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EFFECT OF *Fabp1/Scp-2/Scp-x* ABLATION ON WHOLE BODY AND HEPATIC PHENOTYPE OF PHYTOL-FED MALE MICE

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Abstract

Liver fatty acid binding protein (*Fabp1*) and sterol carrier protein-2/sterol carrier protein-x (*SCP-2/SCP-x*) genes encode proteins that enhance hepatic uptake, cytosolic transport, and peroxisomal oxidation of toxic branched-chain fatty acids derived from dietary phytol. Since male wild-type (WT) mice express markedly higher levels of these proteins than females, the impact of ablating both genes (TKO) was examined in phytol-fed males. In WT males, high phytol diet alone had little impact on whole body weight and did not alter the proportion of lean tissue mass (LTM) versus fat tissue mass (FTM). TKO conferred on dietary phytol the ability to induce weight loss as well as reduce liver weight, FTM, and even more so LTM. Concomitantly TKO induced hepatic lipid accumulation, preferentially 3-fold increased phospholipid (PL) at the expense of decreased triacylglycerol (TG) and total cholesterol. Increased PL was associated with upregulation of membrane fatty acid transport/translocase proteins (FATPs 2,4), cytosolic fatty acid/fatty acyl-CoA binding proteins (FABP2, ACBP), and the rate limiting enzyme in PL synthesis (*Gpam*). Decreased TG and cholesterol levels were not attributable to altered levels in respective synthetic enzymes or nuclear receptors. These data suggest that the higher level of *Fabp1* and *Scp2/Scpx* gene products in WT males was protective against deleterious effects of dietary phytol, but TKO significantly exacerbated phytol effects in males.

Keywords

phytol; branched-fatty acid; FABP1; obesity

INTRODUCTION

Phytol, an important dietary constituent in dairy products and meat of ruminants, is derived from the branched-chain of chlorophyll (1). Disorders in phytol metabolism result in accumulation of phytanic acid and pristanic acid which can lead to multiple pathologies

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including Refsum's disease (2). The protein products of the *Fabp1* gene and both products of the *Scp-2/Scp-x* gene play major roles in protecting mammals from such deleterious effects of phytol (3–7).

FABP1, very highly expressed in liver cytosol, is thought to be protective both by binding lipidic xenobiotics in the cytosol and/or facilitating their metabolism (3). Indeed, FABP1 has high affinity for (8, 9) and enhances the uptake and peroxisomal oxidation (10–13) of phytanic and pristanic acid. Overexpressing FABP1 in cultured cells enhances (11), while ablating FABP1 decreases phytanic and pristanic acid uptake and metabolism both in cultured primary mouse hepatocytes (12) and *in vivo* (14).

Although less prevalent, the two protein products of the *Scp-2/Scp-x* gene (i.e. SCP-2 and SCP-x derived through alternate transcription sites) are also protective by binding, facilitating uptake, and enhancing metabolism of branched-chain fatty acids (3–7). Again, overexpressing SCP-2 or SCP-x in cultured cell lines enhances while ablation of *Scp-x* (15) or *Scp-2/Scp-x* (5–7) inhibits branched-chain fatty acid metabolism.

The levels of the protein product of the *Fabp1* gene as well as the protein products of the *Scp-2/Scp-x* gene not only differ within the liver, but are also markedly dependent on sex. Cytosolic SCP-2 is up to 8 fold less prevalent than FABP1 in liver (3, 16). In contrast, nearly half of SCP-2 and nearly all SCP-x is concentrated in peroxisomes wherein SCP-2 presents bound branched-chain fatty acyl-CoAs to key enzymes for oxidation, including SCP-x which is the only known branched-acyl chain ketoacylthiolase (4, 17–24). It is important to note that hepatic protein levels of FABP1, SCP-2 and SCP-x are higher in livers of male than female mice (14, 25–27). Singly ablating *Fabp1* (12, 14), *Scp-2/Scp-x* (6, 28, 29), or *Scp-x* (15) markedly inhibits hepatic uptake and metabolism of branched-chain fatty acids. However, concomitant upregulation of the non-ablated protein complicates interpretation (15, 29, 30). The impact of ablating both the *Fabp1* and *Scp-2/Scp-x* genes on phenotype and liver lipids in phytol-fed female mice has recently been reported (31). Because of the marked sexual-dimorphism in hepatic expression of *Fabp1* and *Scp-2/Scp-x* genes, the current study focused on determining the effects of TKO on whole body phenotype and liver lipid metabolism in male mice. This would also allow comparison of findings with phytol-fed TKO males herein with those in their female counterparts reported earlier (31).

EXPERIMENTAL PROCEDURES

Animals

Male wild-type (WT) C57BL/6NCr mice were purchased from the National Cancer Institute (Frederick Cancer Research and Development Center, Frederick, MD). Male FABP1/SCP-2/SCP-x null (TKO) mice backcrossed to >10 generations to the C57BL/6NCr background were generated as described earlier (32). Mice were housed at 25 °C, 60–70% humidity, 12:12 h light/dark cycle, and allowed ad libitum access to standard rodent chow. Animal use protocols were approved by the Texas A&M Institutional Animal Care and Use Committee in compliance with the Guide for the Care and Use of Laboratory Animals. Mice were monitored daily for disease or injury, sentinel monitored quarterly, and confirmed free of all known rodent pathogens.

