

Indinavir Induces Acute and Reversible Peripheral Insulin Resistance in Rats

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The use of HIV protease inhibitors (PIs) has been associated with several metabolic changes, including lipodystrophy, hyperlipidemia, and insulin resistance. The etiology of these adverse effects remains unknown. PIs have recently been found to cause acute and reversible inhibition of GLUT4 activity in vitro. To determine the acute in vivo effects of indinavir on whole-body glucose homeostasis, glucose tolerance tests were performed on PI-naïve Wistar rats immediately after a single intravenous dose of indinavir. Glucose and insulin levels were significantly elevated in indinavir-treated versus control rats ($P < 0.05$) during the initial 30 min of the glucose tolerance test. Under euglycemic-hyperinsulinemic clamp conditions, indinavir treatment acutely reduced the glucose infusion rate required to maintain euglycemia by 18 and 49% at indinavir concentrations of 14 and 27 $\mu\text{mol/l}$, respectively. Muscle 2-deoxyglucose uptake was similarly reduced under these conditions. Restoration of insulin sensitivity was observed within 4 h after stopping the indinavir infusion. Indinavir did not alter the suppression of hepatic glucose output under hyperinsulinemic conditions. These data demonstrate that indinavir causes acute and reversible changes in whole-body glucose homeostasis in rats and support the contribution of GLUT4 inhibition to the development of insulin resistance in patients treated with PIs. *Diabetes* 51:937–942, 2002

Inhibitors of the HIV protease (PIs) have been highly successful in controlling HIV replication and have resulted in significant improvements in AIDS-related morbidity and mortality (1). However, despite their clinical successes, it is now recognized that PI therapy is associated with a number of adverse metabolic consequences, including peripheral lipoatrophy, visceral adiposity, hypertriglyceridemia, and insulin resistance (2). Although several possible mechanisms for the development of this PI-associated lipodystrophy syndrome have been proposed, only limited experimental data are available to support any of these hypotheses (rev. in 3).

PIs have recently been shown to selectively inhibit

GLUT4 activity in vitro, providing a potential mechanism for the insulin resistance observed in HIV-positive patients treated with this class of drugs (4). Glucose transport is the rate-limiting step for whole-body glucose disposal in rodents (5–7) and humans (8). GLUT4 is predominantly expressed in tissues responsible for the bulk of whole-body glucose disposal (skeletal/cardiac muscle and fat) (9–12) and is believed to be the principal transporter isoform mediating insulin-stimulated glucose uptake at these sites. GLUT4 inhibition by PIs is observed acutely in both 3T3-L1 adipocytes and *Xenopus* oocytes heterologously expressing GLUT4, and this inhibition is readily reversible by removal of the drug from the incubation media (4). Therefore, if the insulin resistance observed in the HIV PI-associated lipodystrophy syndrome is caused by GLUT4 inhibition, PIs should be capable of producing acute and reversible effects on whole-body glucose disposal.

It has been observed that insulin resistance is a relatively early and prevalent consequence of PI therapy (13). Several investigators have examined insulin sensitivity after several weeks to months of PI treatment (13–15). In a recent report in which oral glucose tolerance tests and euglycemic clamps were performed on healthy volunteers taking indinavir, insulin resistance was observed as soon as 4 weeks after start of therapy (16), before the development of any discernable changes in serum lipids or body fat composition. However, to date, there have been no reported studies examining acute effects (i.e., after a single dose) of any PI on glucose tolerance in PI-naïve animals or humans. To determine the contribution of impaired GLUT4 activity to the development of the insulin resistance associated with PI therapy, we investigated the acute effects of indinavir on whole-body glucose homeostasis and peripheral insulin sensitivity in a rodent model.

