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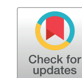
Research article

A colorimetric bioassay for quantitation of both basal and insulin-induced glucose consumption in 3T3-L1 adipose cells



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ABSTRACT

Introduction: The quantitation of glucose consumption in animal cell cultures is mainly based on the use of radiolabeled or fluorescent analogues, resulting in expensive and tedious procedures, requiring special equipment and, sometimes, with potential health and environmental risks.

Objectives: The objective of this work was to evaluate the application of a blood plasma colorimetric assay to quantify glucose consumption in *in vitro* cultures of adipose cells.

Methods: We worked with 3T3-L1 adipose cells differentiated by 7–8 days, which were exposed to different initial glucose concentrations (5.5, 2.8 and 1.4 mM) for variable times, either in the absence or the presence of 100 nM insulin. Using a commercial colorimetric glucose assay, extracellular glucose was determined, and glucose uptake was calculated as the difference between the initial and final glucose concentration.

Results: The colorimetric assay allowed us to quantify glucose uptake in our cell model, observing a linear response over time ($r^2 \geq 0.9303$) to the different glucose concentrations, both in the basal and insulin-induced condition. The insulin-stimulated glucose consumption was higher than basal consumption at all glucose concentrations evaluated, but significant differences were observed at 120-, 360- and 480-min in glucose 5.5 mM ($p \leq 0.01$, $n = 5$), and 240 min in glucose 1.4 mM ($p \leq 0.01$, $n = 5$). A V_{max} of 4.1 and 5.9 nmol/ml/min (basal and insulin-induced, respectively) and a K_m of 1.1 mM (same in basal vs insulin-stimulated) were calculated. The bioassay was also useful in a pharmacological context: in glucose 1.4 mM, glucose consumption showed an effect that depended on insulin concentration, with a calculated EC_{50} of 18.4 ± 1.1 nM.

Conclusions: A simple and low-cost bioassay is proposed to quantify glucose consumption in 3T3-L1 adipose cells.

1. Introduction

Glucose consumption in cells and tissues is a major physiological process, and its alteration is related to diabetes and other diseases [1, 2, 3]. Traditional assays to evaluate glucose uptake have been based on the use of radiolabeled glucose analogues such as [U–^{14}C] glucose, [^{14}C] 2-deoxi-D-glucose and [^{3}H]-2-deoxiglucose among others [4, 5]. Although radioactive methods are highly sensitive, their main disadvantages are the cost of equipment and reagents, and the need for safety protocols and appropriate physical spaces to minimize health and environmental risks. As an alternative, the use of fluorescent glucose derivatives, such as 2-NBDG, has been reported [6, 7, 8]. Nevertheless, this technique depends on the availability of a fluorimeter or a fluorescence microscope for the analyses.

On the other hand, glucose evaluation in clinical samples, such as blood, urine and/or cerebrospinal fluid, has been traditionally based on the use of colorimetric methods [9]. Given the key role of the adipocyte as an energy reservoir, which is reflected in its significant glucose uptake, we hypothesize that a colorimetric assay used for clinical samples should also be useful to evaluate glucose consumption for *in vitro* cultures of adipose cells. Consistent with this, in a recent preliminary study we showed that a blood glucose colorimetric assay was useful to assess the extracellular glucose concentration in 3T3-L1 adipose cells and could also be useful to measure glucose consumption in these cells [10].

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However, the optimal conditions of this assay to the assessment of glucose consumption have not yet been determined. Here, we report the successful application of this blood glucose colorimetric assay to evaluate glucose consumption in 3T3-L1 adipocytes. This assay was also successfully applied to the calculation of the kinetic parameters of the glucose consumption process and to a pharmacological analysis of the concentration-response relationship for insulin stimulation.

2. Materials and methods

2.1. Materials

The 3T3-L1 cell line was purchased from the American Type Culture Collection (ATCC® CL-173™). 3T3-L1 cells are derived from embryonic mouse cells and are committed to differentiating into mature adipose cells [11, 12]. DMEM high glucose (4500 mg/L) medium (cat. 12800017), DMEM no glucose medium (cat. 11966025), FBS (cat. 16000044) and Penicillin/Streptomycin (cat. 15140122) were purchased from GIBCO (Grand Island, NY, USA). Insulin (cat. I0516-5ML), IBMX (cat. I5879-100MG), dexamethasone (cat. D4902-100MG) and Oil Red O (cat. O0625-25G) were purchased from Sigma-Aldrich (Saint Louis, USA). Trypsin-EDTA (cat. L-0930-100) and PBS (cat. L0615-500) were purchased from Biowest (Nuaillé, France). Other materials were purchased from different vendors, including glucose from PanReac (Barcelona, Spain; cat. 141341.1210), sodium bicarbonate from Merck (Darmstadt, Germany; cat. 1063291000) and rosiglitazone from United States Pharmacopeia (USP) (Rockville, USA; cat. 1605817). Finally, the glucose assay kit was purchased from BioSystems (Barcelona, Spain; cat. 11504).

