Modulation by nutrients and drugs of liver acyl-CoAs analyzed by mass spectrometry

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Abstract The profile of liver acyl-CoAs induced by dietary fats of variable compositions or by xenobiotic hypolipidemic amphipathic carboxylates was evaluated in vivo using a novel electrospray ionization tandem mass spectrometry methodology of high resolution, sensitivity, and reliability. The composition of liver fatty acyl-CoAs was found to reflect the composition of dietary fat. Treatment with hypolipidemic carboxylates resulted in liver dominant abundance of their respective acyl-CoAs accompanied by an increase in liver fatty acyl-CoAs. Cellular effects exerted by dietary fatty acids and/or xenobiotic carboxylic drugs may be transduced in vivo by their respective acyl-CoAs.—Kalderon, B., V. Sheena, S. Shachrur, R. Hertz, and J. Bar-Tana. **Modulation by nutrients and drugs of liver acyl-CoAs analyzed by mass spectrometry. *J. Lipid Res*. 2002.** 43: **1125–1132.**

Supplementary key words fatty acids • hypolipidemic drugs • electrospray ionization tandem mass spectrometry • hepatocyte nuclear factor-4α

Long chain fatty acyl-CoAs serve as intermediates in the esterification of fatty acids into triglycerides, phospholipids, and cholesterol esters, or their oxidation to yield energy. In addition to their substrate role, long chain fatty acyl-CoAs, as well as xenobiotic acyl-CoAs endogenously produced by ATP-dependent CoA-thioesterification of the respective xenobiotic free acids (1), may regulate intermediary metabolism by modulating the activity of enzymes (e.g., glucokinase, pyruvate dehydrogenase, acetyl-CoA carboxylase, Δ^9-desaturase, protein kinase C), transporters (e.g., mitochondrial adenine nucleotide translocase, mitochondrial citrate transporter, Na/K ATPase), ion channels (e.g., ATP-sensitive K-channels, endoplasmic reticulum Ca-ATPase) (2–4), or transcription factors (5–9).

The activity of acyl-CoAs as trancriptional modulators is of particular interest. Indeed, long chain fatty acyl-CoAs have recently been reported to specifically bind to hepatocyte nuclear factor 4-α (HNF-4α) and to affect its transcriptional activity as a function of chain length and degree of saturation of the fatty acyl-CoA ligand (6). Thus, saturated fatty acyl-CoAs of C14–C16 activate HNF-4α while (ω-3) polyunsaturated fatty acyl-CoA serve as HNF-4α suppressive ligands. Similarly, the transcriptional activity of HNF-4α is suppressed by the acyl-CoA thioesters of xenobiotic members of the fibrate or Medica series (7). Since HNF-4α controls the expression of liver genes coding for apolipoproteins (e.g., A-I, A-II, A-IV, B, and C-III), vitamin K-dependent coagulation factors (e.g., VII, IX, X), as well as for enzymes and proteins controlling hepatic glucose production (PEPCK, pyruvate kinase, glucose-6-phosphatase), and pancreatic insulin production and secretion (e.g., insulin, glut 2) (10–13), modulation of its transcriptional activity by its acyl-CoA ligands may result in modulating lipoproteins metabolism, blood coagulability, and glucose tolerance in response to carboxylic nutrients or drugs. Indeed, HNF-4α activation by saturated C14–C16 acyl-CoAs may account for the reported increase in blood lipids and coagulability induced by dietary saturated C14–C16 fatty acids, while suppression of HNF-4α by its fatty and xenobiotic acyl-CoA antagonists may account for the hypolipidemic, antithrombogenic beneficial effect exerted by dietary (ω-3) polyunsaturated fatty acids or by hypolipidemic fibrate or Medica drugs (14–18).

Effects exerted by dietary carboxylates and transduced by means of their respective acyl-CoAs require, however, that the acyl-CoAs profile of a concerned cell type should reflect the composition of dietary fatty acids and/or be affected by xenobiotic dietary carboxylates. Since fatty acyl-CoAs are intermediates in metabolic pathways leading from the respective free acids to esterified or oxidized end products, and as xenobiotic free acid prodrugs may not necessarily be effectively thioesterified endogenously to their respective CoA-thioesters (1, 19, 20), the putative relationship between dietary carboxylates and their respec-

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Abbreviations: Medica 14, β,β'-tetramethyltetradecane-α,ω-dioic acid; Medica 16, β,β'-tetramethylhexadecane-α,ω-dioic acid; Medica 18, β,β'-tetramethyloctadecane-α,ω-dioic acid.

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tive intracellular acyl-CoAs is not redundant and must be carefully analyzed. Such analysis calls for a reliable, sensitive, and high resolution method for finger printing the profile of acyl-CoAs and for evaluating the content of individual acyl-CoAs of a given cell type.