RESEARCH DESIGN AND METHODS

Materials. D-3-[³H]glucose was purchased from American Radiolabeled Chemicals (St. Louis, MO). Indinavir was obtained from Merck (Whitehouse Station, NJ). Insulin (Humulin-R) was purchased from Eli-Lilly (Indianapolis, IN). Male Wistar rats (150–200 g) were purchased from Charles River (Wilmington, MA). Microrenathane tubing for venous catheters was obtained from Braintree Scientific (Braintree, MA). PE-50 tubing for arterial catheters was obtained from Becton Dickinson (Franklin Lakes, NJ). Blood glucose levels were determined using a Glucometer Elite XL (Bayer, Tarrytown, NY). Assay kits for nonesterified fatty acid determinations were obtained from Wako Chemicals (Richmond, VA). All other reagents were obtained from Sigma (St. Louis, MO).

Determination of plasma insulin and indinavir levels. Indinavir levels were determined by the high-performance liquid chromatography (HPLC) method of Foisy and Sommadossi (17) using a Waters 626 HPLC system with a Microsorb C-8 column. Samples were run in duplicate on 50 μl serum.

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DOG, [³H]-2-deoxyglucose; GIR, glucose infusion rate; HAART, highly active antiretroviral therapy; HPLC, high-performance liquid chromatography; PI, protease inhibitor.

Standard curves were generated by adding indinavir directly to control rat serum. Insulin levels were determined by radioimmunoassay as previously described (18)

Animal procedures. All animal procedures were approved by the animal studies committee at Washington University School of Medicine. Rats were housed in the animal facility at Washington University and fed a standard rat chow diet and water ad libitum. Catheters were inserted into the left internal carotid artery and right jugular vein under methohexital anesthesia as previously described (19). Rats were allowed to recover from the stress of surgery at least 4 days before performing experiments. All animals weighed between 200 and 300 g at the time experiments were performed. Rats were fasted overnight before each experiment.

Intraperitoneal glucose tolerance tests. An aqueous solution of indinavir (15 mg/kg) or saline was infused through the venous catheter 2 min before the intraperitoneal administration of 50% dextrose (2 g/kg). Blood was sampled via the venous catheter for determination of glucose, insulin, and indinavir levels.

Euglycemic-hyperinsulinemic clamp experiments. Catheters were flushed with normal saline and heparin (40 units/kg) was administered to maintain catheter patency. After determination of fasting blood glucose concentrations, a constant infusion of indinavir (or water for control experiments) was started through the venous catheter at a rate of 5 $\mu\text{l}/\text{min}$ using a Harvard 11 apparatus pump. After 30 min of drug infusion, insulin (40 $\text{mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) in normal saline containing 0.3% BSA was infused at a rate of 5 $\mu\text{l}/\text{min}$ through the venous catheter. At 5-min intervals, $\sim 500 \mu\text{l}$ blood was removed from the arterial catheter into a syringe. Blood (2 μl) was then sampled directly from the catheter for the determination of blood glucose levels. The dead-space blood was then reinfused into the animal. Dextrose (50%) was infused through the venous catheter at a rate sufficient to maintain a plasma glucose level of 110 mg/dl. Insulin sensitivity was determined by the average glucose infusion rate (GIR) over the final 30 min of each 2-h clamp experiment.

Muscle 2-deoxyglucose uptake. At 30 min before the conclusion of 2-h euglycemic-hyperinsulinemic clamp experiments, [^3H]-2-deoxyglucose (DOG) was administered through the arterial catheter. Blood was collected at 5-min intervals for determination of the tracer specific activity. Immediately after euthanasia by sodium pentobarbital administration, rat hind-limb muscles were harvested and placed in liquid nitrogen pending subsequent analysis. The frozen muscle samples (50–80 mg) were ground with a mortar and pestle, boiled in 1.2 ml water for 10 min, and spun in a microcentrifuge at 15,000g for 10 min. Accumulated 2-deoxyglucose-6-phosphate in the supernatant was separated from DOG by ion exchange chromatography using a Dowex 1X-8 (100–200 mesh) anion exchange column as previously described (20).

