A high capacity screen for adipogenic differentiation

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Abstract

Glycerol-3-phosphate dehydrogenase (GPDH) is highly expressed in mature adipocytes. Activity of this enzyme is therefore routinely measured to assess adipogenic differentiation in cell cultures. Existing protocols for GPDH assays require relatively large amounts of cells, and throughput is limited due to multiple steps needed for cell harvest and enzyme extraction. We present here a new protocol allowing GPDH determinations to be performed in 96-well plate format. From the start of cell culture to the final readout all steps are carried out using the same multiwell plate, with a minimum of handling required. Our method is suitable to set up high throughput assays of adipogenic differentiation.

Key words

Glycerol-3-Phosphate dehydrogenase, cellular assay, microtiter format.

Introduction

The presence of adipocytes in a cell culture can be monitored using methods such as staining with lipophilic dyes (Oil Red O or Nile Red), RT-PCR detection of marker genes (1), or biochemical measurement of enzymatic activities.

Among the enzyme activities reported to be modified during adipogenesis (2, 3), GPDH (EC 1.1.1.8) is the most commonly assessed. GPDH activity increases strongly in mature adipocytes (4-6). This cytoplasmic enzyme is involved in the triglyceride biosynthesis pathway, catalyzing the conversion of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (figure 1). Enzyme activity can be measured conveniently by following the oxidation of NADH (β-nicotinamide adenine dinucleotide, reduced form) during the reaction, which can be monitored by absorptiometry at 340 nm (7).

This assay has proven to be instrumental for the comparison of different adipogenic treatments in cell cultures. However, the protocol that was established in the 70's and widely used since then with minor modifications is tedious and does not allow high sample throughput. According to the original protocol, cells are harvested, broken by sonication, and the enzyme-rich cytosolic fraction is isolated by centrifugation (8). Amendments to this sample preparation procedure have been reported (1, 5, 9, 10), however, throughput remained very limited.

The method we propose here bypasses the most time-consuming step of the assay, namely the mechanical homogenization of the samples, while preserving the principle of the enzymatic dosage. Our protocol enables a fast and straightforward determination of

GPDH activity in 96-well format, thus allowing high throughput measurements of adipogenic differentiation.

Material and Methods

Cell culture

The 3T3-L1 preadipocytes were seeded at a density of ca. 10^4 cells per cm^2 in plastic multiwell plates (12-well plate or 96-well culture plates (Nunc, Basel, Switzerland)) and grown in MEMalpha / Ham's F12 medium (1:1) (Life Technologies, Basel) supplemented with 10% fetal calf serum (Life Technologies).

Adipogenic differentiation

To induce adipogenic differentiation, the PPARγ agonist BRL 49653 (synthesized at Novartis) was added to confluent preadipocyte cultures at indicated concentrations together with 0.1 mM 3-isobutyl-1-methylxanthine (IBMX, Fluka, Buchs, Switzerland) (1). In some experiments, cells were treated with Transforming Growth Factor β3 (TGFβ3, synthesized at Novartis) to block the adipogenic action of BRL 49653. Cultures were treated for indicated time periods, usually 3 – 11 days.

GPDH assay

Cells were grown and induced to differentiate in 96-well plates. For enzyme activity determinations, medium was removed and the cells were washed once with phosphate-buffered saline (PBS, calcium and magnesium-free). An ice-cold homogenization solution was then added (100 μL / well ; 20 mM Tris, 1 mM EDTA, 1 mM β-mercaptoethanol (BioRad, Glattbrugg, Switzerland), pH 7.3). Plates were either used directly ("fresh" samples) or stored at –20^{o}C until measurement ("freeze-thaw cycle"). Frozen plates were allowed to thaw at room temperature. Where indicated, mechanical

processing of samples was carried out by 6 consecutive passages through a G21 needle (Becton Dickinson, Basel).

