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Pharmacological inhibition of ALDH1A enzymes suppresses weight gain in a mouse model of diet-induced obesity

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Abstract

Retinoic acid (RA) is known to play a role in weight regulation. Because mice without ALDH1A1, a major RA synthesizing enzyme, are resistant to diet-induced obesity, we tested a hypothesis that pharmacological inhibition of RA synthesis can suppress weight gain in a murine model of diet-induced obesity. C57BL/6J male mice were fed a high fat diet (HFD) for 8 weeks to induce obesity and then randomized to a HFD with or without WIN 18,446, an RA synthesis inhibitor, for an additional 9 weeks. Body weight, body composition, energy expenditure, activity, and food intake were measured. Levels of retinoids, lipids, and genes involved in the metabolism of retinoid and lipids were also determined. Mice treated with WIN 18,446 gained significantly less weight and had decreased adipose tissue weight, adipocyte size, and macrophage infiltration in adipose tissue. In addition, we observed higher UCP1 expression in adipose tissues and decreased expression of RA responsive genes and genes involved in fatty acid synthesis in the livers and lungs of mice treated with WIN 18,446. Thus, pharmacological suppression of RA synthesis via inhibition of ALDH1A1 may be a potential new target for treatment of obesity.

Keywords

Retinoids; Enzymes; Energy expenditure; Animal model; Drug development

Introduction

Approximately one third of American adults are obese [1], placing them at increased risk for diabetes, cardiovascular disease, and certain types of cancer [2]. Although the public health impact and cost of obesity care is well described, clinicians and patients continue to struggle

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to effectively manage obesity. Thus, new targets for obesity pharmacotherapy are needed and have the potential to dramatically impact clinical care and health care costs across the US.

Retinoic acid (RA), a biologically active metabolite of vitamin A (retinol), plays critical roles in health of humans and animals [3]. In the body, RA is synthesized by two-step oxidation processes from retinol [4]. The first reversible oxidation step produces retinal which is then used in the second irreversible oxidation step by the aldehyde dehydrogenases, ALDH1A1, ALDH1A2, and ALDH1A3. Except for vision, the majority of physiological effects of vitamin A are mediated through the binding of RA to and subsequent activation of transcription factors, the RA receptors and retinoid X receptors, which control expression of downstream genes [5].

While studying the effects of RA depletion on spermatogenesis, we observed that mice treated with WIN 18,446, an inhibitor of ALDH1A enzymes, had reduced weight gain and decreased visceral adipose tissue weight and adipocyte size [6]. These phenotypic changes were reversed by co-treatment with RA, suggesting that the inhibition of RA synthesis could suppress weight gain. In agreement with this, others have demonstrated that *Aldh1a1*^{-/-} mice are resistance to diet-induced obesity [7–9] and treatment of Ob/Ob mice with citral, a broad inhibitor of ALDHs decreased visceral adipose tissue [9]. These studies suggested that the absence of *Aldh1a1* reduces tissue RA levels (and increases retinal levels) and thus, alters energy metabolism in these tissues [10, 11].

Based on these findings, we hypothesized that pharmacological inhibition of RA synthesis may be a means of treating obesity and tested this by treating diet-induced obese mice with WIN 18,446, an inhibitor of all three major ALDH1A enzymes [12].

Methods

ALDH1A activity assay in cells

Human lung cancer cell line, H1299, was transfected with *ALDH1A1* (OriGene, SC321535) or *ALDH1A2* [6] using Lipofectamin 3000 (Life Technologies) and selected for G418 resistance. Cells were grown in RPMI1640 supplemented with 10% fetal bovine serum at 37°C with 5% CO₂. For the ALDH1A activity test, transfected cells were plated at ~1×10⁴ cells/well on 96 well plates and allowed to attach overnight. The next day, media was changed to contain 1 μM retinal and variable concentrations of WIN 18,446 (0 – 10 μM) for 24 h. For determination of recovery of enzyme activity, cells were washed with PBS 3 times and then provided with fresh media containing 1 μM retinal. Retinal was prepared in ethanol and WIN 18,446 in DMSO. Final concentrations of ethanol or DMSO were <1%. RA production was measured 24h following WIN 18,446 treatment and 0, 3, 7, 24 and 48 hours following the PBS wash by extracting media with an equal volume of acetonitrile containing the internal standards, all-*trans* RA-*d5* and 13-*cis* RA-*d5* (Toronto Research Chemicals) with UPLC-MS [6, 12]. The running solvents, 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile:methanol (60:40, v:v) (B), were run at a flow rate of 0.55 ml/min with linear gradient: 40% B for 0.5 min, 60% by 1.5 min, 80% by 5 min, 95% by 7 min and 40% by 7.5 min.

