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NMR-Based Metabolomics Reveals That Conjugated Double Bond Content and Lipid Storage Efficiency in HepG2 Cells Are Affected by Fatty Acid *cis*/*trans* Configuration and Chain Length

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ABSTRACT: In the present study the metabolic response to various fatty acids was investigated in HepG2 cells by using a ^{1}H NMR-based approach. To elucidate the effect of *cis*/*trans* configuration, the cells were exposed to either oleic acid (C18:1 *cis*-9), elaidic acid (C18:1 *trans*-9), vaccenic acid (C18:1 *trans*-11), linoleic acid (C18:2), or palmitic acid (C16:0), and multivariate data analysis revealed a strong effect of fatty acid on the lipophilic metabolite fraction. Inspection of the spectra revealed that the difference between the observed responses could be ascribed to the appearance of resonances from conjugated double bonds (5.65, 5.94, and 6.28 ppm) in cells exposed to vaccenic acid, revealing that vaccenic acid upon uptake by the HepG2 cells is converted into a conjugated fatty acid. Upon exposure of the HepG2 cells to either butyric acid (C4:0), caproic acid (C6:0), lauric acid (C12:0), myristic acid (C14:0), or palmitic acid (C16:0), an effect of fatty acid length was also evident, and data indicated that short-chain fatty acids (C4−C6) are immediately converted, whereas medium−long-chain fatty acids (C12−16) are incorporated into triglycerides and deposited in the cells. In conclusion, the present study demonstrates that ^{1}H NMR spectroscopy is a useful method for studying the uptake of fatty acids in in vitro cells.

KEYWORDS: HepG2 cells, proton nuclear magnetic resonance spectroscopy, *trans*-fatty acids, liver metabolism, in vitro metabolomics

INTRODUCTION

The dietary composition of fatty acids is known to have an impact on health; for example, substitution of saturated fat with unsaturated fat seems to be beneficial for insulin sensitivity.[1] However, the exact effects of single fatty acids are far from fully clear. For more detailed investigations of the impact of fatty acids, different categories of fatty acids are often compared, that is, saturation/unsaturation, degree of unsaturation, and *cis*/*trans* configuration as well as different chain length. *cis*/*trans* configurations of unsaturated fatty acids originate from different sources, that is, endogenous fat synthesis in contrast to exogenous sources,[2] where the industrially produced *trans* configuration has been associated with negative impacts on human health.[3] However, in relation to *trans*-fatty acids from ruminant products, positive associations with health effects have been observed.[4,5] Also, the chain length of fatty acids is an important issue in relation to health effects, and especially short-chain fatty acids have been the subject of intense investigation the past decade.[6,7]

However, even though various dietary fatty acids are known to exert different effects on health-related traits, knowledge about the metabolic effects related to each fatty acid is very limited. Metabolomics is an approach that has emerged widely in nutrition research recent years. Especially NMR-based metabolomics has been successfully applied in the elucidation of the metabolic response to various dietary factors such as wholegrain cereals and fibers,[8−10] protein source,[11] and meat intake.[12] Metabolomic applications for the characterization of in vitro cell lines have also been reported.[13−18] However, metabolomic studies on in vitro cells are much more rare than metabolomic studies on biofluids, as studies on in vitro cells offer some additional challenges. Metabolic turnover is high in in vitro cells and, thus, inactivation of enzymes is very important. In addition, it is essential to ensure that the harvesting and handling of cells do not induce any substantial changes that conceal the metabolic effects of interest.[19] Nevertheless, as metabolomics is an explorative approach that can disclose unknown effects, a metabolomic approach could be expected to be a useful tool for elucidating metabolic effects of various fatty acids in vitro, as metabolomic analyses can be carried out without any a priori knowledge about expected effects. In the present study we therefore hypothesized that by applying nuclear magnetic resonance (NMR)-based metabolomics on extracts of HepG2 cells exposed to various fatty acids, new information about metabolic effects associated with different fatty acids could be unravelled. These effects may include different uptake rates, conversion into other products, enhancement/suppression of fatty acid oxidation, or interference with metabolic pathways other than lipid metabolism. We applied the human liver cell line HepG2 for the experiments, because the liver is a key organ in lipid metabolism. The HepG2 cell line holds several liver-specific characteristics, such as plasma protein synthesis and lipid metabolism,[20−22] and is hence regarded as a suitable model for the investigation of hepatic lipid metabolism.

MATERIALS AND METHODS

HepG2 Cultivation and Experimental Setup. HepG2-SF cells adapted to serum-free growth were purchased from Cell Culture Service,

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Germany. The cells were cultivated in 75 cm^2 cell culture flasks during multiplication in serum-free growth medium based on RPMI 1640 (without L-glutamine) (Invitrogen) containing 10% medium supplement Syn Q x 10 (Cell Culture Service), 2 mM L-glutamine (Invitrogen), 20 μg/mL gentamecin (Sigma), 3 μg/mL amphotericin B (Sigma), 100 units/mL penicillin, and 100 μg/mL streptomycin (Sigma). The growth medium was changed every second−third day, and at ∼80% confluence, the cells were subcultivated by trypsination. Cells were grown at 37 °C, in 5% CO_2.