To prepare the enzyme reaction, 90 μL of reaction mix (0.1 M triethanolamine (Fluka), 2.5 mM EDTA, 0.1 mM β-mercaptoethanol, 334 μM NADH (Roche, Basel), pH adjusted to 7.7 with HCl) was added to each well, and plates were pre-incubated for 10 min at 37°C. DHAP (Sigma, Buchs) was added to start the assay (10 μL / well of a 4 mM stock solution in H_2O). A Wallac® 1420 Victor^2 multilabel plate reader was used to measure OD_{340nm} at desired time intervals. The extinction coefficient for NADH under our assay conditions was determined experimentally (ε = 0.002724 μM^{-1}). The plate reader was programmed to keep the temperature of the plates constant at 37°C throughout the duration of the assay. Data were saved on computer and later analyzed using Microsoft Excel.

The protein content of cell cultures was determined in parallel wells with the $^{D}{}_{C}$Protein Assay (BioRad). Results are expressed as mU / μg protein (1 U = 1 μmol NADH / min). For control experiments GPDH from rabbit muscle was used (Roche).

Some experiments were carried out measuring NADH concentration in the classic 1.5-ml cuvette format (Semadeni, Ostermundigen, Switzerland) as described before (1). In this case, sample volume was 1 ml, and OD340nm was measured using a Shimadzu® UV-160 spectrophotometer.

For statistical analysis (fig. 4), we used Student's *t*-test (two-tailed, two samples with equal variance). Linear regression (fig. 2) and calculation of the correlation coefficient was carried out using Microsoft Excel.

Results

According to the established protocol, variations of NADH concentration during GPDH dosage are measured using 1.5-ml polystyrene cuvettes in a standard absorption spectrophotometer. We first tested whether the assay could be transferred to a microtiter plate reader as now available in most laboratories. To this end, we measured NADH concentration gradients (20 μM - 400 μM) using either polystyrene cuvettes or 96-well polystyrene plates, in a final volume of 1 ml and 0.2 ml, respectively. In both cases, perfectly linear relationships were obtained (data not shown). The differences in the device and the plastic material used resulted as expected in different extinction coefficients that could be determined experimentally. We also compared common polystyrene plates to special plates optimized for UV measurements. The latter yielded an up to 80% lower basal OD value measured at 340nm, however, standard polystyrene culture plates provide reliable data in the range of NADH concentrations encountered in this assay (data not shown). We therefore carried out all routine experiments with standard plates.

As the next step, we carried out kinetic measurements in a 96-well plate using samples with increasing GPDH enzyme concentrations (figure 2A). Initial rates were determined graphically and plotted against enzyme concentration (figure 2B). The expected linear relationship was obtained.

To test whether it would be possible to grow cells and to measure their GPDH activity on the same plate, we carried out enzyme assays in wells that had been used or not for cell culture. Half of a culture plate was seeded with 3T3-L1 cells that were grown to confluence, but not induced to differentiate, so GPDH enzyme activity is not detectable.

On the day of the assay, homogenization solution was distributed to both cell-containing and empty wells, and increasing concentrations of GPDH enzyme were added. As presented in figure 3, the presence of cells did not interfere with the determination of enzyme activity. Therefore, if it was possible to extract GPDH activity quantitatively from cells grown in the assay plate, it should become feasible to carry out the enzyme activity determinations in the same plate.

In the published GPDH assay procedures cell extraction is the most time-consuming step. The initial protocol involved sonication of samples, followed by successive centrifugations to isolate the enzyme-rich cytoplasmic fraction (5). These cellular extracts were then used directly, or could be stored after purification at –70^{o}C (4, 11). Other methods make use of syringes to break cells (C. Darimont, personal communication).

As GPDH is a cytoplasmic enzyme, we decided to apply a simple freeze-thaw cycle to achieve enzyme extraction. This approach has proven useful in the past to extract metabolites and soluble proteins from cells [12, and own unpublished results]. To test this method we grew cells in assay plates, induced adipogenic differentiation, and measured GPDH activity following 4 different combinations of sample treatments: lysis at 4^{o}C ('fresh') or lysis plus freeze-thaw cycle ('frozen'), with and without subsequent homogenization (syringe) of extracts, respectively.

