CMO1 Deficiency Abolishes Vitamin A Production from β-Carotene and Alters Lipid Metabolism in Mice*

Susanne Hessel†1, Anne Eichinger§1, Andrea Isken†, Jaume Amengual‡1, Silke Hunzelmann‡, Ulrich Hoeller‡, Volker Elste‡, Willi Hunziker‡, Regina Goralczyk‡, Vitus Oberhauser†, Johannes von Lintig‡3, and Adrian Wyss‡4

From the †Institute of Biology I, Animal Physiology and Neurobiology, Hauptstrasse 1, D-79104 Freiburg, Germany, §DSM Nutritional Products Ltd., R & D Human Nutrition and Health, P.O. Box 3255, CH-4002 Basel, Switzerland, and ¶Frimorfo SA, Chemin du Musée, CH-1700 Fribourg, Switzerland

Carotenoids are currently investigated regarding their potential to lower the risk of chronic disease and to combat vitamin A deficiency in humans. These plant-derived compounds must be cleaved and metabolically converted by intrinsic carotenoid oxygenases to support the panoply of vitamin A-dependent physiological processes. Two different carotenoid-cleaving enzymes were identified in mammals, the classical carotenoid-15,15'-oxygenase (CMO1) and a putative carotenoid-9',10'-oxygenase (CMO2). To analyze the role of CMO1 in mammalian physiology, here we disrupted the corresponding gene by targeted homologous recombination in mice. On a diet providing β-carotene as major vitamin A precursor, vitamin A levels fell dramatically in several tissues examined. Instead, this mouse mutant accumulated the provitamin in large quantities (e.g. as seen by an orange coloring of adipose tissues). Besides impairments in β-carotene metabolism, CMO1 deficiency more generally interfered with lipid homeostasis. Even on a vitamin A-sufficient chow, CMO1−/− mice developed a fatty liver and displayed altered serum lipid levels with elevated serum unesterified fatty acids. Additionally, this mouse mutant was more susceptible to high fat diet-induced impairments in fatty acid metabolism.

Quantitative reverse transcription-PCR analysis revealed that the expression of peroxisome proliferator-activated receptor γ-regulated marker genes related to adipogenesis was elevated in visceral adipose tissues. Thus, our study identifies CMO1 as the key enzyme for vitamin A production and provides evidence for a role of carotenoids as more general regulators of lipid metabolism.

Dietary lipids are precursors for signaling molecules that control many facets in cell physiology. As the classic example, fat-soluble vitamin A (all-trans-retinol) is essential for processes ranging from development to vision and cell proliferation (1–3). Retinol is the precursor for at least two critical metabolites, 11-cis-retinal, the chromophore of visual G-protein-coupled receptors (4), and retinoic acid (RA). All-trans-RA and 9-cis-RA regulate gene expression via heterodimeric nuclear receptors consisting of an RA receptor and a retinoid X receptor (RXR) (5, 6). Both are ligand-dependent transcription factors belonging to the superfamily of nuclear hormone receptors (7). Additionally, RXRs form heterodimers with other members of the nuclear receptor family (8), including the peroxisome proliferator-activated receptors (PPARs).

Because animals, including humans, are unable to synthesize vitamin A de novo, all retinoids (vitamin A and its derivatives) derive from the oxidative cleavage of dietary provitamin A carotenoids, mainly β-carotene (9–11). How this conversion of β-carotene occurs (centric and/or eccentric cleavage) is still a matter of debate (12–14). Recently, two different carotenoid-monooxygenases, CMO1 and CMO2, were molecularly identified in animals, including humans (15). Both belong to a family of structurally related nonheme iron oxygenases, common to all taxa (16–18). Biochemical analyses revealed that CMO1 is a carotenoid-15,15'-oxygenase, which converts β-carotene to retinaldehyde by a centric oxidative cleavage at the C15,C15' double bond (19–24). From this cleavage product, all biologically active retinoids can be produced by endogenous metabolic pathways, including 11-cis-retinal and RA. The CMO1 gene has been shown to be transcriptionally regulated by the action of PPARs and RXRs in both mice and humans (25, 26). This characteristic indicates a regulatory interlink between carotenoid and fatty acid metabolism at this level. The second carotenoid-metabolizing enzyme, CMO2, catalyzes an eccentric oxidative cleavage of carotenoids, such as β-carotene and lycopene, at the C9',C10' double bond (27, 28). The physiological role of this carotenoid oxygenase is still poorly understood.

In most mammals, including humans, dietary provitamin A carotenoids can maintain all vitamin A-dependent physiological functions. But it is still not clear whether dietary vitamin A possesses the full biological activity of the provitamin. There is evidence in the literature that carotenoid oxygenases can tis-

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† These authors contributed equally to this work.

