



Primary murine hepatocytes exposed to fatty acids analyzed by Raman and infrared microscopy

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ABSTRACT

Aim of this work is to present the phenotype and biochemical characteristics of primary freshly isolated murine hepatocytes exposed to oleic and palmitic acids, the most abundant fatty acids (FAs) in human diet and serum. Excess of FAs in the diet is one of the factors that contributes to the non-alcoholic fatty liver disease (NAFLD). The hallmark of NAFLD is the overaccumulation of lipids in hepatic tissue and formation of lipid droplets (LDs). With time NAFLD can progress into more advanced stages leading to liver injury. Hepatocytes as the most abundant cells in the liver are strongly involved in the process of LDs formation, therefore are widely used in research on molecular mechanism of NAFLD development and progression. Primary hepatocytes are rarely used in investigations, due to multistep isolation process and loss of functionality under culture conditions. However, they offer substantial advantages, including preserved phenotype and functions. In order to investigate LDs formation and their content, the Raman and FTIR imaging were employed, as they provide spatial and vibrational information on studied cells in label-free manner.

Introduction

The overabundance of lipid droplets (LDs) in the liver is a hallmark of non-alcoholic fatty liver disease (NAFLD). This condition, also named simply as steatosis, in most cases do not progress into more advanced stages. However, for small group of individuals with NAFLD, may lead to more serious condition named non-alcoholic steatohepatitis (NASH) and subsequently to fibrosis, cirrhosis and eventually hepatocellular carcinoma [1–3]. Despite decades of NAFLD-oriented studies, still much remains to be solved [4].

Liver is composed of several types of highly specialized cells i.e., hepatocytes, Kupffer, stellate (HSCs) and sinusoidal endothelial cells (LSECs). Hepatocytes are highly metabolically active cells including their important role in uptake, turnover and storage of lipidic species [5]. Kupffer cells are the resident macrophages [6], while HSCs possess abundant retinol-rich LDs [7]. LSECs are unique type of endothelial cells with fenestrations, that among other functions regulate the transfer of macromolecules between blood and underlying hepatocytes [8]. The crosstalk between LSECs, HSCs, Kupffer cells and hepatocytes maintain liver integrity and function, however disruption of this process is associated with a development of NAFLD [8].

Number of methods, models and levels of investigation (*in vivo*, *ex vivo*, *in vitro*) were employed, in order to elucidate the pathophysiology

of NAFLD [9,10]. *In vitro* formation of cellular LDs is typically induced by the addition of fatty acids (FAs) to the cell culture medium. Palmitic acid (PA, 16:0) and oleic acid (OA, 18:1) represent the most common saturated and unsaturated FAs in human diet and serum, therefore they have been used to mimic the steatosis at cellular and molecular level [9]. Excess of PA can trigger lipotoxicity in hepatocytes, contrarily, unsaturated FAs are much less toxic, and are able to combat PA induced lipotoxicity [11].

In recent years, techniques of vibrational spectroscopy, e.g. Raman microscopy turned out to be very successful in NAFLD-oriented studies, as they provide the spatial and molecular information [12–14]. As shown in Fig. 1, LSEC, HSC and hepatocyte exhibit distinctive morphological and biochemical phenotype that can be easily identified by Raman imaging, previously presented by K. Kochan et al. [15]. This technique has been already applied for visualisation of micro- and macrovesicular LDs in the murine liver tissue [12,16] as well as high fat diet-induced NAFLD in the isolated liver cells [17,18]. Moreover, we have reported the differential response of LSEC and hepatocytes to OA and PA, using cell lines TSEC and AML-12, respectively [19]. In this work, we aim to define the phenotype of primary freshly isolated murine hepatocytes exposed to FAs (OA and PA) and describe their biochemical content using Raman imaging combined with chemometric analysis. Additionally, we tested the utility of FTIR imaging with enhanced magnification in analysis of hepatocytes.

