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Glycogen synthesis, glycogenolysis, and gluconeogenesis in primary mouse hepatocytes

Media Formulations

A. Media for glycogen synthesis:

Culture medium base: DMEM-Low (Mediatech/Cellgro #10-014; 1g/L glucose, L-Gln, 1mM Na-Pyruvate)
5mM HEPES, pH 7.4@37C
2.0mM L-Glutamine (in addition to that present in stock media)
10nM Dexamethasone
1x Pen-Strep

-Add glucose to reach a final concentration of 15-25mM (or desired concentration)

B. Media for gluconeogenesis

Glucose-free/Phenol-red-free DMEM: 1x media w/NaHCO3 (Mediatech #09-113-PB)
10mM substrate (lactate, pyruvate, glycerol, etc)
10mM HEPES, pH 7.4@37C
10nM Dexamethasone
1x Pen-Strep

-If using forskolin (Sigma #F6886-10MG) to stimulate glucose output and/or induce glycogenolysis, make 10mM stock in DMSO

C. Media for hormone-free depletion of glycogen

Glucose-free/Phenol-red-free DMEM: 1x media w/NaHCO3 (Mediatech #09-113-PB)
5mM HEPES, pH 7.4@37C
10nM Dexamethasone
1x Pen-Strep

Hormone-free depletion of cellular glycogen

Purpose: To reduce the contribution of glucose from glycogenolysis to total hepatic glucose output during gluconeogenesis assay. May also be implemented before initiation of glycogen synthesis, to allow all treatment conditions to begin at a common starting point (i.e. zero glycogen).

1. Wash cells once with glucose-free/phenol-red-free DMEM

2. Add back glucose-free/phenol-red-free DMEM and allow to incubate for 20-60 minutes.

The time needed to deplete >90% of intracellular glycogen depends on the density and health of your hepatocytes, as well as the media/culture conditions. The following table is a rough guideline FOR 12-WELL PLATES ONLY; empirical testing should be performed, and if desired, control wells may be implemented.

Confluence	**Overnight [glucose]**	**Time (min) to deplete**
>90%	<10mM	30-45
>90%	>10mM	45-60
<90%	<10mM	20-30
<90%	>10mM	30-45

Note: Time (min) to deplete assumes 12-well plating of hepatocytes that are healthy (>90% unstained by trypan blue during isolation), no special treatment(s), and standard culture conditions (i.e. no serum).

Glycogen synthesis

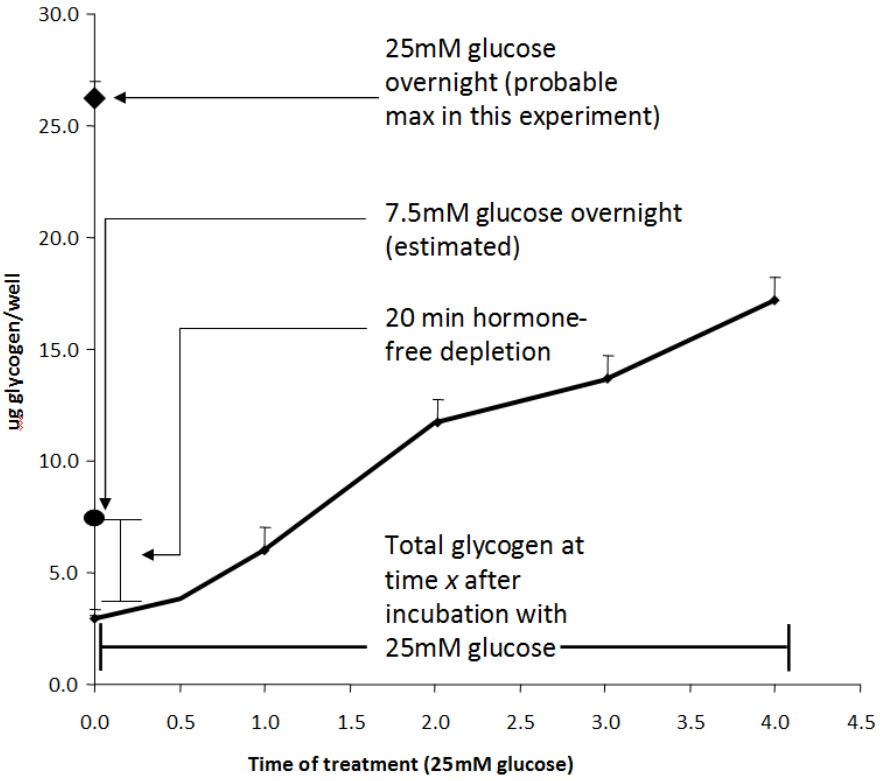
Purpose: To induce synthesis and accumulation of intracellular glycogen. Can also serve as a means to preserve and/or stabilize intracellular glycogen for experiments requiring longer culture times. May be used in conjunction with insulin as an insulin-sensitive readout.

