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Direct Comparison of the Acute In Vivo Effects of HIV Protease Inhibitors on Peripheral Glucose Disposal

Qingyun Yan, MD^{*} and Paul W. Hruz, MD, PhD^{*,†}

^{*}From the Department of Pediatrics, Washington University School of Medicine, St. Louis, MO

[†]From the Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO.

Summary

The clinical use of HIV protease inhibitors (PIs) is associated with the development of peripheral insulin resistance. The incidence and degree of impaired glucose tolerance observed in treated patients vary considerably between drugs, however. To compare the ability of HIV PIs to alter peripheral glucose disposal acutely in a genetically identical model system at therapeutically relevant drug levels, healthy lean male rats previously naive to PI exposure were given ritonavir, amprenavir, lopinavir/ritonavir (4:1), or atazanavir by continuous intravenous infusion to achieve steady state drug levels of 10 or 25 mM rapidly. Under euglycemic hyperinsulinemic clamp conditions, a dose-dependent reduction in the peripheral glucose disposal rate (Rd) was observed with all the PIs except atazanavir. The rank order of sensitivity was ritonavir, lopinavir, and then amprenavir. Changes in skeletal muscle and heart 2-deoxyglucose (2-DOG) uptake correlated with reductions in Rd. All 3 of these PIs also produced significant reductions in 2-DOG uptake into primary rat adipocytes in vitro. Atazanavir had no effect on glucose uptake in vitro or in vivo. The in vivo potency of PIs to impair peripheral glucose disposal acutely correlates with the degree of insulin resistance observed in HIV-infected patients receiving these drugs. Preclinical testing of novel candidate PIs in a rodent model system may be useful in identifying the future risk of altering glucose homeostasis.

Keywords

glucose clamp; adipokines; HIV protease inhibitors; glucose uptake; glucose transporter; insulin resistance; rodents

HIV protease inhibitors (PIs) have contributed greatly to reductions in HIV-associated morbidity and mortality over the last decade and remain a cornerstone of highly active antiretroviral therapy (HAART).¹ Despite their success, however, it is now clear that the use of these drugs is associated with the development of disturbing metabolic changes that greatly increase the lifetime risk for cardiovascular disease and other complications.^{2,3} Insulin resistance is a prevalent and early finding in HIV-infected patients treated with this class of drugs and can occur in the absence of changes in body fat distribution or hyperlipidemia.⁴ Several studies have now established that direct inhibition of the insulin-responsive facilitative glucose transporter isoform 4 (GLUT4) is a primary mechanism by which these drugs acutely alter peripheral glucose disposal.^{5–7}

Correspondence to: Paul W. Hruz.

Reprints: Paul W. Hruz, Washington University School of Medicine, 660 South Euclid Avenue, Campus Box 8208, St. Louis, MO 63110 (e-mail: hruz_p@kids.wustl.edu).

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Whereas the use of all currently available PIs except atazanavir⁸ has been associated with alterations in glucose homeostasis, the percentage of patients affected and the severity of the perturbations in insulin sensitivity differ among the PIs. Indinavir and ritonavir be the most potent at inducing insulin resistance,^{9,10} whereas amprenavir has been reported to have only a modest effect.¹¹ It thus remains unclear whether PIs act in a class-specific manner or whether there are effects that are specific to individual drugs.

Studies in HIV-negative healthy human volunteers have confirmed the prediction made from in vitro data that PIs are capable of inducing acute insulin resistance after a single dose of the drug.^{12,13} Although these studies have established that PIs can acutely alter glucose homeostasis, attempts to compare the effects of PIs in human subjects directly are complicated by factors that are difficult to control. Because of the high degree of protein binding of most PIs,¹⁴ it is difficult to obtain steady-state drug levels rapidly. With repeated dosing, the potential exists for compensatory changes in glucose metabolism in response to acute effects on glucose transport.¹⁵ The serum drug levels achieved during such studies can also vary considerably between individuals. Although clinical trials are extremely valuable in assessing the safety and efficacy of new and existing antiretroviral therapies, such studies present considerable cost and potential risk to volunteer subjects. Whereas the use of animal model systems can never replace the need for comprehensive studies in human subjects, their use can provide important insights into the molecular mechanisms of disease progression and serve as preclinical tools for the evaluation of new candidate drugs.

