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Metabolic interactions of AGE inhibitor pyridoxamine and antioxidant α -lipoic acid following 22 weeks of treatment in obese Zucker rats

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Abstract

Aims—The advanced glycation end product inhibitor pyridoxamine (PYR) and the antioxidant α -lipoic acid (LA) interact to ameliorate insulin resistance in obese Zucker rats following short-term (6-week) treatment. This study was designed to ascertain whether these unique interactive effects of PYR and LA remain manifest following longer-term (22-week) treatment.

Main methods—Female Obese Zucker rats received vehicle (OV), PYR (OP, 60 mg/kg body wt), racemic LA (rac-LA; OM, 92 mg/kg), the R-(+)-enantiomer of LA (R-LA; OR, 92 mg/kg), or combined treatments with PYR and rac-LA (OPM) or PYR and R-LA (OPR), daily for 22 weeks.

Key findings—Individual and combined treatments with PYR, rac-LA, and R-LA significantly ($p < 0.05$) inhibited skeletal muscle protein carbonyls (28–36%), a marker of oxidative damage, and triglyceride levels (21–51%). Plasma free fatty acids were reduced in OM (9%), OR (11%), and OPM (16%), with the greatest decrease (26%) elicited in OPR. HOMA-IR, an index of fasting insulin resistance, was decreased in OP (14%) and OPM (17%) groups, with the greatest inhibition (22%) in OPR. Insulin resistance (glucose-insulin index) was lowered (20%) only in OPR. Insulin-mediated glucose transport in isolated skeletal muscle was improved in OM (34%), OR (33%), OPM (48%) and OPR (31%) groups.

Significance—Important interactions between PYR and LA for improvements in glucose and lipid metabolism in the female obese Zucker rat are manifest following a 22-week treatment regimen, providing further evidence for targeting oxidative stress as a strategy for reducing insulin resistance.

Keywords

Oxidative stress; insulin resistance; skeletal muscle

Introduction

Insulin resistance of skeletal muscle glucose transport activity is an early defect leading to the development of type 2 diabetes (Zierath et al. 2000; Henriksen 2006). While the etiology of

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this muscle insulin resistance is complex and can result from numerous systemic and myocellular defects, one condition that can contribute to the development of insulin resistance is oxidative stress, defined as the imbalance between the cellular production of oxidants and the antioxidant defenses within cells and tissues (reviewed in Evans et al. 2003; Bloch-Damti and Bashan 2005, and Henriksen 2006). Moreover, this oxidative stress-associated insulin resistance can lead to the development of numerous cardiovascular risk factors, such as hypertension, dyslipidemia, atherosclerosis, and central obesity, collectively known as the “insulin resistance syndrome” (DeFronzo and Ferrannini 1991), or the “cardiometabolic syndrome” (Hayden et al. 2006).

Based on this information, numerous investigations have targeted oxidative stress and its sequelae in the design of therapeutic interventions in conditions of insulin resistance (Henriksen 2000, 2006, 2007). We have shown recently that short-term (6-week) treatment of obese Zucker rats, a model of marked whole-body and skeletal muscle insulin resistance that displays many of the pathophysiological characteristics of the cardiometabolic syndrome (Henriksen and Dokken 2006), with pyridoxamine (PYR), an inhibitor of the formation of advanced glycation end products (AGE) (Metz et al. 2003a, 2003b), and the antioxidant α -lipoic acid (LA) (Packer et al. 1995) leads to important interactive effects on metabolic derangements (Muellenbach et al. 2008). For example, 6-week treatment of obese Zucker rats with PYR and the R-(+)-enantiomer of LA (R-LA) in combination caused the largest decreases of fasting plasma glucose, insulin, and free fatty acids (FFA), muscle triglycerides, and whole-body insulin resistance compared to changes brought about by individual treatments with these compounds (Muellenbach et al. 2008). However, it is currently unknown to what degree these unique interactions can be maintained with treatment periods exceeding 6 weeks.

