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"Fluorescent glycogen" formation with sensibility for *in vivo* and *in vitro* detection

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Abstract There are presently many methods of detecting complex carbohydrates, and particularly glycogen. However most of them require radioisotopes or destruction of the tissue and hydrolysis of glycogen to glucose. Here we present a new method based on the incorporation of 2-NBDG (2-{*N*-[7-nitrobenz-2-oxa-1, 3-diazol 4-yl] amino}-2-deoxyglucose), a D-glucose fluorescent derivative, into glycogen. Two kinds of approaches were carried out by using *Clone 9* rat hepatocytes as a cellular model; (1) Incubation of cell lysates with 2-NBDG, carbohydrate precipitation in filters and measurement of fluorescence in a microplate reader (2) Incubation of living hepatocytes with 2-NBDG and recording of fluorescence images by confocal microscopy. 2-NBDG labeled glycogen in both approaches. We confirmed this fact by comparison to the labeling obtained with a specific monoclonal anti-glycogen antibody. Also drugs that trigger glycogen synthesis or degradation induced an increase or decrease of fluorescence, respectively. This is a simple but efficient method of detecting glycogen with 2-NBDG. It could be used to record changes in glycogen stores in living cells and cell-free systems and opens the prospect of understanding the role of this important energy reserve under various physiological and pathophysiological conditions.

Keywords Glycogen · 2-NBDG · Cell free system · TRITC (tetramethylrhodamine isothiocyanate)-conjugated goat anti-mouse IgM · Anti-glycogen antibody

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Introduction

Glycogen is the principal D-glucose storage polymer in vertebrate organisms. Most mammalian cells have glycogen, but only hepatic and skeletal muscle cells are able to storage significant quantities of this molecule [5]. Muscle glycogen only serves as a local source of glucose. However, breakdown of glycogen stores from liver results in a ready supply of glucose for the body tissues [20]. Regulation of glycogen metabolism is complex and is influenced by a variety of factors such as glucose levels or enzymes. Under some pathological conditions, like diabetes and glycogen storage diseases, glycogen storage or degradation is clearly affected. Those diseases as well as the action of hyperglycemic or hypoglycemic drugs could be optimally studied by methods that permit non-destructive glycogen detection.

Incorporation of radioisotopes [1, 9, 10], immunocytochemistry [13] or quantification of glucose after the hydrolysis of glycogen [6] are methods widely used for analyses of glycogen in different cellular models. Routinely, the presence of glycogen can also be examined histochemically in cryosections by the periodic acid-Schiff (PAS) reaction, originally described by MacManus in 1948 [22, 28]. However those procedures present problems, for

instance some of them can not be used in living cells, or they are invasive techniques and repetitive sampling cannot be performed making detailed kinetic studies impossible. In this sense fluorescent probes are more useful even though their specificity is a critical point.

2-NBDG (2-{*N*-[7-nitrobenz-2-oxa-1, 3-diazol 4-yl] amino}-2-deoxyglucose) is a new fluorescent D-glucose derivate developed by Yoshioka *et al.* [34] as probe for the determination of cell viability. It accumulates in living cells and not in dead cells. 2-NBDG was used in studies of cell viability [32] and D-glucose transport in diverse cellular models [17, 19–21, 26, 27, 35] not only microbial cells as *Escherichia coli* [33], yeast [32] but also mammalian cells as vascular smooth muscle cells [18] and even human hepatocarcinoma cells [35]. This dye is not cell permeable and enters in live cells through the same transporters that D-glucose [21, 25], including GLUTs [12], the most extended facilitative D-glucose transporters in mammalian cells [30]. The specificity of 2-NBDG uptake by D-glucose transporters has been demonstrated by its competitive inhibition by D-, but not L-glucose [31–34]. Moreover, the transport of 2-NBDG into cells is inhibited by cytochalasin B, a well-known toxin that inhibits GLUTs [12, 21]. Once the dye is inside the cells it is transformed into a still fluorescent 2-NBDG metabolite [32, 33]. Taking into account that 2-deoxyglucose incorporates into glycogen [7, 29] the purpose of this study was to assess the use of 2-NBDG, as a sensitive probe for monitoring glycogen on hepatocytes.

