Dehydroascorbic acid Transport by GLUT4 in Xenopus Oocytes and Isolated Rat Adipocytes.

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Running title: Dehydroascorbic acid transport by GLUT4.
Abbreviations: 2-DG: 2-deoxyglucose; 3-0MG: 3-0-methyl glucose; CB: cytochalasin B; DHA: dehydroascorbic acid; GLUT: glucose transporter isoform; HPLC: high pressure liquid chromatography; SD: standard deviation; SGLT: sodium dependent glucose transporter
Dehydroascorbic acid (DHA), the first stable oxidation product of vitamin C, was transported by GLUT1 and GLUT3 in *Xenopus laevis* oocytes with transport rates similar to that of 2-deoxyglucose (2-DG), but due to inherent difficulties with GLUT4 expression in oocytes it was uncertain whether GLUT4 transported DHA (J. Biol. Chem. 272:18982-89, 1997). We therefore studied DHA and 2-DG transport in rat adipocytes, which express GLUT4. Without insulin, rat adipocytes transported 2-DG 2-3 fold faster than DHA. Pre-incubation with insulin 0.66 µM increased transport of each substrate similarly: 7-10-fold for 2-DG and 6-8-fold for DHA. Because intracellular reduction of DHA in adipocytes was complete before and after insulin stimulation, increased transport of DHA was not explained by increased internal reduction of DHA to ascorbate. To determine apparent transport kinetics of GLUT4 for DHA, GLUT4 expression in *Xenopus* oocytes was re-examined. Pre-incubation of oocytes for > 4 hours with insulin 1 µM augmented GLUT4 transport of 2-DG and DHA by up to 5-fold. Transport of both substrates was inhibited by cytochalasin B and displayed saturable kinetics. GLUT4 had a higher apparent transport affinity (K_M of 0.98 vs. 5.2 mM) and lower maximal transport rate (V_MAX of 66 vs. 880 pmole/oocyte/10 min) for DHA compared to 2-DG. The lower transport rate for DHA could not be explained by binding differences at the outer membrane face, as shown by inhibition with ethylidene glucose, or by transporter trans-activation, and therefore was likely due to substrate specific differences in transporter/substrate translocation or release. These novel data indicate that the insulin sensitive transporter GLUT4 transports DHA in both rat adipocytes and *Xenopus* oocytes. Alterations of this mechanism in diabetes could have clinical implications for ascorbate utilization.
INTRODUCTION

Cellular accumulation of vitamin C (ascorbic acid, ascorbate) is due to transport of both ascorbate and its oxidized metabolite, dehydroascorbic acid (DHA) (1;2). Although ascorbate is the predominant if not the only form in blood, it is possible that DHA is produced in the extracellular milieu in vivo during oxidative stress (3-5). Experiments in neutrophils demonstrated that the rate of cellular DHA uptake is as much as 30-fold greater than the rate of ascorbate uptake(1;5). Once transported, DHA is immediately reduced intracellularly to ascorbate. DHA uptake followed by intracellular reduction can increase intracellular ascorbate accumulation 5-20-fold within minutes. This process, termed ascorbate recycling, was first demonstrated experimentally in human neutrophils (1;4;6), but may also occur in other cell types if DHA is present extracellularly.

We demonstrated previously that DHA is efficiently transported by glucose transporter isoforms GLUT1 and GLUT3 expressed in Xenopus laevis oocytes, with rates of transport and affinity equal to or greater than that for glucose (7). Isoforms GLUT2, GLUT5, and SGLT1 did not transport DHA, and no glucose transporter isoform transported ascorbic acid (7;8). GLUT4 demonstrated minimal DHA transport activity, and it was not possible to adequately examine DHA transport by this isoform.

GLUT4 is the insulin-sensitive protein responsible for the majority of glucose transport in muscle and adipose tissues. Upon insulin stimulation, intracellular vesicles containing GLUT4 fuse with plasma membranes, greatly increasing glucose transport (9-13). Ineffective recruitment of GLUT4 to the cell surface results in excessive glucose accumulation in the blood and type II diabetes clinically. In addition to aberrant glucose metabolism, some evidence suggests that diabetics may also have decreased cellular concentrations of ascorbate or increased requirements for it (14-17). Considering the importance of GLUT4 in glucose uptake and its possible
implications in ascorbate metabolism, it was worthwhile to pursue investigation of DHA transport by GLUT4.

