Effects of the HIV Protease Inhibitor Ritonavir on GLUT4 Knock-out Mice

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HIV protease inhibitors acutely block glucose transporters (GLUTs) in vitro, and this may contribute to altered glucose homeostasis in vivo. However, several GLUT-independent mechanisms have been postulated. To determine the contribution of GLUT blockade to protease inhibitor-mediated glucose dysregulation, the effects of ritonavir were investigated in mice lacking the insulin-sensitive glucose transporter GLUT4 (G4KO). G4KO and control C57BL/6J mice were administered ritonavir or vehicle at the start of an intraperitoneal glucose tolerance test and during hyperinsulinemic-euglycemic clamps. G4KO mice exhibited elevated fasting blood glucose compared with C57BL/6J mice. Ritonavir impaired glucose tolerance in control mice but did not exacerbate glucose intolerance in G4KO mice. Similarly, ritonavir reduced peripheral insulin sensitivity in control mice but not in G4KO mice. Serum insulin levels were reduced in vivo in ritonavir-treated mice. Ritonavir reduced serum leptin levels in C57BL/6J mice but had no effect on serum adiponectin. No change in these adipokines was observed following ritonavir treatment of G4KO mice. These data confirm that a primary effect of ritonavir on peripheral glucose disposal is mediated through direct inhibition of GLUT4 activity in vivo. The ability of GLUT4 blockade to contribute to derangements in the other molecular pathways that influence insulin sensitivity remains to be determined.

The use of HIV protease inhibitors (PIs)1 as part of combined antiretroviral therapy (cART) has altered the course of the HIV epidemic by greatly reducing disease-related morbidity and mortality (1). The success of PI-based therapies is tempered by the recognition that many PIs contribute to insulin resistance (2). Peripheral and visceral obesity, the hallmark of the metabolic syndrome, is strongly linked to several first generation PIs (3). The development of impaired glucose tolerance has been most directly explained by metabolic syndrome in patients taking PIs (4). In cultured adipocytes, PIs acutely and reversibly inhibit insulin-stimulated glucose uptake at pharmacologically relevant drug levels (5). Furthermore, these in vitro findings correlate with acute and reversible induction of insulin resistance in vivo both in rodents (6) and in HIV-negative volunteers (7).

Despite a high degree of correlation between in vitro and in vivo findings, questions remain regarding the contribution of direct GLUT blockade to changes in glucose homeostasis in treated patients. Impaired insulin signaling (10), reduced insulin secretion (12), and altered adipokine levels (13–15) have also been implicated. To determine the specific contribution of PI-mediated GLUT inhibition to altered glucose homeostasis, we tested the effects of ritonavir on glucose tolerance in transgenic mice lacking GLUT4 (G4KO) (16). We report evidence supporting our hypothesis that GLUT4 inhibition is a primary mechanism leading to changes in glucose tolerance.

EXPERIMENTAL PROCEDURES

Materials—Homozygous GLUT4 knock-out mice (G4KO) on an isogenic C57BL/6J background were a kind gift from Maureen Charron (16). For all experiments, age- and sex-matched control C57BL/6J mice were obtained from Jackson ImmunoResearch Laboratories (Bar Harbor, ME). Ritonavir was obtained in oil-based form (Merck) and resuspended in 100% ethanol (0.79 g/ml) prior to dilution with water to an 11% (v/v) working concentration. 2-[3H]Deoxyglucose (2-[3H]DG) was purchased from Sigma.

Blood Glucose and Insulin Levels—Blood glucose was assayed using the glucose dehydrogenase-based enzymatic assay and quantitated using an Elite XL glucometer (Bayer). Blood insulin levels were assayed in 15 µl of mouse serum using an ELISA according to the manufacturer’s protocol (Crystal Chem Inc., Downers Grove, IL). Intraperitoneal glucose tolerance tests (GTTs) were performed on 3–5-month-old mice following an overnight 16-h fast. Animals were injected intraperitoneally with either ritonavir (10 mg/kg) or vehicle 15 min prior to the intraperitoneal administration of 25% dextrose (1 g/kg). Blood was isolated from the tail vein at the times indicated and assayed for glucose as described above. Intraperitoneal insulin tolerance tests were performed on 3–5-month-old mice follow-

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3 The abbreviations used are: PI, protease inhibitor; cART, combined antiretroviral therapy; GLUT, glucose transporter; 2-DG, 2-deoxyglucose; GTT, glucose tolerance test.
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ing a 6-h fast. Animals were injected intraperitoneally with insulin (0.5 units/kg). Blood was isolated from the tail vein at the times indicated and assayed for glucose as described above. Data are presented as the mean ± S.E. (Student’s unpaired t test).

