urea relative to urinary creatinine (in μM). **c** Ratio of the whole-body synthesis of NO to the relative conversion of systemic arginine into urea

2007). Plasma markers of arginine metabolism (urea, amino acids of the urea cycle) were also affected (Kersten et al. 2001; Makowski et al. 2009; Sheikh et al. 2007), as well as kidney NOS activity (Newaz et al. 2005). All these studies clearly pointed out that PPARα is involved in the regulation of arginine metabolism, even if the underlying mechanisms appear to be complex.

The main objective of our study was to investigate the metabolism of arginine with a focus on NO synthesis, using a whole-body approach. One of the major findings is that PPARα deficiency significantly, markedly, reduced whole-body NO synthesis (Fig. 2). Interestingly, we found that the decrease in NO synthesis in PPARα deficiency could not be ascribed to an increase in the competitive systemic arginase pathway. The major evidence comes from the tracer-based measurements of the systemic conversion of arginine into urea, which was the same in all groups. The role of arginase as a modulator of NOS activity has been advocated in different pathophysiological situations where NO synthesis is thought to be impaired as a result an increased arginase activity and consequent reduction in L-arginine availability (Morris 2009). In contrast, in the present study, PPARα deficiency and the decrease in NO synthesis were associated with an increase in plasma arginine (Table 2), which does not indicate an arginine deficiency. However, arginine metabolism is highly compartmented, and we cannot rule out that the intracellular availability of arginine at the site of NO synthesis may be lowered in PPARα deficiency. The higher plasma arginine concentration in PPARα-null mice was paralleled with a lower plasma citrulline concentration (Table 2). This is consistent with the opposite pattern of variations in plasma arginine and citrulline that was reported in rats treated by pharmacological PPARα agonists (Linz et al. 2009; Sheikh

et al. 2007). Nevertheless, it is doubtful that the decreased plasma concentration of citrulline in PPARα-null mice can be fully accounted for by the impairment of arginine bioconversion via NOS, since this metabolic pathway contributes only marginally to the flux of plasma citrulline.

The molecular mechanisms by which PPARα deficiency impairs arginine channeling and whole-body NO synthesis remain unclear. On the one hand, hepatic mRNA levels of either NOS3 (constitutive) or NOS2 (inducible) were not affected (Fig. 1). It is important to note that the regulation of NO synthesis is so complex that mRNA levels of hepatic eNOS are unlikely to reveal the final modification in the NO synthesis pathway (Fleming and Busse 2003). On the other hand, PPARα deficiency also significantly increased plasma arginine concentration and decreased the mRNA level of arginase 1, which is strongly expressed in the liver, and is the key enzyme of bioconversion of arginine into ornithine and urea. This contrasts with a previous study showing that PPARα deficiency up-regulated hepatic arginase expression (Kersten et al. 2001). A possible explanation for this discrepancy is the fasting duration, which was 5 h in the present study and 24 h in the previous one.

We did not measure arginase activity in the present study, but this activity usually appears to be proportional to the amount of arginase protein, which, in turn, is determined primarily by transcription of the arginase genes (Morris 2009). Therefore, the marked decrease in hepatic arginase expression indicates that the reduction of NO synthesis in PPARα deficiency is not accounted for by an up-regulation of arginase pathway in the liver. It remains, however, very difficult to understand the changes in arginine metabolism using biochemical data from the liver only. In this regard, our measurement of whole-body NO synthesis has the advantage of offering a clear integrative insight into the final overall metabolic process of NO biosynthesis in PPARα deficiency.

In addition, the present study shows that PPARα deficiency affects not only arginine metabolism, but also overall amino acid metabolism, as evidenced by the changes in the plasma amino acid profile (Table 2). The complex pattern of changes that we found contrasts with previous data showing that PPARα deficiency globally decreases the plasma concentration of amino acids, including arginine (Makowski et al. 2009). These discrepancies may result from a difference in the difference of the fasting duration. Considering the high metabolic rates and substrate turn-over in mice (as compared to humans), the 5-h fasting that we used is much more physiological than the 18 h used previously (Makowski et al. 2009). Taken together, this would suggest that the role of PPARα in the prevention of amino acid catabolism is prominent in the adaptation to prolonged food deprivation (18 h), as suggested by Makowski et al. (2009), but is more complex in the regulation of the physiological fed-to-fasted transition.

The nature of dietary fatty interacts with the regulation of arginine metabolism by PPARα

A few in vivo studies suggested that dietary n-3 intake enhances NO synthesis (Harris et al. 1997; Li et al. 2008; Sekine et al. 2007). However, the role of PPARα as a mediator of n-3 PUFA-induced NO synthesis remains to be determined, as well as the underlying changes in the metabolism of amino acids, and especially of arginine. To our knowledge, the present study is the first to reveal that the nature of dietary fatty acids, even in a few amount, modulated some aspects of amino acid metabolism in either WT or PPARα-null mice.

Indeed plasma concentrations of histidine, lysine and α-amino-*N*-butyric acid concentrations were lower in the mice fed the LIN diet than in those fed the COCO diet (Table 2). Besides, the difference between the dietary groups was less pronounced in KO mice than in the WT ones. This is indicative of a classical activation of PPARα by dietary n-3 fatty acids in WT mice, which is partly abolished by PPARα deficiency. This contrasts with a previous study showing that rats treated with a pharmacological PPARα agonist (WY 14,643) exhibited either similar or higher concentrations of plasma amino acids (Sheikh et al. 2007). This discrepancy may be ascribed to the difference in the lipid content of the diets, which was low in our study and high in the rat study (48% in weight).

In contrast with the above-cited amino acids, plasma concentrations of arginine, threonine and urea were not affected by dietary FA in WT mice, but increased more in KO mice fed the LIN diet than in those fed the COCO diet (Tables 2, 3). On the opposite, plasma concentration of ornithine and citrulline decreased in KO mice fed the COCO diet, but not in those fed the LIN diet. Interestingly, in KO mice, utilization of systemic arginine for NO versus urea synthesis also tended to be sensitive to the nature of dietary FA, inasmuch as it was significantly impaired in the LIN group, but not in the COCO group (Fig. 2c). This suggests that PPARα deficiency makes the metabolism of some amino acids, including arginine, sensitive to the nature of dietary FA, as shown previously for lipid metabolism (Morise et al. 2009). Despite that, under our experimental conditions [lipids accounting for only 4.8% (in weight) of the diet], the effects of PPARα deficiency are largely prominent over those of the diet, these findings provide new insights into the pleiotropic effects of PPARα activation, and into the complex interactions between dietary fatty acids, gene transcription, and functional effects.

In conclusion, we have demonstrated for the first time that PPARα deficiency affects whole-body arginine metabolism, resulting in a marked impairment in NO synthesis. Accordingly, PPARα activation would favor NO synthesis, which warrants further study on the potential of PPARα up-regulation to prevent the decrease in NO synthesis that is closely associated with the physiopathology of the metabolic syndrome.

Acknowledgments The authors gratefully acknowledge the contribution of Angélique Foucault-Simonin and Colette Bétoulières for animal care and help with sampling and dissection, Dalila Azzout-Marniche, Véronique Mathé and Catherine Chaumontet for their precious advice on RT-PCR, and of Jean-François Huneau for his contribution to the data discussion. They also thank sincerely Pierre Weill and Guillaume Chesneau (Valorex company, La Messayais, Combourtillé, France) for providing linseed oil, and the Rohm & Hass company for their gift of the special ion-exchange resin. Najoua Guelzim is supported by a doctoral fellowship.

Conflict of interest The authors declare that they have no conflict of interest.

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