Animals and diets

C57BL/6J male mice (n=12) were purchased from the Jackson Laboratory at 6 weeks of age and were acclimated for 1 week in specific pathogen free facility [13] at the University of Washington while feeding on an autoclaved standard chow diet (5053, Purina). At 7 weeks of age, mice were switched to a high fat diet (HFD) containing ~60% calories from fat (5WBN, TestDiet) for 8 weeks to induce obesity. Eight-week following the HFD, six mice were switched to the HFD containing WIN 18,446 (1 mg/g diet, 5WBP, TestDiet) and the other six mice were continued with the HFD for additional 9 weeks. Weights were determined weekly for 14 weeks after the HFD initiation. Only male mice were used in this study as they are more susceptible to diet-induced obesity and are the most frequently used model for this purpose. All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Washington.

Fasting glucose concentration and intraperitoneal glucose tolerance test (IPGTT)

Fasting blood glucose levels were determined at baseline, 4, 8 and 12 weeks, and glucose tolerance test were performed at 13 weeks following the HFD initiation as previously described [13].

Body composition, indirect calorimetry and activity

At 15 weeks post HFD initiation, mice were transferred to the UW Nutrition Obesity Research Center Energy Balance and Glucose Metabolism Core and acclimated for 1 week in metabolic cages with the same diet they were fed before the transfer. During this period, mice were singly housed. The methods for determining body composition, food intake, energy expenditure and activity levels were previously reported [14].

Tissue collection

At the end of the study (17 weeks post HFD), all mice were euthanized by CO₂ asphyxiation followed by cardiocentesis. Tissues were collected under yellow lights to prevent degradation of retinoids. Sera were separated and stored at -80°C until triglyceride and retinoid analyses. Liver, lung, brain, testes, and adipose depots (epididymal, retroperitoneal, mesenteric, inguinal, and brown adipose tissue) were dissected, weighed and small pieces were fixed in 10% phosphate-buffered formalin or snap-frozen in liquid nitrogen and stored at -80°C.

Triglyceride and retinoid analysis

Serum and liver triglycerides were determined using a colorimetric assay [13]. Retinol and retinyl ester levels were determined from liver, lung, and serum following previously reported methods [6].

qRT-PCR

Total RNA was extracted from liver and lung using RNeasy Mini Kit, and cDNA was synthesized with a High capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). qPCR was carried out using SYBR Green PCR master mix (Invitrogen, Carlsbad, CA) with gene specific primers designed using PrimerQuest Tool (Integrated DNA

Technologies, Coralville, IA) or obtained from published literature (Supple Table 1). Expression levels are expressed relative to those in mice fed a HFD.

Immunohistochemistry and Histopathology

At necropsy, tissues were taken for histological analysis and were fixed in 10% phosphate-buffered formalin. Tissue processing, sectioning and hematoxylin and eosin (H&E) staining were performed at the Histology and Imaging Core at the University of Washington. Adipocyte size and inflammation was scored on H&E staining of four adipose depots: epididymal, retroperitoneal, mesenteric, and inguinal (Supple Table 2). Each fat depot was scored individually and then scores summed for each animal; max sum score adipose size=12; inflammation=16. Inflammatory infiltrates were determined by F4/80 IHC to be macrophages in a subset of the mesenteric fat samples (data not shown) using methods previously reported [15]. UCP-1 expression was determined semi-quantitatively from white adipose tissue (mesenteric fat) as previously reported [6]. UCP-1 signal was scored for intensity and extent on a 0–4 scale for each category and scores summed (intensity: 0=none; 1=light; 2=medium; 3=dark; extent: 0=none; 1=few individual scattered cells; 2= foci of 10 or fewer cells; 3 foci of > 10 cells or coalescing foci). Liver lipidoses was determined as previously reported [13].

