The long-term effects of anti-retroviral protease inhibitors on sugar transport in L6 cells

R J Germinario1,2,3,4, S P Colby-Germinario1, C Cammalleri1,2 and M A Wainberg1,3,4

1Lady Davis Institute for Medical Research, SMBD Jewish General Hospital, 3755 Cote St Catherine Road, Montreal, Quebec, Canada
2Concordia University, Department of Biology, Montreal, Quebec, Canada
3McGill AIDS Center, Montreal, Quebec, Canada
4McGill University Department of Medicine, Montreal, Quebec, Canada

(Requests for offprints should be addressed to R J Germinario, McGill AIDS Center, Lady Davis Institute for Medical Research, 3755 Cote St Catherine Road, Montreal, Quebec, Canada H3T 1E2; Email: ralph.germinario@mcgill.ca)

Abstract

The objective of this study was to investigate the long-term effects of anti-retroviral protease inhibitors (PIs) on 2-deoxy-d-glucose (2-DG) transport in L6 cells in vitro. Exposure of L6 cells to saquinavir, ritonavir, indinavir and amprenavir resulted in significant increases in 2-DG transport using PI concentrations of 1–10 µM with continual exposure to PI. After removal of the PI for up to 48 h, 2-DG transport increases did not change and remained at pre-reversal levels. These changes in 2-DG transport were not related to stress-induced sugar transport or to apoptosis. The examination of glucose transporter (GLUT) 1, 3 or 4 translocation with subcellular fractionation indicated that insulin (i.e. 67 nM) could induce the translocation of all the GLUTs to the plasma membrane. Also, ritonavir (10 µM), which leads to a 2-fold increase in 2-DG transport, demonstrated increased GLUT (i.e. 1, 3 or 4) presence in the plasma membrane fraction, in the presence or absence of insulin. This increased 2-DG transport involved transporter presence in plasma membrane preparations and did not affect the ability of insulin to stimulate 2-DG transport with continual PI exposure. The mechanism(s) involved indicates ready reversibility of PI effects on transporters. The mechanism(s) why reversibility of PI-induced 2-DG transport was similar plus or minus PI was not apparent.


Introduction

Protease inhibitors (PIs) exhibit antiviral activity in both primary lymphoid, monocyte cell lines and against a variety of viral strains including clinical isolates with zidovudine resistance (Martin et al. 1995, Winslow & Otto 1995). The PIs are active as administered and a variety of PI with one or more nucleoside reverse transcriptase inhibitors has proven most effective in treating AIDS patients and maintaining low viral loads (Flexner 1998). While this combined anti-retroviral therapy (i.e. highly active anti-retroviral therapy or HAART) has represented a significant advance in the treatments of HIV infection, side-effects of different degrees of severity have been reported such as fat wasting, insulin resistance, central adiposity, hypertriglyceridemia and hyperglycemia (Hammer et al. 1997, Mouton et al. 1997, Carr et al. 1998, 2000, Lo et al. 1998, Virabin & Aquilina 1998, Gervasoni et al. 1999, Mulligan et al. 2000). Further, the removal of these drugs can reverse the metabolic disturbances; however, such an action results in a dramatic reappearance of circulating virus in the bloodstream after up to 2 years of combination drug treatment. Thus, the patient must remain on drug therapy.

Significantly, the cause(s) of these side-effects are unknown but they exhibit a remarkable similarity to the characteristics of metabolic syndrome X (Reaven 1993). Of considerable interest is that all of these changes have been shown to be associated with an increased risk of cardiovascular disease or diabetes (Reaven 1999). Insulin resistance appears to be a major player in the lipodystrophic effects seen in HAART patients. Recent reports have indicated that PIs can decrease the intrinsic activity of the glucose transporter 4 (GLUT 4) (Murata et al. 2000) and cause insulin resistance after short-term PI exposure (Nolte et al. 2001, Hruz et al. 2002) in rat muscle. In the latter two studies, transient insulin resistance was found to be induced by PIs. It was suggested that this modulation of GLUT 4 may relate to the insulin resistance observed with HAART AIDS patients.