This study reports the modulation of liver acyl-CoAs by dietary fat of defined composition as well as by amphipathic carboxylates used as hypolipidemic drugs. The profile of liver acyl-CoAs is evaluated here by using a novel electrospray ionization tandem mass spectrometry (ESI-MS/MS) method for characterizing and quantitating individual long chain fatty and xenobiotic acyl-CoAs.

METHODS

Animals, diets, and drugs

Male albino rats were fed ad libitum for 21 days with either Purina chow, fat depleted diet [99% (w/w) ICN 901682 fat free diet supplemented with 1% (w/w) corn oil], olive oil-enriched diet [79% (w/w) ICN 901682 fat free diet supplemented with 20% (w/w) olive oil (Sigma) and 1% (w/w) corn oil] or fish oil-enriched diet [79% (w/w) ICN 901682 fat free diet supplemented with 20% (w/w) fish oil (Sigma) and 1% (w/w) corn oil]. Olive oil consisted of 83.5% C18:1, 9.4% C16:0, 4.0% C18:2, 2% C18:0, and 0.9% C20:4. Fish oil consisted of 19% C20:5, 11.7% C22:6, 29.6% C18:2, 16.3% C16:0, 15.5% C16:1, 5.9% C14:0, 0.8% C22:0, 0.6% C18:0, and 0.6% C20:4. All synthetic diets were supplemented with 0.12 g (w/w) vitamin E. Alternatively, Purina fed rats were treated for 3 days with 0.1%–0.25% (w/w) β,β'-tetramethylhexadecane-α,ω-dioic acid (Medica 16) as indicated, 0.25% (w/w) nafenopin, or 0.5% (w/w) bezafibrate mixed in Purina chow.

Extraction of liver acyl-CoA thioesters

Rats were anaesthetized with pentobarbital and the livers were quickly freeze clamped and stored in liquid nitrogen. Frozen livers were extracted and processed essentially as described by Deutsch et al. (21). One gram of the fragmented tissue was homogenized for 20 s in 2 ml of 100 mM KH_2PO_4, pH 4.9, using a Polytron homogenizer at room temperature. Added (C17:0)-CoA (20 nmol) served as internal standard. Following the addition of 2 ml of 2-propanol, the homogenate was further homogenized for 20 s followed by adding 0.25 ml saturated $(NH_4)_2SO_4$ and 4 ml of acetonitrile. The emulsion was vortexed for 5 min, centrifuged, and the supernatant diluted with 10 ml of 100 mM KH_2PO_4 (pH 4.9) and loaded at a rate of 1.25 ml/min onto an air-flushed oligonucleotide purification cartridge (Applied Biosystems), equilibrated with 25 mM KH_2PO_4 (pH 4.9). The cartridge was washed with 5 ml of 25 mM KH_2PO_4 (pH 4.9), dried by flushing with air twice, and the acyl-CoAs mixture was eluted by 0.4 ml of 60% acetonitrile in 100 mM KH_2PO_4 (pH 4.9), dried under N_2, and suspended in 200 μL of 2-propanol:1 mM acetic acid 80:20. The salt precipitate was discarded and the supernatant dried under N_2 and resuspended in 150–200 μl of acetonitrile-H_2O, 60:40 (v/v).

Rat liver nuclei were prepared by sucrose gradient centrifugation (22). Nuclear acyl-CoA thioesters were extracted and purified as described above.

Extraction of acyl-CoA thioesters from cultured cells

Cultured cells were washed with saline and scraped into methanol. The dry residue was extracted with chloroform-methanol-H_2O, 40:20:15 (v/v/v) (Folch extraction). The interphase protein was further extracted with methanol-2 M ammonium acetate, 4:1 (v/v) and the extract combined with the upper Folch phase was evaporated to dryness, suspended in 50 mM Tris-HCl buffer pH 8.0, and loaded onto a solid phase C18 cartridge (Varian) equilibrated with 50 mM Tris-HCl, pH 8.0. The column was washed with water and the acyl-CoAs were eluted in methanol, dried under N_2, and suspended in 50 μl of acetonitrile-H_2O, 60:40 (v/v) for MS injection.

