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Autofluorescence discrimination of metabolic fingerprint in nutritional and genetic fatty liver models

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ABSTRACT

Liver tissue autofluorescence (AF) has been characterized in two models with a different potential to undergo disease progression to steatohepatitis: Wistar rats, administered with a methionine, choline deficient diet (MCD), and Zucker (fa/fa) rats, homozygous for a spontaneous mutation of leptin receptor. AF spectra were recorded from liver tissue cryostatic sections by microspectrofluorometry, under 366 nm excitation. Curve fitting analysis was used to estimate the contribution of different endogenous fluorophores (EFs) to the overall AF emission: i) fluorescing fatty acids, a fraction of liver lipids up to now poorly considered and complicated to detect by conventional procedures; ii) lipofuscin-like lipopigments, biomarkers of oxidizing events; iii) NAD(P)H and flavins, biomarkers of energy metabolism and tissue redox state. AF data and biochemical correlates of hepatocellular injury resulted to depend more on rat strain than on intratissue bulk lipid or ROS levels, reflecting a different metabolic ability of the two models to counteract potentially harmful agents. AF analysis can thus be proposed for extensive applications ranging from experimental hepatology to the clinics. AF based diagnostic procedures are expected to help both the prediction of the risk of fatty liver disease progression and the prescreening of marginal organs to be recruited as donors for transplantation. A support is also foreseen in the advancement and personalization of strategies to ameliorate the donor organ preservation outcome and the follow up of therapeutic interventions.

1. Introduction

Autofluorescence (AF) based real-time diagnostic applications in biomedicine provide many clues to the normal or altered state of cells and tissues [1]. Such potential is due to the natural presence of biomolecules acting as endogenous fluorophores (EFs), contributing to the AF overall signal in a close dependence on their nature, amount, and photophysical properties. Since the pioneering studies of Britton Chance and co-workers, AF investigations have relied primarily on NAD(P)H and flavins as biomarkers of energy metabolism and redox state of cells and tissues [2–4]. This has been the case of the monitoring of liver functionality under ischemia and during organ preservation for transplantation [5–7]. More recently, the attention given to additional EFs, such as proteins - specifically collagen and elastin-, vitamin A, porphyrins, fluorescing fatty acids, lipofuscin-like lipopigments and bile components, has opened up the way on more comprehensive metabolic AF investigations [8–10]. Up to now, native lipids have been poorly considered since, in general, they do not fluoresce or give rise to weak AF emission under near-UV excitation. In 1944 lipid oxidized products were indicated by Popper et al. [11], as the responsible for the yellowish fluorescence in liver with nutritional cirrhosis. Next, in 1973 the chromophores produced from the amino/phospholipid peroxidation were shown to give rise to AF signals similarly to peroxidized cell membranes [12]. However, these observations were followed by few AF studies, such as an investigation on the pathogenesis of ceroid lipofuscinosis [13], while the biochemical detection of lipid peroxidation as malonaldehyde products (TBARS) become early a conventional assay to assess liver oxidative events [14]. In fact, oxidative damages due to the hyperproduction of reactive oxygen species (ROS) from the increased β-oxidation of fatty acids were demonstrated to impair organ functionality in surgery and transplantation, besides favoring the progression of liver disorder to steatohepatitis [15].

The renewed attention to liver lipids as mediators of lipotoxicity [16,17] prompted us to investigate them and their oxidized products as additional real time AF diagnostic tools of fatty liver bio-metabolic condition [10,18]. To this aim, we explored the AF ability to assess and discriminate the metabolic fingerprints of two experimental fatty liver models: i) the genetically unaltered Wistar rat administered with a

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methionine/choline deficient diet (MCD), undergoing lipid accumulation, enhanced oxidative stress and subsequent spontaneous progression to steatohepatitis [19–22]; ii) the genetically altered Zucker (fa/fa) rat, exhibiting biochemical alterations comparable to human non-alcoholic fatty liver disease (NAFLD) and unable to undergo progression to fibrosis and steatohepatitis in the absence of additional, external stimuli [23]. Our spectrofluorometric study relied on the EFs assessed to contribute to the overall AF emission of liver tissue, as from the literature and our previous experience [1,2,12,18,24,25]. The AF was excited in the near-UV region, namely 366 nm, because it involves simultaneously all the liver tissue EFs, making a single measurement to provide a comprehensive information relatable to the organ metabolism. Attention was paid in particular to NAD(P)H and flavins as AF biomarkers of tissue energy metabolism and redox state, and to native fluorescing fatty acids and to lipofuscin-like lipopigments as oxidized products. The response of the liver tissue AF to light irradiation was also considered, as an additional tool to differentiate fatty livers depending on the different presence of photosensitive EFs.

2. Materials and Methods

2.1. Animal Model

Four groups of rats were used: 9 week old male Wistar rats (220–250 g) fed for 12 days with the MCD diet (Laboratorio Dottori Piccioni, Gessate, Italy) or with the same diet supplemented with choline and methionine as the lean control, named respectively Wi-MCD and Wi-C groups (n = 4 each); 11 week old male Zucker rats, obese (fa/fa; 375 ± 15 g) and lean (fa/+; 300 ± 15 g), named respectively Zu-F and Zu-L groups (n = 3 each). All rats were maintained under controlled temperature (21 °C), with a 12 h of dark/light cycles, water and food ad libitum. The use of the animals was approved by the Italian Ministry of Health, and the University of Pavia Animal Care Commission (Document: 2/2012). To comply with the principle of Replacement, Reduction, Refinement (three Rs) and European recommendations on the best care in the use of animals and minimizing number, attention was paid to: i) standardizing procedures, each one performed by the same operator to avoid operator variability, ii) obtaining maximum amount of information from the minimum number of animals; iii) obtaining maximum information from each animal (i.e. AF and biochemical analyses from the same liver); iv) minimizing the animal suffering.

