The Effects of Brefeldin A on the Glucose Transport System in Rat Adipocytes

IMPLICATIONS REGARDING THE INTRACELLULAR LOCUS OF INSULIN-SENSITIVE Glut4*

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Insulin activates glucose transport by recruiting Glut4 glucose transporters from an intracellular pool to plasma membrane (PM). To localize intracellular translocating Glut4, we studied the effects of brefeldin A (BFA), which disassembles Golgi and prevents trans-Golgi vesicular budding, on the glucose transport system. Isolated rat adipocytes were treated with and without both BFA (10 μg/ml) and insulin. BFA did not affect maximal rates of either 2-deoxyglucose or 3-O-methylglucose transport or the insulin:glucose transport dose-response curve but did increase basal transport by ~2-fold (p < 0.05). We also measured Glut4 in PM, low (LDM) and high density microsome subfractions. In basal cells, BFA increased PM Glut4 by 58% concomitant with a 18% decrease in LDM (p < 0.05). Insulin alone increased PM Glut4 by 3-fold concomitant with a 56% decrease in LDM. BFA did not affect insulin-induced changes in Glut4 levels in PM or LDM. Most intracellular Glut4 was localized to sub-PM vesicles by immunoelectron microscopy in basal cells, and BFA did not affect insulin-mediated recruitment of immunogold-labeled Glut4 to PM. In summary, 1) in basal cells, BFA led to a small increase in glucose transport activity and redistribution of a limited number of transporters from LDM to PM; 2) BFA did not affect insulin's ability to stimulate glucose transport or recruit normal numbers of LDM Glut4 to PM; and 3) insulin action is predominantly mediated by a BFA-insensitive pool of intracellular Glut4, which localizes to sub-PM vesicles. Thus, the major translocating pool of Glut4 in rat adipocytes does not involve trans-Golgi.

Insulin stimulates glucose transport by rapidly inducing the translocation of intracellular glucose transporters to the cell surface (1, 2). The Glut4 transporter isoform predominates in insulin target tissues (fat and muscle) and mediates the bulk of insulin-stimulatable glucose transport activity. The lack of specific biochemical markers has made it difficult to ascertain the exact intracellular locus of Glut4 in membrane subfractionation experiments (3). Morphological studies employing immunoelectron microscopy (IEM) have localized the major portion of insulin-responsive Glut4 to trans-Golgi (4–6). However, Smith et al. (7) have shown in rat adipocytes that most intracellular Glut4 undergoing insulin-mediated translocation derive from vesicles adjacent to the endoplasmic reticulum (PME). Lange and Brandt (8) found that intracellular translocating Glut4 are concentrated in a cell surface-derived membrane fraction in 3T3-L1 adipocytes by employing subcellular fractionation method together with a hydrodynamic shearing technique.

In the current study, we used brefeldin A (BFA) to test whether the intracellular translocating pool of Glut4 is functionally dependent upon an intact Golgi in rat adipocytes. BFA is a fungal macrocyclic antibiotic, which disrupts the organization of Golgi complex (9), prevents vesicular budding from Golgi and trans-Golgi (10), and leads to retrograde movement of Golgi proteins back to the endoplasmic reticulum. If insulin-responsive Glut4 derive from the Golgi network, BFA should inhibit insulin-stimulated glucose transport and Glut4 translocation.

EXPERIMENTAL PROCEDURES

Materials—Human insulin was kindly supplied by Eli Lilly (Indianapolis, IN). 1-[3-3H]Glucose, 2-deoxy-O-[1,2-3H]glucose, and 3-O-[14C]-methylglucose were purchased from DuPont NEN; bovine serum albumin fraction V was from Armour (Chicago); collagenase type II was from Worthington (Freehold, NJ); and all other reagent grade chemicals were from Sigma. Polyclonal antibodies specific for Glut4 or Glut1 were purchased from East Acres Biologicals (Southbridge, MA) and used for immunoblotting. Monoclonal antibodies for Glut4 were kindly supplied by M. J. Charron (Albert Einstein College of Medicine, NY) and used for immunoelectron microscopy.