Mass spectrometry

Negative ESI-MS/MS mass spectrometry was carried out using a Micromass Quatro II Tandem mass spectrometer at cone voltage of 35V and collision energy of 30 eV, maintaining the electrospray source temperature at 75°C, and adjusting the argon gas pressure inside the collision cell to 5.5×10^{-3} mbr. A 10 μl sample was pumped (Jasco PU-980 pump) into the ESI source through a 10 μl injector loop connected to the ESI source via a fused silica capillary tube. The flow rate of the mobile phase was maintained at 35 μl/min followed by 10 μl/min when the injected sample reached the ESI source and during scanning. For parent ion scanning mode, parents of *m/z* 339 were scanned within the range of *m/z* 450–580 during a scanning time period of 1.22 s. For multiple reaction monitoring (MRM) mode, the mobile phase was introduced and maintained at a flow rate of 35 μl/min, and parents of *m/z* 339 were selected to 15–20 channels of predefined nominal *m/z* to yield the MRM values for 15–20 selected acyl-CoAs. Each sample was analyzed first by parent ion scanning followed by the MRM mode for selected parent ions. Data processing was carried out using the Masslynx program.

Individual acyl-CoAs were identified by their *m/z* as well as by spiking with authentic acyl-CoAs. (C17:0)-CoA (*m/z* = 509.1) served as internal standard for each extract. The ionization sensitivity of individual acyl-CoAs was evaluated both by spiking with known quantities of authentic acyl-CoAs as well as by evaluating the intensity of respective authentic acyl-CoAs in synthetic mixtures subjected to an extraction protocol similar to that of biological extracts. Acyl-CoA intensities in biological extracts were corrected by the respective ionization sensitivities of authentic acyl-CoAs.

The measured content of respective acyl-CoAs was corrected by subtracting the intensity contributed by the ^{13}C-isotopic peak of the acyl-CoA having the same chain length but an additional double bond. The corrected intensity of respective acyl-CoAs was calculated by equation 1:

$$Cm{:}n = (Cm{:}n)' - [Cm{:}(n+1)]F \qquad (Eq.\ 1)$$

where *Cm:n* is the true intensity of an acyl-CoA having *m* carbon atoms in length and *n* double bonds, *(Cm:n)'* is the measured intensity of an acyl-CoA having *m* carbon atoms in length and *n* double bonds, *F* is the molar fraction of an acyl-CoA containing two ^{13}C-isotopes relative to the molar fraction of the respective acyl-CoA consisting of the ^{12}C-isotope only.

The molar fraction of a molecule having *N* carbon atoms and *X* ^{13}C atoms was calculated by the binomial distribution equation 2 (23):

$$\frac{N!}{(N-X)!X!}[0.011]^X[1-0.011]^{N-X} \qquad (Eq.\ 2)$$

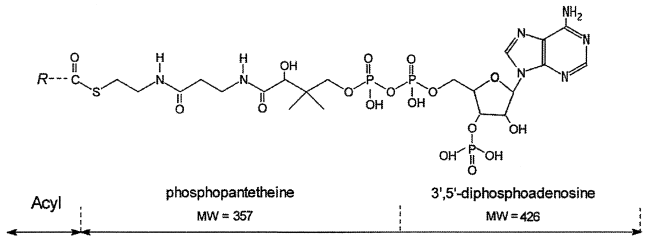
where 0.011 is the natural abundance of ^{13}C.

RESULTS AND DISCUSSION

Quantitation of liver fatty acyl-CoAs by negative ESI-MS/MS

The dehydrated phosphopantethein fragment (*m/z* 357–18) common to all acyl-CoA species results from loss of 3′,5′-diphosphoadenosine (*m/z* 426) together with the acyl

moiety of respective acyl-CoAs (**Scheme 1**), and is usually the most intense acyl-CoA fragment generated by negative ESI (24).



Scheme 1

Scanning for molecular ion parents of *m/z* 339 may therefore provide the profile of a complex acyl-CoA's mixture. Since negative acyl-CoA molecular ions are doubly charged, the respective molecular ion parents of *m/z* 339 should be looked for at *m/z* of (M-2H)/2. Acyl-CoA species of interest identified by scanning for molecular ion parents of *m/z* 339 may be further quantitated by operating the mass spectrometer in the MRM mode for selected *m/z* channels. Both the scanning and MRM modes are exemplified by the profile of liver fatty acyl-CoAs of Purina fed rats (**Fig. 1**), pointing to the resolving power of the concerned methodology. As shown in Fig. 1, the most abundant liver fatty acyl-CoAs in Purina fed rats consist of C18 and C20 species. The C18 cluster mostly consists of (C18:1)-CoA (*m/z* 515) and (C18:2)-CoA (*m/z* 514). The C20 cluster predominantly consists of (C20:4)-CoA (*m/z* 526).

The sensitivity of the concerned methodology allows for fingerprinting the acyl-CoA profile of 50 mg of liver tissue and for detecting individual liver acyl-CoAs as low as 100 pmol.