Materials and methods

Solutions and reactives

Antibodies and fluorescence detection: Mouse anti-glycogen antibody [4]. TRITC (tetramethylrhodamine isothiocyanate)-conjugated goat anti-mouse IgM was purchased from Chemicon International (Hampshire, United Kingdom). NBDG (2-{*N*-[7-nitrobenz-2-oxa-1, 3-diazol 4-yl]amino}-2-deoxyglucose), a fluorescent glucose derivative, which has an excitation wavelength of 480 nm and emits on 535 nm was from Molecular Probes (Leiden, Netherlands).

Drugs: Adrenaline was purchased from B. Braun Medical S.A. (Barcelona, Spain); glucagon was from Novo Nordisk Pharma S.A. (Madrid, Spain); salbutamol was purchased from GlaxoSmithKline S.A. (Madrid, Spain) and insulin from Intervet S.A. (Salamanca, Spain).

Cell culture: Nutrient mixture F-12 Ham Kaighn's modification, Streptomycin sulfate salt and Penicillin G potassium salt were purchased from Sigma (Madrid, Spain) and Foetal Porcine Serum was from Gibco (Barcelona, Spain).

BSA (Bovine Serum Albumin) was purchased from ICN Biomedicals, Inc. (Ohio, U.S.A.). All other chemicals were reagent grade and purchased from Sigma (Madrid, Spain) or Panreac (Barcelona, Spain).

Buffer-lysis solution: 0.9% NaCl, 10 mM Hepes, 0.1% Triton X-100, 0.1% BSA, 5.5 mM D-glucose, pH 7.2.

Buffer solution used for confocal assays (in mM): 138 NaCl, 5.5 D-glucose and 5 KCl; 1 $MgCl_2$, 1.5 $CaCl_2$, 10 Hepes, pH 7.4.

Solution for immunofluorescent protocol: PBS (Phosphate Buffered Saline) (in mM) 137 NaCl, 8.2 Na_2HPO_4, 1.5 KH_2PO_4, 3.2 KCl.

Cell culture

Cells used for the experiments were rat hepatocytes from the cell line *Clone 9* (ECACC N° 88072203). Cells were grown on 60 mm tissue culture plates with a nutrient mixture F-12 Ham Kaighn's modification supplemented with 2.5 g/l $NaHCO_3$, 28 mg/l Streptomycin sulfate salt, 17 mg/l Penicillin G potassium salt and 10% fetal porcine serum pH 7.2. Those hepatocytes were grown in a humidified atmosphere with 5% CO_2 at 37°C.

Fluorescent glycogen detection in a cell-free system

We used the cells 3 or 4 days after they were seeded, when confluence was reached. First hepatocytes were washed three times with buffer-lysis solution and then scraped off the culture plates and transferred into a plastic tube. Those cells were sonicated with one pulse of 30 s in an iced environment. After that, samples were incubated in constant shaking at 37°C with 2-NBDG. At the end of incubation 250 μl of samples were spotted onto GF/C glass microfibre filters (Watmman/Maidstone, United Kingdom), and finally washed with 40 ml ethanol 70%. GF/C filters have a retention rating of 98% for particles of 1.2 μm. Filters were placed in 12 wells plates (Falcon/ Madrid, Spain) and left overnight drying at room temperature in darkness. Then, the retained fluorescence was measured by using a microplate fluorescence reader FL600 (Bio-Tek/Vermont, USA) at 480/25 nm excitation and 535/20 nm emission wavelengths.

Fluorescent glycogen detection in rat hepatocytes: confocal microscopy

Hepatocytes were seeded on coverslips, which were placed in eight wells sterile plates. Coverslips were used when the cells reached confluence and glycogen was detected by using 2-NBDG or by the immunofluorescent method.

Fluorescent detection of glycogen labeled with 2-NBDG

Hepatocytes were washed three times with the buffer solution for confocal assays (cited above in "Materials

and methods") and then incubated in buffer solution with 500 μM 2-NBDG at 37°C.

Fluorescence measurements and images were registered with a 60× oil immersion objective of a Nikon Eclipse TE2000-E inverted microscope attached to the C1 laser confocal system (EZC1 V.2.20 software; Nikon Instruments Europe B.V., The Netherlands). Fluorescent images shown as volume-render projections belong to *Z*-stacks acquired at 0.5-μm intervals and 512×512 pixel resolution.