In this context, the purpose of the present investigation was to determine if these beneficial metabolic actions of PYR and LA, alone and in combination, on markers of oxidative damage, plasma and muscle lipids, whole-body glucose tolerance and insulin sensitivity, and insulin-stimulated glucose transport in skeletal muscle remain manifest in the obese Zucker rat following a longer-term, 22-week treatment regimen. Furthermore, a direct comparison was conducted of the relative actions of the racemic mixture of LA (rac-LA, consisting of 50% R-LA and 50% S-LA) and the purified R-LA, individually and in combination with PYR, on these metabolic parameters following 22 weeks of treatment of obese Zucker rats.

Materials and methods

Animals and treatments

All experimental procedures were approved by the University of Arizona Institutional Animal Care and Use Committee. Female lean Zucker (Fa/-) rats and obese Zucker (fa/fa) rats were obtained at 6–7 weeks of age, with treatments commencing after one week. Animals were housed in a temperature-controlled room (20°–22°C) with a 12:12 hour light/dark cycle at the Central Animal Facility of the University of Arizona, and had free access to chow (Teklad 4% fat mouse/rat diet, Madison, WI) and water. Lean Zucker rats served as age-matched, vehicle-treated lean controls (LV group). The obese Zucker rats were randomly assigned to receive by intraperitoneal injection either vehicle (100 mM Tris buffer, pH 7.4) (OV group), pyridoxamine HCl (PYR, 60 mg/kg body wt; Calbiochem, La Jolla, CA) (OP group), the racemic mixture of α -lipoic acid (rac-LA, consisting of 50% R-(+)-LA and 50% S-(–)-LA; 92 mg/kg body wt; BASF, Ludwigshafen, Germany) (OM group), the purified R-enantiomer of LA (R-LA, 92 mg/kg; trometamol salt, BASF) (OR group), PYR and rac-LA in combination (OPM group), or PYR and R-LA in combination (OPR group), daily for 22 weeks. Body weights were obtained every other day.

Measurement of glucose tolerance and plasma variables

At the end of the 22-week treatment period, animals were food-restricted overnight (chow was restricted to 4 g at 5 pm and was consumed immediately) and subjected to an oral glucose tolerance test (OGTT) using a 1 g/kg glucose feeding by gavage. Blood (~0.25 ml) was collected from a cut at the end of the tail immediately before, and at 15, 30, 60, and 120 min after glucose administration. Whole blood was then thoroughly vortexed with EDTA (18 mM final concentration) and centrifuged at 13,000 X g for 30 s to isolate the plasma. The plasma was then removed and stored at -80°C until analysis. Following the blood collection, animals were administered 2.5 ml of 0.9% saline solution subcutaneously to compensate for plasma loss during the OGTT.

Plasma was analyzed for glucose (Thermo Electron, Pittsburgh, PA), insulin (Linco Research, St. Charles, MO), and free fatty acids (Wako, Richmond, VA.) Fasting whole-body insulin sensitivity was estimated using the homeostasis model assessment of insulin resistance (HOMA-IR) by using the formula: [fasting plasma glucose (mg/dl) X fasting plasma insulin (μU/ml)]/405 (Matthews et al. 1985).

Measurement of glucose transport activity

Treatment of the animals was continued for three additional days following the OGTT, at which time the animals were again food restricted overnight (4 g chow at 5 pm). At 8 am, animals were deeply anesthetized with pentobarbitol sodium (50 mg/kg Nembutal ip, Abbott Laboratories, North Chicago, IL), and soleus and plantaris muscles were obtained. The plantaris muscle was immediately frozen between aluminum blocks cooled to the temperature of liquid nitrogen and stored at -80°C until analysis.

The soleus muscle was used to obtain two strips (25–35 mg), which were prepared for in vitro incubation in the unmounted state without tension. Glucose transport activity, assessed using the intracellular accumulation of the glucose analog 2-deoxyglucose, was measured in the absence or presence of insulin (5 mU/ml, Humulin R, Indianapolis, IN), exactly as described previously (Henriksen and Jacob 1995). This method for assessing glucose transport activity in isolated muscle has been validated (Hansen et al. 1994).

