

Muscling in on GLUT4 kinetics

Jacqueline Stöckli,^{1,*} Daniel J. Fazakerley,^{1,2} Adelle C.F. Coster,^{1,3} Geoffrey D. Holman² and David E. James¹

¹Diabetes and Obesity Program; Garvan Institute of Medical Research; Darlinghurst; Sydney, NSW Australia; ²Department of Biology and Biochemistry; University of Bath; Claverton Down, Bath UK; ³School of Mathematics and Statistics; University of New South Wales; Sydney, NSW Australia

Insulin triggers glucose uptake into muscle and adipose tissue by stimulating the translocation of the glucose transporter GLUT4 from intracellular vesicles to the plasma membrane (PM). Insulin leads to a rapid increase in GLUT4 at the PM from ~5% to 40–50%. This effect is time and dose-dependent, reaching a new steady state after 30 min of insulin stimulation. Previous kinetic analyses in adipocytes has revealed that this is regulated by two mechanisms—increasing the amount of GLUT4 in the endosomal recycling system and increasing the exocytosis rate constant. Fazakerley et al.¹ focuses on GLUT4 kinetics in the L6 skeletal muscle cell line. Despite displaying a similar redistribution of GLUT4 to the cell surface with insulin to that seen in adipocytes, the mechanism for this effect in L6 cells was completely different. Insulin had a modest effect to increase the amount of GLUT4 in the recycling system with the dominant effect being on reduction of the endocytosis rate constant. Similar findings were observed with AMPK agonists. These studies indicate that different cell types are capable of achieving the same cell biological endpoint but using completely distinct mechanisms.

Kinetic Analysis of GLUT4 Trafficking in Fat

GLUT4 translocated to the PM is not retained there, but rather continuously recycles between intracellular recycling compartment(s) and the cell surface. At steady state the amount of GLUT4 at the PM, as well as that in the intracellular pool, does not change. Therefore the amount of

GLUT4 that is internalised is equivalent to the amount of GLUT4 that is exocytosed. This equilibrium can be simply portrayed by the mathematical equation $E \cdot k_{\text{ex}} = P \cdot k_{\text{in}}$, where E is the total amount of GLUT4 in the internal recycling compartment(s), P is the amount of GLUT4 at PM and k_{ex} and k_{in} are the overall rate constants for all exocytosis and endocytosis, respectively. Hence, by studying the system under steady state conditions, where all parameters are constants, it is possible to simplify the kinetic analysis of this process. In this analysis only the overall rate constants for GLUT4 exocytosis or endocytosis are considered. It is possible, and likely, that these rate constants comprise more than one process. For example, k_{ex} maybe a combination of the rate constants for transport of GLUT4 from both the endosomal recycling compartment and the GLUT4 storage compartment.

Initial studies of this process in adipocytes,^{2,5} suggested that the insulin-dependent increase in GLUT4 at the PM was mediated by changing the rate constants that define either exocytosis or endocytosis. The limitation with these studies is that they made the assumption that the total cellular pool of GLUT4 recycles to the PM in the absence and presence of insulin. This assumption was necessary because these early studies relied on the use of photo-activatable glucose analogues to label a pulse of GLUT4 at the PM. Using this method, it was not technically practical to continuously monitor the extend of emptying of the recycling pool because it was not feasible to accurately measure changes over the long time courses that would have been necessary. Thus measurement of the proportion of

Key words: trafficking, kinetics, GLUT4, adipocytes, L6 muscle cell line, AMPK, endocytosis, insulin action, recycling

Submitted: 02/10/10

Accepted: 02/10/10

Previously published online:
www.landesbioscience.com/journals/cib/article/11457

*Correspondence to: Jacqueline Stöckli;
Email: j.stoeckli@garvan.org.au

Addendum to: Fazakerley DJ, Holman GD, Marley A, James DE, Stöckli J, Coster AC. Kinetic evidence for unique regulation of GLUT4 trafficking by insulin and AMP-activated protein kinase activators in L6 myotubes. *J Biol Chem* 2010; 285:1653–60; PMID: 19915010; DOI: 10.1074/jbc.M109.051185.

the total cellular pool of GLUT4 that is actively engaged in cell surface recycling (here referred to as the GLUT4 recycling pool) was not made using this method.

