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Examining Glucose Transport in Single Vascular Smooth Muscle Cells with a Fluorescent Glucose Analog

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Summary

Changes in vascular smooth muscle glucose transport are thought to contribute to the pathogenesis of vascular disease in conditions such as diabetes, yet no single-cell assay for glucose uptake by VSM exists. Therefore, we examined the uptake of the fluorescent glucose analog 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxyglucose (2-NBDG) in isolated pig vascular smooth muscle cells (VSMC) using digital imaging microscopy. Uptake of 2-NBDG by VSMC was inhibited by D-glucose but not by L-glucose, suggesting that 2-NBDG enters VSMC via glucose transporters. Uptake of 2-NBDG was linear in the presence of 10 mM D-glucose (n=6, R^2=0.9408) but not in its absence (n=4, R^2=0.9993), indicating that 2-NBDG is not metabolized and accumulates within the cells. 2-NBDG fluorescence in VSMC was often non-uniform and appeared to represent binding of 2-NBDG to some cytoplasmic component. The present study demonstrates that 2-NBDG is a useful tool for examining vascular smooth muscle glucose uptake at the single cell level.

Key words

Diabetes • Digital imaging microscopy • Fluorescent glucose analogs

Introduction

Glucose transport and metabolism are central to the energetics and function of vascular smooth muscle (VSM) (Hardin and Paul 1995). Indeed, alterations in glucose uptake are associated with the development of diabetic vascular disease (Ruderman *et al.* 1992). Glucose transport and metabolism are also linked to Ca^{2+} handling in smooth muscle (Hardin *et al.* 1992, Kahn *et al.* 1995, Kim and Zemel 1995). Although glucose transport plays an important role in VSM function, its regulation and coupling with cell functions is incompletely understood. For example, the role of hormones in modulating glucose uptake is not completely characterized, as is the functional relationship between glucose uptake and cellular processes such as ion transport.

Small fluorescent analogs of glucose capable of entering cells *via* glucose transporters represent simple and potentially valuable tools for examining glucose uptake at the single cell level. Two fluorescent glucose analogs, 6-NBDG (6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-6-deoxyglucose) (Speizer *et al.* 1985) and 2-NBDG (2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxyglucose) (Yoshioka *et al.* 1996a) have been synthesized and used as indicators of glucose uptake in some specialized situations (Shimada *et al.* 1994, Speizer

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et al. 1985, Yoshioka *et al.* 1996a,c). However, the utility of these compounds for examining vascular function both in health and in disease has not been evaluated. The ability to study glucose uptake in single VSM cells could provide insights into a variety of aspects of glucose transport in this tissue, including its hormonal regulation, its alteration in disease states, and coupling between glucose transport and function. These analogs could also be used to study glucose transport in microvascular VSM cells, which is currently complicated both by the difficulty of harvesting the quantities of cells needed for isotopic studies and by the heterogeneous cellular composition of microvessels. Thus, we sought to determine whether 2-NBDG is a suitable compound for studying glucose uptake in isolated vascular smooth muscle cells (VSMC). To our knowledge, this study is the first to characterize fluorescent glucose uptake in VSMC.

Method

Tissue handling

Tissues were obtained at a local abattoir. Carotid arteries were removed from pigs within ~30 min of slaughter and placed in physiological saline solution (PSS) consisting of (mM): NaCl (116), KCl (4.6), KH_2PO_4 (1.16), $NaHCO_3$ (25.3), $CaCl_2$ (2.5), $MgSO_4$ (1.16), and glucose (5.0), pH 7.4. PSS always included 40 mg/l gentamycin sulfate or 303 mg/l penicillin G plus 100 mg/l streptomycin sulfate to prevent bacterial contamination. Before use, PSS was oxygen- and pH-equilibrated by gassing with 95 % O_2/5 % CO_2. The storage container was kept on ice during tissue collection and transport to the laboratory.

Upon arrival at the laboratory, tissues were transferred to fresh PSS and all loose fat, connective tissue and adventitia removed. Tissues were stored at 4 °C in PSS until use.

