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YM201636, an inhibitor of retroviral budding and PIKfyve-catalyzed PtdIns(3,5)P₂ synthesis, halts glucose entry by insulin in adipocytes

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Abstract

Silencing of PIKfyve, the sole enzyme for PtdIns(3,5)P₂ biosynthesis that controls proper endosome dynamics, inhibits retroviral replication. A novel PIKfyve-specific inhibitor YM201636 disrupts retroviral budding at 800 nM, suggesting its potential use as an antiretroviral therapeutic. Because PIKfyve is also required for optimal insulin activation of GLUT4 surface translocation and glucose influx, we tested the outcome of YM201636 application on insulin responsiveness in 3T3L1 adipocytes. YM201636 almost completely inhibited basal and insulin-activated 2-deoxyglucose uptake at doses as low as 160 nM, with IC₅₀ = 54 ± 4 nM for the net insulin response. Insulin-induced GLUT4 translocation was partially inhibited at substantially higher doses, comparable to those required for inhibition of insulin-induced phosphorylation of Akt/PKB. In addition to PIKfyve, YM201636 also completely inhibited insulin-dependent activation of class IA PI 3-kinase. We suggest that apart from PIKfyve, there are at least two additional targets for YM201636 in the context of insulin signaling to GLUT4 and glucose uptake: the insulin-activated class IA PI 3-kinase and a here-unidentified high-affinity target responsible for the greater inhibition of glucose entry vs. GLUT4 translocation. The profound inhibition of the net insulin effect on glucose influx at YM201636 doses markedly lower than those required for efficient retroviral budding disruption warns of severe perturbations in glucose homeostasis associated with potential YM201636 use in antiretroviral therapy.

Keywords

Insulin resistance; YM201636; PIKfyve; Antiretroviral therapy; Glucose transport; GLUT4; PI 3-kinase; Insulin action

To gain access into the cell interior, replicate and, ultimately, escape the cell, retroviruses recruit and exploit a variety of host cellular factors [1]. The significant drawback associated with the mutagenic potential of targeting retrovirus-unique proteins has led to host-directed drug targeting as a potentially powerful approach in antiretroviral treatment. New classes of antiretroviral medications for HIV treatment targeting cellular components have been satisfactorily used in AIDS patients [2]. It is therefore not surprising that research focused on host proteins important for the retroviral life cycle has recently exploded. A group of evolutionarily conserved proteins, constituting the three subcomplexes (-I, -II and -III) and associated partners of the cellular ESCRT machinery, normally used for multivesicular body formation and cargo degradative sorting through multivesicular endosomes, appear to be

critical in the retroviral life cycle [1,3]. The observation for an arrest of retroviral budding and egress upon disruption of the ESCRT function and documented direct binding between the viral proteins and ESCRT components indicate ESCRT's role in the late stages of the viral life cycle. A subunit of the ESCRT-III complex binds membrane phosphatidylinositol (PtdIns)(3,5)P₂ [4], a low-abundance phosphoinositide synthesized from PtdIns(3)P by PIKfyve [5]. Whereas PIKfyve is not a component of the ESCRT machinery, ultrastructural studies in mammalian cells indicate PIKfyve's role in intraluminal invagination of multivesicular endosomes. PIKfyve and PtdIns(3,5)P₂ have also been implicated in triggering fission/maturation of transport intermediates from early endosomes [5]. Concordantly, disruption of their function is associated with significant constraints in cargo exit from endosome to the TGN or later endosomal compartments [5]. It is, therefore, not surprising that PIKfyve knockdown in host cells has resulted in a marked inhibition of HIV replication [6]. Proper performance of endosome-to-TGN transport is apparently critical for the HIV life cycle, as disrupted functions of other key players in endosome-to-TGN trafficking, including Rab9, the Rab9 effector p40 and TIP47, also inhibit HIV replication or infectivity [6–8]. Significantly, the Rab9 effector p40 interacts with PIKfyve and depends on PIKfyve enzymatic activity for its membrane attachment, mechanistically coupling the PIKfyve-regulated endosome plasticity with trafficking to the TGN [9]. Clearly, these data suggest that use of small-molecule inhibitors targeting PIKfyve-catalyzed PtdIns(3,5)P₂ synthesis in host cells may be a potentially powerful therapeutic strategy for repressing retroviral budding and replication.

