Novel method to differentiate 3T3 L1 cells in vitro to produce highly sensitive adipocytes for a GLUT4 mediated glucose uptake using fluorescent glucose analog

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Abstract Adipocytes play a vital role in glucose metabolism. 3T3 L1 pre adipocytes after differentiation to adipocytes serve as excellent in vitro models and are useful tools in understanding the glucose metabolism. The traditional approaches adopted in pre adipocyte differentiation are lengthy exercises involving the usage of IBMX and Dexamethasone. Any effort to shorten the time of differentiation and quality expression of functional differentiation in 3T3 L1 cells in terms of enhanced Insulin sensitivity has an advantage in the drug discovery process. Thus, there is a need to develop a new effective method of differentiating the pre adipocytes to adipocytes and to use such methods for developing efficacious therapeutic molecules. We observed that a combination of Dexamethasone and Troglitazone generated differentiated adipocytes over fewer days as compared to

the combination of IBMX and Dexamethasone which constitutes the standard protocol followed in our laboratory. The experiments conducted to compare the quality of differentiation yielded by various differentiating agents indicated that the lipid droplet accumulation increased by 112 % and the GLUT4 mediated glucose uptake by 137 % in cells differentiated with Troglitazone and Dexamethasone than in cells differentiated traditionally. The comparative studies conducted for evaluating efficient measurable glucose uptake by GOPOD assay, radioactive ^3H -2-deoxy-D-glucose assay and by non-radioactive 6-NBDG (fluorescent analog of glucose) indicated that the non-radioactive method using 6-NBDG showed a higher signal to noise ratio than the conventional indirect glucose uptake method (GOPOD assay) and the radioactive ^3H -2-deoxy-D-glucose uptake method. Differentiated 3T3 L1 cells when triggered with 2.5 ng/mL of Insulin showed 3.3 fold more glucose uptake in non-radioactive method over the radioactive ^3H -2-deoxy-D-glucose uptake method. The results of this study have suggested that a combination of Dexamethasone and Troglitazone for 3T3 L1 cell differentiation helps in better quality differentiation over a short period of time with increased sensitivity to Insulin. The application of these findings for developing new methods of screening novel Insulin mimetics and for evaluating the immunological responses has been discussed.

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Background

The occurrence of a diabetic condition across the globe has increased dramatically over the last two decades. It is estimated

that about 20 % of the global population is liable to become diabetic in the next 10 years (International Diabetes Federation 2011). Biopharmaceutical companies globally are in a race to combat this and are coming up with many forms of Insulin and Insulin mimetics. In vitro studies for Insulin and Insulin mimetics exploit the concept to use glucose uptake method using adipocytes for these bioassays. These involve the culturing of pre adipocytes to proliferate and differentiate them in chemically defined media. These serve as model systems for investigating the differentiating program in normal conditions or in conditions of diabetes, obesity and Insulin resistance.

During adipocyte differentiation, pre adipocytes exhibit a rise in enzymes that aid in triglyceride accumulation (Coleman et al. 1978a; Yoo et al. 2010). A variety of transcription factors are induced, including CCAAT/enhancer binding protein β (C/EBP β), peroxisome proliferator-activated receptor γ (PPAR γ) and CCAAT/enhancer binding protein α (C/EBP α) during adipogenesis (Morris et al. 1998). The genes regulating triglyceride accumulation in cells are regulated by these (Cowherd et al. 1999; Shao and Lazar 1997; Lin and Daniel Lane 1994; Hostetler et al. 2008; Klemm et al. 2001). Combined gene expression of PPAR γ and C/EBP α has shown synergistic effects on promoting fat cell conversion in myoblasts (Erding et al. 1994). Therefore, it was likely that PPAR γ and C/EBP α function accommodately to establish terminal adipocyte differentiation.