In the study described herein, we have investigated the long-term effects of PI on basal and insulin-stimulated
sugar transport in L6 cells (i.e. a representative culture system of muscle). This study was undertaken to further characterize the mechanism(s) involved in the modulation of 2-DG transport by PIs in a tissue known for ~90% insulin-stimulated glucose disposal (DeFronzo et al. 1981, Baron et al. 1994).

Materials and Methods

L6 myoblasts

Myoblasts were grown in Dulbecco’s minimum essential medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) (Germinario et al. 1978, Bilan et al. 1992). The cells were grown at 37 °C in an atmosphere of 5% CO₂/95% air. Cells were harvested from appropriate culture vessels after incubation for 5 min with 0·02% (w/v) EDTA and 1–2 min with 0·06% (w/v) trypsin (Difco Laboratories, Oakville, Ontario, Canada) at room temperature. When the monolayers became confluent, the 10% FBS–DMEM was removed and replaced with an equal volume of fusion medium (α-MEM containing 2% FBS (v/v)). All experiments were done 4–5 days later when >85% of the control cells were differentiated as assessed visually through morphological changes. Both 10% FBS–DMEM and 2% α-MEM were supplemented with a 100 × penicillin/streptomycin antibiotic solution. L6 cells were recultured every 3 days at about 75% confluence using a split ratio of 1:3 or more.

Insulin-stimulation protocol

Generally, the monolayers of cells were serum deprived for 18 h in serum-free DMEM with 1 mg/ml BSA (0% DMEM). L6 cells were grown to the appropriate stage of differentiation and serum deprived. To determine sugar transport, 2-deoxy-D-[3H]glucose (2-DG) was employed at a concentration of 0·05 mM (specific activity 0·023 µCi/nmol) and 2-DG uptake at linear and rate-limiting conditions were employed as described previously (Germinario et al. 1978, 1989, 1993, Bilan et al. 1992, Pratt et al. 1994, Pratt & Germinario 1994, Tao et al. 1995).

Sugar transport procedures

Details of the experimental protocol employed can be found elsewhere (Germinario et al. 1978, 1989, 1993, Pratt & Germinario 1994, Pratt et al. 1994, Tao et al. 1995). Briefly, cells were plated at 10⁶ cells/cm² on plastic Petri dishes (35 mm diameter; Falcon, Oakville, Ontario, Canada) and grown as described. The cell monolayers were rinsed once in 0% MEM and incubated for 1 h with insulin. Hexose transport was assessed by measuring the uptake of 2-DG in PBS (pH 7·4) containing 0·05 mM 2-DG at 37 °C. There was no PI present in any uptake medium. The radioactive medium was removed and the monolayers were rinsed four times with 4 °C PBS. Since the PIs were dissolved in a dilute solution of DMSO, additional controls were employed to determine the effects of DMSO on 2-DG transport. No differences were seen between 2-DG transport with or without DMSO up to a concentration of 0·5% DMSO. The 2-DG transport in the DMSO test group exhibited an average of 102·8 ± 4% of the 2-DG transport in the non-DMSO group (paired t-test, P > 0·05 (n=24). All control groups are DMSO-containing controls. Further, the use of 10 µM cytochalasin B, a specific inhibitor of carrier-mediated sugar transport, indicated that the increases seen in 2-DG transport were carrier-mediated as the 2-DG transport for all groups employed remained ~15% of the total 2-DG transport in the presence of 10 µM cytochalasin B (data not shown). The cell monolayers were dissolved in 1 M NaOH and aliquots were taken for liquid scintillation counting and protein determination (Lowry et al. 1951).

Cell fractionation and membrane determination

L6 cells from 100 mm diameter Petri plates (Falcon) were treated and plasma membranes prepared and isolated as previously described (Tao et al. 1995). In short, after homogenization with a Dounce homogenizer, a 1000 g centrifugation to remove unbroken cells and nuclei was done. This was followed by a 27 000 g centrifugation yielding the crude membrane pellet from one to three plates which exhibited a 3-fold enrichment of ouabain-sensitive Na⁺/K⁺ ATPase vs the cell homogenate (data not shown).