A novel cell-permeable compound, YM201636, [6-amino-*N*-(3-(4-(4-morpholinyl)pyrido[3',2':4,5]furo[3,2-d]pyrimidin-2-yl)-phenyl)-3-pyridine carboxamide], that inhibits *in vitro* PIKfyve enzymatic activity and PtdIns(3,5)P₂ intracellular synthesis at nanomolar concentrations has been recently identified [10]. Remarkably, treatment of a Moloney leukemia virus-expressing cell line with YM201636 (800 nM) inhibited retroviral release by 80%, suggesting the drug may be of significant therapeutic value [10]. However, PIKfyve has been recently shown to be a positive regulator of the insulin-regulated GLUT4-mediated glucose influx in cultured 3T3L1 adipocytes [11,12]. GLUT4-mediated glucose transport is the rate limiting step in postprandial glucose utilization by adipose and muscle, and its failure is associated with pathogenic features of insulin resistance and type 2 diabetes [13]. Therefore, in this report we directed studies on the effect of YM201636 in insulin-regulated glucose influx and GLUT4 translocation in 3T3L1 adipocytes, a cell line widely used as a prototype of insulin-sensitive adipose tissue. We also inspected the performance of key intermediates in proximal insulin receptor signaling under YM201636 administration in this cell type.

Material and methods

Materials

YM201636 was purchased from Symansis; PtdIns from Avanti Polar Lipids; [γ -³²P]ATP (6000 Ci/mmol) and 2-[1,2-³H]deoxy-D-glucose, from Perkin Elmer; insulin from Eli Lilly; anti-phosphoSer473-Akt or anti-Akt antibodies from Cell Signaling. Anti-PIKfyve antiserum (R7069) was characterized previously [14]. Polyclonal anti-HA antibodies and the pEGFP-HA-GLUT4 construct were kind gifts by Mike Czech and Jeff Pessin, respectively.

Cell cultures and cell treatments

Mouse 3T3L1 fibroblasts (ATCC) were differentiated into adipocytes following a standard differentiation protocol [11]. Adipocytes were used between days 8 and 12 after the onset of the differentiation program. Prior to experiments, adipocytes were serum-deprived for 3 h in DMEM, then incubated with YM201636 at indicated time periods and concentrations at 37

°C, followed by stimulation with insulin (100 nM) for 30 min, in GLUT4 translocation and glucose transport assays, or 10 min, for Akt activation and lipid kinase assays.

2-Deoxyglucose uptake

Glucose transport was determined by measuring 2-deoxyglucose (2DG) uptake as previously described [11]. Briefly, following serum-starvation, 3T3L1 adipocytes (24-well dish) were incubated (30 min) in Krebs–Ringer–Hepes buffer, pH 7.4, in the presence or absence of insulin. 2-[1,2-³H]deoxy-D-glucose was added to a final concentration of 100 μM (0.5 μCi/well) for 5 min at 37 °C. The reaction was stopped by addition of 2DG (20 mM). Cells were washed and lysed in 1% Triton X-100. Aliquots were analyzed for radioactivity and protein concentration. Nonspecific glucose uptake was measured in the presence of 10 μM cytochalasin B and the value was subtracted for each determination. All values were normalized for protein content.

HA-GLUT4-eGFP transfection and fluorescence microscopy

These were performed essentially as described previously [11]. Briefly, 3T3L1 adipocytes were electroporated with the HA-GLUT4-eGFP construct harboring the HA epitope on the first exofacial loop and eGFP on the C-terminus, and seeded on glass coverslips. Twenty-four hours post-transfection, serum-starved cells were preincubated with YM201636, then stimulated with insulin and fixed in formaldehyde. To visualize the cell surface GLUT4 reporter, non-permeabilized cells were stained with anti-HA antibodies followed by Cy3-conjugated goat anti-rabbit IgG. To quantify the reporter surface abundance, images were collected from randomly selected eGFP-positive adipocytes (15–20 cells/condition) by an Olympus 1X81 confocal microscope with a cooled charge-coupled device 12 bit Hamamatsu camera. The exposure times for each fluorescence channel were always set below the saturation threshold of the camera. The area of the selected cell was individually analyzed for an average fluorescence intensity per pixel for Cy3 (cell surface HA-GLUT4-eGFP) and GFP signals (total HA-GLUT4-eGFP) by IPLab Software (Scanalytics). After subtracting the background fluorescence of a non-transfected neighboring cell, quantified in a similar way, the Cy3/GFP ratios were calculated. Slides from separate experiments were analyzed simultaneously.