Since many years, pre adipocyte differentiation has been carried out using chemical stimulants including Dexamethasone, Isobutylmethylxanthine and Insulin in the presence of serum. This method produces well differentiated adipocytes in culture between 12 days and 14 days (Coleman et al. 1978a, b; Balachandran et al. 2008; Chapman et al. 1984; Rubin et al. 1978; Cornelius et al. 1994). In vitro Insulin stimulated GLUT4 (glucose transporter4) mediated glucose uptake is seen using the differentiated adipocytes or myotubes (Erding et al. 1994; Zaid et al. 2008). Insulin stimulated glucose uptake into adipocytes and muscle cells involves translocation of GLUT4 isoform of glucose transporter from intracellular location to the plasma membrane. A small proportion of GLUT4 is also localized within the constitutively recycling endocytic system, but mounting evidence favors the existence of a unique pool of Insulin-regulatable GLUT4 vesicles (Grant et al. 2008; Lloyd et al. 1999; Oatey et al. 1997; Brun et al. 1996). Measurements of glucose uptake in cells have usually employed indirect and direct methods. Conventional method involving indirect assessment of glucose uptake was done by GOPOD. Soon using radioactive glucose isotopes, the conventional method was replaced. The radioisotope method was found to be more sensitive and a direct measure of glucose uptake. The high signal to noise ratio achieved by the use of these isotopes is favorable for kinetic studies of GLUT4 mediated glucose transport. However, the inconvenience and expense

associated with radioactive waste disposal and cleanup tends to impede their use for large-scale screening programs to discover new modulators of glucose uptake. Use of non-radioactive glucose analogs (2-NBDG and 6-NBDG) to measure glucose uptake was practiced in flow cytometer analysis and immunofluorescence techniques for real-time screening experiments. Usage of non-radioactive GLUT4 mediated glucose uptake using 2-NBDG and 6-NBDG in identifying new class of Insulin mimetics was very recently introduced (Jung et al. 2011; Barros et al. 2009).

In this study 3T3 L1 cells have been used as a model to appreciate two aspects of glucose uptake. First, to assess the degree of differentiation using different combinations of the differentiation medium. This approach was initiated to reduce the number of days to achieve well differentiated cells. Second, to obtain a more sensitive, safer and faster method than the radioactive method for glucose uptake. In connection to this, well differentiated 3T3 L1 cells on 7th or 8th day of differentiation using the new approach were taken for GLUT4 mediated glucose uptake assessment using the conventional (GOPOD), radioactive (^3H -2-deoxy-D-glucose) and non-radioactive (6-NBDG) methods. The non-radioactive method showed a 3.3 fold increase in GLUT4 mediated glucose uptake than the ^3H -2-deoxy-D-glucose mediated radioactive method. Further improvement on the current non-radioactive method can help in the development of a more sensitive cell-based method for assessing the in vitro biological function.

Results

Improved differentiation of 3T3 L1 cells

In the current study, five types of differentiation media were used to evaluate the efficient differentiation of 3T3 L1 pre adipocytes in vitro. Treatment with the Insulin-sensitizing agent Troglitazone has always increased transcriptional activation of PPAR γ in 3T3 L1 pre adipocytes (Camp et al. 1999) and Dexamethasone is known to induce adipogenesis by formation of lipid clusters (Li et al. 2006; Zilberfarb et al. 2001). Serum lipids, which are aqueous lipoproteins, are also reported to induce differentiation (Beloor et al. 2010). Combining all such evidences and our previous experience, the 3T3 L1 cells were treated in vitro with different combinations of chemical stimulants to induce adipogenesis (Table 1). Treatment of 3T3 L1 pre adipocytes with different chemical stimulants resulted in different patterns of adipocytes with different degree of lipid accumulation. The differentiated cells stained with Oil Red O indicated that cells treated with Troglitazone and Dexamethasone (Panel A) showed a very high degree of lipid droplet accumulation compared to other groups (Fig. 1).