Reversibility of indinavir effects. Euglycemic-hyperinsulinemic clamps were performed as detailed above, with the exception that the indinavir infusion was started after a steady state for glucose infusion under hyperinsulinemic conditions had been achieved. In control experiments, an equal volume of sterile water was infused in the place of indinavir. Blood was also sampled every 10 min after the GIR had stabilized. After the separation of 50–75 μl plasma for determination of insulin and indinavir levels, packed red cells were resuspended in normal saline and reinfused into the animals. Heparin was redosed at ~ 2 -h intervals. Total blood loss from animals did not exceed 10 ml/kg during the extended clamps.

Measurement of hepatic glucose output. Hepatic glucose output was determined by the tracer dilution method using 3- ^3H -glucose as previously described (21). Blood was sampled for glucose specific activity at 10-min intervals. After determination of basal hepatic glucose output over 40 min, euglycemic-hyperinsulinemic clamp experiments were started by the infusion of insulin at a rate of 40 $\text{mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$. At $t = 120$ min into the clamp experiments, continuous infusion of indinavir at a rate of 0.5 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ was initiated. For control experiments, an equal volume of saline was infused. In a separate set of experiments, the indinavir infusion was started after the basal hepatic glucose output determination, 30 min before the start of the hyperinsulinemic clamp.

RESULTS

Glucose tolerance testing. After a single oral dose of indinavir, plasma drug levels in humans typically reach a peak concentration of 12 $\mu\text{mol}/\text{l}$, with a half-life of ~ 100 min (22). In the rat, indinavir is more rapidly metabolized, with a serum half-life of ~ 20 min (23). Given the reversibility of GLUT4 inhibition by indinavir in vitro, we speculated that in vivo drug levels at the time in which glucose tolerance is assessed could be crucial to detecting alterations in insulin sensitivity. To allow rapid and consistent

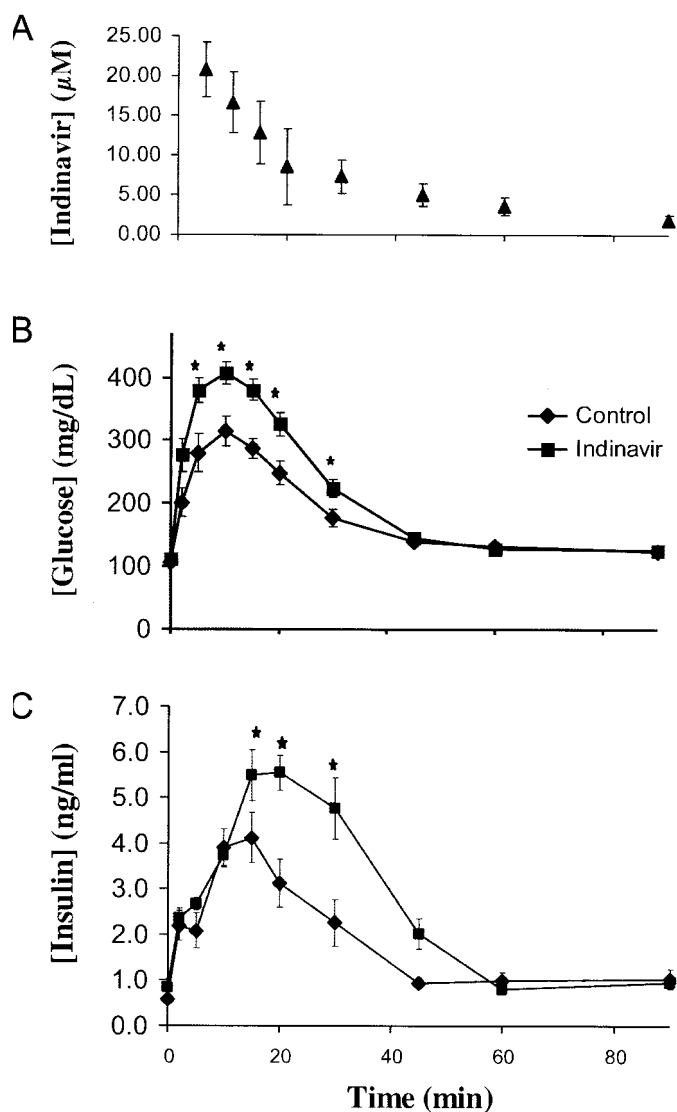


FIG. 1. Glucose tolerance after acute administration of indinavir. Male Wistar rats (200–300 g) were given a single 15 mg/kg i.v. dose of indinavir ($n = 8$) or water ($n = 9$) immediately before the intraperitoneal administration of glucose (2 g/kg). **A:** Indinavir levels achieved as determined by HPLC analysis (17). **B:** Glucose levels as determined using a Glucometer Elite XL glucometer. *** $P < 0.05$, Student's t test.** **C:** Insulin levels as determined by immunofluorescent assay.

