Cyclosporine A and tacrolimus reduce the amount of GLUT4 at the cell-surface in human adipocytes: increased endocytosis as a potential mechanism for the diabetogenic effects of immunosuppressive agents

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Context: Immunosuppressive agents are associated with profound metabolic side effects including new-onset diabetes and dyslipidemia after organ transplantation.

Objective: To investigated the effects of the cyclosporine A (CsA) or tacrolimus on glucose uptake and insulin signalling in human adipocytes and their impact on the regulation of cellular trafficking of the glucose transporter 4 (GLUT4).

Design: Human isolated adipocytes were incubated with therapeutic concentrations of either CsA or tacrolimus, and glucose uptake and expression of insulin signaling proteins were assessed. Furthermore, we studied effects of CsA and tacrolimus on the regulation of cellular trafficking of the GLUT4 in differentiated human pre-adipocytes and L6 cells.

Results: CsA and tacrolimus had a concentration-dependent inhibitory effect on basal and insulin-stimulated ^{14}C-glucose uptake in adipocytes. Although phosphorylation at Tyr1146 of insulin receptor (IR) was inhibited by tacrolimus, the phosphorylation and/or protein levels of the insulin signalling proteins IRS1/2, p85-PI3K, PKB, AS160 and mTORC1, as well as GLUT4 and GLUT1, were unchanged by CsA or tacrolimus. Furthermore, CsA and tacrolimus reduced the GLUT4 amount localized at the cell surface of differentiated human pre-adipocytes and L6 cells in the presence of insulin. This occurred by an increased rate of GLUT4 endocytosis, with no change in the exocytosis rate.

Conclusions: These results suggest that therapeutic concentrations of CsA and tacrolimus can inhibit glucose uptake independent of insulin signalling by removing GLUT4 from the cell surface via an increased rate of endocytosis. Such mechanisms can contribute to the development of insulin resistance and diabetes associated with immunosuppressive therapy. In addition, they may provide novel pharmacological approaches for treatment of diabetes.

Immunosuppressive drugs are associated with hyperglycemia, dyslipidemia and hypertension, which along with obesity are the main features of the metabolic syndrome. In the nontransplant population, metabolic syndrome is associated with increased risk for cardiovascular diseases and diabetes. The calcineurin inhibitors, cyclosporine A (CsA) and tacrolimus, are the main immuno-

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Abbreviations: 2-DG 2-Deoxy-glucose; AS160 Protein kinase B substrate of 160 kDa; CsA Cyclosporine A; GLUT4 Glucose transporter 4; GLUT4myc myc-tagged GLUT4; IR Insulin receptor; IRS1, 2 Insulin receptor substrate 1, 2; mTOR Mammalian target of rapamycin;

suppressive agents used to prevent rejection after solid organ transplantation, as well as for treatment of autoimmune diseases (1). Despite the expected action on the immune system, long-term treatment with either CsA or tacrolimus is associated with metabolic toxic effects, which have a negative impact in the patient's long-term outcome (2). Experimental and clinical studies suggest that CsA and tacrolimus are associated with increased risk for developing new-onset diabetes after transplantation (NODAT) (3). NODAT has been reported to occur in 15%–50% of the transplant recipients and is recognized as a risk factor for cardiovascular diseases, graft rejection and death (4, 5).

CsA and tacrolimus mediate their immunosuppressive action by interrupting Ca^{2+}/calmodulin- calcineurin signaling pathways in T cells, thereby blocking antigen-stimulated gene expression of IL-2, a factor critical for T cell proliferation. Studies in animals and humans indicate that calcineurin inhibitors reduce pancreatic insulin synthesis and secretion (6, 7). Furthermore, recent studies in both mice and humans have indicated that treatment with calcineurin inhibitors is associated with reduced insulin sensitivity in peripheral tissues (8–10). However, the underlying mechanisms for glucose intolerance are not known, and the effects of CsA and tacrolimus in human adipocytes have not been studied previously.

Glucose uptake into muscle and adipose tissue is the major mechanism by which normal whole-body glycaemia levels are maintained (11). This process is stimulated by insulin, and mediated by glucose transporters (GLUT), mainly GLUT4. In the absence of insulin, only 5%–10% of the total cellular GLUT4 content is present at the cell surface in both adipocytes and muscle cells (12, 13). Skeletal muscle is the main tissue responsible for insulin-dependent glucose uptake, whereas adipose tissue accounts for only a small fraction (14). Despite this, mice with adipose-selective depletion of GLUT4 develop impaired glucose tolerance, apparently due to insulin resistance induced in muscle and liver (15). Thus, adipose tissue is crucial in regulating glucose metabolism beyond its intrinsic contribution to whole-body glucose handling and signaling, eg, via adipokines and neuroendocrine pathways, it can contribute to insulin resistance and the development of type 2 diabetes (16).

The main objectives of this study were to investigate the direct effects of therapeutic concentrations of CsA and tacrolimus on glucose uptake and on insulin signaling in human isolated adipocytes. In addition, GLUT4 amount and cellular distribution was addressed. Effects of CsA and tacrolimus on endocytotic and exocytotic rates of the GLUT4 transporter were studied in L6 myoblasts expressing myc-tagged GLUT4 (GLUT4myc).

Materials and Methods

Adipose tissue donors

Human abdominal subcutaneous (n = 44) and omental (n = 11) adipose tissue biopsies were obtained from 44 nondiabetic subjects (19 men/25 women; age 23–70 years; body mass index (BMI) 20–36 kg/m^2). Subjects fasted overnight (>10 hours) and venous blood samples were collected for analysis of glucose, insulin and lipids by routine methods at the Department of Clinical Chemistry, Sahlgrenska University Hospital. Adipose tissue biopsies were performed by needle aspiration from the lower part of the abdomen after local dermal anesthesia with lidocaine (Xylocain; AstraZeneca, Södertälje, Sweden), or by elective abdominal surgery after induction of general anesthesia.