The amount of stain in the cells was measured by eluting the stain from the differentiated cells using standard protocol

Table 1 Composition of various differentiation media used in the study

Components	Differentiation medium A	Differentiation medium B	Differentiation medium C	Differentiation medium D	Differentiation medium E ^a
Dexamethasone (0.25 μ M)	+	–	+	+	+
Troglitazone (40 μ M)	+	+	–	–	–
Serum Lipids (20 μ L/mL)	–	+	+	–	–
IBMX (0.5 mM)	–	–	–	+	+
Insulin (1 μ g/mL)	+	+	+	+	+
DMEM High Glucose Medium	+	+	+	+	+

^a After 48 h of incubation with differentiation medium, DMEM high glucose medium with 1 μ g/mL Insulin was added and incubated for 48 h

(Fig. 2). The absorbance corresponded to the amount of lipid droplet accumulation in differentiated 3T3 L1 cells induced by the respective treatment to differentiate them. 3T3 L1 pre adipocytes treated with the combination of Troglitazone and Dexamethasone showed an improved lipid droplet formation after differentiation in a very short span of time. These results correlated well with other published results about Troglitazone (Camp et al. 1999; Tchoukalova et al. 2000) where it was known to enhance adipogenesis in pre adipocytes. The cells differentiated with other combinations of differentiation stimulants did not show considerable differentiation as compared to treatment with Troglitazone and Dexamethasone (Fig. 1). In addition, Fig. 2 adds value to the results obtained in Fig. 1, showing that the highest lipid droplet formation was in the cells treated with Troglitazone and Dexamethasone.

Differentiation pattern in 3T3 L1 cells

Combination of Dexamethasone and IBMX at various concentrations to differentiate 3T3 L1 cells in culture is practiced by different scientists across the globe. The cells in our laboratory were maintained and cultured in a similar method as discussed earlier using 0.25 μ M Dexamethasone and 0.5 mM of IBMX. The cells that were differentiated with IBMX and Dexamethasone were large and the oil droplets were arranged in loose clusters. In contrast to these cells, the cells differentiated with Troglitazone and Dexamethasone, were small with compact clusters of oil droplets. Figure 3a and b illustrate the arrangement of lipid clusters in the differentiated 3T3 L1 cells.

GLUT4 is a facilitative glucose transporter that is expressed at highest levels in muscle and adipose tissue in mammals. It is the major transporter that regulates glucose uptake into muscle and adipose tissue in response to Insulin after a meal and into skeletal muscle during exercise. A significant proportion of the GLUT4 vesicles in adipocytes reside in an endosomal pool that is continuously recycling between the plasma membrane and the cell interior. Upon stimulation, the GLUT4 gets rapidly translocated to the plasma membrane to facilitate glucose uptake (Lawrence et al. 1992).

In the basal state, GLUT4 cycles slowly between the plasma membrane and the endosomal compartment, thus the majority of the GLUT4 is located within the cell interior. Insulin stimulation triggers a large increase in the rate of GLUT4 vesicle exocytosis (mainly from the endosomal compartment), and a small decrease in the rate of internalization by endocytosis which together result in a net increase in GLUT4 protein content in the plasma membrane, and thus glucose uptake (Ducluzeau et al. 2002).

Increased glucose uptake of cells treated with Troglitazone and Dexamethasone

Induction with Insulin triggers the GLUT4 transporter to relocate to the plasma membrane of the cell thus enabling the uptake of glucose for fat metabolism (Zaid et al. 2008; Lawrence et al. 1992). We have exploited this concept for measuring the in vitro residual glucose using an indirect method like the GOPOD assay where an increased glucose uptake in cells treated with Troglitazone and Dexamethasone was seen. 3T3 L1 cells were differentiated with different combinations of chemical stimulants (refer Table 1). These cells were triggered with Insulin (diluted to different concentrations using Low glucose medium) and the resultant uptake of glucose was measured. Figure 4 shows that 3T3 L1 cells differentiated using the combination of Troglitazone and Dexamethasone showed a higher fold in uptake of glucose than the cells treated with the other combinations. When triggered with 5 ng/mL of Insulin, about 137 % increase in glucose uptake was observed in 3T3 L1 cells treated with a combination of Troglitazone and Dexamethasone as compared to the standard (cells treated with Dexamethasone and IBMX). This observation further asserted our view that the combination of the chemical stimulants Troglitazone and Dexamethasone at concentrations 40 μ M and 0.25 μ M respectively not only showed an increase in lipid cluster accumulation but also showed a rocket high glucose uptake when triggered with Insulin. Thus, further experiments quoted in this paper were performed with the cells differentiated using Troglitazone and Dexamethasone