achievement of desired drug levels, indinavir was administered intravenously to PI-naïve Wistar rats immediately before conducting intraperitoneal glucose tolerance testing. After a single 15-mg/kg i.v. dose of indinavir, we observed indinavir levels of $20.8 \pm 3.4 \mu\text{mol}/\text{l}$ at 5 min, as shown in Fig. 1A with a half-life consistent with previously published reports.

After the administration of 2 g/kg of glucose i.p., serum glucose levels reached a maximum within 15 min, with a steady decrease back to baseline within 60 min in both indinavir-treated and control animals (Fig. 1B). The peak glucose levels achieved over the first 45 min of the glucose tolerance test were significantly higher in the indinavir-treated group. Peak glucose levels reached 407 ± 18 and 314 ± 24 mg/dl in the indinavir-treated and control animals, respectively ($P < 0.02$). Consistent with the hypothesis that indinavir causes acute insulin resistance, insulin levels achieved during the glucose tolerance tests were

TABLE 1
Hepatic glucose output (R_d) under euglycemic-hyperinsulinemic clamp conditions

	Basal	Clamp*	Clamp + indinavir†
R_d ($\text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$)‡	8.3 ± 1.6	28.6 ± 1.5	19.8 ± 0.7
GIR ($\text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$)	0.0	31.6 ± 1.7	22.4 ± 0.6
Hepatic glucose production§	8.3 ± 1.6	-3.0 ± 2.2	-2.6 ± 1.0

Data represent the means \pm SD of three determinations. *40 $\text{mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ glucose clamped at 110 mg/dl; †indinavir infused at $0.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$; ‡ R_d determined by tracer dilution method with $3\text{-}[\text{3}\text{-H}]\text{glucose}$; § R_d - GIR.

nearly twofold higher between 20 and 40 min in the indinavir-treated animals (Fig. 1C). As early as 1 h after the glucose infusion, glucose and insulin levels had returned to normal.

Euglycemic-hyperinsulinemic clamp experiments. Insulin sensitivity was assessed by determining the GIR required to maintain euglycemia under hyperinsulinemic conditions. At an insulin infusion rate of $40 \text{ mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, plasma insulin levels of $660 \pm 47 \text{ } \mu\text{U/ml}$ were achieved. Under these conditions, GLUT4 translocation is maximally stimulated and hepatic glucose production is completely suppressed in normal rats (24). Given the rapid clearance of indinavir after a single intravenous dose and the necessity to maintain constant drug levels during the clamp studies, we administered indinavir by a constant intravenous infusion. At an infusion rate of $0.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, indinavir reached a sustained level of $27.1 \pm 2.6 \text{ } \mu\text{mol/l}$ at 90 min, with a continued slight rise in drug levels of 10–15% per hour thereafter. At an infusion rate of $0.3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, average indinavir levels of $14.1 \pm 1.0 \text{ } \mu\text{mol/l}$ were achieved during the clamp experiments. Suppression of hepatic glucose production at these insulin levels was not affected by indinavir (Table 1). In control animals, plasma free fatty acid levels were acutely reduced by hyperinsulinemia as previously described (25). Although acute indinavir administration inhibited this fall in free fatty acid levels under the hyperinsulinemic conditions, no increase in free fatty acid levels above baseline levels was detected (Fig. 2).