Western analysis procedure

Whole-cell monolayers or membrane fractions were suspended in 0·5 ml PBS with a proteinase inhibitor (phenylmethylsulfonyl fluoride) at a final concentration of 1 mM. Prior to electrophoresis samples were solubilized in 2% SDS (Pierce Chemical Co., Rockford, IL, USA), 10% glycerol, 1 mM EDTA, 0·002% bromophenol blue, 100 mM Tris–HCl (pH 6·8) (Germinario et al. 1993). Electrophoresis was performed as described previously (Germinario et al. 1993, Pratt & Germinario 1994, Pratt et al. 1994). All lanes contained 40 µg/lane of cell protein. Purified erythrocyte GLUT was prepared as described elsewhere (Baldwin et al. 1982). A rabbit polyclonal antibody to the erythrocyte GLUT 1 was prepared using the purified transporter obtained from the preceding procedure. This antibody has been characterized as to its reactivity to the GLUT 1 (Germinario et al. 1978, 1993, Pratt & Germinario 1994, Pratt et al. 1994, Tao et al. 1995). Additionally, membranes were probed with a rabbit anti-mouse GLUT 3 (Alpha Research Diagnostics, San Antonio, TX, USA) or GLUT 4.
Antibody. Briefly, Western blot analysis was carried out as follows: (i) samples were solubilized in Laemmli buffer (Laemmli 1970) and electrophoresis was performed on 10% polyacrylamide slab gels under non-reducing conditions (Andrejchyshyn et al. 1991, Germinario et al. 1993, Pratt & Germinario 1994, Pratt et al. 1994, Tao et al. 1995); (ii) proteins were blotted onto nitrocellulose paper that was blocked with 3% BSA in PBS/0.5% Tween 20 (1 h at 20 °C); (iii) this was followed by incubation with specific antibody overnight at 4 °C; and (iv) detection of GLUT 1, 3 or 4 was performed by enhanced chemiluminescence (ECL kit; Amersham Life Sciences) using horseradish-peroxidase anti-rabbit as a secondary antibody for 1 h at 20 °C. The blot was exposed to XAR film (Eastman Kodak, Rochester, NY, USA). The film was scanned and analyzed using UN SCAN IT gel software (Silk Science, Orem, UT, USA).

Materials

Tritium-labeled 2-DG was purchased from ICN Radiochemical Division (Irvine, CA, USA). 2-DG was purchased from Calbiochem (La Jolla, CA, USA); BSA from Nutritional Biochemicals (Walnut, CA, USA) and phenylmethylsulfonyl fluoride from Sigma. Our thanks go to Hoffman LaRoche for the gift of the PI saquinavir, to Merck for indinavir, to Abbott Pharmaceuticals for ritonavir and to Glaxo Wellcome Inc. for amprenavir.

Statistics

The level of significance chosen for any analysis employed herein was 5%. The tests employed were one-way ANOVAs or two-tailed paired t-tests. All data are ± S.E.M.

Results

We studied the effects of various PIs in L6 cells on 2-DG transport over a range of PI concentrations (Fig. 1A–C). In Fig. 1A, the data showed an increase in 2-DG transport by saquinavir over the range 1–10 µM. The transport increases were >2-fold at all PI concentrations (P < 0.05, one-way ANOVA, n = 3). In Fig. 1B, the indinavir-induced increases seen in 2-DG transport were significantly higher at 10 µM (P < 0.05, one-way ANOVA, n = 3). The difference was on average 1.8-fold greater at

Figure 1 The effect of increasing concentrations of saquinavir, indinavir and ritonavir on basal 2-DG transport in L6 cells. Details as in Materials and Methods. (A) Saquinavir, one-way ANOVA, *P < 0.05, n = 3; triplicate plates in all experiments. Data ± S.E.M. (B) Indinavir, one-way ANOVA, *P < 0.05, n = 3; triplicate plates in all experiments. Data ± S.E.M. (C) Ritonavir, one-way ANOVA, *P < 0.05; n = 3; triplicate determinations in all experiments. Data ± S.E.M.
the highest concentration tested. For ritonavir, similar results were obtained (Fig. 1C) as seen for 10 µM ritonavir, the increase seen being 1.6-fold vs control. Again the results were obtained (Fig. 1C) as seen for 10 µM ritonavir, the highest concentration tested. For ritonavir, similar increases in 2-DG transport seen in L6 cells after PI exposure were not readily reversible. The data in Table 1 for amprenavir-exposed 1.47±1.00. 2-DG transport in all insulin-exposed groups was significantly different from basal (P<0.05, one-way ANOVA, n=4).