1. [Optional] If you wish to deplete your cells of glycogen before initiating synthesis, see *hormone-free depletion of cellular glycogen* (above).

2. To initiate synthesis, simply replace existing media with the high-glucose formulation listed above (*media for glycogen synthesis*). If testing for insulin response, split synthesis media into two aliquots and add desired quantity of insulin (5nM should be more than sufficient, with 1-100nM as a reference range; % insulin response is generally between 30-100%, with an average of 45-60%).

3. Allow cells to accumulate glycogen for 2-5 hours; you may shorten or lengthen this time period depending on your experimental parameters. Generally, the maximum % insulin response can be realized within 3-4 hours.

Notes: For unknown reasons, hepatocytes will continuously lose glycogen in lower glucose media as they remain in culture; however, in the presence of high glucose, they will continuously accumulate glycogen until a threshold is reached, at which time total glycogen will remain constant for a few hours, before beginning to fall.



The graph to the right depicts a time-course of glycogen accumulation. The upper limit of intracellular glycogen for this particular experiment was attained by incubating cells with synthesis media (25mM glucose) overnight, and lysing them the next morning. All other cells were kept in standard culture medium (~7.5mM glucose) overnight, and were depleted of glycogen the following morning by a 20-minute incubation in glucose-free media.

Note that even with ~7.5mM glucose overnight, 20 minutes of depletion was unable to completely ablate glycogen. Note that the initial glycogen content in this condition was not directly measured, and the value plotted is an extrapolation based on observations from several other experiments.

Following depletion, synthesis media was added, and batches of cells lysed at 1-hour intervals (n = 3; +SD). No insulin was present in any wells, at any time.

Had this experiment progressed past four hours, we assume that glycogen would continue to rise until it matched the quantity observed in the overnight 25mM glucose condition.

Glycogenolysis

Purpose: To deplete, using hormones or other exogenous agents, intracellular glycogen. Typically used in conjunction with insulin to assess insulin suppression of glycogenolysis.

1. Choose a glycogenolytic agent; we have had success with glucagon and forskolin; at the appropriate concentration and length of exposure, either of these compounds will elicit satisfactory glycogenolysis, while still allowing an insulin response to be seen.

2. Determine the optimal concentration of your glycogenolytic agent of choice. We have found maximal response with 1-2uM of forskolin; alternatively, 1-10nM of glucagon may work equally well. If using insulin as a counterregulatory hormone, 1-10nM should be sufficient to give a maximal counter-response.

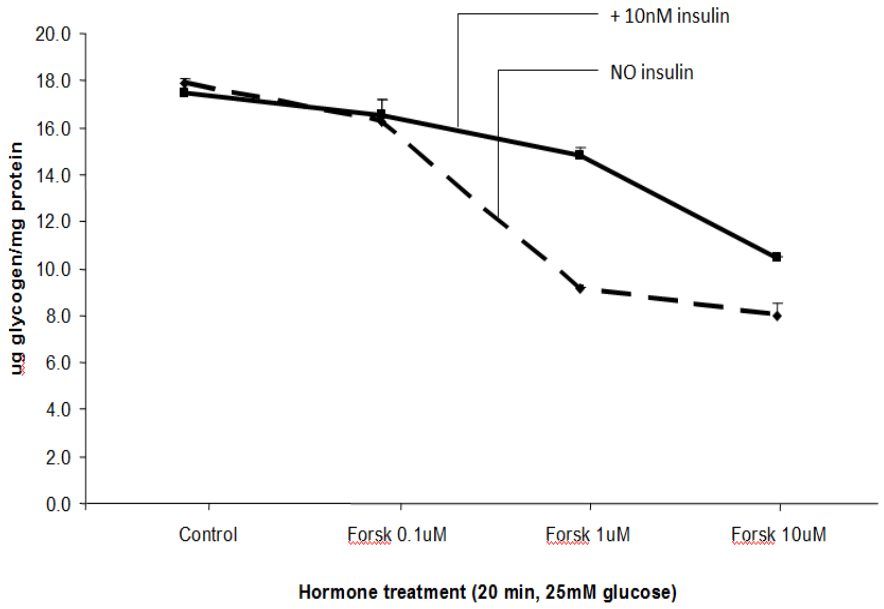
3. Determine the optimal length of hormone treatment. To minimize the contribution of gluconeogenesis to your data, we recommend that you treat for <1hr; however, to induce sufficient glycogenolysis, we recommend that you treat for at least 20 min. We have found that the percent effect of forskolin and insulin/forskolin remain relatively constant over 20-60 minutes. Empirical testing is recommended to determine the optimal time range for your experimental condition(s), as the total quantity of glycogen will also play a role in exposure length.