Fluorescent images are shown as volume-render projections that belong to *Z*-stacks. *Z*-stack is a collection of images superimposed taken by *Z*-scan of the sample at 512×512 pixel resolution. *Z*-scans are acquisitions taken each 0.5 μm along the *Z* axis of the sample by confocal software with a motorized focus.

Immunofluorescent detection of glycogen

Immunofluorescent protocol; cells were fixed with 4% paraformaldehide in PBS 15 min at room temperature, then washed three times with PBS and permeabilized with 2% BSA–0.2% Triton X-100 PBS 5 min on ice. Non-specific binding was blocked with 2% BSA-PBS for 1 h at room temperature. After that, cells were incubated with monoclonal mouse anti-glycogen antibody in 2% BSA-PBS 1 h at room temperature or overnight at 4°C. Then, cells were washed three times in PBS and incubated with TRITC-conjugated goat anti-mouse IgM in 2% BSA-PBS 1 h at room temperature and washed three times with PBS. Finally, coverslips were mounted on slides with 1:1 glycerol:PBS and sealed with nail varnish in order to preserve fluorescence for 4°C storage.

Photographs and fluorescence measurements were taken with a Nikon confocal microscope D-Eclipse C1 with a 60× oil immersion objective using a Helium-Neon laser with an emission wavelength of 543 nm (Espectra-Physics).

Results

In a preliminary approach we studied the possibility that any biochemical incompatibility existed for the incorporation of 2-NBDG into glycogen such as a D-glucose molecule. It was previously described that 2-NBDG is converted into another fluorescent derivative (2-NBDG metabolite) after cells take it. This 2-NBDG metabolite should be 2-NBDG-6-phosphate [33]. In the biochemical pathway towards glycogen synthesis, glucose-6-phosphate (in our experiments 2-NBDG 6-phosphate) is converted successively into glucose-1-phosphate (in our data 2-NBDG 1-phosphate), by phosphoglucomutase (PGM), and then into UPD-glucose (in our experiments UDP-2-NBDG), by UDP-glucose pyrophosphorylase (UDPGPP). This last metabolite is the glycosyl donor in the reaction catalyzed by glycogen synthase. This enzyme utilizes UDP-glucose as one substrate and the non-reducing end of glycogen as another. We present the hypothetical structure of glycogen labeled with 2-NBDG (Fig. 1). It can be seen that C2 is not directly implicated in the glycogen principal bonds.

Fluorescent glycogen detection in a cell free system

First, we determined fundamental parameters such as number of cells and concentration of 2-NBDG in order to discover the optimal conditions for our assays.

Homogenates from diverse number of cells were incubated with 500 μM 2-NBDG for 150 min. Glycogen synthesized by the lysates would precipitate in the filter. The fluorescence that keeps retained in the filters was registered with a microplate fluorescence reader. The highest fluorescence signal was obtained using homogenates from 1.5×10^6 hepatocytes (Fig. 2a). After that, we made a dose–response curve by incubating lysates of 1.5×10^6 cells with different 2-NBDG concentrations and found that the optimal concentration of dye was 500 μM (Fig. 2b).

Experiments described above gave us the adequate experimental conditions to use in the following cell-free assays; 500 μM 2-NBDG and lysates of 1.5×10^6 *Clone 9* hepatocytes.

We carried out a time-course using the above cited conditions for the incubations; 500 μM 2-NBDG and lysates of 1.5×10^6 cells. We observed that fluorescence increased with the incubation time until 150 min (Fig. 3).

Fluorescent glycogen detection in rat hepatocytes

Next step was to study if 2-NBDG labels glycogen in living cells. We incubated the cells with 500 μM 2-NBDG and took images with a confocal microscope at different incubation times in order to asses sub-cellular fluorescence distribution in the cells. We observed changes in the intensity and in the pattern of fluorescence distribution.