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§ To whom correspondence may be addressed: Dept. of Neurobiology and Animal Physiology, Institute of Biology I, Hauptstrasse 1, Albert-Ludwig University, 79104 Freiburg, Germany. Tel: 49-761-203-2539; Fax: 49-761-203-2921; E-mail: lintig@biologie.uni-freiburg.de.

¶ To whom correspondence may be addressed: DSM Nutritional Products, 4303 Kaiseraugst, Switzerland. Tel.: 41-61-688-57-92; Fax: 41-61-688-16-40; E-mail: adrian.wyss@dsm.com.

5 The abbreviations used are: RA, retinoic acid; FFA, serum free fatty acid; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; HPLC, high pressure liquid chromatography; qRT, quantitative real time.
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sue-specifically impact physiological processes. In Drosophila, mutations in CMO1 are associated with blindness (29). In zebrafish, CMO1 is required for RA production in certain developing cells (30). Moreover, evidence for mammals exists that carotenoid derivatives produced by eccentric cleavage of β-carotene, such as β-14-apocarotenal, can influence gene regulation via PPARs and RXRs (31). In humans, mutations in carotenoid monoxygenases have not yet been described in association with any known physiological disorder. Therefore, to learn more about mammalian carotenoid metabolism and function, here we have established and characterized a mouse model with a disruption of the CMO1 gene.

EXPERIMENTAL PROCEDURES

Targeting Construct, Electroporation of Embryonic Stem Cells, and Generation of CMO1<sup>−/−</sup> Chimera—To obtain a homozygous CMO1 knock-out mouse, we used homologous recombination in TC1 ES cells to replace exon 2 and exon 3 of the wild-type CMO1 gene by an IRES-lacZ gene and a neomycin<sup>res</sup> cassette (Fig. 1). Three clones were isolated by PCR screening from a 129SvJ mouse BAC library (Genome Systems, St. Louis, MO). They span three exons and a genomic region of ~19.5 kb of the CMO1 gene. The flanking sequences (5′ arm, 3.9-kb SacI-Apal fragment; 3′ arm, 3.4-kb Apal-Apal) were cloned into the pGl-1 vector. Approximately 8.8 kb, including half of exon 2 and exon 3, were deleted from the wild-type CMO1 allele and replaced by an IRES-lacZ cassette, followed by a neo<sup>res</sup> cassette between two loxp sites (Fig. 1). TC1 ES cells (embryonic stem cells from mouse strain 129SvJ/SvEvTac) were routinely cultured on mitomycin C-treated embryonic fibroblasts, in a humidified atmosphere at 37 °C, 10% CO<sub>2</sub> and subjected to G418 selection. For the electroporation into TC1 ES cells, 0.8 ml of a single cell suspension (5 × 10<sup>4</sup> cells/ml of phosphate-buffered saline without Ca<sup>2+</sup> and Mg<sup>2+</sup>) were placed into a Bio-Rad electroporation cuvette. After the addition of 20 μg of the purified, NotI-linearized targeting construct, the electroporation was performed in a Bio-Rad GenePulser (Bio-Rad) at 240 V, 500 microfarads. After electroporation, the cells were seeded onto neomycin-resistant neomycin<sup>R</sup> ES cells, 0.8 ml of a single cell suspension (5 × 10<sup>6</sup> cells/ml of phosphate-buffered saline without Ca<sup>2+</sup> and Mg<sup>2+</sup>) were placed into a Bio-Rad electroporation cuvette. After the addition of 20 μg of the purified, NotI-linearized targeting construct, the electroporation was performed in a Bio-Rad GenePulser (Bio-Rad) at 240 V, 500 microfarads. After electroporation, the cells were seeded onto neomycin-resistant neomycin<sup>R</sup>

Confirmation of the Genotype by Southern Blot and PCR—The Easy-DNA kit (Invitrogen) was used to isolate genomic DNA from mouse tail biopsies. Genotyping was carried out both by Southern blot analysis and by a combination of different PCRs (Fig. 1). PCR was performed with the following primers: neomycin up, 5′-ATG ATT GAA CAA GAT GGA TTG CAC GCA G-3′; neomycin down, 5′-GAT ATT CGG CAA GCA GGC ATC GCC ATG-3′; Cmo1up, 5′-GAG AGA GCA AGT ACA ACC ATT GGT TTG ATG-3′; Cmo1down, 5′-CTT GTA AGA CTG TAA ATC CTG TTG GAA CAC-3′; LacZ long forward primer (5′-CCT CAA TTT GCT TTI TGG TCA TGG TG-3′) and reverse primer (5′-CTC GCC GCA CAT CTG AAC TTC AG-3′); neomycin long forward primer (5′-AGG TCT AAT TCC ATC AGA AGC-3′) and reverse primer (5′-TCA GCC GCT ACT TCT CTG TTT CGA G-3′).

