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ZOOLOGY

Analytical methods for evaluation of the fatty acid metabolism in rat liver

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ABSTRACT. The liver is an essential organ for body energy homeostasis, controlling the biosynthesis, uptake and the disposal of carbohydrates and lipids. The hepatic steatosis is a common condition frequently associated with metabolic diseases and is characterized by the excessive accumulation of triglycerides in the liver. In recent years, many efforts have been devoted to prevent and treat the hepatic steatosis, but it remains being pointed out as the major cause for chronic hepatic diseases in Western countries. A considerable part of the knowledge about the physiopathology of hepatic steatosis, the effects of diets and drugs on the metabolic capacity of the liver to metabolize fatty acids, as well as the potential therapeutic approaches for hepatic steatosis derived from experimental animal models using rodents. Here, in this article, we present the details of some of the most common techniques used to evaluate fatty acid metabolism in liver of rats, including quantification of total lipid content, measurement of fatty acid oxidation in isolated subcellular fractions and procedures to measure the activities of important lipogenic enzymes. Classical protocols previously described to be performed using samples from other tissues were adapted to liver samples and different techniques with equivalent aims were compared. The principles and the advantages in terms of reliability and costs were discussed and the procedures here described can be applied for a low-cost broad evaluation of the fatty acid metabolism in liver of rats submitted to different experimental conditions.

Keywords: liver; fatty acid/metabolism; fatty liver; hepatic steatosis.

Métodos analíticos para avaliação do metabolismo em ratos hepáticos

RESUMO. O fígado é um órgão essencial para a homeostase energética, controlando a biossíntese, a captação e a eliminação de carboidratos e lipídios. A esteatose hepática é uma condição frequentemente associada a doenças metabólicas e é caracterizada pelo acúmulo excessivo de triacilgliceróis no fígado. Nos últimos anos, muitos esforços têm sido dedicados para prevenir e tratar a esteatose hepática, mas essa condição continua sendo apontada como a principal causa de doenças hepáticas crônicas em países ocidentais. Uma parte considerável do conhecimento sobre a fisiopatologia da esteatose hepática, sobre os efeitos de dietas e drogas na capacidade metabólica do fígado em metabolizar ácidos graxos, bem como sobre as possíveis abordagens terapêuticas para a esteatose hepática, derivam de estudos com modelos animais experimentais usando roedores. Neste artigo, apresentamos os detalhes de algumas das técnicas que podem ser usadas para avaliar o metabolismo de ácidos graxos no fígado de ratos, incluindo a quantificação do conteúdo lipídico total, medida da oxidação de ácidos graxos em frações subcelulares isoladas e procedimentos para medir as atividades de importantes enzimas lipogênicas. Protocolos clássicos previamente descritos para serem realizados utilizando amostras de outros tecidos foram adaptados para amostras de fígado e diferentes técnicas com objetivos equivalentes foram comparadas. Os princípios e as vantagens em termos de confiabilidade e custos foram discutidos e os procedimentos aqui descritos podem ser aplicados para uma avaliação ampla e de baixo custo do metabolismo de ácidos graxos no fígado de ratos submetidos a diferentes condições experimentais.

Palavras-chave: fígado; metabolismo de ácidos graxos; fígado gorduroso; esteatose hepática.

Introduction

The liver plays fundamental roles on body energy metabolism, controlling the biosynthesis, uptake and disposal of lipid and glucose (Zakim, 1990). The liver serves as a buffer of circulating fatty acids (FA) arising from the diet or lipolysis in white adipose tissue. Excessive FA overload leads to excessive esterification and accumulation as triglycerides (TG) in the cytosol of hepatocytes, a condition defined as hepatic steatosis (HS). HS is one of most frequent metabolic risk factors among

the bunch that characterizes the metabolic syndrome associated with obesity and type 2 diabetes (Marchesini et al., 2001).

The severity of HS lies on its silent nature and high evolving potential. The oxidative damage associated with the accumulation of TG in the liver leads to cell death and inflammation, which in turn contribute to the progression of HS to more severe forms of liver diseases (Charlton et al., 2001). The late diagnosis of HS is associated with a worse prognosis and HS is the most frequent cause of chronic liver diseases in Western countries (Rector, Thyfault, Wei, & Ibdah, 2008). For these reasons, many efforts have been diverted to the investigation of mechanisms involved in the pathogenesis of HS and the search for new therapeutic targets. Besides, several drugs which induce hepatotoxicity by impairing the FA oxidation can cause HS (Berson et al., 1998; Kennedy, Unger, & Horowitz, 1996; Natarajan, Eapen, Pullimood, & Balasubramanian, 2006; Ulrich et al., 1998).

