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Measurement of AMPK-induced inhibition of lipid synthesis flux in cultured cells

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Running head: Measurement of de novo lipogenesis

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Abstract

AMP-activated protein kinase (AMPK) is a master regulator of multiple cellular metabolic pathways, including lipid metabolism. One of the well-known substrates of AMPK are acetyl CoA carboxylase (ACC) and 3-hydroxy-3-methylglutaryl-coenzyme A (HMGCoA) reductase, regulatory enzymes of fatty acid and cholesterol synthesis, respectively. The discovery that both of them are inactivated by AMPK suggested the therapeutic potential of AMPK activation in the treatment of metabolic diseases associated with lipid disorders, such as non-alcoholic fatty liver disease (NAFLD). Here we describe a method to measure lipid synthesis flux in intact cells from the saponifiable (including fatty acids) and non-saponifiable (including sterols) fractions of lipid extracts.

Key words: AMP-activated protein kinase, lipogenesis, lipid synthesis, fatty acid synthesis, sterol synthesis, indirect AMPK activators, small molecule AMPK activators, metformin. primary hepatocytes, AMPK knockout

1- Introduction

The regulation of cellular energy metabolism by AMP-activated protein kinase (AMPK) activation is achieved by acute phosphorylation of key enzymes in carbohydrate, lipid and protein metabolism [1]. In the liver, AMPK activation influences several downstream cellular targets involved in fatty acid and cholesterol synthesis. Acetyl-CoA carboxylase (ACC) and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), key enzymes in fatty acid and cholesterol synthesis, respectively, were the first two enzymes shown to be phosphorylated and inhibited by AMPK [2,3]. AMPK inhibits hepatic fatty acid synthesis through the phosphorylation of ACC1 at Ser79, leading to its inactivation and thus determining cytosolic concentrations of the reaction product malonyl-CoA, a key precursor for the generation of new fatty acids [4]. Given these properties, it is not surprising that AMPK has emerged as an attractive therapeutic target for pathological conditions characterized by abnormal hepatic lipid accumulation, such as non-alcoholic fatty liver disease (NAFLD) [5]. Interestingly, recent studies also challenged the therapeutic potential for AMPK activation to limit tumour cell growth and restrict viral replication by inhibiting the cellular lipid accumulation [6-8]. We and others have demonstrated that single and dual treatment with indirect and direct AMPK activators increases ACC phosphorylation leading to the inhibition of de novo lipogenesis in primary hepatocytes [9-14]. Here, we describe a detailed protocol for testing the potential of various pharmaceuticals/nutraceuticals compounds on lipid metabolic flux in cultured cells. The quantitative evaluation of lipid synthesis flux is realized by the use of radiolabeled [1-¹⁴C]-acetate tracer and monitoring the amount of radioactivity into the lipid fractions. Analysis of the saponifiable and non-saponifiable fractions of lipid extracts enables the quantification of fatty acids and sterols synthesis flux, respectively.

2- Materials

2.1.. Cell culture

1. Mouse primary hepatocytes. (see **Note 1**).
2. Cell culture plasticware (6 well plates)
3. Hepatocyte culture medium: Medium 199, 100 nM Dexamethasone, 100 units/mL penicillin, 100 µg/mL streptomycin. Store at 4°C. (see **Notes 2 and 3**).
4. [1-¹⁴C]-acetic acid, sodium salt, 1 mCi/mL (37 MBq/mL), 45-60 mCi/mmol. Store at 4°C. (see **Note 4**).
5. DMSO (Dimethyl sulfoxide). (see **Note 5**).
6. TOFA (5-tetradecyloxy-2-furoic acid).stock solution: 10 mM in DMSO. Store at -20°C. (see **Note 6**).
7. Metformin, HCl stock solution: 500 mM in sterile distilled H₂O. Store at -20°C. (see **Note 7**).
8. Compound 991 stock solution: 10 mM in DMSO. Store at -20°C. (see **Note 8**).
9. 1x Phosphate-buffered saline (PBS): 140 mM NaCl, 2.6 mM KCl; 2 mM Na₂HPO₄, 1.45 mM KH₂PO₄.
10. Cell scrapers.
11. Humidified 5% CO₂ incubator at 37°C.

2.2. Lipid extraction and ¹⁴C-labelled lipid counting

1. 13 × 100 mm borosilicate glass tubes.
2. Polytetrafluoroethylene (PTFE)-lined caps.
3. 40% (w/v) KOH in degassed distilled H₂O (see **Note 9**).
4. Methanol.
5. Water-saturated petroleum ether (boiling point at 40-60°C) (see **Note 10**).