For Southern blot analysis, 20 μg of genomic DNA were digested with KpnI (3′ arm) or EcoRV (5′ arm). After electrophoretic separation, DNA was transferred onto a Zeta probe membrane (Bio-Rad), and hybridization was carried out overnight at 65 °C in ExpressHyb hybridization solution (Clontech) using [α-<sup>32</sup>P]dCTP-labeled 3′ and 5′ probes. The membrane was washed twice with 2× SSC and once with 0.1× SSC, 0.1% SDS at room temperature for 20 min each.

Animals, Diets, and Experimental Procedures—In all experiments, we used C57/BL6;129SvJ-CMO1<sup>tm1dnp</sup> (CMO1<sup>−/−</sup>) mice. For the analysis of vitamin A and lipid metabolism under β-carotene supplementation (β-carotene feeding study), we used mixed background F1 generation of C57/BL6;129SvJ mice (RCC Ltd.) as wild-type controls. For the analysis of vitamin A and lipid metabolism on the vitamin A-sufficient chow, we used C57/BL6 (Charles River) mice as wild-type controls. Animal experiments were performed in accordance with Swiss and German animal protection laws by the guidelines of the local veterinary authorities. Mice were maintained under environmentally controlled conditions (temperature 24 °C, 12 h/12 h light/dark cycle) and had ad libitum access to feed and water in all experiments. For the β-carotene supplementation study, basic feed consisted of the powdered Ssniff<sup>®</sup>EF 1/51 diet (Snniff GmbH, Soest, Germany), containing a residual amount of 0.15 IU/g vitamin A. In the β-carotene feeding study, this chow was supplemented with β-carotene beadlets (10% CWS; DSM Ltd., Sisseln, Switzerland). The final amount of β-carotene in the feed was 1 mg/g. In all other experiments, mice were routinely fed with KLIBA 3430 chow (Provimi Kliba AG, Kaiseraugst, Switzerland) containing 14 IU/g vitamin A. HPLC analysis revealed that this chow contains the carotenoids: β-carotene, zeaxanthin, and lutein. The high fat chow consisted of the KLIBA 3430 enriched with 30% (w/w) soybean oil and was purchased from Provimi Kliba AG (Kaiseraugst, Switzerland). For plasma and serum preparations, first blood was taken from the vena cava under deep anesthesia. The animals were then killed prior to organ removal.

Immunoblot Analysis—For determination of blood RBP4 levels, a commercially available polyclonal antiserum raised against human RBP4 (Dako Cytomation) was used in a dilution of 1:1000. For the tubulin loading control, we used the anti-β-tubulin Ab6046 (Abcam, Cambridge, UK) in a dilution of 1:500. For determination of CMO1, we used an antiserum previously described (30). Secondary antibodies were horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG whole molecules (Sigma). Immunoblots were developed with the ECL system (Amersham Biosciences). Quantification of bands was performed by the Quantity-one (version 4.6) software (Bio-Rad).

HPLC Separation for Retinoids and Carotenoids—Retinoids and carotenoids were extracted from tissues and plasma under...
dim red safety light (600 nm). Briefly, tissues (20–40 mg) were homogenized in 200 μl of 2 M hydroxylamine (pH 6.8) and 200 μl of methanol with a glass homogenizer. For determination of β-carotene and vitamin A blood levels, 200 μl of plasma was added to 200 μl of methanol. Then 400 μl of acetone was added either to plasma or tissue extracts. Extraction of carotenoids and retinoids was performed with petroleum ether. The extraction was repeated three times, and the collected organic phases were dried under a stream of nitrogen and redissolved in HPLC solvent. HPLC separation of carotenoids and retinoids and quantification of peak integrals was performed as previously described (19). Solvents for HPLC and extraction were purchased in HPLC grade from Merck.

Determination of Tissue and Serum Lipids and Glucose Tolerance Test—Determination of the levels of free fatty acids, triglycerides, cholesterol esters, and glutamate pyruvate transaminase in serum and tissues was carried out by the Central Laboratory Unit of the University Hospital Freiburg (Germany). For determination of the total lipid content, liver samples (5–15 mg) were homogenized in phosphate-buffered saline (pH 7.5). Total lipid content was determined by Merckotest 3321 Total Lipids (Diagnostica Merck) according to the manufacturer’s protocol. Glucose tolerance tests were performed by intraperitoneal injection of glucose (1 g/kg body weight) after overnight starvation. Blood glucose levels were determined by a glucometer (Accu-Chek Aviva; Roche Applied Science) according to the manufacturer’s protocol. For this purpose, tail tips of mice were nicked with a fresh razor blade for the collection of blood samples.