This problem has been overcome with the implementation of engineered GLUT4 molecules possessing epitope tags in accessible extracellular positions.^{6,7} Incubation of adipocytes, expressing tagged GLUT4, with saturating concentrations of antibody at 37°C permitted tagging of all GLUT4 molecules in the recycling pool at steady state. Surprisingly, these studies revealed that the size of the recycling pool could be substantially enhanced by insulin.^{8,9} This introduced another possible mechanism by which insulin might modify cell surface levels of GLUT4, independently of changes in the rate constants for GLUT4 exocytosis and endocytosis. This work led to a model whereby in the absence of insulin a large pool of GLUT4 resides in a storage compartment that is static and segregated from the recycling compartment.¹⁰ Insulin dose-dependently releases a substantial amount of GLUT4 into the recycling pool concomitant with a modest increase in the GLUT4 exocytosis rate. Similar studies by McGraw and colleagues^{11,12} have not reported intracellular retention of GLUT4, possibly due to technical differences.⁹

Kinetic Analysis of GLUT4 Trafficking in Muscle

In mammals, muscle plays the dominant role in whole body glucose disposal. In this tissue insulin and exercise lead to GLUT4 translocation. Whereas insulin stimulates GLUT4 translocation via the PI3K/Akt pathway, exercise acts through a different pathway(s), involving, at least in part, stress kinases such as AMPK. Analyses of GLUT4 trafficking in muscle has been difficult due to the complex nature of this tissue and the paucity of muscle cell lines that recapitulate the characteristics of the tissue *in vivo*. Pioneering work by the Klip lab has shown that the L6 cell line is highly insulin responsive and these cells represent the best skeletal muscle model system in the field.¹³ GLUT4 translocates to the PM in response to both, insulin and AMPK agonists in these cells.^{14,15} Hence, it was of considerable interest to determine

the kinetics of GLUT4 trafficking in these cells in response to these different stimuli.

Kinetic Analysis of GLUT4 Trafficking in L6 Myotubes

In Fazakerley et al.¹ the trafficking kinetics of HA-tagged GLUT4 was analysed in L6 myotubes in response to insulin or AMPK agonists. These studies revealed that in muscle cells, like adipocytes, the overall recycling pool size was increased in response to stimulation. However, the amount of GLUT4 in the recycling pool in the absence of stimulation was much higher in L6 myotubes (61%) than in adipocytes (10–20%), as was the GLUT4 exocytosis rate constant ($k_{ex} = 0.06 \text{ min}^{-1}$ vs. $\sim 0.01 \text{ min}^{-1}$, respectively).^{8–10} To offset this marked increase in GLUT4 flux to the PM in the absence of insulin, L6 myotubes have a much more efficient retrieval of GLUT4 from the PM compared to adipocytes ($k_{in} = 0.43 \text{ min}^{-1}$ vs. $\sim 0.07 \text{ min}^{-1}$, respectively) in order to maintain low basal PM levels of GLUT4. This raises the interesting possibility that GLUT4 internalisation may be regulated via discrete pathways in these different cell types.^{16,17} The kinetics of GLUT4 trafficking in L6 myotubes under basal conditions poses an interesting problem for the cell. Given the high flux of GLUT4 to the PM in the basal state, how does this cell type affect changes in PM levels of GLUT4 comparable to those seen in the adipocyte in response to stimulation?

Fazakerley et al.¹ observed two major effects of insulin on GLUT4 trafficking kinetics in L6 myotubes that facilitated a similar increase in GLUT4 at the PM. The first was an increase in the amount of GLUT4 in the recycling pool from 61% to 75% with insulin stimulation. AMPK agonists had a similar effect on their own (77%) but in combination with insulin the increase in the amount of GLUT4 in the recycling pool was additive (88%). This suggests that L6 myotubes may contain two discrete static pools of GLUT4 that can be accessed (or released) by these different stimuli.¹⁸ This differential intracellular segregation of GLUT4 may be the mechanism, which allows muscle cells to respond to different physiological conditions. The additivity observed with

insulin and AMPK agonists also necessitates distinct regulatory mechanisms consistent with the recent suggestion that two related RabGAPs, TBC1D4 and TBC1D1 regulate a later step of GLUT4 translocation in response to insulin and exercise, respectively.^{19,20} It will be of interest to analyse whether there are two pools of GLUT4 vesicles that selectively contain TBC1D4 or TBC1D1 in muscle tissue, one responsive to insulin and the other responsive to energy depletion and AMPK activation.

The second major finding in Fazakerley et al.¹ is that the GLUT4 endocytosis rate constant is a major regulatory node in that it was reduced by 50% in response to all stimuli. There was no additivity between insulin and AMPK agonists in reducing GLUT4 endocytosis, suggesting that they slow endocytosis by the same mechanism. There have also been some studies that have described inhibitory effects of insulin on GLUT4 endocytosis in adipocytes suggesting that this may be a conserved mechanism.^{2,5,12,21} However, the details of this mechanism are not known and it may be that L6 myotubes will provide an ideal model system with which to interrogate the regulation of GLUT4 internalisation. Perhaps the most extraordinary aspect of these observations is that the underlying kinetics of GLUT4 recycling varies quite significantly between adipocytes and L6 myotubes. Despite this, both cell lines achieve a similar transient modulation of cell surface levels of GLUT4 in response to stimuli such as insulin and do so via completely different mechanisms.