As shown in Fig. 3, insulin sensitivity is significantly reduced by acute intravenous indinavir administration. At $14 \text{ } \mu\text{mol/l}$ indinavir, insulin sensitivity was reduced by 18% compared with control animals ($P < 0.0001$). At indinavir levels of $27 \text{ } \mu\text{mol/l}$, a 49% reduction in insulin sensitivity was observed ($P < 0.0001$). Direct measurement of 2-deoxyglucose uptake into rat skeletal muscle under these conditions revealed that indinavir acutely impaired glucose transport into this tissue, consistent with the observed in vitro effects of indinavir on GLUT4 activity (Fig. 4). All of the muscles studied had significant reductions in DOG uptake at the higher indinavir concentration. Although DOG uptake was also reduced at the $14 \text{ } \mu\text{mol/l}$ concentration of indinavir in all of the muscles, statistical significance was not reached for plantaris and gastrocnemius muscle ($P = 0.09$ and 0.051 , respectively). Extensor digitorum longus muscle appeared most sensitive to indinavir, with 45 and 71% reductions in DOG uptake at drug levels of 14 and $27 \text{ } \mu\text{mol/l}$, respectively.

The reversibility of indinavir induced insulin resistance

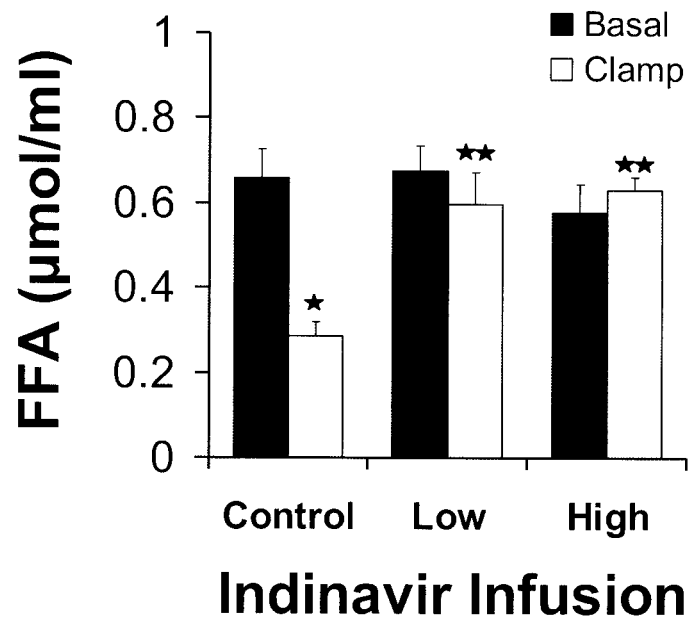


FIG. 2. Effect of indinavir on plasma free fatty acid levels. Serum nonesterified fatty acid levels were determined by colorimetric assay. Basal levels were obtained 30 min after the start of the indinavir infusion, just before the start of the hyperinsulinemic-euglycemic clamps, as described in RESEARCH DESIGN AND METHODS. Clamp levels were sampled at the conclusion of the experiments ($t = 120 \text{ min}$). * $P < 0.05$ compared with basal level, ** $P < 0.05$ compared with control clamp level.

is shown in Fig. 5. After establishing a stable baseline in GIR after 90 min of insulin infusion, indinavir was administered at a constant rate of $0.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$. Consistent with the results shown in Fig. 3, the GIR required to maintain euglycemia was significantly diminished while indinavir was being infused. After the indinavir infusion was discontinued, the GIR required to maintain euglycemia steadily increased over the subsequent 4 h.

DISCUSSION

The demonstration that indinavir produces acute and reversible changes in insulin sensitivity within a rodent model has several implications for the understanding of the etiology of the metabolic changes observed in patients receiving PI therapy. Estimates of the prevalence of insulin resistance in patients receiving this class of drug have varied widely (26). In nearly all published studies, serum PI levels were either not reported or not measured at the time of glucose tolerance testing. As can be seen from our current study, at least initially, the acute effects of indinavir on glucose homeostasis are readily reversible. The failure to detect insulin resistance in previous studies may have been due to glucose tolerance testing at trough levels of PIs, where the effects may be less pronounced. Similarly, previous reports of the effects of PIs in animal models have either not reported PI levels or have observed low drug levels during treatment (27,28). Consideration of plasma drug levels at the time of glucose tolerance testing is particularly important in the rodent model, given the short half-life of indinavir ($\sim 20 \text{ min}$) in these animals (23).