Histology—For paraffin sections, livers were fixed in neutral buffered formalin and processed routinely into paraffin blocks. The embedded tissues were cut into 6-μm slices, mounted on charged adhesive slides, and dried overnight at 50 °C. Slides were then deparaffinized in xylene and rehydrated in graded alcohols to distilled water. Representative histologic sections of each specimen were stained with hematoxylin/eosin (Merck, Germany) according to the standard staining method of Ref. 32 or by Masson’s trichrome stain (Sigma) according to the manufacturer’s instructions. For cryosections, livers were cryoprotected in 30% sucrose overnight. The tissues were embedded in 1.5% agarose and sectioned with a cryostat (Microm HM 500-O) into 12-μm slices. After fixation, the sections were stained with Sudan Red 7B (Sigma) according to Refs. 32 and 33 for general histological stain methods.

RNA Preparation and Quantitative Real Time PCR (qRT-PCR)—Total RNA was isolated from liver and visceral adipose tissues with TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. RNA samples were cleaned up, DNase I-digested with the RNaseasy minikit (Qiagen), and quantified spectrophotometrically. Integrity of the extracted RNA was verified by gel electrophoresis. For cDNA synthesis, 0.5 μg of total RNA (in a final volume of 25 μl) was denatured at 65 °C for 10 min. Then cDNA synthesis was carried out for 15 min at 20 °C and 45 min at 42 °C with reverse transcriptase (MuLV RT; Applied Biosystems). The reaction was stopped at 95 °C for 5 min using a thermal cycler (Progene). For qRT-PCR analysis of target genes, the following primers were used: PPARα, 5′-CGA TGC TGT CCT CCT TGA-3′ and 5′-CTC GCC TGT GAT AAA GCC ATT-3′; ACOX1, 5′-GAT TGG TAG AAA TTG CTG CAA AAA-3′ and 5′-ACG CCA CTT CCT TGC TCT TC-3′; SREBP1c, 5′-CCA GAG GGT GGT CCA AAG-3′ and 5′-AGC TCT TGC AAC ATAT CGG ATC T-3′; FASN, 5′-CCG AGC AAG CAC ACA CAA-3′ and 5′-CAC TCA CAC CCA CCC AGA-3′; CD36, 5′-ATT TAG AAC CGG GCC ACG TA-3′ and 5′-CGC CAA CTC CCA GGT ACA A-3′; FABP4, 5′-ATG TGT GAT GTC TTC TTG GTG G-3′ and 5′-CTG TCT GCC GTG ATT T-3′. As housekeeping gene, we used β-actin (5′-ACG ATT GTG AGG AAC AT-3′ and 5′-GTG GTG GTG GTG AAG CTG TAG CC-3′). qRT-PCR was performed in a total volume of 25 μl, consisting of diluted (1:50) cDNA template, forward and reverse primers (1–2 μM each), and SYBR Green ROX Mastermix (ABgene) with an ABI prism 7000 (Applied Biosystems, Germany). After an initial enzyme activation (95 °C for 15 min), 40 cycles at 60 °C were performed for anneal/extension steps, and fluorescence was measured. In order to verify purity of PCR products, we performed 2% agarose gel electrophoresis. Additionally, a dissociation curve program was performed after each reaction. Relative quantification of target genes was calculated according to Ref. 34, based on the efficiency of each reaction and the crossing point deviation of each sample versus a control and expressed in comparison with a reference gene (β-actin).

Statistical Analysis—The data are presented as means ± S.D. Student’s t test was used to analyze data between the controls and knock-out strains. p values of ≤0.05 were considered significant.

RESULTS

Mouse CMO1 Gene and Targeting Construct—The CMO1 gene appears to be present as a single copy in the mouse (and human) genome. The entire mouse CMO1 gene sequence can be found on the NCBI site on the World Wide Web as a complement (44443023.44480639) at locus NT_078575 (GenBankTM) containing 37,617 bp. CMO1 is expressed in multiple tissues, such as the intestine, testis, liver, spleen, and adipocytes (21, 22, 25).

