Video Article

Measurement of Basal and Forskolin-stimulated Lipolysis in Inguinal Adipose Fat Pads

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Abstract

Lipolysis is a process by which the lipid stored as triglycerides in adipose tissues are hydrolyzed into glycerol and fatty acids. This article describes the method for the measurement of basal and forskolin (FSK)-stimulated lipolysis in the inguinal fat pads isolated from wild type mice fed either normal chow diet (NCD), high fat diet (HFD) or a high fat diet containing 0.01% of capsaicin (CAP; transient receptor potential vanilloid subfamily 1 (TRPV1) agonist) for 32 weeks. The method described here for performing *ex vivo* lipolysis is adopted from Schweiger *et al.*[1] We present a detailed protocol for measuring glycerol levels by UV-Visible (UV/VIS) spectrophotometry. The method described here can be used to successfully isolate inguinal fat pads for lipolysis measurements to obtain consistent results. The protocol described for inguinal fat pads can readily be extended to measure lipolysis in other tissues.

Video Link

The video component of this article can be found at https://www.jove.com/video/55625/

Introduction

Adipose tissues store energy as fat[2] and fatty acid oxidation is required for thermogenesis[3,4]. Fatty acids ingested through diets are packaged along with apoproteins into chylomicrons and delivered to different tissues in the body via blood circulation. Although most cells in the body store a reserve of energy, adipose tissue stores excess energy as fat[5,6]. Lipolysis in adipose tissue is regulated by complex processes and the molecular details of lipolysis still remain vague[7].

Lipolysis is a process by which triglycerides (TGL) stored in adipose tissue are hydrolyzed to produce glycerol and fatty acids (FA) by the enzyme adipose triglyceride lipase (ATGL)[8]. Alterations in basal and stimulated lipolysis is a characteristic feature of obesity. The basal lipolysis is regulated by ATGL activation[9], which converts TGL to diacylglycerol (DAG), that is subsequently hydrolyzed to monoacyl glycerol (MAG). Activation of hormone sensitive lipase (HSL) *via* adenylyl cyclase activates cyclic adenosine monophosphate (cAMP) dependent protein kinase A (PKA) stimulation and causes lipolysis. Measurement of lipolysis, basal and stimulated, is, therefore, important to analyze the activity of proteins involved in this process. Also, unraveling the molecular regulation of lipolysis may be beneficial to develop novel therapeutic strategies against obesity[10]. Since, molecules that stimulate lipolysis and fatty acid oxidation are potential candidates for decreasing fats stored in depots, it is important to employ a robust assay for reproducibility.

Previously published data suggest that activation of TRPV1 protein expressed in white adipose tissue by CAP enhanced basal and FSK (adenylyl cyclase activator)-stimulated lipolysis in inguinal fat pads[11]. Previous research also suggests that long-term activation of TRPV1 by CAP activates PKA[12]. Since the activation of PKA stimulates lipolysis[13,14], measuring both basal and PKA-dependent stimulated lipolysis in inguinal fat pads isolated from NCD or HFD (± CAP)-fed mice after 32 weeks of feeding the respective diets will validate the role of TRPV1 activation in lipolysis.

This article describes an efficient method of determining basal and stimulated lipolysis. Although other methods that employ radioactive isotopes of glycerol and tedious high performance liquid chromatography or gas chromatography/mass spectrometry for measurements[15,16] are available, this method offers a more direct, simple and cost effective technique to determine lipolysis in adipose tissues.

Protocol

All protocols follow the animal care guidelines of the University of Wyoming.

1. Animal Housing and Feeding

NOTE: Adult male wild type mice (C57BL/6) (age 12 to 24 weeks) were bred in the research animal facility as per the Institutional Animal Care and Use Committee (IACUC) approved protocols.

1. Starting from week 6 of age, house mice in groups of four in separate cages and randomly assign them into feeding groups of NCD or HFD (± 0.01% CAP) until week 38 of age.
   NOTE: CAP is an agonist of TRPV1 channel protein expressed in adipose tissues[11,17]. Mix CAP with HFD in a blender inside the hood and transfer the blended mixture to a tray containing small 1 in^2 partitions. Keep the tray inside a -20 °C freezer. Remove the tray containing the HFD + CAP diet from the freezer after 24 h and store in a container at -20 °C freezer until use.
2. House mice in a climate-controlled environment (22.8 ± 2.0 °C, 45 - 50% humidity) with a 12/12-light/dark cycle with access to designated diet and water *ad libitum*.
3. At the end of 38 weeks, dissect inguinal adipose tissues and use for lipolysis experiments (sections 2-7).