We examined DHA transport in isolated rat adipocytes, a well-characterized model of GLUT4-mediated transport, and found that adipocytes transported DHA in an insulin-responsive manner. Because rat adipocytes co-express GLUT1, which also transports DHA, this system could not be used to determine kinetics of GLUT4-mediated DHA transport. On the other hand, *Xenopus laevis* expression of GLUT4 provides a pure transport system, but levels of surface expression can be low (18-20). We therefore examined ways to enhance transporter activity in oocytes, and showed that pre-incubation with insulin increased transport substantially above oocytes not exposed to insulin. This enabled examination for the first time of DHA transport by GLUT4 and characterization of DHA apparent transport kinetics.
EXPERIMENTAL PROCEDURES

Preparation of \[^{14}C\]-Dehydroascorbic acid (DHA).

\[^{14}C\] -DHA was prepared from crystalline \[^{14}C\] -ascorbic acid (NEN-DuPont, 8.0 mCi/mmol) as previously described (4). Briefly, 5 µl of bromine solution (Fluka) was added to 600 µl \[^{14}C\] -ascorbic acid solubilized in ultrapure water at a concentration of 20mM, vortexed briefly and immediately purged with nitrogen on ice and in the dark for 10 min. HPLC with radiomatic detection confirmed 100% conversion of ascorbate to DHA, which could be completely recovered upon reduction with 2,3 dimercapto-1-propanol.

Rat adipocytes

Isolation

Isolation of rat adipose cells was performed as previously described (21;22). Briefly, epididymal fat pads were removed from anesthetized Sprague Dawley rats (Charles River Laboratories) and minced in Krebs-Ringer bicarbonate HEPES buffer, 1% BSA without glucose, containing 200nM adenosine. Type I collagenase (Worthington Biochemical) was added for 50-60min and digested tissue was filtered through a 250 micron nylon screen mesh into a 50 ml conical centrifuge tube. Cells were resuspended and washed 5 times with the Krebs-Ringer buffer described. Cell number was quantified using lipid weight determinations.

Transport protocol

Rat adipocytes were diluted in the Krebs-Ringer buffer described above to 3-4 x 10^6 cells/tube. When indicated, insulin (Humulin R, Ely Lilly) was added at a final concentration of 0.67 µM for 30 min at 37 °C. Radiolabelled compounds were added at the concentrations specified. Transport was stopped after 5 minutes by centrifugation of cells through
dinonylphthalate (ICN), 3.5 ml of scintillant was added, and cell-associated radioactivity was quantified. Radiolabelled compounds (NEN-DuPont) used were: \[^{14}\text{C}] -\text{DHA}; 2-[1,2-\text{H} (\text{N})]-deoxy-\text{D-glucose} \text{ (specific activity 26.2 mCi/mmol). Experiments were also performed at room temperature with comparable results.}

**HPLC analyses of \[^{14}\text{C}] -\text{ascorbic acid and }[^{14}\text{C}] -\text{dehydroascorbic acid}**

Rat adipocyte ascorbate mass and internalized ascorbate and DHA radioactivity were measured using HPLC with in-line electrochemical and radiomatic detection (7;23). To extract intracellular ascorbate and DHA, adipocytes were washed twice with 20 mM TRIS buffer containing 255 mM sucrose and 1 mM EDTA, homogenized with 5 strokes of a dounce homogenizer at 4 °C, and centrifuged at 14,000 rpm for 2 min. The infranatant was removed and centrifuged again for 2 min at 4 °C. The resulting infranatant was removed, added to an equal volume of 90% methanol 1mM EDTA, and centrifuged at 14,000 rpm for 20 min (4 °C). The supernatant was analyzed by HPLC. Oocyte intracellular ascorbate was measured as described (7).

**Xenopus oocytes**

**Plasmids and inserts**

Rat Glut1 and human Glut3, 4 were obtained as plasmid constructs from G. I. Bell and C.F. Burant (University of Chicago, Chicago, IL). Rat GLUT4 was obtained from M. Birnbaum (University of Pennsylvania, Phila, PA). Plasmid constructs were described previously(7;24-28), and mRNA was prepared in vitro by cutting plasmid vectors with appropriate restriction enzymes followed by in vitro transcription utilizing SP6 or T3 mMessage, mMachine (Ambion).

**Oocyte isolation and injection**
Oocytes were isolated from *Xenopus laevis* (Xenopus I, Dexter MI) and injected with mRNA using established methods (29) as described (7). Injection volume and mRNA concentration was 30-50 nl at a concentration of 1mg/ml unless specified. After injection oocytes were maintained at 20 °C in OR-2 medium containing 1mM pyruvate (Sigma) for up to 5 days and OR-2 was changed daily. Experiments were performed 3-5 days after mRNA injection. In some experiments oocytes were incubated in the presence of insulin 1 µM for up to 24 hours prior to transport studies.