A mouse CMO1 genomic sequence was isolated by screening of a 1295vJ mouse BAC library. A targeting vector was constructed to replace half of exon 2 and exon 3 with an IRES-lacZ cassette and a neomycinR4 cassette (Fig. 1A), linearized with NotI and electroporated into ES cells (129S6/SvEvTac). ES cells were injected into C57BL/6 blastocysts. Initial screening for homologous recombination was carried out by Southern blot analysis (Fig. 1C). The male chimeric mice were backcrossed with female C57BL/6 mice to produce heterozygous and subsequently homozygous CMO1+/− mice. The wild-type allele was routinely identified using the primer pair CMO1up and CMO1down (diagnostic fragment 351 bp) and the knock-out gene using CMO1up and lacZlongdown (diagnostic fragment 588 bp) (Fig. 1B). The expression of CMO1 was abolished in the liver of CMO1+/− mice as determined by immunoblotting (Fig. 1D). CMO1+/− mice developed normally, and females and males were fertile on a standard mouse chow, thus excluding a developmental role of CMO1 and β-carotene when preformed vitamin A is available.
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FIGURE 1. Targeting strategy and creation of CMO1−/− mice. A, schematic map for the recombinant and the wild-type (wt) CMO1 alleles. The arrows indicate the positions of the PCR primers used for genotyping. In the recombinant allele, half of exon 2 and exon 3 were replaced by an IRES-lacZ and a neomycin cassette. B, multiplex PCR showing the wild-type genotype, the homozygous CMO1−/− genotype, and the heterozygous CMO1+/− genotype. C, Southern blot analysis of genomic DNA restricted with KpnI and hybridized with the 3′ probe isolated from wild-type (lane 1), CMO1−/− (lane 2), and CMO1+/− (lane 3) mice. D, immunoblot analysis with protein extracts (100 μg/lane) isolated from livers of wild type and homozygous CMO1−/− mice confirms the absence of the CMO1 protein (top). Bottom, tubulin loading control. Primer pairs used for genotyping and the positions of probes for Southern blot analysis are indicated. ex 1, exon 1; ex 2, exon 2; ex 3, exon 3 of the CMO1 wild-type allele. neoR, neomycin resistance; lacZ, β-galactosidase.

β-Carotene Metabolism Is Impaired in CMO1 Deficiency—To analyze the consequences of CMO1 deficiency on a diet that provides β-carotene as the major dietary source for vitamin A, we performed a feeding experiment. To deplete endogenous stores for retinoids (i.e., in the liver), we already subjected control and CMO1−/− breeders to a diet poor in vitamin A (0.15 IU/g). This dietary regimen caused no obvious clinical symptoms related to vitamin A deficiency in breeders or likewise in control and CMO1−/− offspring. Upon weaning, animals were continuously raised on a diet low in vitamin A, but they additionally received β-carotene (1000 ppm). After 16 weeks, the consequence of CMO1 deficiency was already visible upon sectioning. CMO1−/− mice but not wild-type control animals showed an orange coloring in visceral adipose tissue (Fig. 2, A and B), in subcutaneous adipose tissue, and in the intestine (Fig. 2, C and D).

Retinoid production from β-carotene already takes place in intestinal cells. Therefore, we first performed HPLC analysis with intestinal lipid extracts. This analysis confirmed β-carotene accumulation in CMO1−/− mice, whereas in controls besides β-carotene (Fig. 2F), cleavage products, such as retinyl esters and all-trans-retinol, were present (Fig. 2, G and H). This lack of intestinal β-carotene conversion in CMO1−/− mice was accompanied by a ~35-fold increase of β-carotene blood levels (Fig. 2E). To further assess the consequence of CMO1 deficiency, we determined β-carotene and retinoid (retinyl esters and retinol) levels in additional tissues that are known to express the CMO1 gene (Fig. 3). In all of these tissues, levels of β-carotene were increased as compared with control animals. Conversely, tissue vitamin A levels were decreased in CMO1−/− mice as compared with controls. Only in the kidney were retinol levels low and indistinguishable between CMO1−/− and control animals. Thus, CMO1 deficiency impaired β-carotene metabolism, and the provitamin accumulated in several tissues. CMO1−/− Mice Develop Liver Steatosis when β-Carotene Is Provided as the Major Dietary Source for Vitamin A—Next we looked to see whether CMO1 deficiency is associated with any additional phenotype under β-carotene supplementation. We found that livers of CMO1−/− mice had a pale color. Determination of the lipid content of the livers revealed a significant increase over control levels (Fig. 4C). Histological analysis of liver slices showed abnormalities of hepatocytes in centrolobular and midzonal regions (Fig. 4, D and E). These hepatocytes contained large droplets positively staining for lipids (Fig. 4, A and B). HPLC analysis revealed that liver vitamin A (retinyl esters and retinol) was significantly lower as compared with controls (Fig. 4G). Instead, β-carotene accumulation was found in the livers of CMO1−/− mice (Fig. 4H). This impairment in liver vitamin A homeostasis was also seen by an accumulation of the serum retinol-binding protein (RBP4) (Fig. 4, F and I). RBP4 is produced in the liver and secreted in a vitamin A-dependent manner to deliver vitamin A to peripheral target tissues (35). Thus, CMO1−/− mice developed a fatty liver and
displayed depleted vitamin A liver stores when β-carotene was provided as the major dietary source for vitamin A.