Enzymatic dissociation of vascular smooth muscle cells from pig carotid artery

Individual VSMC were isolated by a modification of the procedure of Warshaw *et al.* (1986) as previously described by our group (Hardin and Finder 1998). Briefly, a syringe was used to inflate the artery with an enzymatic dissociation solution containing 496.6 U/ml collagenase, 11.9 U/ml elastase, 60 U/ml deoxyribonuclease I, 1.5 % bovine serum albumin, 0.1 % trypsin inhibitor, 3.99 mM ATP, 0.1 mM isoproterenol, and 1.3 % amino acid standard. Low-Ca^{2+} PSS (as described above, except that $CaCl_2$ was reduced to 0.5 mM) served as the base for the enzymatic dissociation solution. After the artery was inflated with enzyme solution, the free end was tied closed with suture. Filled arteries were incubated in low-Ca^{2+} PSS at 37 °C in a shaking bath for 90 min. Arteries were then cut open at one end and gently squeezed to remove the enzyme solution and the endothelial cells, which had been digested free of the artery wall. Next, arteries were refilled with a second enzymatic dissociation mixture identical to the first, except that the concentration of elastase in the solution was reduced to 4.04 U/ml. The elastase concentration was reduced in this second mixture, as higher elastase concentrations were found to result in damage to VSMC and a reduced cell yield. Refilled arteries were tied closed, placed in low-Ca^{2+} PSS, and incubated at 37 °C for 1 hour.

Following the second incubation, arteries were cut open at one end and the enzyme solution drained. To release VSMC, low-Ca^{2+} PSS was flushed in and out of the artery with a Pasteur pipette inserted into the open end. VSMC harvested from each artery were kept separate until microscopic examination of the cell suspension for quality. Cell suspensions consisting mainly of long, spindle-shaped VSMC were chosen for study and combined.

Synthesis of 2-NBDG

2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxyglucose (2-NBDG) was synthesized from 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD chloride) and glucosamine as described by Yoshioka *et al.* (1996c). The synthesis was started by combining 10 ml of 300 mM $NaHCO_3$ containing 0.5 g glucosamine with 20 ml methanol containing 0.5 g NBD chloride. The mixture was then placed in the dark and stirred overnight. The resulting dark brown solution was passed through Whatman No.1 filter paper to remove precipitate. The liquid was then passed over a silica gel column (Wakogel C-200, particle size 75-150 μm) and eluted with ultrafiltered water (18 MΩ resistance). Bright yellow fractions containing 2-NBDG were collected for further purification. Selected fractions were lyophilized to powder in a Speed Vac (Savant Instruments, Farmingdale, NY). The resultant dry material was weighed and then resuspended in MOPS-buffered PSS containing (in mM): NaCl (116), KCl (4.6), MOPS (26.2), $CaCl_2$ (2.5), $MgSO_4$ (1.16), pH 7.4. The volume of

MOPS-buffered PSS used for resuspension was calculated to result in a 5 mM solution of 2-NBDG. Typically, the resuspended 2-NBDG was a light brown in color and contained some particulate matter. Therefore, the solution was refiltered using a 0.2 μm syringe filter to yield a clear, bright yellow liquid. When examined in a fluorescence spectrophotometer (Hitachi F-3010, Hitachi Ltd., Tokyo), the 2-NBDG synthesized in our laboratory exhibited an absorption maximum at 480 nm and an emission maximum at 540, consistent with previously reported values of 475 nm and 550 nm (Yoshioka 1996c). 2-NBDG solution was stored at 4 °C until use, as freezing appeared to result in breakdown of the product.

Digital imaging fluorescence microscopy

These experiments utilized a widefield epifluorescence microscope (Nikon Diaphot, Garden City, NY) equipped for digital imaging of fluorescent probes. Major components of this system included a liquid-cooled CCD camera (Photometrics Ltd., Tucson, AZ), Ludl z-stepper motor and 6-position filter wheel (Ludl Electronic Products, Hawthorne, NY), 150 W xenon arc lamp and liquid light guides, and vibration isolation table. Computer equipment included MicroTome and Volume Scan software and a digital signal processing board for integrated control of microscope components, image acquisition and deconvolution (VayTek Corp., Fairfield, IA). ImagePro Plus software (Media Cybernetics, Silver Spring, MD), was used for quantitative analysis of images. For experiments in which the transport properties of 2-NBDG were studied, we utilized bandpass excitation of 485±15 nm, a 505 nm long-pass dichroic mirror, and a long-pass emission filter providing greater than 90 % transmittance at wavelengths between 520 and 700 nm (Omega Optical, Brattleboro, VT). This emission filter improved sensitivity more than 4-fold over a standard fluorescein emission filter of 535±35 nm. Images for these studies were acquired using a 100x oil immersion objective. The sarcolemma was defined by the transmitted light image and the area of interest saved for later application to the 2-NBDG images. Images collected in a 12-bit format were analyzed with MicroTome software or converted to 8-bit before analysis using ImagePro Plus software.

