2-(3,4-Dihydro-2H-pyrrolium-1-yl)-3oxoindan-1-olate (DHPO), a novel, synthetic small molecule that alleviates insulin resistance and lipid abnormalities

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ABSTRACT

Type-2 diabetes is growing at epidemic proportions world-wide. This report describes the effect of a novel, synthetic, small molecule 2-(3,4-dihydro-2H-pyrrolium-1-yl)-3oxoindan-1-olate (DHPO), on metabolic abnormalities in genetic and dietary mouse models of type-2 diabetes. DHPO (20 mg/kg/d i.p. for 21 days) attenuated fasting blood glucose, improved glucose disposal and corrected dyslipidemia in genetic (leptin deficient, ob/ob) and dietary (high-fat-fed) mouse models of insulin resistance. In addition, DHPO augmented 2-deoxy-D-glucose (2DG) uptake in gastrocnemius muscles of wild-type mice and in cultured myotubes. The increase in 2DG-uptake was associated with an increase in the phosphorylation of AMPK (thr-172) and its downstream effector acetyl-CoA carboxylase without any changes in the phosphorylation of Akt of insulin receptor. The AMPK inhibitor, compound C attenuated DHPO-induced glucose-uptake whereas the PI3-kinase inhibitor Wortmannin was less effective. In addition, DHPO failed to augment glucose-uptake in the gastrocnemius muscle from AMPK-α2-transgenic (kinase-dead) mice. Taken together, these results suggest that DHPO is a novel small molecule that alleviates impaired glucose tolerance and lipid abnormalities associated with type-2 diabetes.

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1. Introduction

Insulin resistance concomitant with obesity, type-2 diabetes, hypertension, and other features of the metabolic syndrome is a major risk factor for cardiovascular diseases, the leading cause of mortality and morbidity [1]. The pathogenesis of type-2 diabetes involves progressive development in insulin resistance associated with a defect in insulin secretion, leading to overt hyperglycemia. The molecular mechanisms involved in the process leading to type-2 diabetes remain elusive. Indeed, disruptions in skeletal muscle glucose-uptake, insulin signaling, and glycogen synthesis are evident in insulin resistant subjects up to two decades before their developing diabetes [2]. Insulin resistance is characterized by the impairment of postprandial glucose-uptake by muscle with endogenously secreted insulin. In patients with this condition, insulin levels are 2–4 folds higher than in nondiabetics [3]. Defects

in the insulin receptor function, signaling, glucose transport, and glycogen synthesis are believed to contribute to insulin resistance [4].

Although management of insulin resistance and type-2 diabetes by diet and exercise helps delay or prevent the onset of diabetes [5], for many patients, achievement of tight glucose control is difficult, requiring therapy with drugs which augment the action of insulin. However, compounds which improve insulin sensitivity and glucose intolerance are somewhat limited warranting the discovery and characteriziation of novel molecules targeting various pathways involved in the pathogenesis of type-2 diabetes. During our screening for lipoxygenase inhibitors we serendipitously identified DHPO, 2-(3,4-dihydro-2H-pyrrolium-1-yl)-3oxoindan-1-olate as a lead compound with potent antioxidant properties [6]. The objective of this study was to evaluate the impact of DHPO on cellular and animal models of insulin resistance. Our studies reveal that DHPO markedly improved carbohydrate and lipid abnormalities associated with insulin resistant conditions. Furthermore, investigations into cellular signaling mechanisms suggest that DHPO may be causing its effects *via* the augmentation of the AMP-activated protein kinase (AMPK) signaling pathway.

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2. Materials and methods

2.1. Synthesis of DHPO

To a solution of ninhydrin (2.0 mmol) in methanol, proline (2.0 mmol) was added and the product obtained was separated by vacuum filtration, washed and dried. DHPO was also synthesized by solid state reaction by triturating proline (2.0 mmol) and ninhydrin (2.0 mmol) on a water bath at 40 °C. The completion of the reaction was ascertained by TLC (Silica gel G F254 plate; solvent system: 5% (v/v) methanol in carbon tetrachloride) by monitoring the disappearance of ninhydrin at 254 mm. The structure was characterized by elemental and spectral analysis [6] (Online Fig. 1).

2.2. Animals and treatment protocol

All animal treatment procedures described in this study were approved by the Animal Care and Use Committee at University of Wyoming (Laramie, WY). Animals were housed under well-controlled conditions of temperature (22 ± 2 °C), humidity ($55 \pm 5\%$) and a 12 h/12 h light–dark cycle with access to food and water ad libitum. For the genetic model, homozygous B6.V lep<ob>/J male mice (5-weeks old) were obtained from the Jackson Laboratory (Bar Harbor, ME). For the dietary model, 3-month-old male C57/bL6 mice were randomly assigned to standard chow or high-fat diets (Research Diets, New Brunswick, NJ) for 16 weeks. Both ob/ob ($n = 20$) and the high-fat-diet-fed mice ($n = 20$) were randomly assigned to receive DHPO or vehicle via intraperitoneal injection at a dose of 20 mg/kg/day for 14 and 21 days, respectively. The dosage was determined by pharmacokinetic studies described below.