Liver Steatosis Is Related to CMO1 Deficiency and Develops Independently of the Vitamin A Status of the Diet—β-Carotene derivatives, such as RA, can influence liver fatty acid metabolism (e.g. genetic disruption of RA signaling can cause liver steatosis and steatohepatitis) (36). We found that CMO1<sup>−/−</sup> mice developed a fatty liver when β-carotene was provided as the major precursor for vitamin A. Therefore, we next wondered whether this liver phenotype can be prevented by feeding mice with preformed vitamin A. To address this question, we bred and raised mice on a vitamin A-sufficient standard chow (14 IU of vitamin A/g). In a first set of experiments, we performed Masson’s trichrome staining for collagen of liver sections of CMO1-deficient animals with different ages (n = 2 per age) (Fig. 5, A–C). We found that hepatocytes of 3-month-old animals already contained some lipid droplets (Fig. 5B). Droplets were larger and more widespread in livers of 12-month-old animals (Fig. 5C), but this analysis revealed that this alteration was not accompanied by enhanced collagen deposition, a characteristic of liver fibrosis (37).

For a developmental and more defined analysis, we used 25-week-old CMO1<sup>−/−</sup> mice and age-matched controls (n = 10/genotype), raised and bred under the same environmental and dietary conditions. CMO1<sup>−/−</sup> mice had significantly elevated levels of total lipids as compared with control animals (Fig. 5D). Additionally, livers of CMO1<sup>−/−</sup> mice contained significantly more triglycerides (Fig. 5E). Histology of liver slices

![Graph](image-url)
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Again revealed lipid droplets in hepatocytes in CMO1−/− mice but not in control animals (data not shown). Thus, CMO1−/− mice developed liver steatosis on a vitamin A-sufficient chow.

To exclude the possibility that CMO1 deficiency interferes with liver vitamin A homeostasis even when preformed vitamin A is available with the diet, we performed HPLC analysis to measure retinoid levels. Liver vitamin A (retinyl esters and all-trans-retinol) was in the same range in CMO1−/− and control animals (3 μmol ± 0.2 × g−1 in both genotypes). Additionally, levels of RBP4 in liver protein extracts were comparable in both genotypes (Fig. 4, F and I). We also determined the levels of circulating retinol and β-carotene in the blood. In CMO1−/− mice, we found low but significant β-carotene serum levels (0.02 ± 0.005 μM), whereas β-carotene was below detection levels in control animals. Interestingly, serum all-trans-retinol levels were slightly but significantly reduced in both female and male CMO1−/− mice as compared with controls (Fig. 5F).

PPARγ Signaling Is Altered in Adipocytes of CMO1−/− Mice—CMO1 has been identified as a PPAR- and RXR-responsive target gene in mice (25). These lipid-activated nuclear receptors play an essential role in regulating fatty acid metabolism (38). β-Carotene-derived retinaldehyde has been recently shown to protect against diet-induced obesity by inhibiting PPARγ activity (39). Retinaldehyde is also the precursor for RA and 9-cis-RA, which activate RA receptors and RXRs. Furthermore, putative CMO2-derived β-carotene cleavage products can inhibit PPAR activities as well as RXR activities in cell culture (31). We found CMO2 mRNA expression in the liver and visceral adipose tissue of both CMO1−/− and control animals (Fig. 6A). Therefore, we now wondered whether the expression of genes involved in fatty acid metabolism is altered in CMO1 deficiency. To address this question, we performed qRT-PCR analysis for genes of both fatty acid catabolism and synthesis (Fig. 6B). In the liver, mRNA levels of PPARα were comparable between genotypes. Fatty acid oxidase 1 (ACOX1) mRNA levels were significantly elevated in CMO1−/− mice. Fatty acid synthase (FASN) and SREBP1c mRNA levels were within the same range in the different genotypes. In contrast, levels of stearoyl-CoA-desaturase 1 mRNA were significantly decreased in livers of CMO1−/− mice. We next analyzed the mRNA levels of PPAR target genes in visceral adipose tissue (Fig. 6C). This analysis revealed that mRNA levels of the fatty acid-binding protein 4 (FABP4) and the scavenger receptor CD36 were significantly elevated in CMO1−/− mice. Thus, CMO1−/− mice show altered mRNA expression of genes involved in fatty acid metabolism in the liver and increased mRNA levels of PPARγ-regulated target genes in visceral adipose tissue determined by qRT-PCR. Values are means ± S.D.; n = 9/genotype; *, p ≤ 0.01; **, p = 0.001. C, relative mRNA levels of marker genes of PPARγ-regulated target genes in visceral adipose tissue determined by qRT-PCR. Values are means ± S.D.; n = 5/genotype; *, p ≤ 0.01. AU, arbitrary units; vAT, visceral adipose tissue; ACOX1, acyl-CoA-oxidase 1; CD36, scavenger receptor CD36; FABP4, fatty acid-binding protein 4; FASN, fatty acid synthase; SCD1, stearoyl-CoA desaturase 1; SREBP1c, sterol regulatory element-binding protein 1c; wt, wild type.