To compare the relative potency of various PIs to induce insulin resistance in vivo, we investigated the acute effects of PIs on peripheral glucose disposal in a rodent model system in which serum drug levels, genetic background, and environment could be tightly controlled. We report the first direct comparison of the in vivo potencies of PIs and provide evidence that this model system can be useful in predicting adverse effects on glucose homeostasis before clinical trials.

MATERIALS AND METHODS

Materials

2-Deoxyglucose (2-DOG)-1-[3H]-glucose was purchased from Sigma (St. Louis, MO). Atazanavir was obtained from Bristol-Myers Squibb (Princeton, NJ). Kaletra (lopinavir/ritonavir) and Norvir (ritonavir) were obtained from Abbott (Chicago, IL). Agenerase (Amprenavir) was obtained from GlaxoSmithKline (Research Triangle Park, NC). Insulin (Humulin-R) was purchased from Eli-Lilly (Indianapolis, IN). Male Wistar rats (125–150 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 rat adiponectin and rat leptin determination were obtained from ALPCO (Windham, NH). Assay kits for rat insulin determinations were purchased from Linco (St. Charles, MO). Assay kits for rat free fatty acid (FFA) determinations were obtained from Wako Chemicals (Richmond, VA).

Assays

PI levels were determined by the high-performance liquid chromatography (HPLC) method of Foisy and Sommadossi¹⁶ using a Waters 600 HPLC system with a Microsorb C-8 column. Standard curves were generated by adding PIs directly to control rat serum. Using a Model 680 XR Micro plate reader (Bio-Rad, Hercules, CA), insulin, leptin, and adiponectin levels were determined by enzyme-linked immunosorbent assay (ELISA) (A450 nm) using the

manufacturer's specifications. FFA (A540 nm) was determined by the Wako nonesterified fatty acid (NEFA) C test method.

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 maintained in a controlled room with a 12-hour light and dark cycle. The animals were supplied with a standard rat chow diet and water ad libitum. Catheters were inserted into the left external carotid artery and right jugular vein under methohexital anesthesia as previously described.¹⁷ Rats were allowed to recover from the stress of surgery for at least 5 days before performing experiments. All animals weighed between 205 and 220 g at the time experiments were performed. Rats were fasted overnight before each experiment.

Drug Preparation

PIs were extracted from the commercially available capsules and dissolved in ethanol. Because lopinavir was obtained from Kaletra capsules, 20% ritonavir was present in each of the assays examining this PI. The concentrations of active drug were determined by HPLC assay under the same conditions as the serum PI assays. Standard curves were obtained using pure drug obtained from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program.

Primary Rat Adipocyte 2-DOG Uptake

Adipocytes were prepared from Wistar rat epididymal fat pads as described previously.¹⁸ The fat pads were minced and digested with 2 mg/mL of type 1 collagenase for 1 hour at 37°C in Krebs-Ringer bicarbonate HEPES buffer (KRBH; 120 mM of NaCl, 4 mM of KH₂PO₄, 1 mM of MgSO₄, 1 mM of CaCl₂, 10 mM of NaHCO₃, 200 nM of adenosine, and 30 mM of HEPES, pH 7.4) containing 1% fraction V bovine serum albumin (BSA) and 2.5 mM glucose. The dispersed cells were washed with KRBH containing 3% BSA, and a 20% (vol/vol) cell suspension was prepared in the same buffer.

Adipocytes were stimulated with the addition of 1 μ M of insulin for 30 minutes at 37°C. 2-DOG uptake measurements were performed by adding 100 μ L of the cell suspension to 100 μ L of KRBH containing 3% BSA as well as the indicated concentrations of indinavir. After incubating the mixture for 1 minute at 37°C, the assay was initiated with the addition of 20 μ L of 2-DOG label solution (final concentration of 50 μ M, 0.5 μ Ci per assay). After 1 minute at 37°C, the assay was quenched by adding cytochalasin B to a final concentration of 0.4 mM. The cells were separated by spinning the reaction through dionylphthalate oil as described previously,¹⁸ and the intracellular radioactivity was quantified. Nonspecific uptake was measured in the presence of 50 μ M of cytochalasin B and subtracted from the experimental values.