To determine the mechanism(s) of the 2-DG transport increase in the L6 cells (Fig. 2D), we examined the translocation of the GLUT 1, 3 and 4 into the plasma membrane fraction of L6 cells in the presence and absence of 67 nM insulin and/or 10 µM ritonavir (Fig. 2A–C). In Fig. 2A, Western blots of the plasma membrane fraction for GLUT 1 showed the increase in GLUT 1 into the plasma membrane of insulin-, ritonavir-, and ritonavir and insulin-exposed cells. In short, insulin induced an average 1.5-fold increase in 2-DG transport (Fig. 2D). In the presence of 10 µM ritonavir, a nearly 2-fold increase in 2-DG transport was observed (Fig. 2D), while increases in band density were observed for the GLUT 1, GLUT 3 (Fig. 2B) or GLUT 4 (the lowest being GLUT 4)

![Figure 2](https://via.placeholder.com/150)

**Figure 2** The effect of insulin, ritonavir and insulin plus ritonavir on 2-DG transport and glucose transporter translocation into a plasma membrane fraction derived from L6 cells. Details as in Materials and Methods. (A) An example of a Western blot probing for GLUT 1. The groups probed were no insulin control, +67 nM insulin (+Ins) or 10 µM ritonavir (Rit) and insulin and ritonavir (Rit+Ins). One-way ANOVA, n=5. Data ± S.E.M. *P<0.05. (B) An example of a Western blot probing for GLUT 3. The groups were as described in (A). One-way ANOVA, n=5 or 6. Data ± S.E.M. *P<0.05. (C) An example of Western blot probing for GLUT 4. The groups were as described in (A). One-way ANOVA, n=5 or 6. Data ± S.E.M. *P<0.05. (D) The effects of the groups employed on 2-DG transport. One-way ANOVA, n=8, triplicate plates in all experiments. Data ± S.E.M. *P<0.05.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Basal</th>
<th>With insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.338 ± 0.058</td>
<td>0.733 ± 0.117*</td>
</tr>
<tr>
<td>Saquinavir</td>
<td>0.705 ± 0.132b</td>
<td>1.132 ± 0.147*</td>
</tr>
<tr>
<td>Indinavir</td>
<td>0.617 ± 0.098b</td>
<td>1.17 ± 0.294*</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>0.441 ± 0.1b</td>
<td>0.765 ± 0.132*</td>
</tr>
<tr>
<td>Amprenavir</td>
<td>0.76 ± 0.17b</td>
<td>1.12 ± 0.22a</td>
</tr>
</tbody>
</table>

Table 2 Effects of insulin on PI-exposed L6 cells 2-DG transport (nmole 2-DG/mg protein/5 min). All data ± S.E.M.

Table 1 The transport of 2-DG after removal of PIs from the L6 cells for 24 and 48 h. All data ± S.E.M.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>2-DG transport (nmole/mg protein/5 min)</th>
<th>2-DG transport ratio after reversalb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ritonavir (10 µM)</td>
<td>0.217 ± 0.002</td>
<td>1.06 ± 0.003</td>
</tr>
<tr>
<td>Saquinavir (10 µM)</td>
<td>0.334 ± 0.004*</td>
<td>0.97 ± 0.007</td>
</tr>
<tr>
<td>Indinavir (10 µM)</td>
<td>0.281 ± 0.001*</td>
<td>0.98 ± 0.005</td>
</tr>
<tr>
<td>Ritonavir (10 µM)</td>
<td>0.36 ± 0.01*</td>
<td>0.92 ± 0.005</td>
</tr>
</tbody>
</table>

Table 1 The transport of 2-DG after removal of PIs from the L6 cells for 24 and 48 h. All data ± S.E.M.

aTreatment with drugs was at plating (3–4 days) and during 4 days of induction.

bReversal represents time after removal of drugs. Control represents 2-DG transport during reversal time. The ratios represent 2-DG transport in reversed divided by the drug-exposed control.