Lipid kinase assay, TLC and HPLC analyses

Assays of PIKfyve and class IA PI 3-kinase activities were performed as previously described [14,15]. Briefly, following 3T3L1 adipocyte serum-starvation and insulin stimulation, cell lysates containing protease inhibitors were clarified (14,000g, 15 min, 4 °C) and then subjected to immunoprecipitation with anti-PIKfyve antibodies (16 h, 4 °C). Washed beads were mixed with 100 μM PtdIns and preincubated for 15 min (37 °C) with YM201636 (100 nM) or vehicle in the assay buffer (50 mM Tris–HCl, pH 7.5, 1 mM EGTA and 10 mM MgCl₂). The kinase assay (50 μl final volume) was carried out for 15 min at 37 °C with 15 μM ATP and [γ-³²P]ATP (30 μCi). Lipids were extracted, spotted on TLC glass plates (Whatman, K6-SILICA, 250 μm), resolved by a chloroform/methanol/water/ammonia solvent system and detected by autoradiography. Due to insufficient resolution between PtdIns(3)P and PtdIns(5)P under these conditions, the silica corresponding to monophosphorylated PtdIns radioactive spots was scraped from the TLC plate, and following deacylation, subjected to HPLC analysis for PtdInsP separation and quantitation as detailed elsewhere [14]. Fractions, collected every 0.25 min, were analyzed for radioactivity. Individual peak radioactivity, quantified by area integration and presented as a percentage of the summed radioactivity from the ³²P-labeled Gro-Pins3P and Gro-Pins5P peaks, was compared with that obtained at basal conditions.

Immunoblotting

Following cell treatments, 3T3L1 adipocyte lysates were collected in RIPA buffer supplemented with protease and phosphatase inhibitors and analyzed by immunoblotting as detailed elsewhere [11].

Statistical analysis

Data are expressed as means $\pm$ SEM. Statistical analysis was performed by Student's *t* test, with $p < 0.05$ considered as significant.

Results

Upon differentiation to adipocyte phenotype, the 3T3L1 cell line expresses both GLUT1 and GLUT4 glucose transporters. About 90% of stimulated glucose transport by low doses of insulin (100 nM) is due to GLUT4, whereas the basal glucose entry is mostly through GLUT1, with only a small contribution of GLUT4 [16,17]. Importantly, short preincubation of 3T3L1 adipocytes with different concentrations of YM201636 (0–4 μ M) induced a marked inhibition of both basal and insulin-activated 2DG uptake in a dose-dependent manner (Fig. 1A). At concentrations as low as 160 nM, YM201636 nearly completely inhibited the net insulin effect, with a 50% inhibition of the net insulin response observed at 54 ± 4 nM (Fig. 1B).

Mechanistically, the insulin effect on activating glucose uptake is primarily due to rapid movements of GLUT4 from intracellular storage compartment to the fat/muscle cell surface [18,19]. To test whether arrested insulin responsiveness of glucose transport by YM201636 was due to a perturbed GLUT4 translocation process, we examined the drug's effect in cells expressing a GLUT4 reporter by immunofluorescence microscopy. The doubly labeled HA-GLUT4-eGFP is a widely used GLUT4 reporter molecule, which allows evaluating both the transporter translocation and plasma membrane fusion in non-permeabilized cells, because the HA-tag positioned on the GLUT4 exofacial loop is accessible extracellularly [18]. Intriguingly, insulin-stimulated cell surface HA-GLUT4-eGFP accumulation was not affected at YM201636 doses that nearly completely inhibited 2DG uptake (Fig. 2). A YM201636 concentration as high as 800 nM was required to produce a 45% inhibition of cell surface HA-GLUT4-eGFP accumulation. These data demonstrate a disparity of more than one order of magnitude between the YM201636 doses required for inhibition of insulin-activated GLUT4 translocation and 2DG, with the latter being the more sensitive process.