Questions have been raised regarding the levels of PIs required to achieve significant inhibition of GLUT4 activity in vitro, particularly in consideration of PI binding to

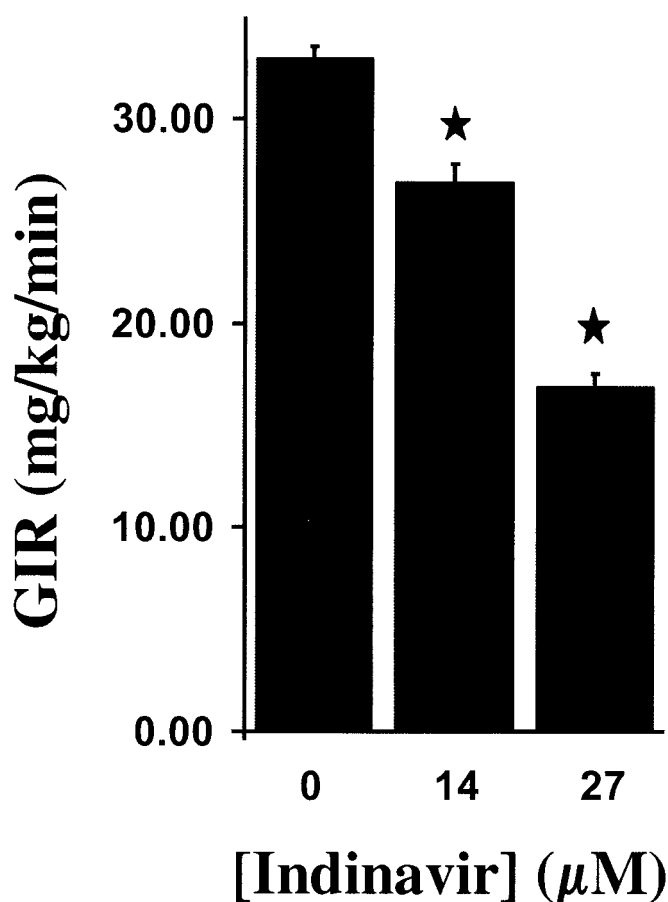


FIG. 3. Acute effects of indinavir on peripheral insulin sensitivity. Catheters were inserted into carotid arteries and jugular veins of 200- to 300-g male Wistar rats at least 4 days before the experiments. Euglycemic-hyperinsulinemic clamps were performed using $40 \text{ mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ regular human insulin. The rate of infusion of 50% dextrose was adjusted to maintain plasma glucose levels at 110 mg/dl. Insulin sensitivity was determined by the average GIR over the final 30 min of each 2-h experiment. $n = 12, 10,$ and 6 for control and 14 and 24 $\mu\text{mol/l}$ indinavir, respectively. Data represents the means \pm SE. * $P < 0.0001$, Student's t test.

serum proteins (29). In humans receiving indinavir as part of highly active antiretroviral therapy (HAART), the drug reaches peak concentrations of $12 \mu\text{mol/l}$ within 1 h after dosing and is metabolized with a serum half-life of ~ 100 min. Pharmacokinetic studies have established that $\sim 60\%$ of indinavir in human serum is protein bound (22). With the lower indinavir infusion rate used in this study ($0.3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$), the drug levels achieved were only slightly above this range ($14 \mu\text{mol/l}$). Because indinavir binding to serum proteins may differ between rats and humans, it is not possible to directly correlate the degree of insulin resistance observed in the rats with effects in humans at comparable drug levels. However, in individual patients, these peak drug levels are certainly achievable. Furthermore, this degree of impairment in glucose disposal (18%) represents a significant perturbation in whole-body glucose homeostasis. Previous studies have demonstrated that much smaller reductions in glucose disposal under the euglycemic-hyperinsulinemic clamp conditions in humans can be associated with impaired glucose tolerance and type 2 diabetes (30). Because indinavir is capable of producing acute impairment of insulin sensitivity in vivo,