Manipulating liver fatty acyl-CoAs by diets

The ESI-MS/MS method makes it possible to characterize and quantitate liver fatty acyl-CoAs as a function of the dietary composition of fatty acids. Feeding rats with a fat free diet as compared with the regular Purina diet (4% fat) resulted in an increase in the relative abundance of fatty acyl-CoAs of (C16:1) and (C18:1) (**Fig. 2**), thus reflecting the higher activity of stearoyl-CoA desturase induced under fat free conditions (25). The deficiency in dietary linoleic (C18:2) acid resulted in a significant decrease in liver (C18:2)-CoA and metabolites thereof, e.g., (C20:4)-CoA. Similarly, the deficiency in dietary α-linolenic (C18:3) acid resulted in a significant decrease in the relative abundance of its (C20:5, ω-3)-CoA and (C22:6, ω-3)-CoA metabolites. Hence, the liver fatty acyl-CoAs profile maintained by a fat free diet reflects the dietary composition as well as changes in enzymatic elongation and desaturation activities induced by lack of dietary fat.

The effect of the fatty acid composition of dietary fat on the liver fatty acyl-CoAs profile was further evaluated by feeding rats with a diet enriched with defined fatty acids as compared with fat free diet. Feeding rats with oleic acid (C18:1, ω-9)-enriched diet resulted in a significant increase in relative abundance of liver (C18:1)-CoA with a concomitant decrease in (C14:0)-CoA, (C16:0)-CoA, and (C16:1)-CoA (Fig. 2), thus indicating that even under conditions of enriching the diet with a nonessential fatty acid that may be generated in vivo, the composition of liver fatty acyl-CoAs still reflects the dietary source. Furthermore, the profile of liver fatty acyl-CoAs could be dramatically manipulated by enriching the diet with the essential eicosapentaenoic (C20:5, ω-3) and docosahexaenoic (C22:6, ω-3) fatty acids of fish oil (Fig. 2). Thus, feeding rats the fish oil diet resulted in about 20-fold increase in liver (C20:5)-CoA and (C22:6)-CoA, constituting now the

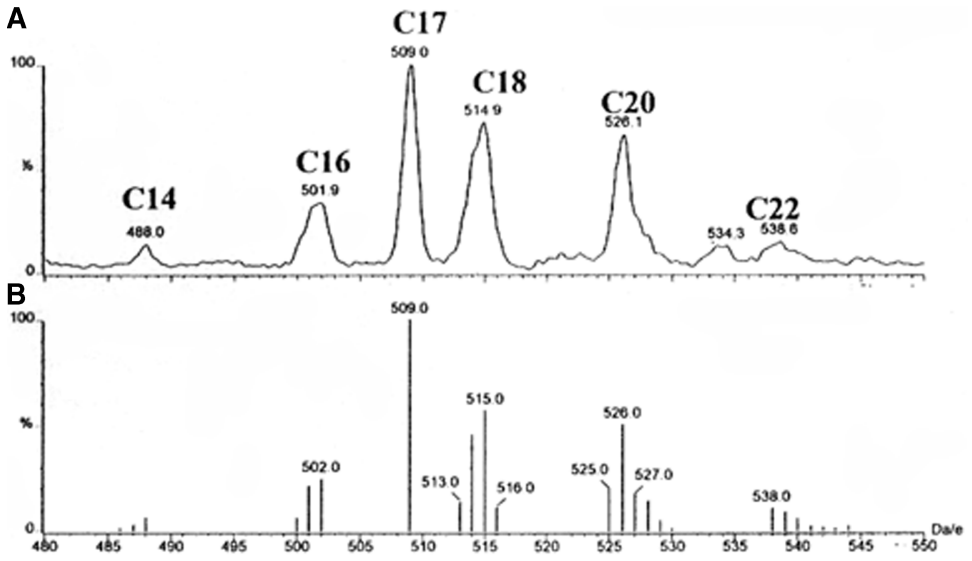


Fig. 1. Liver fatty acyl-CoAs of Purina-fed rats. Liver extraction and analysis of its acyl-CoAs by electrospray ionization tandem mass spectrometry were as described in Methods. One representative spectrum. A: Parent ion scanning. B: Multiple reaction monitoring (MRM) mode.