In experiments that were designed to investigate possible perinuclear distribution of 2-NBDG, 2-NBDG and the nuclear stain DAPI were imaged in the same cell. The optical properties of DAPI (excitation 375±20 nm, emission 452 nm) permitted excellent spectral separation from 2-NBDG. These experiments utilized bandpass excitation of 375 nm for DAPI and 485 nm for 2-NBDG, 455 and 530 nm bandpass dichroic mirrors, and 452±20 nm and 528±24 nm bandpass emission filters providing 70 and 90 % transmittance at the wavelengths studied (Omega Optical, Brattleboro, VT). These conditions allowed both DAPI and 2-NBDG to be monitored, although they were not optimal for the detection of 2-NBDG. Camera shutter time was optimized for the detection of 2-NBDG (10 s) and DAPI (1 s). Fluorescence images were acquired at five focal planes separated by 1.0 μm in the z-axis to permit deconvolution analysis. In addition, a transmitted light micrograph was taken for each field of cells at the middle focal plane. All images were acquired using a 40x oil immersion objective. Out-of-focus fluorescence was removed to yield high-resolution images by deconvolution utilizing the nearest neighbor algorithm employing a theoretical point spread function as described previously (Graier *et al.* 1998). This processing mode represents a "digital confocal microscope," but allowed us to utilize ultraviolet excitation for DAPI.

Time course of 2-NBDG uptake in the absence of glucose

Several drops of cell suspension were placed on a coverslip fixed to the bottom of the superfusion chamber. Cells were allowed to settle for several minutes to let them adhere to the coverslip. The chamber was then superfused with HEPES-buffered PSS containing (in mM): NaCl (138), KCl (5), $CaCl_2$ (2), $MgCl_2$ (1), HEPES (10), pH 7.4. Once non-adherent cells had washed away, a cell or group of cells with typical spindle morphology was chosen for study as we have previously described (Hardin and Finder 1998).

Once a cell was selected for study, both superfusion and suction were stopped, leaving the chamber filled with HEPES-buffered PSS. A 5 mM solution of 2-NBDG (100 μl) was carefully added to the chamber to avoid disturbing cells. Bath volume before 2-NBDG addition was 391±39.4 μl (mean ± SEM, n=7). Thus, the final concentration of 2-NBDG in the chamber was 1 mM.

Cells were incubated in 1 mM 2-NBDG for 5 min. Thereafter superfusion and suction were restarted. Cells were superfused with HEPES-buffered PSS for 5 min to clear 2-NBDG from the bath and eliminate background fluorescence. Following washout, an image of the cell was acquired. The 2-NBDG incubation, washout, and imaging procedures were then repeated three times. Thus, the same cell was imaged following 5, 10, 15, and 20 min of total exposure to 2-NBDG.

Because additional exposure of 2-NBDG to the excitation beam resulted in significant photobleaching, no further time points were examined. Transmitted light images of cells were acquired after each exposure to 2-NBDG to verify that the cell had not been mechanically disturbed by the solution changes.

Average fluorescence intensity (I_{avg}) of the cells at each time point was determined with ImagePro Plus software. The outline of the cell was traced using the transmitted light image. This area was used as the area of interest for which I_{avg} was calculated. Because deconvolution of the images resulted in images which were too dim to analyze, I_{avg} was calculated using non-deconvolved images.

Time course of 2-NBDG uptake in the presence of D-glucose or L-glucose

Experiments in which the effect of D-glucose or L-glucose on 2-NBDG uptake by VSMC was examined were performed as above. In these studies, HEPES-buffered PSS was replaced by HEPES-buffered PSS containing either 10 mM D-glucose or 10 mM L-glucose for superfusion and washout.

Selection of cells for data analysis

The solution changes performed during the course of the experiments sometimes dislodged the cell of interest, causing it to move. Such cells were not included in the data analysis. Cells that showed little or no fluorescence, likely due to non-viability, were also excluded. One aberrant cell in the L-glucose group, which exhibited similar fluorescence intensity after the first two exposures to 2-NBDG, a nearly 3-fold increase over this level following the third exposure, and a subsequent ~1.5-fold decrease after the fourth exposure, was also excluded from the data analysis. This cell was regarded as an outlier, since this behavior was not noted in any other cell studied.