Biochemical assays

Pieces (25–40 mg) of frozen plantaris muscle were used for the measurement of protein carbonyl levels, a biomarker for tissue oxidant status, using the spectrophotometric assay of Reznick and Packer (1994). Triglyceride concentrations were determined in other pieces (30–40 mg) of plantaris muscle using the extraction method of Folch et al. (1957) and the processing method of Frayn and Maycock (1980), as modified by Denton and Randle (1967). Tissue was homogenized in extraction buffer (20:10:3 mixture of chloroform:methanol:butylated hydroxytoluene) and incubated at 4°C for 16 hours. Separation of phases was obtained after addition of 0.9% saline, with centrifugation at 3,000 X g for 60 min. The lower (organic) phase was evaporated to dryness under N₂ gas at 60°C for 60–90 min. The sample was reconstituted in extraction buffer and triglyceride concentration was determined spectrophotometrically using an enzymatic colorimetric assay (Sigma Chemical).

Additional pieces of plantaris muscle were used for the assessment of phospho-Akt1/2 ser⁴⁷³, and GLUT-4 protein expression, using immunoblotting with commercially available antibodies, as described previously (Dokken et al. 2005; Ort et al. 2007).

Statistical Analysis

All values are expressed as means ± SE. The significance between the OV group and the respective obese groups treated with PYR, rac-LA, R-LA, the combination of PYR and rac-

LA, or the combination of PYR and R-LA was assessed by one-way analysis of variance (ANOVA) with a post hoc Dunnett test using SPSS computer software (version 16.0, Chicago, IL). The significance of differences between the OV group and LV group was assessed by using an unpaired Student's t-test. A level of $p < 0.05$ was set for statistical significance.

Results

Body weights

Initial and final body weights (in grams) of the obese compound-treated groups (initial weights: OP: 166 ± 10 , OM: 168 ± 8 , OR: 164 ± 5 , OPM: 142 ± 10 , and OPR: 168 ± 5 ; final weights: OP: 584 ± 23 , OM: 571 ± 11 , OR: 566 ± 19 , OPM: 566 ± 19 , and OPR: 592 ± 11) did not differ from the OV group (171 ± 5 and 591 ± 18). Likewise, the gains in body weight over the 22-week treatment period for the OP, OM, OR, OPM, and OPR groups (418 ± 26 g, 403 ± 9 g, 402 ± 20 g, 424 ± 13 g, and 424 ± 13 g, respectively) were not different compared to that of the OV group (420 ± 14 g). In contrast, the initial and final body weights of the LV group (111 ± 4 and 259 ± 7) were significantly less ($p < 0.05$) than those of the OV group.

Skeletal muscle oxidative damage

Protein carbonyls in skeletal muscle were assessed as a biomarker of oxidative damage (Fig. 1). Compared to the LV group, protein carbonyls in plantaris muscle of the OV group were 2.5-fold greater ($p < 0.05$). Inhibition of muscle protein carbonyls relative to the OV group was elicited by individual treatments with PYR (28%), rac-LA (28%), or R-LA (36%), and with combined treatments with PYR and rac-LA (28%) and with PYR and R-LA (36%) (all $p < 0.05$).

Fasting plasma glucose, insulin, and free fatty acids and measure of insulin resistance

Fasting plasma glucose, insulin, and FFA values in the OV group were significantly greater ($p < 0.05$) compared to those of the LV group (Fig. 2), indicative of the marked derangements in metabolic regulation of the obese Zucker rat. Fasting plasma glucose was diminished ($p < 0.05$) by individual treatment with PYR (9%) and by combined treatment with PYR and R-LA (11%), while fasting plasma insulin was significantly reduced compared to the OV group in the obese animals that received either the combination of PYR and rac-LA (7%) or the combination of PYR and R-LA (7%) (both $p < 0.05$). Moreover, significant diminutions ($p < 0.05$) in fasting plasma FFA were observed in the obese animals treated individually with rac-LA (9%) or R-LA (11%) or with the combined treatment with PYR and rac-LA (16%). The greatest decrease in fasting plasma FFA was elicited by combined treatment with PYR and R-LA (26%).

The HOMA-IR value, an index of fasting whole-body insulin resistance (Matthews et al. 1985), was substantially greater (14-fold, $p < 0.05$) in the OV group compared to the LV group (Fig. 2). This index of fasting whole-body insulin resistance was significantly reduced ($p < 0.05$) in the obese animals treated individually with OP (14%) or with the combined treatment with PYR and rac-LA (17%), and to the greatest extent by combined treatment with PYR and R-LA (22%).