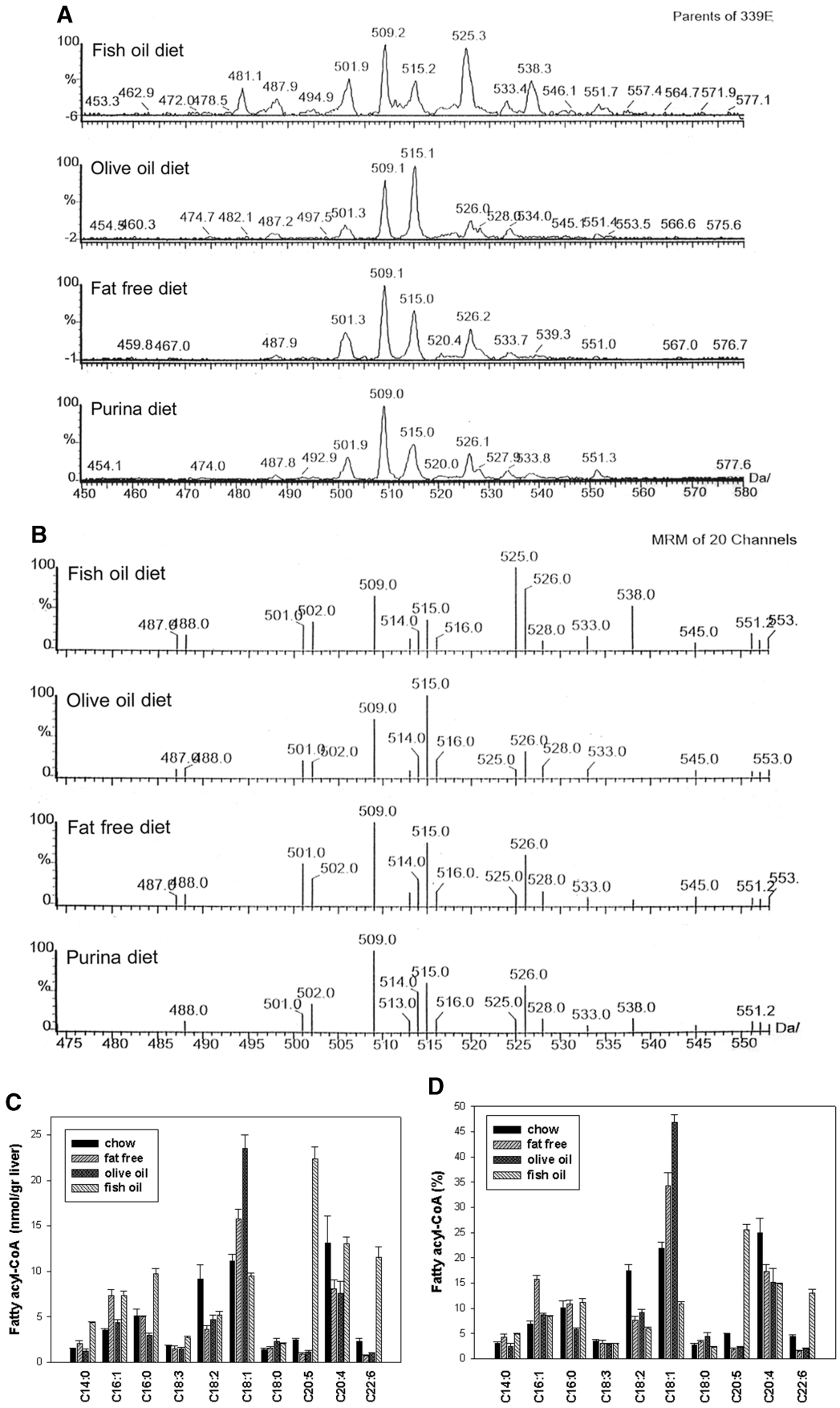


Fig. 2. Manipulation of liver fatty acyl-CoA profile by dietary fat. Male albino rats were fed with regular (Purina), fat-free, olive oil-enriched, or fish oil-enriched diets as described in Methods. Liver extraction and analysis of its acyl-CoAs were as described in Methods. A: Parent ion scanning. One representative spectrum for each dietary mode. B: MRM mode. One representative spectrum for each dietary mode. C: Liver fatty acyl-CoAs (nmol/g tissue) of the four diets. Total identified fatty acyl-CoAs amounted to 51.6 ± 5.7, 46.5 ± 2.4, 50.2 ± 2.4, and 87.8 ± 4.1 nmol/g for chow, fat free, olive oil and fish oil diets, respectively. Mean ± SE (n = 3–4 for each dietary mode). D: Percent abundance of liver fatty acyl-CoAs of the four diets. Mean ± SE (n = 3–4 for each dietary mode).

most abundant acyl-CoA species of liver acyl-CoAs. Moreover, feeding fish oil resulted in ~2-fold increase in total liver acyl-CoAs, and in particular in the C14-CoA and C16-CoA clusters. Thus, the profile of liver fatty acyl-CoAs may be manipulated by dietary fats as a function of their fatty acids composition. Hence, liver transcriptional effects exerted by dietary fatty acids may be transduced in vivo by their respective acyl-CoAs. In particular, the expression of liver HNF-4-controlled genes may be transcriptionally activated or suppressed in vivo by saturated or (ω-3) polyunsaturated dietary fatty acids, respectively (6).