4. When performing the actual assay, we suggest that you treat the cells in the media in which they have been cultured overnight; if this is not possible or practical, allow cells to acclimate to any fresh media for at least two hours, to minimize the effect of changing the media on cellular glycogen content. In our experience, the concentration of glucose in the media only affects the absolute glycogen values, and not the percent hormone/agent effects; this may or may nor hold true under your experimental conditions.

5. Insulin (alone) controls are not necessary, as no appreciable stimulation occurs within one hour; they may still be included if desired.

6. When stimulating with hormone, if using insulin to counter forskolin/glucagon, we suggest you first add insulin, and then forskolin. There is no need to pretreat with insulin. If practical, both hormones may be mixed together and added concurrently. Simply add hormone(s), swirl to mix, and place the plate back in the incubator.

7. At the end of the treatment period, dump all media and lyse with 0.75% SDS for protein.



As shown in the figure to the left, there is an optimal ratio of forskolin (or glucagon) to insulin. If the concentration of forskolin is too high, insulin cannot counteract its effects; however, if the concentration of forskolin is too low, very little glycogen is released, and the insulin effect, while high on a percentage basis, is negligible on an absolute ug basis.

Data in triplicate -/+ SE; cells incubated overnight in 25mM glucose.

Gluconeogenesis

Purpose: To determine the quantity and/or rate of gluconeogenesis (GNG) in primary hepatocytes. Sensitive to agonists/antagonists, and can be used as an insulin-sensitive readout.

If measuring more than basal output, choose the type and concentration of agonist/antagonist agents. Forskolin is recommended as a means to augment gluconeogenesis (due to its consistency and stability), although glucagon will also work; insulin will antagonize either agent. The concentration of agonist depends upon whether you wish to study acute (presumably non-genomic) or long-term (presumably genomic) insulin suppression of agonist-induced gluconeogenesis.

For reasons not clearly understood, insulin does not appear to suppress the basal (no agonist) level of GNG. To see an insulin effect on gluconeogenesis requires the presence of an agonist; the length of time needed to observe said insulin effect depends on the concentration of agonist.

[Forskolin]	[Insulin]	Time needed to observe insulin suppression of forskolin effect
<2uM	1-10nM	Immediate; limited by sensitivity of glucose quantitation assay
>10uM	1-10nM	>8hrs; typically between 12-16hrs

At low (<2uM) concentrations of forskolin, insulin acutely suppresses forskolin-induced GNG, typically between 20-50%. However, actual stimulation (of GNG by forskolin) tends to be very weak.

At high (10-25uM tested) concentrations of forskolin, insulin is unable to acutely inhibit GNG; however, if cells are exposed to both hormones for longer periods of time (overnight), insulin often is able to suppress forskolin-induced GNG significantly (50-100%).

Note that we have not conducted any tests to conclusively prove that the discrepancies observed are attributable to non-genomic vs. genomic factors.

A. General measurement of GNG

1. Wash cells with glucose-free/phenol red-free media, and deplete cells of glycogen (see *hormone-free depletion of cellular glycogen*).

2. Add *media for gluconeogenesis*. If running the assay for short periods of time, or if short collection intervals (<4hrs) will be used, add the minimum volume of media that will cover the cells (~300uL /well for a 12-well plate).