As indicated in figure 4, we obtained comparable concentration-response patterns for BRL 49653 measured in fresh or frozen samples. Cell homogenization indeed improved GPDH recovery in fresh samples. With mechanical disruption, values were significantly higher than without. However, there was no statistically significant difference between the results from syringe-homogenized fresh samples and the treated or untreated frozen

samples. It is likely that freezing/thawing in homogenization buffer destroys cell structure sufficiently to quantitatively release soluble cytoplasmic enzymes. We also established that frozen samples could be stored at –20^{o}C for prolonged periods of time without loss of enzyme activity. We confirmed these observations with the following cell systems undergoing adipogenic differentiation: 3T3-F442a murine preadipocytes, rat primary bone marrow stromal cells, and primary human trabecular bone cells (data not shown).

Thus, by breaking cell membrane with a simple freeze/thaw cycle, we could adapt the GPDH enzyme activity assay to a 96-well plate format that is in principle suitable for high throughput screening.

Since its development, we applied this method to rapidly characterize a series of growth and differentiation factors on several cell systems. As an example, we show in figure 5 the anti-adipogenic activity of TGFβ exerted on 3T3-L1 cells. Cells were treated for 6 days with 0.1 mM IBMX and different concentrations of BRL 49653, and varying concentrations of TGFβ3 were added. TGFβ3 inhibited GPDH activity dose-dependently as described in the literature (13).

Discussion

Enzymatic determination of GPDH activity is very useful to study adipogenic differentiation in vitro. We have established experimental conditions that allowed adaptation of this assay to microtiter plate format and high throughput screening.

While the use of a plate reader for the GPDH assay has already been reported (14), it must be noted that in this case the multiwell plate was only used for OD reading at the end of the assay procedure. Cell samples had to be generated and processed separately.

In our method, the same multiwell plate is used from cell culture to assay readout, requiring only a minimum of handling at the individual steps. This procedure not only increases throughput, but also lowers experimental error considerably, as transfer and centrifugation steps or mechanical treatment of cells are omitted. The fact that plates can be stored at –20^{o}C for prolonged times and thus pooled for determination of enzyme activity is an additional positive point. Previous articles mentioned that samples had to be analyzed freshly (5, 6, 8, 10, 11), or after storage at –70^{o}C (4, 9) only after extensive sample processing.

All published protocols require more cell material than our assay. Decreasing the culture requirements from 12-well plates (11, 13) or 35-mm dishes (9, 15, 16) to 96-well plate format is a clear advantage. It allows testing of compounds or any biological material available only in small amounts. Although we have not tested this, we expect that it should be possible to further decrease sample volumes by adapting the assay to 384-well plate format.

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Figure legends

Figure 1 :

Principle of the dosage of GPDH activity based on the oxidation of NADH.

Figure 2 :

GPDH activity measurement. A) OD_{340nm} values measured over time for increasing concentrations of GPDH enzyme. B) GPDH activity values determined by the initial slope of OD_{340nm} kinetic curves. The r^2 correlation coefficient was calculated by linear regression analysis.

Figure 3 :

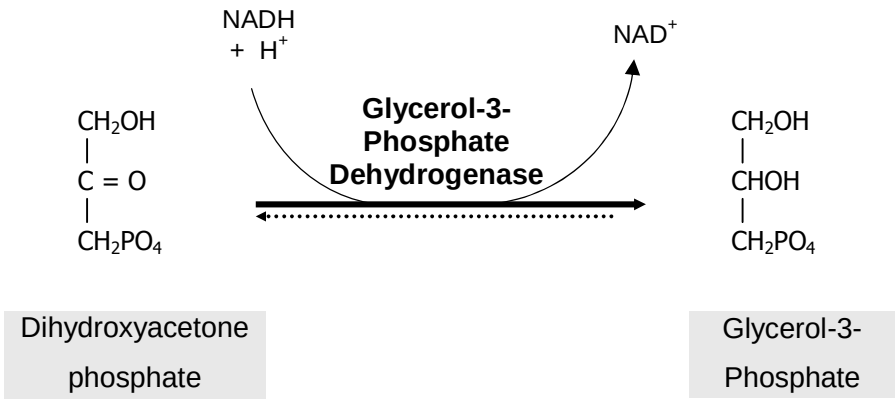
GPDH activity values measured in wells containing a cell layer (dark circles) or not (empty circles). Determinations were done in triplicate. Data are presented as mean ± SEM.