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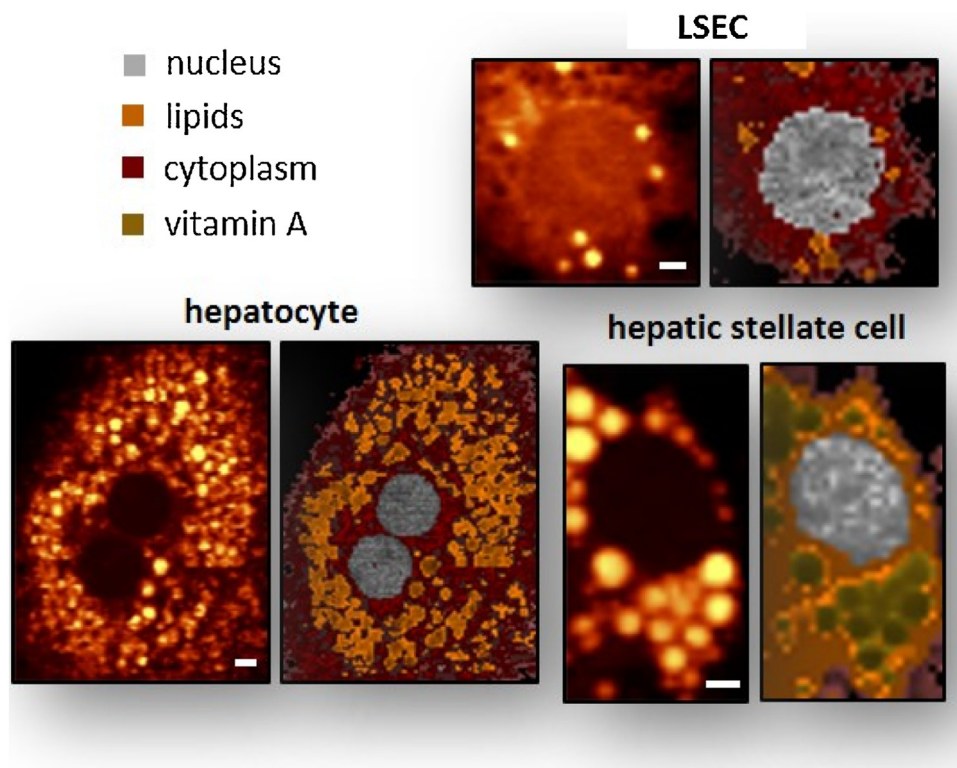


Fig. 1. Raman imaging of liver cells: LSEC, hepatocyte and HSC. The images were constructed by an integration of Raman bands over the selected spectral region: 3050–2800 cm^{-1} (organic matter, on the left) and false-colour images produced by k-means cluster analysis (KMC, on the right). Scale bar equals 2 μm .

Experimental

Cells isolation and incubation with FAs

All procedures involving animals were conducted in accordance with the Guidelines for Animal Care and Treatment of the European Union, and were approved by the 1st Local Animal Ethics Commission in Krakow (decision no. 292/2015). In this study male C57BL/6 male mice fed AIN-93G diet (a standard rodent) was used. The isolation of hepatocytes were

performed according to protocol described elsewhere [20]. Afterward, cells were kept in William's E medium (Lonza) under 37 °C and 5% CO_2 conditions to spread out. In the next step hepatocytes were incubated with either OA, PA (Sigma Aldrich) and an equimolar mixture of both FAs (OA + PA) conjugated with Bovine Serum Albumin (BSA, Sigma Aldrich) with final concentration at 200 μM for each FAs. After 18 h, cells were fixed with a solution of glutaraldehyde (2%, 4 min) and hold in phosphate buffer (PBS, Gibco), pH = 7 at 4 °C. For IR imaging, the same samples were gently washed twice with distilled water and air-dried.