Phytol Dietary study

Seven week old male WT or TKO mice were placed on a modified AIN-76A phytol-free, phytoestrogen-free pelleted control diet (5% calories from fat, diet no. D11243, Research Diets, New Brunswick, NJ) for one week. This phytol-free and phytoestrogen-free control AIN-76A rodent diet was chosen to preclude complications from: i) dietary phytol and its metabolites present at significant levels in standard rodent chows (29). Phytol and its metabolites (phytanic acid, pristanic acid) are potent ligand activators of RXR α (33, 34) while phytol metabolites activate PPAR α (9, 29, 30, 35–37)—nuclear transcription factor that form a heterodimer pair to markedly enhance transcription of multiple genes in lipid metabolism (9, 29, 30, 35–37); ii) dietary estrogens which can interfere with gender comparisons (38, 39). For one additional week the mice were then either continued on this diet or placed on a modified AIN-76A pelleted rodent diet supplemented with 0.5% phytol (5% calories from fat, diet no. D01020601, Research Diets, New Brunswick, NJ).

Whole Body Phenotype

Body weight and food intake were measured on alternate days. At the beginning (day 0) and end (day 8) of the dietary study, dual-energy X-ray absorptiometry (DEXA) was performed using a Lunar PIXImus densitometer (Lunar Corp., Madison, WI) as described to obtain lean tissue mass (LTM) and fat tissue mass (FTM) (26, 40). The instrument was calibrated with a phantom mouse of known bone mineral density and fat tissue mass as described (26, 41). Blood was collected at the end of the study by cardiac puncture for subsequent processing to serum and storage at -80°C . Mice were euthanized according to AVMA Guidelines for the Euthanasia of Animals as described earlier (40), and livers were harvested, weighed, snap frozen with dry ice, and stored at -80°C s.

Liver Homogenization and Protein Analysis

Liver samples (~0.1 gram) were extensively minced, 0.5 mL PBS (pH 7.4) added, and homogenized using a motor-driven pestle (Tekmar Co, Cincinnati, OH) operating at 2000 rpm. Liver homogenate protein concentration was evaluated using the Bradford protein micro-assay (Bio-rad, Hercules, CA) in accordance with the manufacturer's instructions using Costar 96-well assay plates (Corning, Corning, NY) and the BioTek Synergy 2 micro-plate reader (BioTek Instruments, Winooski, VT).

Liver and Serum Lipid Analysis

Aliquots of liver homogenate (5 mg protein from above) were also used for quantitating lipids including total cholesterol (TC), free cholesterol (C), triacylglycerol (TG), and phospholipid (PL) after solvent-extraction, thin layer chromatography (TLC), and quantitation as in (42). Alternately lipid classes were determined directly in the homogenate using diagnostic kits available from Wako Chemicals (Richmond, VA). Quantitation did not significantly differ between the two methods. To determine cholesteryl ester concentration (CE), free (non-esterified) cholesterol concentration (C) was subtracted from the total cholesterol. Serum β -hydroxybutyrate (β -Hydroxybutyrate LiquiColor, β -OHB) and high density lipoprotein cholesterol (Direct HDL-Cholesterol, HDL-C) were determined using diagnostic kits from Stanbio Laboratory (Boerne, TX). Serum apolipoprotein B (apoB) and

apolipoprotein A-I (apoA1) were quantitated with diagnostic kits from Diazyme Labs (Poway, CA). Serum non-HDL-C was calculated by subtracting serum HDL-C from serum TC.

Liver Protein Expression Analysis by Western Blotting

Western blot analysis was performed on liver homogenates as described in (27) using the following antibodies: Rabbit and goat polyclonal antibody to mouse acyl-CoA-binding protein (ACBP) (SC-23474), apoA1 (SC-23606), bile salt export pump (BSEP) (SC-17294), carnitine palmitoyltransferase 1a (Cpt1a) (SC-31128), carnitine palmitoyltransferase 2 (Cpt2) (SC-20671), cellular retinoic acid binding protein 1 (CRABP I) (SC-10062), fatty acid transport protein 4 (FATP4) (SC-5834), intestinal fatty acid binding protein (FABP2) (SC-16063), LDL-Receptor (LDL-R) (SC-11826), liver $\times$ receptor (LxR) (SC-1201), multidrug resistance protein (MDR) (SC-8313), retinoid $\times$ receptor α (RxR α) (SC-553), sterol regulatory element-binding protein 1 (SREBP1) (SC-367) and sterol regulatory element binding protein 2 (SREBP2) (SC-8151) were obtained from Santa Cruz Biotechnology (Dallas, TX). Rabbit and mouse polyclonal antibody to mouse acetyl-CoA acetyltransferase (ACAT2) (ab66259), apoB (ab31992), fatty acid transport protein 2 (FATP2) (ab83763) and fatty acid transport protein 5 (FATP5) (ab89008) were purchased from Abcam (Cambridge, MA). Sheep polyclonal antibody to bovine catalase (W90080C) was purchased from BioDesign (Dublin, OH). Rabbit polyclonal antibody to mouse FABP1 (11294), p-thiolase (12464), SCP2 (12249), and SCPx (11306) were prepared as described (26, 43). Goat polyclonal antibody to mouse glutathione s-transferase (GST) (27457701V) was purchased from GE Life Sciences (Pittsburgh, PA). Bacterial polyclonal antibody to mouse 3 α -hydroxysteroid reductase (3 α HSD) (H9117-01) was purchased from US Biological (Peabody, MA). Rabbit polyclonal antibody to mouse PPAR α (PA1-822A) was purchased from Pierce Antibody (Rockford, IL). Rabbit polyclonal antibody to mouse scavenger receptor class B member 1 (SR-B1) (NB400-104) was purchased from Novus Biological (Littleton, CO).

Antibodies to housekeeping proteins were used as a loading control to normalize protein expression were obtained as follows: glyceraldehyde 3-phosphate dehydrogenase (GADPH) (SC-13063) and cytochrome c oxidase subunit IV (COX4) (ab16056) were from Abcam (Cambridge, MA) while mouse monoclonal antibody to mouse GAPDH (MAB374) was from Millipore (Billerica, MA). Secondary antibodies were as follows: Alkaline phosphatase-conjugated goat polyclonal antibody to rabbit IgG (product # A3687) and rabbit polyclonal antibody to goat IgG (product # A4187) were from Sigma-Aldrich (St. Louis, MO). Alkaline phosphatase-conjugated rabbit polyclonal antibody to mouse IgG (product # ab6729-I) was from Abcam (Cambridge, MA). Protein levels were quantified as described previously (31). Where pure protein was available (FABP1, SCP2, ACBP), quantitative analyses by standard curves were produced from Western blots and analyses of band intensities was performed as described (31).