Adipocyte Isolation and Glucose Transport Studies—The epididymal fat pads were excised from adult male Sprague-Dawley rats (Harland, Indianapolis, IN), weighing 175–225 g, weighed, and digested by collagenase (1 mg/ml, final concentration) at 37 °C for 1 h as described previously (11). Digestion was performed using glucose- and insulin-free buffer containing 25 mM HEPES, 115 mM NaCl, 0.8 mM MgSO₄, 1.4 mM CaCl₂, 5.4 mM KCl, 1.0 mM NaH₂PO₄, 1.0 mM sodium pyruvate, and 1% bovine serum albumin at pH 7.4. After digestion, isolated cells were filtered through a fine nylon mesh, washed three to four times, and resuspended in the same buffer. Cells were incubated with or without BFA for 30–60 min, followed by treatment with or without insulin for 30 min. Glucose transport activity was determined as initial rates of 2-deoxyglucose (2-DG) or 3-O-methylglucose (3-OMG) uptake. 2-DG uptake was measured over 3 min (2–3 × 10⁶ cells/ml) as described previously (11). 3-OMG uptake was assayed by a modification of the method of Whitesell and Gliemann (12), as described previously by Foley et al. (13).

Membrane Subfractions and Immunoblot—PM and low (LDM) and

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high (HDM) density membrane subfractions were prepared from rat adipocytes using a modification of the differential ultracentrifugation method of McKeel and Jarett (14) as described previously (6). 35S protein from each fraction was solubilized in Laemmli sample buffer (15) and resolved by SDS-polyacrylamide gel electrophoresis (1.5-mm slab gels, 7% polyacrylamide). Proteins were electrophoretically transferred to nitrocellulose filters and incubated with Glut4- or Glut1-specific polyclonal antibodies (1:1000) followed by125I-protein A. Relative levels of immunoreactive Glut4 and Glut1 were quantitated by densitometric analysis of autoradiographs.

Immunoelectron Microscopy—Isolated rat adipocytes were incubated for 1 h at 37 °C with no addition or with 10 μg/ml BFA. Half of each set was then incubated for 30 min with no addition or with 100 ng/ml insulin. The cells were fixed and prepared as described previously (7, 16) for both routine electron microscopy, to assess the effects of BFA on Golgi structure, and immunoelectron microscopy, to examine effects of insulin and BFA on the cellular distribution of Glut4. Monoclonal antibodies MC2A and MC2A against Glut4 carboxyl- and amino-terminal, respectively, were employed. Immunolabeling and quantitative morphometric analysis of Glut4 associated with the PM and sub-PM

![Figure 1](image1.png)  
**FIG. 1. Effect of BFA on 2-deoxyglucose transport.** Equal numbers of isolated rat adipocytes were treated without and with the indicated concentrations of BFA for 30 min followed by incubation in the absence and presence of a maximal insulin concentration (100 ng/ml) for 30 min. At the end of incubation, initial rates of 2-DOG uptake were measured. The data are mean ± S.E. of three experiments.

![Figure 2](image2.png)  
**FIG. 2. Effect of BFA on dose-response curve for insulin stimulation of glucose transport.** Equal numbers of isolated rat adipocytes were treated without and with BFA (10 μg/ml) for 30 min followed by incubation in the absence and presence of indicated insulin concentration for 30 min. At the end of incubation, initial rates of 2-DOG were measured. The data are mean of six experiments.
invaginations and vesicles, and cytoplasmic vesicles were performed as described previously (7).

RESULTS

Glucose Transport Activity—We first studied whether BFA could affect glucose transport activity in intact cells. Isolated rat adipocytes were preincubated with increasing concentrations of BFA (0–10 μg/ml) for 30 min and then treated in the absence and presence of a maximal insulin concentration (100 ng/ml) for 30 min. At the end of incubation, initial rates of 3-OMG were measured. The data are mean ± S.E. of four experiments.