2.3. DPHO pharmacokinetics

Pharmacokinetic studies were performed in overnight fasted C57BL/6 mice and the serum concentrations of DHPO were determined by liquid chromatography–mass spectrometry (LC/MS). Briefly, aliquots of serum were extracted with acetonitrile, and injected into the LC/MS: mobile phase: 0.3% HCHO and acetonitrile (40:60); stationary phase: Waters C_{18} column (100 mm × 4.6 mm) 0.5 μm; flow-rate: 0.3 ml/min; retention time: 0.51 min; capillary voltage 3.35 kV; cone voltage 40 V; extractor voltage 3 V; RF lens voltage 1.6 V. The source and desolvation temperatures were 110 and 40 °C respectively, whereas the desolvation and cone gas flow were 800 and 20 l/h, respectively. The selected mass-to-charge ratio (m/z) transition of DHPO ions $[M-2]^-$ used in the single ion reaction was 212.3, at a dwell time set at 500 ms.

2.4. Intraperitoneal glucose tolerance test

At the end of the treatment period both ob/ob mice and high-fat-fed mice were subjected to intraperitoneal glucose tolerance test (GTT) as described previously [7]. Briefly, the mice were fasted (~12 h), and glucose challenge was initiated with intraperitoneal injection of glucose (1 g/kg). Glucose levels were determined in blood drops obtained by clipping the tail of the mice immediately before glucose challenge, as well as at 30, 60, and 120 min intervals using AUCC-CHEK Advantage Glucose Analyzer (Roche Diagnostics, IN).

2.5. Determination of serum lipids

Serum levels of triglycerides, total cholesterol and high-density-lipoprotein (HDL) were measured using commercial kits (from Equal Diagnostics, Exton, PA) per manufacturer's protocol.

2.6. Cell culture and treatment

The skeletal muscle cell line C2C12 from adult mouse hind-legs (American Type Culture Collection) was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin under a humidified atmosphere of 5% CO_2 in air and maintained at low confluence. Differentiation was induced in sub-confluent cells by replacing the media with one that contained low serum as reported previously [8]. After differentiation myotubes were rendered quiescent by incubating them with serum-free medium for 12 h and during the last 1 h of quiescence, myotubes were subjected to DHPO-treatment. At the end of the treatment period, the cells were lysed with RIPA buffer containing protease and phosphatase inhibitors, sonicated, centrifuged ($12,000 \times g$ for 20 min at 4 °C), protein concentration of the supernatant determined, and subjected to Western blotting.

2.7. 2-Deoxy-glucose-uptake assay

A 2-deoxy-glucose-uptake assay was performed as previously described [9,10]. Briefly, after serum starvation for 4 h, C2C12 myotubes were washed with Krebs–Ringer phosphate HEPES buffer (KRPH buffer; 10 mM phosphate buffer, pH 7.4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 136 mM NaCl, 4.7 mM KCl, 10 mM HEPES (pH 7.6)) and then incubated without or with DHPO in KRPH buffer for 30 min at 37 °C in the presence of 2-deoxy-[^{3}H]-glucose (0.2 μCi). At the end of the incubation period, the cells were washed three times with ice-cold PBS. The cells were then lysed in PBS containing 0.2 M NaOH, and glucose-uptake was assessed by scintillation counting. For ex vivo glucose-uptake assay, gastrocnemius muscles were dissected out from mice, incubated for 30 min in Krebs Henseleit buffer supplemented with 8 mM glucose and 0.1% bovine serum albumin in the presence or absence of DPHO in an atmosphere containing 95% O_2–5% CO_2. Glucose-uptake was measured as described above except that the muscle strips were freeze-dried, weighed, treated with 1N NaOH prior to determination of the radioactivity count.