2.2. Cell culture

Cells were grown in T75 cell culture flasks in complete medium (DMEM high glucose medium supplemented with 10% FBS, 1.5 g/L sodium bicarbonate, 100 U/mL penicillin and 100 g/mL streptomycin) at 37 °C in 5% CO_2. Medium was changed every two days. At a confluence of 60–80%, cells were treated with 0.25% Trypsin-EDTA and subcultured in 24-well plates (to document cell differentiation) or 48-well plates (to evaluate glucose consumption). All these plates were pretreated overnight at 4 °C with 20 mg/ml gelatin, and the initial density of cells was 8,000 cells/cm^2.

2.3. Cell differentiation

Cells seeded on 24 or 48 well plates were grown in complete medium up to 100% confluence. At day 2 post confluence, cells were changed to differentiation medium (complete medium supplemented with 10 μg/ml insulin, 0.1 μM dexamethasone, 0.5 mM IBMX and 2 μM rosiglitazone) by 48 h, and then returned to complete medium for 7–8 days of differentiation, with media changes every other day [13, 14, 15].

2.4. Lipid staining

Adipose cells were stained with Oil red O, a chemical reagent with affinity for neutral lipids and cholesterol esters which allows the observation of intracytoplasmic lipids in these cells [16]. A stock solution of 0.35% Oil red-O in isopropanol was prepared, filtered (0.22 μm) and stored at 4 °C. At the time of use, a working solution was prepared by diluting the stock solution in distilled water at 3:5 ratio and filtering again. Cells were first washed with 10% formaldehyde in PBS and then fixed with the same solution for 1 h at room temperature. Subsequently, two washes were performed with 60% isopropanol and the plates were let dry at room temperature. Adipocytes were then stained with Oil Red O's working solution for 15 min at room temperature, followed by 3–4 washes with distilled water. Finally, plates were let dry at room temperature and stained cells were photographed using a Nikon Eclipse TE300 inverted microscope from the *Laboratorio de Microscopía Óptica de la Universidad Nacional de Colombia*.

2.5. Glucose consumption

Adipocytes differentiated by 7 days in 48-well plates were fasted in DMEM with no glucose for 1 h, and then placed in DMEM at a fixed initial glucose concentration (1.4, 2.8 or 5.5 mM), either in the absence (basal consumption) or in the presence of 100 nM insulin (stimulated consumption).

At the time indicated in each figure, the extracellular glucose concentration was evaluated using a glucose assay kit, which is based on the method described by Trinder [17]. Briefly, this method uses two coupled enzymatic reactions (glucose oxidase and peroxidase) to transform glucose into quinoneimine, which is detected spectrophotometrically at 500 nm. The reaction was adapted for a 96-well format, where 2 μL of sample (or standard) and 200 μL of reagent were mixed and was incubated for 10 min at room temperature (16–25 °C) as described by the manufacturer. A 1 mg/ml glucose solution was used as standard. The absorbance at 492 nm was read in a BioRad microplate reader and analyzed with the Microplate Manager®6 software. Glucose consumption was calculated as the difference between initial glucose (IG) and extracellular glucose (EG) according to the equation:

$$Consumption = IG - EG \tag{1}$$

2.6. Kinetic parameters for glucose consumption

Consumption rates were calculated from the time-course curves for glucose consumption. The relationship between consumption rate and glucose concentration was adjusted to a saturation model, according to the following equation:

$$y = \frac{V_{max} * x}{K_m + x} \tag{2}$$

where, V_{max} refers to maximum rate and K_m to the Michaelis constant, respectively.

