Insulin Stimulated GLUT4 translocation – size is not everything!

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Abstract
Insulin regulated trafficking of the facilitative glucose transporter GLUT4 has been studied in many cell types. The translocation of GLUT4 from intracellular membranes to the cell surface is often described as a highly specialised form of membrane traffic restricted to certain cell types such as fat and muscle which are the major storage depots for insulin-stimulated glucose uptake. Here we discuss evidence that favours the argument that rather than being restricted to specialised cell types, the machinery through which insulin regulates GLUT4 traffic is present in all cell types. This is an important point as it provides confidence in the use of experimentally tractable model systems to interrogate the trafficking itinerary of GLUT4.

Introduction

A major action of insulin is to increase uptake of glucose into fat and muscle. The finding that this is brought about through a change in localisation of a single protein, the facilitative glucose transporter GLUT4, from intracellular membranes to the cell surface initiated three decades of research into understanding how this is achieved [1]. This remains an important research goal as this response is the major mechanism by which circulating plasma glucose levels are restored following post prandial increases, and is defective in the disease states of insulin resistance and Type 2 diabetes [1]; characterisation of GLUT4 trafficking is therefore likely to identify potential therapeutic targets for these pathophysiologies.

Studying GLUT4 under true physiological conditions in humans is challenging to say the least and therefore our current working model of the transporter's trafficking itinerary has been constructed using data obtained from a variety of model systems, ranging from isolated tissues to cell types that do not express GLUT4 unless engineered to do so. While debate remains about details of the model, it is generally accepted that GLUT4 is retained intracellularly continually cycling through numerous compartments of the endosomal system, and that insulin alters this trafficking itinerary to result in increased numbers of GLUT4 transporter proteins present on the plasma membrane [1-6] (Figure 1). The membranes from which GLUT4 is delivered to the cell surface upon receipt of an insulin signal are termed insulin responsive vesicles (IRVs). Despite the large amount of data from other systems that have contributed to the map presented in Figure 1, the prevailing view in the field is that this insulin-responsive trafficking of GLUT4 is a characteristic property of fat and muscle cells [1].

Heterologous expression of GLUT4 in a variety of cell types indicates that most, if not all, eukaryotic cells possess the molecular machinery to sequester GLUT4 intracellularly. Perhaps the most striking example of this comes from studies expressing GLUT4 in yeast which show that the transporter is retained intracellularly using the same molecular

machinery required to sequester GLUT4 in insulin sensitive cells [7-10]. These studies indicate that GLUT4 trafficking is regulated by machinery that existed prior to the evolution of GLUT4 in tissues specialised to clear large amounts of glucose from their environment in response to an insulin signal. This contention is supported by the presence of molecules instrumental in GLUT4 sorting, such as the insulin-responsive aminopeptidase IRAP, and the multiligand sorting receptor sortilin, in cell types lacking expression of GLUT4 [11,12]. Furthermore, insulin-stimulated delivery of GLUT4 to the plasma membrane has been demonstrated in a variety of cell types, including fibroblasts, HeLa and CHO cells [13,14], which are not deemed classical 'insulin target' cells, offering the view that the mobilisation of GLUT4 to the surface is not a specific property of adipose and muscle cells, but rather exists in all eukaryotic cells.

Although cell lines such as fibroblast, HeLa and CHO cells do translocate ectopically expressed GLUT4 from internal membranes to the cell surface upon insulin stimulation, the response is not as large as 'physiologically relevant systems'[13,14]; this promulgates the most common argument against the hypothesis that machinery responsible for regulating GLUT4 traffic is not unique to cells expressing GLUT4, but rather is present in the vast majority of cell types and subject to additional layers of regulation in a physiological context. One possibility is that the field is misled by thinking that the larger the insulin response the more physiologically relevant it is, whereas in reality the magnitude may vary depending on tissue- and species-specific physiological demands and/or the experimental system(s) used. Comparison of fold increases in glucose transport and/or GLUT4 translocation to the cell surface reported for a variety of experimental systems in response to insulin indicates that this criticism may not be valid as these range from ~2-fold to 20-fold (Figure 2); below we discuss the differences of insulin response in different systems.

Variations in Magnitude of GLUT4 Response.

