Brefeldin A Inhibits Insulin-induced Glucose Transport Stimulation and GLUT4 Recruitment in Rat Adipocytes*

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GLUT4, the major insulin-responsive glucose transporter isoform in rat adipocytes, rapidly recycles between an intracellular pool and the plasma membrane in the basal and insulin-stimulated states. To gain insight into the route of this GLUT4 recycling, we studied the effects of brefeldin A (BFA) on glucose transport and glucose transporter subcellular distribution in rat adipocytes in the absence and in the presence of insulin. 3-O-Methyl-D-glucose equilibrium exchange measurements revealed that BFA inhibits insulin-stimulated glucose transport by as much as 80%, whereas the inactive BFA analog, B36, was without effect. The inhibition was reversible and was a saturable function of BFA concentration with an apparent $K_i$ of less than 1 μM. In the absence of insulin, on the other hand, BFA caused a slight (up to 2-fold) increase in glucose transport. Subcellular fractionation and semiquantitative immunoblotting analysis revealed that BFA inhibits insulin-induced redistribution of GLUT4 from microsomes to the plasma membranes, with a dose dependence similar to that for glucose transport inhibition. BFA also caused a slight increase in the plasma membrane GLUT4 level in the absence of insulin. BFA did not affect the subcellular distribution of GLUT1 in these experiments. These findings strongly suggest that GLUT4 recycling in rat adipocytes involves a BFA-sensitive, coat protein-mediated, membrane-budding step, which is distinct between the constitutive and the insulin-induced pathways.

GLUT4 was shown to be associated with clathrin at the cell surface of 3T3-L1 adipocytes (7), suggesting that GLUT4 recycling includes internalization through clathrin-coated endocytosis. Evidence obtained with rat brown adipocytes (8) and muscle cells (9) also indicated that GLUT4 occurs intracellularly not only in small endocytic vesicles but also in large tubulovesicular structures morphologically indistinguishable from TGN or endomembrane compartments. This is analogous to the recycling pathways of many cell surface receptors, including those of insulin (10), transferrin (11), and epidermal growth factor (12) receptors.

Brefeldin A (BFA) (13), a fungal metabolite, is known to interfere with transport vesicle formation of a number of vesicle-mediated intracellular protein transport pathways (13-15). Typically, BFA blocks protein transport from the endoplasmic reticulum to the Golgi and between the Golgi cisternae by inhibiting membrane budding and transport vesicle formation catalyzed by specific cytosolic coat proteins including β-COP (16). BFA also affects the morphology of the TGN and endosomes that are mediated by a coat protein similar to β-COP. This suggestion is further supported by the recent findings that β-adaptin, a component of the AP-1/clathrin coat assembly of TGN, is homologous to β-COP (18, 19) and that BFA rapidly disassembles the clathrin coats-adaptin complex at TGN. BFA, on the other hand, apparently does not inhibit the receptor-mediated endocytosis (19, 20), indicating that the AP-2/clathrin assembly-assisted membrane budding at the cell surface is not affected by BFA (15, 21). More recently, BFA was shown to enhance apical endocytosis in polarized MOCK cells, apparently by dissociating certain coat proteins at the apical membrane that regulate the rate of endocytosis (22).

To test the possible involvement of a BFA-sensitive, protein-mediated membrane-budding mechanism in GLUT4 recycling pathways in rat adipocytes, we examined, in the present study, whether BFA affects glucose transport and subcellular distribution of glucose transporters in adipocytes. We demonstrate that BFA inhibits insulin-stimulated glucose transport as much as 90% and inhibits insulin-induced GLUT4 recruitment to a similar extent. We also show that BFA, in the absence of insulin, stimulates glucose transport up to 2-fold and increases the plasma membrane GLUT4 level to similar extents. These findings, together with other BFA effects currently known, strongly suggest that the insulin-stimulated GLUT4 recycling pathway in rat adipocytes involves two distinct, BFA-sensitive membrane steps, one at the TGN or endosomes for externalization and the other at the plasma membrane for internalization, and these membrane-budding steps show different sensitivities to BFA for constitutive and insulin-stimulated GLUT4 recycling.