Liver mRNA Transcription Analysis by Real-time qRT-PCR

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was performed on total mRNA isolated from livers and purified using an RNeasy minikit

(Quiagen, Valencia, CA) as described earlier (44). The following primers were obtained from Applied Biosystems (Foster City, CA) for determining liver transcription of mouse glycerol-3-phosphate acyltransferase (GPAT) (*Gpam*; Mm00833328_m1), 1-acylglycerol-3-phosphate-O-acyltransferase (AGPAT) (*Agpat2*; Mm00458880_m1), Lipin (*Lpin2*; Mm00522390_m1), diacylglycerol acyltransferase (DGAT) (*Dgat2*; Mm00499536_m1), sodium-taurocholate cotransporting polypeptide (NTCP) (*Slc10*; Mm01302718_m1), organic anion-transporting polypeptide 1 (OATP1) (*Slco1*; Mm01267414_m1), organic anion-transporting polypeptide 2, OATP2 (*Slc22a7*; Mm00460672_m1). qRT-PCR mRNA data were normalized to 18S RNA (a housekeeping gene) as in (45) and made relative to male WT mice on phytol diet set to one.

Statistics

Data were expressed as the mean $\pm$ standard error of the mean (SEM) with *n* indicated in table and figure legends. Statistical analysis was performed by t-test (GraphPad Prism, San Diego, CA, San Jose, CA), with *p* < 0.05 considered statistically significant.

RESULTS

Whole Body Phenotype

Although dietary phytol did not significantly change whole body weight in WT male mice, the loss of all three gene products of the *Fabp1* and *Scp-2/Scp-x* genes (i.e. TKO) decreased body weight by 18%, while phytol-fed WT mice did not exhibit significant weight loss (Table 1). The body weight loss in phytol-fed TKO vs phytol-fed WT male mice correlated with a decrease (19%) in food consumption (Table 1). In WT males, phytol-diet alone also decreased food consumption but to significantly lesser extent compared to control-diet, i.e. 11% vs 19% (*p* < 0.05) (not shown). This suggested that the phytol-diet induced weight loss in TKO mice was attributable only in part to food aversion. To determine if altered energy metabolism also contributed to phytol-fed TKO mice's body weight loss, energy conversion efficiency was calculated [g body weight gain/kcal food consumed]. In male TKO mice phytol-diet significantly decreased the energy conversion efficiency by -0.276 ± 0.048 g/kcal (Table 1). In contrast, in male WT mice phytol-diet decreased energy conversion efficiency only slightly, i.e. -0.022 ± 0.007 g/kcal (Table 1)—albeit still a loss when compared to control-diet fed WT male which exhibited a positive energy conversion efficiency, i.e. $+0.027 \pm 0.015$ g/kcal (not shown). Taken together, these data indicated that the greater phytol-diet induced weight loss in male TKO vs phytol-fed male WT mice was attributable primarily to decreased energy conversion efficiency.

Concomitantly, phytol-fed male TKO (Table 1), but not female phytol-fed TKO (46), mice also exhibited decreased liver weight (i.e. 35%) as compared to their phytol-fed WT counterparts (Table 1). Even when taking into account the decreased weight gain of phytol-fed TKO vs phytol-fed WT mice, phytol-diet decreased liver weight/body weight ratio (i.e. 29%) in TKO vs WT mice (Table 1).

Finally, PIXImus dual-energy x-ray absorptiometry (DEXA) scans revealed phytol-diet increased fat tissue mass (FTM) modestly in WT mice, but markedly decreased that in TKO

mice (Table 1). This was even more evident with the change in FTM as %, which showed phytol-diet increased % FTM in WT mice by 30% but decreased that in TKO mice by 9%—a nearly 40% difference (Table 1). Finally, DEXA scans demonstrated phytol-diet decreased lean tissue mass (LTM) only slightly in WT mice, and only decreased LTM by 1.8g in TKO mice (Table 1).

In summary, dietary phytol elicited a significantly greater loss in body weight in TKO than WT male mice. Decreased food intake accounted only in part for this weight loss. Instead, the greater weight loss in phytol-fed TKO mice was also associated with a significantly decreased energy conversion efficiency as compared to that exhibited by phytol-fed WT mice. Finally, the loss of body weight appeared to be proportional to similar % losses of FTM and LTM.

Hepatic Glyceride Lipids

TKO increased hepatic total lipid nearly 50% in phytol-fed males (Table 2). This increase was due to over 3-fold increased phospholipid at the expense of neutral lipid, especially triacylglycerol (Table 2). Liver accumulation of phospholipid correlated with 2–3 fold increased transcription of *Gpm* and *Agpat* (Table 3). These enzymes regulate the production of phosphatidic acid from which both phospholipid and triacylglycerol are derived. However, increased level of fatty acid containing lipid (especially phospholipid) was not attributable to any significant increase in SREBP1 which regulates transcription of the rate limiting enzymes in *de novo* fatty acid synthesis (Fig 1E). Although TKO induced a significant increase in transcription of *Lipe* and *Dgat*, key enzymes in hydrolyzing phosphatidate to diacylglycerol for conversion to triacylglycerol (Table 3). Since phospholipid increased while triacylglycerol decreased (Table 2), this suggested that loss of FABP1 and SCP2 resulted in redirecting fatty acid targeting into phospholipid (47, 48) or potentially increased toxicity of phytol due to loss of these proteins that facilitate phytol metabolism.