Examination of perinuclear distribution of 2-NBDG

For experiments in which a possible perinuclear distribution of 2-NBDG was investigated, VSMC were obtained from pig carotid artery as described above, except that cells were harvested in HEPES-buffered PSS. 2-NBDG utilized in these experiments was obtained from Molecular Probes (Eugene, OR), rather than synthesized in our laboratory. Cells were incubated with 25 μM DAPI and 1 mM 2-NBDG for 30 min at 37 °C. At the end of the incubation period, cells were pelleted by centrifugation and resuspended in HEPES-buffered PSS to remove background fluorescence. Cells were allowed to settle onto coverslips coated with Cell-Tak (Collaborative BioMedical Products, Bedford, MA) before images were collected. Images were acquired as described above.

Measurement of lactate production by carotid arteries

Carotid arteries were obtained and dissected as described above. Artery segments used for measuring lactate production weighed 0.3415±0.0134 g (n=24). Arteries were placed in individual chambers and incubated (3 h at 37 °C) in MOPS-buffered PSS containing 0, 5, 10, 20, 30, or 50 mM glucose (n=4 for each concentration). The solutions were bubbled with air throughout the incubation.

At the end of the incubation period, arteries were removed from the incubation solution, blotted dry on filter paper, and weighed. The incubation solution was frozen in liquid nitrogen for lactate assay.

Lactate production was assayed spectrophotometrically. Carotid incubation solution (20 μl) was added to 980 μl of lactate assay buffer containing 160 mM hydrazine, 400 mM glycine, 625 μM NAD, and 10 U/ml lactate dehydrogenase, pH 9.0-9.1. The change in absorbance was read at 340 nm.

Reagents

Collagenase was obtained from Worthington Biochemical (Lakewood, NJ). Elastase was purchased from Calbiochem (La Jolla, CA). Wakogel was obtained from Wako Pure Chemical Industries Ltd. (Japan). DAPI and 2-NBDG used in combination with DAPI were obtained from Molecular Probes (Eugene, OR). All other reagents were from Sigma Chemical (St. Louis, MO).

Statistical analysis

Fluorescence data were analyzed using a one-tailed Student's t-test for two samples assuming unequal variances. All statistical calculations were performed using Microsoft Excel 97 software. P values ≤ 0.05 were considered significant.

Results

D-glucose inhibits 2-NBDG uptake by VSMC

We examined the effect of D-glucose and L-glucose on 2-NBDG uptake by VSMC to ascertain whether 2-NBDG utilizes glucose transporters to enter VSMC. To determine if 2-NBDG uptake by VSMC is

inhibited by D-glucose, VSMC received four sequential five-minute exposures to 1 mM 2-NBDG in the presence of 10 mM D-glucose. VSMC exposed to 2-NBDG in the presence of 10 mM D-glucose (n=6) were significantly less fluorescent (p=0.028) than VSMC exposed to 2-NBDG alone (n=4) (Fig. 1). Thus, D-glucose appears to inhibit entry of 2-NBDG into VSMC by competing with 2-NBDG for glucose transporters.

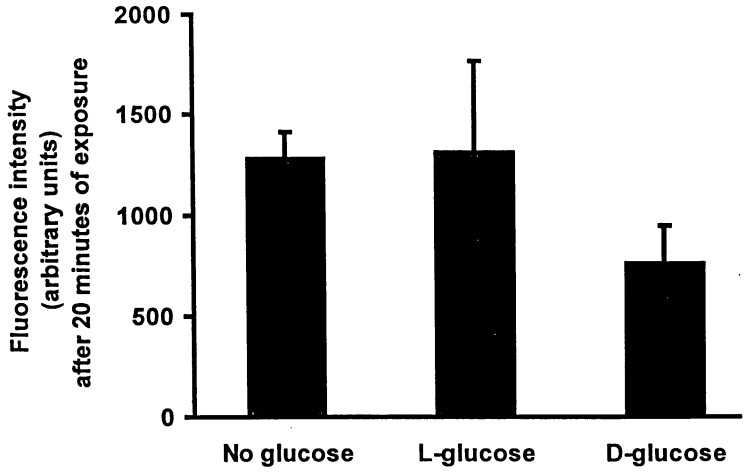

Fig. 1. *D-glucose, but not L-glucose, inhibits uptake of 2-NBDG by vascular smooth muscle cells. Cells received four sequential five-minute exposures to 1 mM 2-NBDG in the presence of no additional glucose (n=4), 10 mM D-glucose (n=6) or 10 mM L-glucose (n=2). Average fluorescence intensity of cells (arbitrary units) was calculated following 20 min of total exposure to 2-NBDG. Cells treated with 2-NBDG alone are more fluorescent than cells treated with 2-NBDG in the presence of 10 mM D-glucose (p=0.028). On the contrary, average fluorescence intensity of cells treated with 2-NBDG in the presence of 10 mM L-glucose is not significantly different from the control (no glucose) condition (p=0.477). Results are presented as the mean ± SEM.*