2.7. Concentration-response relationship for insulin

Adipocytes differentiated for 7 days in 48-well plates were exposed to different insulin concentrations (0–200 nM) in DMEM with 1.4 mM glucose. After 240 min, glucose consumption was assessed as described previously (Eq. 1) and the relationship between the fractional glucose consumption and insulin concentration was fitted to the four-parameter logistical equation (Eq. 3) as follow:

$$y = d + \frac{(a - d)}{1 + \left(\frac{x}{c}\right)^b} \tag{3}$$

where a and d respectively refer to minimum and maximum glucose consumption, c refers to EC_{50} and b is the Hill coefficient (or Hill slope) of the curve in the area of 10–90% response.

2.8. Analytical parameters LOD and LOQ

From a stock solution of 5.55 mM glucose (1 mg/ml), a set of serial dilutions was prepared in DMEM medium. The glucose concentration of each dilution was then measured with the Glucose reagent (BioSystems), and calibration curves were built. The Lower limit of Detection (LOD) and Limit of Quantitation (LOQ) were calculated from the regression analysis applied to these curves, according to:

$$LOD = \frac{3*S_b}{m}$$

$$LOQ = \frac{10*S_b}{m}$$

where m is the calibration sensitivity (curve slope) and S_b is the standard deviation of blank.

2.9. Statistical analysis

To assess whether the data were parametric, a homoscedasticity analysis, using the Hartley F_{max} test [18], and a normality analysis, using the Kolmogorov-Smirnov test [19], were performed. The comparison between basal and insulin-stimulated consumption was made with the *t* test [20] for parametric data, or with the Mann-Whitney test [21] for nonparametric data. The comparison between different treatment times was made by ANOVA [22], followed by a multiple comparison analysis with the Tukey test [23] for parametric data, or the Kruskal-Wallis test [24] followed by the Dunn test [25] for nonparametric data. Statistical analyses and graphical representations were performed with GraphPad Prism version 6.0c for Mac [26].

3. Results

3.1. In vitro culture and differentiation of 3T3-L1 cells

Figure 1 shows images of the cells at day 8 of differentiation. Differentiated cells (C, D) had a larger size than control cells (A, B). In addition, intracytoplasmic lipid droplet accumulation was observed only in differentiated cells, which was confirmed by Oil Red O staining (Compare B and D). These results confirm the mature adipose phenotype of our differentiated cells.

3.2. Analytical parameters LOD and LOQ

To evaluate the analytical parameters of the glucose test, calibration curves of absorbance versus glucose concentration were constructed, and parameters were calculated as described in methodology. Figure 2 shows a linear relationship across the entire range of glucose concentrations evaluated (from 0 to 5550 nmol/ml), with a correlation coefficient (r^2) of 0.9959 (Figure 2A). The low concentration region of this curve (0–350 nmol/ml) is shown in Figure 2B. Based on this analysis, a LOD of 132.5 nmol/ml and a LOQ of 401.5 nmol/ml were calculated. We highlight that all glucose consumption data presented in this paper were obtained by working within the linear range of this assay and above the LOQ.

3.3. Glucose consumption in 3T3-L1 cells

Glucose consumption in adipocytes differentiated for 7 days on 48-well plates was then evaluated at different initial concentrations of glucose, either in the absence (-Ins) or in the presence of 100 nM insulin (+Ins). The results are shown in Figure 3. As expected, the insulin-stimulated consumption was higher than basal consumption for all conditions of initial glucose assessed. However, differences between basal and stimulated consumption were not statistically significant to all incubation times. At 5.5 mM glucose, significant differences were observed at 120-, 360- and 480-min incubation (Figure 3A). Surprisingly, at 2.8 mM glucose, differences were not significant at any time, although a clear trend of higher consumption in response to insulin were observed (Figure 3B). At 1.4 mM glucose, significant differences were only observed at 240 min (Figure 3C). Where significant differences were observed, the insulin-stimulated consumption turned out to be 1.5–2.5-fold higher than basal consumption, depending on the specific condition, a result that was reproducible between different trials.

In addition, glucose consumption was linear over time, both in basal and insulin-stimulated conditions. In fact, the regression analysis revealed a linear correlation between 0 and 480 min, with Pearson coefficients (r^2) ranging from 0.9303 to 0.9969 for the different glucose conditions evaluated (Figure 4).