Hepatic Cholesterol and Cholesteryl Ester

TKO decreased total cholesterol (Fig. 1A) and even more so unesterified free cholesterol (Fig. 1B) in phytol-fed male mice. The increased hepatic cholesterol was not attributable to any significant increase in hepatic expression of SREBP2, the nuclear receptor that increases transcription of enzymes in *de novo* cholesterolgenesis (Fig. 1F). Increased expression of hepatic ACAT2, the key enzyme in cholesteryl ester synthesis (Fig. 1D), would have been expected to increase cholesteryl ester levels. However, this was not observed (Fig. 1C), likely due to loss of FABP1 and/or SCP2, both of which stimulate cholesteryl ester formation (49, 50).

Serum Lipoproteins

TKO decreased serum levels of both free cholesterol (Fig. 2A) and cholesteryl ester (Fig. 2B) in phytol-fed male mice. The reduced serum cholesterol level was specifically associated with decreased HDL cholesterol (Fig. 2C). Serum level of apoA1, the major apoprotein found in HDL, was not altered (Fig. 2D). As a result, the ratio of apoA1/HDL cholesterol was significantly increased—suggesting decreased particle size (Fig. 2E). The

hepatic level of SR-B1, the liver receptor for HDL, was increased significantly in the TKO (Fig. 2I).

In contrast, TKO did not significantly alter serum level of non-HDL cholesterol (Fig. 2F), serum level of apoB which is the major apoprotein in LDL (Fig. 2G), or hepatic level of LDL-R which is the liver LDL receptor (Fig. 2J). Consequently, TKO did not alter the ratio of apoB/non-HDL cholesterol in phytol-fed males (Fig. 2H).

Hepatic Bile Acid Transport

Since serum cholesterol levels are regulated by bile acid cotransport of HDL-derived cholesterol into bile (51–53), the impact of TKO on expression of key proteins of the hepatic bile acid system was examined in phytol-fed male mice.

TKO markedly decreased hepatic transcription of two key basolateral transporters of bile acids, *Oatp1* and *Oatp2* (Table 4). However, this was counteracted by concomitant upregulation of the other major basolateral bile acid transporter *Ntcp* (Table 4). However, this was not due to altered hepatic expression of the nuclear regulatory protein LxR (Fig. 3E). TKO completely abolished hepatic expression of FABP1 (Fig. 4D), the major murine cytosolic bile acid binding/transport protein in liver, as well as abolished hepatic expression of SCP-2 (Fig. 4H) and SCP-x (Fig. 5C;D), both of which are also involved in bile acid transport and peroxisomal formation. Concomitant upregulation of other cytosolic bile acid binding/transport proteins (3 α HSD, GST) may represent a potential compensatory mechanism(s) at least in part for this loss (Fig. 3A, C). Finally, TKO increased hepatic expression of the canalicular membrane protein for phospholipid transport into bile MDR (Fig. 3B), but did not affect the canalicular bile salt export protein BSEP (Fig. 3D).

Taken together, these findings suggested that TKO altered hepatic expression of proteins in the bile acid reuptake and cytosolic transport pathway which may have contributed to lower serum HDL cholesterol levels in phytol-fed male mice.

Hepatic Fatty Acid Uptake, Cytosolic Transport, and Oxidation

The contribution of altered fatty acid uptake, transport and oxidation to hepatic glyceride accumulation in phytol-fed male TKO mice was examined. With regards to integral membrane proteins involved in fatty acid uptake, TKO trended to increase hepatic expression of the major plasma membrane fatty acid transport protein, FATP5 (Fig. 4A), and significantly increased that of the two intracellular membrane fatty acid transporters, i.e. FATP2 and FATP4 (Fig. 4B, C) in phytol-fed male mice. Complete loss of the cytosolic fatty acid binding proteins, FABP1 (Fig. 4D) and SCP2 (Fig. 4H) in phytol-fed TKO male mice elicited concomitant compensatory upregulation of other known FABP family members including FABP2 (Fig. 4E) and CRABP1 (Fig. 4F) as well as in ACBP (Fig. 4G) which is not a FABP family member but is also involved in cytosolic transport and oxidation of fatty acyl-CoAs. With regards to hepatic expression of proteins in fatty acid oxidation, TKO markedly increased expression of two key enzymes in mitochondrial fatty acid β -oxidation (CPT1A, CPT2) in phytol-fed male mice (Fig. 5A, B). TKO abolished hepatic expression of a major peroxisomal enzyme in phytol oxidation (SCP-x, Fig. 5C; D), but did not alter catalase (Fig. 5E) or slightly increase p-thiolase (Fig. 5. F), other peroxisomal proteins. TKO

did not alter hepatic expression of PPAR α (Fig. 5G), but slightly decreased that of RXR α (Fig. 5H)—a nuclear receptor heterodimer pair regulating transcription of fatty acid β -oxidative enzymes. The net effect of the TKO-induced loss of FABP1, SCP2, and SCP-x along with compensatory upregulation of other proteins in fatty acid β -oxidation in phytol-fed male mice was unaltered serum level of β -hydroxybutyrate (β -OHB, Fig. 5I). Serum β -OHB is a measure of hepatic fatty acid oxidation.

Taken together, these findings suggested that the TKO-induced increase in hepatic phospholipid was not attributable to a net decrease in fatty acid β -oxidation in phytol-fed male mice.