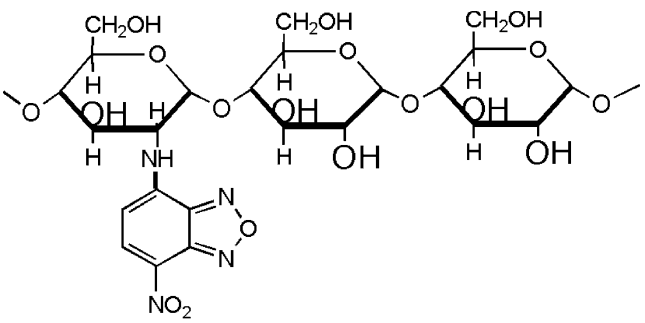

Fig. 1 Hypothetical structure of 2-NBDG labeled glycogen

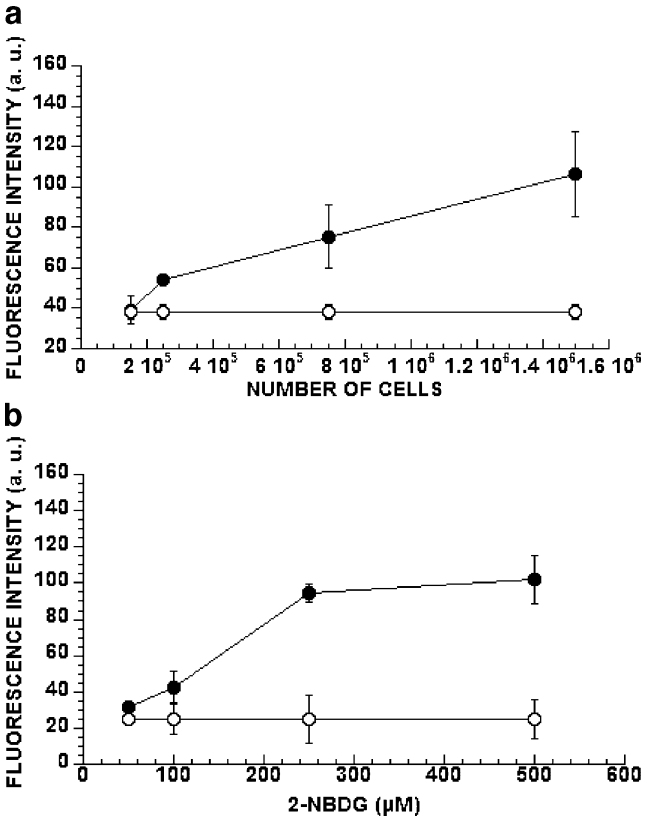

Fig. 2 Kinetic analysis of 2-NBDG incorporation to glycogen. **a** Influence of the number of hepatocytes used to prepare the cell lysates in the fluorescence retained in the filters. Cell lysates obtained from different number of cells were incubated with 500 μM 2-NBDG for 150 min and fluorescence (expressed in arbitrary units) was registered as described in "Materials and methods" (*black circles*). Autofluorescence was measured in cell lysates without 2-NBDG (*white circles*). **b** Dose–response curve of 2-NBDG. Cell lysates from 1.5×10^6 hepatocytes were incubated with different concentrations of 2-NBDG for 150 min (*black circles*) and fluorescence retained in the filters was expressed in arbitrary units (a.u.). Autofluorescence was measured in cell lysates without 2-NBDG (*white circles*). Results are the mean ± SEM from $n=3$ determinations

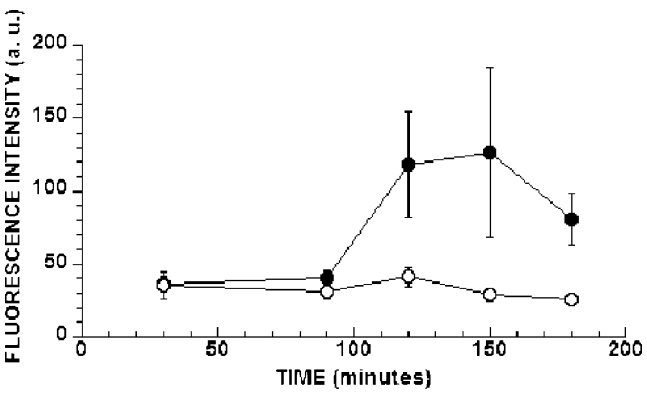

Fig. 3 Time course of 2-NBDG incorporation to glycogen. Cell lysates from 1.5×10^6 hepatocytes were incubated with 500 μM 2-NBDG for different periods of time and fluorescence retained in the filters was expressed in arbitrary units (a.u.) (*black circles*). Autofluorescence was measured in cell lysates without 2-NBDG (*white circles*). Data are the mean ± SEM of five independent experiments

Fluorescence appears diffuse in hepatocytes when the cells are incubated with 2-NBDG for 30, 60 or 180 min (Fig. 4a, b and c respectively). The higher fluorescent signal is registered at 180 min of incubation. After that time 2-NBDG was removed. Subsequent images obtained after another 180 min (Fig. 4d) and 360 min (Fig. 4e) revealed a decrease in fluorescence in the most part of the cell, however there are small granules where 2-NBDG fluorescence seemed to be accumulated.