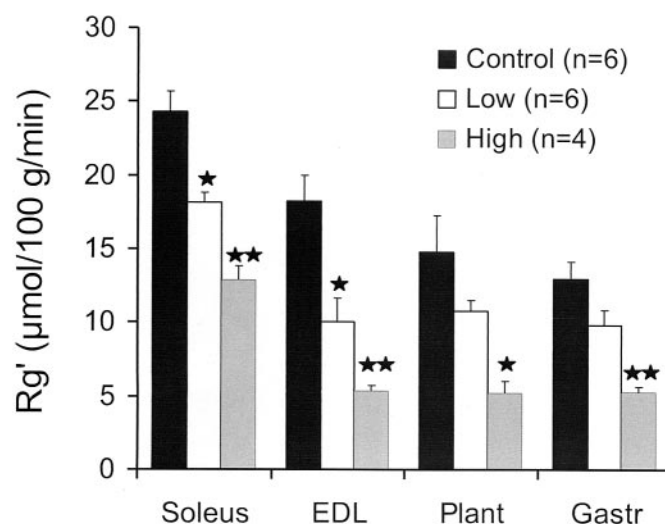


FIG. 4. Effect of indinavir on muscle 2-deoxyglucose uptake. DOG was injected into the arterial catheters 30 min before the conclusion of the euglycemic-hyperinsulinemic clamp experiments. Hindlimb muscles were removed from the rats immediately after euthanasia with sodium pentobarbital and were analyzed for accumulation of radiolabeled 2-deoxyglucose 6-phosphate, as described in RESEARCH DESIGN AND METHODS. EDL, extensor digitorum longus; Plant, plantaris; Gastr, red gastrocnemius; R_g' , glucose metabolism index. Values represent the means \pm SE. * $P < 0.05$; ** $P < 0.001$, Student's t test.

the timing of drug dosing in relation to meals may have direct consequences on glucose tolerance. Indinavir is typically dosed three times daily, with recommendations to take the drug at least 1 h before or 2 h after meals to facilitate drug absorption (31). When taken before meals, glucose loading would take place precisely at the time of peak indinavir levels, when insulin resistance is maximal. Although extension of our study to humans will be required to confirm this prediction, increased postprandial blood glucose excursions and impaired glucose tolerance with indinavir dosing before meals may necessitate reconsideration of current dosing guidelines.

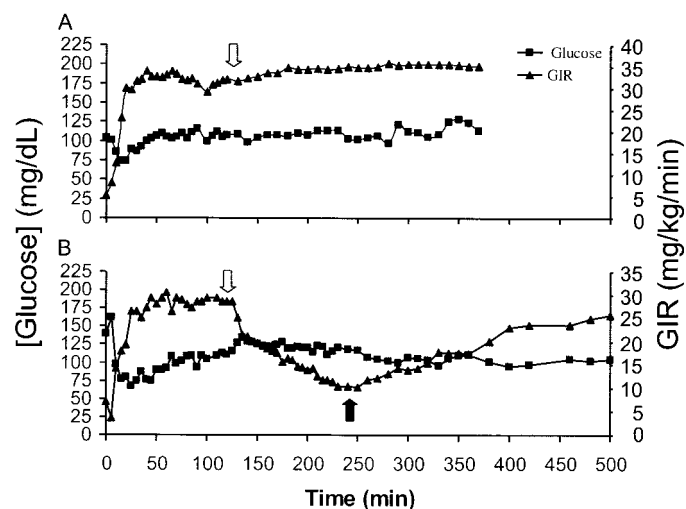


FIG. 5. Reversibility of insulin resistance induced by acute indinavir administration. Euglycemic-hyperinsulinemic clamps were initiated as described for Fig. 3. At the time indicated by the open arrow, a constant intravenous infusion of indinavir was started at a rate of $0.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$. The infusion was discontinued as indicated by the solid arrow. Indinavir levels reached levels of $25\text{--}30 \mu\text{mol/l}$ during the period of drug infusion.