Fig. 1 Differentiation of 3T3 L1 in vitro: comparison of various differentiation protocols. 3T3 L1 adipocytes were treated with five types of differentiation media (DM) for 48 h. These differed in their composition where- **a** DM with 0.25 μ M Dexamethasone, 40 μ M Troglitazone and Insulin at 1 μ g/mL. **b** DM with 40 μ M Troglitazone, serum lipids at 20 μ L/mL and Insulin at 1 μ g/mL. **c** DM with 0.25 μ M Dexamethasone, serum lipids at 20 μ L/mL and Insulin at 1 μ g/mL. **d** DM with 0.25 μ M Dexamethasone, IBMX at 0.5 mM and Insulin at 1 μ g/mL. **e** DM with 0.25 μ M Dexamethasone, IBMX at 0.5 mM and Insulin at 1 μ g/mL. This particular DM combination required a medium change with DMEM high glucose medium with 1 μ g/mL Insulin after 48 h. Adipocytes obtained above were washed and fixed. After permeation with 0.5 % triton-X 100, they were stained with Oil Red O and photographed digitally. The photographs included were representatives from an array of images obtained from experiments conducted on three different days across three different passages. (Data not shown)

	Differentiation medium A	Differentiation medium B	Differentiation medium C	Differentiation medium D	Differentiation medium E [†]
Dexamethasone (0.25μM)	+	-	+	+	+
Troglitazone (40μM)	+	+	-	-	-
Serum Lipids (20μL/mL)	-	+	+	-	-
IBMX (0.5mM)	-	-	-	+	+
Insulin (1μg/mL)	+	+	+	+	+

[†] After 48 hours of incubation with differentiation medium, DMEM high glucose medium with 1 μ g/mL insulin was added and incubated for 48 hours.

at concentrations 40 μ M and 0.25 μ M respectively in the differentiation medium.

Insulin mediated glucose uptake—a comparison between radioactive and non-radioactive approaches

For an Insulin-related glucose uptake study, we chose to work with three readout methods for cell-based assays, and to investigate the most sensitive of the three. Differentiated 3T3 L1 cells were charged with different concentrations of Insulin and assessed for glucose uptake as described earlier.

Figure 5 shows the uptake level of glucose using the radioactive (3 H-2-deoxy-D-glucose), non-radioactive (6-NBDG) and the indirect (GOPOD) method. Across the different concentrations of 6-NBDG tested, a common trend observed was the increase in signal with an increase in concentration of 6-NBDG used. The maximum concentration of 6-NBDG tested was 400 μ M, and it was seen that even at such a high concentration, there was no saturation or quenching of signal.

From the results obtained, it was clear that there was a drug dose-dependent increase in the signal obtained

Fig. 2 Differentiation in 3T3L1 cells: comparison of various differentiation protocols- 3T3 L1 adipocytes were treated with five types of differentiation media (DM) for 48 h and stained with Oil Red O. This stain was eluted from the differentiated cells. The absorbance of this elutant was read at 490 nm. The absorbance value obtained is directly

proportional to the amount of Oil Red O taken into the cells, which in turn is directly proportional to the extent of differentiation caused by that particular differentiation medium. All data are mean $\pm$ SE ($n=3$, three experimental repeats)

across all the three methods. However, on considering 2.5 ng/mL of Insulin (for the purpose of Insulin-related Immunogenicity assays, the working range chosen is close to 3 ng/mL of Insulin), the non-radioactive 6-NBDG method showed a magnitude of 9.3 in comparison with 2.9 in a radioactive method and 2.8 in the indirect glucose uptake method (GOPOD). This translated

to the signal obtained in the 6-NBDG method being approximately 3.3 fold more than the one obtained in the radioactive ^3H -2-deoxy-D-glucose method. At 2.5 ng/mL of Insulin, the GOPOD method detected the glucose uptake to be around 51 %, while that in 6-NBDG method was approximately 89 %. Hence it was clearly seen that the most sensitive method among the three

Fig. 3 Difference in the arrangement of lipid clusters: comparison of differentiation yielded by Dexamethasone and Troglitazone with the standard- 3T3 L1 adipocytes were treated with differentiation media (DM) A and D for 48 h and stained with Oil Red O. Micrographs of cells stained with Oil Red O show that **a** DM with IBMX and Dexamethasone which has been considered as the standard, shows large and

distributed lipid clusters. **b** In comparison, DM with Dexamethasone and Troglitazone shows small and compact lipid clusters. The photographs included were representatives from an array of images obtained from experiments conducted on three different days across three different passages. (Data not shown)