L-glucose does not appear to inhibit 2-NBDG uptake by VSMC

L-glucose, a non-transportable form of glucose, should be expected to have little inhibitory effect on entry of 2-NBDG into VSMC if 2-NBDG uptake occurs *via* glucose transporters. Therefore, VSMC received four sequential five-minute exposures to 1 mM 2-NBDG in the presence of 10 mM L-glucose. At 20 min of exposure to 2-NBDG, I_{avg} of L-glucose treated cells was indistinguishable from controls (Fig. 1).

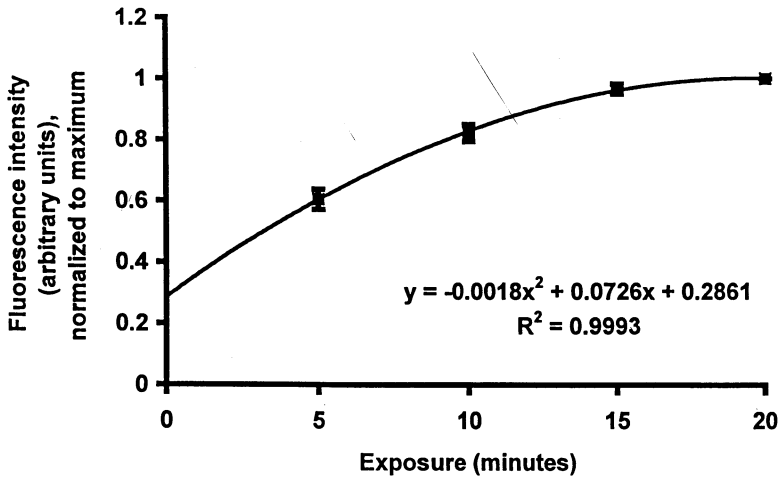

Fig. 2. *2-NBDG uptake saturates by 15 min total exposure to 2-NBDG in the absence of D-glucose (R^2=0.9993, n=4). Data for each cell have been normalized to the maximum fluorescence intensity of that cell and are shown in arbitrary units. Results are presented as the mean of the normalized values ± SEM. Some error bars are obscured by the data points.*

2-NBDG uptake is non-linear in the absence of D-glucose, but not in its presence

The data were also examined to determine if 2-NBDG uptake was linear. Linearity of 2-NBDG uptake was evaluated by normalizing the average fluorescence intensity of the cell at each time point to the maximum fluorescence intensity of that cell. The normalized data show that 2-NBDG uptake by VSMC becomes non-linear by 15 min of total exposure to 1 mM 2-NBDG in the absence of D-glucose (n=4, R^2=0.9993) (Fig. 2). The non-linearity of 2-NBDG uptake suggests that VSMC are unable to metabolize this compound, since non-metabolizable analogs of glucose 6-phosphate should accumulate in cells and inhibit further transport. The calculated line for fluorescence intensity versus exposure time does not pass through the origin, suggesting that an initial rapid uptake of 2-NBDG, that cannot be resolved within the time scale of our experiments, may occur. This rapid uptake phase could reflect either another transport process or a slight binding of 2-NBDG to the cell surface.

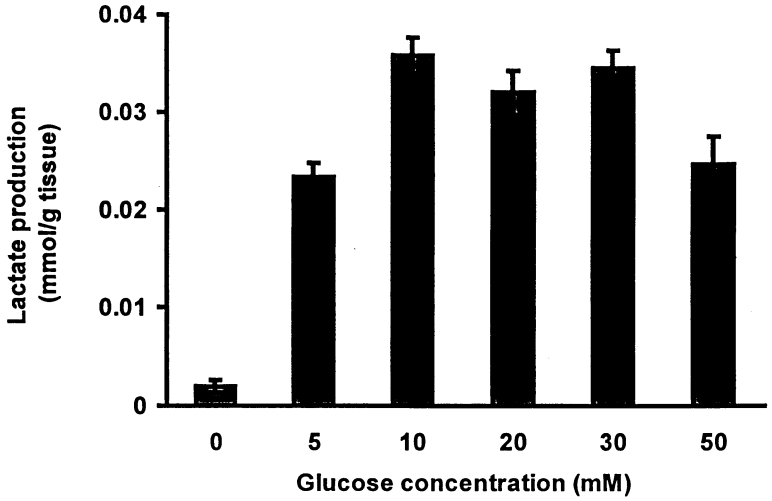

Fig. 3. *Glucose uptake, as measured by lactate production, is linear over the range of 0-10 mM in pig carotid arteries (R^2=0.978, n=4 arteries at each concentration). Arteries were incubated with 0-50 mM glucose for 3 h at 37 °C and glucose uptake was evaluated by assaying lactate production. Results are presented as the mean for each concentration ± SEM.*