Clinical and biochemical characteristics of the adipose tissues donors are shown in Table 1. Some of the subjects donating adipose tissue in this study were also part of a previous published study elucidating metabolic effects of rapamycin (17). Subjects with diabetes, other endocrine disorders, systemic illnesses or malignancy, as well as ongoing medication with systemic glucocorticoids and immune modulating therapies were excluded from the study. The study protocol was approved by the Regional Ethics Review Boards in Gothenburg and Uppsala. Written informed consent was received from all subjects.

Isolation of human adipocytes and primary preadipocytes

Isolated human adipocytes were obtained from biopsies after collagenase type II digestion (from *Clostridium histolyticum*, Sigma Chemical Co., St. Louise, MO, USA), in Hank's medium (Invitrogen Corporation, Paisley, UK) containing 6 mM glucose, 4% BSA (Sigma), 0.15 μM adenosine (Sigma), pH 7.4 for 60 minutes at 37°C in a shaking water-bath. Isolated adipocytes were filtered through a 250 μm nylon mesh and were washed four times in Hank's medium without glucose (4% BSA, 0.15 μM adenosine and pH 7.4). The average cell diameter was measured in isolated adipocytes (18).

Human primary preadipocytes (n = 4) were isolated and cultured in DMEM:F12 supplemented with 10% FBS and 1% PEST in Lab Tek II chamber glass slides (Nalgene Nunc International, Naperville, IL, USA). Confluent preadipocytes were differentiated and were incubated with or without CsA or tacrolimus (100 nM) for 75 minutes, and 6 nM insulin was added in the last 20 minutes. Cells were used to GLUT4 immunofluorescence analysis. See Supplemental Material and Methods for further details on primary preadipocytes isolation and differentiation procedure.

Culture of adipocytes and glucose uptake assessment

To investigate the effects of short-term preincubations (15 minutes) with either CsA (Sigma, 1–1000 nM) or tacrolimus (Sigma, 1–1000 nM) on glucose uptake, isolated subcutaneous

NODAT New onset diabetes after transplantation; p70S6K1 p70 ribosomal S6 kinase; PEST Penicillin-streptomycin; PI3K Phosphatidylinositol 3-kinase; PKB Protein kinase B

Table 1. Characteristics of the adipose tissue donors ($n = 44$).

Variable	Value		
Sex (male/female; n)	19/25		
Age (years)	50	±	13
Body mass index (kg/m^2)	27.7	±	4.0
Waist-hip ratio (WHR)	0.92	±	0.09
Systolic blood pressure (mmHg)	132	±	18
Diastolic blood pressure (mmHg)	83	±	11
Subcutaneous adipocyte diameter (μm) [a]	99.8	±	11.7
Omental adipocyte diameter (μm) [b]	88.4	±	19.7
HbA_{1c} (mmol/mol, IFCC) [c]	34	±	4
Glucose (mmol/liter)	5.1	±	0.6
Insulin (mU/liter)	8.6	±	6.0
HOMA-IR [d]	1.7	±	1.1
Body fat mass (%)	32.1	±	7.1
Triglycerides (mmol/liter)	1.2	±	0.4
Cholesterol (mmol/liter)	6.0	±	1.3
LDL-cholesterol (mmol/liter)	3.9	±	1.1
HDL-cholesterol (mmol/liter)	1.7	±	0.6

Data are means ± SD

[a] $n = 44$; [b] $n = 11$; [c] Normal range 27–46 mmol/mol (IFCC standard); [d] Homeostasis model assessment-estimated insulin resistance, calculated as: fasting insulin (mU/liter) × fasting glucose (mM)/22.5; HbA_{1c}, glycosylated haemoglobin; LDL, low-density lipoprotein; HDL, high density lipoprotein

and omental adipocytes (n = 8–17 and n = 8, respectively) were diluted to a lipocrit of 5% in Hank's medium (4% BSA, 0.15 μM adenosine and pH 7.4) without glucose. Measurement of cellular D-[U-^{14}C]-glucose uptake was performed according to a previously validated technique for human adipocytes, that reflects rate of transmembrane glucose transport (19). See Supplemental Material and Methods for further details.

To further investigate the effects of long-term preincubations (20h) of either CsA or tacrolimus on glucose uptake, the subcutaneous and omental adipocytes (n = 8 and n = 5, respectively) were incubated in DMEM (6 mM glucose, 10% FBS, 1% PEST, Invitrogen) in the absence or presence of either CsA or Tacrolimus (100 nM), at 37°C under a gas phase of 5% CO_2 with gentle agitation (~30 rpm) in a culture chamber (20). After incubation the cells were washed and diluted in Hank's medium without glucose to measure glucose uptake, as above. The viability of adipocytes was not significantly affected after 24 hours incubation with CsA or tacrolimus (90 to 100%, p=ns), when compared to untreated cells.

Immunoblotting of adipocyte lysates

For immunoblotting analysis, isolated subcutaneous adipocytes were diluted in Hank's medium (4% BSA, 0.15 μM adenosine and pH 7.4) with 5.6 mM glucose, and were preincubated with either CsA or tacrolimus (100 nM) for 15 minutes (n = 5) or 20 hours (n = 4–6). Cell lysates were prepared and immunoblotting was performed as described in Supplemental Materials and Methods.