2. Preparing Mice for the Experiments

1. Anesthetize mice by injecting ketamine and xylazine mixture (10 mg/kg and 80 mg/kg body weight, respectively). Inject 0.01 mL of the mixture/10 g body weight of mouse.
2. Confirm deep anesthesia by a firm toe pinch. If there is a pedal reflex, test the mouse again after at least 30 s.
3. Use vet eye ointment to prevent dryness of eyes during anesthesia. Do not leave mice unattended during any of the procedures.
4. Euthanize mice by injecting a high dose of ketamine and xylazine mixture injection (10 mg/kg and 80 mg/kg body weight, respectively) mixture (0.01 ml/10 g body weight) followed by cervical dislocation.
   NOTE: This method of euthanasia is approved by the IACUC of the University of Wyoming.

3. Isolation of Inguinal Adipose Fat Pads

1. Place the mouse (fed with NCD or HFD (± CAP) for 32 weeks) lying on its left for the procedure.
   NOTE: By placing the mouse on the left side, the left forelimb and left hindlimb will be on the dissection platform, while the right forelimb and right hindlimb will be facing away from the platform.
2. Sterilize the skin surface with a 2 $inch^2$ gauze pad soaked in about 2.5 mL of 70% ethanol. Make a 2 - 3 mm lateral cut through the skin using a scalpel to reveal the underlying fatty layer.
3. Make a 1 cm cut (depending on the size of the mouse) through the skin using a scalpel just below the rib cage across the dorsal surface joining the two lateral incisions.
4. Peel the skin flap by dragging it carefully using sterile forceps and leave the subcutaneous pad intact by not cutting the fat pads. This is the fat pad lying under the skin.
5. Dissect the fat pad carefully from the underlying muscle and fascia using a pair of scissors. Pull the fat pad as it is cut from the underlying muscle.
   NOTE: The weight and size of the fat pads depends on the type of mice (NCD or HFD (± CAP)-fed).
6. Use tweezers to transfer it to a Petri dish containing phosphate buffered saline (PBS) until the lipolysis experiments, at room temperature (~15 min).
7. Isolate the fat pads from the right side of the mouse as described in steps 3.2-3.6.

4. Basal Glycerol Release from Inguinal Fat Pads

1. Use sharp scissors to cut about 20 mg of fat tissue into 5 to 8 pieces.
2. **Incubate the cut pieces of inguinal fat pads in 200 µL of incubation medium (Dulbecco's Modified Eagel's Medium (DMEM) containing 2% fatty acid free bovine serum albumin (BSA)) at 37 °C, 5% CO_2 and 95% humidity for 60 min.**
   1. Collect the incubation medium and freeze at -80 °C for lipolysis assay.
      NOTE: The cut pieces are used for determination of protein concentration after lipid extraction.
   2. Transfer the cut fat pieces with the help of tweezers into 1 mL of extraction solution (chloroform:methanol (2:1, v/v) and 1% glacial acetic acid) and incubate for 60 min at 37 °C under vigorous shaking at 100 rpm. Discard the fat extraction solution.
      NOTE: This step will extract fat from the cut pieces. Discard the fat extraction solution as it will interfere with the protein determination.
   3. Transfer the tissue (from step 4.2.2) using tweezers into a sterile microfuge tube containing 500 µL of lysis solution (0.3 N NaOH containing 0.1% sodium dodecyl sulfate; SDS) and incubate overnight (12 h) at 55 °C under vigorous shaking at 100 rpm.
3. Determine the protein concentration of tissue (step 4.2.3) using the bicinchoninic acid (BCA) reagent and BSA as standard[18].
4. **Thaw the frozen medium (step 4.2.1) on ice and determine the glycerol content of the medium using a free glycerol reagent as described in protocol sections 6 and 7[6].**
   1. Extrapolate the concentration of glycerol in samples from the standard curve plotted using glycerol standards[19] and express lipolysis as nanomoles glycerol per mg protein per h[20].