DISCUSSION

The protein expression products of the *Fabp1* and *Scp-2/Scp-x* genes are highly involved in the uptake, cytosolic transport, and metabolism of straight chain fatty acids (3, 54–56) as well as branched-chain lipids (phytanic acid, cholesterol, bile acids) (12, 42, 57–60). As such, these genes contribute to regulating both hepatic and serum lipid accumulation (3). While individually ablating the *Fabp1* and *Scp-2/Scp-x* genes has provided significant insight into their potential roles in hepatic lipid metabolic response to dietary phytol (6, 12, 14, 28, 29), concomitant upregulation of the non-ablated gene, especially FABP1 in *Scp-2/Scp-x* gene ablated mice, has complicated interpretation (15, 29, 30). Additional complexity arises from the fact that hepatic expression of these proteins is highly sex-dependent. Livers of female mice have significantly lower levels of FABP1, SCP-2 and SCP-x than males (14, 25–27). Therefore, to further understand the contributions of these genes on the impact of dietary phytol on phenotype, especially lipid metabolism, mice ablated in both *Fabp1* and *Scp-2/Scp-x* genes (TKO) were developed (59, 61, 62). Since male WT mice express much higher levels of these proteins, it was important to determine if male TKO mice may be even more sensitive to dietary phytol as compared to their female TKO counterparts. The data provided the following new insights:

First, TKO markedly exacerbated the impact of dietary phytol on whole body phenotype in male mice. Since phytol-diet alone decreased whole body weight gain by 19% in male TKO, but not WT, mice, this suggested that the TKO-induced weight loss in male mice was not associated with avoidance of phytol-containing food. Instead, it correlated with TKO inducing significantly negative food conversion efficiency in phytol-fed males (i.e. -0.276 ± 0.045 g/kcal). Similarly, phytol-diet alone also decreased body weight gain in female TKO mice by 18% but not WT mice—an effect again not attributed to decreased preference for phytol-containing diet but instead to significantly decreased food conversion efficiency (i.e. -0.172 ± 0.032 g/kcal) (46). Thus, a major difference between male and female phytol-fed mice is the fact that TKO decreased food conversion efficiency by 38% more in males than females. The greater decrease in food conversion efficiency of phytol-fed male than female TKO mice correlated with the significantly higher normal expression of FABP1, SCP-2 and SCP-x in livers of male than female mice (14, 25–27). Overexpressing FABP1 in cultured cells is known to enhance (11), while ablating FABP1 decreases phytanic and pristanic acid metabolism in cultured primary mouse hepatocytes (12) and *in vivo* (14). Likewise, overexpressing SCP-2 or SCP-x in cultured cell lines enhances while ablation of

Scp-x (15) or *Scp-2/Scp-x* (5–7) inhibits branched-chain fatty acid metabolism. The greater magnitude of FABP1, SCP-2, and SCP-x loss in male TKO mice results in a nearly 6-fold increased hepatic accumulation of phytol metabolites in response to phytol-diet, but only 20% increased phytol metabolite accumulation in phytol-fed female TKO mice (63). Since phytol-metabolites are the most potent known ligand activators of PPAR α (9, 35, 37), the greater accumulation of phytol-metabolites in phytol-fed male TKO mice resulted in increased hepatic expression of mitochondrial fatty acid β -oxidative enzymes (CPT1A, CPT2) and a trend towards increased serum β -hydroxybutyrate in males as shown herein, but not in phytol-fed TKO females (46). Greater mitochondrial fatty acid β -oxidation would have been expected to result in greater weight loss phytol-fed TKO males than females as was indeed observed.

With regards to the respective roles of the loss of FABP1 vs loss of SCP-2 and SCP-x, comparisons with studies examining the impact of individually ablating these genes on weight gain provide further insights. Individually ablating *Fabp1* (14) or *Scp-2/Scp-x* (29) similarly decreased body weight of phytol-fed males. Consistent with the fact that hepatic FABP1 level is 8-fold higher than that of SCP2 (3, 16), this suggested that the loss of complete function of both *Fabp1* and *Scp-2/Scp-x* genes was not additive in male mice due to concomitant upregulation of FABP1 in *Scp-2/Scp-x* ablated mice which may have at least in part compensated for the loss of SCP-2 and SCP-x (15, 27, 29, 30). Concomitant upregulation of FABP1 was not observed in female *Scp-2/Scp-x* null mice (27).

Second, TKO induced weight loss in phytol-fed male mice was not attributed to preferential loss of lean tissue mass (LTM) or fat tissue mass (FTM), since the % decrease in each (i.e. 6–9%) was similar. Likewise, in phytol-fed female TKO mice the decrease in % LTM and % FTM was also proportional rather than preferential (46). Finally, it is important to note that the loss of both LTM and FTM mass was not attributable to loss of FABP1 since individually ablating *Fabp1* did not exacerbate the effect of phytol diet on either LTM or FTM in phytol-fed males (14). In contrast, singly ablating *Scp-x* elicited markedly decreased FTM and LTM in phytol-fed males (15). This suggested a more complex interplay wherein the protein products of the *Fabp1* gene and *Scp-2/Scp-x* gene differentially impacted LTM and FTM, respectively, in phytol-fed male mice.

Third, in phytol-fed male mice TKO increased hepatic accumulation of total lipid, specifically phospholipid, at the expense of decreasing triacylglycerol. TKO increased hepatic total lipid and phospholipid by 44% and 3.4-fold, respectively, in phytol-fed males. While a similar hepatic lipid phenotype was observed in phytol-fed female TKO mice, the magnitude of lipid accumulation was much greater—a 1.8- and 4.9-fold increase in total lipid and phospholipid, respectively (46). The less extensive hepatic lipid accumulation in phytol-fed male than female TKO mice may be attributed to the higher normal expression of FABP1, SCP-2 and SCP-x in livers of male than female mice (14, 25–27) resulting in greater hepatic fatty acid oxidation in the phytol-fed male TKO livers noted above. It should be noted, however, that the greater hepatic lipid accumulation in phytol-fed female than male TKO mice was not due to differences in the impact of TKO on hepatic expression of lipogenic genes (*Gpam*, *Agpat*, *Lipin*, *Dgat*)—all of which were overall increased to a very similar extent in both male (shown herein) and female (46) phytol-fed TKO mice. The

increased hepatic phospholipid in both phytol-fed TKO males shown herein and in the corresponding females (31) may be attributed not only to increased transcription of key enzymes in phosphatidic acid synthesis, but even more so to loss (greater loss in males than females) of FABP1 and SCP-2 that may have resulted in redirecting fatty acids towards phospholipids (47, 48) and/or potentially increased toxicity of phytol. Thus, overall the impact of TKO on hepatic lipid metabolism was qualitatively similar in the two sexes, but quantitatively greater in the male phytol-fed TKO mice.