To investigate the possibility that accumulation of 2-NBDG within cells inhibits 2-NBDG transport, we examined lactate production in pig carotid arteries to determine the concentration range over which glucose uptake is linear in VSMC. Since vascular smooth muscle is largely glycolytic, lactate production is a good indicator of glucose uptake by this tissue (Hardin and Paul 1995). In pig carotid arteries, lactate production remained linear over the range of 0-10 mM glucose (R^2=0.978, n=4) (Fig. 3). Thus, 2-NBDG uptake becomes non-linear at much lower concentrations than does glucose uptake. These data are consistent with inhibition of transport produced by the intracellular accumulation of a non-metabolizable compound, as well as with a low affinity of the glucose transporter for 2-NBDG.

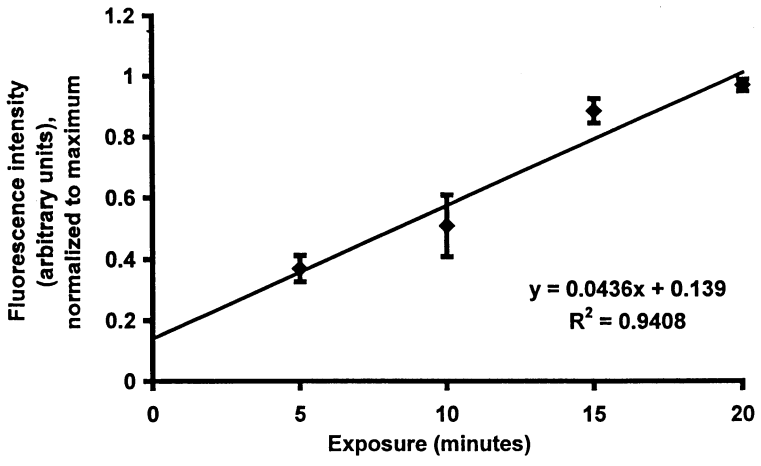

Fig. 4. *2-NBDG uptake is linear in the presence of 10 mM D-glucose (R^2=0.9408, n=6). Data for each cell have been normalized to the maximum fluorescence intensity of that cell and are shown in arbitrary units. Results are presented as the mean of the normalized values ± SEM. Some error bars are obscured by the data points.*

To further investigate this issue, we examined the linearity of 2-NBDG uptake in VSMC that received four sequential five-minute exposures to 2-NBDG in the presence of 10 mM D-glucose. In the presence of 10 mM D-glucose, which inhibits 2-NBDG transport, 2-NBDG uptake remained linear for at least 20 min of total exposure to 2-NBDG (R^2=0.9408, n=6; data normalized as described above) (Fig. 4). Thus, it appears that 2-NBDG is non-metabolizable and accumulates within VSMC, inhibiting its own transport.