5. FSK-stimulated lipolysis

1. **Preincubate about 20 mg of inguinal fat pad (5 to 8 cut pieces) obtained from NCD or HFD (± CAP)-fed wild type mouse in 200 µL DMEM containing 2% fatty acid free BSA, 10 µM FSK and 5 µM Triacsin C at 37 °C in 5% CO_2 and 95% humidity for 60 min.**

1. Transfer the tissue pieces using tweezers to an identical medium and incubate for a further 60 min at 37 °C, 5% CO_2 and 95% humidity. Collect the incubation medium and store at -80 °C until the lipolysis assay.
   NOTE: Stimulation of lipolysis causes a rapid release of fatty acids and glycerol within 15 min, which is linear during the first hour, and plateaus thereafter. Since the rate of stimulated lipolysis is higher and stable between the first and second hour of FSK stimulation, this protocol measured lipolysis in the stimulated state following the second incubation period as described previously[1]. So, both the experimental steps (5.1 and 5.1.1) are necessary.

2. To extract fat from inguinal fat pads, transfer the cut pieces of inguinal fat pads (obtained from NCD or HFD (± CAP)-fed mice) from step 5.1 with the help of tweezers into 1 mL of extraction solution (chloroform:methanol (2:1. v/v) and 1% glacial acetic acid) and incubate for 60 min at 37 °C under vigorous shaking at 100 rpm. Discard the extracted fat.
3. Transfer the tissue (from step 5.2) using tweezers into a sterile microfuge tube containing 500 µL of lysis solution (0.3 N NaOH containing 0.1% SDS) and incubate overnight (12 h) at 55 °C under vigorous shaking at 100 rpm.
4. Determine the protein concentration and glycerol content of tissue as described in steps 4.3 and 4.4, respectively.

6. Preparation of Free Glycerol Reagent

1. Reconstitute the glycerol reagent[19] in 40 mL of deionized water in an amber colored glass vial, place a stopper on the vial and mix well by inverting 10 times. Do not mix by shaking.
2. Store the vial at 4 °C inside a refrigerator protected from light by covering the vial completely with aluminum foil.
3. Proceed to the preparation of glycerol standard (section 7).

7. Preparation of Glycerol Standard and Determination of Glycerol Content

1. Make a stock of 1 mM by diluting 35 µL of the supplied stock with 65 µL of deionized water.
   NOTE: The manufacturer supplied glycerol standard stock is 2.8 mM glycerol.
2. Take five 1-cm path length disposable methacrylate cuvettes. Label the cuvettes using a marker as 0, 1.25, 2.5, 5 and 10 nmol standard.
3. Add 0, 1.25, 2.5, 5 and 10 µL of 1 mM glycerol standard to the respective labeled cuvettes. Make up the volume of each cuvette to 10 µL with deionized water.
4. Make 1 to 10 dilution of all samples (from step 4.4 or 5.1.1) by adding 10 µL of the sample to 90 µL of deionized water into fresh 500-µL prelabeled centrifuge tubes. Add 10 µL of the diluted samples into the cuvettes labeled with the respective sample identification number.
5. Switch on the UV-VIS spectrophotometer and set the wavelength to 540 nm.
6. Warm the free glycerol reagent to room temperature by keeping the vial (step 6.2) at room temperature for 15 min.
7. Set up a series of labelled cuvettes as blank "0" nmol standard (step 7.3), standards (step 7.3) and samples (step 7.4).
8. Add 10 µL of each of the standard and sample into the respective labeled cuvette. Use "0" nmol standard as blank.
9. Add 0.8 mL of free glycerol reagent into each cuvette, containing the glycerol standards using a 1 mL pipette.
10. Cover the cuvette with a 1.5 cm square plastic paraffin film and mix the contents by inverting the cuvette for 3 times and set aside at room temperature for 10 min.
11. Place the cuvette in the UV-VIS spectrophotometer and record the absorbance (540 nm wavelength).
12. Plot the standard curve using the concentrations of the standards (nmol) in X-axis and the recorded absorbance in the Y-axis.
13. Calculate the concentration of glycerol in the samples (nmol) by extrapolation using the standard curve plotted in step 7.12.
14. Multiply the concentration of samples (nmol/cuvette) with the dilution factor 10 (refer to step 7.4) and 20 (total volume)
15. Divide the concentration of glycerol in each sample (step 7.13) by the mg of protein calculated by the BCA method.
    NOTE: Since the inguinal fat pad samples are incubated for 60 min, represent the results as nmol of glycerol/mg protein/h.