Fig. 4 Comparison of various differentiation protocols: Graphical representation of % glucose uptake with increasing concentrations of Insulin trigger—Cells differentiated with the five differentiation protocols are tested for glucose uptake at increasing concentrations of Insulin (5, 10, 20 and 40 ng/mL). % Glucose uptake for these Insulin treatments has been calculated considering 0 ng/mL of Insulin (only

medium blank) to yield 0 % glucose uptake. It can be seen that the obtained % glucose uptake rises with the treated dose in a linear fashion. From the results obtained, it was clear that Differentiation medium A has resulted in a higher %glucose uptake when compared to the standard treatment of Medium D. All data are mean $\pm$ SE ($n=3$, three experimental repeats)

tested was the 6-NBDG method. Figure 6 illustrates the magnitude obtained while three different glucose uptake methods were compared with a 2.5 ng/mL Insulin trigger.

Insulin-mediated glucose uptake—resultant of GLUT4 activity

To further confirm that the increase in glucose uptake in adipocytes was due to GLUT4 activity, the effect of the GLUT4 inhibitor Cytochalasin B was tested (Fig. 7). The actin cytoskeleton has been proposed to play an important role in Insulin-stimulated GLUT4 translocation. Cytochalasin B, being a microfilament disrupting agent, is known to inhibit GLUT4 translocation across the plasma membrane (Ducruzeau et al. 2002).

The dose-dependent decrease in glucose uptake in 3T3 L1 cells with increasing concentrations of Cytochalasin B clearly showed that the glucose uptake observed was indeed GLUT4 mediated. There was no indication of cell death or loss of cell viability observed, despite the increasing concentrations of the microfilament disrupting agent, proven by our experiments to test the viability of cells after Cytochalasin B treatment. We observed that the cells treated with Cytochalasin B when subjected to viability test, indeed showed proliferation even at the highest concentration of Cytochalasin B tested. Thus, the decrease in glucose uptake was solely due to inhibition of GLUT4 translocation and not due to toxicity leading to cell death. Figure 8 shows the results of the MTS assay for cell viability.

Discussion

3T3 L1 cells are extensively used as a model to study adipogenesis and GLUT4 trafficking in vitro. However, one typical matter of concern is the long duration required for the cells to differentiate to adipocytes in culture. A combination of Dexamethasone and IBMX was used by many investigators at different concentrations for the last several years. As the need to produce new Insulin mimetic molecules increases, there is a requirement for bioassays which are fast, robust, safe and sensitive. In this study, we have made an effort both to reduce the time of adipogenesis in vitro and to come up with a robust, safe and sensitive bioassay for glucose uptake.

Earlier studies have hypothesized that Dexamethasone, a synthetic glucocorticoid enhances pre adipocyte differentiation. This compound has been used by researchers routinely as a component of differentiation media in protocols for pre adipocyte differentiation. Treatment with Dexamethasone in the presence of serum has shown to increase the number of C/EBP α and AD-3 immunoreactive cells (Tchoukalova et al. 2000; Shugart and Umek 1997). Usage of a mixture of Troglitazone, Dexamethasone and Insulin in this study reduced the number of days required for adipocyte generation from 12–14 days to 7–8 days. Among the five combinations tested, we found the combination of Dexamethasone at 0.25 μ M, Troglitazone at 40 μ M and Insulin at 1 μ g/mL to be the most effective combination in terms of increased lipid droplet accumulation as well as increased adipocyte numbers.