In order to confirm that 2-NBDG is incorporated into glycogen we made immunofluorescent assays with a monoclonal anti-glycogen Ig M that was detected with a TRITC-conjugated secondary antibody goat anti-mouse Ig M. Hepatocytes that were incubated in the absence of D-glucose overnight showed a very low fluorescence (Fig. 5a). After 3 h of incubation with 6 mM D-glucose (Fig. 5b) hepatocytes

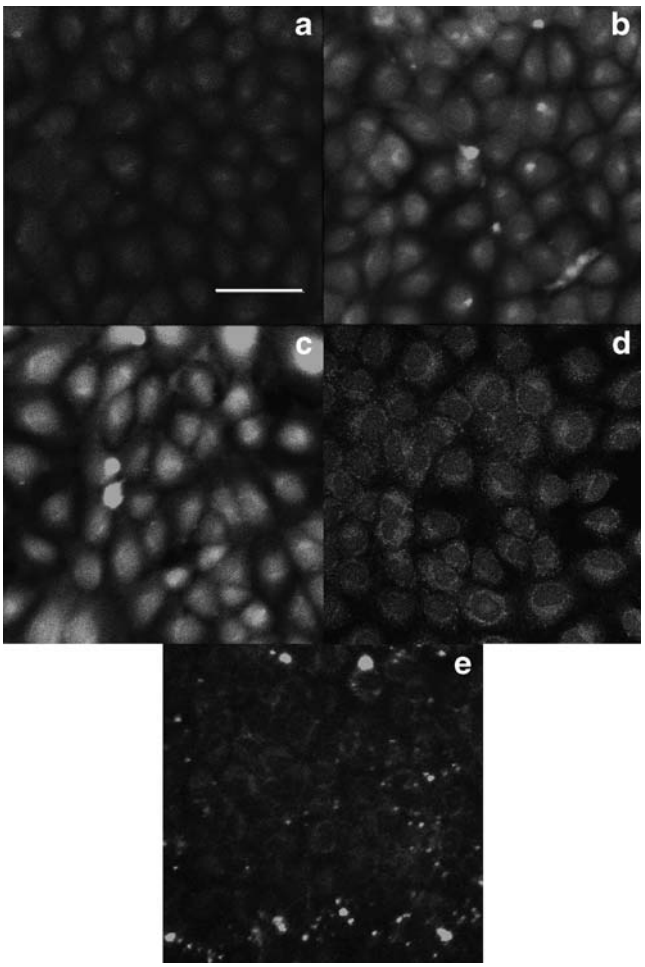

Fig. 4 Confocal microscopy images of hepatocytes incubated along 30 (**a**), 90 (**b**), 180 min (**c**) with 2-NBDG. In **d** and **e** cells were incubated for 180 min with 2-NBDG and then the dye was removed. Photo **d** was obtained after another 180 min incubation with no dye and photo **e** after another 360 min without 2-NBDG. Scale bar=50 μm. All photos (**a**–**d**) are in the same magnification. Images are projections from representative volume renders of three independent experiments

