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Research paper

A nonradioisotope chemiluminescent assay for evaluation of 2-deoxyglucose uptake in 3T3-L1 adipocytes. Effect of various carbonyls species on insulin action

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ABSTRACT

We have developed a rapid nonradioisotope chemiluminescent assay adapted to high-throughput screening experiments, to evaluate glucose uptake activity in cultured cells. For chemiluminescence quantification of 2-deoxyglucose, we used a luminol oxidation reaction after an enzymatic dephosphorylation of 2-deoxyglucose-6-phosphate. All reactions were performed at 37 °C by consecutive addition of reagents, and the assay is able to quantify 2DG in picomole per well. To confirm the reliability of this method, we have evaluated the dose–effect of insulin, GLUT4 inhibitors and insulin-sensitizing agent on 2DG uptake into 3T3-L1 cells. The results obtained with the assay for 2DG uptake in vitro in the absence or presence of insulin stimulation, were similar to those obtained by the previous radioisotopic and enzymatic methods. We have also used this assay to evaluate the effect of various reactive carbonyl and oxygen species on insulin-stimulated 2DG-uptake into adipocytes. All reactive carbonyl species tested decreased insulin-stimulated glucose uptake in a time- and dose-dependent manner without affecting basal glucose uptake in 3T3-L1 cells. 4-hydroxynonenal was found to be the most potent in the impairment of glucose uptake. This new enzymatic chemiluminescent assay is rapid and useful for measurement of 2DG uptake in insulin-responsive in cultured cells.

1. Introduction

The route of glucose entry into the body plays an important role in the distribution of administered glucose and overall glucose homeostasis. Its stimulation by insulin is of exquisite sensitivity and responsiveness. The two major transporters expressed in adipose tissue/skeletal muscle are specific glucose transporter GLUT4 and the ubiquitous transporter GLUT1. The molecular mechanisms underlying the glucose transport and its regulation are similar for adipose and muscle cells. Consequently, adipocytes are more widely used for transport studies than myocytes due to their more accessibility and more pronounced insulin responsiveness. Uptake of glucose in cultured cells is commonly determined by using non-metabolizable radioactive hexoses, such as 3-*O*-methylglucose (3MG) or radioisotope [14C or 3H]-labelled 2-deoxy-D-glucose (2DG) analogues [1,2]. 2DG is transported into cells via identical transporters than glucose, where it is phosphorylated to a stable and membrane impermeable derivative, 2-deoxy-D-glucose-6-phosphate (2DG6P), by hexokinase or glucokinase, which may accumulate at a specific intracellular compartment, ensuring less rapid equilibration, slower kinetics and more convenient measurement of 2DG transport, compared to 3MG [3].

These radioisotope-based monitoring methods allow the cumulative measurement of cellular glucose at a fixed time point and facilitate significant advances in biomedical studies and diagnosis of various diseases, especially metabolic diseases [4,5]. However, routine use of these radiolabelled analogues is costly and requires a specialized institution where isotopes can be handled. Consequently, many efforts have been spent during the last decade in the development of assay using non-radioactive tracers, which are compatible with the analysis of glucose transport with sufficient insulin responsiveness.

Hence, fluorescence-based monitoring of cellular glucose uptake is a suitable alternative of radioisotope-based monitoring system in laboratory research. The fluorescent probe 6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-6-deoxy-D-glucose (6-NBDG) was first

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introduced in 1985 for the study of the human erythrocyte glucose transporter [6]. It cannot be phosphorylated by hexokinase contrary to 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG), introduced in 1996 for the study of *Escherichia coli* and first used in mammalian cells in 2000 [7,8]. A fluorescent analogue of 2-NBDG, was synthesized and has been used in various studies, especially for exploring cellular metabolic functions associated with GLUT systems [9–12]. However, 2-NBDG has poor photophysical properties in aqueous solution and does not compete strongly with D-glucose during cellular uptake under physiological conditions.

To overcome the limitations of 2-NBDG, such as weak fluorescence intensity, high treatment dosage, and non compatibility in physiological conditions, Yamamoto and co-workers have developed a method based on the assay of 2DG6P, which is monitored by the resazurin–diaphorase amplified detection of NADPH produced during the oxidation of 2DG6P by glucose-6-phosphate dehydrogenase (G6PDH). This assay system could detect the fluorescence intensity induced by uptake of 2DG into adipocyte cells as well as in muscle tissue [13]. However, this fluorimetric assay system is time consuming and involves many steps including particularly an inconvenient heat treatment at 85 °C to destroy endogenous NAD(P)H, NAD(P) and enzymes. Other authors have also reported an enzymatic method for measurement of 2DG uptake in both insulin-responsive tissues and differentiated 3T3-L1 adipocytes based on previously developed methods [14,15] and adapted to photometric analysis. This assay is similar than the previously enzymatic methods but for photometric quantification of the small amount of 2-deoxyglucose 6-phosphate that accumulates in cells, it introduces glucose-6-phosphate dehydrogenase, glutathione reductase, and 5,5-dithiobis(2-nitrobenzoic acid) to the recycling amplification reaction of NADPH. Whereas this assay is adapted and optimized for the measurement of 2DG uptake in some tissues and cultured cell, it also requires a time-consuming heat treatment (95 °C).

