Adipocyte Subcellular Fractionation

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Materials and Reagents

1. Fat tissue
2. Bovine serum albumin (BSA)
3. (-)-N6-(R-Phenyl-isopropyl)-adenosine
4. Adenosine
5. Sodium orthovanadate
6. Ammonium bicarbonate
7. Sucrose
8. Tizma base
9. Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)
10. Potassium chloride (KCl)
11. Sodium phosphate
12. Sodium pyrophosphate
13. Monopotassium phosphate
14. Iodoacetate
15. EDTA
16. EGTA
17. Sodium fluoride
18. Glucose
19. Calcium Chloride
20. HEPES (ICN Biomedicals)
21. Glucose
22. Protease inhibitors (Roche Diagnostics)
23. NaCl 0,9% solution (see Recipes)
24. Collagenase solution (see Recipes)
25. KRHLP solution (see Recipes)
26. KRHG solution (see Recipes)
27. PES homogenization buffer (see Recipes)
28. Tris-EDTA solution (see Recipes)
29. Stock solutions (see Recipes)

Equipment

1. Beckman centrifuges and ultracentrifuges (Beckman Coulter)
2. Rotors: JA-21, SW-41, TLS-55, and TLA-100 (Beckman Coulter)
3. Probe sonicator
4. Teflon/glas homogenizer (Thermo Fisher Scientific)
5. Gauze bandage
6. Beckman UltraClear tubes (Nalgene & Beckman Coulter)

Procedure

1. The fat tissue is rinsed with isotonic NaCl solution (0.9%) immediately after harvesting.
2. The tissue is cut to small pieces and incubated with collagenase (1 ml/g fat) for 1 h at 37 °C.
3. KRHLP-1% BSA buffer is added and the cells are filtered first through a single and then a double layer of gauze bandage.
4. The cells are washed with KRHLP-1% BSA buffer until the lower phase is clear.
5. The cells are diluted with the same buffer up to 10-15% and preincubated with PIA (1 µl/ml cell suspension) and adenosine (0.5 µl/ml) for 10-15 min at 37 °C. The buffer is changed to PES buffer (homogenization buffer) containing 2 mM sodium orthovanadate and protease inhibitors. Up to 20-30% cells.
6. The cells are homogenized at RT with 5 strokes in a Teflon/glass homogenizer.
7. The homogenate is transferred to Nalgene tubes and centrifuged for 20 min at 4 °C (JA-21, 14,000 rpm).

   From now on all steps are done at 4 °C. With cold spatula the fat on the top of the tubes is removed.

   The supernatant contains intracellular membrane vesicles (microsome fraction) and soluble proteins (cytosol fraction).

   The pellet contains in addition to the plasma membrane, mitochondria and nuclei.
8. The supernatant is transferred to Beckman UltraClear tubes and centrifuged for 75 min (SW-41, 35,000).
9. The super containing cytosolic proteins is transferred to 15-ml tubes and frozen. The pellet (microsome) is resuspended in 200 µl Tris-EDTA/2 mM sodium orthovanadate.
10. The pellet is resuspended in 200 µl Tris-EDTA/2 mM sodium orthovanadate and placed carefully on 1.12 M sucrose solution (400 µl). Rinse the pellet tube with 400 µl Tris-EDTA/2 mM sodium orthovanadate and put on the sucrose solution. (600 µl of suspension can be loaded on the 400 µl of 1.12 M sucrose).

11. The sucrose tube is centrifuged for 60 min (TLS-55, 46,000 rpm). The plasma membrane will stay at the top of the sucrose solution while the mitochondria and the nuclei will sediment.
12. The plasma membrane band (400-600 µl) is transferred to a new tube and diluted up to 1,000 µl with Tris-EDTA/2 mM sodium orthovanadate. Vortex! 10% is saved as plasma membrane sample and the rest is for caveolae preparation.
13. The plasma membrane fraction and the caveolae fraction are pelleted (20 min, TLA-100, 69,000 rpm). The plasma membrane sample is resuspended in 200 µl Tris-EDTA/2 mM vanadate and frozen.
14. Caveolae sample is resuspended in 200 µl carbonate buffer (pH 11) (or 50 mM NH_4HCO_3 + 2 mM sodium orthovanadate) and transferred to sonication tube. Caveolae tube is rinsed with additional 200 ml and the volume in the sonication tube is adjusted to 2,000 ml.
15. The sonication probe is cooled before and sonication is performed at 16 micron (3x 20 sec, with 60 sec intervals).
16. The sonicated sample is diluted with 2 ml 90% sucrose and placed at the bottom of an ultracentrifuge tube containing 5-35% discontinuous sucrose gradient.
17. The tube is centrifuged for 16-20 h (SW-41, 39,000).
18. A light-scattering band (caveolae-enriched fraction) confide to the 5-35% sucrose interface is collected (1 ml) and diluted in Tris-EDTA + 2 mM sodium orthovanadate up to 4 ml.
19. Caveolae are pelleted for 20 min (TLA-100, 69,000 rpm).