Representative Results

To evaluate the effect of CAP on basal and stimulated lipolysis, this research measured lipolysis in inguinal adipose fat pads isolated from NCD or HFD (± CAP)-fed wild type mice. The representative results for the basal and FSK-stimulated lipolysis for the inguinal fat pads are given in **Table I**. Basal and FSK-stimulated glycerol release in the presence of Triacsin C, which inhibits acyl coA synthetase and prevents the regeneration of TGL. As shown in **Figure 1**, HFD suppressed the FSK-stimulated lipolysis, and CAP increased both basal and FSK-stimulated lipolysis. Triacsin C was used to inhibit the regeneration of TGL from glycerol and fatty acids[11]. All data are expressed as means ± S.E.M. Comparisons between groups are analyzed using one-way ANOVA and *post hoc* analyses were performed using Student's *t* test. Sample sizes are set to determine whether the mean value of an outcome variable in one group differed significantly from that in another group. A p-value < 0.05 is considered as statistically significant.

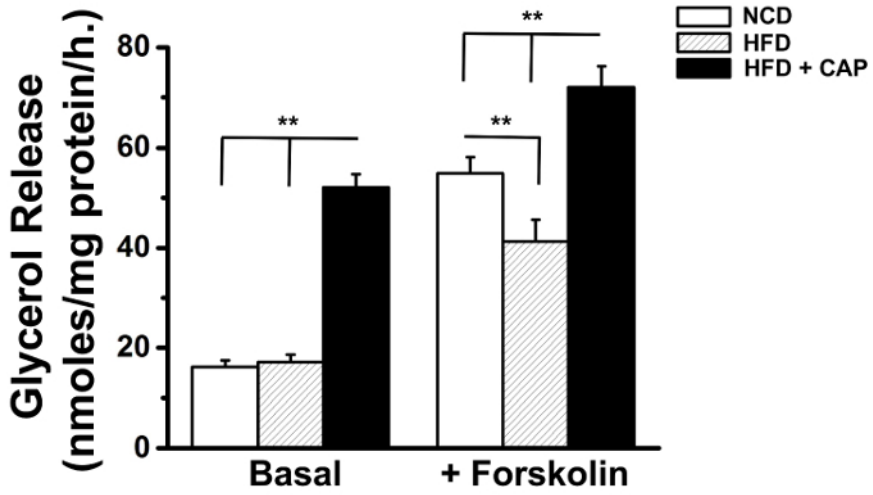

Figure 1: CAP Increases Basal and FSK-stimulated Glycerol Release in Inguinal Fat Pads. Bar graphs represent the mean ± S.E.M. of basal and FSK (10 μM)-stimulated glycerol release (nmol/mg protein/h) in the inguinal fat pads isolated from NCD or HFD (± CAP)-fed wild type mice. **Represents statistical significance for p-value <0.05 for n = 6. Please click here to view a larger version of this figure.

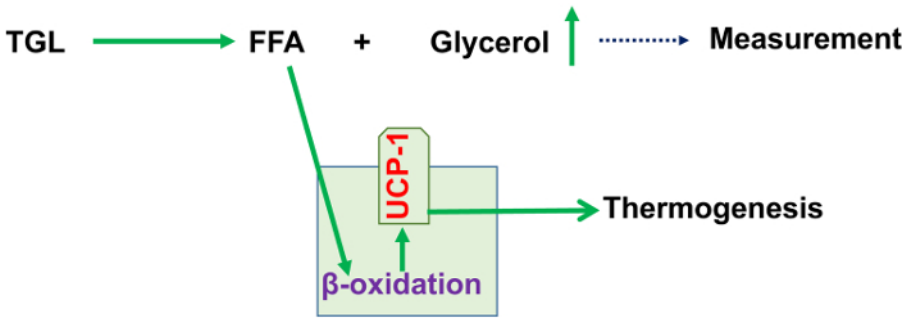

Figure 2: Hydrolysis of Triglycerides (TGL) Results in the Generation of Free Fatty Acids (FFA) and Glycerol. FFA undergoes mitochondrial β-oxidation. This, along with the upregulation of uncoupling protein 1 (UCP-1) stimulates thermogenesis. Glycerol release is measured by the protocol described in the article. Please click here to view a larger version of this figure.