An essential step in the insulin-signaling circuit that integrates signals issued by the activated insulin receptor with GLUT4 translocation is the phosphorylation and activation of Akt/PKB [19]. Therefore, we examined the effect of YM201636 on insulin-induced Akt/PKB phosphorylation. Lysates derived from 3T3L1 adipocytes treated with different concentrations of YM201636 prior to insulin stimulation were analyzed by immunoblotting with anti-phosphoSer473-Akt (Fig. 3). Unaltered Akt phosphorylation in response to insulin was seen at lower doses of YM201636 (160 nM). However, 800 nM YM201636 resulted in a 55% inhibition of Akt-Ser473 phosphorylation and no detectable changes in total Akt (Fig. 3). These data show a striking similarity to the YM201636 dose required for inhibition of GLUT4 cell surface accumulation in response to insulin, suggesting that reduced Akt activation mechanistically underlies the observed inhibition of GLUT4 cell surface translocation by YM201636.

Akt activation results mainly from a direct association with PtdIns(3,4,5)P₃ produced by the wortmannin-sensitive class IA PI 3-kinase, activated in response to insulin [18,19]. We have

reported previously that subpopulations of insulin-activated wortmannin-sensitive class IA PI 3-kinase associate with PIKfyve in 3T3L1 adipocytes [14,15]. Therefore, to reveal whether the reduced Akt phosphorylation could be linked to YM201636 inhibition of insulin-induced class IA PI 3-kinase activation in a cellular context, lysates of insulin-stimulated 3T3L1 adipocytes were immunoprecipitated with anti-PIKfyve antibodies and assayed for PI 3-kinase and PIKfyve activities in the presence or absence of 100 nM YM201636. Consistent with previous studies [14,15], TLC analyses coupled with subsequent HPLC runs for identification of radiolabeled PIP, revealed a marked increase of PtdIns(3)P in response to insulin (Fig. 4). By contrast, the PIKfyve product PtdIns(5)P was unaffected by insulin as reported previously and confirmed herein (Fig. 4B). As expected for a PIKfyve-specific inhibitor, YM201636 inhibited product synthesis by PIKfyve (Fig. 4). Unexpectedly, however, at 100 nM, YM201636 also markedly abrogated the insulin-dependent activation of PI 3-kinase, as well as the basal activity, evidenced by nearly complete elimination of PtdIns(3)P increment following HPLC resolution of PIPs (Fig. 4B). These data demonstrate that, in addition to PIKfyve, YM201636 also inhibits the insulin-dependent class IA PI 3-kinase activation at nanomolar doses, and indicate that the significant reduction in Akt phosphorylation and GLUT4 cell surface translocation by insulin in the presence of YM201636 (800 nM) could also result from the class IA PI 3-kinase inhibition.

Discussion

PIKfyve, the sole enzyme for PtdIns(3,5)P₂ synthesis, plays a critical role in endosome processing in the course of cargo transport [5]. Several trafficking pathways, both constitutive and regulated, traversing the endosomal network are particularly sensitive to PIKfyve dysfunction. The observation for inhibition of retroviral assembly and budding out of host cells upon PIKfyve silencing has brought a considerable promise for potential efficacy of PIKfyve pharmacological targeting in antiretroviral therapy [6]. Recently, a screen for cell-permeable small-molecule inhibitors has identified a compound, YM201636, that powerfully inhibits PIKfyve-catalyzed PtdIns(3,5)P₂ synthesis [10]. Importantly, YM201636 was found to inhibit retroviral release from infected cells, suggesting its applicability as a retroviral therapeutic [10]. These findings pose the important question about the effect of YM201636 on the fundamental biological process of glucose transport. This becomes prominent in the light of recent observations for the requirement of PIKfyve enzymatic activity in insulin-regulated GLUT4 translocation and glucose transport [11,12]. The current study provides a key novel finding for an unexpected nearly complete arrest of both basal and insulin-induced glucose influx in cultured adipocytes at YM201636 concentrations as low as 160 nM. At this concentration, however, the insulin-regulated GLUT4 translocation remained unaltered and required as high as 800 nM YM201636 for a 45% inhibition. These results, taken together with other recent findings and considerations, have two important implications. First, as the arrest of glucose influx is apparent at YM201636 concentrations that are substantially lower than those required for an effective inhibition of retroviral budding (>800 nM; [10]), a gross perturbation of both basal and postprandial glucose uptake and disruption of the overall glucose homeostatic mechanism could be anticipated in potential YM201636 preclinical and clinical trials. Second, because 160 nM YM201636 reduces the intracellular PtdIns(3,5)P₂ levels in 3T3L1 adipocytes by only 15–20%, a decrease for which there is only ~25% inhibition of insulin-activated glucose transport and unaltered basal transport as seen under PIKfyve knockdown [11, and not shown], YM201636 likely inhibits a high-affinity PIKfyve-unrelated target, whose activity is required for stimulation of glucose uptake. The doubly labeled HA-GLUT4-eGFP reporter is accessible extracellularly through the HA-tag present at the transporter exofacial loop [18]. Therefore, the documented nearly complete inhibition of insulin-stimulated glucose influx by YM201636 in the background of unchanged HA-GLUT4-eGFP amounts