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1 The abbreviations used are: TGN, trans-Golgi network; BFA, brefeldin A; PM, plasma membrane; 30MG, 3-O-methyl-D-glucose; NM, nuclei/mitochondria; β-COP, β-coat protein.

2 M. Robinson, personal communication.
the membrane budding at TGN being more sensitive in insulin-stimulated adipocytes, and the membrane budding at the plasma membrane is selectively blocked in basal adipocytes.

EXPERIMENTAL PROCEDURES

Materials—BFA was obtained from Epicenter Technologies (Madison, WI) and stored at 20 °C in ethanol as 10 mg/ml stock solution. W36, an inactive, structural analog of BFA, was a generous gift from Dr. Richard D. Klausner, NIH. 3-O-[14C]Methyl-D-glucose and 125I-protein A were purchased from DuPont NEN. Cytochalasin B was from Sigma.

Isolation and Subfractionation of Adipocytes—Adipocytes were isolated as described (23) from epididymal fat pads of male Sprague-Dawley rats (body weight, 150–200 g). Isolated adipocytes were homogenized in STE buffer (0.25 M sucrose, 2 mM EDTA, and 10 mM Tris-HCl, pH 7.4, with 10 μM phenylmethylsulfonyl fluoride), and centrifuged at 16,000 × g for 15 min at 4 °C, and the resulting pellet was used as microsomes (post-PM microsomes). Adipocyte total membranes were obtained as pellets by centrifuging the homogenates at 150,000 × g for 45 min at 4 °C

Glucose Transport Assay—Equilibrium exchange time course of 1 mM 3-O-methyl-D-glucose (30MG) by adipocytes suspended in a Krebs-Ringer buffer, pH 7.4 (15–25% cytocrit), was followed by measuring 3-O-[14C]methyl-D-glucose uptake at six different time points for 2–30 s. The 1-min tracer uptake by the cells in the presence of 2 μM cytochalasin B was also measured and used to calculate the uptake at t = 0. The tracer uptake at 15 min was also measured and used to calculate equilibrium. The time courses observed were analyzed according to the equilibrium tracer exchange kinetics in a closed, two-compartment system, from which first order rate constants (k) were calculated as described (24).

RESULTS

The effects of BFA on the basal and insulin-stimulated rat adipocyte glucose transport were studied by measuring [14C]30MG equilibrium exchange (Figs. 1 and 2). The glucose transport function in freshly isolated adipocytes used here was increased by 7–8-fold after a 20-min incubation with 7 nM insulin at 37 °C. When adipocytes were preincubated in the presence of 3 μM BFA for 20 min, however, the insulin-stimulated glucose transport was much reduced (by about 60%) (Fig. 1), demonstrating that BFA inhibits the insulin-stimulated glucose transport function in rat adipocytes. The effect was reversible; more than 90% of the insulin-stimulated transport function was restored after removal of BFA by repeated washings (not shown). A similar incubation of adipocytes with B36 (1–10 μM), an inactive analog of BFA (15), caused no detectable effect on the insulin-stimulated glucose transport (not shown), indicating that the inhibition is specific to BFA and not due to nonspecific cell damage. The inhibition was increased with an increasing BFA concentration in a saturable manner; the insulin-stimulated, glucose transport increment was reduced by more than 60% with 1 μM BFA and was almost totally abolished at 10 μM BFA (Fig. 2).

In the absence of insulin, the 20-min incubation with 10 μM BFA unexpectedly caused a slight by significant stimulation in 30MG exchange in adipocytes (Fig. 1). This stimulation of the basal glucose transport function was also a saturable function of BFA concentration (Fig. 2). An apparent maximum of 2-fold stimulation in basal transport was observed after incubation with 10 μM BFA, where basal and insulin-stimulated transport activities were practically identical (Fig. 2).