*Transport protocol*

Transport of [14C]DHA, 2-[1,2,3H (N)]-deoxy-D-glucose (NEN-DuPont, 26.2 mCi/mmol), and 3-O-[methyl-3H] D-glucose (75.2 mCi/mmol) was examined by incubating groups of 10-20 oocytes at 23 °C in OR-2 containing different concentrations of freshly prepared [14C]- DHA (0.6-5.5 µCi/ml) or sugar (0.5-1.0µCi/ml labeled sugar with added non-labeled sugar) for up to 10 min. After incubation, oocytes were washed immediately 4 times with 4ml of ice-cold phosphate buffered saline containing 0.1mM phloretin. Inhibitors or competitors were added to the incubation as indicated. Individual oocytes were either dissolved in 500 µl 10% SDS and internalized radioactivity was measured using scintillation spectrometry, or oocytes were frozen to –70 °C in 50µl of 60% methanol 1 mM EDTA for later HPLC analysis.

*Oocyte plasma membrane isolation and immunoblot analysis*

Oocytes plasma membranes were prepared using established protocols(30). Briefly, oocytes manually dissected without collagenase treatment were injected with 30 ng of GLUT4 mRNA or sterile water and maintained for 3 days at 20 °C in OR-2 medium containing 1mM pyruvate. Prior to plasma membrane isolation oocytes were incubated for 4 hours in the presence...
or absence of 1µM insulin. Plasma membrane complexes were purified from groups of 30 oocytes by repeated Dounce homogenization and gravity sedimentation(30). Prepared plasma membrane complexes were solubilized overnight at 4°C in 0.4%SDS, 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2mM EDTA and 0.1 volume 20% Triton X-100 (20). Equal amounts of solubilized plasma membrane complexes were analyzed on SDS/4-20%/polyacrylamide gels. Proteins were transferred to a PVDF membrane and incubated with polyclonal GLUT4 antisera (Alpha Diagnostic International San Antonio TX). Detection was by colorimetric measurement of alkaline phosphatase activity. Densitometric analyses of colorimetric signals were performed using a flatbed scanner (Hewlett Packard ScanJet 5100C) and Scion Image analysis software (Scion corporation, Fredrick MD).

Statistics and Kinetic Calculations

Data are expressed as the arithmetic mean ± the standard deviation (SD) of 10-20 oocytes at each data point, unless otherwise indicated. SD is not displayed when smaller than the symbol size. Transport kinetics were analyzed by best-fit analysis of data points utilizing curve fitting (Jandel Scientific, San Rafael, CA) and Eadie-Hofstee transformation which gave comparable results.
RESULTS

DHA and 2-DG transport in adipocytes

DHA transport by GLUT4 was first studied in isolated rat adipocytes, cells that express GLUT4. In adipocytes without insulin the ratio of GLUT4 to GLUT1 in plasma membrane is approximately 2:1. Upon insulin stimulation this ratio changes to approximately 10:1(9). Because GLUT4 is disproportionately up-regulated by insulin, adipocytes are a preferred cell system to study GLUT4-mediated transport. If DHA were transported by GLUT4, DHA transport would be insulin stimulated. Adipocytes, pre-incubated with or without insulin, were incubated with either $[^{14}C]$ -DHA or $[^3H]$ -2-DG (30, 100, and 300 µM) and uptake was measured (Fig. 1A, 1B). A linear concentration-dependent increase in uptake for each substrate occurred, and insulin pre-incubation increased both 2-DG and DHA uptake by 6-8-fold. Ratios of 2-DG to DHA uptake in the basal state and stimulated state were approximately 3:1 (Fig. 1C), indicating that transport of each substrate increased proportionately with insulin. Intracellular DHA was completely reduced to ascorbate, as determined by HPLC (data not shown).

It was next examined whether DHA transport in adipocytes was inhibited by cytochalasin B, a potent inhibitor of hexose transport. Insulin-stimulated and non-stimulated adipocytes were incubated with 100 µM $[^{14}C]$ -DHA or $[^3H]$ -2-DG with or without cytochalasin B, and uptake was measured (Fig. 2). Insulin pre-incubation increased total uptake of both substrates by 5- and 7-fold. Cytochalasin B inhibited basal and insulin stimulated uptake of both DHA and 2-DG. With insulin, cytochalasin B-inhibitable transport increased by 7.7 and 8.7-fold for DHA and 2-DG, respectively. Taken together, the data in figures 1 and 2 suggest that DHA is transported by GLUT4 in adipocytes.
DHA and 2DG transport in Xenopus laevis oocytes