Recipes

1. NaCl 0,9% solution
   9 g NaCl in 1,000 ml H_2O
2. Collagenase solution (pH 7.5)

KRH-stock	2 ml	5 ml
HEPES-stock	1,6 ml	4 ml
BSA-stock	5 ml	12.5 ml
Glucose	144 µl	360 µl
Adenosine	20 µl	50 µl
collagenase	12 mg	30 mg
Add water to	10 ml	25 ml

3. KRHLP solution (pH 7.5)

	1% BSA	3.5% BSA
KRHLP-stock	20 ml	20 ml
H_2O	40 ml	-
BSA-stock	14.3 ml	50 ml
HEPES-stock	16 ml	16 ml
Glucose	1,44 ml	1.44 ml
Adenosine	0.2 ml	0.2 ml
Add water to	100 ml	100 ml

4. KRHG solution (pH 7.5)

	1% BSA	3.5% BSA
KRH-stock	20 ml	20 ml
H_2O	40 ml	-
BSA-stock	14.3 ml	50 ml
HEPES-stock	16 ml	16 ml
Glucose	1.44 ml	1.44 ml
Adenosine	0.2 ml	0.2 ml
Add water to	100 ml	100 ml

5. PES homogenization buffer (pH 7.4)

NaH_2PO_4	10 mM	0.276 g	for	1 L
EDTA	1 mM	0.372 g	for	1 L
Sucrose	0.25 M	85.58 g	for	1 L
NaF	25 mM	105 mg	for	100 ml
Na_2PP_i	1 mM	45 mg	for	100 ml
EGTA	0.5 mM	19 mg	for	100 ml
Iodoacetate	4 mM	74 mg	for	100 ml

6. Tris-EDTA solution (pH 7.4)

Tris	10 mM	0.6055 g
EDTA	1 mM	0.186 g
Add water to		500 ml

7. Stock solutions
   a. KRH/KRHLP

	KRH-stock	KRHLP-stock
NaCl	35.1 g	35.1 g
KCl	1.75 g	1.75 g
KH_2PO_4	0.82 g	0.34 g
$MgSO_4 \cdot 7H_2O$	1.48 g	1.48 g
Solve in 900 ml H_2O		
$CaCl_2 \cdot 2H_2O$	1.48 g	1.48 g
Add water to	1,000 ml	1,000 ml

b. HEPES

37.23 g HEPES

900 ml H_2O

pH 7.3 (NaOH)

Add water to 1,000 ml

c. Glucose - 139 mM

2.5 g. glucose in 100 ml H_2O

d. Adenosine - 100 µM

13.4 mg adenosine in 500 ml H_2O

e. PIA - 100 µM (-)-N6-(R-Phenyl-isopropyl)-adenosine (adenosine analog)

1 mM stock: 1.93 mg in 5 ml EtOH abs

1µM stock: 1 ml 1 mM dilute in 9 ml H_2O (total volume is 10 ml)

f. Sucrose 1.12 M

3.83 g sucrose in Tris-EDTA to total volume of 10 ml.

References

1. Aboulaich, N., Vainonen, J. P., Stralfors, P. and Vener, A. V. (2004). Vectorial proteomics reveal targeting, phosphorylation and specific fragmentation of polymerase I and transcript release factor (PTRF) at the surface of caveolae in human adipocytes. *Biochem J* 383(Pt 2): 237-248.
2. Gustavsson, J., Parpal, S., Karlsson, M., Ramsing, C., Thorn, H., Borg, M., Lindroth, M., Peterson, K. H., Magnusson, K. E. and Stralfors, P. (1999). Localization of the insulin receptor in caveolae of adipocyte plasma membrane. *FASEB J* 13(14): 1961-1971.