Enzyme activity assay for retinoic acid synthesis in liver and lung

Activity of RA synthesis enzymes were determined using homogenates of liver and lung. Liver and lung (50–80mg) were homogenized in 4mL of 25 mM Tris buffer (pH 7.4) containing 0.25M sucrose, 1mM DTT per gram of tissue with a Polytron. Tissue homogenates were centrifuged at $15,000 \times g$ for 15min, and protein concentrations were determined from the supernatant using a Bio-Rad protein assay kit (cat # 500-0006). Enzyme activity was determined by measuring RA production from 10 μ g supernatant protein in an activity assay buffer (10 mM HEPES, 150 mM KCl, 2mM EDTA, pH 7.5) with 5 μ M all-*trans* retinal and 2mM NAD after 5 min incubation at 37°C. Enzyme activity was quenched by addition of an equal volume of acetonitrile containing 10 nM all-*trans* RA-*d*5 as internal standard. All assays were performed in triplicates.

Statistical analysis

Statistical analyses were carried out using Prism (Graph Pad, La Jolla, CA) statistical software. Student t-test was used for comparison between two groups with statistical significance defined as having a p-value of 0.05 or less.

Results

WIN 18,446 strongly inhibits both human ALDH1A1 and ALDH1A2 in cells

To determine the kinetics of WIN 18,446 mediated inhibition of ALDH1A1 and ALDH1A2, we measured RA production in H1299 cells that express only ALDH1A1 or ALDH1A2 following treatment with WIN 18,446 and retinal. RA production was decreased in response to the increasing concentrations of WIN 18,446 24h following the treatment in both cell lines (Fig. 1). The strong inhibitory effects of WIN 18,446 remained after removal of WIN

18,446 from the media, and RA production was suppressed in a dose-dependent manner even 48 hours after the removal of the WIN 18,446.

WIN 18,446 treatment suppresses weight gain in a mouse model of diet-induced obesity

To test the efficacy of ALDH1A inhibition in the treatment of obesity, diet-induced obese C57BL/6J mice were treated with a HFD with or without WIN18,446. Mice fed a HFD gained weight rapidly, increasing to ~250% of initial weight by 14 weeks following the diet initiation. In comparison, weight of mice treated with WIN 18,446 plateaued even though they continued to consume the HFD (Fig. 2A). At five weeks post WIN 18,446 treatment, glucose tolerance test showed significantly reduced glucose at 15 min following the i.p. glucose injection (Fig. 2B). However, the AUC of blood glucose were not significantly different between the two groups (data not shown).

WIN 18,446 decreases fat mass and increases energy expenditure

Body composition of mice were determined at 8 weeks following the WIN 18,446 treatment. The results suggest that the lower total body weight is mainly due to a reduction in fat mass (Table 1). When fat mass and lean mass were expressed as % body weight, WIN 18,446 treated mice showed lower body fat percent and higher lean mass percent (Table 1). Because *Aldh1a1*^{-/-} mice were resistant to obesity partly due to increased energy expenditure associated with thermogenesis [9], we determined whether WIN 18,446 treatment results in similar changes in obese mice. Energy expenditure and activity were determined over a three-day period (3 light/dark cycles) in metabolic cages at 9 weeks post WIN 18,446 treatment. Mice treated with WIN 18,446 had significantly higher energy expenditure while activity levels did not differ significantly between the two groups (Fig. 2C and D). WIN 18,446 treatment did not influence food intake (2.9 ± 0.12 g/d vs. 2.9 ± 0.11 g/d; HFD vs. HFD + WIN 18,446), suggesting WIN 18,446 did not affect taste or appetite.

WIN 18,446 treatment influences adipose tissue phenotype

Because WIN 18,446 treatment significantly influenced adipose weight, we examined histological changes in adipose tissue. Mice fed a HFD containing WIN 18,446 had significantly smaller adipocytes compared to those fed a HFD (Fig. 3A and B) and had lower adipocyte inflammation (Fig. 3C). In addition, WIN 18,446 treatment significantly increased UCP-1 expression in adipose tissues (Fig 3D, E).