L6 Myotubes: A Muscle Cell Line to Study GLUT4 Trafficking

It will be of fundamental interest to determine if the regulatory mechanisms for GLUT4 trafficking described here in L6 myotubes are conserved *in vivo* in skeletal muscle. One major difference is that the level of the GLUT4 protein is much higher in skeletal muscle than in L6 cells. This has also been reported for other muscle cell lines such as C2C12 cells.^{22,23} While the reason for this is not known, it is notable that the expression of GLUT4 in muscle is tightly regulated by neural innervation. Denervation of skeletal

muscle leads to a dramatic loss of GLUT4 protein expression²⁴ and possibly of other factors that modulate GLUT4 trafficking, within days. Regardless, it is clear that L6 myotubes recapitulate some of the physiological characteristics of skeletal muscle tissue, and represent a good skeletal muscle cell line model.

Reflections

As is often the case in biological research, the data reported in Fazakerley et al.¹ may be in conflict with some previously published data. One of the distinguishing features of Fazakerley et al. is the rigorous nature of the kinetic analysis. Ultimately, all kinetic studies rely on curve fitting from which the kinetic parameters are derived. Many studies do not report on the accuracy of the kinetic parameters associated with fitting. The derived kinetic parameters can be dramatically influenced not just by the quality of the data but also by the acquisition of sufficient data at key points along the curve to prevent biased or constrained curve fitting. In Fazakerley et al.¹ the size of the GLUT4 recycling pool was not assumed but rather experimentally determined. The fits were very accurate due to the number of data points included over a long time course, which is reflected by the low standard errors (determined from the 95% confidence intervals) of the derived kinetic parameters. The endocytosis rate constant k_{in} is inferred from the kinetic parameters, determined by an antibody uptake experiment, with the equation $k_{in} = E \cdot k_{ex} / P$. In Fazakerley et al.¹ k_{in} was additionally confirmed by direct measurement of GLUT4 internalisation.

However the issue of the appropriateness of the model when fitting has not been completely solved. The equations used by Fazakerley et al.¹ assume single exponents are associated with the release of GLUT4 from the recycling pool and its refilling. It is more likely that double exponents feature in the combined movements of GLUT4 between the PM and

two intracellular pools. Kinetic methods are needed that can separate out the rates of release and refilling of the specialised GLUT4 pool from those associated with GLUT4 docking, fusion and reinternalisation at the PM.

The questions that remain are: How do L6 myotubes achieve additivity in response to a combination of stimuli? How does insulin stimulation decrease the endocytosis rate constant? Which kinetic parameters are affected in insulin resistant cells? How do L6 cells compare to real muscle? This final question will be a major challenge.