Insulin-stimulated glucose transport and GLUT4 translocation have been quantified in many systems, but have mainly focussed on adipose, muscle and cardiac tissue as these represent the major site of insulin action on glucose transport. The ease and historical context of primary rat adipose cells drove their use as the model of choice through the 1960's and 1970's until the development of the murine adipocyte cell line 3T3-L1 in 1975 offered a tractable cell culture alternative. These two cell types routinely exhibit very large increases in glucose transport, with 10- to 30-fold increases reported, concomitant with broadly comparable levels of insulin-stimulated GLUT4 translocation [15,16]. Electron microscopy immunolabelling studies in rodent brown fat, primary adipocytes and cardiomyocytes all have revealed similarly large insulin-dependent increases in GLUT4 delivery to the cell surface [17,18].

While few would doubt the utility and importance of these models, it is worth placing these large insulin-induced increases in context. Studies in human adipose cells reveal much smaller effects of insulin (e.g. [19-21]); with many groups reporting insulin-stimulated effects on transport of the order of only 2- to 3-fold (Figure 2). How can we resolve this apparent paradox? Why should human and rodent adipose tissues exhibit such marked differences in the magnitude of their responses to insulin? A comparison of human and rat adipocytes revealed that the basal rates of glucose transport between the two cell types were similar [19]. By contrast, the maximal rate of glucose transport in human fat was found to be 15-fold less than that observed in rat cells [19]. Using specific photolabels, this study showed that human adipocytes have less GLUT4 than their rodent counterparts and more importantly showed that as a fraction of the total cell GLUT4, more was at the surface of human adipocytes in the absence of insulin than was the case in rat cells [19]. This suggests that the relatively low fold-increase in glucose transport in human compared to rat adipocytes may be related to the metabolic requirements of adipose tissues in these two species. Regardless of the *why*, it is clear that a relatively modest 3-fold increase in glucose transport in human adipocytes is sufficient. Such data support the notion that the large increases in insulin-stimulated glucose transport in rodent adipocytes are the exception, not the rule.

Such modest increases (~3-fold) in insulin-stimulated glucose transport are also reported in rodent brown fat [22,23]; consistent with this, tissue-specific overexpression of GLUT4-myc in brown fat in a transgenic mouse increased the magnitude of insulin-stimulated glucose transport to around 5- to 6-fold [24].

Analysis of pathways and networks between humans and rodents has revealed that even small, species-specific changes can have profound effects on metabolic network functionality [25]. In the context of carbohydrate metabolism, a recent review of human and rodent models reveals species-specific differences at 'every level of glucose regulation', including gene/protein expression and tissue-specific differences [26]. Viewed in this context, the large fold-effects of insulin on rodent adipocytes may reflect a peculiar metabolic adaptation of that species, and need not therefore represent the 'gold standard' by which all other insulin-stimulated transport events are quantified. This would hardly be surprising given the different genetic makeup and feeding strategies of humans and rodents, a point we return to below.

Studies of insulin-stimulated glucose transport in intact skeletal muscle are complicated by a need to consider exercise status, tissue blood flow and capillary perfusion [27,28]. Studies have quantified the effects of insulin on muscle using a range of models, including muscle strips and isolated myoblast cultures where issues of blood flow and capillary diffusion are minimized, and the typical effects of insulin are of the order of 2- to 6-fold depending on the system in question [29-34]. Assessment of GLUT4 translocation in muscle and muscle cell

lines has revealed insulin-stimulated increases at the cell surface of between 2- and 6-fold in human and rat muscles, respectively, in which the levels of cell surface GLUT4 were measured using affinity labelling approaches [29,32-34]. In muscle cell lines, the extent of translocation is frequently smaller, with insulin-stimulated translocation of GLUT4-myc in L6 cells typically around 2.5-fold [35]. Interestingly, studies in which comparisons of glucose transport have been examined in myotubes alongside muscle cell culture models (L6, C_2C_{12}) also report modest increases in glucose transport (less than 2-fold) [36]. Similarly, 2.25-fold increases in glucose transport were observed in rat FDB muscle strips and primary myoblast cultures, parallel assay of glucose transport in L6 cells revealed 1.7-fold increases in response to insulin [37]. Estimates of glucose uptake in isolated cardiomyocytes typically fall into the 2- to 3-fold effects of insulin [22,23] (although much greater increases are observed *in situ* in the heart which likely reflect contributions of capillary delivery and contraction [17,38]). These studies offer the view that insulin-stimulated glucose transport in many 'physiologically relevant' cell types is of the order of 2- to 6-fold. The magnitude of insulin-stimulated delivery of GLUT4 to the surface of undifferentiated fibroblasts, CHO and HeLa cells is 2-3 [13,14,39,40], well within the range of that reported for human adipocytes and skeletal muscle (Figure 2).