Manipulating liver acyl-CoAs by drugs

The ESI-MS/MS method reported here also makes it possible to characterize and quantitate liver fatty and xenobiotic acyl-CoAs in response to treatment with xenobiotic amphipathic carboxylates. As shown in **Fig. 3**, feeding rats with the hypolipidemic Medica 16 (18) resulted in Medica 16-CoA becoming the dominant liver acyl-CoA species, reaching a content of about 2-fold of total liver fatty acyl-CoAs of nontreated animals. A similar dominant abundance of the xenobiotic acyl-CoA was also observed upon feeding rats with either nafenopin or bezafibrate serving as examples for hypolipidemic fibrate drugs (**Table 1**). Moreover, in addition to the xenobiotic acyl-CoA becoming the most abundant acyl-CoA species, all xenobiotics affected both the composition and content of liver fatty acyl-CoAs (Table 1, **Fig. 4**). Thus, Medica 16 treatment resulted in a 1.5-fold increase in liver total fatty acyl-CoAs content with a specific increase in the relative abundance of (C18:0)-CoA at the expense of decrease in (C16:0)-CoA and (C14:0)-CoA. Similarly, treatment with fibrate drugs resulted in a 2-fold increase in total liver fatty acyl-CoAs content with a specific increase in (C16:1)-CoA and (C18:1)-CoA at the expense of (C18:2)-CoA. The increase in liver fatty acyl-CoAs content in response to hypolipidemic drugs was linearly dependent on the liver content of the xenobiotic acyl-CoA (**Fig. 5**). Hence, the liver acyl-CoA profile is dramatically affected by treatment with hypolipidemic xenobiotic amphipathic carboxylates.

The sensitivity of the ESI-MS/MS method makes it also possible to evaluate the nuclear content of xenobiotic acyl-CoAs. Thus, treating rats with Medica 16 resulted in nuclear Medica 16-CoA (**Fig. 6**). This nuclear acyl-CoA could not be accounted for by contamination with cytosolic Medica 16-CoA as verified by its absence in nuclei derived from nontreated animals that, prior to their isolation by sucrose gradient centrifugation, were mixed with liver cytosol derived from Medica 16-treated rats.

The sensitivity of the ESI-MS/MS method may also allow for detection of acyl-CoAs in cell cultures incubated with the respective acids. Thus, incubating COS-7 cells with

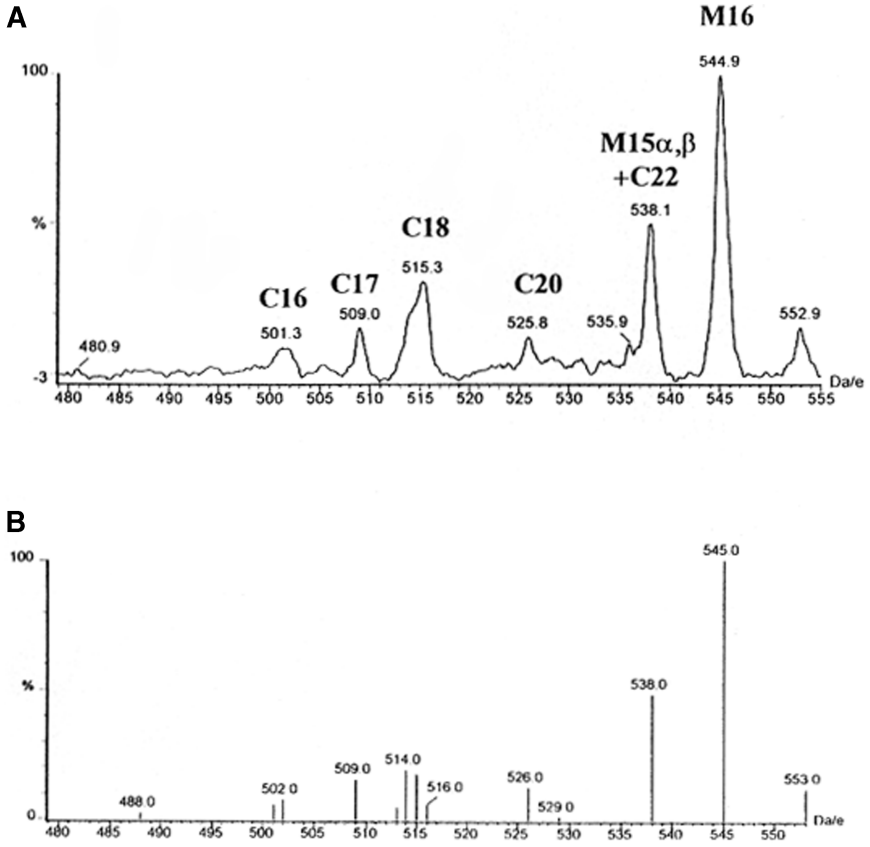


Fig. 3. Effect of β,β'-tetramethylhexadecane-α,ω-dioic acid (Medica 16) on liver acyl-CoA profile. Purina fed rats were treated with 0.25% (w/w) Medica 16 as described in Methods. One representative spectrum. A: Parent ion scanning. B: MRM mode. M15α,β (2-dimethyl-13-diemthyl- α,ω-pentadecanedioic acid) is an M16 metabolite whose *m/z* coincides with that of C22:6.