Based on these results, consumption rates and kinetic parameters for this process were calculated. Figure 5 shows the relationship between consumption rate and glucose concentration, where a good fit to a saturation model is observed (r^2 = 0.9624 and 0.9967 for basal and insulin-stimulated consumption, respectively). From this analysis, the

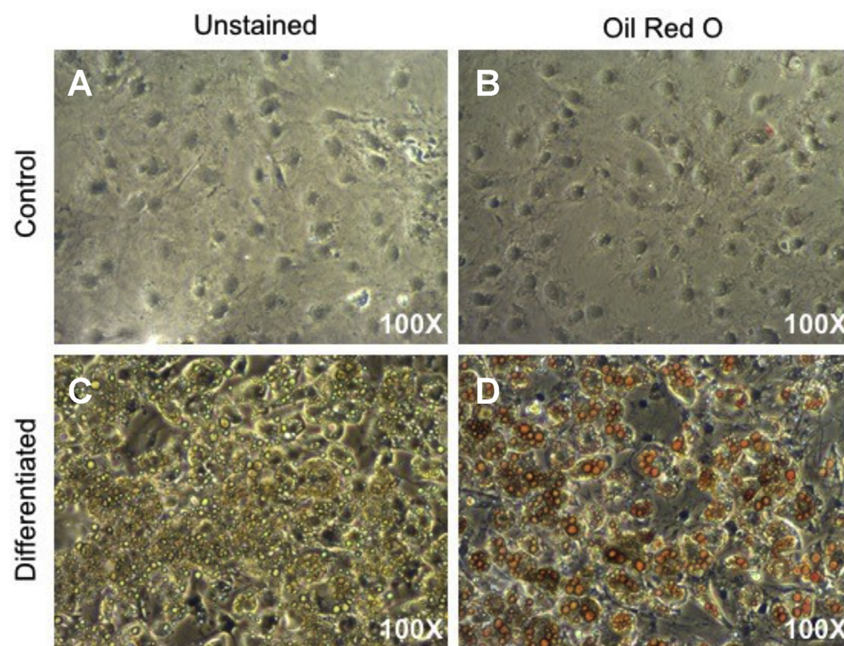

Figure 1. Differentiation of 3T3-L1 cells. The cells were grown in 24-well plates and differentiated as described in methods. Representative images of control cells (A, B) and differentiated cells (C, D) are shown at day 8 of differentiation. Both undyed cells (A, C) and lipid stained cells with red oil O (B, D) are shown. (Magnification 100x).

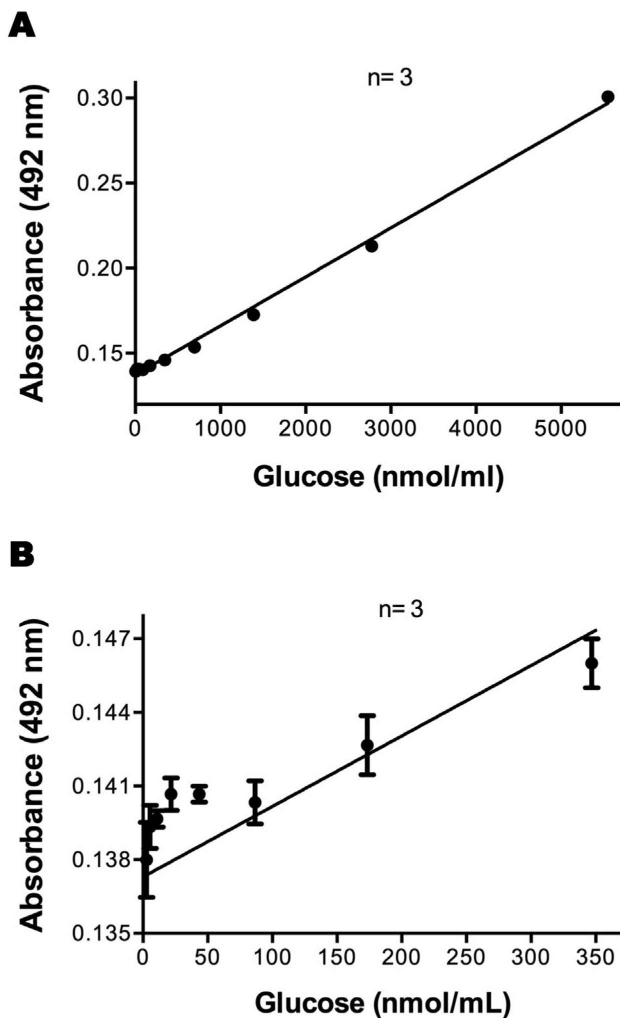

Figure 2. Calibration curve for glucose test. A set of serial glucose dilutions in DMEM no glucose medium was prepared and the glucose concentration of each dilution was determined using a colorimetric glucose reagent (Biosystems, Barcelona, Spain) as described in methodology. (A) Curve showing the entire range of concentrations evaluated (0–5550 nmol/ml). (B) Curve showing only the range of low concentrations (0–350 nmol/ml). The average and standard mean error (SEM) of three tests are represented, each performed in triplicate (n = 3). Symbols represent our experimental data, and the curve represents the regression curve. Linear regression equation: $y = 2.876x + 0.1373$ ($r^2 = 0.9959$).