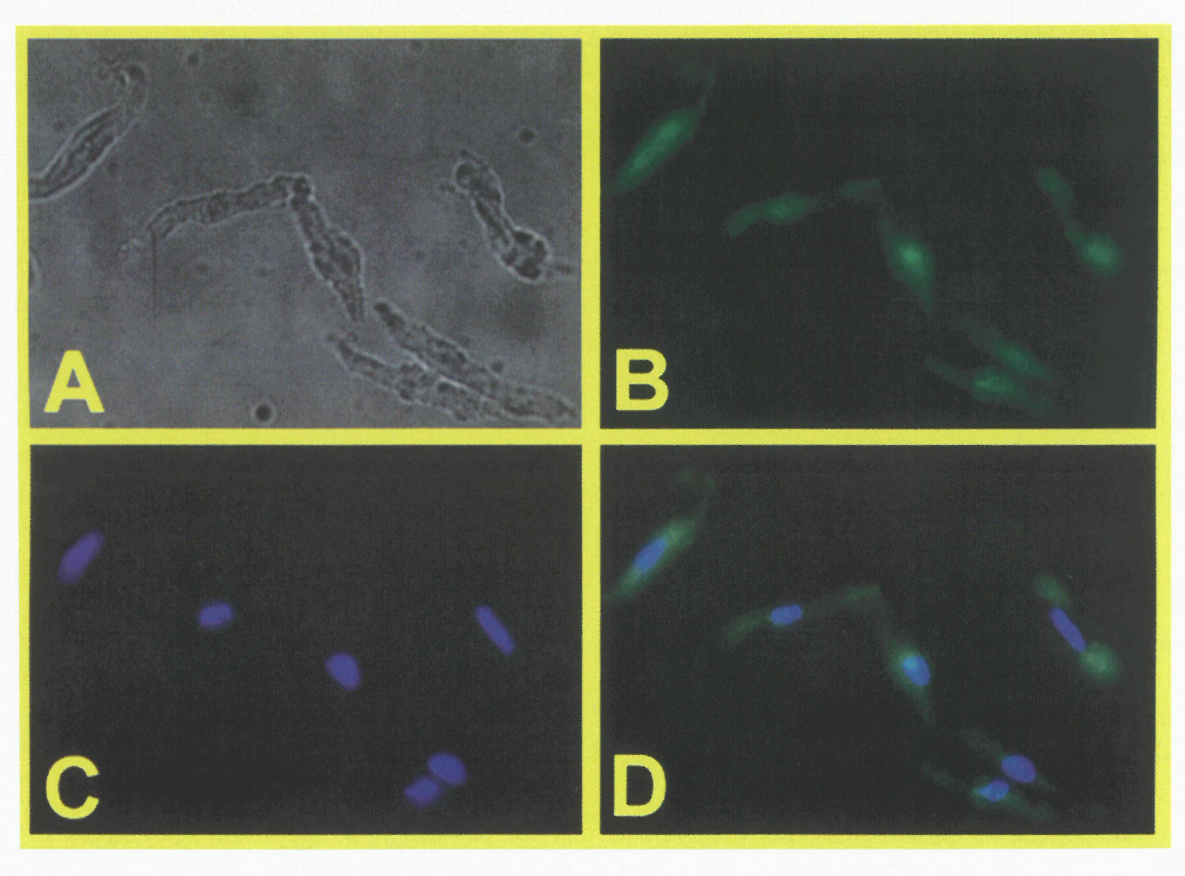

Fig. 5. *2-NBDG fluorescence is localized in the perinuclear region. Cells were incubated with 1 mM 2-NBDG and 25 μM DAPI. A, transmitted light image of cells. B, 2-NBDG staining (note the increased staining in the perinuclear region). C, DAPI staining, showing position of nuclei. D, combined image showing both 2-NBDG (green) and DAPI (blue).*

Localization of 2-NBDG fluorescence in VSMC

Of the cells in which 2-NBDG transport was studied, 58 % (7 of 12) were non-uniformly labeled by 2-NBDG. In some cells, localization appeared to be perinuclear. Punctate fluorescence and fine striations were also observed in some cells. In order to determine if 2-NBDG was localized to the perinuclear region, we treated cells with both 2-NBDG and the DNA stain DAPI. In most cells, 2-NBDG fluorescence was stronger in the perinuclear region of the cell than at its periphery (Fig. 5). Four of six cells (67 %) demonstrated perinuclear localization of 2-NBDG; one cell demonstrated exclusion of 2-NBDG from the perinuclear region; and one cell demonstrated little or no localization of 2-NBDG fluorescence. The images shown in Figure 5 have not been deconvolved (the low intensity of the 2-NBDG signal prevented the production of high-quality deconvolved images). However, deconvolution analysis demonstrated that the increased intensity of 2-NBDG fluorescence in the perinuclear region was not due to a thickness artifact (data not shown).

Discussion

We have described for the first time a single-cell method for determination of glucose uptake in vascular

smooth muscle cells. The transport process has some fundamental characteristics that are similar to those described for other cell types.

2-NBDG uptake by VSMC is competitively inhibited by D-glucose

2-NBDG uptake by vascular smooth muscle cells, as measured by $I_{avg.}$, was significantly inhibited in the presence of 10 mM D-glucose. In contrast, I_{avg} of cells exposed to 2-NBDG in the presence of 10 mM L-glucose did not appear to be significantly different from control. Although the low number of cells studied in the presence of L-glucose makes it difficult to draw firm conclusions from these experiments, it appears likely that L-glucose does not inhibit uptake of 2-NBDG by vascular smooth muscle cells. In contrast, D-glucose did inhibit 2-NBDG transport. Thus, the present study suggests that 2-NBDG utilizes cellular glucose transporters to enter VSMC, in agreement with data from other cell types (Yoshioka *et al.* 1996a,c).