Influence of WIN 18,446 treatment in liver

Aldh1a1^{-/-} mice showed alterations in lipid metabolism in liver [10] and thus, we examined structural changes in liver by histology and determined their triglyceride levels. Varying degree of lipidosis was seen in liver of all mice regardless of WIN 18,446 treatment (Supple Fig1A). In agreement with histological analysis, liver triglyceride levels were not different between the two treatment groups (Suppl Fig 1B). Interestingly, serum levels of triglycerides tend to be lower in mice treated with WIN 18,446, although this did not reach statistical significance (Supple Fig 1C).

Aldh1a1^{-/-} mice were reported to have decreased levels of triglyceride production in the liver that were associated with lower expression levels of fatty acid synthase (*Fasn*) and

acetyl-coA carboxylase (*Acc-1/Acaca*) and sterol regulatory element binding protein 1 (*Srebp-1/Srebf1*) [10]. In agreement with this, we observed significantly decreased expression levels of *Acc-1* in liver of WIN 18,446 treated mice (Supple Fig 1E). However, WIN 18,446 treatment did not alter *Srebp-1* expression levels (Supple Fig 1F) and only mildly decreased *Fasn* (Supple Fig 1D).

Influence of WIN 18,446 on retinoid metabolism

To determine whether WIN 18,446 influence retinoid storage and metabolism, we determined retinol and retinyl ester concentrations in serum, liver, and lung. WIN 18,446 treatment did not change serum retinol levels or retinol and retinyl ester levels in the liver (Supple Table 3). However, both retinol and retinyl ester levels in lung were significantly reduced by WIN 18,446 treatment. Interestingly, while both *Aldh1a1* and *Aldh1a2* mRNA expression levels in liver and lung were not affected by WIN 18,446 treatment, two RA responsive genes, *Cyp26a1* and/or *Lrat* were significantly reduced in liver and lung in response to WIN 18,446 treatment (Fig. 4). Significant reduction in *Cyp26a1* and/or *Lrat* expression levels suggests that RA levels in these tissues might be lower in WIN 18,446 treated mice compared to HFD fed mice. Thus, we examined whether the WIN 18,446 treatment inhibit RA production by *in vitro* enzyme assays using tissue homogenates as an enzyme source (Supple Fig. 2). WIN 18,446 treatment significantly suppresses RA synthesis in liver while it did not induce significant inhibition in lung.

Discussion

We have previously shown that mice fed a control diet (AIN 93M) containing WIN 18,446 had lower body weight that is associated with a change in white adipose tissue (WAT) weight and phenotypes [6]. Co-administration of RA reversed phenotype induced by WIN 18,446 [6], suggesting that inhibition of RA synthesis by ALDH1A enzymes may be a strategy to regulate body weight. WIN 18,446 is a potent inhibitor of RA synthesis by ALDH1A1 and ALDH1A2 *in vitro* [6, 12]. ALDH1A2 inhibition by WIN, 18,446 is not reversible by dialysis, and ALDH1A2 activity was not protected significantly by the presence of substrate [6], suggesting that WIN 18,446 is likely an irreversible inhibitor of ALDH1A2. We tested inhibitory effects of WIN 18,446 against ALDH1A1 and ALDH1A2 using H1299 cells stably transfected with each enzyme. Because H1299 does not have any significant endogenous ALDH activity [16], RA produced in the transfected cells can be attributed to either ALDH1A1 or ALDH1A2. Both enzymes were strongly inhibited by WIN 18,446 in a dose-dependent manner 24 h after the treatment, and removal of the WIN 18,446 did not reverse the dose dependent inhibition of ALDH1A1 or ALDH1A2 up to 48 hours after the removal (Fig. 1). These data suggest that recovery of enzyme activity likely depended on enzymes that did not interact with WIN 18,446 for the first 24h and/or newly synthesized enzymes. More importantly, WIN 18,446 can inhibit two major RA synthesis enzymes in cells, providing us a pharmacological tool to test efficacy of ALDH1A inhibition in the treatment of obesity.

WIN 18,446 treatment was effective in attenuating weight gain in mice fed a HFD and resulted in significant weight differences starting at 3 weeks following the treatment (Fig.