Glucose tolerance and insulin sensitivity

The glucose and insulin responses during the oral glucose tolerance test for the various groups are shown in Fig. 3 (top). These data were used to derive the integrated areas under the curve for glucose (AUC_G) and insulin (AUC_I) for the various groups, which are shown in Fig. 3 (bottom) and are quantitatively compared. The AUC_G was 44% greater ($p < 0.05$) in the OV group compared to the LV group (Fig. 3). Compared to the OV group, the AUC_G was reduced ($p < 0.05$) in the OP group (6%), the OM group (7%), the OR group (10%), and the OPM group

(11%), with the greatest reduction in this variable observed in the OPR group (16%). The AUC_i was markedly greater (12-fold, $p < 0.05$) in the OV group compared to the LV group (Fig. 3). Unlike the AUC_g, the long-term treatment with PYR and LA did not alter the AUC_i of the obese animals.

The glucose-insulin index (defined as the product of the AUC_g and the AUC_i) is an index of whole-body insulin resistance in the glucose-fed state (Cortez et al. 1991). Compared to the LV group, the glucose-insulin index was 17-fold higher in the OV group (Fig. 3, bottom), reflective of the profound insulin resistance of these obese animals. While trends for a diminution (10–13%) of the glucose-insulin index were observed in the OP, OM, OR, and OPM groups, the only statistically significant reduction in this parameter was seen in the OPR group (20%, $p < 0.05$).

Muscle glucose transport activity

The basal rates of 2-DG uptake in the soleus muscle did not differ among the various groups (Fig. 4, left panel). The insulin-mediated increase in 2-DG uptake above basal was markedly greater in the isolated soleus muscle of the LV group compared to the OV group (Fig. 4, right panel). In the OP group, there was a small, non-significant increase in the increase in 2-DG uptake above basal due to insulin (10%). In the soleus of the obese animals, the increase in 2-DG uptake above basal due to insulin was significantly ($p < 0.05$) increased following the 22-week treatment rac-LA (34%), R-LA (33%), and combined treatment with PYR and rac-LA (48%) or with PYR and R-LA (31%).

Tissue triglyceride concentrations

Triglycerides in the plantaris muscle of the OV group were 3-fold greater than in the LV group (Fig. 5). Compared to the OV group, muscle triglycerides were reduced ($p < 0.05$) in the OP group (51%), the OM group (21%), the OR group (29%), the OPM group (23%), and the OPR group (31%).

Akt phosphorylation and GLUT-4 protein expression

Neither the level of Akt ser⁴⁷³ phosphorylation, whether expressed in absolute terms or relative to the plasma insulin concentration, nor the protein expression of GLUT-4 or Akt1/2 in the obese plantaris muscle was altered by any of the interventions (data not shown).

Discussion

The present investigation provides the novel finding that long-term (22-week) treatment of obese Zucker rats, a rodent model of marked whole-body and skeletal muscle insulin resistance, with the AGE inhibitor PYR and the antioxidant LA elicits significant enhancements of metabolic regulation, and that important interactions between PYR and LA in facilitating these metabolic improvements are manifest in these animals. Specifically, the 22-week treatment with PYR and LA, alone or in combination, significantly reduced muscle tissue oxidative damage, as skeletal muscle protein carbonyls were decreased in all active treatment groups (Fig. 1). Moreover, in these same groups, muscle triglycerides levels were similarly decreased (Fig. 5). With regard to individual PYR treatment, this 22-week intervention induced a significant diminution of fasting plasma glucose (Fig. 2) and fasting insulin resistance (as reflecting by HOMA-IR) (Fig. 2) and improved glucose tolerance (Fig. 3). Treatment with either rac-LA or R-LA individually caused a significant decrease in fasting plasma FFA (Fig. 2), improved glucose tolerance (Fig. 3), and was associated with enhanced insulin-mediated glucose transport activity in isolated soleus muscle (Fig. 4).

The most critical observations in the present study relate to the important interactive effects between PYR and LA on these metabolic parameters. A decrease in fasting plasma insulin was detected only in the OPM and OPR groups (Fig. 2), and the greatest absolute decreases in fasting plasma FFA and HOMA-IR were seen in the group receiving PYR and R-LA in combination (Fig. 2). Moreover, the greatest enhancement of glucose tolerance and the only significant reduction in whole-body insulin resistance (as reflected by the glucose-insulin index) were elicited in this group receiving both PYR and R-LA (Fig. 3).