References

1. Fazakerley DJ, Holman GD, Marley A, James DE, Stöckli J, Coster AC. Kinetic evidence for unique regulation of GLUT4 trafficking by insulin and AMP-activated protein kinase activators in L6 myotubes. *J Biol Chem* 2010; 285:1653-60.
2. Jhun BH, Rampal AL, Liu H, Lachaal M, Jung CY. Effects of insulin on steady state kinetics of GLUT4 subcellular distribution in rat adipocytes. Evidence of constitutive GLUT4 recycling. *J Biol Chem* 1992; 267:17710-5.
3. Holman GD, Lo Leggio L, Cushman SW. Insulin-stimulated GLUT4 glucose transporter recycling. A problem in membrane protein subcellular trafficking through multiple pools. *J Biol Chem* 1994; 269:17516-24.
4. Satoh S, Nishimura H, Clark AE, Kozka JJ, Vannucci SJ, Simpson IA, et al. Use of bismannose photolabel to elucidate insulin-regulated GLUT4 subcellular trafficking kinetics in rat adipose cells. Evidence that exocytosis is a critical site of hormone action. *J Biol Chem* 1993; 268:17820-9.
5. Yang J, Holman GD. Comparison of GLUT4 and GLUT1 subcellular trafficking in basal and insulin-stimulated 3T3-L1 cells. *J Biol Chem* 1993; 268:4600-3.
6. Czech MP, Chawla A, Woon CW, Buxton J, Armoni M, Tang W, et al. Exofacial epitope-tagged glucose transporter chimeras reveal COOH-terminal sequences governing cellular localization. *J Cell Biol* 1993; 123:127-35.
7. Quon MJ, Guerre-Millo M, Zarnowski MJ, Butte AJ, Em M, Cushman SW, et al. Tyrosine kinase-deficient mutant human insulin receptors (Met1153→Ile) overexpressed in transfected rat adipose cells fail to mediate translocation of epitope-tagged GLUT4. *Proc Natl Acad Sci USA* 1994; 91:5587-91.
8. Govers R, Coster AC, James DE. Insulin increases cell surface GLUT4 levels by dose dependently discharging GLUT4 into a cell surface recycling pathway. *Mol Cell Biol* 2004; 24:6456-66.
9. Muretta JM, Romenskaia I, Mastick CC. Insulin releases Glut4 from static storage compartments into cycling endosomes and increases the rate constant for Glut4 exocytosis. *J Biol Chem* 2008; 283:311-23.
10. Coster AC, Govers R, James DE. Insulin Stimulates the Entry of GLUT4 into the Endosomal Recycling Pathway by a Quantal Mechanism. *Traffic* 2004; 5:763-71.
11. Karylowski O, Zeigerer A, Cohen A, McGraw TE. GLUT4 is retained by an intracellular cycle of vesicle formation and fusion with endosomes. *Mol Biol Cell* 2004; 15:870-82.
12. Martin OJ, Lee A, McGraw TE. GLUT4 distribution between the plasma membrane and the intracellular compartments is maintained by an insulin-modulated bipartite dynamic mechanism. *J Biol Chem* 2006; 281:484-90.
13. Khayat ZA, Patel N, Klip A. Exercise- and insulin-stimulated muscle glucose transport: distinct mechanisms of regulation. *Can J Appl Physiol* 2002; 27:129-51.
14. Patel N, Khayat ZA, Ruderman NB, Klip A. Dissociation of 5' AMP-activated protein kinase activation and glucose uptake stimulation by mitochondrial uncoupling and hyperosmolar stress: differential sensitivities to intracellular Ca^{2+} and protein kinase C inhibition. *Biochem Biophys Res Commun* 2001; 285:1066-70.
15. Lee YS, Kim WS, Kim KH, Yoon MJ, Cho HJ, Shen Y, et al. Berberine, a natural plant product, activates AMP-activated protein kinase with beneficial metabolic effects in diabetic and insulin-resistant states. *Diabetes* 2006; 55:2256-64.
16. Antonescu CN, Diaz M, Femia G, Planas JV, Klip A. Clathrin-dependent and independent endocytosis of glucose transporter 4 (GLUT4) in myoblasts: regulation by mitochondrial uncoupling. *Traffic* 2008; 9:1173-90.
17. Robinson LJ, Pang S, Harris DS, Heuser J, James DE. Translocation of the glucose transporter (GLUT4) to the cell surface in permeabilized 3T3-L1 adipocytes: effects of ATP, insulin, and GTPgamma S and localization of GLUT4 to clathrin lattices. *J Cell Biol* 1992; 117:1181-96.
18. Ploug T, van Deurs B, Ai H, Cushman SW, Ralston E. Analysis of GLUT4 distribution in whole skeletal muscle fibers: identification of distinct storage compartments that are recruited by insulin and muscle contractions. *J Cell Biol* 1998; 142:1429-46.
19. Chavez JA, Roach WG, Keller SR, Lane WS, Lienhard GE. Inhibition of GLUT4 translocation by Tbc1d1, a Rab GTPase-activating protein abundant in skeletal muscle, is partially relieved by AMP-activated protein kinase activation. *J Biol Chem* 2008; 283:9187-95.
20. Cartee GD, Funai K. Exercise and Insulin: Convergence or divergence at AS160 and TBC1D1? *Exerc Sport Sci Rev* 2009; 37:188-95.
21. Blot V, McGraw TE. GLUT4 is internalized by a cholesterol-dependent nystatin-sensitive mechanism inhibited by insulin. *EMBO J* 2006; 25:5648-58.
22. Kotliar N, Pilch PF. Expression of the glucose transporter isoform GLUT 4 is insufficient to confer insulin-regulatable hexose uptake to cultured muscle cells. *Mol Endocrinol* 1992; 6:337-45.
23. Tortorella LL, Pilch PF. C2C12 myocytes lack an insulin-responsive vesicular compartment despite dexamethasone-induced GLUT4 expression. *Am J Physiol* 2002; 283:514-24.
24. Henriksen EJ, Rodnick KJ, Mondon CE, James DE, Holloszy JO. Effect of denervation or unweighting on GLUT-4 protein in rat soleus muscle. *J Appl Physiol* 1991; 70:2322-7.