Much of what is currently known about disturbances on liver FA metabolism derived from studies performed in rodents. Several techniques have been used to evaluate the HS in these animals, including histological analyses like the techniques for lipid staining, commonly employed to reveal the accumulation of lipid in the cells, but little inform about its pathogenesis. TG in the liver can originate from plasma albumin-bound FA and also from *de novo* production (Fungwe, Cagen, Wilcox, & Heimberg, 1992). These processes are controlled by hormones as insulin and glucagon through regulation of transcription factors and a specific set of genes (Horton, Goldstein, & Brown, 2002; Paquette, Wang, Jankowski, Gutkowska, & Lavoie, 2008). The measurements of gene expressions by qPCR for mRNA detection and Western blotting for protein levels have been amply used to understand the molecular mechanism implicated in HS (Paquette et al., 2008). On the other hand, measurements of the liver lipid content, the FA oxidation capacity of isolated mitochondria and peroxisomes, and the activities of lipogenic enzymes have proven to be important tools for the assessment of the liver FA metabolism and should not be overlooked in the studies of the pathogenesis of HS.

Regarding the liver lipid content, there are several procedures described to extract lipids from biological materials and they frequently have in common the utilization of mixtures of polar and non-polar organic solvents. Here, we present two modified protocols of classical techniques, the Folch's method originally described to extract lipids from animal tissues (Folch, Lees, & Sloane Stanley, 1957) and the Srivastava's method designated to extract lipids from human skeletal muscle tissue (Kumar Srivastava, Pradhan, Mittal, Kumar, & Nagana Gowda, 2006). Both are based on the use of mixtures of methanol: chloroform and, after some modifications, have been used in liver samples in our laboratory, leading to reproductive and reliable results and allowing not only to determine the total amount of lipids, but also the quantifications of TG and cholesterol.

Simple protocols for the isolation of mitochondrial and peroxisomal subcellular fractions, from a single liver, are presented for measuring the hepatic capacity of FA oxidation. In mitochondria, the β-oxidation capacity was assessed by polarographic measurements of oxygen consumption, in a closed incubation system and in peroxisomes, by fluorimetric measurements, based on the formation of a fluorescent probe sensitive to hydrogen peroxide.

Once having assessed these two pathways of liver lipid disposal, it is equally important to investigate the existence of alterations in the lipogenic enzyme activities. This importance is highlighted by studies performed with animal models of obesity associated with HS that revealed that in some instances, (Campos et al., 2012; Garcia-Ruiz et al., 2006; Perez-Carreras et al., 2003; Rector et al., 2010) but not always (Brady, Brady, Romsos, & Hoppel, 1985; Lazarin Mde et al., 2011; Lee et al., 2001; Noland et al., 2007; Riu, Bosch, & Valera, 1996; Sanyal et al., 2001; Turner et al., 2007), the liver lipid accumulation is associated with a decreased capacity of mitochondrial or peroxisomal β-oxidation. Thus, in some conditions, the liver FA metabolism is diverted to the synthesis, thereby favoring the liver TG accumulation. This appears to be the case of the HS associated with estrogen deficiency, for example (D'Eon et al., 2005; Paquette et al., 2008; Volzke et al., 2007).

Here, we present two protocols frequently used to measure the Fatty Acid Synthase (FAS) activity and we compared the influence of the previous nutritional state of the animals on the enzyme activity. In addition to FAS, the Glucose-6-Phosphate Dehydrogenase (G6PD) activity was also measured, in fed rats. The protocols currently used to measure G6PD activity differ with respect to the substrates used – glucose 6-phosphate alone or in the presence of 6-phosphogluconate – and reasoning was done, also with this respect. As a whole, the methods allow a complete preliminary investigation of liver FA metabolism disturbances and represent an important tool to guide studies involving HS.

Material and methods

Material and reagents

The following reagents were purchased from Sigma Chemical Co. (St. Louis, USA): ATP, ADP, phenylmethylsulfonyl fluoride (PMSF), and 2,4-dinitrophenol (DNP). All the other reagents were of the best available grade. The apparatus for mitochondrial incubation was manufactured at the University of Maringá.

Animals

Thirty male Wistar albino rats (195 – 230 g) obtained from the Central Animal House of the *Universidade Estadual de Maringá* were used for the study. They were kept in rat cages in well-ventilated room, temperature of 27–30°C, 12 $12h^{-1}$ light/dark cycles, with free access to tap water and dry rat pellet (Nuvilab®, São Paulo, Brazil). All experiments were conducted in strict adherence to the guidelines of the Ethics Committee for Animal Experimentation of University of Maringá (statement 050/2011).