For experimental use the cells were seeded at a density of ∼14000 cells/cm^2 in 75 cm^2 cell culture flasks (Nunc Nunclon). The cells proliferated for 3 days before exposure to experimental medium. Experimental medium was prepared as follows: A 20 mg/mL bovine serum albumin (BSA) (fatty acid free, Sigma) stock solution was made in growth medium. The BSA stock solution was sterile filtered using 0.22 μm syringe filters, and aliquots were made equaling the number of experimental media (2 × 6). Fatty acids (100 mM in ethanol or pure ethanol for control) were diluted into the BSA stocks to a final concentration of 1 mM. Two sets of experimental medium were made, one for investigation of *cis*/*trans* effects (oleic acid (∼99%, Sigma Aldrich), elaidic acid (>99%, Fluka), vaccenic acid (>99%, Sigma), linoleic acid (>99%, Sigma), and palmitic acid (>99%, Sigma) as well as control) and one for investigation of effects of chain length (butyric acid (>99%, Aldrich), caproic acid (>99.5%, Aldrich), lauric acid (>98%, Aldrich), myristic acid (>99%, Sigma), palmitic acid (>99%, Sigma), and control). The structures of the fatty acids used in the experiments are shown in Figure 1. The fatty acid/BSA solutions were incubated for 2−4 h at 37 °C and stored at 4 °C until further use. The fatty acid/BSA solutions were diluted in growth medium, obtaining a final fatty acid concentration of 100 μM and a BSA concentration of 2 mg/mL. The final concentration of ethanol in the experimental medium was ∼17 mM, which did not have any impact on cell viability according to preliminary tests (data not shown). The experimental media were equilibrated to 37 °C before addition. Total exposure time was 3 days with a change of the experimental medium halfway. For each experimental condition 10 biological replicates were produced for investigation of *cis*/*trans* effects and 9 biological replicates for investigation of chain-length effects.

HepG2 Harvesting and Extraction for NMR Analysis. Medium was aspirated from cells in the culture flask and placed on ice, and the cells were rinsed twice with 5 mL of ice-cold phosphate-buffered saline (PBS). The cells were flash frozen by immersing the culture flask in a metal tray with liquid nitrogen. A total of 3 mL of ice-cold methanol was added to the cell culture flask in two rounds, and cells were scraped off the flask surface with a rubber cell scraper after each methanol addition. After scraping, the cell suspension was transferred to glass vials, and 3 mL of ice-cold $CHCl_3$ was added to each cell sample, thoroughly vortexed, and left on ice for 10 min before the addition of 3 mL of ice-cold H_2O. The samples were left for phase separation at 4 °C overnight and centrifuged for 30 min at 1000*g* at 4 °C. Five milliliters of methanol/H_2O phase and 2 mL of $CHCl_3$ phase were transferred to separate glass vials and dried by vacuum centrifugation.

NMR Analysis. Prior to NMR analyses water-soluble extracts were dissolved in D_2O containing 0.0025% (w/v) sodium trimethylsilyl-[2,2,3,3-$^2H_4$]-1-propionate (TMSP) as an internal standard, and lipid extracts were dissolved in $CDCl_3$ containing 0.05% w/w tetramethylsilane (TMS). The NMR measurements were performed at 298 K on a Bruker Avance III 600 spectrometer, operating at a ^1H frequency of 600.13 MHz and equipped with a 5 mm ^1H TXI probe (Bruker BioSpin, Rheinstetten, Germany). ^1H NMR spectra of water-soluble extracts and lipid extracts were obtained using single 90° and 30° pulse experiments, respectively. For the water-soluble extract, water suppression was achieved by irradiating the water peak during the relaxation delay of 5 s. A total of 64 transients of 16K data points spanning a spectral width 12.14 ppm were collected for water-soluble extracts. For lipid extracts each ^1H NMR spectrum contained a total of 64 scans with a size of 32K data points and a spectral width of 16.02 ppm. Spectra were referenced to the TMSP or TMS signal at 0 ppm.

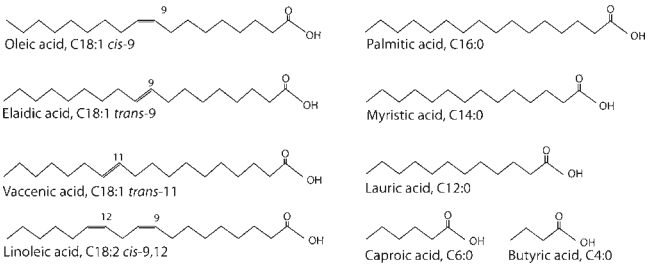

Figure 1. Structural representations and formulas of fatty acids used in the experiments.