Treatment with Dexamethasone increases the number of C/EBP α immunoreactive pre adipocytes without changing

Fig. 5 Insulin mediated glucose uptake: a comparison between radioactive and non-radioactive approaches—3T3L1 cells differentiated with Differentiation medium A were treated with different concentrations of Insulin and tested for glucose uptake. **a** In the radioactive method, differentiated cells were serum starved and washed with KRPH buffer. The starved cells are exposed to Insulin drug dilutions for 1 h. After 1 h, ^3H -2-deoxy-D-glucose was added. The cells were lysed and the lysate was transferred to isoplates to measure the resultant radioactivity. **b** In the non-radioactive method, using the fluorescent method, the cells were grown and differentiated in black well

microclear plates. Different concentrations of 6-NBDG (20 μM , 50 μM , 100 μM , 200 μM and 400 μM) were tested to detect the glucose uptake triggered by different concentrations of Insulin. Cells were washed and the resultant fluorescence measured. **c** In the non-radioactive method, using the GOPOD method, washed cells were triggered with Insulin dilutions in low glucose medium for 22 h. The resultant glucose uptake was measured by the glucose oxidase peroxidase method. For all the above approaches, the response was reported after negation with the corresponding blank values. All data are mean $\pm$ SE ($n=3$, three experimental repeats)

Fig. 6 Different methods used to detect glucose uptake: a comparison of magnitude yielded by each method—Previous figures have shown that there is a dose-dependent increase in the glucose uptake yielded by the three methods tested. However, on considering the dose 2.5 ng/mL, the highest response is clearly seen in the non-radioactive method (fluorescent method). The radioactive method and the non-

radioactive method (GOPOD method) have shown almost similar magnitudes. This is a graphical representation of the glucose uptake values yielded by the dose of 2.5 ng/mL of Insulin in the three different methods tested. All data are mean $\pm$ SE ($n=3$, three experimental repeats)

C/EBP α protein concentration per cell. On the contrary, Troglitazone does not change the number of C/EBP α -positive cells but moderately increases C/EBP α protein expression mainly during the late phase of differentiation, suggesting a slight increase in C/EBP α protein

concentration per cell during terminal differentiation (Tchoukalova et al. 2000).

The combination of both Dexamethasone and Troglitazone has shown to increase the number of PPAR γ -positive cells without changing PPAR γ protein levels. Both PPAR γ

Fig. 7 The glucose uptake in 3T3L1 cells being GLUT4 mediated: Effect of Cytochalasin B on glucose uptake—Various concentrations of Cytochalasin B was tested on 3T3 L1 cells at concentrations of 5, 10 and 20 μ M as indicated in “Materials and Methods”. The Insulin trigger subsequently added is seen to be inhibited with increase in concentrations of Cytochalasin B. All data are mean $\pm$ SE ($n=3$, three experimental repeats)

Cytochalasin B (μ M/mL)	t-value	p-value (DF = 3)
0	3.0816	0.0541
5	4.0068	0.0279
10	3.8427	0.0311
20	4.0568	0.0270

Fig. 8 Measure of Cell proliferation in Cytochalasin B treated 3T3 L1 cells: evidence to show that Cytochalasin B is non-toxic—Various concentrations of Cytochalasin B were tested on 3T3 L1 cells at concentrations of 5, 10 and 20 μM . The treated cells were then assayed for cell viability using the MTS viability method, where they showed an indication of proliferation when compared to the positive control. The positive control consisted of a preparation of dead cells. It is seen that the GLUT4 inhibitor is not cytotoxic. All data are mean $\pm$ SE ($n=3$, three experimental repeats)

Cytochalasin B (μM)	t-value	p-value(DF = 3)
5	5.0892	0.0147
10	5.4823	0.0119
20	4.8196	0.0170
Positive control	5.0028	0.0154

and C/EBP α act at different phases of differentiation. PPAR γ appears to be sufficient to trigger the adipogenic program by activating a subset of critical adipogenic genes, but C/EBP α is required for establishment of Insulin-sensitive glucose transport (Tchoukalova et al. 2000).

The stimulating effect of Troglitazone on C/EBP α protein expression during the late phase of differentiation is found to be one of the potential mechanisms for increasing the sensitivity of Insulin transduction although it has been shown that PPAR γ does not require the presence of C/EBP α to induce factors involved in Insulin signaling, such as GLUT4 (Camp et al. 1999). Thus this increase in cellular levels of PPAR γ and C/EBP α have made the differentiated 3T3 L1 cells to take up 140 % more glucose and a 112 % more lipid droplets accumulation when compared with the standard combination of IBMX and Dexamethasone.