on the adipocyte surface points to differential sensitivity of these insulin effects to YM201636 inhibition, suggesting separate targets in insulin-activated glucose influx and GLUT4 translocation, each inhibited by YM201636 but at different doses. Similar dissociation between insulin-stimulated glucose uptake and GLUT4 translocation has been previously observed with the PI 3-kinase inhibitor wortmannin in both adipose and muscle cells [20,21]. Whereas the identity of the high-affinity target in the wortmannin inhibition of glucose uptake is still inconclusive, it should be emphasized that PIKfyve activity is wortmannin-insensitive [14]. From the perspective of insulin signaling to GLUT4 and glucose transport, the identity of the here-predicted PIKfyve-independent, high-affinity target of YM201636 and whether it is related to the high-affinity target of wortmannin remain important objectives for future studies.

Our observation for reduced insulin-dependent Akt activation at YM201636 doses resembling those required for inhibition of GLUT4 translocation is consistent with current views for Akt acting upstream of GLUT4 [18,19]. Intriguingly, the original work on YM201636 has shown no significant changes of the serum-stimulated Akt phosphorylation in NIH3T3 cells at 800 nM YM201636 [10], whereas our data in 3T3L1 adipocytes demonstrated >50% reduction of phosphoSer473Akt at this concentration. Because we observed a similar decrease in the phosphoThr308Akt phosphorylation in response to insulin stimulation of 3T3L1 adipocytes (not shown), the above-mentioned discrepancy is most likely related to variations in the cell types and extracellular stimuli. Our data for reduced Akt phosphorylation are also in line with the documented dramatic inhibition of the insulin-dependent activation of class IA PI 3-kinase. Thus, in addition to PIKfyve, YM201636 at 100 nM completely inhibited class IA PI 3-kinase activation in response to insulin, reducing also the basal activity (Fig. 4). Our observation for arrested class IA PI 3-kinase activation by insulin is unexpected as Jefferies et al. have shown that the 110 α catalytic subunit of class IA PI 3-kinase resists YM201636 even at micromolar concentrations, ID₅₀ = 3.3 μ M, a dose ~100-fold higher than that required for the PIKfyve inhibition [10]. These conflicting results are likely associated with the source of the class IA PI 3-kinase used in the two studies. Whereas in [10], a non-stimulated recombinantly-produced GST-fusion of the 110 α catalytic subunit is examined, we monitored the YM201636 effect on the insulin-dependent activation of class IA PI 3-kinase immunopurified from native 3T3L1 adipocytes, conditions reflecting more authentically the in vivo function shown to require the 110 β catalytic subunit [22]. Our data clearly demonstrate that inhibition of insulin-induced activation of class IA PI 3-kinase by YM201636 occurs in parallel to the PIKfyve inhibition. Thus, the dual inhibitory effect of YM201636 on both PIKfyve and insulin-activated class IA PI 3-kinase makes unclear the relative contribution of the PIKfyve inhibition to the reduced Akt phosphorylation and GLUT4 translocation in response to insulin.

