SOP_Inhibitor titration intact cells

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Abstract: To investigate the control distribution of a biochemical pathway by inhibitor titrations, both local perturbations in reaction-level kinetics and changes in steady-state system flux must be measured. Culture titrations were performed with irreversible glycolytic inhibitors, allowing whole-cell flux and in vitro enzyme activity to be conducted using the same treated cells. Briefly, cell cultures were exposed to a glycolytic inhibitor for a set incubation period, whereafter the medium was refreshed to remove all non-bound inhibitor. As the formation of an irreversible covalent bond prevents dissociation of the protein-inhibitor complex, residual enzyme activity was expected to remain constant throughout the duration of the experiment. Accordingly, experimental measures of glycolytic flux could then be obtained from the treated intact cells, whereafter a cytosolic extract could be produced for in vitro determination of endogenous enzyme activity.

Materials:

Reagents*:* High glucose (HG; 4.5g glucose/L) Dulbecco's modified Eagle's medium (DMEM); low glucose (LG; 1g glucose /L) DMEM; heat-inactivated fetal bovine serum (FBS); antibiotic antimycotic solution (PenStrep; 100 U/mL penicillin, 0.1 mg/mL streptomycin and 0.25 µg/mL amphotericin B; Sigma-Aldrich); 0.5% Trypsin-EDTA (Gibco); sterile calcium/magnesium free phosphate buffered saline (PBS, Sigma-Aldrich); 50 mM Tris-HCl buffer (pH 7.0)

Equipment: Cell Incubator (37C, 5% CO2, humidified), 96 well plates 100 mm dishes TC-coated

Procedure:

Preperation:Cells were seeded into suitable plates/ culture dishes and grown overnight in HG DMEM supplemented with 10% FBS and 1% v/v PenStrep to a confluence of 80% in the cell incubator (standard conditions, 37°C in a humidified atmosphere with 5% CO_2).

Inhibitor treatment: Thereafter, the medium was aspirated and the cells were briefly washed with LG DMEM. Cultures were then incubated with micromolar concentrations of inhibitor (iodoacetic acid (IAA) or 3-bromopyruvate (3BrP)) in LG DMEM for 60 min under standard conditions.

Flux sampling: Following treatment, the medium was replaced with 7 mL of fresh low glucose DMEM and 100 µL samples were extracted at regular intervals over a 8-hour period. Samples were stored at -20°C for further use in flux determination assays. After 8h the cells were harvested and stored at -80°C prior cell lysis and enzyme activity measurements.

Cell harvest: Medium was removed and the cells were incubated with 0.05% w/v Trypsin-EDTA in sterile PBS for 5–10 min. The trypsin was then quenched by addition of twice the corresponding volume of DMEM supplemented with 10% v/v FBS and the cells were centrifuged at 100 × g for 3 min at room temperature. After discarding the supernatant, the cell pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.0), followed by another centrifugation step. Washing was repeated, whereafter the cell resuspensions were transferred into Eppendorf tubes and stored at -80 °C.