Culture of L6 cells and assessment of 2-deoxy-D-[^{3}H] glucose uptake and plasma membrane GLUT4

L6 cells that stably expresses the GLUT4 protein containing a fourteen amino acid epitope human c-myc, were kindly provided by Dr. A. Klip (The Hospital for Sick Children, Toronto, Ontario, Canada), and were cultured as previously described (21). The L6 cells were maintained in MEM-α (Invitrogen) supplemented with 10% FCS (at 37°C, 5% CO_2). For immunofluorescence analysis cells were grown in Lab Tek II chamber glass slides. For glucose uptake and plasma membrane preparation, L6 cells were differentiated in medium supplemented with 2% FCS into myotubes within 7 days after seeding. The cells were serum-starved for 3–4 hours and were incubated with or without CsA or tacrolimus (100 nM) for 75 minutes, with or without 100 nM insulin (Humulin R, Lilly, Solna, Sweden) during the last 20 minutes. 2-Deoxy-D-[^{3}H] glucose (2-DG) uptake was measured according to a previously validated technique for L6 cells (22). Plasma membrane preparations were performed using a commercial protein extraction kit (abcam, Cambridge, UK), to analyze GLUT4 translocation to the plasma membrane. Equal amount of protein (6–8 μg) were subjected to immunoblotting against anti-GLUT4. Anti-Na^+/K^+ ATPase (Cell Signaling) was used as a plasma membrane fraction marker. See Supplemental Material and Methods for further details.

GLUT4myc internalization (endocytosis) and externalization (exocytosis) in L6 cells

L6 cells expressing GLUT4myc allow reproducible measurements of the cell surface myc-epitope, and they are well-characterized for assessment of GLUT4 traffic (21, 23). GLUT4myc internalization and externalization was measured in L6 myoblasts by an antibody-coupled colorimetric absorbance assay as previously described (23), with some modifications. See Supplemental Material and Methods for further details.

Immunofluorescence microscopy of differentiated human preadipocytes and L6-GLUT4 myc cells

Following incubation, the cells were washed with ice-cold PBS and then fixed in 4% paraformaldehyde/PBS (Histolab Products AB, Gothenburg, Sweden) for 20 minutes. After fixa-

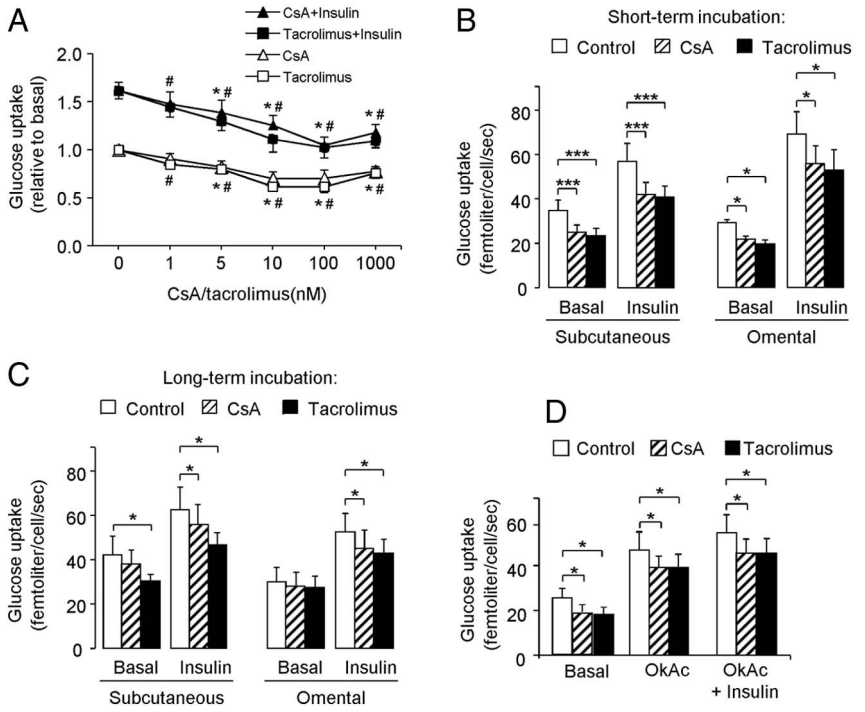

Figure 1. Cyclosporine A (CsA) and tacrolimus reduce glucose uptake in subcutaneous and omental adipocytes. **A:** Freshly isolated human subcutaneous adipocytes (n = 5–9) were preincubated during 15 minutes with or without different concentrations of CsA or tacrolimus (1–1000 nM) and the incubation continued with or without insulin stimulation (6 nM) for an additional 15 minutes, before D-[U-^{14}C]-glucose was added, and glucose uptake was then assessed during the following 45 minutes. *$P < .05$ and #$P < .05$ CsA and tacrolimus treated vs. untreated. **B and C:** Freshly isolated human subcutaneous and omental adipocytes were preincubated short-term (15 minutes) (B) and long-term (20 hours) (C) with CsA or tacrolimus (100 nM) and thereafter glucose uptake was measured as previously explained. (B, n = 17 and n = 8; C, n = 8 and n = 5 for subcutaneous and omental adipocytes, respectively). Total incubation times were 75 minutes (short-term) and 21 hours (long-term). *$P < .05$ and ***$P < .001$ CsA and tacrolimus treated vs. untreated. **D:** Freshly isolated human subcutaneous adipocytes were preincubated during 15 minutes with CsA or tacrolimus (100 nM) and the incubation continued with okadaic acid (1 μM) as well as with or without insulin (6 nM) for an additional 15 minutes, and thereafter as previously reported (n = 5). *$P < .05$ CsA and tacrolimus treated vs. untreated. OkAc, okadaic acid.

tion, part of the L6-GLUT4myc cells were permeabilized with 0.1% Triton X100 (Sigma) in PBS for total cell immunofluorescence and part were left nonpermeabilized for cell surface immunofluorescence. Cells were blocked with 20% FCS in PBS during 30 minutes. Differentiated human preadipocytes were probed with anti-GLUT4 antibody (1:200, antibody recognizing several epitopes in various regions of GLUT4 potentially including extracellular parts, from Millipore Corporation, Darmstadt, Germany), while L6-GLUT4 myc cells were probed with anti-c-myc (diluted 1:100, Sigma). The secondary antibody (Alexa Fluor 594 goat antirabbit IgG, 1:500, Molecular Probes, Eugene, OR, USA), was added for 1 hour. The cells were washed, labeled with DAPI for nuclear staining and mounted in vectashield H-1000 (Vector Laboratories, Burlingame, CA, USA) for immunofluorescence microscopy. Images were processed with NIH ImageJ software (Java-based public version). See Supplemental Material and Methods for further details.