Figure 4 :

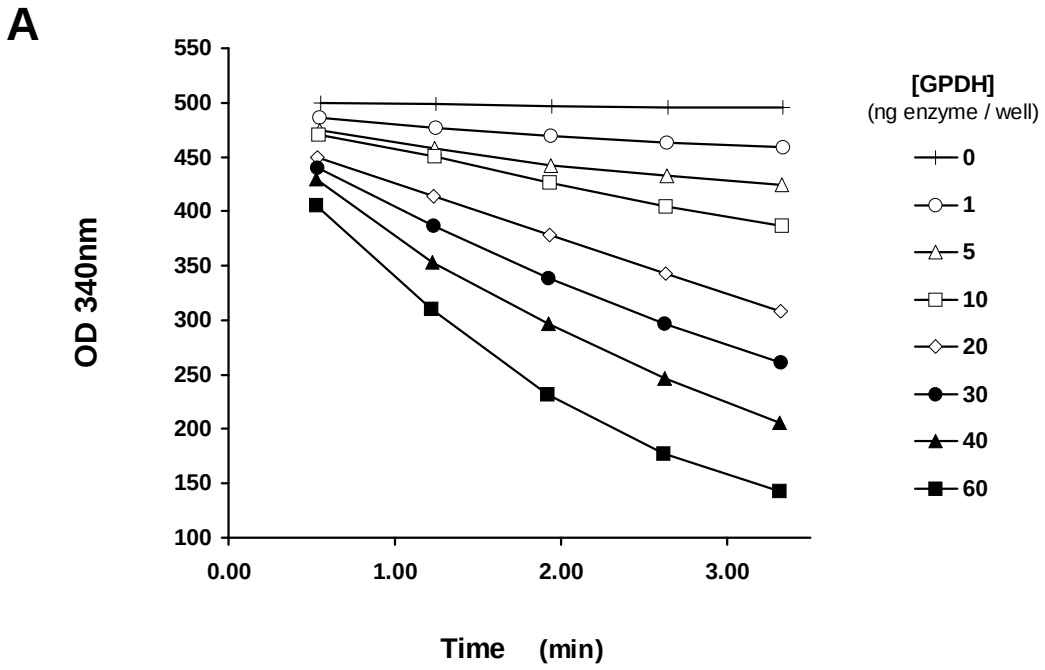
GPDH activity measured in fresh and frozen cultures of 3T3-L1 cells after 11 days of treatment (A) or 9 days of treatment (B). m.d.: mechanical disruption (syringe). Data are presented as mean ± SEM (n=3). Asterisks indicate statistical significance (**P<0.01, *P< 0.05) compared to the 'Fresh + m.d.' condition (A) or the 'Fresh' condition (B).

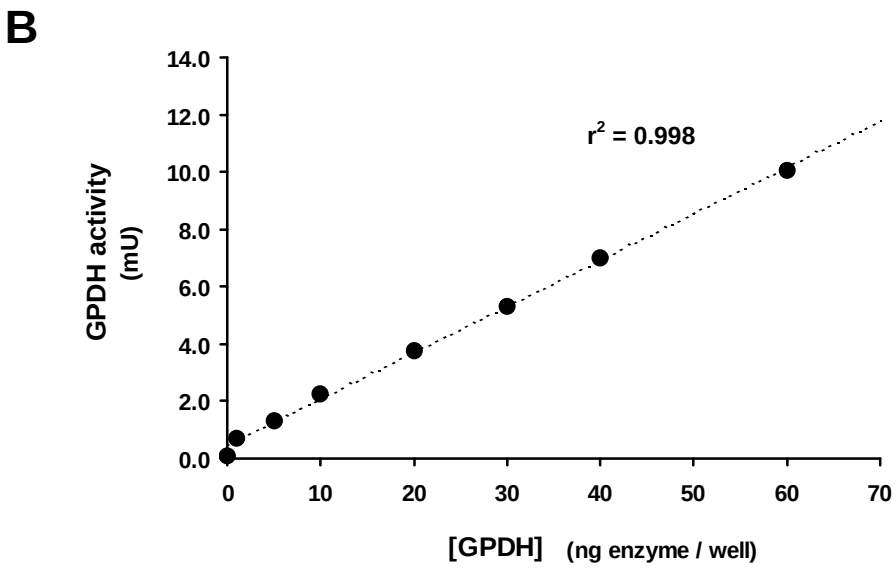
Figure 5 :

Dose-dependent inhibition of GPDH activity by TGFβ3 measured in 3T3-L1 cultures after 6 days of treatment. Data are presented as mean ± SEM (n=5).