The ^1H NMR spectra obtained on water-soluble extracts were divided into 0.007 ppm integral regions and integrated in the region of 0.5−9.5 ppm. The reduced spectra were normalized to the whole spectrum to remove any concentration effects. Before further data analysis, the signals from residual water (4.7−5.0 ppm) as well as residual methanol (3.34−3.38 ppm) were manually cut out of both data sets, ending with data sets containing 1170 independent variables. The ^1H NMR spectra obtained on the chloroform phase were likewise subdivided into 0.007 ppm integral regions and integrated in the region of 0.3−6.5 ppm. Due to extensive shift in the singlet around 1.6 ppm in NMR spectra from samples exposed to fatty acids of different chain lengths, the region from 1.55 to 1.62 ppm consisting of 13 variables was further manually reduced to 1 variable. The new variable was multiplied with the square root of the number of reduced variables, because this procedure was found to result in peak magnitudes resembling those of the original spectra before variable reduction. This was done to obtain approximately the same weight of the peak in the subsequent multivariate data analysis as without variable reduction. This resulted in a total of 1215 independent variables in the chloroform data set containing samples exposed to fatty acids of different chain lengths. The data set of chloroform-soluble metabolites from *cis*/*trans*-exposed samples included 1221 independent variables. Due to errors in the sample preparation, a few samples were left out in the final data sets. From the data set of water-soluble metabolites from cells exposed to fatty acids of different chain lengths, a total of 12 samples were left out (3 × control, 2 × C4, 3 × C6, 2 × C12, 1 × C14, 1 × C16), whereas from the data set of chloroform-soluble metabolites from cells exposed to *cis*/*trans*-fatty acids 2 control and 1 palmitic acid-exposed samples were left out. Multivariate data analysis was performed using Unscrambler software version 9.2 (Camo, Oslo, Norway). Principal component analysis (PCA) was applied to the centered, area-normalized, and Pareto-scaled data to explore any clustering behavior of the samples.

RESULTS

$CHCl_3$ Cell Extracts. A representative ^1H NMR spectrum obtained on the $CHCl_3$ extract of fatty acid exposed HepG2 cells is shown in Figure 2. Several signals are observed, which have been assigned to various lipid groups on the basis of existing literature.[23,24] Inspection of the ^1H NMR spectra reveals clear differences between cells exposed to different fatty acids. In particular, fatty acids with different *cis*/*trans* configuration give rise to pronounced differences in the ^1H NMR spectra of $CHCl_3$ cell extracts. These spectral differences are apparent with regard to occurrences of different peaks as well as different peak magnitudes. The most pronounced differences between the different *cis*/*trans*-fatty acids include a signal around ∼1.9−2.15 ppm representing