Statistical Analysis

Differences between control and treated conditions in human adipocytes were performed pair-wise using Wilcoxon signed-rank test or paired Student's *t* test, as appropriate. Thus, comparisons between treated and untreated adipocytes were performed pair-wise within the same subject and experiment to minimize confounding variables. Unpaired Student´s *t* test was used for differences between control and treated conditions in L6 cells. Results are given as mean ± SEM, or as indicated. A *P*-value < 0.05 was considered statistically significant. Statistical analyses were performed using the SPSS package version 18 (SPSS Inc., Chicago, IL).

Results

Cyclosporine A (CsA) and tacrolimus inhibit glucose uptake in human adipocytes

Short-term incubation of subcutaneous isolated adipocytes with CsA (5–1000 nM) or tacrolimus (1–1000 nM) significantly decreased basal and insulin-stimulated (6 nM) glucose uptake in a dose depended manner by up to 40% (Figure 1A). The inhibitory effect on insulin-stimulated glucose uptake was similar when a lower concentration of insulin (0.15 nM) was used (data not shown). In omental adipocytes, similar effects were observed after short-term incubation, and both basal and insulin-stimulated (6 nM) glucose uptake were significantly decreased by up to 30% (Figure 1B) upon treatment with either drug. After long-term incubation with CsA or tacrolimus, basal glucose uptake was inhibited by 10 and 20%, respectively, while insulin-stimulated glucose uptake was inhibited by 11 and 25%, in subcutaneous adipocytes (Figure 1C). In omental adipocytes, neither CsA or tacrolimus significantly affected basal glucose uptake, while insulin-stimulated glucose uptake was inhibited by ~15%. Moreover, short-term preincubation with CsA or tacrolimus (100 nM) inhibited glucose uptake stimulated by okadaic acid by ~20% (Figure 1D).

Effects of CsA and tacrolimus on insulin signaling

CsA and tacrolimus did not change total insulin receptor (IR) protein levels after 15 minutes or 20 hours preincubations (Figure 2A and B). Instead, tacrolimus reduced insulin-stimulated IR Tyr1146 phosphorylation at both incubation times by ~30% ($P<0.05$, Figure 2A and

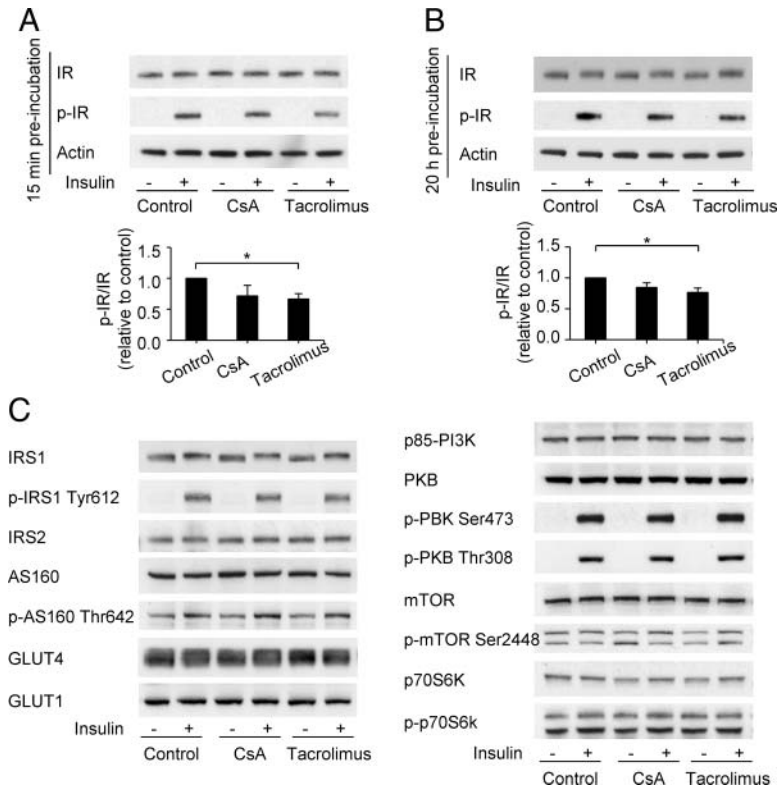

Figure 2. Effects of Cyclosporine A (CsA) and tacrolimus on insulin signaling proteins. Freshly isolated human subcutaneous adipocytes were incubated without (control) or with CsA and tacrolimus (100 nM) for 15 minutes **(A)** or 20 hours **(B-C)**, prior to insulin stimulation (6 nM) for additional 15 minutes. Immunoblotting analysis showed that tacrolimus reduces insulin-stimulated IR Tyr1146 phosphorylation after short- (A) or long-term (B) incubation (n = 4–6). Data are mean ± SEM of densitometry analysis of insulin-stimulated IR Tyr1146 phosphorylation after CsA and tacrolimus treatment compared to untreated adipocytes (set to 1) and normalized to the respective protein levels (p-IR/IR). $*P < .05$ and $**P < .01$ CsA and tacrolimus treated vs. untreated. **(C)** CsA or tacrolimus had no effect on total IRS1/2 and phosphospecific antibody against p-IRS1 Tyr612, total p85 subunit of PI3K, total PKB and phosphospecific antibodies against p-PKB Ser473 and Thr308, total AS160 and phosphospecific antibody against p-AS160 Thr642, total GLUT4, total GLUT1, total mTOR and p70S6K and phosphospecific antibodies against p-mTOR Ser2448 and p-p70S6K Thr421/Ser424. n = 4–6. Results show representative blots. Densitometry analyses are shown in Supplemental Figure 1.