Lipid extraction according to the Folch method

Pieces of liver weighing 150-200 mg were transferred to 2 mL Eppendorf tubes and stored in a freezer (-80°C). In the day of the experiments, volumes of cold methanol were added (1.0 mL 150 mg^{-1} liver) and the samples were homogenized using a bead-beating tissue homogenizer, during one or more cycles of 1 minute. In this system, the tissue was homogenized by adding one steel ball (4 mm diameter) inside the tube and vortex agitation (Figure 1A). The homogenates were kept on ice and volumes of 1.0 mL were transferred to glass tubes maintained sealed with rubber caps. After adding more 330 μL methanol and 2.66 mL chloroform, the samples were vortexed for 15 seconds and allowed to rest at 4°C for a period of 4 hours. Afterwards, volumes of 1.6 mL cold saline (0.9% NaCl) were added to the tubes, which were capped again and the mixtures were vortexed for another 15 seconds. After uncapping the tubes, the samples were centrifuged at 13,250 g at 4°C for 15 minutes. This procedure led to the complete separation of a lower organic phase and an upper aqueous phase, and between them, a compact, wafer-shaped layer was formed (Figure 1B). The upper phase was removed by vacuum aspiration until reaching the interphase. The compact interphase was displaced easily, volumes of 2.0 mL of the lower phase were collected (Figure 1C) and transferred to previously weighed glass tubes, which were handled always using surgical gloves. Chloroform was evaporated with nitrogen gas at room temperature. This step was time-consuming, depending on the number of samples, since each sample required approximately 30-40 min. of drying time. The use of a multiport evaporating system speeds up this process greatly. After dried, each tube was weighed again, and the total lipid content was calculated by subtracting the final weight from the initial one. The results were expressed as grams of lipids 100 $grams^{-1}$ of liver wet weight. Then, the samples were dissolved in 2% Triton X-100 (100 uL) and vortexed for TG and cholesterol measurements, using commercial kits and following the instructions of the manufacturer.

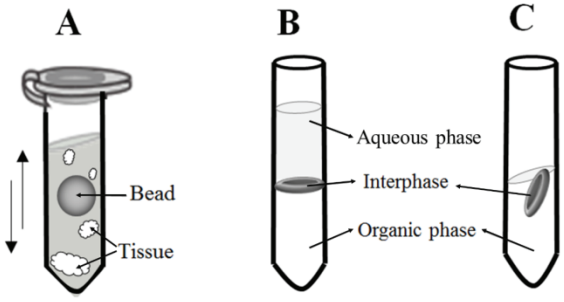

Figure 1. Liver lipid extraction by gravimetry.

Lipid extraction according to the Srivastava method

The Srivastava method is similar to the previous one. The same precautions regarding the temperatures and sealing of the tubes were taken. However, in this method, the ratio methanol: chloroform was 1:3 and sonication steps were included. For this method, pieces of 100 mg of rat liver were transferred to Eppendorf tubes and stored in a freezer (-80°C). In the day of the experiments, after adding 1.0 mL cold methanol, the samples were homogenized using the same bead-beating system and kept on ice. Volumes of 0.5 mL homogenate were transferred to glass tubes with 0.5 mL cold methanol and kept closed. Samples were vortexed thoroughly for 30 seconds and sonicated in a sonication bath (with ice), for 45 minutes. After adding 3 mL chloroform, samples were vortexed and kept at 4°C for 4 hours. Then, the vortexing and sonication steps were repeated, volumes of 2 mL saline were added and the mixtures were vortexed again. Samples were centrifuged at 13,250 g at 4°C for 15 minutes. Two phases were formed, with a similar, compact interphase. Volumes of 2 mL of the lower phase were collected and transferred to previously weighed glass tubes. Chloroform was evaporated with nitrogen gas and the tubes were weighed again to determine the amount of lipids. The results were also expressed as grams of lipids 100 $grams^{-1}$ of liver wet weight and after weighing the tubes, the lipid samples were dissolved in 100 μL

2% Triton X-100, by vortexing and sonicating and immediately used for TG and cholesterol measurements, using commercial kits. In some cases, it would be preferable to express the lipid contents per mg of protein. In this case, the liver protein content could be measured in the initial homogenate, before adding the chloroform.

Isolation of mitochondria and peroxisomes

For isolation of the mitochondrial fraction, the liver was removed, minced and homogenized in a Dounce homogenizer in 8 volumes of a cold medium containing 200 mM mannitol, 76 mM sucrose, 2 mM Tris, 0.2 mM EGTA and 0.1 mM PMSF, pH 7.4. The resulting homogenate was filtered through two layers of gauze, separated in two tubes (approximately 50 mL/tube) and centrifuged at 600 g at 4°C for 10 min. The supernatant was subjected to a second centrifugation at 7,080 g for 10 min. to precipitate the mitochondrial fraction. After two cycles of washing, resuspension and centrifugation of the pellet at 6,392 g for 10 min., the mitochondria were resuspended in the same medium until a final protein concentration of about 40-50 mg mL^{-1} (Bracht, Ihii-Iwamoto, & Salgueiro-Pagadigorria, 2003).

For isolation of peroxisomes, the Natarajan method (Natarajan et al., 2006) was adapted and this fraction was obtained from the same liver, through the centrifugation of the post-mitochondrial supernatants at 15,000 g for 5 min., to avoid contamination by mitochondria, and then at 39,000 g for 10 min. for the precipitation of the fraction containing peroxisomes, which was resuspended and homogenized with Dounce homogenizer in a cold medium containing 250 mM sucrose, 1 mM EDTA, 0.1 mM PMSF and 10 mM Tris, pH 7.3. This suspension was centrifuged again at 15,000 g for 10 min. to remove any remaining mitochondrial contamination. Thereafter, the supernatant was centrifuged at 39,000 g for 10 min., to obtain the purified peroxisomal fraction, which was resuspended and homogenized to a final protein concentration of about 20 mg mL^{-1}.