Xenopus oocytes present an ideal means to specifically examine DHA transport by GLUT 4. In oocytes adequate membrane expression of GLUT4 can be problematic due to its poor insertion in the plasma membrane (19;20). For this reason and because previously reported DHA transport was very low in GLUT4-expressing oocytes (7), it was necessary to increase transport rates to study DHA transport kinetics. Oocytes and mammalian cells might have similar insulin-responsive intracellular pathways for translocation of transmembrane proteins (31). Therefore, we tested whether insulin enhanced DHA and 2-DG transport in GLUT4-expressing oocytes. These oocytes were incubated from 1-24 hours with insulin 1 µM, washed, and incubated with either radiolabelled DHA or 2-DG (Fig. 3A, 3B). Both DHA and 2-DG transport increased up to 5-fold after 4 hours of insulin pre-incubation. Transport of both substrates was completely inhibited by 10 µM cytochalasin B (data not shown). Control water-injected oocytes showed very little transport and were unresponsive to insulin pre-incubation. Pre-incubation with 1µM insulin produced the maximum effect on both DHA and 2-DG transport (Fig. 3C, 3D). The effect of insulin was specific for GLUT4, because oocytes expressing either GLUT1 or SGLT1 showed no increased transport in response to insulin (Fig. 3E).

We tested whether the effect of insulin on increasing DHA and 2-DG transport was due to increased GLUT4 translocation to the oocyte plasma membrane. Oocytes previously injected with or without GLUT4 were incubated for 4 hours with insulin, and GLUT4 in isolated oocyte plasma membranes was quantitated by Western blot (Fig. 4). Insulin increased GLUT4 in the membrane approximately 5 fold, based on densitometric analyses, in agreement with the transport findings.

Based on these results, DHA transport experiments in GLUT4-expressing oocytes were performed after a 4-hour pre-incubation with insulin 1 µM except as indicated. mRNA injection
amount and post-injection incubation time influenced DHA and 2-DG transport. Transport of both substrates increased linearly with injected amounts of mRNA from 0.3-40 ng (data not shown), and 30 ng of mRNA was used for injections. Transport increased linearly with substrate incubation time from days 2 to 5 after mRNA injection (data not shown). Experiments were performed using oocytes 3-5 days post-injection, because oocyte fragility increased after day 5.

*Transport kinetics and transport properties of DHA and 2DG in GLUT4-expressing oocytes*

To determine kinetics injected oocytes were pre-incubated with insulin, washed, and incubated with different concentrations of $[^{14}C] \text{-DHA}$ or $[^{3}H] \text{-2-DG}$. Intracellular substrate uptake was quantified and apparent transport kinetics constants were determined by Eadie-Hofstee transformation of the data (Fig. 5A, 5B, insets). For DHA the apparent $K_M$ was 0.98 mM and the apparent $V_{MAX}$ was 66 pmole/oocyte/10 min, and for 2-DG the apparent $K_M$ was 5.2 mM and the apparent $V_{MAX}$ was 880 pmole/oocyte/10 min. Non-linear curve fitting gave similar results.

Because of the difference in maximal transport rates between DHA and 2-DG in GLUT4-expressing oocytes, uptake rates of DHA and 2-DG were compared to those in oocytes expressing GLUT1 or GLUT3. At comparable extracellular concentrations, 2-DG uptake was 6-12-fold greater than that of DHA in GLUT4-expressing oocytes (Fig. 6A and 6B). In contrast, oocytes expressing GLUT1 or GLUT3 transported both substrates equally (Fig. 6B). Maximal transport rates of DHA for GLUT1- and GLUT3-expressing oocytes were approximately 100 pmole/oocyte/min compared to a maximal rate of 6.6 pmole/oocytes/min for GLUT4-expressing oocytes. The apparent transport affinity of GLUT4 for DHA was similar to that of GLUT1 and GLUT3 (7) and higher than that for 2-DG (lower $K_M$), but the rate of GLUT4 mediated transport was at least 6-fold lower for DHA compared with 2-DG. The maximal transport rate differences
between DHA and 2-DG could not be attributed to differences in expressed transporter on the cell surface because oocytes used in experiments with either substrate were injected and incubated in the same way at the same time. We therefore examined whether differences in transport rate between DHA and 2-DG could be attributed to other factors.

Decreased transport of DHA could not be attributed to intracellular reduction of dehydroascorbic acid to ascorbate. Measurement of intracellular ascorbic acid using HPLC demonstrated 100% reduction of DHA acid to ascorbate at all extracellular concentrations of DHA examined. Furthermore, using GLUT1 and GLUT3-expressing oocytes, intracellular reduction of DHA to ascorbate began to diminish at approximately 70-100 pmol/min/oocyte, which is at least 10-fold higher than the maximal rate of transport seen in GLUT4-expressing oocytes (7) (data not shown).