B). In addition, CsA or tacrolimus did not change the protein levels or the insulin-stimulated phosphorylation of insulin receptor substrate (IRS) 1, protein kinase B (PKB), PKB substrate of 160 kDa (AS160), mammalian target of rapamycin (mTOR) or its downstream target p70S6K (Figure 2C and Supplemental Figure 1). Similar results were obtained for IRS2, p85-PI3K, GLUT4 and GLUT1 protein levels.

CsA and tacrolimus reduce the amount of GLUT4 at the cell surface in human adipocytes

Fluorescence labeling with anti-GLUT4 in the plasma membrane of adipocytes is shown in Figure 3. Insulin increased by ~4-fold the amount of GLUT4 at cell surface, compared with basal (Figure 3). Incubation with CsA or tacrolimus (100 nM) significantly reduced the amount of insulin-stimulated GLUT4 at the adipocyte surface by ~60% (Figure 3), and had no effect on basal amount of GLUT4 at cell surface (data not shown).

CsA and tacrolimus reduce insulin-stimulated glucose uptake and plasma membrane GLUT4 levels in L6 cells

Short-term incubation of L6 cells with CsA or tacrolimus (100 nM), significantly reduced insulin-stimulated (100 nM) glucose uptake by ~20% (Figure 4A), but had no effect on basal glucose uptake. Based on the inhibitory effects of CsA and tacrolimus on insulin-stimulated glucose uptake in L6 cells, we further investigate whether the treatment could modulate the levels of GLUT4 at the plasma membrane, measured following fractionation of L6 cells. CsA and tacrolimus significantly reduced the plasma membrane levels of GLUT4 by ~40% (Figure 4B). In addition, nonpermeabilized and permeabilized L6-GLUT4myc cells were exposed to antimyc antibody for immunofluorescence analysis of cell-surface and total GLUT4myc, respectively (Figure 4C and D). In nonpermeabilized L6 cells, a low level of surface GLUT4 was detected in the basal state, and the intensity of the staining increased by 2-fold ($P < .01$) upon insulin stimulation (Figure 4C). Pretreatment of the cells with either CsA or tacrolimus reduced the amount of insulin-stimulated GLUT4myc at the cell surface (Figure 4C), by 20%–30% ($P < .05$). In contrast, insulin did not significantly alter the total GLUT4myc, and neither CsA or tacrolimus had any effect (NS, Figure 4D).

CsA and tacrolimus increase the GLUT4 endocytosis in L6 cells

To further investigate the effects of CsA and tacrolimus on the GLUT4 cycling with the plasma membrane, we studied the effects of CsA and tacrolimus in the GLUT4 exocytosis and endocytosis. GLUT4myc internalizes with similar rate constants for endocytosis in basal and insulin-stimulated conditions (k_{en}= 0.29 and 0.30 minutes^{-1}, respectively, Figure 5A and B). CsA or tacrolimus increased the insulin-stimulated rate of GLUT4myc endocytosis (k_{en}

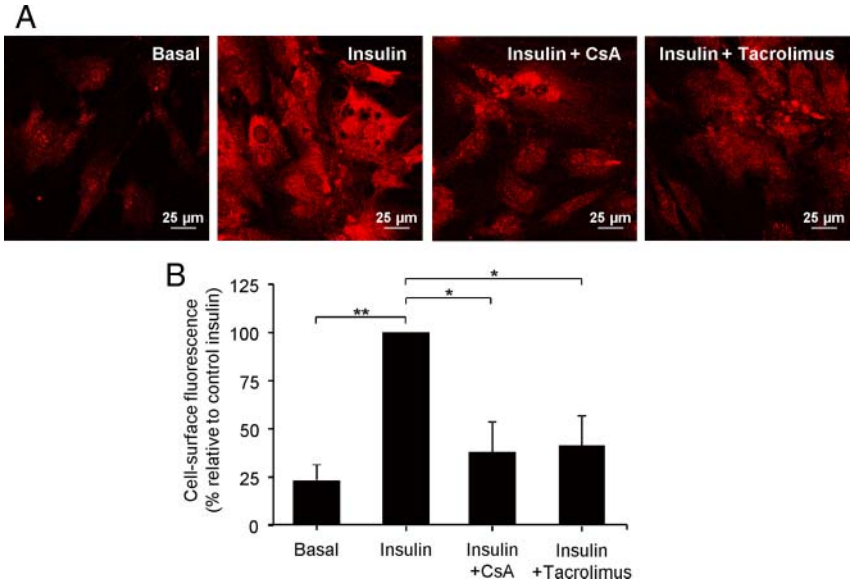

Figure 3. Cyclosporine A (CsA) and tacrolimus inhibit the amount of GLUT4 localized at the adipocyte surface. Human preadipocytes differentiated into adipocytes were serum starved and incubated without or with CsA or tacrolimus (100 nM) during 75 minutes, and insulin (6 nM) was added in the last 20 minutes. Cells were fixed and probed with anti-GLUT4 and with Alexa Fluor 594 goat antirabbit IgG**. A**: Immunofluorescence was detected using laser confocal microscopy. A representative image of 4 separate experiments is presented. Bars represent 25 μm. **B:** The fluorescence intensity (mean pixel density per are unit) was quantified and is represented as % relative to insulin. $*P < .05$ and $**P < .01$.

insulin = 0.30 minutes^{-1}, $k_{en\ insulin+CsA}$ = 0.39 minutes^{-1} and $k_{en\ insulin+tacrolimus}$ = 0.44 minutes^{-1}, Figure 5B), but had modest effect on the basal endocytosis ($k_{en\ basal}$ = 0.29 minutes^{-1}, $k_{en\ CsA}$ = 0.31 minutes^{-1} and $k_{en\ tacrolimus}$ = 0.33 minutes^{-1}, Figure 5A). In addition, CsA and tacrolimus, significantly decreased the fraction of cell surface GLUT4myc remaining at the cell surface by up to 30%, after 10 or 20 minutes incubations and in insulin-stimulated conditions ($Y_{insulin} \sim 0.30$, $Y_{CsA+insulin} \sim 0.19$ and $Y_{tacrolimus+insulin} \sim 0.21$, Figure 5B). Insulin treatment increased by ~ 2-fold the GLUT4myc exocytosis, but neither CsA or tacrolimus effect the basal or insulin-stimulated GLUT4myc exocytosis (Figure 5C).