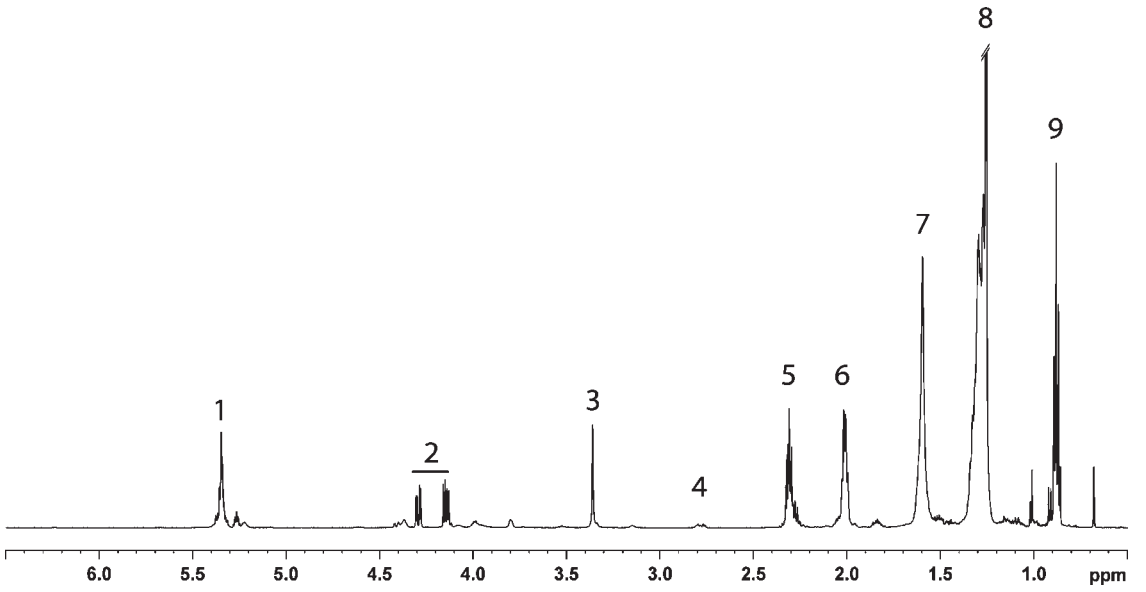

Figure 2. Representative 600 MHz ^{1}H NMR spectrum obtained on $CHCl_3$ extract of HepG2 cells exposed to palmitic acid. Assignments: 1, −C**H**═C**H**−; 2, glyceryl moiety; 3, choline protons; 4, ═CH—C**H**$_2$—CH═; 5, −CH$_2$—C**H**$_2$—COOH; 6, −**H**$_2$C—CH═CH−; 7, −C**H**$_2$—CH$_2$—COOH; 8, CH$_2$ protons; 9, CH$_3$ protons.

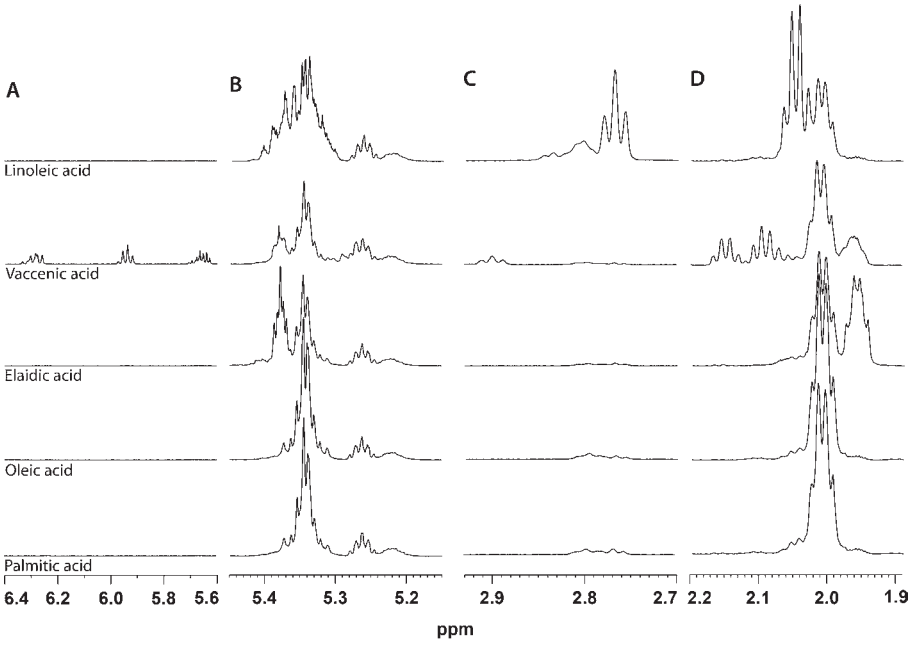

Figure 3. Expanded regions of ^{1}H NMR spectra obtained on $CHCl_3$ extract of HepG2 cells exposed to linoelic acid, vaccenic acid, elaidic acid, oleic acid, and palmitic acid: (A) 5.8−6.3 ppm region, where signals from conjugated double bonds appear after exposure to vaccenic acid; (B) 5.15−5.45 ppm region representing −C**H**═C**H**− signals; (C) 2.7−2.9 ppm region representing ═CH—C**H**$_2$—CH═ signals; (D) 1.9−2.2 ppm region representing −**H**$_2$C—CH═CH− signals.

protons next to a double bond (−**H**$_2$C—CH═CH−) and a signal around ∼5.3−5.4 ppm representing double-bond protons (−C**H**═C**H**−) (Figure 3). In addition, exposure to vaccenic acid gives rise to additional peaks at 5.65, 5.94, and 6.28 ppm, which can be assigned to conjugated double bonds (Figure 3). This assignment was verified by a ^{1}H NMR spectrum obtained on a conjugated linoleic acid standard, which also showed resonances at 5.65, 5.94, and 6.28 ppm (data not shown). Furthermore, significant spectral differences concerning peak magnitude are present around 1.3 ppm, representing methylene protons (−C**H**$_2$−), and a signal around 1.6 ppm representing methylene protons in β-position to the carboxyl acid group (−C**H**$_2$—CH$_2$—COOH−). For fatty acids representing different chain lengths, the spectral differences are exclusively due to different peak magnitudes, with the most pronounced differences being observed around 1.3 and 1.6 ppm (data not shown).