The effect of insulin, ritonavir and insulin plus ritonavir on 2-DG transport and glucose transporter translocation into a plasma membrane fraction derived from L6 cells. Details as in Materials and Methods. Saquinavir, indinavir, ritonavir and amprenavir were present throughout.

Saquinavir, indinavir, ritonavir and amprenavir represent 10 µM of each PI. L6 cells were serum deprived overnight and then exposed to 67 nM insulin for 60 min prior to determination of 2-DG transport. All data ± S.E.M.
Anti-retroviral protease inhibitors and sugar transport · R J GERMINARIO and others

A

GLUT 1

B

GLUT 3

C

GLUT 4

D

3H-2DG Transport

nmol/mg/5min

Control

Insulin

Ritonavir

Ritonavir + insulin

Treatment
(Fig. 2C). This was seen for the plasma membranes of the GLUT 4 ritonavir plus insulin group. This was suggestive of some effect on the insulin group vs the insulin plus ritonavir group, although the differences were not significant \((P>0.05,\) one-way ANOVA). Regarding the magnitude of the changes seen in 2-DG transport, a number of studies showing the same magnitude of changes in 2-DG transport have demonstrated differences in band densities for GLUT 1, 3 and 4 (Andrejchyshyn et al. 1991, Germinario et al. 1993, Pratt & Germinario 1994, Pratt et al. 1994, Tao et al. 1995). In PI-treated cells (i.e. ritonavir or indinavir) no differences in whole-cell transporter content (i.e. for the GLUT 1, 3 and 4) were observed (data not shown).

**Discussion**

The data presented herein indicate that long-term PIs have a considerable effect on 2-DG transport in L6 cells. The increased 2-DG transport rates were seen over a range of concentrations including physiological levels, i.e. known to occur in vivo \((1–10\,\mu\text{M})\). Further, the myotube system in the presence of insulin exhibited increased 2-DG transport in vitro. The L6 myotube system is a muscle-like cell line and hence it is a reflection of effects of PIs on muscle. The 3T3-L1 adipocyte reflects the fat cell and does not dispose of large amounts of glucose in vivo as the muscle does. The 3T3-L1 adipocyte, however, did not exhibit increased insulin-stimulated 2-DG transport after short-term exposure to PI and showed decreased basal 2-DG transport (Murata et al. 2000). Controversy does exist as others (Ranganathan & Kern 2002) have shown increased basal 2-DG transport in 3T3-L1 adipocytes in the presence of indinavir and saquinavir. Also, they showed little or no effect of PI on insulin-stimulated 2-DG transport. An important consideration here is that the 2-DG uptake medium employed in our study lacked the presence of PI. Further the PI effect on 2-DG transport is readily reversible (Nolte et al. 2001, Hruz et al. 2002). It must be noted that on 3T3-L1 cells, the conditions used in the cited study (Murata et al. 2000) employed considerably higher PI concentrations \((20–100\,\mu\text{M})\) that those employed herein on L6 cells \((1–10\,\mu\text{M})\), and more importantly shorter exposure times \((6\text{ min to }4\text{ h vs }6–8\text{ days in the present study})\). It is of interest that the increased 2-DG transport seen in L6 cells is different from in vitro studies on muscle contraction (Nolte et al. 2001) and PI-induced decreases on muscle uptake (Nolte et al. 2001, Hruz et al. 2002). In the latter studies cited, PI exposure times again were short \((<2\text{ h to }4\text{ h})\) and readily reversible. In our studies PI exposure was for several days during muscle differentiation at physiological concentrations of PI so the results are not directly comparable. This is indicative of another mechanism being involved. In the former studies indinavir appears to be interacting with the transporter directly negatively affecting GLUT 4 intrinsic activity. In our study, long-term exposure to PI did not affect glucose transport intrinsic activity (Joost et al. 1986, Calderhead & Lienhard 1988, Clancey & Czech 1990, Clancey et al. 1991, Harrison et al. 1991, Germinario et al. 1992, Pratt et al. 1994, Murata et al. 2000, Pessin & Saltiel 2000) perhaps related to the lack of reversibility of the PI effect. Some factors have been shown to increase intrinsic activity (Joost et al. 1986, Calderhead & Lienhard 1988, Clancey & Czech 1990, Clancey et al. 1991, Harrison et al. 1991, Germinario et al. 1992) while others decrease the intrinsic activity of GLUTs (e.g. GLUT 4) (Pratt et al. 1994, Murata et al. 2000). Included in the latter group are PI effects in 3T3-L1 adipocytes (Murata et al. 2000).