Discussion

In this study we demonstrate that CsA and tacrolimus, at therapeutic concentrations commonly used in clinic, inhibited glucose uptake in human subcutaneous and omental adipocytes and in L6 muscle cells. This appears to be independent of the insulin signaling cascade, and removal of GLUT4 from the cell surface via increased rate for endocytosis may be a novel mechanism for the diabetogenic effects of the calcineurin inhibitors. These mechanism clearly differ from that of another class of immunosuppressive agents, namely mTOR inhibitors, and we recently reported major alterations in insulin signaling and action in human adipocytes exposed to rapamycin (17).

Most the studies that have examined the diabetogenic effects of CsA and tacrolimus, indicate that these calcineurin inhibitors inhibit pancreatic insulin synthesis and secretion (6), but a reduction in peripheral insulin sensitivity has also been suggested (9, 10). The reduction of glucose uptake in human adipocytes after incubation with either CsA or tacrolimus, as well as in L6 cells, suggests that these drugs may contribute to the development of NODAT by inhibiting glucose uptake in insulin-sensitive cells. Furthermore, using immunofluorescence we were able to show that CsA and tacrolimus significantly contribute to reduced insulin-stimulated presence of GLUT4 in the plasma membrane of differentiated human preadipocytes and L6 cells. Due to the challenge to obtain enough adipocytes to isolate the plasma membrane, we decided to further explore the effects of both drugs in the presence of GLUT4 in the plasma membrane in a cell line, the L6 cells. Plasma membrane isolated from L6 cells further confirmed that both drugs significantly contribute to reduced insulin-stimulated presence of GLUT4 in the plasma membrane. This data gives new insight to the mechanism by which calcineurin inhibitors impair glucose handling in peripheral tissues.

Results from several randomized clinical trials and meta-analyses, have suggested that tacrolimus is more diabetogenic than CsA (24, 25), although this has not been confirmed by others (26). In our study, however, the dose response relationships with respect to impairment of glucose uptake were similar for the two drugs. These effects of CsA and tacrolimus could be detected at low concentrations (1–5 nM) that were even below the recommended therapeutic ranges for both CsA (40–80 nM) and tacrolimus (6–12 nM) (27) and with similar dose-response effects.

In addition, we show that CsA and tacrolimus inhibit glucose uptake stimulated by okadaic acid. Okadaic acid, a protein phosphatases type 1 (PP1) and 2A (PP2A) inhibitor, stimulates adipocyte PKB kinase activity and glucose transport independent of PI3K activation (28). These observations support that CsA and tacrolimus mediate their effects independent or downstream of PI3K activation.

A reduction in IR Tyr1146 activation by ~30% was found in adipocytes incubated with tacrolimus, when