6-NBDG assays were initially developed as fluorescent probes to monitor GLUT1 receptor kinetics in vitro (Speizer et al. 1985). Data suggesting the use of the novel 6-NBDG method as a sensitive method to screen Insulin mimetics was reported successfully (Jung et al. 2011). The binding of Insulin to its receptor triggers multiple signalling pathways that contribute broadly in cellular growth, differentiation and in the glucose metabolism. GLUT4 mediated glucose transport with an Insulin trigger is widely studied in 3T3 L1 adipocytes and C2C12 myotubes. A delay between GLUT4 exocytosis and Insulin-stimulated glucose uptake in L6-GLUT4-*myc* myotubes was studied suggesting that

activation of GLUT4 precedes glucose transport (Somwar et al. 2001). GLUT4 mediated glucose uptake was demonstrated using a radioactive and a non-radioactive method. However, in comparison with the radioactive glucose uptake method, the non-radioactive 6-NBDG method is safe and sensitive. As safer and sensitive methods are more demanding, the current method is found to contain more scope for developing a functional bioassay for screening neutralizing antibody and potency evaluation.

Conclusion

In summary, we have described the development of a new method that can differentiate 3T3 L1 pre adipocytes to adipocytes in a short span of time showing more sensitivity towards glucose uptake. NBDG fluorescent analogues of glucose have been used to monitor glucose uptake but have not been utilized as tools for bioassay development. Our research paper describes the successful employment of NBDG as a screening tool to develop a sensitive method using a safe, cost-effective and efficient cell-based screen for neutralizing antibody. Thus, this novel NBDG based screening protocol along with the 3T3 L1 cells differentiated with Troglitazone and Dexamethasone has the potential to discover new approaches in biopharmaceutical industry to support trials on safety and efficacy assessment.

Materials and methods

Reagents Dulbecco's Modified Eagle's Medium, Fetal Bovine Serum (FBS), Penicillin-Streptomycin, 0.25 % Trypsin-EDTA, 6-NBDG and HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) were obtained from Invitrogen by Life Technologies; Bovine Insulin, Dexamethasone (DEX), Isobutyl Methyl Xanthine (IBMX), Glucose Oxidase/Peroxidase (GOPOD), Serum lipids (catalogue no. L4646) and Dulbecco's Phosphate Buffered Saline (DPBS) were obtained from Sigma Aldrich (Saint Louis, MO). Troglitazone was obtained from Calbiochem, USA (Catalogue no. 648469).

Cell culture 3T3 L1 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in DMEM (high glucose) supplemented with 10 % FBS (Invitrogen Canada Inc.), 10,000 units of Penicillin G and 10,000 µg/mL Streptomycin sulphate (Invitrogen), and 10 mM HEPES at 37 °C in 5 % CO₂ atmosphere.

Differentiation of 3T3 L1 cells Pre adipocytes were induced to differentiate after 48 h of confluence in 96-well plates (Greiner) into adipocytes. The cells were incubated with five types of differentiation media (Table 1) for 48 h. The differentiation pattern was studied both by observing the morphological changes and by estimating oil droplet accumulation in the cells. The differentiated adipocytes in culture after 7–8 days were taken for Oil Red O staining and to evaluate for its ability to uptake glucose by GOPOD method.

Oil Red O staining The staining procedure was performed as mentioned previously with few modifications (Zacarias Ramirez et al. 1992). Briefly, differentiated adipocytes (as mentioned above) were washed with DPBS and fixed with formalin. The cells were permeated using 0.5 % Triton-X 100 and stained with Oil Red O at a final concentration of 0.14 mM. The stain from the cells was eluted using 100 % isopropanol and the absorbance of the eluted stain was read at 490 nm. Digital photographs of the stained cells were obtained using Nikon digital imaging system.