Some glucose transport isoforms exhibit increased transport activity due to substrate trans-activation (32;33). Because DHA is completely reduced and phosphorylation is not rate limiting within the time course of these experiments(25) (7)(data not shown), we tested whether increased transport of 2-DG by GLUT4 could be due to intracellular activation of the transport process. Control and GLUT4-expressing oocytes were loaded overnight with 20 mM 3-OMG, a non-metabolized glucose analog, and transport of $[^{14}\text{C}]$-DHA or $[^{3}\text{H}]$-2-DG was examined. Uptake rates of both substrates were similar regardless of 3-OMG loading (Fig. 7). Loading of oocytes with 3-OMG was confirmed using 3-O-[methyl-$^{3}\text{H}$] D-glucose and measuring intracellular accumulation of radiolabel in a parallel set of GLUT4-expressing oocytes (data not shown).

It was also tested whether binding affinity to the external face of the transport protein was different for DHA or 2-DG. Incubation of GLUT4-expressing oocytes with 500 µM of either $[^{14}\text{C}]$-DHA or $[^{3}\text{H}]$-2-DG in the presence of increasing amounts of the exo-facial glucose
inhibitor 4,6-O-ethylidene-α-glucose resulted in a similar concentration-dependent inhibition of uptake for both substrates (Fig. 8). These results suggest that DHA and 2DG binding to the external face of GLUT4 are comparable.

In summary, GLUT4-expressing oocytes transported DHA with an apparent affinity higher than that of 2-DG, but with a 6-12 fold lower maximal transport rate. This could not be attributed to intracellular reduction of DHA, substrate trans-activation, or decreased DHA binding at the extracellular surface. Therefore, the lower transport rate is likely due to differences between DHA and 2-DG in either transmembrane translocation or intracellular release.

The relevance of DHA transport by GLUT4 in vivo depends upon both DHA availability and the presence of other substrates such as glucose that could inhibit transport. We tested whether DHA uptake would occur in GLUT4-expressing oocytes in the presence of glucose at physiologic concentrations and at higher concentrations that occur in diabetes mellitus. GLUT4-expressing oocytes were incubated with DHA (100 µM) for 10 min in the presence of D-glucose (0-30 mM) (Fig. 9). Uptake was inhibited approximately 50% by glucose within the physiologic concentration range of 3-5 mM, while higher concentrations inhibited DHA uptake by up to 92%. These data show that concentrations of glucose within the physiologic range, and 30-50 fold in excess of DHA, only partially inhibited DHA uptake in oocytes.
DISCUSSION

This study presents new information showing that GLUT4 transported dehydroascorbic acid, the oxidized form of ascorbate. Transport was initially examined using isolated rat adipocytes because of their abundance of GLUT4. Rat adipocytes express GLUT1 as well as GLUT4, and in response to insulin both are translocated to the cell surface. Upon insulin stimulation, GLUT4 concentration in plasma membrane is approximately 10 fold greater than GLUT1, and increased glucose transport after insulin stimulation can be attributed predominantly to GLUT4(9). We report here that DHA and 2-DG were transported by rat adipocytes without insulin, insulin pre-incubation increased uptake of both DHA and 2-DG 6-8 fold, and the rate of DHA transport was approximately 3 fold less than that of 2-DG independent of insulin. These novel data support the conclusion that DHA transport in adipocytes was mediated by GLUT4.

To validate that GLUT4 could transport DHA, GLUT4 was expressed in Xenopus oocytes. Advantages of oocytes are that specific GLUT transporters can be tested individually, endogenous glucose and DHA transport is nearly undetectable in control oocytes, and the human GLUT4 construct could be tested. These experiments showed conclusively for the first time that GLUT4 transported DHA, that the affinity of GLUT4 for DHA was similar to that of GLUT 1 and GLUT3, and that the affinity of GLUT4 for DHA was higher than for 2-DG.

Oocyte expression of GLUT4 has been reported (18-20;31;34;35), but measurement of hexose transport has been problematic because GLUT4 expression on the oocyte surface is generally less than that of other glucose transport proteins(7;20). Experiments with DHA as a substrate are further complicated by low specific activity of synthesized [14C] -DHA and its lability, making long incubations impossible.

These problems were solved by increasing GLUT4 transport activity in Xenopus oocytes. Insulin pre-incubation ≥ 4 hours increased transport activity of both DHA and 2-DG by up to 5-
fold, and the maximum effect was achieved with 1 μM insulin. Insulin increased GLUT4 translocation to the oocyte plasma membrane by approximately 5 fold, as expected. Although oocytes do not have insulin receptors, our results are consistent with those showing that insulin can act through IGF-1 pathways (18). Previous studies have shown insulin to increase GLUT-4 mediated transport of 2-DG in oocytes 2-fold by increasing $V_{\text{MAX}}$, although the longest insulin pre-incubation time was 1.5 hours (31;35). Other investigators examined GLUT4-mediated transport (25;36) in oocytes but insulin effects were not reported (37). It is possible that other investigators did not detect insulin stimulation of GLUT4 translocation because the insulin incubation time was too short. Although the mechanisms by which insulin increased the amount of GLUT4 in oocyte membranes are not fully understood (31;35;38), enhanced GLUT4 transport activity induced by insulin was essential for measurement of DHA apparent transport kinetics.