Fourth, TKO decreased hepatic cholesterol (primarily free but not esterified cholesterol) and decreased both free and esterified cholesterol in serum of phytol-fed male mice. In contrast, singly ablating *Fabp1* increased hepatic free cholesterol while decreasing cholesteryl ester, but increased serum esterified cholesterol to increase total cholesterol, in phytol-fed males (14). This suggested that loss of SCP-2 and SCP-x, rather than FABP1, may have had a greater impact on the hepatic and serum cholesterol phenotype of phytol-fed TKO male mice. The decreased serum cholesterol in phytol-fed male TKO mice was especially noteworthy because it correlated with decreased serum HDL-cholesterol which, in the face of unaltered serum apoA1 level, markedly increased serum apoA1/HDL cholesterol ratio. Increased ratio of apoA1/HDL cholesterol was consistent with smaller HDL particle size—a factor known to exert potent protection against atherogenic LDL (55, 56). Overall, decreased serum and hepatic cholesterol in TKO male mice is likely attributed to both dietary and knock out effects. While phytol-diet alone has no impact on hepatic total cholesterol (both free and esterified cholesterol) in male wild-type mice (26), TKO alone increases total hepatic cholesterol (both free and esterified cholesterol) (59). The latter finding of TKO's impact in the context of control-chow differed markedly from the TKO-induced decrease in hepatic cholesterol in the context of phytol-diet shown herein. Similarly, while phytol-diet alone also had no impact on serum cholesterol (both free and esterified cholesterol) (26), TKO alone only slightly increased only serum free cholesterol, but not HDL-cholesterol or non-HDL-cholesterol, in control-chow fed male mice (not shown). Again, the latter finding of TKO's impact on serum cholesterol in the control-fed males was in marked contrast to the TKO-induced decrease in serum cholesterol and HDL-cholesterol noted herein with phytol-fed males. Ablation of SCP-2 increases cholesterol efflux from the liver into HDL particles due to concomitant upregulation of FABP1. However, when both FABP1 and SCP-2 are ablated, cholesterol efflux is decreased (32). In TKO mice, there is no concomitant upregulation of FABP1 to facilitate the upregulation of cholesterol efflux from the liver as nascent HDL. In addition, due to ablation of SCP-x the TKO male mice accumulate phytanic acid, a potent PPAR α agonist (63). While in humans activation of PPAR α increases serum HDL cholesterol, in mice PPAR α activation decreases serum HDL cholesterol due to decreased expression of apoA-I and apoA-II genes in the liver (64). PPAR α activation may also be responsible for decreased hepatic free cholesterol in males. It has been suggested that there is transcriptional upregulation of *Cyp7a1* upon PPAR α activation in mice, where CYP7A1 is the major rate limiting enzyme in the synthesis of bile acid from cholesterol, thus decreasing hepatic cholesterol (65). Finally, it is important to note that these effects of TKO on decreasing cholesterol (liver and serum) in phytol-fed male mice did not extend to their female counterparts which exhibited unaltered hepatic cholesterol (both free and esterified) and instead increased serum cholesterol (both free and esterified) (31). The

increased serum cholesterol in phytol-fed TKO female mice was attributed to increased serum apoB and non-HDL-cholesterol concomitant with decreased hepatic apoB—suggesting increased secretion of nascent VLDL which then contributed to increased serum level of non-HDL (i.e. primarily LDL in fasting mice) (31). While the molecular basis for the marked difference in cholesterol phenotype of male vs female phytol-fed TKO remains to be elucidated, it may be related to females' much lower basal FABP1, SCP-2 and SCP-x levels concomitant with higher basal accumulation of branched-chain fatty acids (e.g. phytanic acid) as compared to their male counterparts (63). In addition, PPAR α effects on lipid metabolism are known to be influenced by estrogen which inhibits the actions of PPAR α agonists (e.g. phytanic acid) (64).

Fifth, TKO resulted in complete loss of FABP1, SCP-2, and SCP-x—key proteins in fatty acid uptake, cytosolic transport, and β -oxidation—in phytol-fed male mice. However, these losses were at least in part compensated for by concomitant upregulation of other membrane fatty acid transport proteins (FATP2, FATP4), cytosolic fatty acid/fatty acyl-CoA transport proteins (FABP2, CRABP1, ACBP), and enzymes in fatty acid β -oxidation (CPT1, CPT2, p-thiolase). Consequently, the serum level of β -hydroxybutyrate (β -OHB, a physiological marker of fatty acid β -oxidation) was unaltered. Compensatory mechanisms also resulted in unaltered serum β -OHB level in phytol-fed female TKO mice (31). Taken together, these data suggested that the marked hepatic accumulation of total lipid (especially phospholipid) in phytol-fed TKO male and female mice was not associated with decreased fatty acid oxidation.