Euglycemic-Hyperinsulinemic Clamp Experiments

Catheters were flushed with normal saline and heparin (40 U/kg) was administered to maintain catheter patency. After determination of fasting blood glucose levels, a constant infusion of PI was started through the venous catheter at a rate of 10 mL/min using a Harvard 11 apparatus pump. After 30 minutes of drug infusion, insulin (10 mU $\cdot$ kg⁻¹ $\cdot$ min⁻¹) in normal saline containing 0.3% BSA was infused through the venous catheter. At 10-minute intervals, 500 μ L of blood was removed from the arterial catheter into a syringe. Blood (5 μ L) 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 100 to 110 mg/dL. Insulin

sensitivity was determined by the average infusion rate during the final 30 minutes of each 90-minute clamp experiment.

Skeletal Muscle and Cardiac 2-DOG Uptake

[3H]-2-DOG (50 μ Ci) was administered through the arterial catheter 30 minutes before the conclusion of the euglycemic-hyperinsulinemic clamp experiments. Blood was collected at 5-minute intervals for determination of the tracer-specific activity. Immediately after euthanasia by sodium pentobarbital administration, rat hind limb muscles (extensor digitorum longus [EDL] and soleus) and hearts were harvested, washed with PBS, and placed in liquid nitrogen pending subsequent analysis. Frozen muscle samples (50–80 mg) were ground with a mortar and pestle, boiled in 1.2 mL of water for 10 minutes, and spun in a microcentrifuge at 15,000 g for 10 minutes. Accumulated 2-DOG-6-phosphate in the supernatant was separated from 2-DOG by ion exchange chromatography using a Dowex 1X-8 (100–200 mesh) anion exchange column.

RESULTS

Primary Rat Adipocyte Glucose Uptake

To determine the relative potencies of PIs in altering GLUT4 activity in vitro, we examined the acute effects of 4 PIs (amprenavir, lopinavir/ritonavir, ritonavir, and atazanavir) on 2-DOG uptake using freshly isolated insulin-stimulated rat adipocytes. This cell type predominantly expresses the GLUT4 glucose transporter.¹⁹ As shown in Figure 1A, all the PIs except atazanavir significantly inhibited zero-trans-2-DOG uptake at therapeutically relevant drug levels. In the presence of 10 mM of amprenavir, ritonavir, or lopinavir, glucose transport was reduced by 24%, 31%, and 40%, respectively, relative to the vehicle-treated controls.

The reduction in glucose uptake was even greater (35%–58%) in the presence of 25 mM of these PIs. Because of the limited solubility of these hydrophobic compounds in the assay medium, it was not possible to definitely establish the dissociation constant of an enzyme-inhibitor complex (K_i) values for these drugs. Similar to indinavir, which has a K_i of 15 mM,⁵ all these drugs had estimated inhibitory constants in the low micromolar range. Atazanavir failed to induce any change in glucose transport activity.

Lopinavir was tested together with ritonavir because both drugs are always used in a combined 4:1 ratio in the clinical setting. As shown in Figure 1B, whereas 2.5 mM of ritonavir alone did not significantly alter glucose transport activity, 5 mM of this PI produced a 15% reduction in 2-DOG uptake. In combination with lopinavir, the 2 drugs produced additive effects on transport activity.

Peripheral Glucose Disposal

All the PIs tested possess a high degree of protein binding in serum, with peak drug levels in the low micromolar range (5–15 mM).²⁰ To determine whether the acute effects of these drugs on glucose uptake in vitro correlate with altered glucose disposal in vivo, we performed euglycemic-hyperinsulinemic clamp experiments on healthy male Wistar rats previously naive to PI exposure. With continuous intravenous infusion of the PIs to age- and weight-matched animals, steady-state drug levels were rapidly achieved and maintained throughout the clamp experiments as shown in Table 1. With the concomitant infusion of exogenous human insulin, steady-state hormone levels were achieved near the upper end of values obtained during a typical glucose tolerance test in these animals.¹⁷ With the clamping of serum glucose levels at 110 mg/dL during insulin infusion, peripheral glucose disposal rates (R_d s) were found to be significantly lower with each of the PIs except atazanavir (Fig. 2). At “therapeutic” drug levels (8–12 mM), inhibition of glucose disposal was 18.4%, 30.6%, and 51.5% for amprenavir,

lopinavir/ritonavir (4:1), and ritonavir, respectively. The same rank order of sensitivity (ritonavir > lopinavir > amprenavir) was also observed at higher drug levels (25–30 mM).