6. Bromophenol blue stock solution: 0.2 % (w/v) in distilled H₂O.
7. Hydrochloric acid (HCl) fuming 37% (12 N). (see **Note 11**).
8. 5 mL polyethylene pipette dropper.
9. Vortex
10. Oven at 80°C.
11. Chemical fume hood.
12. 20 mL liquid scintillation vials.
13. Liquid scintillation cocktail.
14. Radiometric beta counter.

3- Methods

3.1. Stimulation of cells

1. Mouse primary hepatocytes plated at a density of 0.4×10^6 cells/well in 6-well plates are cultured in 1.8 mL of serum-free hepatocyte culture medium at 37°C in an incubator providing saturated humidity and 5% CO₂. (see **Notes 1, 2 and 3**).
2. Prepare a 10-fold concentrated mix of radiolabelled tracer in M199 medium sufficient for the number of wells used. Use 1,2 μ L [¹⁴C]-acetic acid (1.2 μ Ci).with 198.8 μ L M199 medium per well. (see **Note 12**).
3. Add AMPK activators at various concentrations or equivalent volume of vehicle and 200 μ L of 10x tracer mix/well. Wells incubated with 20 μ M TOFA could serve as positive control of lipid synthesis inhibition. (see **Note 6**).
4. Incubate exactly for 3 hours at 37°C in an incubator providing saturated humidity and 5% CO₂.

3.2. Harvesting and lysing of the cells

1. Wash the cells 3 times with 2 mL of ice-cold PBS.
2. Harvest cells by gently scrapping wells in 0.5 mL of PBS.
3. Transfer to glass tubes containing 1 mL of 40% KOH and 2 mL of methanol.
4. Rinse wells with 0.5 mL PBS and combine. Cap the tubes and vortex for 1 minute.
5. Heat at 80°C for 1 hour then allow tubes to cool completely at room temperature.

3.3. Lipid extraction

3.3.1. Extraction of non saponifiable lipids (see **Notes 13 and 14**)

1. Add 3 mL of water-saturated petroleum ether in each tube. (see **Notes 15**).
2. Cap the tubes and vortex for 1 minute.
3. Centrifuge at 1000 x g for 2 minute to separate aqueous and organic phases (see **Note 16**).
4. Transfer the upper organic phase with a 5 mL pipette dropper to 20 mL scintillation vials.
5. Repeat extraction with 3 mL of water-saturated petroleum ether and combine the upper phases.
6. Evaporate to dryness the organic extract from scintillation vials under a chemical fume hood. (see **Note 17**).
7. Add 10 mL of organic scintillation fluid to the dried residues. Vortex thoroughly to dissolve lipids.
8. Count incorporation of ^{14}C into the non saponifiable fraction. (see **Note 18**).

3.3.2. Extraction of saponifiable lipids (see **Note 19**)

1. Add 50 μL of 2% bromophenol blue to the lower aqueous fraction from 3.3.1 **step 4**.

2. Perform saponification of lipids by acidifying to the lower aqueous fraction with 700 μ L of 37% HCl and vortex for 1 minute (see **Note 20**).
3. Add 3 mL of water-saturated petroleum ether in each tube. (see **Notes 15**).
4. Cap the tubes and vortex for 1 min.
5. Centrifuge at 1000 x g for 2 min to separate aqueous and organic phases (see **Note 16**).
6. Transfer the upper organic phase with a 5 mL pipette dropper to new 20 mL scintillation vials.
7. Repeat extraction with 3 mL water-saturated petroleum ether and combine the upper phases.
8. Evaporate to dryness the organic extract from scintillation vials under a chemical fume hood. (see **Note 17**).
9. Add 10 mL of organic scintillation fluid to the dried residues. Vortex thoroughly to dissolve lipids.
10. Count incorporation of ^{14}C into the saponifiable fraction. (see **Note 19**).
11. Determine specific activity by counting 50 μ L of labelled media. (see **Note 18**).

[Figure 1 near here]

4- Notes

1. The method for the measurement of lipid synthesis described here can be adapted to other cell types because lipid synthesis is active in most cultured cells. An important point to respect is the number of cells in each well since there is no internal control to normalize data. If the cells studied are available in small numbers, the protocol can be

adapted to 12- or 24-well plates. However, we found that the 6-well plate format give better results in term of reproducibility.