Lipolysis (nmoles glycerol /mg of protein/ h)	NCD (n = 6)	HFD (n = 6)	HFD + CAP (n = 6)
Basal	16.22 ± 1.28	17.16 ± 1.48	52.07 ± 2.66
FSK Stimulated (with Triacsin C)	54.88 ± 3.27	41.23 ± 4.43	72.04 ± 4.16

Table 1: Effect of CAP on Basal and FSK Stimulated Lipolysis in Inguinal Fat Pads. Mean basal and FSK-stimulated glycerol release ± S.E.M measured in inguinal fat adipose tissue obtained from NCD, HFD or HFD + CAP-fed wild type mice.

Discussion

The breakdown process of TGL into glycerol and fatty acids is catalyzed by ATGL[9] during basal lipolysis and orchestrated by an array of proteins including the activation of adenylyl cyclase/PKA-dependent pathway during stimulated lipolysis[21,22,23]. Enhancement of lipolysis increases the plasma levels of fatty acids for transportation and energy use[24]. Fatty acids are taken up by mitochondria as acetyl CoA, which is used to produce energy.

The degree and extent of lipolysis can efficiently be measured by glycerol release from adipose tissues (**Table 1** and **Figure 1**). Hydrolysis of TGL produces fatty acids and glycerol. Previous research showed that dietary supplementation of CAP significantly enhanced the basal as well as FSK-stimulated glycerol release from inguinal and brown fat pads[11,17]. The increased production of fatty acids from enhanced lipolysis could serve as feed to produce heat and thermogenesis since CAP also significantly increased the expression of mitochondrial UCP-1 in the inguinal fat pads. Consistent to this notion, previous research suggests that CAP enhanced the expression of peroxisome proliferator activated receptor alpha (PPARα) in inguinal fat pads, which plays a critical role in the mitochondrial fatty acid oxidation and in the transcriptional upregulation of UCP-1[25,26,27]. Consequently, CAP also increased metabolic activity, heat production and energy expenditure in HFD-fed mice[28,29]. Further, HFD feeding suppressed stimulated lipolysis while CAP increased basal and FSK-stimulated PKA-dependent lipolysis as well as enhanced insulin

sensitivity[30]. Therefore, as described by the model in **Figure 2**, it is reasonable to interpret that CAP increases basal and stimulated lipolysis without causing insulin resistance as the excess fat is burned to produce heat by mitochondrial UCP-1 upregulation.

This article describes the method of measuring glycerol release from white adipose tissues. Although several methods using stable isotopes of glycerol are available, they require either high performance liquid chromatography or gas chromatography/mass spectrometry for measurements[15,16]. Also, glycerol radio-tracer measurements are associated with non-specificities[31]. Alternate methods include determining mRNA and protein levels of lipases and regulatory proteins involved in lipolysis. Colorimetric methods are also available to determine the concentration of long chain fatty acids in circulation. The method described here is very efficient in analyzing glycerol release from adipose tissues as it can be done using a plate reader capable of fluorescence and absorbance measurement. Also, the simplified analytical method for glycerol release described in this article provides improved stability and precision[32].

Standard operating procedures should be practiced as this method involves surgical techniques to isolate fat pads. This protocol recommends the use of fat free BSA. This may cause air bubble formation, which will interfere with absorbance reading. Therefore, care should be taken while handling samples to prevent air bubbles. Precaution should be taken not to shake or invert samples during BSA treatment, unless such a step is recommended in the protocol. Also, the fat extraction step from inguinal fat is critical since the presence of fat will interfere with inguinal adipose tissue protein determination. Additionally, during the preparation of glycerol standards, it is important to mix the contents by inversion and not shake the vial.

The method described here can be used to successfully isolate inguinal fat pads for several assays including lipolysis measurements. The lipolysis protocol described for inguinal fat pads can readily be extended to the measurement of lipolysis in other tissues. The methods are optimized for all kinds of fat pads, as well as preadipocytes.

In summary, this article describes the method for measuring basal and FSK-stimulated glycerol release in inguinal adipose fat pads isolated from NCD or HFD (± CAP)-fed mice. The presented data suggest that TRPV1 activation by CAP increased the basal and FSK-stimulated lipolysis and prevented the accumulation of lipids in the adipose tissues. These data demonstrate a critical role of TRPV1 protein in the regulation of lipolysis in white adipose tissue.

Disclosures

The authors have nothing to disclose.

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