3. Due to batch-to-batch variations in the rate of glucose output, it is difficult to recommend aliquot volumes for measurement of glucose. It is advisable to remove the entire volume of media- if additional time points are to be collected, simply refresh the media. For a 12-well plate containing 300uL of media, without agonist, ~0.01-0.02ug glucose/uL media/hr is typical.

4. Continue to refresh media for as many time points as desired.

B. Acute insulin suppression of forskolin-induced GNG (generally does not work)

Treat cells as described in (A), with the following modifications:

1. Make forskolin-alone media

2. Make forskolin + insulin media

3. If desired, as a control, make insulin-alone media (not needed for short-term GNG).

4. Take aliquots at 1-hour intervals, refreshing the media each time.

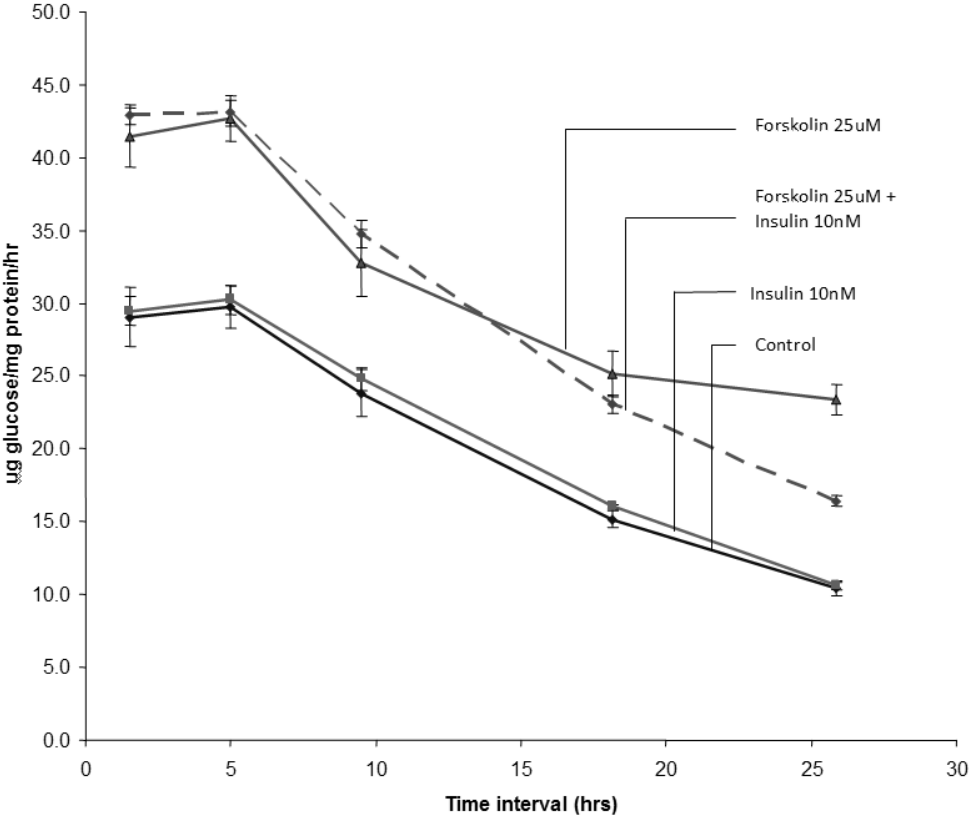
C. Long-term insulin suppression of forskolin-induced GNG

Treat cells and make media as described in (A) and (B), respectively, with the following modifications:

1. Use higher [forskolin]

2. Have an insulin-alone control; the longer treatment time often results in slight, but significant changes in total protein in insulin-treated lanes.

3. Hourly aliquots are not necessary, due to the length of time needed to see an insulin effect. You may with to collect a single aliquot on the first day (2-6hrs), before leaving the cells to incubate overnight. Refresh the media after overnight incubation, and collect several 2-hour aliquots thereafter.



Typical dynamics of long-term insulin suppression of forskolin-induced GNG. Note the progressive decrease in average hourly rate of glucose production. In all experiments, the rate decreases over time; percent effects of hormones may or may not change, depending on individual mouse characteristics and cell health/confluence.

Acute/short-term insulin suppression of forskolin-induced GNG resembles figure shown for insulin suppression of forskolin-induced glycogenolysis with forskolin at 1uM (see above).