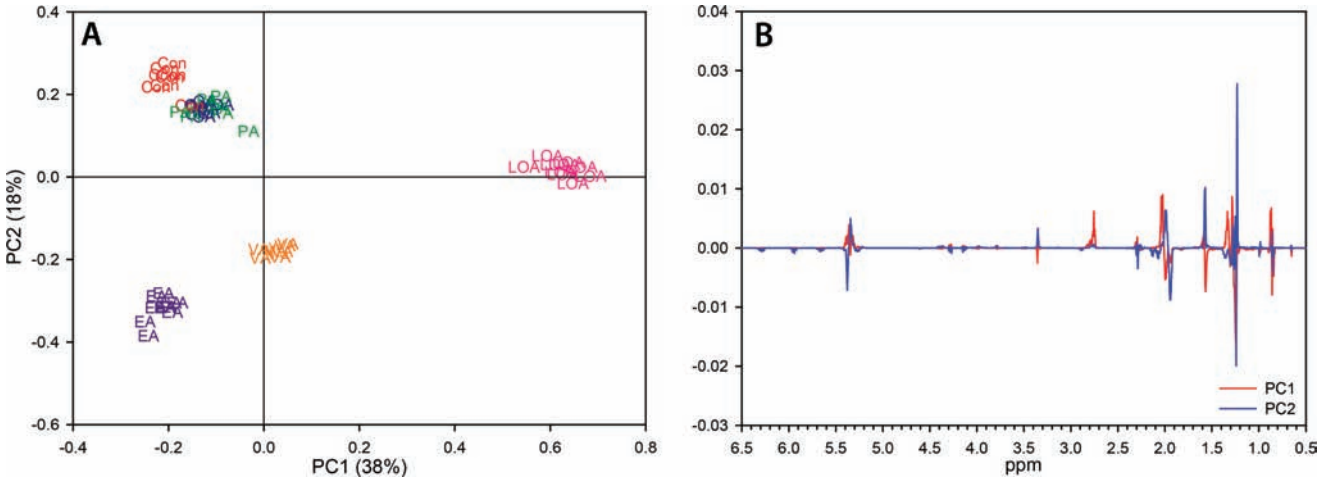

Figure 4. (A) PCA score plot showing the first and second principal components obtained for $CHCl_3$ cell extracts upon exposure to linoleic acid (LOA), vaccenic acid (VA), elaidic acid (EA), oleic acid (OA), palmitic acid (PA), and control (Con). (B) Corresponding first loading line plot after back-scaling of the pareto-scaled loadings.

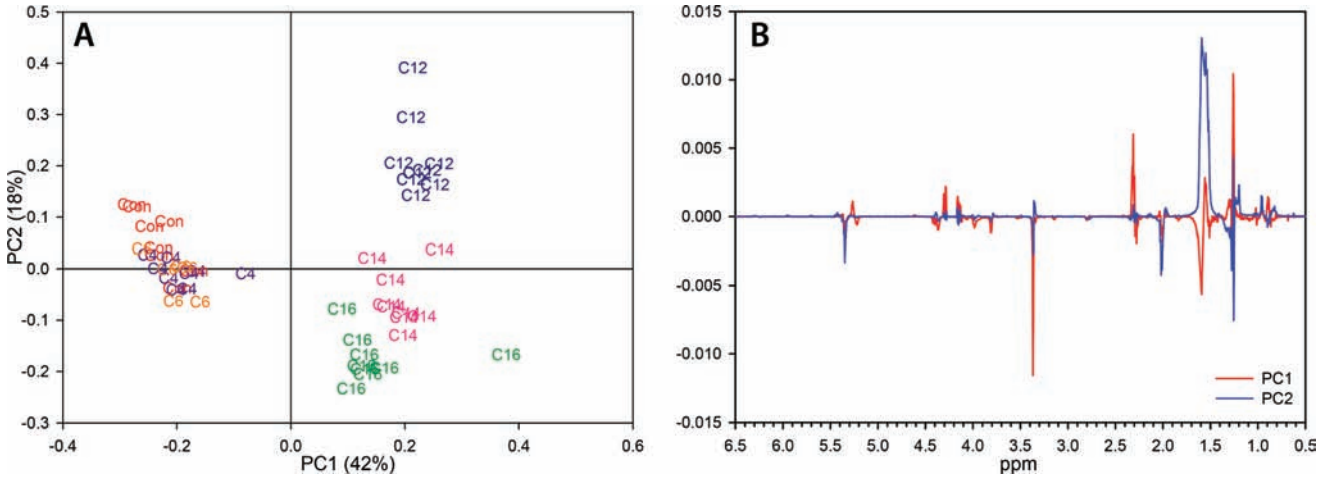

Figure 5. (A) PCA score plot showing the first and second principal components obtained for $CHCl_3$ cell extracts upon exposure to butyric acid (C4), caproic acid (C6), lauric acid (C12), mystiric acid (C14), and palmitic acid (C16). (B) Corresponding first loading line plot after back-scaling of the pareto-scaled loadings.