For liver injury (41), were significantly higher in the CMO1−/−/H9252 mice showed alterations in fatty acid metabolism, we finally asked whether CMO1−/− mice are more susceptible to diet-induced obesity. To approach this question, we used 20-week-old female CMO1−/− and control mice (n = 5/genotype). The average weight of these mice was comparable at the beginning of the study. They were then fed with the standard chow supplemented with 30% fat in the form of soybean oil. We monitored weight gain weekly during a period of 8 weeks. CMO1−/− mice gained significantly more weight as compared with control animals (Fig. 7G). Analysis of the total lipid content of livers showed that it increased in both geno-
serum cholesterol ester levels were specifically increased in CMO1 deficient mice (Fig. 7J, K and L). Consistent with lipid accumulation in the liver, the levels of serum FFA were also significantly increased (Fig. 7L), as already observed in CMO1 deficient mice raised with the fat-unsupplemented standard chow.

**DISCUSSION**

All natural vitamin A derives from certain carotenoids with provitamin A activity. Although the role of vitamin A derivatives is well established in various cell physiological aspects, little is known about the impact of the provitamin A on these processes. Recently, two different carotenoid-cleaving enzymes, CMO1 and CMO2, have been molecularly identified in mammals, including humans (22–24, 27). Ubiquitous expression of these enzymes in tissues suggests that besides vitamin A bound to RBP4, circulating carotenoids may tissue-specifically influence vitamin A-dependent processes. We here established a mouse model with a homozygous genetic disruption of the CMO1 gene to learn more about β-carotene metabolism and functions. On a diet providing β-carotene as major retinoid precursor, these mutant mice accumulated the provitamin in large quantities in several tissues accompanied by a decrease in vitamin A levels. In controls, no such β-carotene accumulation became detectable, but tissue vitamin A levels were significantly higher. This difference between genotypes was already obvious in intestinal β-carotene metabolism. Although in CMO1 deficient mice large amounts of dietary β-carotene accumulated, in controls, besides trace amounts of β-carotene, retinoids were found. Consistent with this lack of intestinal β-carotene conversion to retinoids, serum levels of the provitamin were highly increased in CMO1 deficient mice and reached levels up to 14 μM. This finding clearly indicates that CMO1 in the wild-type situation already plays a major role in the conversion of β-carotene to retinoids in the intestine. However, some β-carotene was also absorbed intact in control animals. Thus, widespread provitamin A accumulation in CMO1 deficient mice supports the
idea that β-carotene tissues specifically can impact physiological processes. In this context, it is notable that humans naturally absorb large quantities of β-carotene intact and can accumulate carotenoids in various tissues, including testis, ovary, and liver, where mRNA of CMO1 was detected (43). Moreover, the observation that in CMO1 deficiency, β-carotene accumulates in large quantities and tissue retinoid levels drop down rather excludes that the second carotenoid oxygenase, CMO2, contributes to vitamin A production systemically or tissue-specifically.

Besides gross impairments in β-carotene metabolism, CMO1 deficiency affected lipid metabolism more generally. In CMO1−/− mice, the total liver lipid content was elevated, and a fatty liver phenotype was histologically evident with hepatocytes showing large lipid droplets. Liver steatosis in CMO1 deficiency developed independently of the vitamin A status of the chow. CMO1−/− mice showed liver steatosis when liver stores for vitamin A were depleted under β-carotene supplementation but also when raised on a vitamin A-sufficient standard chow. Under the latter dietary condition, the capacity to store retinoids was not altered in CMO1−/− mice, but liver steatosis was obvious with increased levels of triglycerides. Thus, we provide evidence that liver steatosis is directly related to CMO1 deficiency. Consistent with this assumption, mice with impaired liver vitamin A storage show no liver steatosis (35).