2A). WIN 18,446 treatment reduced both fat and lean mass in mice, resulting in significant weight suppression (Table 1). However, percent fat mass was reduced while percent lean mass was increased with WIN 18,446 treatment, suggesting that fat mass decrease is likely the main contributor to the weight suppression in these mice. While it is unclear why lean body mass is reduced in mice treated with WIN 18,446, plausible explanation may include reduction in intramuscular fat that has been reported with weight loss [17]. These results are consistent with our hypothesis that ALDH1A inhibition may be a target for treatment of obesity. In support of our hypothesis, *Aldh1a1*^{-/-} mice are resistant to diet induced obesity. While it is currently unknown how ALDH1A1 deficiency suppresses weight gain, some of the changes seen in *Aldh1a1*^{-/-} mice suggest plausible mechanisms including increased lipolysis and thermogenic capabilities of WAT [11]. *Aldh1a1*^{-/-} mice fed a HFD had significantly smaller adipocytes likely due to decreased adipogenesis as well as increased lipolysis [9]. Similarly, we found that mice fed a HFD containing WIN 18,446 had significantly smaller adipocytes compared to those fed a HFD (Fig. 3A, B). Additionally, the phenomenon known as “browning” of WAT [18] has been observed in *Aldh1a1*^{-/-} mice. These mice have increased body temperature and expression in WAT of uncoupling protein 1 (UCP-1), a classic BAT marker associated with thermogenesis [11]. We have previously reported WIN 18,446 treatment also significantly induced UCP-1 expression in WAT, which could be down-regulated by RA treatment to the level found in mice fed a control diet [6]. In the current study, we also observed increased UCP-1 expression in adipose tissue and increased energy expenditure in mice fed a HFD containing WIN 18,446 (Fig. 2C, Fig. 3D, E). These data demonstrate that inhibition of RA synthesis by a pharmacological inhibitor increases energy expenditure partly due to increased UCP-1 expression similarly to what was observed in *Aldh1a1*^{-/-} mice.

In addition to the anti-obesity phenotype, *Aldh1a1*^{-/-} mice showed favorable metabolic changes, including lower serum TG levels associated with reduced liver TG production [10]. Similarly, mice treated with WIN 18,446 have significantly lower levels of circulating TG, an effect that again was reversed by RA treatment [6], demonstrating the beneficial effects of RA reduction on lipid metabolism. While serum TG levels were generally lower with WIN 18,446 treatment even when mice were fed a HFD, they were not statistically significantly different from those fed a HFD due to one outlier (Supple Fig. 1C). Although we did not detect any significant changes in hepatic lipodosis or liver TG levels associated with WIN 18,446 treatment (Supple Fig. 1A, B), lipidogenic enzymes such as fatty acid synthase and acetyl coA carboxylase 1 were decreased in liver of animals treated with WIN 18,446 (Supple Fig. 1D, E), similarly to what was reported in *Aldh1a1*^{-/-} mice [10].

WIN 18,446 treatment was associated with increased levels of retinol in circulation and liver and decreased levels of retinol and retinyl esters in the lung in mice fed a control diet [6]. However, in the current study, WIN 18,446 treatment did not result in significant changes in retinol in circulation or in liver (Suppl Table 3). In the previous study, we formulated the diet to include WIN 18,446 at 2 mg/g diet while in this study we lowered the treatment dose to 1 mg/g diet because of a longer treatment period (4 weeks vs. 9 weeks). Thus, it is possible that lower levels of WIN 18,446 in the current study resulted in weaker inhibition of ALDH1A enzymes as inhibition of ALDH1A is dose-dependent (Fig. 1). While levels of retinol and retinyl ester, precursors of RA, in liver did not change significantly, expression of

two RA responsive genes, *Cyp26a1* and *Lrat* were strongly suppressed in WIN 18,446 treated mice (Fig. 4C, D), suggesting that WIN 18,446 treatment reduced RA levels in the liver, resulting in functional consequence, i.e. decreased RA responsive gene expression. Decreased expression levels of *Cyp26a1* in liver were also reported in *Aldh1a1*^{-/-} mice [10]. In agreement with this finding, we also demonstrated that liver homogenates from the mice fed a HFD containing WIN 18,446 produced significantly lower levels of RAs from retinal in comparison to those from mice fed a HFD (Supple Fig.2A). Since WIN 18,446 does not alter *Aldh1a1* and *Aldh1a2* expression levels in the liver (Fig. 4A and B), this data suggest that the treatment inhibits catalytic function of the enzymes rather than suppressing expression of these enzymes.