Figs. 3 and 4 illustrate the effects of BFA on steady-state distribution of GLUT4 between the intracellular pool and the plasma membrane of rat adipocytes, measured by subcellular fractionation and semiquantitative immunoblotting analysis. The adipocyte preparation used here typically responds to insulin (7 nM, 20 min at 37 °C) by increasing GLUT4 level in a crude plasma membrane fraction (NM-PM) 4–5-fold, with a
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**Fig. 3.** Effects of BFA on steady-state, subcellular distribution of GLUT4 in rat adipocytes shown in immunoblot autoradiograph. Relative contents of GLUT4 in crude plasma membranes (PM/NM) and post-PM microsomes (H/LDM) were quantitated by immunoblot using 40 μg of membrane protein/lane. Rat adipocytes were treated without or with varying concentrations of BFA in the absence (−) and in the presence (+) of 7 nM insulin for 20 min at 37°C. This experiment is representative of three independent experiments.

**Fig. 4.** Semiquantitative immunoblotting analysis of BFA effects on the plasma membrane (PM/NM) GLUT4 contents of basal and insulin-stimulated adipocytes. Blot intensities were quantitated by densitometric scanning using a laser densitometer and expressed in a relative quantity to that of the basal adipocytes with no BFA treatment. Values are the mean of three independent experiments with S.E. shown in bars.

A significant reduction of post-PM microsomal (H/LDM) GLUT4 content (Figs. 3 and 4). These insulin-induced changes in NM-PM and H/LDM GLUT4 pool sizes were reduced when adipocytes were first incubated with BFA for 20 min at 37°C (Figs. 3 and 4), the treatment that inhibited insulin-stimulated glucose transport function referred to above (Figs. 1 and 2), indicating that BFA blocks insulin-induced GLUT4 recruitment. The inhibition was dose-dependent and saturable with an increasing concentration of BFA. An incubation with 10 μM BFA totally blocked insulin-induced GLUT4 recruitment (Figs. 3 and 4). A similar incubation of adipocytes with 10 μM B36 did not affect insulin-induced GLUT4 recruitment (not shown), indicating that the inhibition is specific to BFA. BFA by itself in the absence of insulin, on the other hand, caused a significant (almost 2-fold) and reproducible increase in the plasma membrane GLUT4 content of basal adipocytes (Figs. 3 and 4). This effect was also a saturable function of BFA concentration, where an apparent maximal 2-fold increase was observed at 10 μM BFA. The total cellular GLUT4 levels of the basal and insulin-stimulated adipocytes, as estimated by immunoblots of total membrane fractions (not shown), were not affected by BFA in these experiments. Immunoblots using antibodies specific to GLUT1 failed to detect any significant BFA effect on plasma membrane GLUT1 levels both in the basal and insulin-stimulated adipocytes (not shown).

**Fig. 5.** Effects of AlF4− on glucose transport in insulin-stimulated adipocytes in the presence and absence of BFA. Rat adipocytes were incubated in a Krebs-Ringer buffer in the presence (+) or absence (−) of AlCl3 and NaF for 20 min prior to further incubation for 30 min, with or without 7 nM insulin and/or 3 μM BFA. Cells were then subjected to 30MG (1 mM) equilibrium exchange flux measurements as described in the footnotes of Figs. 1 and 2. Flux rates were expressed in relative quantities by normalizing each first-order rate constant (k) to that of the basal, untreated adipocytes. For AlF4− treatments, three sets of AlCl3-NaF mixture were used, giving final concentrations of 50 μM and 30 mM (1+), 75 μM and 45 mM (2+), and 100 μM and 60 mM (3+), respectively.