PCA was carried out on NMR spectra obtained on lipophilic cell extracts to elucidate the overall effect of exposure to fatty acids with various *cis*/*trans* configurations (Figure 4). A strong grouping of the samples according to *cis*/*trans* configuration is observed in the score scatter plot. Cells treated with linoleic acid are most strongly distinct from the other fatty acid exposures (Figure 4A). Analysis of the corresponding back-scaled loading plots reveals that the grouping of fatty acids with various chain lengths mainly can be ascribed to differences in the signals representing CH_2 and CH_3 protons in fatty acids (~1.3 and ~0.7 ppm), a signal at ~1.6 ppm representing methylene protons in β-position to the carboxyl acid group ($-C\mathbf{H}_2-CH_2-COOH-$), a signal at ~2.0 ppm representing protons next to a double bond ($-\mathbf{H}_2C-CH{=}CH-$), a signal at ~2.8 ppm representing protons between two double bonds ($=CH-C\mathbf{H}_2-CH=$), and a signal at ~5.3 ppm representing double bonds ($-C\mathbf{H}{=}C\mathbf{H}-$) (Figure 4B).

PCA was likewise performed to elucidate the overall effect of fatty acid chain length on the lipophilic metabolite profile of cell extracts. A strong grouping of the samples into two different fatty acid groups is observed in the score scatter plot. The group consisting of control cells, cells exposed to butyric acid (C4) or caproic acid (C6), was clearly distinct from the group of cells exposed to lauric acid (C12), mystiric acid (C14), or palmitic acid (C16) (Figure 5A). Analysis of the corresponding back-scaled loading plots reveals that the grouping of fatty acids with various chain lengths mainly can be ascribed to differences in the 1.3 ppm signal representing CH_2 protons in fatty acids, a signal at ~1.6 ppm representing methylene protons in β-position to the carboxyl acid group ($-C\mathbf{H}_2-CH_2-COOH-$), a signal at ~2.3 ppm representing protons next to a double bond ($C\mathbf{H}_2-CH{=}CH$), a signal at ~3.35 ppm representing choline protons ($N(CH_3)_3$), signals around 4.0−4.5 ppm representing the glycerol backbone in fatty acids, and a signal at ~5.3 ppm representing double bonds ($-C\mathbf{H}{=}C\mathbf{H}-$) (Figure 5B).

Aqueous Cell Extracts. A representative ^{1}H NMR spectrum obtained on an aqueous extract of HepG2 cells is shown in Figure 6. In the ^{1}H NMR spectra of aqueous cell extracts multiple

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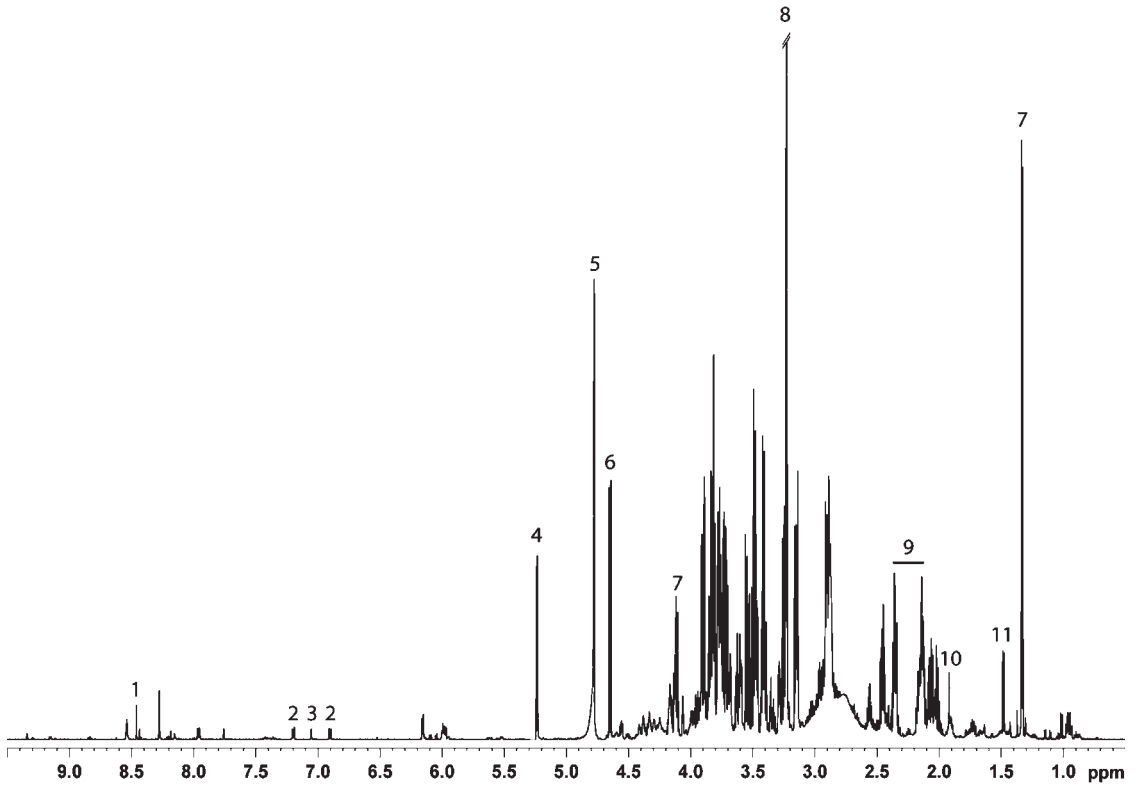