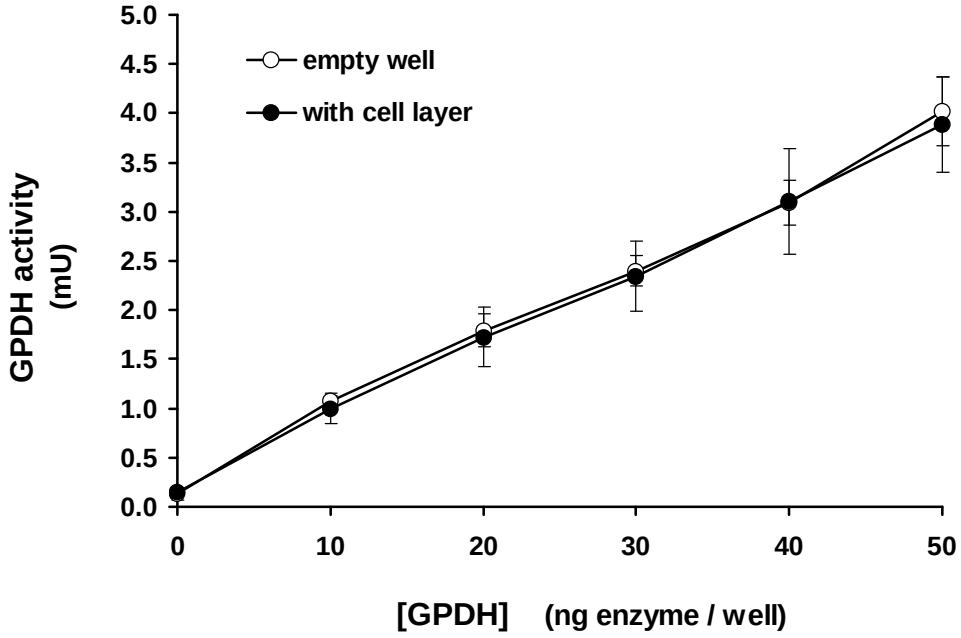


Sottile & Seuwen Fig.1



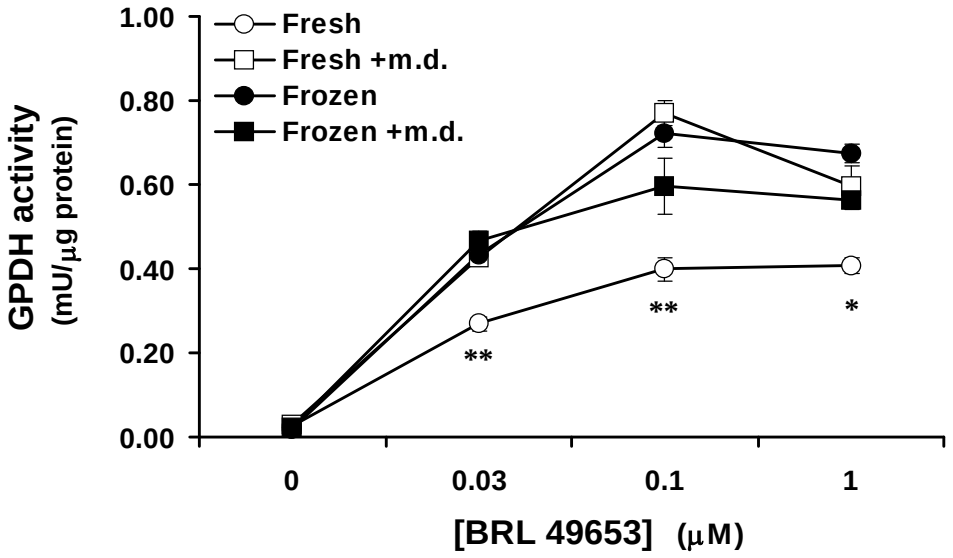


Sottile & Seuwen Fig.2



Sottile & Seuwen Fig.3

A