AlF4−, an activator of heterotrimeric G proteins (26), is known to inhibit the BFA-induced dissociation of β-COP from the Golgi (27). When adipocytes were treated with BFA together with AlF4−, the inhibition of insulin-stimulated glucose transport function by BFA was significantly less compared with the inhibition caused by BFA alone in the absence of AlF4− (Fig. 5). This AlF4− effect was dose-dependent, abolishing more than 40% of the BFA-induced inhibition of the glucose transport stimulation by insulin (Fig. 5). The incubation with AlF4− alone caused a reduction of insulin-stimulated glucose transport by as much as 40% (Fig. 5), indicating that more than 80% of the BFA effect was abolished by AlF4−. AlF4− caused a significant cell clustering in the absence of insulin, which made flux measurements less reproducible. Our preliminary data (not shown) failed to reveal any clear AlF4− effect on basal adipocyte glucose transport function either without or with BFA (3 μM).

**DISCUSSION**

The data presented here clearly demonstrate that BFA affects rat adipocyte glucose transport function, both in the absence and presence of insulin, but in opposite directions. For insulin-stimulated adipocytes, BFA inhibits glucose transport and blocks the insulin-induced GLUT4 recruitment from its intracellular storage pool to the plasma membrane without reducing total cellular GLUT4 content. The blockade of the insulin-induced redistribution appears to be specific to GLUT4, as the insulin-induced GLUT1 recruitment was not significantly affected by BFA. Both the transport inhibition and the blockade of GLUT4 redistribution by BFA were reversible and were saturable functions of BFA concentration with more than 60% of the maximal effects being observed at 1 μM BFA. In the absence of insulin, on the other hand, BFA at this same concentration range stimulates glucose transport, and this stimulation is accompanied by a readily demonstrable increase in the plasma membrane GLUT4 level. No changes in microsomal or total cellular GLUT4 contents were apparent, again indicating...
that the increased plasma membrane GLUT4 level of BFA-treated basal adipocytes is due to a recruitment of GLUT4 from an intracellular pool to the cell surface rather than new synthesis of GLUT4. These insulin-like effects of BFA on glucose transport and GLUT4 redistribution are rather modest (2-fold) compared with the corresponding insulin effects (7–8 and 4–5-fold, respectively).

The paradoxical, dual effects of BFA on the steady-state plasma membrane GLUT4 levels shown here indicate that both the constitutive and insulin-stimulated GLUT4 recycling pathways include a BFA-sensitive step or steps and further suggest that two distinct, BFA-sensitive mechanisms regulate the basal and insulin-stimulated GLUT4 recycling in rat adipocytes. It is interesting to note that okadaic acid, a serine/threonine phosphatase inhibitor, exerts similar dual effects on glucose transport in rat adipocytes, stimulating basal glucose transport and inhibiting insulin-stimulated glucose transport (28, 29).

Chakrabarti et al. (30) have recently described the BFA effects on glucose transport function and GLUT4 subcellular distribution in 3T3-L1 cells that are significantly different from those we observed here with rat adipocytes. In 3T3-L1 cells, BFA inhibited basal 2-deoxyglucose uptake, as well as insulin-stimulated 2-deoxyglucose uptake, and the inhibition of insulin-stimulated uptake was less than 60%, even in the presence of BFA as high as 300 μM. Furthermore, BFA caused no change, or a slight increase, in the insulin-induced GLUT4 recruitment in 3T3-L1 adipocytes. Together, these results suggest that BFA inhibits glucose transport in 3T3-L1 adipocytes by inhibiting the intrinsic activity of glucose transporters but not their subcellular redistribution; thus, there is no indication for an involvement of a BFA-sensitive step in the insulin-induced, GLUT4 recycling in 3T3-L1 adipocytes. The nature of the difference in BFA effects on insulin-induced GLUT4 translocation between rat adipocytes and 3T3-L1 cells is not immediately clear. As in rat adipocytes, however, BFA caused a significant (almost 2-fold) increase in plasma membrane GLUT4 level in basal 3T3-L1 adipocytes, with a concomitant reduction in intracellular GLUT4 level (30). There was no significant indication in our studies with rat adipocytes that BFA affects glucose transporter intrinsic activity.