Interestingly, we show increased 2-DG transport with changes in membrane-located GLUTs in L6 cells after PI exposure. One could speculate that other factors increase 2-DG transport (Hayashi 2000). For example, the increase might be indicative of PI-induced metabolic stress, which can lead to increased 2-DG transport. We have examined this and we have found no differences in total cellular GLUT 1 (an indicator of metabolic stress) (Hayashi et al. 2000), GLUT 3 or GLUT 4 in the presence or absence of ritonavir or indinavir (data not shown). Thus, this stress-associated gene (i.e. glucose transport) is not affected (Hayashi et al. 2000). Additionally, the L6 myotube is resistant to apoptosis as PI exposure and high concentrations of atinomycin D do not induce apoptosis in myotubes, using activation of caspase 3 as an indicator of such (data not shown). It is entirely possible that other intracellular signaling molecules are perturbed after exposure to PIs. Some investigators have demonstrated no effects on insulin receptor substrate-1, phosphatidylinositol 3-kinase or protein kinase B (PKB) (Murata et al. 2000) in 3T3-L1 adipocytes after PI treatment. Others have shown that nelfinavir decreases PKB phosphorylation by insulin (Rudich et al. 2001). To date no studies have been done with long-term PI in muscle in vivo either in the basal state or after insulin stimulation. The experimental protocols of the preceding study (Murata et al. 2000) were quite short-term compared with ours \((e.g. 6\text{ min to }4\text{ h exposure vs }6\text{ days in our study})\) and the PI was present in the uptake medium. Regarding the non-reversibility of PI-induced sugar transport, the possible involvement of transport regulatory proteins such as the atypical PKCs (\(\alpha, \beta, \gamma\)) in this system are of considerable interest (Bandyopadhyay et al. 1997, Standaert et al. 1999). Recently, several investigators have indicated the involvement of PKB in insulin resistance and diabetic complications (Ishii et al. 1996, Bossemmaier et al. 1997). The results observed herein indicated that 2-DG transport is increased by continual exposure to PI in L6 cells. Interestingly, PKC \(\beta_1\) knockout mice have increased basal sugar transport, which led the investigators to conclude that this form of PKC may be a negative regulator of sugar transport.
transport (Standaert et al. 1999). It may be that PKC β2 is affected by PI, which would affect basal sugar transport. The complexities of sugar transport regulation are well established and the involvement of the atypical PKCs (Ishii et al. 1996, Banyopadhyay et al. 1997, Bosenmaier et al. 1997, Standaert et al. 1999) in basal sugar transport and insulin-stimulated sugar transport leads to possible multiple sites of interaction of the PI. At this point, we do not know what specific mechanism(s) is involved.

In summary, PIIs resulted in increased 2-DG transport in L6 cells, which was not readily reversed after PI removal. Further, insulin-stimulated 2-DG transport was not decreased by long-term PI exposure in L6 cells (PI reversibility). Finally, in L6 cells, insulin- or ritonavir-induced increases in 2-DG transport did appear to involve the translocation of either GLUT 1, 3 or 4.

Acknowledgement

This work was supported in part by a grant from CANFAR.

References


Received in final form 27 May 2003
Accepted 29 May 2003