![Graph showing effects of BFA on glucose transport](image)

**FIG. 3.** Effect of BFA on 3-O-methylglucose transport. Equal numbers of isolated rat adipocytes were treated without and with BFA (10 μg/ml) for 30 min followed by incubation in the absence and presence of a maximal insulin concentration (100 ng/ml) for 30 min. At the end of incubation, initial rates of 3-OMG were measured. The data are mean ± S.E. of four experiments.

![Graph showing reversibility of BFA effect](image)

**FIG. 4.** Reversibility of BFA effect. Isolated rat adipocytes were treated without and with BFA (10 μg/ml) for 30 min followed by incubation in the absence and presence of maximal insulin concentration (100 ng/ml) for 30 min. At the end of incubation, the cells were washed three to four times and resuspended in the same but BFA-free buffer for another 30 min before 2-DOG assay was performed. The data are mean ± S.E. of three experiments.

concentrations of 5 and 10 μg/ml, increased basal 2-DOG transport rates by ~2-fold (p < 0.05). In addition, full dose-response curves, shown in Fig. 2, demonstrated that BFA at 10 μg/ml did not significantly affect the insulin ED_{50} for stimulation of 2-DOG uptake. We also examined whether BFA could alter basal and insulin-stimulated transport rates of 3-OMG, a glucose analog that is transported but not phosphorylated upon entry into the cell. Again, BFA (10 μg/ml) produced a moderate increase in basal transport (64%, p < 0.05) but did not affect maximal insulin-stimulated 3-OMG transport rates (Fig. 3). The increase in basal 2-DOG transport was fully reversed...
within 30 min after BFA removal, as shown in Fig. 4. However, as might be expected, insulin-stimulated 2-DOG transport was not affected by the addition or removal of BFA. In other experiments not shown, adipocytes were first stimulated with maximal insulin (100 ng/ml, 30 min, 37°C) and then exposed to 10 μg/ml BFA for 30 min. Pretreatment with insulin did not alter the results; BFA still was unable to affect insulin-stimulated 2-DOG transport.

Immunoblot Analysis—To examine whether BFA altered the subcellular localization of glucose transporters, PM, LDM, and HDM subfractions were prepared from cells preincubated with or without BFA and insulin, and transporter levels were quantitated by immunoblot analysis of membrane proteins. As shown in Fig. 5B, in basal cells, BFA increased Glut4 in the PM fraction by 58% concomitant with a 18% decrease in LDM (both p < 0.05). In cells treated with insulin alone, Glut4 was increased by ~3-fold in PM and was decreased by 56% in LDM. Importantly, BFA did not affect levels of Glut4 in PM from insulin-treated cells, or the Glut4 decrement in LDM. In both basal and insulin-stimulated cells, BFA did not alter HDM Glut4 content (Fig. 5B; p = not significant). The effect of BFA on glucose transporter distribution in basal cells was specific for Glut4; BFA had no significant effect on Glut1 subcellular distribution (data not shown).

From the data in Fig. 5B, it is clear that even maximal insulin was unable to recruit all intracellular Glut4 in LDM to the PM; 40–45% of Glut4 remains associated with LDM as has been consistently reported by multiple investigators. Although BFA caused an 18% decrease in LDM Glut4 in both basal and insulin-stimulated cells, the absolute decrement in LDM Glut4 as a consequence of insulin stimulation was similar in cells treated with and without BFA. Therefore, the BFA-sensitive pool of LDM Glut4 appears to be distinct from insulin-responsive translocating Glut4.

Immunoelectron Microscopic Studies—Table I shows quantitative morphometric analysis of immunolabeled Glut4 using two monoclonal antibodies. Studies using the amino-terminal antibody MC1A revealed that the majority of intracellular Glut4 was localized to sub-PM vesicles or PM invaginations in basal cells. Insulin treatment caused a significant translocation of sub-PM Glut4 to the cell surface. The carboxyl-terminal antibody MC2A preferentially recognizes insulin-activated
Glut4 (7), and studies using MC2A also revealed a large increase in “activated” Glut4 associated with the PM upon insulin stimulation (Table I, Fig. 6, A and B). Quantitative analysis by IEM was not able to statistically resolve small changes in PM and LDM Glut4 as a consequence of BFA treatment in basal cells. Importantly, BFA had no effect on either translocation (MC1A) or activation (MC2A) of Glut4 by insulin (Table I; Fig. 6C). Routine electron microscopy demonstrated that BFA treatment under these experimental conditions disrupted the Golgi complex in rat adipocytes (data not shown).