Utilizing oocytes expressing GLUT4, we compared zero-trans uptake kinetics of DHA and 2-DG. The apparent transport affinity of GLUT4 for DHA was 0.98 mM, similar to that of GLUT1 (1.1 mM) and GLUT3 (1.7 mM) (7). The apparent transport affinity of GLUT4 for 2-DG was considerably lower, 5.2 mM, but this is comparable to other values for GLUT4 (25;35;39). Despite its higher apparent transport affinity for DHA, however, GLUT4 transported DHA at a substantially slower rate than 2-DG both in oocytes and in rat adipocytes. For example, in oocytes expressing GLUT4 uptake of 2DG was 6-12 fold greater than uptake of DHA, while in oocytes expressing either GLUT1 or GLUT3 uptake of 2-DG and of DHA were essentially equal.

We examined several possible explanations to account for the lower transport rate of DHA compared to 2-DG by GLUT4 in oocytes. Substrate transport by glucose transport proteins may involve either a single binding site which translocates from outside to inside the cell, termed simple-carrier transport, or two sites which are independently present on each side of the membrane, termed fixed-site carrier transport (40;41). Following these mechanisms, apparent
transport affinity, measured as zero-trans uptake, is a function of substrate binding to the transport protein at the extracellular face, membrane translocation of the substrate, and substrate release leading to the return of the transport protein for further extracellular substrate binding. We showed that extracellular binding of DHA and 2-DG were the same by performing inhibition experiments with ethylidene glucose, a glucose analog not translocated by glucose transporters. Because the intracellular side of the transport protein may also be available to bind substrate, we examined whether the differences in transport might be attributed to a trans-activation effect. Once inside oocytes, DHA is rapidly reduced to ascorbate, which does not interact with glucose transport proteins (7). Therefore, only 2-DG could possibly interact with intracellular binding sites. Using oocytes pre-loaded with 20 mM 3-OMG, we demonstrated that intracellular sugar had no effect GLUT4 transport of either 2-DG or DHA. These data are consistent with previous reports that GLUT4, unlike GLUT1, does not demonstrate mechanisms of transactivation (32;33). Taken together, the findings suggest that the different rate of uptake of DHA compared to 2-DG is likely due differences in membrane translocation and/or intracellular substrate release. To clarify this issue, additional biochemical tools are needed that are not currently available, such as a non-reducible DHA analog or a high affinity ligand that inhibits DHA but not 2-DG transport.

DHA transport by GLUT4 may be a novel aspect of ascorbate recycling, in which DHA formed by oxidation of extracellular ascorbate is rapidly transported into cells and immediately reduced to ascorbate intracellularly. Ascorbate recycling allows cells to accumulate ascorbate rapidly (4), and may be a new mechanism for ascorbate accumulation in GLUT 4 expressing cells such as adipocytes, skeletal muscle cells, or cardiac myocytes. Because GLUT 4 transports DHA as shown in this paper, GLUT1 and GLUT3 transport DHA (7), and nearly all cells possess at least one of these transporters, we predict that most cells will transport DHA if available.
The contribution of GLUT 4-mediated transport of DHA to ascorbate accumulation in vivo is unknown. Plasma concentrations of DHA in healthy people cannot be distinguished from zero (3). DHA was detected in plasma of diabetic patients, but this may be due to assay artifact (2; 42; 43). DHA plasma concentrations, however, may not indicate substrate availability to tissues. Cellular transport may be a consequence of local formation of DHA extracellularly, which would not be reflected by plasma measurements (2). Local oxidation of ascorbate to DHA is followed by its rapid uptake into neutrophils (4). It is unknown whether similar local oxidation occurs in the extracellular milieu of tissues that express GLUT 4. Transport of ascorbate itself may be responsible for ascorbate accumulation under conditions without oxidation. With oxidative stress, DHA uptake and subsequent intracellular reduction might contribute to ascorbate accumulation (1; 2).