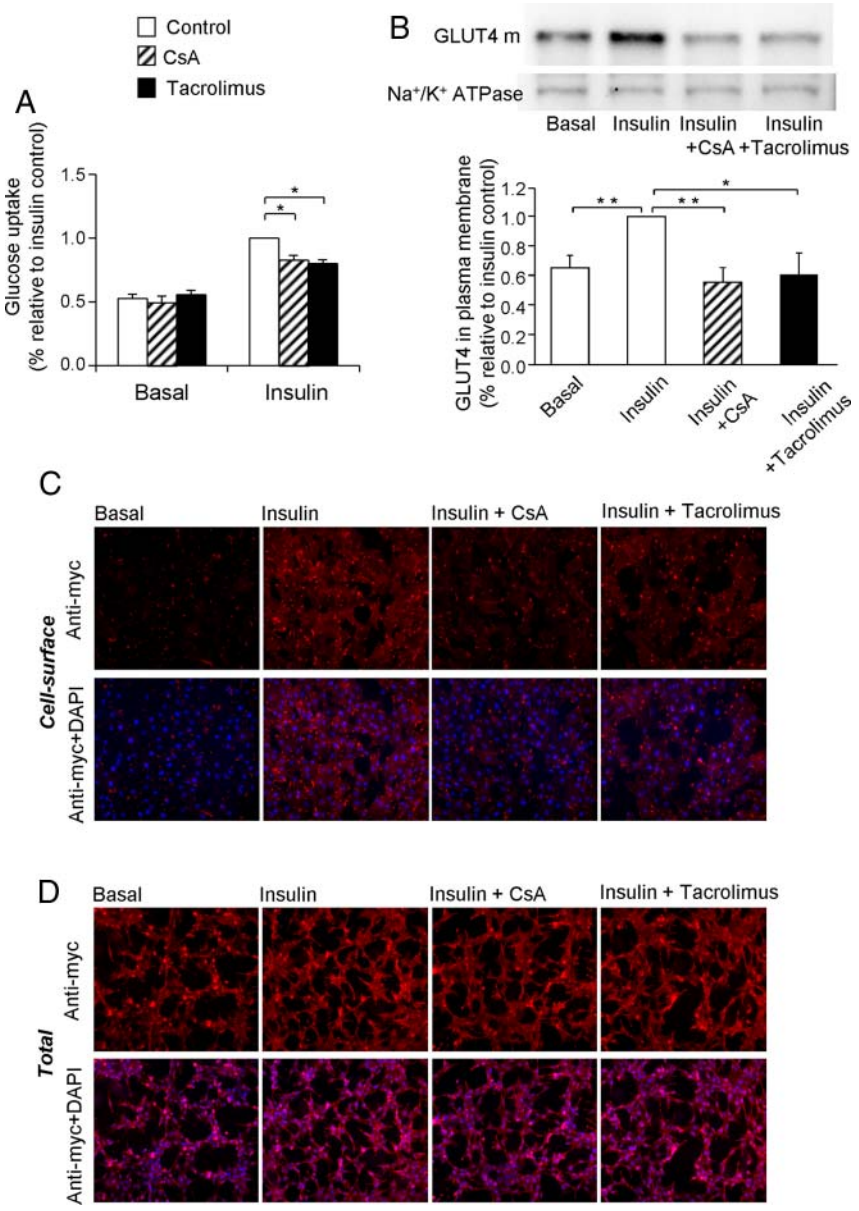

Figure 4. Cyclosporine A (CsA) and tacrolimus reduce glucose uptake and the amount of GLUT4 at the plasma membrane in L6 cells. L6 cells that stably express GLUT4myc were preincubated in the presence or absence of CsA or tacrolimus (100 nM) for 75 minutes, and 100 nM insulin was added in the last 20 minutes. Cell were used to **(A)** measure 2-deoxy-D-[^{3}H] glucose uptake (n = 8) and **(B)** changes in plasma membrane GLUT4 protein levels (n = 6). A representative western-blot is shown. The blots were quantified and the histogram shows the densitometry analysis of the Western-blots. Na^+/K^+ ATPase was used as a plasma membrane fraction marker. Data are presented as mean ± SEM and they are expressed as % relative to insulin. *$P < .05$, ** $P < .01$. **(C-D)** L6 cells were processed for immunofluorescence analysis with antimyc antibody (red) at the cell-surface in nonpermeabilized L6-GLUT4myc cells (n = 5, **C**) and for total myc in permeabilized L6-GLUT4myc cells (n = 3, **D**). Cell nuclear DNA was stained with DAPI (blue). Immunofluorescence was detected using fluorescence microscopy. The cell-surface myc fluorescence intensity was quantified and normalized to the respective cell number and calculated as % relative to insulin-stimulated cells (set to 1). Basal: 0.54 ($P < .01$), Insulin+CsA: 0.81 ($P < .05$), Insulin+tacrolimus: 0.75 ($P < .01$) compared to insulin alone.

compared with untreated adipocytes. However, the concentration of insulin required to maximally activate glucose transport elicits only about 15% of the maximal receptor kinase activity (29), compatible with the spare receptor concept (30). Indeed, the observed inhibitory effect on the phosphorylation of the IR, was not associated with any change in expression or phosphorylation of the proximal insulin signaling cascade proteins (IRS1/2, p85-PI3K, PKB, mTOR, p70S6K), or GLUT4 and GLUT1 protein levels. Taken together, these data indicate that the inhibitory effects of CsA and tacrolimus on glucose uptake are not mediated through effects on the early steps of the insulin signaling cascade. Therefore we propose that the reduced insulin-stimulated glucose uptake observed in human adipocytes treated with either CsA or tacrolimus could be due to effects on the intracellular trafficking of GLUT4, independent of the insulin signaling machinery. We also demonstrated that in L6 cells, CsA and tacrolimus increased the insulin-stimulated rate of GLUT4 endocytosis, but they had no effect on the exocytosis trafficking. We believe that this mechanism may be similar in human adipocytes, as CsA and tacrolimus also reduced the amount of GLUT4 at the cell surface in these cells. However, different effects of CsA and tacrolimus on basal glucose uptake in human adipocytes and L6 cells could be due to the different cell models, or differences in the GLUT4 internalization in these cells (31). Chronic insulin treatment has been reported to reduce glucose transport and to double the rate constants for glucose endocytosis in rat adipocytes (32). These effects are ameliorated by inclusion of metformin in the culture medium, and this might be mediated by an AMPK-dependent reduction in endocytosis (32, 33). In addition, metformin was shown to improve immunosuppressive agents-induced hyperglycemia in a rodent model (34). Whether metformin can prevent the CsA and tacrolimus effects on glucose uptake in human adipocytes should be addressed in future studies. It could thus be hypothesized that targeting this endocytic pathway with metformin or with novel pharmacological

approaches may be a way to increase cell-surface GLUT4. Since transmembrane glucose transport is considered to be rate-limiting for glucose disposal in diabetes, such a treatment could mitigate impaired glucose metabolism caused by calcineurin inhibitors and, moreover, it could potentially improve glycaemic control in other types of diabetes, including type 2.

The question remains, about how CsA and tacrolimus increase the rate of GLUT4 endocytosis. The effect of CsA and tacrolimus on other proteins involved in docking, fusion and endocytosis of GLUT4 vesicles (eg, adaptor protein 2, transferrin receptor) should be further studied, since changes in the protein composition of GLUT4 vesicles may alter their subcellular distribution (35). Another intracellular mechanism required for insulin-mediated GLUT4 trafficking is dynamic remodelling of actin filaments (36). Interestingly, calcineurin regulates actin dynamics in renal podocytes (37), and mice lacking calcineurin Aβ have reduced insulin-stimulated glucose uptake and develop insulin resistance (38). Calcineurin is a serine phosphatase, known to be involved in the regulation of phosphodiesterases and consequently in cAMP degradation (38). Therefore, it has a potential role in hormonal signaling and regulation of metabolism. It is also attempting to speculate that calcineurin inhibition could affect cytoskeleton function and thus alter vesicle trafficking. The significance of calcineurin GLUT4 trafficking may be addressed by silencing calcineurin expression in human cells in future experiments.