kinetic parameters of the process (V_{max} and K_m) were calculated (Table 1). As observed, V_{max} in insulin-stimulated consumption was 1.4-fold higher than in basal conditions, while K_m had the same value in both conditions.

Finally, our bioassay was evaluated in terms of its behavior in a pharmacological analysis looking at the concentration-response relationship for insulin stimulation. Our result shows the expected dependence between glucose consumption and insulin concentration (Figure 6). From these data, an EC_{50} of 18.4 ± 1.1 nM was calculated. This result also indicates that our results above (Figure 3), where 100 nM insulin was used to stimulate glucose consumption, were carried out in the area of maximal response of this assay.

4. Discussion

In this work, a colorimetric glucose test marketed for blood and urine samples was applied to quantify glucose consumption in adipocytes. Our work was carried out in 3T3-L1 cells, which were first differentiated into adipocytes by treatment with a mix of previously described

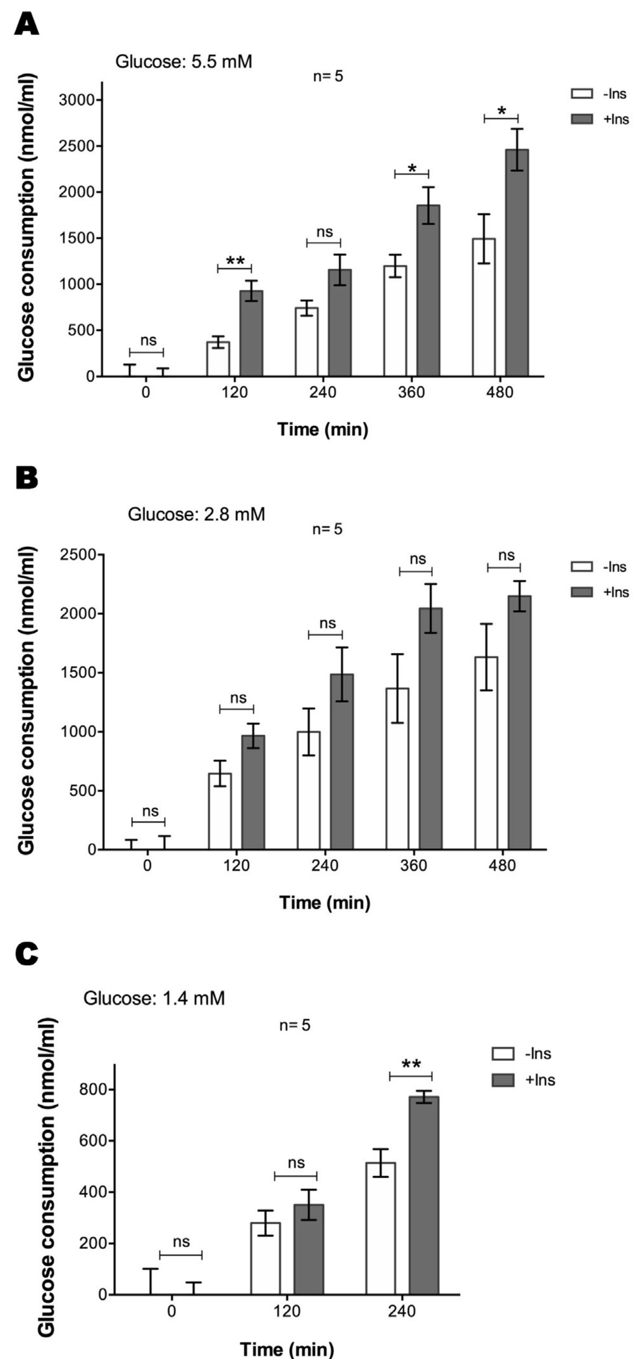

Figure 3. Glucose consumption in 3T3-L1 adipocytes. Cells grown in 48-well plates and differentiated for 7 days were fasted (no glucose) for 1 h and then incubated in DMEM medium at different glucose concentrations: 5.5 mM (panel A), 2.8 mM (panel B) and 1.4 mM (panel C), as described in the methods. At each indicated time, both basal (-Ins) and insulin-stimulated glucose consumption (+Ins) were evaluated. The stimulation was with insulin 100 nM. The data represent the average and standard error of the mean (SEM) of five different experiments, each performed in triplicate (n = 5). In C, data only up to 240 min are shown because at longer times they were below the LOQ of this bioassay. ns, not significant ($p > 0.05$); *$p \leq 0.05$, **$p \leq 0.01$.