One might speculate about the mechanisms underlying these important interactions between PYR and R-LA for the enhancement of whole-body glucose tolerance and insulin sensitivity in the obese Zucker rat following the 22-week treatment regimen. Oxidative stress is reduced both by PYR, via inhibition of receptors for AGE (RAGE), the synthesis of AGE products, and free radical formation (Voziyan et al. 2005), and by R-LA, acting directly as a free radical scavenger (Packer et al. 1995) and as a modulator of cellular redox status (Han et al. 2008). The effects of R-LA to diminish oxidative stress are mediated by the reduction of the compound to dihydrolipoic acid (Moini et al. 2002), a process catalyzed by thioredoxin reductase and E3 enzyme (dihydrolipoamide dehydrogenase) of keto-acid dehydrogenase complexes in the mitochondrion (Haramaki et al., 1997). The decrease in oxidative stress could explain some of the effects of the compounds for improving insulin action (Henriksen 2006). In addition, R-LA treatment induced a reduction in plasma FFA, with the greatest diminution of this variable being seen in the OPR group (Fig. 2). This decrease in plasma FFA in the OPR group could contribute to the robust improvements in glucose tolerance and insulin sensitivity observed in this group, as the decline in plasma FFA should be associated with enhanced insulin action on whole-body glucose disposal, due to a lesser activation of novel PKC isoforms and greater IRS-1-dependent insulin signaling in skeletal muscle (Yu et al. 2002). It should be noted, however, that these improvements in whole-body insulin action with PYR and LA, alone or in combination, were not associated with enhancements of *in vivo* insulin signaling functionality (Akt ser⁴⁷³ phosphorylation) or GLUT-4 protein expression in skeletal muscle of these obese animals. A limitation of this study is that insulin signaling functionality was not assessed *in vitro* in skeletal muscle under insulin-stimulated conditions.

Other explanations for the interactions between PYR and R-LA are certainly possible, and should be investigated in future studies of these compounds. Indeed, the metabolic consequences of the 22-week treatment of the obese Zucker rats with LA could be mediated by their actions as redox modulators independently of any effects on free radicals, a concept recently advanced by Jones (2008) in the context of a “redox hypothesis” whereby oxidative stress is defined in terms of a disruption of thiol elements of redox circuits critical for normal cellular functions.

There are some interesting comparisons of the metabolic responses elicited in the present 22-week treatment regimen with PYR and LA and those facilitated in our previous study utilizing a much shorter (6-week) treatment period (Muellenbach et al. 2008). In both studies, the interventions with PYR and LA, alone or in combination, caused similar decreases in muscle protein carbonyls. However, 6 weeks of treatment with PYR alone did not alter muscle triglycerides or fasting plasma FFA, whereas the 22-week treatment with PYR led to significant decreases in both variables (Fig. 2). In general, the interactions between PYR and R-LA were less pronounced in the obese animals treated for 22 weeks (present study) compared to those treated for 6 weeks (Muellenbach et al. 2008) for reductions in fasting plasma glucose, insulin, and FFA and the HOMA-IR and for improvements in glucose tolerance and whole-body insulin sensitivity. These findings indicate that, in the insulin-resistant obese Zucker rat, the substantial improvements in variables related to whole-body insulin resistance associated with shorter-term (6-week) treatment with PYR and LA in combination become less pronounced following a longer-term (22-week) treatment period.

In the present investigation, direct comparisons of the interactive effects of PYR and rac-LA and those of PYR and R-LA were possible. Whereas rac-LA and R-LA individually elicited similar modifications of the metabolic parameters measured, in many cases the interactions of PYR and R-LA were more robust than the interactions of PYR and rac-LA. For example, the effect of PYR and R-LA in combination to lower fasting plasma FFA was superior to that of combined PYR and rac-LA (Fig. 2). Moreover, the greatest reductions in AUC_g and the only significant lowering of whole-body insulin resistance (the glucose-insulin index) were observed in the OPR group (Fig. 3). The underlying mechanism(s) for these observations remains elusive. However, it is known that the R-enantiomer of LA has a greater metabolic action than the S-enantiomer (Streeper et al. 1997), and the absolute dose of R-LA is twice as great in the OPR group compared to the OPM group. How the R-LA interacts with PYR to elicit these metabolic effects in the obese Zucker rat should be addressed in future investigations.