"The" GLUT4 Compartment

Another area of criticism that studies of GLUT4 traffic in model systems is subject to is that they don't contain the specialised compartment from where GLUT4 translocates to the cell surface in response to insulin. Here, the field is perhaps guilty of a circular argument: GLUT4 in rodent adipocytes undergoes up to 20-fold translocation because these cells have a specialised compartment; undifferentiated fibroblasts, CHO and HeLa cells exhibit only a 2-3-fold effect because they 'lack' the specialised compartment. Perhaps a clearer way to describe this is that the 'specialised compartment' isn't specialised but is present in all cell types (see below) but is more extensive in rodent adipocytes because of the need to adapt those tissues to metabolic need (mentioned above).

Following its cloning, GLUT4 was expressed in a range of cell types, including neurones and fibroblasts. In all cases, GLUT4 was retained intracellularly in small vesicles scattered throughout the cytosol. Heterologous expression of GLUT4 in PC12 cells revealed that GLUT4 was targeted to small vesicles distinct from the two classes of storage vesicles previously characterised in these cells; secretory granules and synaptic vesicles [41], leading to the hypothesis that GLUT4 is targeted to a organelle that is present in a wide range of cell types including CHO and HeLa cells. Expression of GFP-tagged GLUT4 in undifferentiated fibroblasts, CHO or HeLa cells revealed that insulin was able to drive translocation of GLUT4 to the surface, via mechanisms with many of the same properties, such as wortmannin sensitivity (signalling through IGF-1 receptors which these cells express), observed in rodent adipocytes [13,14,39,40]. A key difference between the translocation of GLUT4 observed in

these systems and that seen in rodent adipocytes is the magnitude of the response, an observation that has been used to argue that HeLa cells lack the machinery to 'correctly' target GLUT4. Rather we contend that the machinery does exist in these cells, but the capacity of these cells to sort GLUT4 into its compartment is less than in rodent adipocytes, but instead more akin to that in human cells (which HeLa are). This contention is underscored by the observation that the magnitude of response in HeLa cells is close to that observed in human adipose cells and many muscle cell types, including the widely used L6 myoblast line.

Further thoughts

GLUT4 translocation is often cited as being a highly specialised form of membrane traffic and while this is apt in that it has evolved to respond quickly to changes in environment it is important bear in mind that other systems appear to use overlapping machinery and similar mechanisms to control cell surface delivery under changing physiological conditions. Examples include regulated trafficking of aquaporin water channels and ion pumps in the kidney and while the signalling pathways that regulate these events may differ the similarity of the trafficking machinery indicates that different tissues use IRV-type membranes to modulate physiologies in response to changing environmental conditions [42].

GLUT4 is expressed in tissues not classically viewed as major insulin targets, including defined regions of the brain and nerve terminals. Action potential firing recruits GLUT4 present at hippocampal nerve terminals to the synaptic surface [43], consistent with the finding that electrical activity at nerve terminals drives the glycolysis necessary to sustain synaptic vesicle recycling [44]. In a striking parallel with studies of GLUT4 in adipocytes, the GLUT4 is stored in a compartment distinct from synaptic vesicles (c.f. the study in PC12 cells cited above) from which it is mobilised to the cell surface [43]. The increases reported are of the order of 2- to 3-fold, much smaller than the increases in synaptic vesicle exocytosis [43]. Similarly, GLUT4 is expressed in many brain regions [45], and also in the placental syncytiotrophoblast [46]. Insulin drives delivery of GLUT4 to the surface of these cells, and in both cases the magnitude of the response is of the order of 2- to 2.5-fold [45,46].

These studies suggest that it is likely that *all* eukaryotic cells have the appropriate machinery to sequester and re-locate GLUT4 to the cell surface. Our contention is that this machinery is not equally developed in all cell types, meaning that the extent of translocation varies, and that some cells have evolved subtle modifications of the system to facilitate alternative regulatory circuits. Examples include differences in the relative importance of the Akt-target and Rab GTPase activating protein AS160 (TBC1D4) and its homolog TBC1D1 in adipocytes and muscle [47], respectively. The expression of both these isoforms in skeletal muscle allows fine-tuning of GLUT4 translocation under different physiological conditions, neatly illustrating how subtle modifications of the system can be exploited [48].