Measurement of [1-¹⁴C]-acetate incorporation into lipids is a very sensitive method to assess variations of lipid synthesis induced by AMPK activation or other stimuli.

When this measure is coupled with the use of AMPK-deficient cells such as AMPK α 1 α 2 KO hepatocytes [15], it is a powerful functional test to determine the AMPK dependence on lipid synthesis inhibition induced by various drugs or stimuli (**Figure 1**),.

2. Medium 199 contains 0.6 mM sodium acetate. If an alternative medium is used, supplement or adjust the sodium acetate concentration to 0.6 mM.
3. Dexamethasone is specifically used in primary culture of hepatocytes to limit dedifferentiation [16].
4. We use [1-¹⁴C]-acetic acid, sodium salt from Perkin Elmer (NEC084h001MC). Use of radioactive material requires a legal authorization and needs precautions such as designate and label areas for working with radioactive material, use of a chemical fume hood to handle radioactive sources and wear appropriate personal protective equipment. Radioactive waste requires a specific management.
5. In unstimulated cells, the vehicle (DMSO, ethanol, etc.) should be used as a control.
6. TOFA is a competitive inhibitor of acetyl-CoA carboxylase used as a positive control of lipid synthesis inhibition.
7. Metformin is an indirect AMPK activator which stimulates AMPK activity by increasing intracellular AMP/ATP ratio through inhibition of complex I of the mitochondrial respiratory chain [17,18].

8. Compound 991 is a small-molecule that directly activates AMPK through an AMP-independent mechanism by binding at the interface between the carbohydrate-binding domain of the regulatory β subunit and the catalytic α subunit [19].
9. Distilled H₂O is degassed under vacuum to remove CO₂ and to avoid the formation of K₂CO₃.
10. Water-saturated petroleum ether is prepared by mixing one volume of distilled H₂O with one volume of petroleum ether in a glass bottle. After shaking and decanting, water-saturated petroleum ether corresponds to the upper phase.
11. Concentrated HCl can be replaced by concentrated H₂SO₄ (12 N).
12. [1-¹⁴C]-labelled acetate concentration in medium is 0.6 $\mu\text{Ci}\cdot\text{mL}^{-1}$ and cold acetate concentration is 0.6 mM.
13. Non-saponifiable fraction corresponds to neutral lipids, predominantly sterols including cholesterol.
14. Extraction of the non-saponifiable fraction can be skipped (Section 3.3.1 steps 1 to 8) and acidification directly carries out. In this case, extracted lipids after saponification correspond to total lipids (sterols and fatty acids).
15. Petroleum ether is extremely flammable. Precautions should be taken in its handling and storing. Vapours are harmful to eyes, respiratory system and skin. Consequently, this solvent should be handling only under a chemical fume hood and proper personal protective should be wearing as chemical resistant gloves, laboratory coat and safety glasses.
16. Centrifugation may be optional because aqueous and organic phases can separate rapidly after a few minutes of decanting.
17. Evaporation of organic extracts in vials takes several hours. Usually, we keep the uncapped vials under a chemical fume hood overnight. Evaporation can be accelerated

through the use of an evaporator system which blows down a stream of dry air or nitrogen gas in the vials.

18. Counting are disintegrations per minute (dpm). The blank value obtained from wells without cells but treated as other wells is deducted for each sample. We recommend to perform each condition in triplicate for each experiment. Data are normalized to the protein amount per well. For this, reserve at least a 6-well plate of cells incubated without [1-¹⁴C]-labelled acetate to assess the amount of proteins per well. The results can be expressed as a percentage of basal (unstimulated condition) or in absolute values (nanomoles of [1-¹⁴C]-acetate incorporated/mg of proteins/hour) using the specific activity. Specific activity corresponds to the quantity of radioactivity (in dpm) per nmole of acetate in medium. Specific activity is calculated by dividing the number of total dpm measured per well (in 2 mL) by the number of nmoles of acetate per well ($0.60 \text{ mM} \times 2 \text{ mL} = 1220 \text{ nmoles}$, the amount of acetate provided by the tracer is negligible). The value of specific activity thus allows to convert the dpm count values deducted of blank in nmoles of [1-¹⁴C]-acetate incorporated in lipids. By dividing these data by the incubation time (3 hours) and the amount of proteins per well (mg), the results can be expressed in nmoles of [1-¹⁴C]-acetate incorporated in lipids/mg of proteins/hour.
19. Saponifiable fraction mainly corresponds to fatty acids.
20. pH indicator (blue bromophenol) should turn yellow to assure complete saponification.