differentiation agents. According to this procedure, mature adipocytes were obtained by day 7 of their differentiation. The consumption of glucose in these cells was determined by quantifying the amount of glucose in the extracellular culture medium using the colorimetric test. To identify optimal bioassay conditions, glucose consumption was

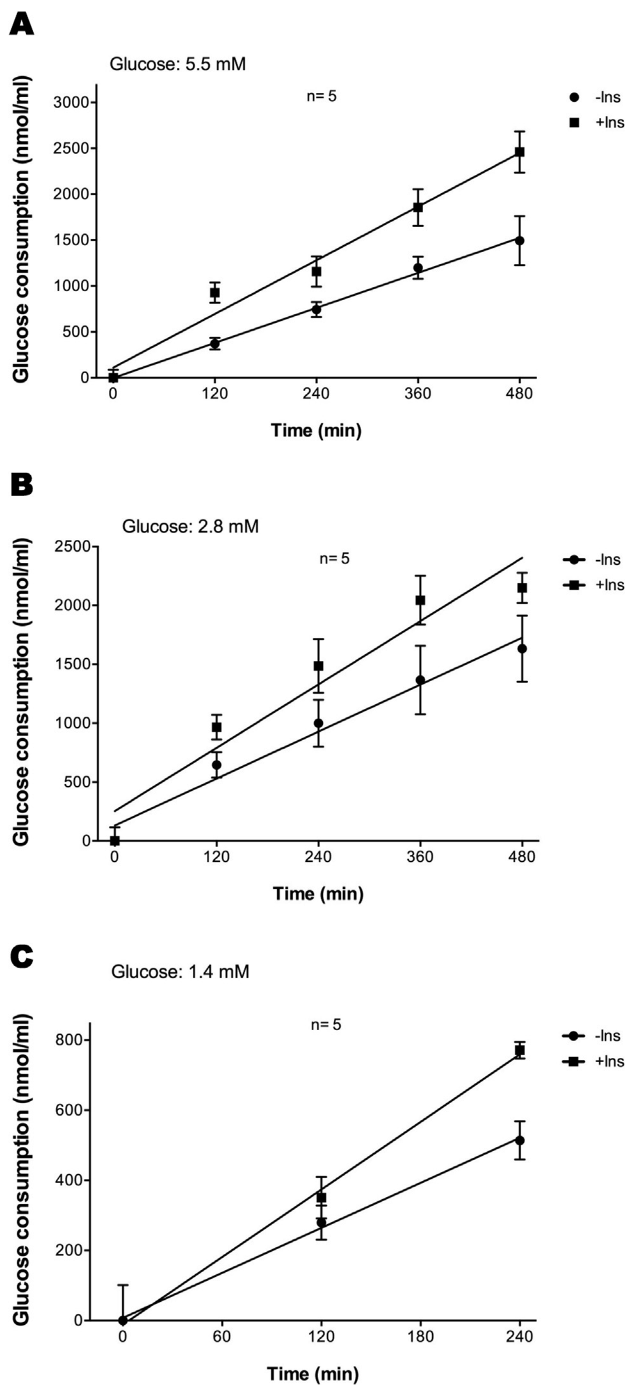

Figure 4. Time course for glucose consumption in 3T3-L1 adipocytes. A linear regression analysis was performed with the data obtained for glucose consumption. The average and standard error of the mean (SEM) of five experiments is presented, each performed in triplicate (n = 5). Equations (and r^2 values) describing these curves are: (**A**) for 5.5 mM glucose: $y = 3.180x - 1.556$, $r^2 = 0.9969$ (-Ins); $y = 4.875x + 110.5$, $r^2 = 0.9767$ (+Ins). (B) for 2.8 mM glucose: $y = 3.320x + 131.9$, $r^2 = 0.9718$ (-Ins); $y = 4.480x + 253.9$, $r^2 = 0.9303$ (+Ins). (C) for 1.4 mM glucose: $y = 2.141x + 7.583$, $r^2 = 0.9831$ (-Ins); $y = 3.214x - 11.67$, $r^2 = 0.9853$ (+Ins).

