Glucose up-take assay.v2

1. Fast mice the day before the experiment (at 5:00 pm) by changing to a new cage. Keep the water!!! It is reasonable to harvest 8-12 mice per day.

2. Anesthetize the mice by i.p. injection of Nembutal at 90 mg/kg.

3. Dissect the muscles of interest carefully without stretching them and rinse the muscle in **Dissection buffer** (2 ml/sample) briefly.

4. Incubate the muscles individually in glass scintillation vials containing 2 ml of Krebs-Henseleit bicarbonate buffer (KHB) with 8 mM glucose, 32 mM mannitol with (**Recovery (+) buffer)** or without 60 μU/ml insulin (**Recovery (-) buffer**) with a gas phase of 95% O_2-5% CO_2, in a shaking incubator at 37°C for 30 min for recovery.

5. Rinse the muscles for 10 min at 37°C in 2 ml of oxygenated KHB containing 40 mM mannitol and with insulin (**Rinse buffer**) if it is present during the previous incubation to remove glucose from the extracellular space.

6. Incubate the muscles for 20 min at 37°C in flasks containing 2 ml of KHB with 4 mM 2-deoxy-[1,2-^{3}H]glucose (2-DG; 1.5 μCi/ml) and 36 mM [^{14}C]mannitol (0.2 μCi/ml) with insulin if it is present during the previous incubation (**Incubation buffer**), with a gas phase of 95% O_2-5% CO_2, in a shaking incubator.

7. Blot the muscles on filter paper dampened with incubation medium and store on ice in an eppendorf tube.

8. Weigh the muscles and add 0.5 ml of 0.5 N NaOH. Incubate at 60°C for 1 hours and shake occasionally.

9. Count 100 ul of the muscle extracts in duplicates in scintillation vials containing 4 ml of scintillation fluid with channels preset for simultaneous 3H and 14C counting.

10. To obtain reading for 10 ul of incubation buffers.

11. Determine the intracellular water content of the muscles by subtracting the measured extracellular space water from total muscle water. Total muscle water is assumed to be 80% of muscle weight, which is the average value for rat epitrochlearis muscles.

Solutions

1. Krebs Buffer (KHB) (200 ml)

Add 20 ml stock I to 160 ml of Milli-Q H2O. Gas with 95% O2/5% CO2 on ice for 20 min.

Add 20 ml of Stock II, gas for additional 10 min.

-check pH, should = 7.2-7.4

2. Dissection Buffer (50 ml)

364.5 mg (40 mM) Mannitol $C_6H_{14}O_6$ F.W. = 182.17 [Sigma M-9546]

50 ml KHB

3. KHB-BSA (150 ml)
150 mg (0.1%) of RIA Grade Bovine Serum Albumin (BSA) [Sigma A-7888]
150 ml KHB, let dissolve and keep on ice.

4. Recovery (-) (40 ml)
57.7 mg (8 mM) Glucose $C_6H_{12}O_6$ F.W. = 180.2 [Sigma G-7528]
233.2 mg (32 mM) Mannitol
40 ml KHB-BSA. Keep on ice.

Insulin Dilution
Stock Insulin = 100 Units/ml [Eli Lilly & Co. VL-7510]
Dilution #1 = 10 µl of Stock Insulin + 990 µl of KHB-BSA = 1 Units/ml
Dilution #2 = 15 µl of Dilution #1 + 985 µl of KHB-BSA**.** Keep on ice.

5. Recovery (+) (20 ml)
20 ml Recovery (-)
80 µl of Insulin Dilution #2. Keep on ice.

6. Rinse (-) (40 ml)
291.5 mg (36 mM) Mannitol
40 ml KHB+BSA. Keep on ice.

Rinse (+) (20 ml)
20 ml Rinse (-)
80 µl of Insulin Dilution #2
Keep on ice.

7. Incubation (-) Solution (40 ml)
26.3 mg (4 mM) 2-Deoxy-D-Glucose $C_6H_{12}O_5$ F.W. = 164.2 [Sigma D-6134]
262.7 mg (36 mM) Mannitol
104 µl Deoxy-D-Glucose, 2-[1, 2-^{3}H] {American Radiolabeled Chemicals, Inc., ART-103}
120 µl Mannitol, D-[1-^{14}C] {American Radiolabeled Chemicals, Inc., ARC-127}
40 ml KHB-BSA. Keep on ice.

8. Incubation (+) Solution (20ml)
20 ml Incubation (-) Solution
80 µl of Insulin Dilution #2
Keep on ice.

Stock solutions:
1. KHB Stock I (500 ml)
NaCl 33.9 g; KCl 1.72 g; KH2PO4 0.79 g; NaHCO3 10.63 g. Keep at 4°C.

2. KHB Stock II (500 ml)
$CaCl_2 \cdot 2H_2O$ 1.84 g; $MgSO_4 \cdot 7H_2O$ 1.43 g. Keep at 4°C.