Type II diabetes is characterized by insulin insensitivity, decreased GLUT4 translocation to the cell membrane, decreased glucose transport, and accumulation of glucose in plasma. Although unresolved, a number of investigators have suggested that ascorbate requirements of diabetics may be increased (14-17). The data here provide several potential mechanisms by which this may occur. DHA uptake followed by internal reduction could be one mechanism accounting for ascorbate accumulation in GLUT4 expressing tissues. If in diabetics DHA uptake is diminished due to decreased GLUT4 at the cell membrane, ascorbate intracellular concentrations might decline. It is also possible that DHA clearance by GLUT1, GLUT3, or GLUT4 may decline due to high extracellular glucose concentrations, and DHA residence time outside cells may increase. Because DHA is labile, irreversible extracellular degradation of DHA may occur and ascorbate reducing equivalents would be lost. Another possibility is that insulin-induced increases in GLUT1 at the plasma membrane may partially mediate DHA uptake. In oocytes, the rate of GLUT1-mediated DHA transport is approximately 20 fold faster than that
mediated by GLUT4, although such comparisons are not ideal because transport rate differences in oocytes might be due to differences in transporter expression(20). If the rate of DHA transport by GLUT 1 greatly exceeds that by GLUT4, a modest increase in GLUT1 at the plasma membrane could mediate DHA accumulation. In diabetes if insulin-induced GLUT1 recruitment to the plasma membrane is diminished(44), DHA uptake would be diminished. All of these possibilities are dependant on formation of DHA in vivo, which remains to be proven (2;43), and on DHA reduction pathways in normal and diabetic tissues. Considering the possible implications of ascorbate recycling for the determination of ascorbate requirements for all individuals, and especially those with aberrant glucose metabolism or increased oxidant loads, these concepts deserve further investigation.
Figure 1: Concentration dependent uptake of 2-DG and DHA uptake in basal and insulin stimulated rat adipocytes. Rat adipocytes were pre-incubated in the presence (○, ▽) or absence (●, ▼) of insulin (0.67 µM) at 37 °C for 30 min. Afterwards either [3H]-2-DG (Panel A) or [14C] -DHA (Panel B) was added for 5 min at 37 °C, cells were washed, and radioactivity quantified. Intracellular radioactivity was measured by HPLC and found to be 100% [14C]- ascorbate. Panel C: The relative rates of uptake of Radiolabelled DHA and 2-DG for basal (□) and insulin stimulated cells (■) at each substrate concentration are plotted. Results for both panels are mean ± SD of 4 samples.

Figure 2: Insulin stimulation of 2-deoxyglucose and DHA uptake in rat adipocytes. Rat adipocytes were pre-incubated for 30 min at 37 °C in the presence (insulin) or absence (basal) of 0.67 µM insulin. Afterwards, cells were incubated with 100 µM [14C] –DHA (■) or [3H] -2-DG (□) for 5 min at 37 °C with or without cytochalasin B (400 µM) (22). Cells were washed as described in methods and cell associated radioactivity was measured. Results are mean ± SD of 4 samples.

Figure 3: Insulin stimulation of DHA and 2-DG uptake in Xenopus oocytes injected with GLUT4 mRNA: Xenopus oocytes previously injected with either with GLUT4 mRNA (filled symbols) or water (open symbols) were pre-incubated with insulin (1 µM) for up to 24 hours (Panels A and B) or with different amounts of insulin for 4 hours (Panels C and D) followed by incubation with 500 µM [14C] -DHA (Panels A and C) or [3H] -2-DG (Panels B and D) for 10 min at room temperature. Panel E: Oocytes injected with either GLUT4 (▼), GLUT1 (□) or SGLT1 (■) were pre-incubated with insulin as above followed by incubation with 500 µM [3H] -2-DG (GLUT1) or [14C] -glucose (SGLT1). Control values for 2-DG transport by GLUT4 and GLUT1
were 18.5 ± 8 and 198 ± 98 pmole/10 min/oocyte, respectively and glucose transport by SGLT1 was 139 ± 19 pmole/10 min/oocyte. Results are mean of 10-20 oocytes ± SD.

Figure 4: Detection of GLUT4 in Xenopus oocyte plasma membranes. Thirty oocytes per group previously injected with either 30 ng of GLUT4 mRNA or sterile water were incubated for 4 hours in the presence or absence of insulin 1μM. After preparation of oocyte plasma membranes, equal amounts were loaded for Western Blot analyses. GLUT4 was visualized using a polyclonal GLUT4 antibody.

Figure 5: DHA and 2-DG uptake in GLUT 4 injected oocytes. Oocytes injected with GLUT4 mRNA (●,▼) or uninjected oocytes (▲) were pre-incubated for 4 hours with 1 μM insulin, washed and incubated with either [14C] -DHA (Panel A) or [3H] -2-DG (Panel B) for 10 min at room temperature, washed and intracellular radioactivity quantified. Inset: Eadie-Hofstee transformation of uptake data. Apparent transport kinetics constants were K_M of 0.98 mM vs. 5.2 mM and V_MAX of 66 pmole/10 min/oocyte vs. 880 pmole/10 min/oocyte for DHA and 2-DG, respectively. Data represents mean ± SD of 10-20 oocytes.