Figure 6. Representative 600 MHz ^{1}H NMR spectrum obtained on methanol extract of HepG2 cells exposed to palmitic acid. Tentatively assigned peaks: formate (1), tyrosine (2), histidine (3), α-glucose (4), residual water (5), β-glucose (6), lactate (7), choline (8), glutamine (9), acetate (10), and alanine (11).

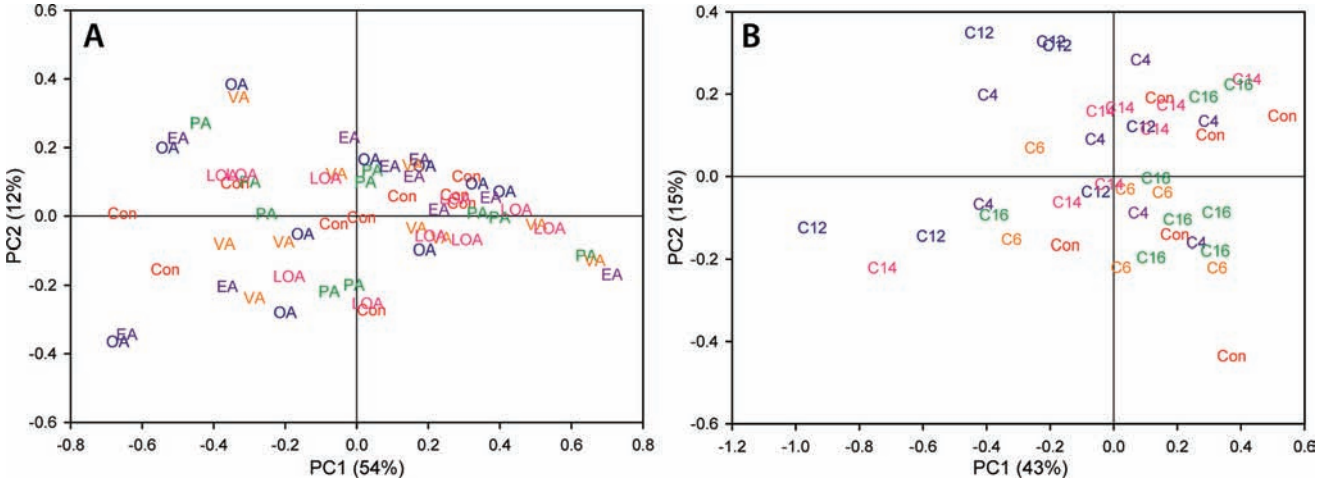

Figure 7. (A) PCA score plot showing the first and second principal components obtained for methanol cell extracts upon exposure to linoelic acid (LOA), vaccenic acid (VA), elaidic acid (EA), oleic acid (OA), and palmitic acid (PA). (B) PCA score plot showing the first and second principal components obtained for methanol cell extracts upon exposure to butyric acid (C4), caproic acid (C6), lauric acid (C12), mystiric acid (C14), and palmitic acid (C16).

signals are observed, which have tentatively been assigned to various metabolites on the basis of existing literature[25] and the Human Metabolome Database.[26] To elucidate the effect of different fatty acid exposures on the metabolite profile of aqueous cell extracts, PCA was performed. Analysis of the obtained score plots revealed no clear grouping of the samples according to type of fatty acid. Figure 7 shows the score scatter plots for PC1 and PC2. Sample grouping in higher PCs was also elucidated, but also here no indications of grouping according to type of fatty acid were observed (data not shown).

DISCUSSION

In recent years metabolomics, which aims at describing the entire set of metabolites in a cell or organism, has proven useful in many areas of life sciences. Metabolomic applications for the