The acute *in vivo* effect of indinavir on glucose tolerance in rats is entirely consistent with direct inhibition of GLUT4 being the mechanism of insulin resistance. Although our current data cannot be directly extrapolated to other PIs, *in vitro* studies have demonstrated that PIs as a class are all capable of producing acute and reversible inhibition of GLUT4 activity in 3T3-L1 adipocytes *in vitro* (4). Consideration of the K_i (inactivation constant) for GLUT4 inhibition as well as the therapeutically achieved drug levels for each of the PIs will be required to assess the contribution of GLUT4 inhibition to the insulin resistance observed with these other drugs. The majority of insulin-stimulated glucose disposal occurs into muscle. Similar effects of PIs on glucose uptake have recently been reported in isolated skeletal muscle (32). This does not exclude the possibility that in addition to direct inhibition of GLUT4 activity, additional mechanisms also contribute to impaired glucose tolerance. Mice specifically lacking GLUT4 in adipocytes have been found to have peripheral insulin resistance *in vivo*, with normal insulin sensitivity in isolated skeletal muscle (33). This suggests that the secretion of some adipocyte-derived factor from these cells causes peripheral insulin resistance. Interestingly, these adipocyte-specific GLUT4 knockout animals did not have any abnormalities in total fat content or distribution. The chronic absence of GLUT4-mediated glucose uptake in these animals is in contrast to the reversible insulin resistance that we hypothesize is taking place in PI-treated patients.

It is unlikely that alterations in plasma free fatty acids directly contribute to the acute insulin resistance observed in these studies. Even when free fatty acids are infused into animals at pharmacological levels, insulin resistance is not observed until >3 h into the infusion (25). However, acute indinavir administration did prevent the normal suppression of nonesterified fatty acids under hyperinsulinemic conditions. It is not clear whether this effect is related, directly or indirectly, to GLUT4 inhibition. It remains possible that under chronic treatment with indinavir, elevated free fatty acids secondarily contribute to insulin resistance. The changes that have been observed with prolonged exposure to PIs, including redistribution of body fat and development of hypertriglyceridemia, may also contribute to insulin resistance. The insulin resistance produced via these mechanisms is unlikely to be as readily reversible as that observed as a result of direct GLUT4 inhibition.

A direct connection between PIs and the development of other adverse effects associated with HAART is less clear than the association with insulin resistance. Lipodystrophy has been observed in patients who have never been exposed to PIs (34–36). It is likely that PIs have multiple cellular targets, each of which contribute independently or synergistically to the phenotypic changes observed. However, given the immediate effects of PIs in causing insulin resistance, it is possible that reversible insulin resistance as a result of inhibition of GLUT4 activity in adipocytes directly or indirectly contributes to the development of changes in body fat. It is now recognized that in addition to its traditional role as an energy storage depot, fat functions as an important endocrine tissue (37,38). As

discussed previously, significant insulin resistance is observed at peak indinavir levels. However, lesser effects on glucose sensitivity may be present at trough PI levels. The contrasting changes in peripheral versus visceral fat may be due to differences in the ability of these two tissues to compensate (or overcompensate) for this alternating state of insulin sensitivity by releasing one or more adipocyte-derived endocrine or paracrine factors. The hyperinsulinism resulting from acute GLUT4 inhibition may also contribute to changes in fat distribution. This may be due specifically to the induction of insulin resistance at the terminal step in insulin-stimulated glucose uptake. Because insulin signaling itself is not acutely affected by PIs (4), the effects on adipose tissue may be mediated by sustained insulin effects on lipid metabolism and/or stimulation of mitogenic insulin signaling pathways.

In addition to providing new insight into the mechanism for HAART-associated insulin resistance, the acute and readily reversible effects of indinavir on insulin sensitivity may also provide a novel tool in the study of perturbations in normal glucose homeostasis. This may facilitate the search for new classes of drugs capable of counteracting or reversing insulin resistance at a site distal to insulin signaling and GLUT4 translocation.

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