Skeletal Muscle Glucose Uptake

To assess the direct effect of PIs on glucose uptake into skeletal muscle, which accounts for most peripheral glucose disposal,²¹ [3H]-2-DOG was injected into the arterial catheter 30 minutes before the termination of the clamp experiment. The accumulation of the phosphorylated sugar in oxidative and nonoxidative muscle was determined so that direct comparisons on acute changes could be obtained between each of the PIs. As shown in Figure 3A, 2-DOG uptake was significantly reduced with lopinavir and ritonavir in soleus muscle, which contains predominantly type 1 slow-twitch fibers, at therapeutically relevant drug levels.

Only modest and statistically insignificant reductions in glucose uptake were observed in EDL muscle, which contains a higher percentage of type 2b fast-twitch fibers (see Fig. 3B). Uptake into both fiber types was significantly reduced using higher concentrations of lopinavir and ritonavir. As with total peripheral glucose disposal, 25 mM of atazanavir, which is nearly fourfold higher than typical peak drug levels in treated patients, had no effect.

In addition to examining skeletal muscle glucose uptake, the uptake of 2-DOG was measured in the rat heart. Similar to adipose and skeletal muscle tissue, rodent myocytes express GLUT1 and GLUT4.²² In response to exercise and insulin, GLUT4 translocates to the plasma membrane. As shown in Figure 3C, the pattern of glucose transport inhibition under hyperinsulinemic-euglycemic clamp conditions was similar to that seen with skeletal muscle. Lopinavir and ritonavir significantly inhibited glucose transport. Although amprenavir inhibited glucose uptake to a lesser extent, statistical significance was not achieved ($P = 0.07$). Atazanavir had no effect, even at drug levels of 25 mM. The rank order of sensitivity was ritonavir, lopinavir, and then amprenavir.

Adipokine Levels

Serum FFA levels were not elevated in any of the treatment groups. A statistically significant reduction in the normal suppression of FFA levels in response to hyperinsulinemia was observed with atazanavir, ritonavir and lopinavir/ritonavir (4:1) at the highest drug levels, similar to that seen with indinavir.¹⁷ Although compensatory changes in serum adiponectin levels have been reported to occur with more chronic administration of PIs to human subjects,¹⁵ under the short duration of the current studies, no significant changes in leptin or adiponectin levels were observed (Table 1). Thus, the differences observed in peripheral glucose disposal and muscle glucose uptake could not be attributed to differences in serum adipokine levels.

DISCUSSION

We have previously shown that in cultured 3T3-L1 adipocytes, the PIs indinavir, ritonavir, and amprenavir are all capable of acutely inhibiting 2-DOG uptake.⁶ In these studies, however, the PI levels used far exceeded the maximal drug levels that are typically achieved during the treatment of HIV-infected patients. Furthermore, PIs act as isoform selective inhibitors of GLUT4. The measurement of glucose uptake in 3T3-L1 adipocytes, which express GLUT1 and GLUT4 in nearly equal amounts, may significantly underestimate inhibitory activities. Studies in primary rat adipocytes established that acute effects on glucose transport occur at physiologically relevant drug levels.²³ The K_i for indinavir in adipocytes (15 mM) is near the peak drug levels typically achieved during clinical use. Thus, observed effects of this drug on glucose disposal are highly dependent on the serum drug levels achieved during glucose tolerance testing or euglycemic-hyperinsulinemic clamps. Given the different pharmacokinetic

properties of each of the currently available PIs, direct comparisons between the glucose uptake inhibitory properties has been difficult. The current study provides a direct comparison of the acute effects of PIs under tightly controlled experimental conditions.