Determination of the mitochondrial β-oxidation capacity

Here, we present a simple method for measuring the liver mitochondrial β-oxidation by the amounts of oxygen consumed by intact mitochondria oxidizing FA, through polarographic measurements, using a Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH, USA). The mitochondrial incubation system (Figure 3, Panel A) consists of an acrylic chamber, sealed with a lid containing a central orifice through which the substrates and mitochondria (0.6-1.2 mg mL^{-1}) can be added.

Volumes of 2.0 mL of the incubation medium, containing 2.0 mM monobasic potassium phosphate, 10 mM HEPES, 0.1 mM EGTA, 130 mM potassium chloride, 5 mM magnesium chloride, 0.1 mM 2,4-dinitrophenol, 2.5 mM L-malate and 50 mg% FA-free bovine-serum albumin, pH 7.2 (Garland, Shepherd, Nicholls, & Ontko, 1968), were added to the central chamber and maintained under constant agitation by a magnetic bar and heated at 37°C by circulation of water through an external chamber. The oxygen concentration in the incubation medium was monitored by an oxygen electrode connected to this system. After the addition of the mitochondria-enriched fraction (0.3 mg protein mL^{-1}), the reactions were initiated by the addition of 20 μM octanoyl-CoA, 20 μM palmitoyl-CoA or 20 μM stearoyl-CoA, each one in the presence of 2.0 mM L-carnitine or 20 μM palmitoyl-L-carnitine. In these experiments, β-oxidation rates were measured in the presence of DNP because, under these conditions, the rate of β-oxidation is no longer retroactively controlled by the mitochondrial transmembrane potential and occurs at its maximum rate. The use of octanoyl-CoA and palmitoyl-L-carnitine, on the other hand, circumvents the step requiring CPT-I for the oxidation of these FA, and the rate of oxidation becomes exclusively dependent on the activity of the enzymes present in the mitochondrial matrix. Considering that, at 37°C, the oxygen is dissolved in water at concentrations of 190 µM, the rate of oxygen consumption was calculated and expressed as nmol/min.mg of mitochondrial protein.

Determination of the peroxisomal β-oxidation capacity

The peroxisomal fatty acyl-CoA oxidase activities were measured fluorimetrically using a modification (Taguchi, Ogura, Takanashi, Hashizoe, & Honda, 1996) of the Small method (Small, Burdett, & Connock, 1985). The assay was based on the determination of H_2O_2 production in a reaction catalyzed by exogenous peroxidase, which was coupled to the oxidation of DCFH-DA into the highly fluorescent compound DCF. The DCFH-DA was prepared daily in ethanol at a concentration of 5mM. Volumes of 2 mL of a medium containing 11 mM monobasic potassium phosphate, pH 7.4, 40 mM aminotriazole, 0.02% Triton X-100 and 150 µg mL^{-1} horseradish peroxidase, were added to a cuvette. The reaction was carried out at 30°C and under constant agitation. After the addition of the

peroxisome-enriched fraction (0.3 mg protein mL^{-1}), this mixture was pre-incubated in the dark for 5 min. to allow the aminotriazole to inhibit the catalase activity. DCFH-DA was added at a final concentration of 15 µM and incubated again for more 3 min., considering that some impurities in the peroxidase cause a small amount of oxidation of DCFH-DA. After this time, there was a slow rate of auto-oxidation and the reaction was initiated by addition of the substrate palmitoyl-CoA (final concentration of 30 μM). The enzyme activity was monitored in real time by recording the variation in fluorescence (excitation 503 nm; emission 529 nm) over a period of 10 minutes. Rates were then corrected for substrate blank. The activity of fatty acyl-CoA oxidase was expressed as pmol DCF produced min. mg^{-1} of peroxisomal protein. DCF formation was quantified from data of a standard curve over the range of 0.0 to 1 nmol prepared in TRIS buffer 0.1 M pH 8.0. The fluorescence emitted by each concentration was measured (excitation 503 nm; emission 529 nm).

Determination of the fatty acid synthase (FAS) activity

The expression and activity of FAS are profoundly decreased in fasted animals (Kelley, Nelson, & Hunt, 1986; Moustaid, Beyer, & Sul, 1994). For this reason, the activity of FAS was measured using livers from fed or refed rats. For the latter, the animals were fasted for 48 hours, starting at 8:00 am, and had free access to food for the next 48 hours before the experiment. After removed, the livers were homogenized with Dounce in 5 volumes of 0.1 M potassium phosphate buffer, pH 7.4 and the homogenate was filtered through gauze, centrifuged at 30,000 g for 30 minutes and the supernatant was used as FAS source.

In addition to varying the nutritional status of the animals (fed or refed), two techniques for measurement of FAS activity from rat liver were compared (Kelley et al., 1986; Nepokroeff, Lakshmanan, & Porter, 1975). These two spectrophotometric methods measure the malonyl-CoA- and acetyl-CoA-dependent rate of oxidation of NADPH at 340 nm and can be used for purified enzymes or crude preparations. However, in these crude preparations, a blank was run in parallel to make the appropriate corrections, as described later.