**DISCUSSION**

Studies designed to localize the intracellular, insulin-sensitive, translocating pool of Glut4 have been controversial. Our experiments, using BFA, support the conclusion that the Golgi apparatus and trans-Golgi are not the major functional sources of Glut4 undergoing insulin-mediated translocation to PM in rat adipocytes. BFA is a fungal antibiotic that has been extensively used in studies of intracellular membrane trafficking. BFA causes a morphological disassembly of Golgi apparatus. It blocks transport of proteins into post-Golgi compartments in the cell and redistributes Golgi-resident proteins back into endoplasmic reticulum (18). BFA blocks constitutive secretion by preventing the formation of non-clathrin-coated vesicles required for vesicular transport through Golgi cisternae (10) as well as clathrin-coated vesicles that bud from trans-Golgi network (19). Molecular mechanisms of BFA toxicity are not known; however, β-cop, ADP-ribosylation factor (components of non-clathrin-coated vesicles), as well as γ-adaplin (a component of clathrin-coated vesicles) are unable to bind to Golgi membranes and are released into cytosol. Blockage of vesicle assembly prevents anterograde transport of proteins from endoplasmic reticulum through Golgi to the cell surface. Thus, if intracellular translocating Glut4 derives from trans-Golgi, we would expect to see that insulin-stimulated glucose transport and Glut4 translocation are inhibited by BFA. However, in this study, we demonstrated that BFA did not affect insulin’s ability to stimulate glucose transport or translocate normal numbers of intracellular Glut4 to PM.

We also found that BFA led to a small (~2-fold) increment in basal glucose transport activity, which corresponds with redistribution of a limited number of Glut4 from LDM to PM. The data in Fig. 5 define two functional pools of intracellular Glut4. BFA-sensitive Glut4 constitute a small pool of intracellular Glut4, which is recruited from LDM to PM subfractions upon treatment with doses of BFA known to disrupt Golgi. However, BFA did not affect insulin’s ability to maximally stimulate glucose transport activity or translocate normal numbers of LDM Glut4 to PM. Thus, the BFA-sensitive pool is distinct from the absolute net decrement in LDM Glut4 observed as a consequence of maximal insulin stimulation. This latter insulin-responsive component comprises the largest pool of intracellular Glut4. The combined biochemical and immunocytochemical data indicate that the insulin-responsive pool is not located in trans-Golgi and more likely resides in PM invaginations, cell surface-connected vesicles, or sub-PM endosomal compartments (7), which could sediment or cofractionate in the LDM subfraction. These ideas are consistent with data obtained using a hydrodynamic shearing technique applied to 3T3 cells before homogenization (8). This procedure yielded a low density surface-derived vesicle fraction, which would sediment in LDM if shearing was not applied. It was shown that the low density surface-derived vesicle fraction contained nearly 60% of the cellular glucose transporters and the total insulin-sensitive transporter pool.

Other authors have recently examined effects of BFA on Glut4 translocation. Chakrabarti et al. (19) studied effects of BFA on glucose transport and transporter translocation in