Interestingly, the 22-week intraperitoneal treatment with PYR in the present study produced more metabolic improvements in the obese Zucker rat compared to a 6-week PYR treatment regimen (Muellenbach et al. 2008). The dose of PYR (60 mg/kg body wt) corresponded to that of the oral PYR treatment investigations of Stitt et al. (2002) and Alderson et al. (2003), which resulted in reduced retinal microvascular damage in the streptozotocin-diabetic rat and improved cardiovascular function and prevention of dyslipidemia in the obese Zucker rat, respectively. After 6 weeks of PYR treatment, the only metabolic improvements elicited in the obese Zucker rat was a significant diminution in the AUG_i and a trend for a decreased glucose-insulin index (Muellenbach et al. 2008). In contrast, after 22 weeks of this PYR treatment in the same animal model, there were significant reductions of fasting plasma glucose (Fig. 2), fasting insulin resistance (HOMA-IR) (Fig. 2), and the AUG_g (Fig. 3) and a trend for a decrease in the glucose-insulin index (Fig. 3). These data indicate that, at least in this rodent model of insulin resistance and pre-diabetes, treatment regimens longer than 6 weeks are required in order to realize the metabolic benefits of PYR.

Conclusion

In conclusion, the present investigation provides evidence for important interactions between the AGE inhibitor PYR and the antioxidant LA (either the racemic mixture or the purified R-enantiomer) following a long-term (22-week) treatment regimen in the obese Zucker rat, resulting in improvements in glucose and lipid metabolism in this rodent model of insulin resistance and pre-diabetes. These results support targeting oxidative stress as a long-term strategy for reducing whole-body and skeletal muscle insulin resistance.

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Fig. 1. Effects of a 22-week treatment of obese Zucker rats with PYR and LA on muscle protein carbonyls. Data are presented as the mean $\pm$ SE for 4–6 animals per group. * $P < 0.05$, LV vs. OV group; ** $P < 0.05$, obese compound-treated group vs. OV group.

Fig. 2.

Effects of a 22-week treatment of obese Zucker rats with PYR and LA on fasting plasma glucose, insulin, and free fatty acids and on fasting insulin resistance. Data are presented as the mean $\pm$ SE for 4–6 animals per group. HOMA-IR units are mg/dl $\times$ μ U/ml. * $P < 0.05$, LV vs. OV group; ** $P < 0.05$, obese compound-treated group vs. OV group.

Fig. 3.

Effects of a 22-week treatment of obese Zucker rats with PYR and LA on glucose and insulin responses during an oral glucose tolerance test. The top panels show the glucose and insulin responses to a 1 g/kg oral glucose load. Error bars for the glucose and insulin responses are obscured by the symbols. The bottom panels show the integrated areas under the curve (AUC) for glucose and insulin derived from data in the top panels, as well as the glucose-insulin index, a measure of insulin resistance, was calculated as the product of the glucose AUC and the insulin AUC. Data are presented as the mean $\pm$ SE for 4–6 animals per group. * $P < 0.05$, LV vs. OV group; ** $P < 0.05$, obese compound-treated group vs. OV group.

Fig. 4.

Effects of a 22-week treatment of obese Zucker rats with PYR and LA on glucose transport activity in soleus muscle. 2-Deoxyglucose uptake in soleus strips was measured in the absence of insulin (open bars) and in the presence of 5 mU/ml insulin (cross-hatched bars) (left panel). The increase in 2-DG uptake above basal due to insulin (filled bars) is also presented (right panel). Data are presented as the mean $\pm$ SE for 4–6 animals per group. * $P < 0.05$, LV vs. OV group; ** $P < 0.05$, obese compound-treated group vs. OV group.

Fig. 5. Effects of a 22-week treatment of obese Zucker rats with PYR and LA on muscle triglyceride levels. At the end of the 22-week treatment period, triglyceride concentrations were determined in plantaris muscles. Data are presented as the mean $\pm$ SE for 4–6 animals per group. * $P < 0.05$, LV vs. OV group; ** $P < 0.05$, obese compound-treated group vs. OV group.