characterization of cells in vitro have also been reported.[13−18] However, to our knowledge, the present study is the first to elucidate the metabolic response to dietary fatty acids in human cells in vitro. In the present study the HepG2 cell line was cultivated and exposed for 3 days to 100 μM of various fatty acids representing both different chain lengths and *cis*/*trans* configurations. Proton NMR spectroscopy of $CHCl_3$ extracts revealed a strong effect of fatty acid on the lipophilic metabolite profile. This result reveals that exposure to different fatty acids has an impact on the fatty acid profile in the cell. Consequently, the fatty acid exposure must have resulted in an incorporation of the fatty acids in the cells, and the present study demonstrated that the applied ^{1}H NMR technique is useful for investigating the uptake of fatty acids in cell cultures. In lipid research ^{13}C NMR spectroscopy is commonly applied,[27−32] which probably should be ascribed to the fact that ^{13}C NMR spectroscopy has larger chemical shift dispersion, making it easier to detect differences. However, a major advantage with ^{1}H NMR spectroscopy is the high sensitivity, which means that a ^{1}H NMR spectrum can be applied many times more rapidly than a ^{13}C NMR spectrum. It is therefore intriguing that the present study revealed that ^{1}H NMR spectroscopy is applicable for the study of fatty acid metabolism in cell cultures, for which the low sensitivity of ^{13}C NMR spectroscopy could be a challenge.

Although most of the effects observed in the ^{1}H NMR spectra of cells upon fatty acid exposure could be ascribed to uptake of the fatty acids, intriguingly the present study revealed the presence of conjugated double bonds in cells exposed to vaccenic acid (Figure 3). This result reveals that vaccenic acid upon uptake by the HepG2 is converted into a conjugated fatty acid, probably conjugated linoleic acid (*cis*-9,*trans*-11). Noticeably, our previous FTIR study applied on a similar experiment revealed that vaccenic acid exposure resulted in the presence of new absorption bands at 981 and 946 cm^{-1}, which could be ascribed to double-bond conjugation.[33] Consequently, evidence for the conversion of vaccenic acid to conjugated linoleic acid by HepG2 cells has been obtained both by the application of FTIR study and by ^{1}H NMR spectroscopy as shown in the present study.

$CHCl_3$ extracts of cells exposed to fatty acids with different chain lengths from C4 to C16 revealed a clear grouping of short-chain fatty acids (C4−C6) and medium−long-chain fatty acids (C12−16) (Figure 5A). Thus, the metabolic response observed in the HepG2 cell line was clearly dependent on fatty acid chain length and indicates a different response to short-chain fatty acids (C4−C6) compared with medium−long-chain fatty acids (C12−16). The result may be ascribed to the fact that short-chain fatty acids (C4−C6) undergo β-oxidation immediately, whereas medium−long-chain fatty acids (C12−16) are deposited in the cells. This explanation is in agreement with the fact that PCA loadings revealed a higher intensity of the NMR signal from glycerol backbone (∼4.0−4.5 ppm) in cells exposed to medium−long-chain fatty acids (C12−16) (Figure 5B). Intriguingly, exposure to medium−long-chain fatty acids (C12−16) was also associated with a decrease in the intensity of the NMR signal from choline (∼3.35 ppm) (Figure 5B), which probably should be ascribed to the fact that a higher amount of triglycerides in the cells reduces the contribution from cell membrane material. Consequently, there are several indications that short-chain fatty acids (C4−C6) are immediately converted, whereas medium−long-chain fatty acids (C12−16) are incorporated into triglycerides and deposited in the cells.

The dietary composition of fatty acids is known to exert different effects on human health;[1] however, knowledge about the metabolic effects related to specific fatty acids is limited. One of the major advantages with metabolomics is the fact that it is an explorative approach that can be applied without any a priori knowledge about expected effects. Consequently, metabolomics could be expected to be a useful technique for exploring the metabolic response to different fatty acids. In the present study the metabolic response was elucidated by examination of the aqueous extract of HepG2 cells upon exposure to various fatty acids. However, neither visual inspection of the obtained aqueous ^{1}H NMR spectra nor the application of PCA to the NMR data could reveal any clear effect of type of fatty acid on the metabolite profile of the HepG2 cells (Figures 6 and 7). It must therefore be concluded that even though the present study revealed that exposure of HepG2 cells to different fatty acids (100 μM) in 3 days resulted in fatty acid uptake, it did not induce any differences in the metabolic response detectable by ^{1}H NMR spectroscopy of aqueous cell extracts. The reason that we did not observe any effect of fatty acid chain length or *cis*/*trans* configuration could be that the only metabolic effect related to the 3 days of fatty acid exposure is an effect on β-oxidation of fatty acids and the end product acetyl-CoA is not detectable by ^{1}H NMR spectroscopy. It could be of great interest to investigate if a more targeted approach using ^{13}C NMR spectroscopy after exposure to ^{13}C-enriched fatty acids could provide more information useful for the elucidation of individual fatty acid metabolism. Furthermore, future applications of LC-MS-based metabonomics could be of interest to see if more sensitive techniques would be able to detect an effect of fatty acid type on the metabolic fingerprint. Such investigations could be valuable in reaching a deeper understanding of the differential effects of fatty acids evident on the whole body level. Furthermore, a mapping of potential differential metabolic effects of various fatty acids would be a useful starting point for a thorough investigation of the significance of fatty acid combinations on the effect of single fatty acids.

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