Hyperinsulinemic-Euglycemic Clamp Experiments—Weight-matched G4KO and C57BL/6J mice were anesthetized with ketamine/xylazine (87 and 13.4 mg/kg intraperitoneally), and catheters (MRE 025, Braintree Scientific Inc., Braintree, MA) were implanted into both the right internal jugular vein and femoral artery and allowed to recover for 5–7 days. Following a 5-h fast, catheters were flushed with normal saline, and heparin (20 units/kg) was administered to maintain catheter patency. After determination of fasting blood glucose levels, a constant infusion of ritonavir (0.35 mg/kg/min) was started through the venous catheter at a rate of 0.5 μl/min using a Harvard 11 apparatus pump. After 30 min of drug infusion, insulin (20 milliunits/kg/min) in normal saline containing 0.1% BSA was infused through the venous catheter. At 10-min intervals, 100 μl of blood was removed from the arterial catheter into a syringe. Blood (5 μl) was then sampled directly from the catheter for the determination of blood glucose levels. Blood glucose was assayed using a Contour TS glucometer. The dead space blood was then reinfused into the animal. Dextrose (25%) was infused through the venous catheter at a rate sufficient to maintain a plasma glucose level of 100–110 mg/dl. Peripheral glucose disposal (Rglu) was determined by the average glucose infusion rate during the final 30 min of each 120-min clamp experiment.

Skeletal Muscle and Adipose Tissue 2-DG Uptake—2-[3H]DG (5 μCi) was administered through the arterial catheter 30 min before the conclusion of hyperinsulinemic-euglycemic clamp experiments. Blood was collected at 0, 3, 5, 10, 15, 20, 25, and 30 min for determination of the tracer-specific activity (8). Immediately after euthanasia by cervical dislocation, hind limb muscles and epididymal fat were harvested, washed with PBS, and placed in liquid nitrogen pending subsequent analysis. Frozen tissue samples were ground with a mortar and pestle, boiled in 1.2 ml of water for 10 min, and spun in a microcentrifuge at 15,000 × g for 10 min. Accumulated 2-DG 6-phosphate in the supernatant was separated from 2-DG by ion exchange chromatography using a Dowex 1-X8 (100–200 mesh) anion exchange column (8).

Adipokine and Leptin ELISAs—Serum leptin and adiponectin levels were determined using a commercially available mouse ELISA kit (Millipore) according to the manufacturer’s protocol. Serum samples for adiponectin were diluted 1000-fold prior to assay.

Drug Assays—Serum PI levels were determined by the HPLC method of Foisy and Sommadossi (17) using a Waters 626 HPLC system with a Microsorb C-8 column (Waters Corp., Milford, MA). Samples were run in duplicate in 50 μl of serum. Standard curves were generated by adding pure PI standards directly to control mouse serum.

RESULTS

Glucose Tolerance in G4KO Mice Is Not Acutely Altered by Ritonavir—In the acute setting, HIV PIs such as ritonavir are hypothesized to impair glucose handling by targeting and inhibiting the insulin-sensitive glucose transporter GLUT4 in fat (6) and muscle (18), although several alternative mechanisms have been proposed (11, 19–21). If the adverse effects of PIs on glucose homeostasis are mediated primarily through GLUT4, then these effects should be ameliorated on a GLUT4-null background. To characterize physiologic responses to glucose load, 3–5-month-old whole body GLUT4 knock-out mice (G4KO) (16) underwent intraperitoneal GTTs in the presence or absence of ritonavir (10 mg/kg). As shown in Fig. 1A, administration of ritonavir before the start of the GTT in age-matched control C57BL/6J mice (WT) significantly altered glucose excursion at the 15- and 30-min time points compared with vehicle treatment. Blood glucose before and after glucose load was significantly higher in G4KO mice compared with control C57BL/6J mice (Fig. 1B), consistent with a loss of insulin-sensitive glucose uptake upon GLUT4 ablation (fasting blood glucose: WT, 126 ± 15 mg/dl (n = 7); and G4KO, 331 ± 45 mg/dl (n = 7); p < 0.01). Importantly, administration of ritonavir to G4KO mice, in contrast to control mice, did not further exacerbate the glucose intolerance. No significant differences in the results of GTTs were observed when the mice were separated by sex. Qualitatively similar results between the genotypes were also obtained when ritonavir was administered at the start of the GTT (data not shown). HPLC analysis of serum samples taken at the 30-min time point, the peak of the PI effect in control mice, revealed a mean ritonavir concentration of 16.8 ± 2.0 μl/ml (n = 3), which is near the reported peak therapeutic concentration (22). Taken together, these data support the con-
with vehicle treatment (WT: vehicle, 251 ± 15 mg/dl; and ritonavir, 386 ± 25 mg/dl; p < 0.01), but importantly, this rise was not accompanied by a compensatory rise in serum insulin levels (WT: vehicle, 0.52 ± 0.06 ng/ml; and ritonavir, 0.45 ± 0.09 ng/ml; p = 0.5), indicating relative β-cell dysfunction. As expected, blood glucose levels were not affected, as the G4KO mice are virtually insensitive to changes in circulating insulin due to loss of the GLUT4 isoform (G4KO: vehicle, 404 ± 31 mg/dl; and ritonavir, 373 ± 28 mg/dl; p = 0.43) (Fig. 2B). Insulin levels were significantly lower in ritonavir-treated G4KO mice (G4KO: vehicle, 1.94 ± 0.4 ng/ml; and ritonavir, 0.8 ± 0.18 ng/ml; p < 0.01). These data indicate that the β-cell does not respond appropriately with an increase in serum insulin levels during PI-induced insulin resistance.