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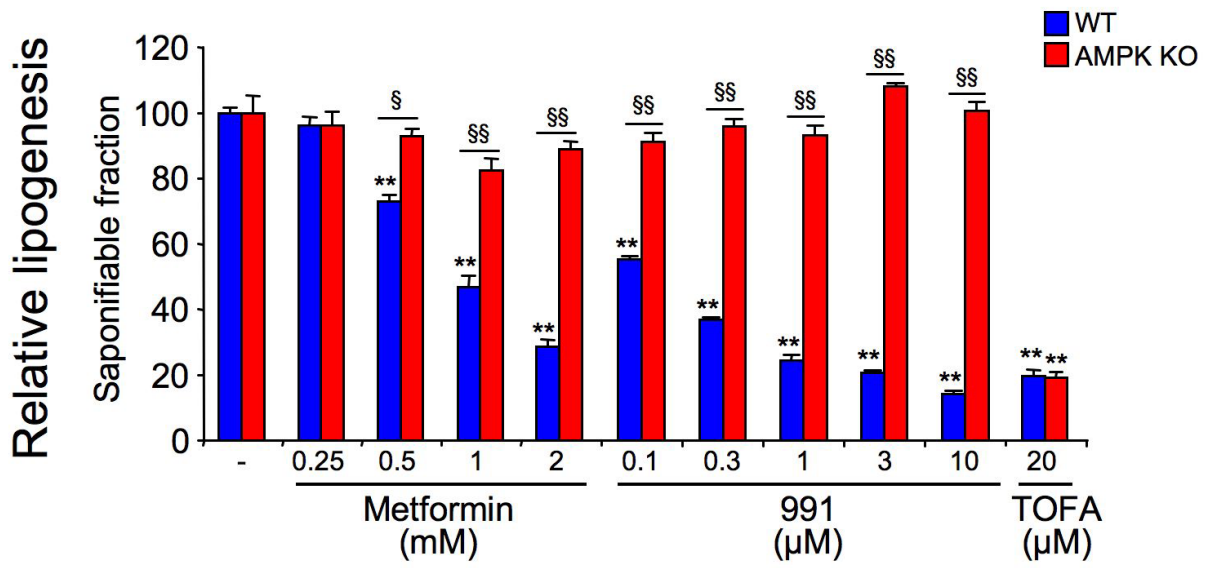
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Figure Caption

Figure 1. Effects of metformin and small-molecule 991 on lipid synthesis rates in WT and AMPK-deficient hepatocytes. After plating, WT and AMPK $\alpha 1 \alpha 2$ knockout primary hepatocytes were cultured for 16 hours in serum-free M199 medium containing antibiotics and 100 nM dexamethasone. Hepatocytes were then incubated for 3 hours in fresh medium in the absence or in the presence of increasing concentrations of metformin (0.25, 0.5, 1 or 2 mM), an indirect AMPK activator, or 991 (0.1, 0.3, 1, 3 or 10 μ M), a small-molecule direct activator of AMPK, or TOFA (20 μ M), a competitive inhibitor of acetyl-CoA carboxylase and [1- 14 C]-acetate tracer was added directly in medium. Rates of fatty acid and sterol synthesis were assessed from incorporation of [1- 14 C]-acetate into saponifiable (**A**) and non-saponifiable (**B**) lipids respectively. Results were normalized to protein content and presented as a percentage of [1- 14 C]-acetate incorporated in WT or AMPK KO hepatocytes incubated in the absence of compounds. Results are representative of three independent experiments. Data are mean \pm SEM. * $P < 0.05$, ** $P < 0.001$ compared with WT or AMPK KO hepatocytes incubated in the absence of compounds and $^{\S}P < 0.01$, $^{\S\S}P < 0.001$ compared with WT hepatocytes incubated in the same conditions by two-way ANOVA with Bonferroni post hoc test. Whilst TOFA inhibits lipid synthesis both in WT and AMPK-deficient hepatocytes, metformin and 991 inhibit [1- 14 C]-acetate incorporation in lipids in concentration-dependent manner only in WT hepatocytes. These results demonstrate that the inhibition of hepatic lipid synthesis in response to metformin and 991 is strictly dependent of AMPK.

A



B