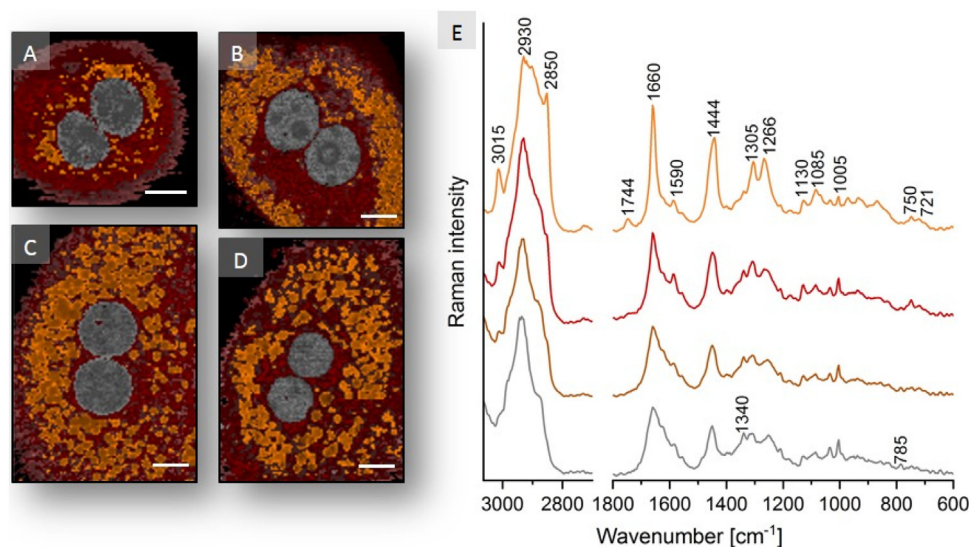


Fig. 2. KMC analysis of hepatocytes. False color images of primary hepatocytes (A) control, (B) incubated with OA, (C) with PA and (D) with an equimolar mixture of OA and PA. (E) Corresponding average Raman spectra of control hepatocyte. Colors of the spectra corresponds to the color of the classes in the false-color image. Scale bars equal 10 μm .

Raman and FTIR imaging

Raman imaging was performed using WITec alpha 300 spectrometer (Ulm, Germany). All spectra were recorded with a laser excitation wavelength 532 nm, a 63× immersion objective (Zeiss Fluor, NA = 1.0) and a back-illuminated CCD camera. Sampling density was 0.5 μm and integration time for single spectrum was 0.4 s. IR images were recorded using a Varian 620-IR microscope coupled to a 670-IR spectrometer with a 128 × 128 pixel focal plane array (FPA) detector. Collection of IR images was performed in transmission mode. HD FTIR spectroscopic imaging used a 15× Cassegrain objective and condenser optics with NA of 0.62 and a projected FPA pixel size of 1.1 × 1.1 μm^2 giving a measured area of 141 × 141 μm^2 . All FTIR spectra were recorded by co-adding of 128 scans and in the range of 3800 to 900 cm^{-1} with a spectral resolution of 4 cm^{-1} .

Data analysis

Raman data were preprocessed and analyzed in WITec project Plus software with the following steps: routine cosmic ray removal, baseline correction using autopolynomial of degree 3. Preprocessed Raman data were analyzed using cluster analysis with k-means (KMC) method in the spectral range of 1800–600 cm^{-1} . KMC results were obtained with Manhattan distance mode. As a result, similar spectra were assigned to the same class (cluster), while each class represents cell compartments with different spectral characteristic. FTIR spectral pre-processing and data analysis was performed using a CytoSpec v.2.00.02 software 22. After noise reduction with 15 PCs, second-derivative spectra were calculated (Savitzky–Golay algorithm, 9 points of smoothing). KMC method was

performed in the spectral range of 1800–900 cm^{-1} , with the Ward's algorithm and spectral distances were computed as D-values. Two classes were adjusted to extract cells and background, next the number of classes was adjusted in order to discriminate spectrally different cell areas.

Results and discussion

Primary hepatocytes incubated with FAs were deemed as a model of lipids overabundance occurring during NAFLD development. Fig. 2 displays false-color images of healthy (control) hepatocyte (A), incubated with OA (B), PA (C), and an equimolar mixture of OA and PA (D). KMC analysis enabled to define 4 cell areas with different biochemical composition found based on its spectral signature. Fig. 2E presents color-coded average spectra of each discriminated class obtained for control hepatocyte. The orange spectrum corresponds to the class rich in LDs, manifested by the presence of bands assigned to unsaturated lipids (ULs) at 3015, 1660, 1266 cm^{-1} and saturated lipids (SLs) at 1444 and 1305, as well as the band originated at 1744 cm^{-1} typically assigned to triacylglyceroles (TAGs), and a low intensity band at 721 cm^{-1} from phospholipids containing choline [21]. Similarly, the dark-red class corresponds to cell area also rich in lipids, however the bands of hemoproteins at 1590 and 750 cm^{-1} are more noticeable, suggesting presence of mitochondria [22]. Additionally, in average spectra of dark red and brown class, the band at 1744 cm^{-1} is not visible. The average spectrum of grey class exhibit the marker band of DNA&RNA at 785 cm^{-1} , therefore it was assigned to nuclei [23].