TABLE 1. The effect of hypolipidemic drugs on liver acyl-CoAs content

Treatment	Nontreated	Medica 16	Nafenopin	Bezafibrate
Xenobiotic acyl-CoA	–	112.7 ± 23.9[a]	40.0 ± 4.5[a]	100.1 ± 30.0[a]
Total fatty acyl-CoAs	64.9 ± 4.6	101.3 ± 12	123.3 ± 17.9	120.4 ± 12.3

Conditions as in Figs. 3 and 4. Mean ± SE (n = 3–5 for each treatment group).
[a] Significant as compared to nontreated animals.

either β,β'-tetramethyloctadecane-α,ω-dioic acid (Medica 18), Medica 16, or β,β'-tetramethyltetradecane-α,ω-dioic acid (Medica 14) (26) resulted in parent ions of 709.6, 681.7, and 653.6 *m/z*, representing the hydrated acylphosphopantetheine fragments of the respective acyl-CoAs resulting from loss of 3′,5′-diphosphoadenosine (*m/z* 426) (not shown). It is worth noting, however, that parents of *m/z* 339 analyzed in cell culture samples consisted of the respective single charged acyl phosphopantetheine fragments rather than the respective doubly charged acyl-CoA molecular ions identified in liver extracts. The cause for that difference remained unexplained, as spiking cell culture samples with authentic acyl-CoAs resulted in molecular ion parents for the authentic acyl-CoAs. This shortcoming may interfere with spiking cell cultures with an internal acyl-CoA standard.

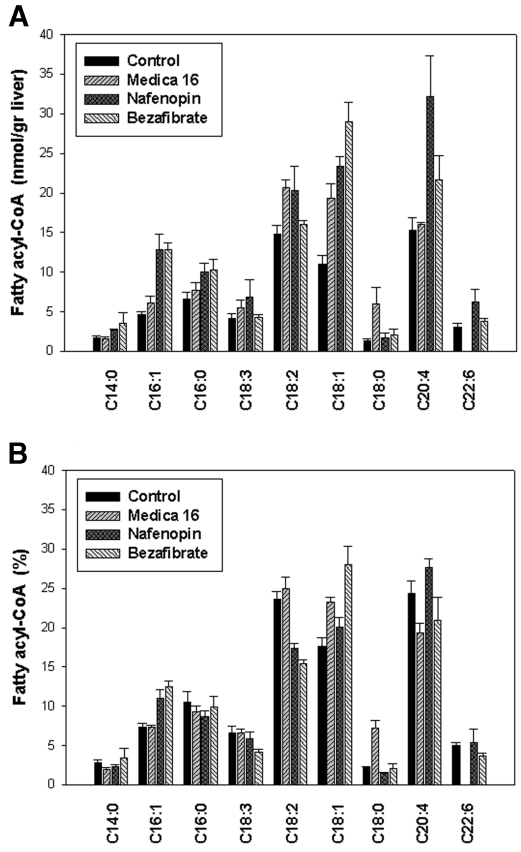


Fig. 4. Manipulation of liver fatty acyl-CoA profile by hypolipidemic drugs. Purina-fed rats were treated with 0.25% (w/w) Medica 16, nafenopin, or bezafibrate as described in Methods. Liver extraction and analysis of its acyl-CoAs were as described in Methods. Mean ± SE (n = 3–4 for each treatment mode). *m/z* corresponding to (C22:6)-CoA coincides with the CoA thioester of an M16-CoA metabolite and was therefore not included in the respective profiles for M16. A: Liver fatty acyl-CoAs composition (nmol/g tissue). B: Percent abundance of liver fatty acyl-CoAs.