These data provide support for the association between the acute inhibition of GLUT4 intrinsic activity in vitro and altered peripheral glucose disposal in vivo. All the PIs that inhibited glucose transport in vitro also significantly inhibited glucose disposal in vivo. The difference in magnitude of inhibition in the in vitro setting is most likely attributable to differences in the solubility of these drugs in an aqueous solution. Whereas ritonavir and lopinavir are virtually insoluble in water, amprenavir is slightly soluble.²⁰ Because atazanavir has the greatest aqueous solubility of the 4 PIs tested, it is unlikely that the failure to observe any inhibitory effects is related to solubility issues. It is also possible that the differences in the magnitude of glucose transport inhibition observed in vitro versus in vivo are contributed by differences in protein binding in these 2 systems. Whereas PIs are highly bound to serum proteins in a similar manner in rats and human beings,^{24,25} slight differences in the availability of free drug may lead to significant changes in susceptibility to GLUT4 inhibition. Thus, caution must be made in directly extrapolating rodent data to human beings.

Despite significant differences in drug exposure, patient population, and measurements used among the published clinical studies that have evaluated PI-related changes in insulin sensitivity, a good correlation exists between our animal data and clinically observed changes in glucose homeostasis. Most notably, the lack of GLUT4 inhibition in vitro or significant changes in glucose disposal in vivo found with atazanavir agrees with the clinical observation that this PI does not clinically alter insulin sensitivity.

Our studies also demonstrate that therapeutic levels of amprenavir produce smaller effects on peripheral glucose disposal than ritonavir or lopinavir/ritonavir (4:1). This is in agreement with the findings of a controlled prospective clinical study that reported only a small but statistically insignificant trend toward insulin resistance with amprenavir after 48 weeks of treatment.¹¹ In contrast, more acute treatment of human subjects with lopinavir/ritonavir (4:1) produced a nearly 25% reduction in the glucose disposal-to-insulin ratio.⁸

With long-term exposure to PIs, together with reverse transcriptase inhibitors, as part of HAART, additional metabolic changes in adipokine levels, hyperlipidemia, and lipodystrophy may occur independent of the acute effects of PIs on glucose transport. Efforts to prevent these adverse metabolic effects rest on successful avoidance of the most proximate effects of these drugs on specific cellular targets. It remains to be determined whether GLUT4 inhibition directly contributes to the other metabolic changes observed during HAART. Growing evidence suggests that drugs such as atazanavir that do not alter GLUT4 activity also do not induce hyperlipidemia. Thus, it is possible that the identification of additional PIs that do not acutely inhibit glucose transport may also lead to the prevention of hyperlipidemia and lipodystrophy. In addition to providing a means to compare the relative effects of PIs in vivo directly, testing of PIs in this rodent model system provides the opportunity to investigate tissue-specific effects of these drugs in a manner that is difficult, if not impossible, using human subjects. The current observation of the differential effects of PIs on glucose uptake in soleus versus EDL muscle provides further evidence that the acute in vivo effects of PIs are the result of GLUT4 inactivation. Soleus muscle contains predominantly slow-twitch type 1 fibers, which have higher levels of GLUT4 expression and are more dependent on glucose transport than EDL muscle.²⁶ Thus, the increased sensitivity of soleus muscle to PIs is consistent with an inhibitory effect mediated through alterations in GLUT4 intrinsic activity.

The observation of reduced cardiac glucose uptake in the presence of PIs represents the first evidence that this tissue may also be directly affected by these drugs. A major concern regarding

the cumulative perturbations in glucose and lipid homeostasis observed with PIs is that these metabolic changes may lead to significant cardiovascular morbidity.

Acute effects of these drugs on cardiac myocyte glucose uptake might also contribute to this risk, particularly under acute ischemic conditions. Normally, under nutrient-deprived conditions, the heart effectively shifts to utilization of ketones,²⁷ thereby conserving hepatic glucose production for the brain. The shift to ketone utilization occurs over several hours.²⁸ Because the binding affinities of PIs are near peak therapeutic drug levels, it is possible that with fluctuating peak and trough drug levels, impaired glucose transport is intermittent, preventing sustained induction of ketogenesis.²⁹

The availability of a reliable assay to predict the potential of new candidate PIs to induce insulin resistance in vitro and/or within an isogenic animal model system should greatly facilitate efforts to prevent this adverse effect before the initiation of costly clinical trials. This model system is also likely to provide a facile means to test pharmacologic strategies aimed at ameliorating insulin resistance in patients receiving the currently available PIs.