FAS, according to Nepokroeff method

FAS activity was measured by the incubation of 50 μL of the 30,000 g supernatant (or approximately 0.6 mg protein) in a reaction mixture containing 0.5 mM Potassium Phosphate Buffer, pH 7.0, 33 μM acetyl-CoA, 100 μM NADPH, 1 μM EDTA and 1 μM β-mercaptoethanol. After a pre-incubation period of 5 minutes at 30°C, in order to obtain maximum enzymatic activity, the reaction was started by the addition of 100 μM malonyl-CoA. The final volume was 1.0 mL.

The oxidation of NADPH was followed at 340 nm for 10 minutes, while the cuvette chamber was maintained at 30°C. Full-scale detection of the recorder tracing was set at 0.2 absorbance unit. A control, without malonyl-CoA was run in parallel to discount the spontaneous oxidation of NADPH or its oxidation by other enzymes. The results were expressed as NADPH oxidized min. mg^{-1} protein using the molar extinction coefficient, ε=6.22 mM^{-1} cm^{-1}.

FAS, according to Kelley method

FAS activity was measured by the incubation of 50 μL of the 30,000 g supernatant (or approximately 0.6 mg of protein) in a reaction mixture containing 0.5 mM Potassium Phosphate Buffer, pH 7.0, 66 μM acetyl-CoA, 200 μM malonyl CoA and 200 μM NADPH (final volume of 1.0 mL). Here, the substrates were pre-incubated and the reaction was started by adding the enzyme. The oxidation of NADPH was followed at 340 nm for 10 minutes at 30° C. Full-scale detection of the recorder tracing was set at 0.2 absorbance unit. A blank without malonyl-CoA was run in parallel. The results were expressed as NADPH oxidized $min.^{-1}$ mg protein using the molar extinction coefficient, ε=6.22 mM^{-1} cm^{-1}.

Determination of the glucose 6-phophate dehydrogenase (G6PD) activity

To measure the G6PD activity, the livers from fed and fasted rats were homogenized with Dounce in 5 volumes of a medium containing 0.1M Tris/HCl and 1 mM EDTA, pH 7.6. The homogenate was filtered through gauze and centrifuged at 13,000 g for 15 minutes and the supernatant was used as enzyme source (Tian, Pignatare, & Stanton, 1994).

The activity of the enzyme G6PD was measured in volumes of 50 μL supernatant (0.2 mg protein mL^{-1}). Samples were added to a cuvette containing buffer (50 mM TRIS 1.0 mM $MgCl_2$, pH 8.1), final volume of 1.0 mL. The enzyme activity was determined spectrophotometrically by measuring the rate of increase in absorbance at 340 nm, due the production of NADPH from $NADP^+$, over a 5-min. registration time (Tian et al., 1998). G6PD catalyzes the conversion of glucose 6-phophate (G6P) to 6-phosphogluconolactone, which is rapidly hydrolyzed to 6-phosphogluconate (6PG),

the substrate for the 6-phosphogluconate dehydrogenase (6PGD), the second enzyme in the pentose phosphate pathway. That is why the activity of G6PD was determined by subtracting de production of NADPH in the presence of 6PG from that in the presence of both substrates (G6P + 6PG). The accuracy of this calculated activity was evaluated by means of the performance of three different series of experiments: in the presence of 0.2 mM G6P alone or in the presence of 0.2 mM 6PG. In another series of experiments, only 0.2 mM 6PG was added. Substrates were added to the cuvette and afterwards, 0.1 mM of $NADP^+$ was added. The molar extinction coefficient of NADPH (ε= 6.22 mM^{-1} cm^{-1}) was used and the results were expressed as NADPH produced $min.^{-1}$ mg protein.

Results

Fat liver accumulation can be easily assessed by extraction and measurement of lipids. Figure 2 presents the results of total liver lipid content, determined according by Folch's and Srivastava's methods (Panel A), as well as the quantification of TG (Panel B) and cholesterol (Panel C) in the lipids extracted from the livers through these methods. These two methods did not differ in their capacity of extracting total lipid content, which in both reached values of approximately 5%. However, when measuring the TG and cholesterol in these extracted lipids, the Srivastava's method was more effective and detected an amount approximately 60% higher than those extracts according to the Folch's method.

To investigate the liver fatty acid disposal, the peroxisomal and mitochondrial β-oxidation capacities were evaluated. Figure 3 illustrates the isolated mitochondria incubation system (Figure 3A) and the results for the capacities to oxidize octanoate, palmitate and stearate (Figure 3B). These fatty acids were utilized as the acyl-CoA derivatives in the presence of L-carnitine. In another series of experiments, palmitoyl-L-carnitine was used. Under these conditions, octanoyl-CoA and stearoyl-CoA exhibited similar and the lowest rates of oxidation. These were followed by the palmitoyl-CoA and palmitoyl-L-carnitine, which presented rates about 23 and 41% higher than the former.

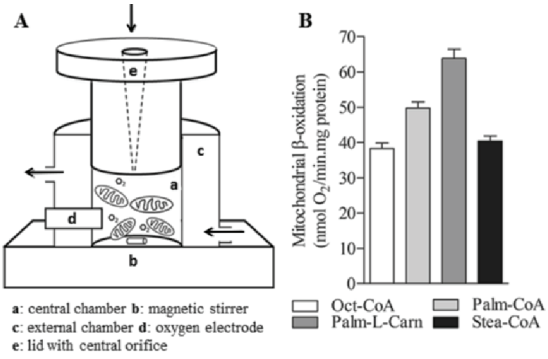

Figure 3. Mitochondrial β-oxidation.