Rats were anaesthetized (Pentobarbital i.p. injection, 40 mg/kg) before the abdomen was opened by a median incision to expose the liver, and sacrificed after liver isolation to collect tissue samples [26]. These were immediately frozen in liquid N_2 and stored at −80 °C until processed for biochemical assays, or mounted to cut tissue sections by cryostat for AF analysis and Nile red fluorochromization.

2.2. Liver Tissue AF Microspectrofluorometry

AF emission spectra were recorded from unfixed, unstained liver tissue sections (30 μm thick) under epi-illumination, by means of a microspectrograph (Leitz, Wetzlar, Germany) mounting an Optical Multichannel Analyzer with a 512-element intensified diode array detector (mod. 1420/512, EG&G - PAR, Princeton, NJ). AF was excited by a 100 W/Hg lamp (Osram, Berlin, Germany), combined with KG1-BG38 anti-thermal filters. Measurement conditions were: for AF, 366 nm band-pass interference excitation filter (FWHM 10 nm, T366 = 25%) and 390 nm dichroic mirror (T366 < 2%), spectra recorded in the 400–680 nm range; for Nile red [18], 436 nm band-pass interference excitation filter (FWHM 10 nm, T_{436} = 40%), TK 450 dichroic mirror (T_{436} < 2%), 480 nm long-pass filter. Spectra were recorded in the 480–750 nm range. Areas to be measured were chosen under bright field observation at low light intensity. Attention was paid to avoid centrilubular and portal areas and the AF signals of collagen and elastin from their typical connective structures. Spectra were collected with a Leitz 25× objective (NA 0.60), from liver tissue parenchima areas (4×10^4 μm^2) selected by a field iris diaphragm. The measuring field area was adapted only in the case of lipid droplets, to select only the vesicles containing accumulated lipids. Each spectral acquisition lasted for 10 sequential scans of 200 ms each, (total measuring time of 2 s). AF photobleaching studies were performed under continuous 366 nm excitation (light fluence of 6.36 mW cm at the focal plane, for a total light dose of 12.72 mJ cm^{-2} per acquired spectrum), by recording sequential pairs of spectra from the same measurement area.

The optimal excitation of flavins and lipofuscin-like lipopigments is typically at wavelengths longer than 366 nm. However, the higher quantum yield of pure flavin-adenine dinucleotide in PBS solution under excitation at 460 nm than 366 nm (5:1) did not prevent its reliable detection at the shorter wavelength [25,27]. The reliable detection of lipofuscin-like lipopigments under 366 nm excitation was also confirmed by a combined imaging and microspectrofluorometric analysis of a liver tissue section with particularly abundant lipopigments [28].

The contribution of each EF to the overall AF spectral emission was estimated through an iterative non-linear curve-fitting procedure (PeakFit; SPSS Science, Chicago, IL) based on the Marquardt-Levenberg algorithm [29]. Before fitting analysis, spectral peaks were normalized to 100 a.u., and wavelengths converted into wavenumbers, being the theoretical models for line spreading based upon frequency. The emission profile of each individual EF was represented by Half-Gaussian Modified Gaussian (GMG) spectral functions. Each EF GMG function is defined by previously assessed spectral parameters, in terms of peak center wavelength position (λ) and full width at half intensity maximum (FWHM): NAD(P)H_{free} (λ = 463 nm; FWHM = 115 nm), NAD(P)H_{bound} (λ = 444 nm; FWHM = 105 nm), flavins (λ = 526 nm; FWHM = 81 nm), vitamin A (λ = 488 nm; FWHM = 102 nm), fatty acids (I.E. arachidonic acid, λ = 470 nm; FWHM = 90 nm), protein emission tail (λ < 440 nm) and lipofuscin-like lipopigments (λ ≈ 587 nm; FWHM ≈ 80 nm). The spectral function of proteins and of lipofuscin-like lipopigments were made free to adapt to achieve a satisfying spectral combination, considering the variability of their emission signals depending on their heterogeneous chemical composition, oxidation, and crosslink degree [30,31]. The GMG functions were combined to attain the best matching between the curve representing the sum of the spectral functions and the experimentally measured spectrum [25]. To this end a preliminary manual operation was followed by the Peakfit algorithm data processing, based on the finding of the true absolute minimum value of the sum of squared deviations. The goodness of the result was verified in terms of residual analysis and coefficient of determination (r^2). An adjustment up to 5% of spectral parameters was allowed when a good fitting was not otherwise attained. NAD(P)$H_{bound,\ free}$ [2] and flavin AF data were used to calculate the redox ratios [7]. For a more familiar presentation of the combination of original spectra and fitting curves, the results from fitting analysis were re-converted to wavelengths.

2.3. Biochemical Assays

Liver tissue assays: ATP/ADP amounts were quantified with a commercial luciferase kit (ATPlite, Perkin-Elmer, Milan, Italy) using a standard curve; total glutathione (GSH) was measured by an enzymatic method (Glutathione Assay Kit, Cayman Chemical Co., Ann Arbor, MI, USA); lipid peroxidation was measured as thiobarbituric acid reactive substances (TBARS) formation according to the method of Esterbauer and Cheeseman [32], using malondialdehyde (MDA) as standard; ROS were quantified by the DCFH-DA method based on the ROS-dependent oxidation of DCFH to DCF, as already described in detail [33]; protein content was assayed by the method of Lowry et al. [34]. Serum contents of alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) were measured by conventional procedures. Lipids were quantified according to Lyn-Cook et al. 2009 [35]. Frozen tissue samples (50–70 mg each were homogenized in