**Insulin Does Not Reduce Glucose Levels in G4KO Mice**—To assess the effect of ritonavir on peripheral glucose uptake, the glucose-lowering effect of intraperitoneal insulin injection (0.5 units/kg) was determined using insulin tolerance tests on 6-h fasted mice. As predicted from previous studies on the acute effect of the PI indinavir on rodents (8), the glucose-lowering effect of insulin was blunted in control mice injected with ritonavir (10 mg/kg) (Fig. 3A). Ablation of GLUT4 eliminated the insulin-sensitive component of glucose uptake, and therefore, assaying insulin sensitivity in the G4KO mice was without effect even at concentrations up to 2.5 units/kg (Fig. 3B and data not shown). Although average glucose levels were slightly higher 15 and 30 min after insulin injection in the G4KO mice, the values were not statistically different between treatment groups. Thus, the glucose intolerance in the control mice likely reflects both impaired β-cell function and a reduction in peripheral insulin sensitivity.

**Peripheral Glucose Disposal Is Not Altered by Ritonavir in G4KO Mice**—To more directly determine the extent to which the observed effects of ritonavir on glucose tolerance are mediated through effects on peripheral glucose disposal, hyperinsulinemic-euglycemic clamps, the established gold standard measurement of peripheral insulin sensitivity (24), was determined in wild-type and G4KO mice exposed to ritonavir by continuous intravenous infusion (0.35 mg/kg/min). Previous studies have established that this infusion rate provides steady-state ritonavir blood levels of 10–15 μM (25). As shown in Fig. 4A, glucose $R_{d}$ was reduced by 68% in ritonavir-treated wild-type mice (30 ± 11 mg/kg/min) compared with vehicle-treated animals (99 ± 12 mg/kg/min). As shown in Fig. 4B, the transport of 2-DG into skeletal muscle was inhibited 70% by ritonavir in wild-type mice. Consistent with the lack of insulin-responsive glucose transport, glucose $R_{d}$ in vehicle-treated G4KO mice was significantly lower than that in wild-type animals (37 ± 9 mg/kg/min). Whole body glucose disposal and soleus muscle 2-DG uptake were not different between ritonavir- and vehicle-treated G4KO mice. The uptake of 2-DG into

**Glucose-stimulated Insulin Secretion Is Inhibited by Ritonavir**—To assess the contribution of in vivo β-cell responsiveness to PI-induced changes in glucose tolerance, serum insulin levels were measured 30 min following co-injection with glucose (1 g/kg) and either ritonavir (10 mg/kg) or vehicle. As shown in Fig. 2A, ritonavir caused an expected increase in blood glucose levels in WT mice compared to vehicle (WT: vehicle, 251 ± 15 mg/dl; and ritonavir, 386 ± 25 mg/dl; p < 0.01), but importantly, this rise was not accompanied by a compensatory rise in serum insulin levels (WT: vehicle, 0.52 ± 0.06 ng/ml; and ritonavir, 0.45 ± 0.09 ng/ml; p = 0.5), indicating relative β-cell dysfunction. As expected, blood glucose levels were not affected, as the G4KO mice are virtually insensitive to changes in circulating insulin due to loss of the GLUT4 isoform (G4KO: vehicle, 404 ± 31 mg/dl; and ritonavir, 373 ± 28 mg/dl; p = 0.43) (Fig. 2B). Insulin levels were significantly lower in ritonavir-treated G4KO mice (G4KO: vehicle, 1.94 ± 0.4 ng/ml; and ritonavir, 0.8 ± 0.18 ng/ml; p < 0.01). These data indicate that the β-cell does not respond appropriately with an increase in serum insulin levels during PI-induced insulin resistance.