ALDH1A enzymes catalyze irreversible oxidation of retinal to RAs, and thus, loss of this enzyme would result in an increase in retinal and a decrease in RA levels in tissues. It was suggested that increased retinal contributes to weight suppression through an increase in thermogenic activity [9, 11] while decreased in RA results in improved glucose and lipid metabolism in *Aldh1a1*^{-/-} mice [10]. *Aldh1a1*^{-/-} mice have increased levels of retinal in adipose tissues and high levels of retinal (300 nM – 1 µM) can act as an antagonist of PPAR and RXR pathway [9]. However, we did not detect any significant amounts of retinal in liver, lung or adipose tissues of mice treated with WIN 18,446 in control [6] or HFD. It is possible that this discrepancy is due to less efficient extraction of retinal in our method as we did not condense it with hydroxylamine to produce stable oximes [19, 20]. Alternatively, a decrease in RA following WIN 18,446 treatment suppress weight gain as co-treatment of RA in mice treated with WIN 18,446 gain weight similarly to control mice [6]. Because retinal can be converted to retinol and retinyl esters in tissues, role of RA reduction in weight regulation can be further tested in *Aldh1a1*^{-/-} mice fed a HFD with and without supplemental RA.

In summary, we demonstrated that pharmacological suppression of RA synthesis may be a target to treat obesity. However, WIN 18,446 may not be suitable for obesity treatment due to its side effects, including blocked spermatogenesis and alcohol intolerance [6, 21]. Thus, we propose that development of specific and potent inhibitor of ALDH1A1 is needed for a safe treatment for obesity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Production of retinoic acid in H1299 cells expressing (A) ALDH1A1 and (B) ALDH1A2. Cells were treated with WIN 18,446 and retinal for the first 24 hrs and then washed and replenished with new media containing only retinal (indicated with arrows) for additional 48 hrs. Data are expressed as mean $\pm$ SEM.

Figure 2.

(A) Body weight curve, (B) glucose tolerance test, (C) energy expenditure, and (D) activity in mice fed a high fat diet with or without WIN 18,446. HFD, high fat diet (n=6), HFD + WIN 18,446 (n=6). Data are expressed as mean $\pm$ SEM or individual data points with bars representing mean $\pm$ SEM. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$; unpaired Student's t-test

Figure 3.

(A) H&E stained adipose tissues, (B) adipocyte size and (C) degree of inflammation in four adipose depots, (D) immunohistochemistry staining of UCP1 in mesenteric adipose tissues, and (E) UCP-1 expression levels in mesenteric fat. Mes, mesenteric; Epi, epididymal; Ing, inguinal; Retro, retroperitoneal fat. Original magnification for A and D, 10X. Individual data points are shown with bars representing mean $\pm$ SEM. **, $p < 0.01$; unpaired Student's t-test.

Figure 4. mRNA expression of genes involved in retinoid metabolism in liver (A-D) and lung (E-H) of mice fed a high fat diet with and without WIN 18,446. Expression levels are shown as relative to those found in mice fed a HFD after normalization by β -actin or HPRT. HFD, high fat diet; WIN, WIN 18,446. Individual data points are shown with bars representing mean $\pm$ SEM. *, p<0.05; **, p<0.01; unpaired Student's t-test.

Table 1

Body composition

	HFD (n=6)	HFD + WIN 18,446 (n=6)
Body weight (g)	43.5 ± 1.1	35.2 ± 1.7 ^{**}
Fat mass (g)	17.0 ± 1.2	10.2 ± 1.7 [*]
Lean mass (g)	24.8 ± 0.1	23.1 ± 0.3 ^{***}
Fat (%)	38.7 ± 1.8	28.1 ± 3.6 [*]
Lean (%)	57.2 ± 1.6	66.4 ± 3.3 [*]

^{*}
p<0.05;

^{**}
p<0.01;

^{***}
p<0.001