Measure of glucose uptake

- (i) **Non-radioactive method (Direct uptake):** 3T3 L1 cells (seeded in black, micro clear 96-well plates at a concentration of 25,000 cells/well), differentiated using Troglitazone and Dexamethasone were serum starved for 1 h in DMEM low glucose medium (Sigma Aldrich). Insulin drug dilutions at different concentrations (ng/mL) were added to the serum starved cells with appropriate controls and were incubated at 37 °C for 1 h. To facilitate the uptake, 6-NBDG diluted in

DPBS was added at concentrations of 20, 50, 100, 200 and 400 µM and the cells were further incubated. After incubation, cells were washed thrice with DPBS and the resultant fluorescence was measured (Excitation at 485/20 nm and Emission at 528/20 nm) using a fluorescent micro plate reader (Biotek Synergy 2).

- (ii) **Non-Radioactive method (Indirect uptake using GOPOD):** 3T3 L1 cells were differentiated with different combinations of the differentiation medium (Table 1). The cells were washed and triggered with Insulin drug. Insulin drug was diluted in Low Glucose Medium (Sigma Aldrich) supplemented with 10 % FBS (Invitrogen), 2 mM L-Glutamine (Invitrogen) and 10 mM HEPES. Cells exposed to assay medium (without drug) served as a control. After 22 h, a glucose uptake assay was done using 4-aminoantipyrine (Sigma Aldrich) and N-ethyl-N-sulfo-propyl-M-toluidine (NENST, Sigma Aldrich) as the substrates and GOPOD as the enzyme. Residual glucose was calculated by a measure of absorbance at 550 nm (Biotek Synergy 2). Based on the value of residual glucose obtained, percentage glucose uptake was calculated.
- (iii) **Radioactive method (Direct uptake):** Assays were performed as described earlier with modifications (Lakshmanan et al. 2003). Differentiated 3T3L1 cells (as described for non-radioactive method) were serum starved for 2 h in DMEM low glucose medium with 0.1 % BSA. The cells were washed with Kreb's Ringier Phosphate HEPES (KRPH) buffer (140 mM NaCl, 5 mM KCl, 1.3 mM CaCl₂, 1.2 mM KH₂PO₄, 2.5 mM MgSO₄, 5 mM NaHCO₃, 25 mM HEPES and 0.1 % BSA). Insulin dilutions (diluted in KRPH buffer) at different concentrations (ng/mL) were added to the cells and incubated at 37 °C for 1 h with appropriate controls. To facilitate glucose uptake in the cells further to the Insulin trigger, ³H-2-Deoxy-D-Glucose (Perkin Elmer, USA) at a final concentration of 0.01 µCi was added. Following an hour's incubation, the cells were lysed using 0.5 N NaOH with 0.5 % Triton X-100 (Sigma) and the cell lysate was transferred to Isoplates (Perkin Elmer) to measure the radioactivity using a Liquid Scintillation Counter (Chameleon V LSC Plate reader).

Glucose uptake inhibition by GLUT4 inhibitor This experiment was designed to demonstrate the GLUT4 mediated glucose uptake activity in differentiated 3T3 L1 cells. Glucose uptake stimulated by 10 ng/mL of Insulin (for both radioactive and non-radioactive method) was inhibited by the addition of 5, 10 and 20 µM concentrations of Cytochalasin B (1 h pre-treatment with Cytochalasin B) with appropriate controls.

Measurement of cell viability In order to determine whether the Cytochalasin B treatment was toxic to the assayed cells or not, the above treated cells were also assayed for measuring cell proliferation by a colorimetric cell viability assay using MTS (Promega).

Statistical analysis Data are presented as Mean $\pm$ SE using the general linear model procedure on personal computers. Using the Student's *t*-test, *t*-values were first computed and then *p* values were generated using GraphPad prism software. *p* values of less than 0.05 were considered to be significant.

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Competing interests The authors declare that they have no competing interests.

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Authors' contribution

DV designed and conducted experiments on Insulin sensitivity (non-radioactive), Cell viability, differentiation and jointly wrote the manuscript. HS designed and conducted experiments on Insulin sensitivity (non-radioactive fluorescent analog), differentiation and jointly wrote the manuscript. MSP performed experiments on Insulin sensitivity (radioactive) and GLUT4 inhibition. SS performed Oil Red O staining, experiments on dye elution and differentiation. SKA was associated in Insulin sensitivity (radioactive) and cell preparation. KD and MND initiated the projects, reviewed all designed experiments and the manuscript. All authors helped in discussing, reading and proofreading the final manuscript.