evaluated over time at different glucose concentrations (1.4, 2.8, and 5.6 mM), either under basal or insulin-stimulated conditions. The results showed that insulin-induced glucose consumption was higher (1.5–2.5-fold) than that of basal consumption, with statistically significant differences that depended on the initial glucose concentration and the duration of incubation. In fact, significant differences in glucose consumption were observed at ≥120 min in 5.5 mM glucose and at 240 min in 1.4 mM glucose. Interestingly, no significant differences were observed at any time (0–480 min) at 2.8 mM glucose, although there was a clear tendency towards greater consumption in the insulin-stimulated condition. Based on these results, two optimal experimental conditions were established to evaluate glucose consumption in 3T3-L1 adipocytes with our bioassay: *(i)* 5.5 mM glucose for 120 min; *(ii)* 1.4 mM glucose for 240 min, with 100 nM insulin stimulation in both cases.

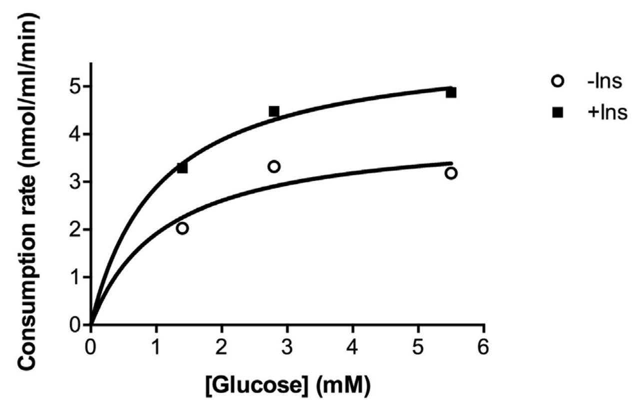

Figure 5. Relationship between consumption rate and glucose concentration. Based on our glucose consumption results over time, consumption rates were calculated at different glucose concentrations (0–5.5 mM), either under basal (-Ins) or insulin-stimulated (+Ins) conditions. Experimental data were fitted to a saturation model, as described in the methods.

To evaluate the behavior of our bioassay in a pharmacological context, concentration-response experiments were conducted for insulin stimulation. At 1.4 mM glucose, an EC_{50} value of 18.4 ± 1.1 nM was calculated. This value is consistent with EC_{50} values reported by other authors using both radioactive (~2 nM) or fluorescent methods (~30 nM) [27, 28, 29]. In addition, the concentration-response curve obtained with our bioassay clearly shows that 100 nM insulin induces a maximal pharmacological response. This same insulin concentration has been used by other authors [30, 31, 32]. Thus, the concordance between our pharmacological data and those reported by other authors using radioactive and fluorescent methods validates the colorimetric bioassay described here.

The bioassay described here was also useful to calculate the kinetic parameters V_{max} and K_m for glucose consumption. According to the results, insulin-stimulated V_{max} was 1.4 times higher than basal V_{max} (5.9 vs 4.1 nmol/ml/min, respectively), while K_m remained the same (1.1 mm) under both conditions. These results are consistent with those of different authors using 2-deoxyglucose (2DG) as a radioactive probe who have also reported an increase in V_{max} [33, 34, 35, 36]. Furthermore, our calculated K_m (1.1 mM) are in the same order as K_m values previously reported by the same authors (2–7 mM) [33, 34, 35, 36].

Glucose consumption is a multistage process involving a first rate-limiting step of membrane transport carried out by GLUT proteins [37, 38, 39, 40, 41], followed by metabolism mediated by enzymes of the glycolytic and downstream pathways [42, 43]. The glucose analogue 2-deoxyglucose (2DG) is taken up by cells and phosphorylated to 2-deoxyglucose-6-phosphate (2DG6P) by glucokinase (GK) but is not further metabolized and accumulates inside cells proportional to its uptake. Recently, during the execution of our work, a colorimetric assay to evaluate glucose uptake based on the use of 2-deoxyglucose (2DG) was marketed [44]. In this assay, glucose uptake is quantified by determining the intracellular concentration of 2DG6P through different enzymatic reactions that eventually produce TNB (5-thio-2-nitro-benzoate), which is detected at 412 nm. This procedure involves a stage of cell lysis in a dry ice/ethanol bath or in liquid nitrogen, which is not required in our bioassay. In this sense, our method seems to be simpler than this other method [44]. Another difference lies in the time required to perform the

Table 1. Kinetic parameters of glucose consumption.