2-NBDG is incompletely metabolized in VSMC

When glucose was absent from the superfusion solution, 2-NBDG uptake by VSMC became non-linear by 15 min of exposure to 2-NBDG. In the presence of 10 mM D-glucose, however, 2-NBDG uptake remained linear for at least 20 min. As discussed above, D-glucose inhibits 2-NBDG uptake by VSMC, thereby reducing the accumulation of 2-NBDG in the cell. These data suggest that 2-NBDG is non-metabolizable, and that accumulation of this compound within VSMC inhibits further transport. This is consistent with previous studies of 2-NBDG transport in *Escherichia coli* which suggest that 2-NBDG may be phosphorylated by hexokinase, but is metabolized no further (Yoshioka *et al.* 1996b). In this context it should be noted that the structurally similar glucose analog 6-NBDG is completely non-metabolizable, since the fluorophore is attached to carbon 6, replacing the hydroxyl which is normally phosphorylated by hexokinase (Speizer *et al.* 1985).

2-NBDG fluorescence is localized in VSMC

Most of the cells examined in the transport portion of this study displayed localized 2-NBDG fluorescence, suggesting that this compound may bind to unidentified intracellular structure(s). Z-scans demonstrated that the fluorescence was not surface-associated (data not shown). Further experiments in which 2-NBDG fluorescence was detected in combination with the nuclear stain DAPI, confirmed that 2-NBDG has a perinuclear distribution in most cells. These observations are consistent with previously reported data for the related compound 6-NBDG, which accumulates in red blood cells above its equilibrium concentration due to binding to some component of the cytoplasm, possibly hemoglobin (Speizer *et al.* 1985). Since VSMC lack hemoglobin, the localized fluorescence observed in this study suggests that 2-NBDG can also interact with other constituents of the cytoplasm.

The difference in the position of the fluorophore in the 2-NBDG used in the present study and the 6-NBDG used by Speizer *et al.* (1985) suggests an interesting possibility. As discussed above, 2-NBDG appears to be phosphorylated by hexokinase, whereas 6-NBDG is not. Thus, the localization observed in the present study may be due to binding of 2-NBDG to hexokinase, and may reflect hexokinase localization. However, this suggestion is speculative, as it is currently unknown whether 2-NBDG localization reflects specific interactions with hexokinase, or nonspecific interactions with a variety of cytoplasmic proteins. Alternatively, the perinuclear distribution of 2-NBDG may be due to an as-yet-uncharacterized interaction between the fluorophore and internal cellular membranes such as the sarcoplasmic reticulum and/or the nuclear membrane.

Significance and Conclusion

Despite the potential usefulness of small fluorescent glucose analogs such as 2-NBDG and 6-NBDG, these compounds have not been extensively studied. 2-NBDG and 6-NBDG have been reported to have similar transport properties in a variety of species and cell types (Shimada *et al.* 1994, Speizer *et al.* 1985, Yoshioka *et al.* 1996a,c). However, the uptake of fluorescent glucose analogs by vascular smooth muscle has not previously been examined. Vascular smooth muscle is a highly glycolytic tissue in which glucose transport and metabolism is central to normal function (Hardin and Paul 1995). Furthermore, alterations in vascular smooth muscle glucose transport play an important role in the pathogenesis of diabetic vascular disease (Ruderman *et al.* 1992). The use of 2-NBDG allows glucose transport in vascular smooth muscle to be visualized at the level of the single cell for the first time. This technique will enable glucose transport measurements in small microvessels and other artery samples from diseased animals and humans from which few cells are available.

The present study expands upon previous work in other cell types to demonstrate that 2-NBDG uptake can be used as a single-cell glucose uptake assay in isolated vascular smooth muscle cells. Examination of VSM glucose uptake in vascular smooth muscle at the single cell level should provide insights into how this central process is altered in disease states such as diabetes, as well as into how glucose uptake rate affects other cellular processes.

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Abbreviations used

ATP, adenosine 5'-triphosphate
DAPI, 4',6-diamidino-2-phenylindole
HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-(2-ethanesulfonic acid)
I_{avg}, average fluorescence intensity
MOPS, 3-(*N*-morpholino)propanesulfonic acid
NAD, β-nicotinamide adenine dinucleotide
2-NBDG, 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxyglucose
6-NBDG, 6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-6-deoxyglucose
PSS, physiological saline solution
VSM, vascular smooth muscle
VSMC, vascular smooth muscle cells

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