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white adipose tissue from WT mice (5.7 μmol/100 g/min) was <10% of skeletal muscle glucose uptake. The effect of ritonavir on 2-DG uptake was more modest (17%) in this tissue and did not reach statistical significance (data not shown). The low amount of white adipose tissue in G4KO mice precluded accurate measurement of the effect of ritonavir on 2-DG uptake from this tissue in vivo. Nevertheless, these data indicate that direct inhibition of white adipose tissue glucose transport is not primarily responsible for the acute effect of ritonavir on glucose homeostasis in the WT mice.

**Adipocytokine Levels Are Not Acutely Altered by Ritonavir in G4KO Mice**—Although skeletal muscle is directly responsible for the majority of peripheral glucose disposal, drug-induced effects on adipose tissue could still potentially impair glucose homeostasis indirectly through changes in adipocytokine secretion. To determine whether the acute effects of ritonavir on glucose tolerance are influenced by changes in adiponectin or leptin secretion, we first measured serum levels of these adipokines in WT mice before and after treatment with ritonavir (1%, w/w) in standard rodent chow for 12 h overnight. Total food intake was not different between experimental groups during this interval. As shown in Fig. 5A, leptin levels were significantly reduced in WT mice following ritonavir treatment. Consistent with the reduced fat content in G4KO mice (16), leptin levels were significantly lower in G4KO compared with WT animals. Ritonavir treatment did not further influence serum leptin levels in G4KO mice. As shown in Fig. 5B, ritonavir treatment did not significantly alter adiponectin levels in either WT or G4KO mice.

**DISCUSSION**

Metabolic side effects and the development of viral resistance to HIV PIs continue to represent the most serious limitations to the use of these agents in antiretroviral treatment regimens. Knowledge of the precise molecular interactions that contribute to the development of insulin resistance is a key to the rational design and development of novel PIs that maintain clinical efficacy without the induction of glucose intolerance.
Previous studies have supported an important role of PI-induced GLUT4 blockade in the development of altered glucose homeostasis during cART. Although in vitro data have established that PIs act as acute and reversible non-competitive inhibitors of GLUT4 (7) and that these drugs act through direct interaction with the transport protein (26), the existence and influence of additional molecular targets have remained unclear. Furthermore, although excellent correlation between PI-mediated insulin resistance in vitro and in rodent models and healthy human volunteers has been established (27), each of these systems alone has been insufficient to prove that the in vivo effects on peripheral glucose disposal are mediated primarily through the direct inhibition of facilitative glucose transport. In this study, the demonstration that glucose tolerance is not acutely affected by ritonavir in mice lacking GLUT4 provides confirmation of a central role of GLUT4 blockade in these in vivo effects.

Because GLUT4 mediates the most distal step in insulin-stimulated glucose uptake, it is difficult to assess changes in more proximal signaling events following genetic or pharmacologic ablation of this transporter. Therefore, given the severe base-line insulin resistance present in GLUT4 knock-out mice, our findings cannot completely exclude the possibility that ritonavir has additional effects on glucose homeostasis in addition to those mediated directly through GLUT4 blockade.

Glucose transport has been previously implicated in the regulation of leptin secretion from primary adipocytes (28). This earlier study used several nonselective GLUT inhibitors over 1–4 days. The lower serum leptin levels observed in G4KO mice, together with the failure to observe further changes in leptin levels following ritonavir treatment of the transgenic animals, further support a role of GLUT4 in leptin secretion from adipocytes. However, it remains unclear whether or to what degree the observed changes in serum leptin levels contribute to the observed changes in peripheral glucose disposal. Adiponectin does not appear to contribute to the acute changes in peripheral glucose disposal induced by ritonavir.

Because these studies were all performed in the setting of acute drug exposure, it remains possible that additional long-term effects of ritonavir also indirectly impact insulin sensitivity. When administered chronically to rodents and humans, ritonavir is also known to induce hyperlipidemia (29), which in acute drug exposure, it remains possible that additional long-term effects of ritonavir also indirectly impact insulin sensitivity. When administered chronically to rodents and humans, ritonavir is also known to induce hyperlipidemia (29), which in acute drug exposure, it remains possible that additional long-term effects of ritonavir also indirectly impact insulin sensitivity. When administered chronically to rodents and humans, ritonavir is also known to induce hyperlipidemia (29), which in acute drug exposure, it remains possible that additional long-term effects of ritonavir also indirectly impact insulin sensitivity. 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