The peroxisomal capacity of oxidation of palmitoyl-CoA was measured fluorimetrically over a 10-min. observation period and the results are presented in Figure 4. The time courses of increases in fluorescence due the β-oxidation of the palmitoyl-CoA and auto-oxidation of DCFH-DA are shown in Figure 4A, as well as the standard curve of DCF obtained by varying the concentrations from 50 to 1,000 nM, which was used to determine the DCF coefficient calibration (Figure 4B). This coefficient was 0.6 and was used to calculate the rate of peroxisomal β-oxidation in pmol DCF produced $min.^{-1}$ mg of protein (Figure 4C).

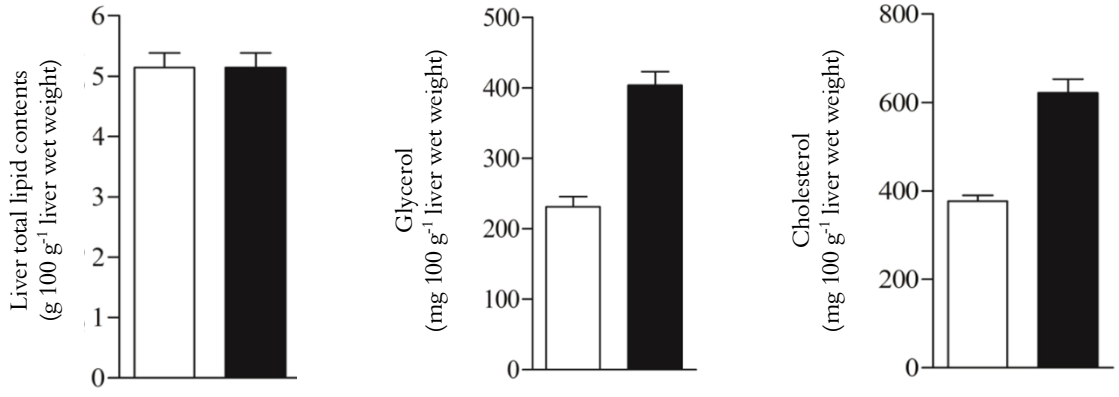

Figure 2. Liver lipid content.

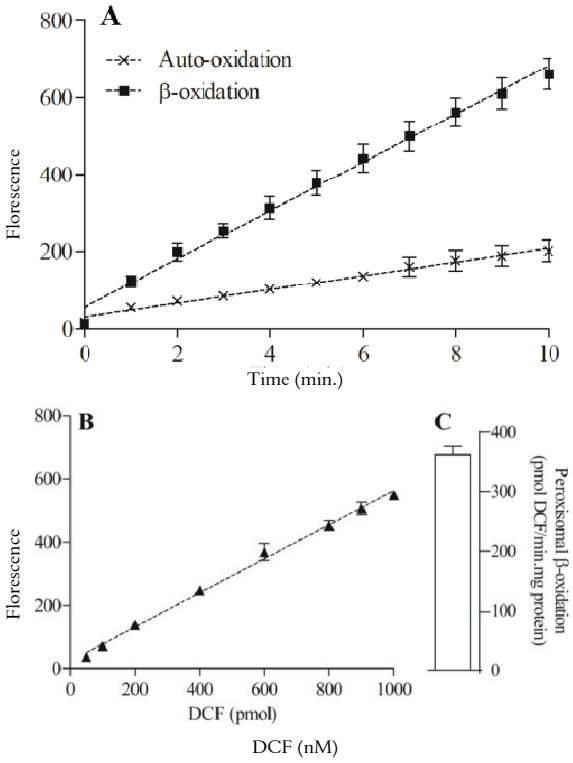

Figure 4. Peroxisomal β-oxidation

The results of the FAS activity measurements obtained by using two different protocols (Nepokroeff and Kelley methods) and in two nutritional conditions, fed and refed rats, are presented in Figure 5. As can be seen, these protocols yielded two similar results and, in both, the FAS activity was higher in refed animals, by approximately 75%.

The G6PD activities were assessed in livers from fed rats and the results are shown in Figure 6. Figure 6A are shows the time courses of NADPH production after the addition of G6P and 6PG separately or in combination. The difference between the amounts of NADPH produced in the presence of G6P + 6PG and those obtained only in the presence of 6PG, attributable to the catalytic activity of G6PD, is also presented. In Figure 6B, the rates of NADPH production in these four conditions are shown. As can be seen, when only G6P was used as substrate, the amounts of NADPH produced were about twice higher than those obtained by subtracting the NADPH produced in the presence of 6PG from those in the presence of both substrates. Similar experiments were conducted in livers of fasted rats, but as they yielded similar results, they are not shown.