It has long been known that adipocytes vary in size and insulin sensitivity and this has recently received further attention with the identification of a protein that links adipocyte size with insulin sensitivity [49]. Interestingly, adipocyte size, rather than the degree of obesity, has been shown to predict Type 2 diabetes [50]. This has been supported by a recent study that showed weight gain in insulin-sensitive humans resulted in significant maladaptive changes in adipocytes which correlate with insulin resistance [51]. Such studies argue that variations in the magnitude of insulin response exist within tissues, and that small variations in this response can have big consequences.

There are also species-dependent modifications to GLUT4 trafficking which might underly the observed variation in the magnitude of insulin response. This is perhaps not surprising considering the different feeding strategies of humans and mice. In humans there is a specialised clathrin heavy chain isoform, CHC22, that performs GLUT4-sorting functions served by the canonical clathrin heavy chain CHC17 in rodent [52], and that this involves a species-specific modification of the GLUT4 trafficking itinerary involving direct sorting of GLUT4 from the ER-Golgi intermediate compartment to IRVs [39]. Recent work from the same group has revealed that ancestral human dietary changes influenced selection of allotypes that affect CHC22's role in metabolism, further adding credence to the idea that cell- and species-specific modulations of this trafficking pathway are functionally significant [53]. Thus, while the wealth of data from rodent cells clearly demonstrates their utility in providing key molecular insight into insulin action, a role that will continue, such studies exemplify the need for human adipocyte and muscle cell lines to maximise the field’s translational capacity.

Why does this matter?

The ultimate goal of research into GLUT4 traffic is to arm ourselves in the battle against insulin-resistance and Type-2 diabetes. Identifying therapeutic targets via high throughput screening and/or genome editing in humans is not currently possible for many reasons. It is however, possible to employ genome wide, unbiased screens in HeLa cells. This approach was recently used to identify genes required for GLUT4 translocation in HeLa cells [40]. Importantly, hits from this screen have been validated with gene deletion studies in mouse adipocytes [40].

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Annotated references

43 **This study shows that during action potential firing GLUT4 delivery to the cell surface from intracellular stores provides a glycolytic regulatory system to meet increased energy demands. This seminal study reveals a novel paradigm for GLUT4 trafficking regulation.

39 *This study revealed that in human cells, CHC22 clathrin mediates membrane traffic from the ER-to-Golgi Intermediate Compartment, which is needed for GLUT4 sequestration during GLUT4 pathway biogenesis.

40 *This study is the first reported use of HeLa cells expressing GLUT4 in a genome-wide screen for novel effectors. The authors validate their study by identifying Exocyst components as regulating GLUT4 translocation and confirming this data in cultured adipocytes.

28 **This study examined glucose transport in human muscles and observed that during submaximal insulin stimulation muscle membrane permeability to glucose in humans increases twice as much in previously exercised compared to rested muscle. The novel methodology described will allow more detailed analysis of glucose transport in muscle and the impact of exercise.

48 *This study identifies cross-talk between TBC1D4 and TBC1D1 in the regulation of glucose transport in skeletal muscle, and defines a key role for AMP activated protein kinase.

53 *This study undertook detailed genetic analysis of CHC22 allotypes through evolution to reveal subtle modifications arising from single amino acid changes which impact GLUT4 trafficking.

Figure 1. Map of GLUT4's trafficking itinerary. Details GLUT4's trafficking itinerary have been extensively reviewed over the years (e.g. [1-6]), and while many aspects of this remain a topic of debate it is generally accepted that GLUT4 is retained intracellularly continually cycling through numerous compartments of the endosomal system including the *trans* Golgi network (TGN), and that insulin increases (+) the rate of exocytosis and decreases (-) endocytosis, resulting in an overall increases in the number of GLUT4 proteins present at the cell surface. IRVs/GSVs (insulin responsive vesicles/GLUT4 storage vesicles) represent the compartment(s) from which GLUT4 translocates to the cell surface. 15 laboratories collaborated in person (by workshop) to generate this map which is reproduced from http://www.ucl.ac.uk/research/domains/food-metabolism-society