On a vitamin A-sufficient chow, liver steatosis became more pronounced with age. Additionally, we found that CMO1−/− mice gained significantly more weight than C57BL/6 controls when a high fat diet was fed. This weight gain was associated with a more pronounced fatty liver. C57BL/6 has been shown to be more susceptible to diet-induced liver steatosis than 129SvJ mice (44). F2 129SvxC57BL/6 intercrosses, representing the genetic background of CMO1−/− mice, behave in this respect like C57BL/6 mice (45). Serum triglyceride levels were comparable between CMO1−/− and C57BL/6 control mice both on the standard and on the fat-supplemented standard chow. Serum cholesterol ester levels increased in CMO1-deficient animals fed with the fat-supplemented chow. Most interestingly, serum FFAs were significantly increased in CMO1 deficiency under each dietary condition. Since the rate of hepatic uptake of FFAs is unregulated, this pathology may cause liver steatosis in CMO1−/− mice.

The CMO1 gene has been identified as a PPAR- and RXR-activated target gene (25). PPARα activates genes involved in the oxidation of fatty acids in the liver (46), and PPARγ activates genes in anabolic lipid pathways, particularly in adipocytes (47). CMO1 activity on β-carotene results in retinaldehyde production. This cleavage product can be converted to RA. In the liver, RA signaling can influence fatty acid metabolism as well as gluconeogenesis (48–50). Our data rather exclude the possibility that liver steatosis in CMO1−/− mice is caused by major impairments in glucose metabolism. CMO1−/− mice behaved normally in glucose tolerance assays. Additionally, mRNA levels of ACOX1, essential for fatty acid oxidation, were up-regulated, and importantly SCD1 mRNA levels were significantly decreased as compared with controls. Systemic SCD1 deficiency has been shown to protect against diet-induced obesity (51). Additionally, in the liver, elevated SCD1 activity plays a role in the onset of diet-induced hepatic insulin resistance (52), but possible effects of CMO1 deficiency on the regulation of glucose metabolism require more detailed analysis in future research.

Several studies demonstrate that RA signaling plays a role for adipocytes. In cell culture, an enhanced differentiation of adipocytes has been observed in retinol-depleted serum (53). At the molecular level, a cross-talk between PPARγ and RA signaling during adipocyte differentiation is well established (54, 55). However, not only RA but also retinaldehyde, which directly derives from β-carotene cleavage via CMO1, has been recently shown to antagonize PPARγ activity. In vivo, elevated retinaldehyde levels in adipocytes can prevent diet-induced obesity (39). Indeed, in CMO1 deficiency, mRNA levels of PPARγ-regulated target genes, such as CD36 and FABP4, were significantly increased in visceral adipose tissues.

Thus far, our data best fit a model in which PPAR-induced CMO1 expression (i.e. in adipocytes), leads to the production of β-carotene cleavage products, such as retinaldehyde and/or RA. These β-carotene derivatives may fine tune the cross-talk between nuclear receptors that regulate fatty acid metabolism (i.e. by antagonizing PPARγ activities). In addition, the second carotenoid cleavage enzyme, CMO2, must be considered in this process. This enzyme is expressed in the liver and, as shown here, in adipose tissues. CMO2 catalyzes an eccentric cleavage of carotenoids. Those carotenoid cleavage products can repress PPARγ and PPARα as well as RXR activities in cell culture (31). Here, we found no evidence that CMO2 contributes to the conversion of the bulk of β-carotene accumulated in CMO1 deficiency. Considering that signaling molecules are required in very small quantities, we cannot exclude the possibility that CMO2 activity on β-carotene may also influence the activity of nuclear receptors. Notably, in our study, we used chow diets containing trace amounts of various naturally occurring carotenoids. Therefore, CMO2 activity on other carotenoids different from β-carotene must also be taken into account. Interestingly, it has been recently shown that nonprovitamin A carotenoids, such as lycopene, can influence PPARγ and CMO1 mRNA expression in rats (56).

In summary, our study identifies CMO1 as the key enzyme for the conversion of β-carotene to vitamin A in mammals. In addition, we provide evidence that carotenoids via carotenoid-oxygenases influence lipid metabolism more generally. In the future, CMO1−/− mice represent a valuable model to elucidate the essential details in this process. Carotenoids in staple food are the major source for vitamin A in humans, and vitamin A deficiency is still a global health problem. Therefore, the need for a better understanding of carotenoid metabolism and functions is of unquestionable relevance for human health.

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