Discussion

The first step to evaluate the liver FA metabolism is a measurement of the liver lipid content. Therefore, in this study, we presented two modified protocols of classical techniques for liver lipid extraction. In both, methanol: chloroform mixtures were used, given their ability to dissolve lipids and efficiently disrupt cell membranes. Despite of the disadvantage of using chloroform, which is denser than water and will remain at the bottom of the tube, these techniques have proven to be more reliable and to conduct to more reproducible results than others which use ternary mixtures of hexane- or heptane-isopropanol-water. The use of these solvents has the advantage of being less toxic and the preferable distribution of the non-polar phase containing the lipids on the top, which facilitates its collection. However, this method is relatively less efficient and more suitable for extraction of fatty acids from plasma or tissues with low lipid content (Dole, 1956; Dole & Meinertz, 1960; Hara & Radin, 1978; Li, Yang, Bai, & Liu, 2014).

Although the Folch method has been sometimes the target of criticism, it leads to reliable results and, in addition, has the advantages of speediness, low costs and reproducibility. In fact, it conducts to results similar to those obtained by the Srivastava method, as demonstrated in this study. Nevertheless, the technique of Folch is simpler and more adequate to measure total lipids, which could be enough in many cases. When one intends to evaluate the contents of TG and cholesterol separately, it is preferable to use the Sirvastava method, as revealed by the comparison of the results obtained by the two methods with samples from the same liver.

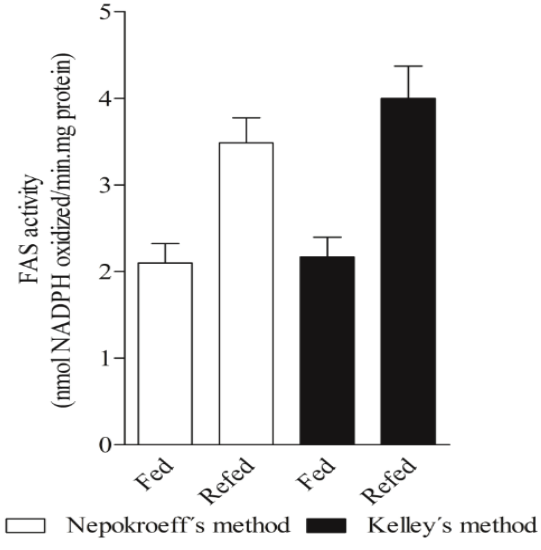

Figure 5. FAS activity.

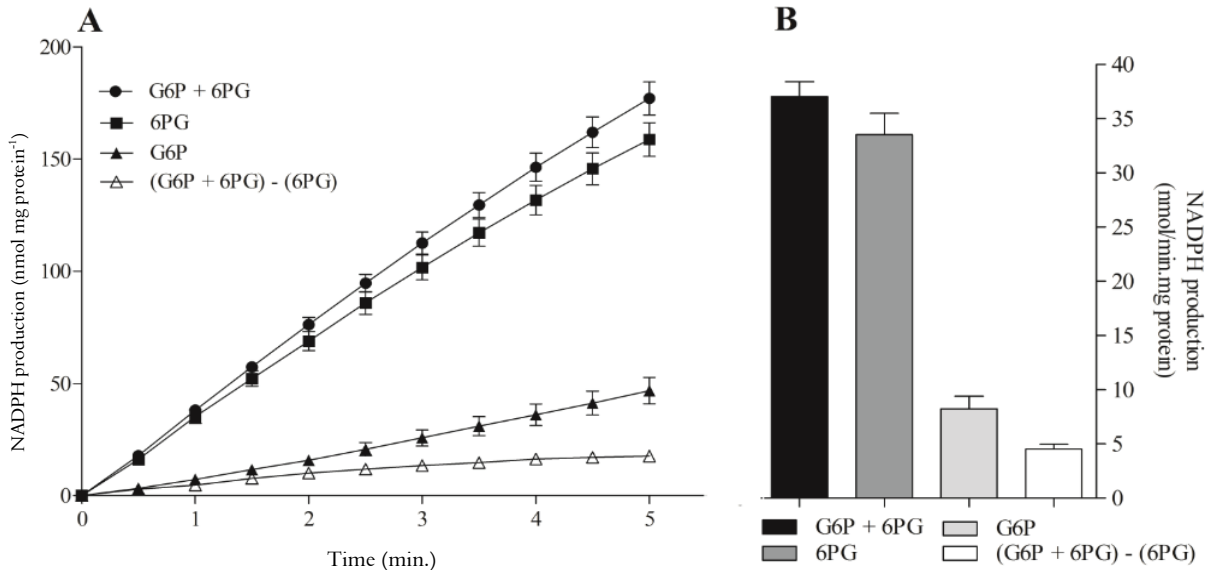

Figure 6. G6PD activity.