Uses and implications

Characterization and quantitation of liver acyl-CoAs as described here extends the negative fast atom bombardment (FAB) ionization mass spectrometry methodology previously described by Zirrolli et al. (24) and applied to synthetic mixtures of acyl-CoAs. FAB analysis of acyl-CoAs results in *m/z* 339 parent ions consisting mainly of the acylphosphopantetheine fragments of respective acyl-CoAs rather than the respective molecular ions. Generating a stable molecular ion in the ion source rather than the respective acylphosphopantetheine fragment yields better sensitivity and makes it possible to quantitate the respective acyl-CoAs by spiking with acyl-CoA standards. It should be noted, however, that since the parent molecular ions are recorded by *m/z* (M-2H)/2, the resolution between respective acyl-CoAs is reduced by a factor of two. Thus, two acyl-CoAs differing by one double bond will differ by only 1 *m/z*. Characterization and quantitation of complex acyl-CoAs mixtures using ESI-MS/MS as reported here surpasses presently available methodologies (e.g., HPLC (27), electron capture GC/MS) (28) by its better resolution and reliability, minimal interference by other ingredients in the biological sample, minimal workup due to separation/derivatization, and shorter processing and analysis time. Furthermore, the method al-

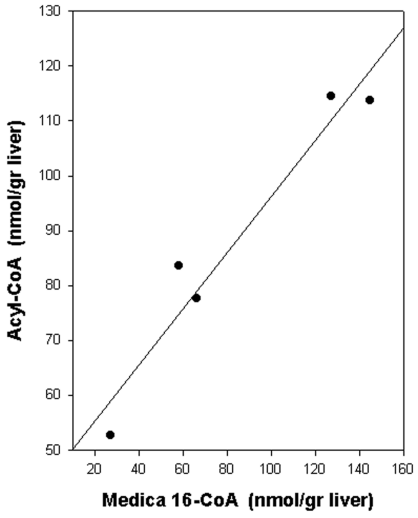


Fig. 5. Liver fatty acyl-CoA content as function of liver Medica 16-CoA concentration. Conditions as in Figs. 3 and 4.

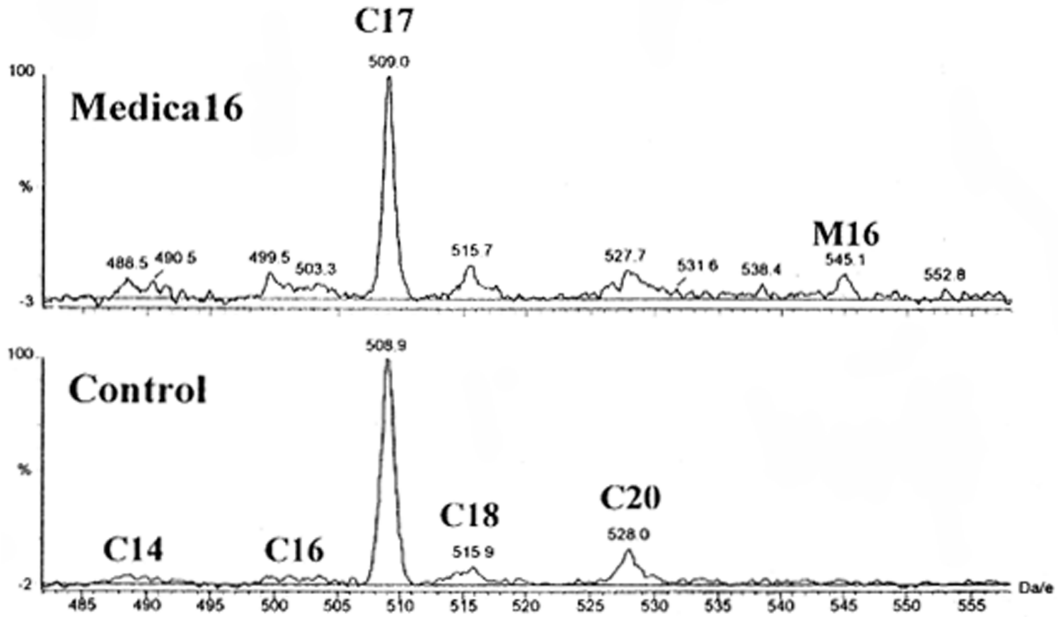


Fig. 6. Liver nuclear Medica 16-CoA content. Conditions as in Fig. 3. Rat liver nuclei were prepared, extracted, and processed as described in Methods.

lows for identifying respective acyl-CoAs by their mass characteristics.

The results reported here are in line with previous reports (29, 30) and may indicate that changes in the composition of dietary fatty acids are reflected by respective changes in the composition of liver fatty acyl-CoAs. Moreover, the pool of liver acyl-CoAs may be extensively modulated by xenobiotic amphipathic carboxylic drugs due to the dominant liver abundance of respective xenobiotic acyl-CoAs accompanied by modulating the composition and content of liver fatty acyl-CoAs. Hence, transcriptional events exerted by dietary fatty acids and/or xenobiotic carboxylic drugs may be transduced in vivo by their respective acyl-CoAs.

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