Figure 6: Relative uptake of 2-DG and DHA in Xenopus oocytes. Oocytes were pre-incubated for 4 hours with insulin 1 μM, washed, and incubated with either [14C] -DHA or [3H] -2-DG. Panel A: Uptake of [14C] -DHA (●) or [3H] -2-DG (▼) (0.1-3 mM) after incubation of GLUT4-expressing oocytes at room temperature for 10 min. Panel B: Ratio of 2-DG/DHA uptake for oocytes expressing either GLUT4 (■), GLUT1 (▲) or GLUT3 (▲). Data represent mean ± SD of 10-20 oocytes.
Figure 7: Effect of 3-OMG loading on DHA and 2-DG uptake in *Xenopus* oocytes. GLUT4-injected or control uninjected oocytes were pre-incubated for 24 hours with insulin 1 µM with (■) or without (□) 20 mM 3-O-methyl-glucose. Oocytes were then washed and incubated with either [14C]-DHA or [3H]-2-DG (500 µM) for 10 min at room temperature and internalized radioactivity was quantified. 3-OMG loading was confirmed with parallel experiments using [3H]-3-OMG (not shown). Data represent mean ± SD of 10-20 oocytes.

Figure 8: Inhibition of DHA or 2-DG uptake by the exo-facial glucose inhibitor, 4,6-O-ethylidene-α-glucose. GLUT4-injected oocytes were pre-incubated with insulin 1 µM for 4 hours (1 µM) and then incubated with 500 µM [14C] -DHA (●) or [3H] -2-DG (▲) for 10 min at room temperature in the presence of increasing concentrations of ethylidene-glucose (0.5-100 mM). Oocytes were washed and internalized radioactivity was quantified. Data are expressed as a function of transport in the presence of no inhibitor. Substrate uptake under these conditions was 15 ± 7 and 96 ± 41 pmoles/10min/oocyte for [14C] -DHA or [3H] -2-DG, respectively. Data represent means ± SD of 10-20 oocytes.

Figure 9: Inhibition of DHA uptake by D-glucose. Oocytes were pre-incubated with insulin 1 µM for 4 hours, washed, and then incubated with [14C]-DHA (100 µM) for 10 min at room temperature with increasing amounts of D-glucose (0-30 mM). Oocytes were washed and internalized radioactivity was quantified. Data represent mean ± SD of 10-20 oocytes.
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[\textsuperscript{3}H]-2-DG or [\textsuperscript{14}C]-DHA uptake (pmole/5min/4 \times 10^5 cells)

**Fig. 1 A and B**

**A**

**B**
Fig. 1C

Ratio 2-DG/DHA uptake vs. Substrate concentration (µM)

C

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Fig. 2

$[^3]H\text{-2-DG}$ uptake (pmole/5min/4 $\times 10^5$ cells)

$[^{14}]C\text{-DHA}$ uptake (pmole/5min/4 $\times 10^5$ cells)

- Basal
- Basal + CB
- Insulin
- Insulin + CB
Fig. 3

(A) [14C]-DHA uptake (pmole/10 min/oocyte) vs. Insulin pre-incubation time (hours).

(B) [3H]-2-DG uptake (pmole/10 min/oocyte) vs. Insulin pre-incubation time (hours).
Fig 3.

Hexose uptake (% of no insulin control)

Insulin pre-incubation time (hours)

GLUT1

GLUT4

SGLT1
Figure 4

|        | Sham | GLUT4 |
|--------|------|-------|
| insulin| -    | +     |
|         | -    | +     |

[Image of a gel with bands indicating the presence or absence of a protein.]
Fig 5.

[Graph showing the relationship between DHA concentration (mM) and [14C]-DHA uptake (pmole/10 min/oocyte). The graph includes a linear regression inset showing a decrease in V/S ratio with increasing DHA concentration.]
Fig. 6

A

Uptake (pmole/10 min/oocyte)

Substrate concentration (mM)

B

Ratio 2-DG/DHA uptake

Substrate concentration (mM)
Fig. 7

[\[^{14}\text{C}]\text{-DHA or}^{[3}\text{H}]\text{-2-DG uptake (pmole/10 min/oocyte)}]
Fig. 8

2-DG or DHA Uptake (% of control)

Ethylidene-glucose (mM)
Fig 9.
Dehydroascorbic acid Transport by GLUT4 in Xenopus Oocytes and Isolated Rat Adipocytes
Steven C. Rumsey, Rushad Daruwala, Hadi Al-Hasani, Mary Jane Zarnowski, Ian A. Simpson and Mark A. Levine

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