Limitations of our study include its descriptive nature. Moreover, the same subjects were not used for all analysis and different cell types (primary human adipocytes, differentiated humans preadipocytes and L6 cells) were used for experiments on cellular GLUT4 localization and trafficking. In addition, the in vitro models do not include the complex cross-talk between tissues occurring in the regulation of glucose metabolism in vivo (16). Some in vivo results supporting our present findings come from recent rat studies where CsA (39, 40) or tacrolimus treatment (7) caused glucose intolerance and inhibited adipocyte glu-

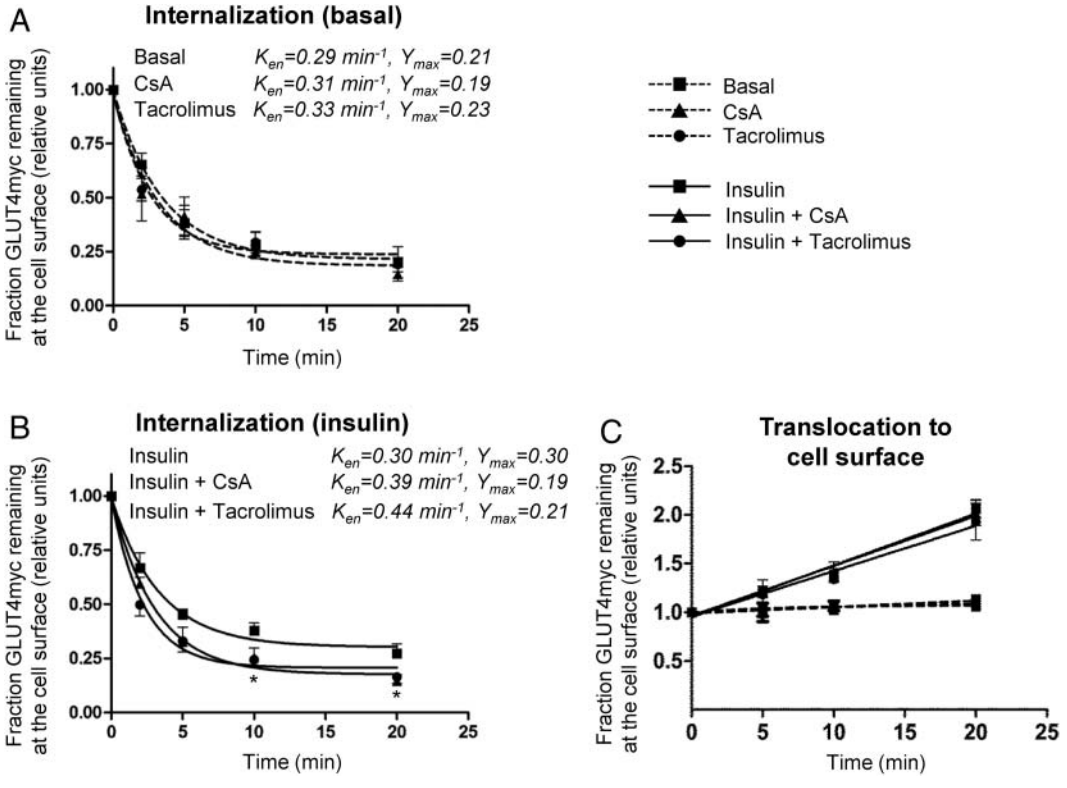

Figure 5. Cyclosporine A (CsA) and tacrolimus increase insulin-stimulated endocytosis of GLUT4myc in L6 cells, and have little effect on the exocytosis. Confluent L6-GLUT4myc cells were incubated in the absence or presence of 100 nM insulin at 37°C for 30 minutes and thereafter with antimyc antibody for 1 hour to label cell surface GLUT4myc. To measure the time course of internalization, cells were then rewarmed to allow endocytosis in the absence **(A)** or presence of 100 nM insulin **(B)** and CsA or tacrolimus (100 nM) for 2–20 minutes. At the indicated times cells were put on ice and the myc antibody-labeled GLUT4myc remaining on the surface was measured with the optical densitometry detection assay. **C:** To measure the time course of stimulation, the cells were rewarmed to allow endocytosis in absence of insulin during 30 minutes. Cells were then incubated in absence or presence of insulin (100 nM) and CsA or tacrolimus (100 nM) for 5, 10 or 20 minutes. Cells were placed on ice again and the Myc antibody-labeled GLUT4myc remaining on the surface was analyzed by the OPD optical densitometry detection assay. The amount of GLUT4myc remaining at the cell surface at any time point is expressed as a percentage of the cell surface GLUT4myc level at 0 minutes of incubation. Data points are mean ± SEM of 4–5 separate experiments each performed in duplicates. *Line*, single exponential fits. Rate constants for endocytosis (k_{en}) were calculated from single exponential fits. *$P < .05$ CsA or tacrolimus and insulin-treated vs. insulin.

cose uptake, without affecting insulin-stimulated phosphorylation of key insulin signaling proteins.

In conclusion, we have demonstrated that the calcineurin inhibitors CsA and tacrolimus, at therapeutic concentrations, impair glucose uptake in human subcutaneous and omental adipocytes and in L6 muscle cells. The effects appear to be independent of the insulin signaling cascade. Our data in L6 cells also suggest that CsA and tacrolimus reduce amount of GLUT4 at the plasma membrane in the presence of insulin and this process is mediated via enhanced rate of GLUT4 endocytosis. That may contribute to impaired glucose handling in peripheral tissues, and diabetes occurring in organ-transplanted patients. Conversely, these findings could potentially point to novel pharmacological mechanisms to inhibit GLUT4 endocytosis and increase its cell surface availability. Such a treatment approach would enhance cellular glucose uptake and improve glycaemic control in diabetes caused by calcineurin inhibitor therapy. Potentially, it may also be effective in other forms of diabetes.

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