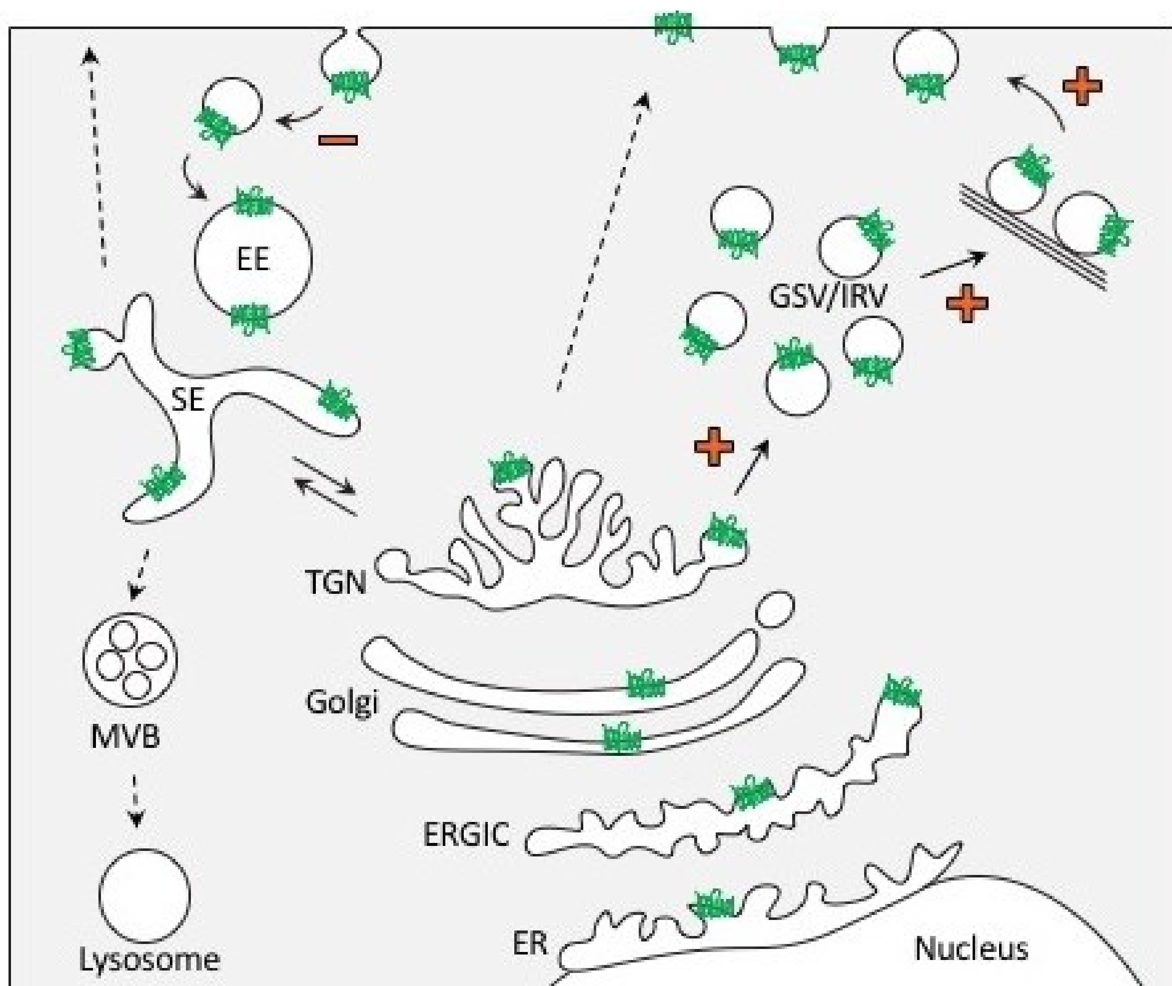

Figure 2. Comparison of insulin-stimulated GLUT4 translocation to the surface of different cells. Shown are values of the fold increase in plasma membrane GLUT4 in response to insulin measured using different experimental systems. Rat and murine (3T3-L1) adipocytes exhibit large responses, typified by the studies cited [15,16]; these were chosen as examples where glucose transport and GLUT4 translocation were measured in parallel and are in general reflective of typical results from many groups. Indicative studies in other systems are presented; these include studies in rodent and human muscles [15, 16, 19-21], myotubes in culture, isolated muscle strips or different examples of muscle cell lines. Exemplar citations are shown in square brackets; we apologise to our colleagues in the field whose studies were not cited due to space limitations.