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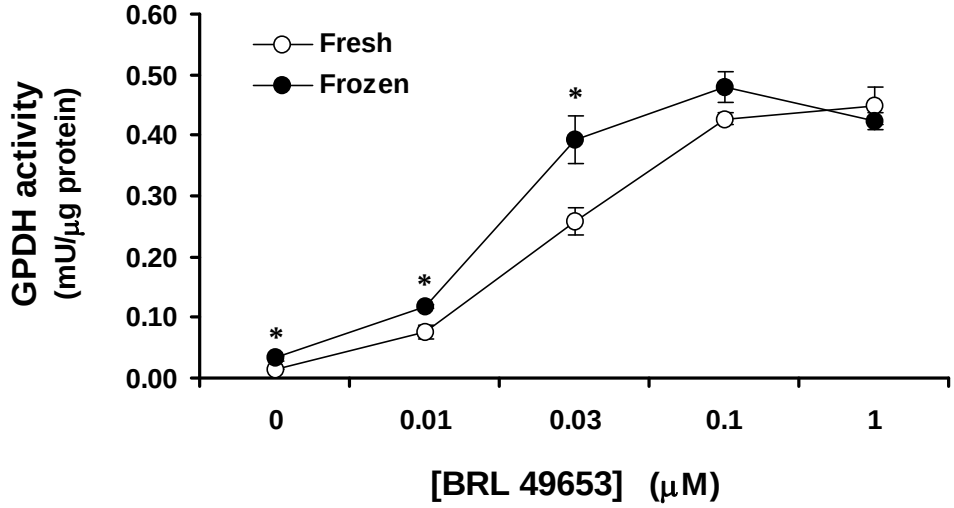
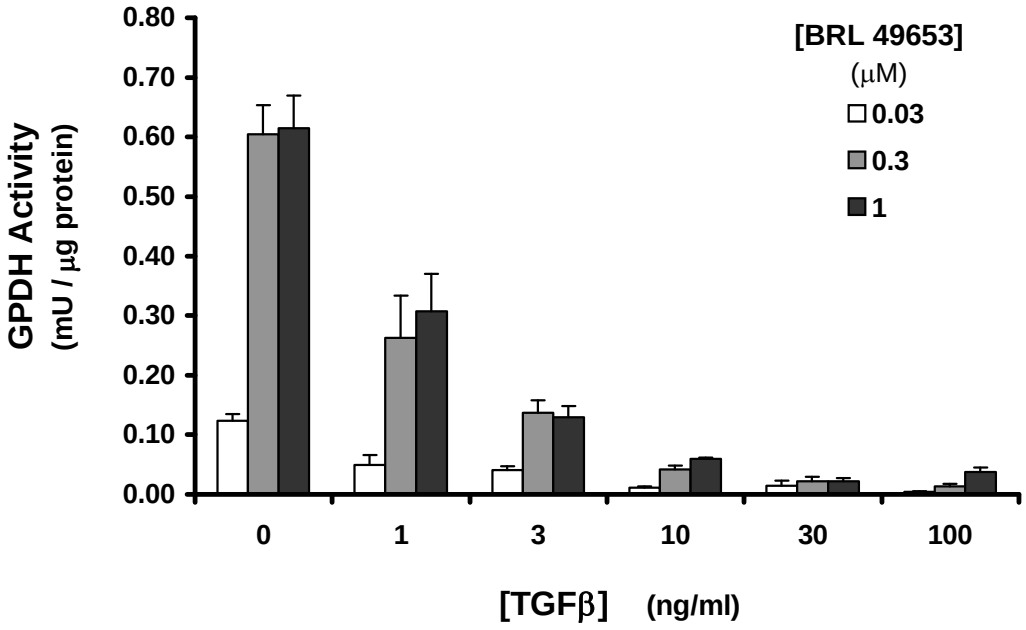


Sottile & Seuwen Fig.4



Sottile & Seuwen Fig.5