The Raman images in Fig. 3 show the distribution of organic matter, nucleic acids, ULs and SLs and cholesteryl esters, respectively. On the

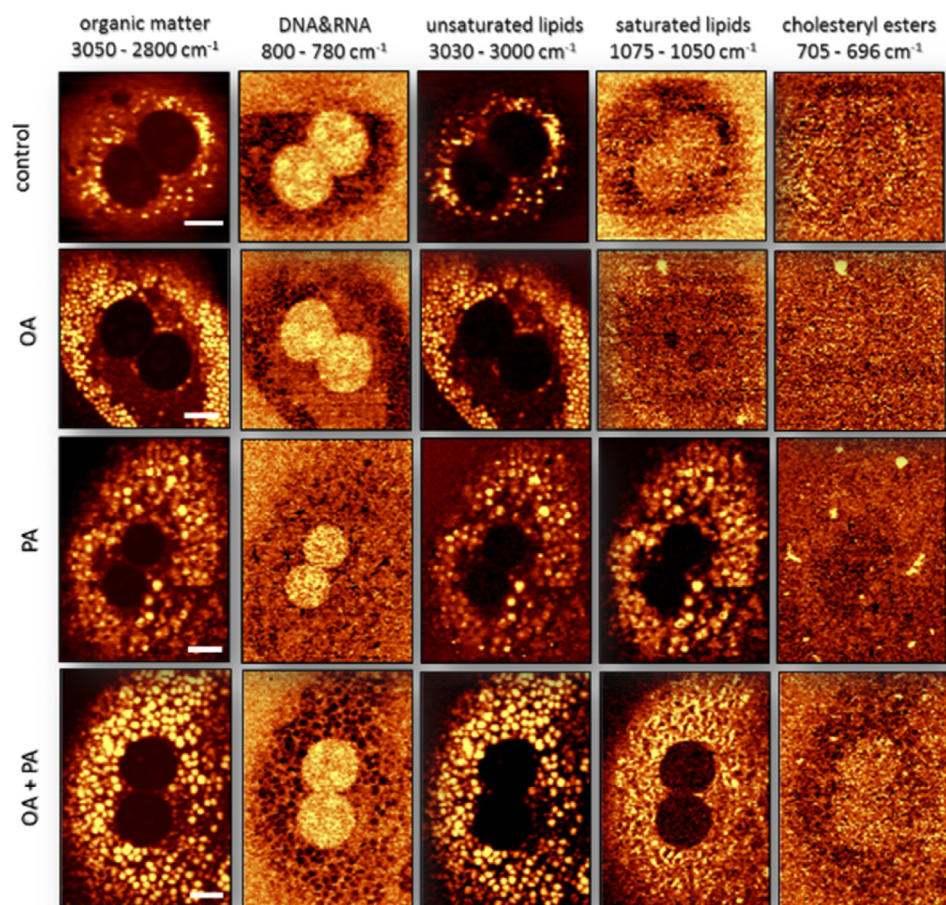


Fig. 3. Raman images of primary hepatocytes. In the row from the top: control, cells incubated with oleic acid, with palmitic acid and with an equimolar mixture of OA and PA, obtained by an integration of Raman bands over the selected spectral regions: 3050–2800 cm^{-1} (organic matter), 800–780 cm^{-1} (DNA&RNA), 3030–3000 cm^{-1} (unsaturated lipids), 1075–1050 cm^{-1} (saturated lipids) and 705–696 cm^{-1} (cholesteryl esters), respectively. Scale bar equals 5 μm .

other hand, LDs in control cells mainly consist of ULs, with no detectable signal from SLs neither from cholesteryl esters. Similar observation was made after incubation of cells with OA, however small spots of SLs and cholesteryl esters were identified. In contrary, after an uptake of PA the SLs as well as ULs were detected, accompanied with few deposits of cholesteryl esters. Saturated and unsaturated FAs have different effect on cells. While OA is easily esterified and accumulated in LDs, PA poorly undergoes conversion to TAGs, becoming lipotoxic in higher concentrations [24]. In order to attenuate effect of PA uptake, an enzymatic desaturation is promoted [25]. As a result, both ULs and SLs observed in LDs (Fig. 3). After incubation with equimolar mixture of OA and PA, distribution of ULs was similar to the above displayed images, however distribution of SLs seems to be a residue of taken up PA. This may be explained by promotion of PA incorporation into TAGs by exogenous or endogenously generated unsaturated FAs [24]. Previously reported effect of FAs incubation with AML-12 cells, are in agreement with presented here, however with minor differences [19]. Primary hepatocytes considering at least their size, forms more LDs, that may be related with their higher ability of FAs uptake and subsequent metabolism. The characteristic distribution of SLs after treatment with OA and PA was not observed in AML-12 cells [19].