Parameter	-Ins	+Ins
V_{max} (nmol/ml/min)	4.1	5.9
K_m (mM)	1.1	1.1

The consumption rate was calculated at different glucose concentrations and the data were fitted to a saturation model (Eq. 2) as described in Methods. Abbreviations: *Ins*, Insulin; V_{max}, maximum rate; K_m, Michaelis constant.

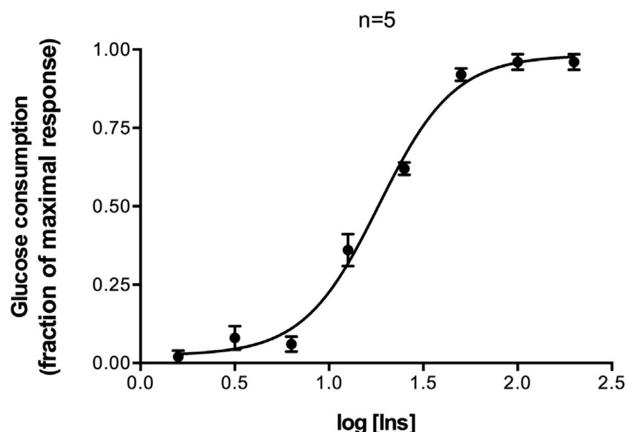

Figure 6. Concentration-response curve for insulin-stimulated glucose consumption in 3T3-L1 adipocytes. After 1 hour of glucose starvation in 48-well plates, adipocytes were incubated in glucose 1.4 mM at different insulin concentrations (0–200 nM) for 240 min, and glucose consumption determined with the colorimetric bioassay described in the methods. The average and SEM of five experiments is presented, each by quadruplicate (n = 5). The line represents the best data fit to a 4-parameter logistic regression model as described in the methods.

assay. Considering the post glucose-fasting steps, the 2DG method requires ≥200 min, while our method takes 130 min when using 5.5 mM glucose. Therefore, our bioassay is also more convenient. Finally, another important difference between both methods is that 2DG is a non-metabolizable probe, so the 2DG-based method is focused on the transport step [37]. In contrast, our method encompasses not only the transport step but all steps of the consumption process (transport and metabolism), which makes our K_m an apparent Michaelis constant.

A key advantage of our bioassay is its low cost. Considering the reagent and materials required for the glucose test, we calculate a cost of approximately US$ 0.40 per sample (staff, taxes and cell culture materials excluded), which is significantly lower than any currently available assay. Additionally, the cost of a microplate reader is lower than any equipment required for radioactive or fluorescence methods. In addition, not using radioisotopes minimizes the health risk to staff and the risk of environmental contamination. It is a simple bioassay because it does not require cell lysis, protective equipment, or special protocols for the management of hazardous substances.

One potential disadvantage of our bioassay is that culturing the cells in 48-well plates makes our procedure more laborious than in 96-well plates. Our first attempts to reproduce our results in a 96-well format were not successful. Secondly, in terms of the duration of our method, it takes more time (130 min) than any radioactive and fluorescent method (~30 min) [37]. Finally, we observed considerable variability between replicas, however, three to four replicas per sample were enough to achieve reproducible results in our work.

Taken together, our results clearly show the usefulness of the colorimetric bioassay described here to evaluate glucose consumption in adipose cells. Therefore, we propose this bioassay as a simple and low-cost alternative to the currently available methods. We envision multiple applications for this bioassay, including clinical, educational, and research applications. The evaluation of pharmacological agents and the identification of new molecular targets related to diabetes and obesity are an important focus. In fact, by applying this bioassay we have identified molecular targets potentially related to insulin resistance in 3T3-L1 cells (manuscript in preparation). It will be interesting to evaluate the behavior of our bioassay in other cellular contexts, such as primary adipocytes, muscle cells and others. It will be also interesting to evaluate the behavior in a 96-well plate format, which would make the procedure easier and could lead to its potential automation.

Declarations

Author contribution statement

P. Rivera: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

D. Gomez and A. Ondo-Méndez: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

C. Gómez-Alegría: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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