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FIGURE 1.

Acute effects of PIs on adipocyte glucose uptake. Each of the indicated PIs was added to insulin-stimulated primary rat adipocytes 1 minute before adding 50 mM of [3H]-2-DOG. Cells were incubated for 1 minute at 37°C with gentle agitation before quenching the reaction by separating the cells and reaction mixture through dinonylphthalate as described in the Methods section. Panels A and B represent separate sets of experiments. A, Lopinavir/ritonavir samples contained 2.5 and 6.25 mM of ritonavir together with 10 and 25 mM of lopinavir, respectively. Each data point represents the mean $\pm$ SE of 4 assays. *Statistical significance relative to vehicle-treated controls ($P < 0.001$, analysis of variance of the mean).

FIGURE 2.

Acute effects of PIs on peripheral glucose disposal. Euglycemic-hyperinsulinemic clamps were performed using $10 \text{ mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ of insulin. PIs were administered by constant intravenous infusion to maintain drug levels of 5 to 10 mM for “therapeutic” doses and 25 to 35 mM for “high” doses (see Table 1). Data represent the mean $\pm$ SE of 4 independent experiments per group. * $P < 0.0001$ as determined by analysis of variance of the mean.

FIGURE 3.

Acute effects of PIs on muscle glucose uptake. $[3\text{H}]\text{-DOG}$ was injected into the arterial catheters 30 minutes before the conclusion of the euglycemic-hyperinsulinemic clamp experiments. Muscles were removed from the rats immediately after euthanasia and were analyzed for accumulation of radio-labeled 2-DOG-6-phosphate, as described in the Methods section. A, Soleus muscle. B, EDL muscle. C, Cardiac tissue. Rg' indicates glucose metabolism index. Values represent the means $\pm$ SE of 4 independent experiments. * $P < 0.05$ as determined by analysis of variance of the mean.

TABLE 1.**Euglycemic Hyperinsulinemic Clamps**

	Drug Level (μM)	Rat Weight (gram)	Glucose (mg/dL)	Insulin (ng/mL)	Leptin (pg/dL)	Adiponectin (ng/dL)	FFA (mmol)
Control	0	215 ± 0.28	105 ± 3.99	5.03 ± 1.26	431.2 ± 41.8	3835.2 ± 363.6	0.51 ± 0.15
Atazanavir	25.46 ± 2.16	214 ± 5.26	108 ± 2.59	6.94 ± 1.04	472.9 ± 186.5	2516.0 ± 289.3	1.03 ± 0.07*
Amprenavir	11.26 ± 1.23	219 ± 5.59	105 ± 3.94	6.36 ± 0.81	326.3 ± 170.4	2845.6 ± 328.6	0.59 ± 0.07
	29.56 ± 5.54	216 ± 0.81	111 ± 4.87	5.47 ± 1.51	327.8 ± 49.0	3892.6 ± 271.6	0.78 ± 0.07
Lopinavir/ Ritonavir	10.68 ± 0.57/1.51 ± 0.39	210 ± 4.47	108 ± 4.67	4.85 ± 0.97	375.0 ± 155.1	3687.9 ± 220.5	0.51 ± 0.01
	25.74 ± 1.53/3.54 ± 1.07	219 ± 0.97	112 ± 4.06	8.11 ± 0.93	562.1 ± 283.1	3675.3 ± 662.8	0.86 ± 0.05*
Ritonavir	8.72 ± 0.59	209 ± 9.90	109 ± 2.35	5.45 ± 0.21	323.2 ± 96.0	3070.5 ± 218.4	0.49 ± 0.11
	34.79 ± 3.89	214 ± 2.46	106 ± 8.87	9.11 ± 3.64	534.1 ± 285.0	5282.0 ± 452.8	1.02 ± 0.09*

Data represent the mean ± SD, n = 4 for each group.

* $P < 0.001$ as determined by ANOVA.