In this study, we have developed a rapid chemiluminescent method for measurement of 2DG uptake in insulin-responsive 3T3-L1 adipocytes adapted to 96-well microplates. The principle of our method is based on 2DG determination in luminol oxidation reaction after an enzymatic dephosphorylation of 2DG6P. To confirm the reliability of this method, we have evaluated the dose–effect of insulin, cytochalasin B (hexose uptake inhibitor) [16], wortmannin, genistein (GLUT4 inhibitors) [17,18], LY294002, (inhibitor of translocation of GLUT thorough PI3-K inhibition) [19], and troglitazone (insulin-sensitizing agent) [20,21] on 2DG uptake into 3T3-L1 cells. Our method is limited for in vitro use but is more rapid and convenient than previous adapted for in vivo study.

Stress induced by reactive aldehydes, such as 4-hydroxynonenal (HNE) and acrolein is implicated in the development of insulin resistance in 3T3-L1 adipocytes. These carbonyls compounds were found to modify the structure of insulin and impair metabolic processes by inhibiting glucose uptake in primary cultures [22–24]. Methylglyoxal (MG), a metabolite of sugar, is a highly reactive dicarbonyl that was also found to decrease the glucose uptake in L6 skeletal muscle cells [25]. From these studies, we have also evaluated the effect of various reactive carbonyl species on insulin-stimulated 2DG-uptake into adipocytes using our new chemiluminescent assay.

We limited the validation of the assay to the measurement of 2DG uptake in cell cultures because glucose exists in the living body and then 2DG and glucose need to be measured separately to use 2DG uptake assay in in vivo study. All reactions were performed at 37 °C in one 96-well microplate by the sequential addition of reagents, and the results obtained were similar to those obtained by the previous method.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were of analytical grade and purchased from Sigma–Aldrich (st. Louis, MO, USA) except HNE which was purchased from Cayman (Ann Arbor, MI, USA). Reagents I and II named as Glucofax I and Glucofax II respectively were fully packaged and purchased from Yelen company (Ensues la Redonne, France).

2.2. Culture of 3T3-L1 adipocytes

3T3-L1 fibroblasts were obtained from ATCC and were maintained at 37 °C under 5% CO_2 with DMEM (4.5 g/L of glucose) supplemented with 10% foetal bovine serum. Differentiation of 3T3-L1 preadipocytes into mature adipocytes was made in T150 cm^2 flasks. Two days after confluence, cells were incubated for 2 days with DMEM supplemented with 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and insulin (1 μg/ml) then for 4 more days with DMEM supplemented with 10% FBS and insulin alone. At this time, over 80% of the cells displayed the characteristic lipid-filled phenotype. Differentiated adipocytes were gently detached from the plate by Accutase (PAA Laboratories, Les Mureaux, France) treatment as describe previously [26], seeded (3.5×10^4 cells/well) at confluence in 96-well culture dishes and cultured for two more days in DMEM supplemented with 10% FBS. This reseeding procedure of adipocytes eliminates the differentiation variability occurring when cells are directly differentiated in 96-well plates. The differentiated 3T3-L1 adipocytes were incubated with serum and glucose-free DMEM at 37 °C under 5% CO_2 for 4 h and then washed twice with Krebs–Ringer–phosphate–Hepes (KRPH) buffer (20 mM Hepes, 5 mM KH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 136 mM NaCl, and 4.7 mM KCl at pH 7.4) containing 0.2% bovine serum albumin and warmed at 37 °C.

2.3. 2DG uptake assay

The 3T3-L1 adipocytes were incubated at 37 °C for 30 min with 100 μl of KRPH buffer containing 0.2% BSA. Then KRPH buffer was removed and cells were incubated for 20 min with 170 μl of 100 nM insulin diluted in KRBH buffer, after which 19 μl of 10 mM 2DG was added and the cells were incubated for 20 min. Cells were then washed four times with cold PBS buffer. Preliminary experiments showed that washing four times reduced the amount of extra cellular 2DG to an undetectable level. The washed cells were lysed with 60 μl of dephosphorylating reagent I containing Triton X-100 (0.5%) and alkaline phosphatase (10 units/ml) in Tris/HCl buffer (100 mM, pH 8.6). After 60-min incubation at 37 °C, 20 μL of cell lysates were collected and transferred into 96-well plate. Then 100 μl of reagent II containing glucose oxidase (100 U/ml) and luminol (0.2 mg/ml)/HRP (0.07 U/ml) was added, and after 10-min incubation, the chemiluminescence was recorded on a microplate *luminometer TECAN Infinite 200* (*TECAN*, *Männedorf*, *Switzerland*). 2DG uptake was quantified from the linear plot of luminescent signal versus concentration of authentic 2DG6P (Fig. 1).

For experiments with 2DG uptake inhibitors, the protocol was slightly different. Washed cells were incubated 20 min at 37 °C with 160 μl of inhibitors at indicated concentration, after which 17 μl of 1 μM insulin was added (100 nM at end concentration). After 20-min incubation, 20 μl of 10 mM 2DG was added and the cells were incubated for 20 min. Cells were then washed four times with cold standard PBS buffer and uptake of 2DG into the cells was quantified by the enzymatic chemiluminescent assay described above. Each treatment was compared with controls, and statistical