In summary, the data presented herein suggested that as compared to their female counterparts the greater sensitivity of male mouse whole body phenotype to dietary phytol may be more highly dependent on hepatic expression of *Fabp1* gene and *Scp-2/Scp-x* gene products. Furthermore, this sensitivity correlated with the much higher expression of FABP1, SCP-2, and SCP-x in males than females (14, 25–27). Hepatic levels of FABP1, SCP-2, and SCP-x are also highly responsive to both natural and fibrate activators of PPAR α (3). In fact FABP1 facilitates trafficking of these ligands into the nucleus (66–71), directly binds PPAR α therein (72–74), and facilitates ligand transfer/activation of PPAR α transcriptional activity (70, 71, 75, 76). In human subjects a SNP resulting in a FABP1 T94A substitution markedly diminishes the ability of ligands to promote human FABP1 translocation into hepatocyte nuclei and abolishes ligand-mediated activation of PPAR α (77–79). Taken together, these findings indicate the importance of considering sex in the effectiveness of nutraceutical and pharmaceutical activators of nuclear receptors involved in fatty acid metabolism. Further teasing out the mechanistic basis for the differential responses of male vs female mice to dietary phytol as well as other xenobiotics will be a major challenge in the field. Several recent reviews suggest the involvement of both genetic and epigenetic factors (80–83).

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Abbreviations

ACAT2	acyl-CoA cholesterol acyltransferase-2
ACBP	acyl-CoA-binding protein
<i>Agpat2</i>	1-acylglycerol-3-phosphate-O-acyltransferase-2
apoA1	apolipoprotein A-I
apoB	apolipoprotein B
BSEP	bile salt export pump
BW	body weight
C	free cholesterol
cholesterol-E	cholesteryl ester
COX4	cytochrome c oxidase subunit IV
CPT1a or 2	carnitine palmitoyltransferase 1a or 2
CRABP I	cellular retinoic acid binding protein 1
DEXA	dual-energy X-ray absorptiometry
<i>Dgat2</i>	diacylglycerol acyltransferase
FABP1	liver fatty acid binding protein (L-FABP)
FABP2	intestinal fatty acid binding protein
FATP2	4 and 5, fatty acid transport protein 2, 4 and 5
FTM	fat tissue mass
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GPAT/<i>Gpam</i>	glycerol-3-phosphate acyltransferase
GST	glutathione s-transferase
HDL-C	high density lipoprotein cholesterol
3αHSD	3 α -hydroxysteroid reductase
β-OHB	β -hydroxybutyrate
LDL-R	low density lipoprotein receptor
<i>Lpin2</i>	phosphatidate phosphatase (lipin-2)
LTM	lean tissue mass
LxR	liver $\times$ receptor

MDR	multidrug resistance protein
non-HDL-C	non-HDL cholesterol
<i>Ntcp</i>	sodium-taurocholate cotransporting polypeptide
<i>Oatp1 or 2</i>	organic anion-transporting polypeptide 1 or 2
PL	phospholipid
PPARα	peroxisome proliferator activated receptor alpha
qRT-PCR	quantitative real-time polymerase chain reaction
RxRα	retinoid $\times$ receptor α
SCP-2	sterol carrier protein-2
SCP-x	sterol carrier protein-x
SEM	standard error of the mean
SR-B1	scavenger receptor class B member 1
SREBP1 and 2	sterol regulatory element-binding protein 1 and 2
TG	triglyceride/triacylglycerol
TKO	<i>Fabp1/Scp-2/Scp-x</i> gene ablated mouse
WT	wild-type mouse

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Figure 1. Effect of *Fabp1/Scp-2/Scp-x* Gene Deletion (TKO) on hepatic Cholesterol Level and Target Genes in Cholesterol Metabolism in Phytol-fed Mice

Male WT and TKO mice were fed a 0.5% diet phytol diet and livers collected for determination of total cholesterol (A), free cholesterol (B), and cholesteryl ester (C) as described in Methods. Western blotting of liver homogenate was determined as in Methods to obtain relative hepatic protein levels of ACAT2 (D), SREBP1 (E) and SREBP2 (F). The housekeeping genes GAPDH or COX4 was used as a loading control to normalize protein expression for SREBP1 and SREBP2, respectively, while COX4 was the loading control to normalize protein expression for ACAT2. Inset in Panels D-F show representative western blots of relative protein expression in each mouse group. Means $\pm$ SE; n= 8 animals per group; *p < 0.05 between phytol-fed WT versus phytol-fed TKO mice.

Figure 2. *Fabp1/Scp-2/Scp-x* Gene Deletion (TKO) Alters Serum Lipid, Lipoproteins, and Liver Lipoprotein Receptor Levels in Phytol-fed Mice

Male WT and TKO mice were fed a 0.5% phytol diet and serum collected as described in Methods. Serum levels of free cholesterol (A), cholesteryl ester (B), HDL cholesterol (C), apoA1 (D), apoA1/HDL-C (E); non-HDL-C (F), apoB (G), and apoB/non-HDL-C (H) as well as liver expression of SR-B1 (I) and LDL-R (J) were measured as described in Methods. The housekeeping gene COX4 was used as a loading control to normalize protein expression for SR-B1 and LDL-R. Inset in Panels I to J show representative western blots of relative protein expression in each mouse group. Means $\pm$ SE; n= 8 animals per group; *p < 0.05 between phytol-fed WT versus phytol-fed TKO mice.

Figure 3. Impact of *Fabp1/Scp-2/Scp-x* Gene Deletion (TKO) on Hepatic Levels of Proteins for Bile Acid Transport in Phytol-fed Mice

Male WT and TKO mice were fed a 0.5% phytol diet, livers collected, and western blotting performed as described in Methods to measure the relative hepatic levels of 3aHSD (A), MDR (B), GST (C), BSEP (D), and LxR (E). The housekeeping protein COX4 was used as a loading control to normalize protein expression. Inset in Panels A–E show representative western blots of relative protein expression in each mouse group. Means $\pm$ SE; n= 8 animals per group; *p < 0.05 between phytol-fed WT versus phytol-fed TKO mice.