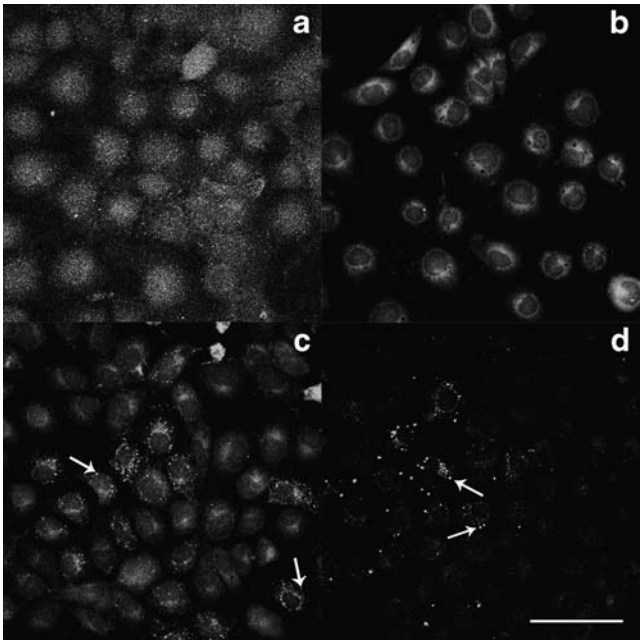

Fig. 5 Fluorescence images of confocal microscopy of *Clone 9* hepatocytes incubated overnight without glucose and then incubated for 0 h (**a**), 3 h (**b**), 6 h (**c**) and 9 h (**d**) with 6 mM D-glucose. At the end of the incubation, cells were processed for immunofluorescence analysis with the anti-glycogen antibody and the TRITC-conjugated secondary antibody as indicated in "Materials and methods." Scale bar=50 μm. All photos (**a**–**d**) are in the same magnification. *White arrows* point to representative accumulations of fluorescent labeling. Images are projections from representative volume renders of three experiments

showed an increase in fluorescence distributed around the nucleus. Punctuate fluorescence was found after 6 h incubation with 6 mM D-glucose (Fig. 5c) even this pattern of fluorescence became more evident after 9 h incubation (Fig. 5d).

In Fig. 6 we compare immunofluorescent imaging and 2-NBDG labeling. Figure 6a,b and c are confocal images of hepatocytes (incubated overnight in the absence of D-glucose) obtained following the immunofluorescence protocol. Figure 6a shows hepatocytes after 3 h of incubation with 6 mM D-glucose, Fig. 6b after 6 h of incubation with 6 mM D-glucose and Fig. 6c after 9 h of incubation with 6 mM D-glucose. Figure 6d,e and f are confocal images of hepatocytes (incubated overnight in the absence of D-glucose) obtained following the 2-NBDG loading protocol. After 3 h incubation with 500 μM 2-NBDG and 5.5 mM glucose hepatocytes show a great fluorescence (Fig. 6d) indicating a loading of 2-NBDG. However once 2-NBDG was washed from the medium and hepatocytes were incubated for another 3 or 6 h with 6 mM glucose (Fig. 6 e and f) appears some spots of intense fluorescence distributed in a similar way to immunofluorescent images (Fig. 6b and c respectively). However, the general diffuse fluorescence was clearly disappearing in Fig. 6e and f. This effect is due to the fact that all molecules of 2-NBDG that are not incorporated into glycogen enter in the glycolysis pathway and are metabolized to non-fluorescent derivates.

Effect of different drugs on the incorporation of 2-NBDG into glycogen

To check if we could use 2-NBDG as a label to analyze glycogen metabolism, we made some experiments adding drugs that induce increase on glycogen synthesis (such as insulin) or degradation (such as adrenaline, glucagon and