Hepatocytes are the largest of liver cells, with an average size of 40 μm , therefore they were deemed as suitable objects to be studied also with FTIR imaging. The comparison of Raman and FTIR imaging of hepatocytes incubated with OA is displayed at Fig. 4A–D, respectively. As already presented, Raman imaging together with subsequent KMC analysis enable to achieve differentiation of cell areas such as nuclei or LDs.

The segmentation of cells into nuclear and cytoplasmic area was performed with the support of KMC analysis. The major spectral differences of color-coded average second-derivative IR spectra of obtained classes were observed in the intensity of lipid-related bands at 3015, 2960, 2850 and 1744 cm^{-1} in comparison to the intensity of

amide I band (1654 cm^{-1}). Low intensity bands of nucleic acids at 1080 and 1050 cm^{-1} can be identified in the spectrum of area that match the localization of nuclei [26]. Although the comparison of two classes in the KMC image of FTIR measurement vs. 4 classes obtained for Raman image, may not seem to be reasonable, the segmentation of FTIR image into more classes provided no extra spectral information except difference of lipidic bands intensity. Moreover, such structures as LDs were indistinguishable. It should be noted that, theoretical spatial resolution of Raman image in setup used here equals 360 nm, while for the FT-IR imaging performed with enhanced resolution, the pixel size is approx. of $\sim 1.1 \times 1.1 \mu\text{m}^2$ [26]. On the other hand, FTIR imaging allow for the measurement of larger numbers of cells in shorter time. In showed here example (Fig. 4A, C), Raman image acquisition took 60 min, while FTIR image was recorded within 10 min.

Advantages and disadvantages Raman and FTIR imaging in analysis of hepatocytes depends on the information that is desirable to obtain. If the detailed analysis of subcellular content is considered, the Raman microscopy should be applied, due to better spatial resolution. However, relatively long acquisition time, limits the number cells to be analyzed. On the other hand, when general information about the composition of cellular lipids is of interest, then FTIR imaging is a method of choice, due to possibility to analyze high number of cells. To sum up, simultaneous application of both techniques provides complementary information on studied cells.

Declaration of Competing Interest

The authors report no declarations of interest.

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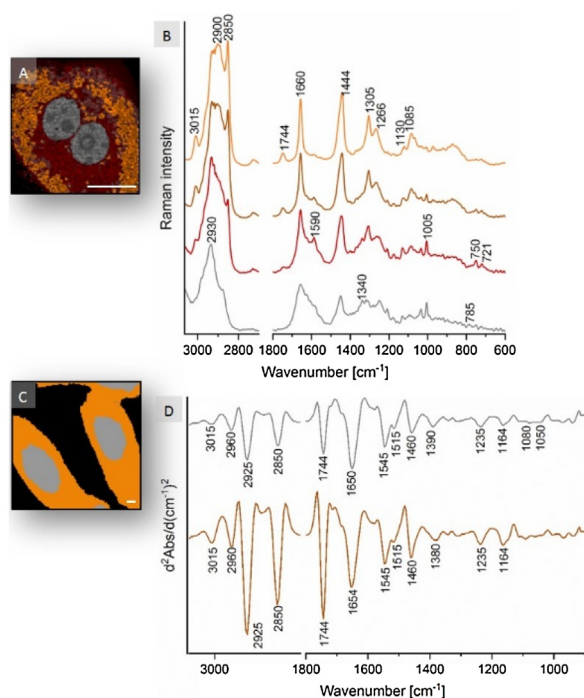


Fig. 4. KMC analysis of Raman and infrared spectra of hepatocytes incubated with oleic acid. (A) false-color image obtained by Raman imaging and (B) corresponding average Raman spectra, (C) false-color image obtained by infrared imaging and (D) corresponding average derivative infrared spectra. The colors of the spectra corresponds to colors of the classes in false-color images. Scale bar equals 10 μm .

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