Figure 4. Effect of *Fabp1/Scp-2/Scp-x* Gene Deletion (TKO) on Hepatic Levels of Membrane and Cytosolic Proteins Mediating Fatty Acid Transport and Targeting in Phytol-fed Mice

Male WT and TKO mice were fed a 0.5% phytol diet, livers collected, and western blotting was performed as described in Methods in order to measure hepatic levels of FATP5 (A), FATP2 (B), FATP4 (C), FABP1 (D), FABP2 (E), CRABP1 (F), ACBP (G) and SCP2 (H). The housekeeping proteins COX4 (A–B) and GAPDH (C, E, F) were used as loading controls to normalize protein expression. Insets show representative western blots of relative protein expression in each mouse group. Quantitative western blotting was performed to measure the expression of FABP1 (D), SCP2 (B) and ACBP (C) as described in Methods. Means $\pm$ SE; n = 8 animals per group; *p < 0.05 between phytol-fed WT versus phytol-fed TKO mice.

Figure 5. *Fabp1/Scp-2/Scp-x* Gene Deletion (TKO) Impacts Hepatic Levels of Proteins in Fatty Acid Oxidation in Phytol-fed Mice

Male WT and TKO mice were fed a 0.5% phytol diet, followed by collection of liver and serum as described in Methods. Western blotting was performed to determine hepatic expression of CPT1A (A), CPT2 (B), SCP-x (C and D), catalase (E), p-thiolase (F), PPARα (G) and RxRα (H) as described in Methods. The housekeeping proteins COX4 (A, E, G) and GAPDH (B, C, D, H) were used as a loading controls to normalize protein expression. Insets show representative western blots of relative protein expression in each mouse group. Serum concentration of β-hydroxybutyrate (β-OHB) (I) was determined as in Methods. Means $\pm$ SE; n = 8 animals per group; * p < 0.05 between phytol-fed WT versus phytol-fed TKO mice.

Table 1
Effect of *Fabp1/Scp-2/Scp-x* gene ablation (TKO) on whole body phenotype of phytol-fed male mice

Food consumption, body weight change, energy conversion efficiency (body weight change/kcal food consumed), liver weight and liver/weight/body weight of male phytol-fed wild type (WT) and TKO mice were obtained as described in Methods. Changes in tissue mass (g) and % for whole body fat tissue (FTM) and lean tissue (LTM) were determined by Lunar PIXImus dual-energy x-ray absorptiometry (DEXA) as described in Methods. Values represent the mean $\pm$ SEM, n=8.

	WT	TKO
BW Change (%)	-0.93 ± 0.73	$-18.20 \pm 2.23^*$
Total Food Consumption	19.8 ± 0.57	$16.0 \pm 0.96^*$
Energy Conversion Efficiency (g/kcal)	-0.022 ± 0.007	$-0.276 \pm 0.048^*$
Liver Weight (g)	1.51 ± 0.07	$0.98 \pm 0.14^*$
Liver Weight/Body Weight (g)	0.07 ± 0.004	$0.05 \pm 0.006^*$
FTM Change (g)	0.785 ± 0.213	$-0.234 \pm 0.176^*$
FTM Change (%)	29.65 ± 8.12	$-9.22 \pm 6.95^*$
LTM Change (g)	-0.064 ± 0.484	$-1.803 \pm 0.592^*$
LTM Change (%)	-0.37 ± 2.81	$-10.67 \pm 3.51^*$

* $p < 0.05$ for TKO vs. WT.

Table 2
Effect of *Fabp1/Scp-2/Scp-x* gene ablation (TKO) on hepatic lipids of phytol-fed male mice

Levels of total lipid, neutral lipid, phospholipid, and triacylglycerol were measured in livers of male phytol-fed wild type (WT) and TKO mice as described in Methods. Values represent the mean $\pm$ SEM, n=8.

	WT	TKO
Total Lipid (nmol/mg Protein)	501.5 $\pm$ 15.1	726.9 $\pm$ 49.0 *
Neutral Lipid (nmol/mg Protein)	210.5 $\pm$ 11.2	136.2 $\pm$ 10.2 *
Phospholipid (nmol/mg Protein)	152.6 $\pm$ 16.2	523.3 $\pm$ 45.5 *
Triacylglycerol (nmol/mg Protein)	127.8 $\pm$ 5.7	71.3 $\pm$ 9.1 *

* p < 0.05 for TKO vs. WT.

Table 3
Effect of *Fabp1/Scp-2/Scp-x* gene ablation (TKO) on mRNA expression of hepatic genes in triacylglycerol synthesis in phytol-fed mice

mRNA levels of *Gpam*, *Agpat2*, *Lpin2* and *Dgat2* were measured by qRT-PCR in livers from male phytol-fed wild type (WT) and TKO mice as described in Methods. Values represent the mean $\pm$ SEM, n=8.

	WT	TKO
<i>Gpam</i>	1.00 $\pm$ 0.02	2.00 $\pm$ 0.16 *
<i>Agpat2</i>	1.00 $\pm$ 0.03	2.96 $\pm$ 0.59 *
<i>Lpin2</i>	1.00 $\pm$ 0.04	5.82 $\pm$ 0.61 *
<i>Dgat2</i>	1.00 $\pm$ 0.03	4.28 $\pm$ 1.21 *

*
p < 0.05 for TKO vs. WT.

Table 4
Effect of *Fabp1/Scp-2/Scp-x* gene ablation (TKO) on hepatic mRNA expression of genes in bile acid transport

mRNA levels of *Oatp1*, *Oatp2*, and *Ntcp* were measured by qRT-PCR in livers from male phytol-fed wild type (WT) and TKO mice as described in Methods. Values represent the mean $\pm$ SEM, n=8

	WT	TKO
<i>Oatp1</i>	1.000 $\pm$ 0.081	0.006 $\pm$ 0.001 *
<i>Oatp2</i>	1.000 $\pm$ 0.023	0.265 $\pm$ 0.049 *
<i>Ntcp</i>	1.000 $\pm$ 0.089	3.250 $\pm$ 1.070 *

*
p <0.05 for TKO vs. WT.