In summary, the data in this report demonstrate that the novel small-molecule inhibitor of PIKfyve, YM201636, arrests basal and insulin-induced glucose influx in cultured adipocytes at low nano-molar concentrations, likely by affecting a PIKfyve-unrelated target. Importantly, these doses are significantly lower than the effective antiretroviral dose, warning of the hazards associated with a potential use of YM201636 in antiretroviral therapy. At higher concentrations, YM201636 reduces GLUT4 cell surface translocation by insulin, which may result from inhibition of both PIKfyve and insulin-induced activation of class IA PI 3-kinase.

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Fig. 1.

Effect of YM201636 on basal and insulin-induced glucose transport. Serum-starved 3T3L1 adipocytes were treated with the indicated concentrations of YM201636 (30 min), then stimulated with or without insulin (100 nM; 30 min) followed by 2DG assay. (A) Data from three independent experiments in triplicates (mean $\pm$ SEM); (B) the net insulin effect above basal calculated for each dose of YM201636 and expressed as a percent of the net insulin effect in cells not treated with YM201636; * $p < 0.001$.

Fig. 2.

Effect of YM201636 on basal and insulin-induced GLUT4 translocation. 3T3L1 adipocytes, electroporated with HA-GLUT4-eGFP cDNA were serum-starved, treated with YM201636 (30 min), then stimulated with insulin (100 nM; 30 min) as indicated. Cells were analyzed by immunofluorescence microscopy. Shown is quantitation of the ratio of cell surface HA (Cy3)-signal to total GFP fluorescence in the HA-GLUT4-eGFP-expressing cells from three independent experiments, in which 10–20 cells/condition/experiment were analyzed by quantitative fluorescence microscopy as described in Materials and methods (mean $\pm$ SEM; *different vs. insulin-stimulated control, $p < 0.001$; #different vs. insulin-stimulated control, $p < 0.025$).

Fig. 3.

Effect of YM201636 on Akt phosphorylation by insulin. Serum-starved 3T3L1 adipocytes were treated with YM201636 (30 min), then stimulated with insulin (100 nM; 10 min) or left untreated as indicated. Aliquots (50 μ g) of the cell lysates, collected with protease and phosphatase inhibitors, were analyzed by immunoblotting with anti-phosphoSer473-Akt and anti-Akt antibodies. (A) Chemiluminescence detections of a representative blot with stripping between the antibodies. No phosphoSer47-Akt is seen in the absence of insulin. (B) Quantitation of the band intensity of the insulin-stimulated condition under different YM201636 concentrations normalized for total Akt, and presented as a percentage of the insulin-stimulated control (no YM201636, only vehicle); mean $\pm$ SEM, $n = 3$; [#]different vs. insulin-stimulated control, $p < 0.025$; ^{*}different vs. insulin-stimulated control, $p < 0.001$.

Fig. 4.

YM201636 inhibits both PIKfyve and insulin-activated class IA PI 3-kinase. Serum-starved 3T3L1 adipocytes were stimulated with or without insulin (100 nM; 10 min) as indicated. Anti-PIKfyve immunoprecipitates were incubated with YM201636 (100 nM; 15 min) or with vehicle and subjected to lipid kinase assay in the presence [γ -³²P]ATP. (A) A representative autoradiogram of a TLC plate from three independent experiments. (B) PtdInsP samples recovered from the TLC plate in (A) (arrows) were deacylated and separated on HPLC column. Positions of ³²P-GroPins3P (3P) or ³²P-GroPins5P (5P) were determined from parallel runs of the respective deacylated ³²P-labeled PtdInsP standards. In the absence of YM201636 (—), insulin increased GroPins3P to $546 \pm 39\%$ in comparison to

basal GroPins3P in panel (a) ($p < 0.001$; $n = 3$), as calculated by peak area integration. In the presence of YM201636 (+), there was an insignificant insulin-dependent increase ($111 \pm 16\%$) of GroPins3P vs. basal GroPins3P in panel (a), indicating severe inhibition of the insulin-dependent activation of PI 3-kinase by YM201636. YM201636 also inhibited the basal PI 3-kinase activity, evidenced by the $52 \pm 6\%$ decrease in basal GroPins3P in panel (c) vs. GroPins3P in panel (a) ($p < 0.001$; $n = 3$).