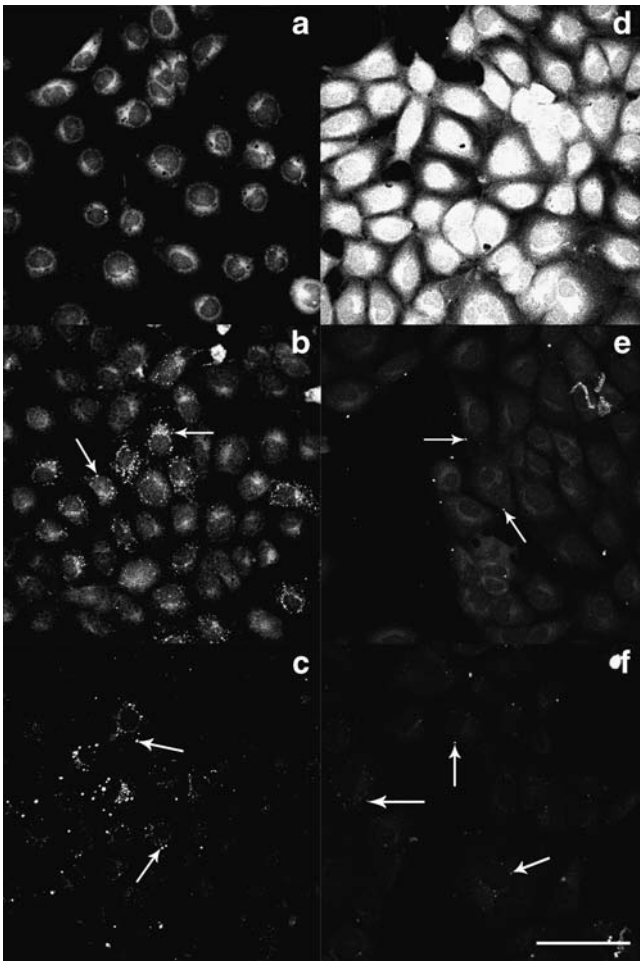

Fig. 6 Comparison of confocal images of hepatocytes obtained following the immunofluorescence protocol (**a**, **b** and **c**) and the loading protocol with 2-NBDG (**d**, **e** and **f**). In both cases cells were incubated overnight without glucose. **a**, **b** and **c** show images obtained with hepatocytes incubated with 6 mM glucose for 3 h, 6 h and 9 h respectively and then processed by the immunofluorescent protocol. Hepatocytes of the images **d**, **e** and **f** are incubated for 3 h with 500 μM 2-NBDG and 5.5 mM glucose, and then 2-NBDG was extracted from the incubating medium. After that, hepatocytes in **e** were incubated another 3 h with 6 mM glucose; in **f** hepatocytes were incubated another 6 h with 6 mM glucose. Scale bar=50 μm. All photos (**a**–**f**) are in the same magnification. *White arrows* point to representative accumulations of fluorescent labeling. Images are projections from representative volume renders of three experiments

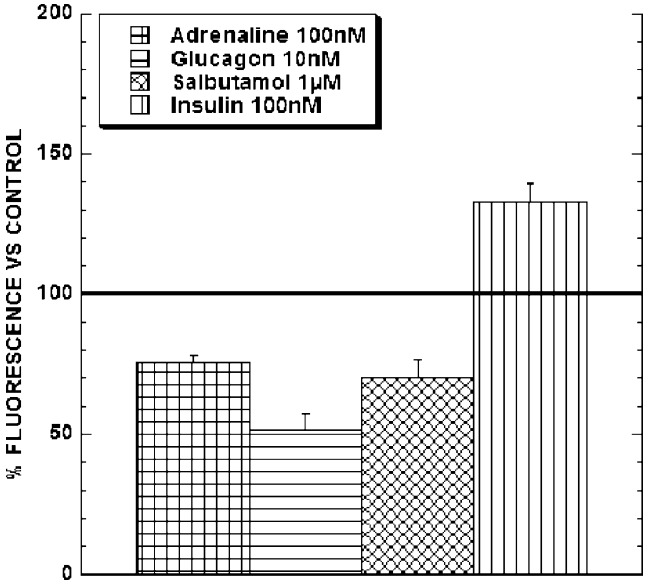

Fig. 7 Effect of different drugs on fluorescent glycogen. Cell lysates from $1{,}5\times10^6$ hepatocytes were incubated for 120 min with 500 µM 2-NBDG. Then cell lysates were incubated with different drugs for 30 min. After that, reactions were stopped as described in "Materials and methods" and fluorescence of filters was registered. Results are plotted as percentage of fluorescence *versus* control (fluorescence obtained with untreated samples). Results are the mean ± SEM from $n=5$ determinations

salbutamol). Drugs were added to the cell lysates after 120 min of incubation with the 2-NBDG. Then we incubated 30 min with the drugs and registered the fluorescence retained in the filters. Adrenaline, glucagon and salbutamol caused a decrease in fluorescence (statistically significant differences in a *T* student test with $\alpha<0.05$ for adrenaline and glucagon). Among the drugs used glucagon showed the strongest effect in decreasing the fluorescence intensity. Samples treated with insulin showed higher fluorescence than controls (statistically significant differences in a *T* student test with $\alpha<0.05$). Figure 7 summarizes the effects of each drug used, expressing results as fluorescence percentages *versus* controls.