When the mechanisms by which drugs, diets or hormonal disorders induce HS are the major aim, it is also essential to evaluate the hepatic ability of eliminating fatty acids by the major oxidative pathways, mitochondrial and peroxisomal as well as the channeling of fatty acids for β-oxidation between these two organelles (De Craemer, Pauwels, & Van den Branden, 1995; Noland et al., 2007). From the same rat liver homogenate, mitochondrial- and peroxisomal-enriched fractions were isolated by differential centrifugation. The incubation of mitochondria with the substrates in the presence of DNP allowed the β-oxidation to occur at its maximum rate. The use of medium-chain octanoyl-CoA and long-chain palmitoyl-L-carnitine, which freely enter into mitochondria, without requiring CPT-I and other proteins involved in the carnitine shuttle (Fromenty & Pessayre, 1995; McGarry, Mills, Long, & Foster, 1983), makes it possible to evaluate the activities of the medium- and long-chain fatty acid dehydrogenases. When comparing the mitochondrial capacity of oxidizing palmitoyl-CoA and palmitoyl-L-carnitine, on the other hand, the rate-limiting step of the oxidation of this long-chain FA, *i.e*, the entry of palmitoyl-CoA via CPT-I, could be evaluated (Noland et al., 2007).

On the extreme opposite of the FA oxidation, the liver also functions as the major site for the synthesis of endogenous lipids, which are stored in the cytosol of hepatocytes or secreted into the blood as lipoproteins (Geelen, Harris, Beynen, & McCune, 1980). All the excess of energy from carbohydrates and proteins are stored mainly as TG and consequently the lipogenic activity of the liver is profoundly influenced by the nutritional and hormonal status of the animal. The rate of lipid synthesis and the activity of lipogenic enzymes are decreased during starvation or intake of a fat-rich diet, whereas re-feeding of a fat-free carbohydrate-rich diet to starved animals markedly enhances the rate of lipid synthesis and the activity of lipogenic enzymes (Geelen et al., 1980; Herzberg, 1983; Moustaid et al., 1994; Wakil, Stoops, & Joshi, 1983).

There are several studies in which the total capacity of fatty acid synthesis was measured in isolated hepatocytes, by providing lactate or acetate as lipogenic substrates (Beynen, Buechler, Van der Molen, & Geelen, 1982; Caro & Amatruda, 1981; 1982; Clark, Rognstad, & Katz, 1974). However, these techniques involve the isolation of primary hepatocytes, the use of ^{14}C- or ^{3}H-labeled substrates and lipid extraction, which implies in high costs and difficulty in reproduction. Furthermore, in these methods, the liver total lipogenic capacity is measured and then the results are not conclusive. Therefore, these methods could be used as preliminary, but for a better understanding of the FA metabolism, a direct assessment of the lipogenic enzyme activities should be conducted.

In this study, we reviewed techniques of measurement of the activities of the two most important enzymes involved in liver lipogenesis, namely, FAS and G6PD, in the supernatants of crude liver homogenates. Fatty acid synthase (FAS) catalyzes the biosynthesis of saturated fatty acids from simple precursors (*de novo* lipogenesis). The primary product of the FAS reaction is palmitate (C16:0), but stearate (C18:0) and shorter fatty acids

may also be produced. FAS substrates are acetyl-CoA, which functions as a primer for the reaction, and NADPH, which provides reducing equivalents. The fatty acid is elongated from the initial acetyl-CoA by repeated condensations with malonyl-CoA, the third substrate, which donates two carbons in each cycle of condensation. Thus, palmitate synthesis requires seven cycles of malonyl-CoA addition to an acetyl-CoA primer to yield a saturated, 16-carbon fatty acid (Burton, Haavik, & Porter, 1968).

The liver FAS activity was assessed using two different protocols (Kelley et al., 1986; Nepokroeff et al., 1975). Moreover, as the activity and expression of this enzyme are affected by the nutritional status of the animal, these assays were performed in rats under two alimentary conditions: fed and refed, that is, after one cycle of starvation-refeeding. The results demonstrated that, in refed rats, the FAS activity increased approximately twofold in the liver, irrespectively of the method used (Kelley's or Nepokroeff's) and this is in agreement with previous reports (Kochan, Karbowska, & Swierczynski, 1997). Those authors also tested whether by increasing the number of the starvation-refeding cycles, the activity of FAS was further increased. But even after eight of such cycles no changes were observed in the activity of this enzyme.

Conclusion

Our results also demonstrated that there was no difference in the activity of FAS measurements by using Kelley's or Nepokroeff's. Hence, the difference of these two methods lies on the costs. By using half the amount of substrate, malonyl-CoA and acetyl-CoA, the Nepokroeff's method is much less expensive.

The assays in which the activity of the G6PD was evaluated, either by the NADPH produced after the addition of G6P or by subtracting the NADPH produced in the presence of 6PG from that produced in the presence of both substrates (G6P + 6PG), revealed that the former yielded higher values (approximately 50%). And this makes sense, since around half of the NADPH produced, in this condition, is due to the activity of 6PGD. However, is also important to consider that the rate of NADPH production by the 6PGD under this condition is limited by the supply of 6PG by G6PD. Following this rationale, when one intends to investigate possible changes in the activity of G6PD performing the experiment by adding only G6P as substrate, the NADPH production still reflect indirectly the activity of G6PD, which is a rate-limiting enzyme of the pentose phosphate pathway.

Overall, the techniques described here will be useful as a guide to perform preliminary studies on the liver FA metabolism from which a hypothesis could be elaborated to guide more specific investigations in the search for the mechanisms involved in liver FA metabolism disorders.

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