We have previously shown that GLUT4 in rat adipocytes rapidly recycles between an intracellular pool and the plasma membrane in the absence and in the presence of insulin, and its steady-state plasma membrane pool size is determined by two first-order rate constants, one for internalization (endocytosis) and the other for externalization (exocytosis) (6). The reduction in the plasma membrane GLUT4 content by BFA observed here with insulin-stimulated adipocytes could be a result of reduced GLUT4 externalization and/or increased GLUT4 internalization. Both the externalization and internalization of a membrane protein would involve transport vesicle formation via membrane budding at the donor membrane as an essential step. Evidence currently available strongly suggests that BFA affects this step; BFA inhibits secretory protein transport by blocking the nonclathrin, β-COP-mediated membrane budding at the endoplasmic reticulum and the Golgi for transport vesicle formation (14, 15). This BFA effect is apparently not restricted to the protein transport at the cis side of the Golgi, because TGN and recycling endosomal compartments are also affected (17, 19–22). BFA blocks protein secretion at the apical surface of MCK cells without affecting protein movement between the endoplasmic reticulum and the Golgi (21). BFA also blocks immunogold transcytosis in MCK cells, apparently by blocking transcytotic vesicle formation from basolateral early endosomes (20). These findings strongly suggest that BFA inhibits insulin-induced GLUT4 recruitment by blocking GLUT4 externalization and that the GLUT4 externalization in insulin-stimulated adipocytes may involve membrane budding at TGN mediated by a BFA-sensitive, coat protein assembly similar to β-COP if not β-COP itself (15–18). This is consistent with the recent observation that β-COP is present not only at the cis side of the Golgi (15) but also at TGN during a low temperature transport block (16). Furthermore, AIF, a well known activator of the trimeric G proteins (26), is known to prevent the BFA-induced dissociation of β-COP from budding Golgi membranes (27). Our demonstration in this study that the pretreatment with AIF effectively prevents adipocytes from the inhibition by BFA of insulin-stimulated glucose transport function (Fig. 5) further suggests the involvement of a membrane-budding step at TGN mediated by a β-COP-like, coat protein assembly in GLUT4 externalization for insulin-stimulated pathway. It is relevant to note here that AIF does not affect the association of a small GTP-binding protein, the ADP-ribosylation factor, to Golgi that is known to be required for subsequent binding of β-COP for Golgi membrane budding (37).

The increased, steady-state plasma membrane GLUT4 level in BFA-treated basal adipocytes, on the other hand, is not readily explainable from the BFA effects currently known. It could result from an increased externalization or a reduced internalization, and there has been no clear demonstration that BFA increases protein externalization or decreases endocytosis. BFA was shown to increase surface levels of mannose-6-phosphate/insulin-like growth factor II receptors in fibroblasts (17, 32). However, BFA increased the externalization and internalization rates equally in this case, and the increased surface receptor pool size was apparently due to an increased intracellular receptor pool size available for recycling. Such an increase in GLUT4 storage pool size, however, was not detectable in the present study.

The effects of BFA on membrane trafficking are not only multi-targeted (15) but also cell type-specific (20); thus, care must be taken in applying existing information to interpret its actions on a poorly characterized system such as GLUT4 recycling. Nevertheless, existing data uniformly point to the premise that BFA affects membrane budding and transport vesicle formation at donor membranes where the membrane budding is catalyzed by members of a family of coat proteins. It is tempting to propose as a working hypothesis that two distinct, BFA-sensitive coat assemblies mediate vesicle budding for GLUT4 recycling pathways in rat adipocytes, one at the plasma membrane controlling GLUT4 internalization (endocytosis) for the basal and insulin-stimulated GLUT4 recycling and the other at the storage organelle regulating the GLUT4 externalization for the insulin-stimulated GLUT4 recycling selectively. The biochemical identification of these putative, BFA-sensitive coat protein assemblies would facilitate our understanding of the GLUT4 internalization and externalization pathways and their regulation at the molecular level.

REFERENCES

Brefeldin A Inhibits Insulin-induced Glucose Transport