Discussion

Previous studies reported the incorporation of 2-deoxyglucose into the glycogen molecule [1, 7, 8, 26, 29]. Taking into account that 2-NBDG is a fluorescent derivative of 2-deoxyglucose the goal of this work was to assess the use of 2-NBDG as a sensitive probe for monitoring glycogen in hepatocytes. The starting point was to study if the 2-NBDG gets into the glycogen synthesis pathway as a non-fluorescent D-glucose molecule. For this purpose we chose hepatocytes, which are the major producers of glycogen reserves with a highly developed metabolic route. We performed the experiments in two kinds of systems: (1) a cell-free system and (2) culture cells. In both cases after incubation with 2-NBDG we found a time-dependent increase in fluorescence. In the cell-free system, based on the fact that glycogen is selectively collected on the filters by the ethanol precipitation method [15], this increase of fluorescence indicates a progressive incorporation of 2-NBDG to the glycogen synthesized that could be clearly detected after 90 min.

In the experiments in living Clone 9 hepatocytes, while 2-NBDG is in the incubation media confocal images show cells with a high and diffuse fluorescence. This effect is due to the uptake of the dye by hepatocytes as it was demonstrated in other cellular models [17–21, 26, 27, 33]. Also we assure that specific D-glucose transporters are responsible of the uptake of 2-NBDG by studying the inhibition of this transport by cytochalasin (data not shown). After the initial loading with 2-NBDG, the dye was washed out and cells were incubated with D-glucose for 3 to 6 h showing a change in the pattern of fluorescence distribution. Even though the intensity of fluorescence in hepatocytes decreased due to metabolization of 2-NBDG in non-fluorescent fragments by entering in the glycolysis pathway, fluorescent granules corresponding to labeled glycogen were more evident. The unspecific surface labeling is minimum and could be lost during the previous washes of the cells based on the fact that in the *Z*-scans of our experiments the fluorescence was not surface-associated. These agree with data obtained by Lloyd *et al.* [18] studying the 2-NBDG incorporation in vascular smooth muscle cells.

Recently, glycogen sub-cellular distribution in hepatocytes was studied by using a specific antibody against glycogen [9, 11, 16] developed by Baba [4]. A drawback of labeling glycogen by this immunochemistry protocol is that hepatocytes must be fixed. However it was a specific way to demonstrate that fluorescence we found in granules in hepatocytes is due to labeled glycogen. In the immunofluorescent assays hepatocytes were overnight without D-glucose in order to reach the minimal glycogen storage conditions before the experiment starts. Images showed a granulated fluorescence in hepatocytes that increased after being incubated with D-glucose for different periods of time. This distribution of fluorescence is very similar to distribution obtained in hepatocytes incubated with 2-NBDG supporting the fact that 2-NBDG gets into the glycogen molecule. Differences in fluorescence intensity can be explained because immunofluorescence labels all glycogen made in the incubation time while 2-NBDG only labels partly the present glycogen. We must keep in mind that we labeled the glycogen by 2-NBDG incorporation. The heterogeneity on spot size can be explained by the fact that 2-NBDG as well as D-glucose incorporation rate is different in every glycogen particle of the cells because growing extremes of glycogen are not the same in all particles. Paying attention to the structure of glycogen, it has variable ramifications depending on its granule size.

Moreover, small "young" glycogen particles can potentially incorporate more D-glucose residues than big particles.

Glycogen labeled with 2-NBDG is also the target of drugs that modify glycogen metabolism. Adrenaline, glucagon and salbutamol, drugs that increase glycogen degradation [2, 14, 23, 24], diminished fluorescence signal in lysates of hepatocytes indicating a degradation of labeled glycogen. On the other hand, those cell free systems incubated with insulin, a drug that causes glycogen synthesis [3], showed a moderate increase in the fluorescence. In this case, glycogen synthesis causes an increase in 2-NBDG incorporation to this molecule.

There are many methods of detecting complex carbohydrates, and particularly glycogen, in mammalian tissue. However, most of them require radioisotopes [1, 7, 29], immunocytochemical techniques and fixation of cells [4, 13, 28] or destruction of the tissue and hydrolysis of glycogen to glucose after which standard methods of measurement of free glucose may be used [6]. Results demonstrate that 2-NBDG is a sensitive probe that allows a noninvasive detection of glycogen avoiding fixation of cells. The *in vivo* incorporation of "fluorescent glucose" into glycogen in the cell opens the prospect of understanding the role and function of this important energy reserve under various physiological and pathophysiological conditions. It is also a new method for a rapid testing of drugs that modify the incorporation or release of glucose from glycogen.

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