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**Table I**

| Antibody  | Condition | Plasma membrane | Sub-PM vesicles and invaginations | Cytoplasmic vesicles |
|-----------|-----------|-----------------|----------------------------------|----------------------|
| MC1A Basal | 0.3 ± 0.1 | 1.8 ± 0.5       | 0.1 ± 0.0                        |
| BFA       | 0.5 ± 0.1 | 1.9 ± 0.5       | 0.1 ± 0.0                        |
| Insulin   | 2.3 ± 0.7 | 1.1 ± 0.3       | 0.1 ± 0.0                        |
| BFA + insulin | 2.2 ± 0.7 | 1.2 ± 0.4       | 0.1 ± 0.0                        |

| MC2A Basal | 0.5 ± 0.2 | 0.1 ± 0.1       | 0.1 ± 0.0                        |
| BFA       | 0.6 ± 0.2 | 0.1 ± 0.0       | 0.1 ± 0.0                        |
| Insulin   | 4.8 ± 1.3 | 0.1 ± 0.0       | 0.1 ± 0.0                        |
| BFA + insulin | 4.8 ± 1.2 | 0.1 ± 0.0       | 0.1 ± 0.0                        |

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**Fig. 6. Immunogold labeling of Glut4 in isolated rat adipocytes.** Isolated rat adipocytes were treated without and with BFA (10 μg/ml) for 1 h followed by incubation in the absence and presence of maximal insulin concentration (100 ng/ml) for 30 min. At the end of incubation, the cells were prepared for IEM and immunostained with MC2A, the monoclonal antibody to the carboxyl-terminal peptide of Glut4. Antibody binding sites were detected with gold-labeled protein A. A: basal cells; B: insulin-treated cells; C: cells treated by both BFA and insulin. pm, plasma membrane; L, central lipid droplet; arrowheads, sub-PM invaginations or vesicles.
3T3-L1 cells. Similar to our data in rat adipocytes, these authors found that BFA increased PM Glut4 by approximately 2-fold and did not impair insulin’s ability to increase cell surface Glut4 concentration. However, BFA was found to inhibit both basal and insulin-stimulated glucose transport activity by up to ~60% with a half-maximal effect being observed at 10 μg/ml. They explained that BFA may directly inhibit the intrinsic activity of glucose transporters. We found no evidence in rat adipocytes that 10 μg/ml BFA inhibited intrinsic activity of glucose transporters. The reason for the difference in BFA effects between 3T3-L1 cells and rat adipocytes is not clear but could be explained by utilization of different cell systems or more generalized cellular toxicity at high BFA concentrations.

Lachaal et al. (20) have also studied the effects of BFA on glucose transport and transporter translocation in rat adipocytes. In their studies, BFA at 1 μg/ml inhibited insulin-stimulated glucose transport as well as redistribution of Glut4 from microsomes to PM. These data are not confirmed in the current study. There were some differences in experimental design. Lachaal et al. (20) used 3-OMG equilibrium exchange to measure glucose transport activity and did not subfractionate total microsomes into LDM and HDM while studying Glut4 subcellular redistribution. However, it is not clear whether these differences can fully explain the discrepancies between the results. In the current study, we have performed morphological investigations in addition to biochemical experiments, and both lines of investigation are consistent with the conclusion that BFA does not interfere with insulin’s ability to stimulate glucose transport activity at Glut4 translocation.

In summary, we have shown that 1) in basal cells, BFA led to a small increase on glucose transport activity and to the redistribution of a limited number of Glut4 from LDM to the PM subfraction; 2) BFA did not affect insulin’s ability to stimulate transport or recruit normal numbers of Glut4 to PM; 3) there are two functional pools of intracellular Glut4 in rat adipocytes, a large insulin-responsive pool, which is unaffected by BFA, and a smaller BFA-sensitive pool; and 4) BFA-insensitive Glut4, which translocate in response to insulin, are localized by IEM in sub-PM vesicles. In several human diseases characterized by insulin resistance such as obesity and Non-insulin-dependent Diabetes Mellitus, cellular depletion of Glut4 is a major mechanism of insulin resistance in adipocytes (21). However, defects in translocation of Glut4 may be responsible for insulin resistance in skeletal muscle (22, 23). In addition, we have shown that abnormalities in Glut4 trafficking may contribute to insulin resistance in adipocytes from women with gestational diabetes (24). Precise localization of the intracellular Glut4 pool and a better understanding of Glut4 trafficking will permit elucidation of translocation defects causing insulin resistance in human diseases.

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