2.8. Western blot analysis

The expression and phosphorylation of each protein were analyzed by Western blot analysis [7]. In brief, the harvested cell lysates were subjected to 7.5% SDS-polyacrylamide gel electrophoresis (1:30 bis:acrylamide). Proteins were transferred to a nitrocellulose membrane, and the membranes were then blocked for 1 h at room temperature with 5% non-fat dry milk in Tris buffered saline (TBS) containing 0.1% Tween 20. Immunostaining to detect each protein was achieved with an over-night incubation (4 °C) with a 1:1000 dilution of anti-phospho (Ser-473) Akt (1:1000), anti-phospho (thr-308) Akt (1:1000), IRβ (1:1000), anti-IRS-1 (1:1000), anti-GLUT-4 (1:1000), anti-phospho (thr-172) AMPK (1:1000), AMPK (1:1,000) and anti-beta-actin (1:5000). Proteins were visualized after subsequent incubation with a 1:5000 dilution of anti-mouse or rabbit IgG conjugated to horseradish peroxidase and a SuperSignal Chemiluminescence detection procedure (Pierce Biotechnology Inc.). All antibodies used in this study were from Cell Signaling Technology Inc. (Beverly, MA). Protein concentrations were determined using a bicinchoninic acid assay (Pierce Biotech. Inc.). Three independent experiments were performed for each condition and the intensity of immune-reactive bands was analyzed with a Bio-Rad calibrated densitometer.

2.9. Glucose transporter-4 (GLUT-4) determination

Skeletal muscle membrane proteins were extracted using a membrane protein extraction kit (Biovision) as described by us

previously [9]. The membrane protein was subsequently used for Western blot analysis of glucose transporter-4.

2.10. AMPK kinase activity determination

AMPK kinase activity was determined using AMPK KinEASE[TM] FP Fluorescein Green Assay kit (Millipore). AMPK active enzyme (BIOMOL, Plymouth Meeting, PA) was incubated with different concentrations of DHPO and activity was determined as per the protocol supplied by the manufacturer.

2.11. Data analysis

Data are expressed as mean ± SEM and statistically evaluated using one-way ANOVA followed by Bonferroni *post hoc* test using Graph Pad Prism (Graph Pad Software, Inc., San Diego, CA, USA). A '*p*' value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. DHPO pharmacokinetics

Bioavailability of DHPO in mice was determined following intraperitoneal injections of DHPO (20 mg/kg body weight) and assessing serum DHPO concentrations by HPLC/MS. Pharmacokinetic parameters of DHPO including half-life ($t_{1/2}$), volume of distribution (Vd), clearance, and area under the plasma concentration–time graph (AUC) are given in Online Table 1. The percentage bioavailability of DHPO was 47.7 ± 4.3 following intraperitoneal administration.

3.2. Effect of DPHO on body mass, blood glucose and serum insulin in obese and high-fat-fed mice

As anticipated body, heart, liver, and kidney masses were significantly higher in ob/ob mice compared to lean mice (Online Table 2). In addition, obese mice had higher fasting blood glucose and serum insulin levels compared to the lean counterparts

Table 1
General parameters of vehicle and DHPO-treated high-fat-fed mice.

	Standard chow	HF diet (vehicle)	HF diet (DHPO)
Body mass (g)	26.3 ± 0.9	35.7 ± 5.3[*]	27.0 ± 1.8[#]
Heart mass (g)	0.16 ± 0.0	0.19 ± 0.01	0.17 ± 0.01
Liver mass (g)	1.64 ± 0.07	1.78 ± 0.20	1.64 ± 0.20
Kidney mass (g)	0.32 ± 0.03	0.38 ± 0.08	0.34 ± 0.01
Spleen mass (g)	0.13 ± 0.01	0.12 ± 0.03	0.25 ± 0.11
Blood glucose (mmol/l)	7.11 ± 0.58	10.47 ± 0.74[*]	6.10 ± 0.77[#]
Serum insulin (ng/ml)	0.34 ± 0.02	1.69 ± 0.16[*]	0.74 ± 0.19[#]

Data are means ± SEM.
[*] $p < 0.05$ versus standard chow.
[#] $p < 0.01$ versus high-fat-diet-fed mice.

indicating the presence of insulin resistance. In contrast, obese mice treated with DPHO had significantly lower serum insulin levels compared to the vehicle-treated obese mice. However, fasting blood glucose concentrations did not differ significantly between the DPHO and vehicle-treated obese mice. In the dietary model, high-fat-feeding resulted in elevated body mass compared to the mice that received the standard chow (Table 1) without any significant changes in the masses of various organs (heart, liver, kidney and spleen) following high-fat-feeding. DHPO-treatment resulted in a significant reduction in the overall body mass gain caused by high-fat-feeding. This decrease in weight-gain was not associated with a decrease in food-intake (data not shown). In addition, DPHO-treatment lowered the elevated basal blood glucose and serum insulin levels in high-fat-diet-fed mice. In obese mice, in contrast, there were no changes in the basal blood glucose levels.

3.3. Effect of DHPO on whole-body glucose tolerance

Following an acute intraperitoneal glucose challenge, blood glucose concentrations in lean mice began to drop after peaking at 30 min and returned to nearly baseline values after 120 min (Fig. 1A). In contrast, obese mice exhibited severe hyperglycemia and impaired glucose tolerance as evidenced by the high
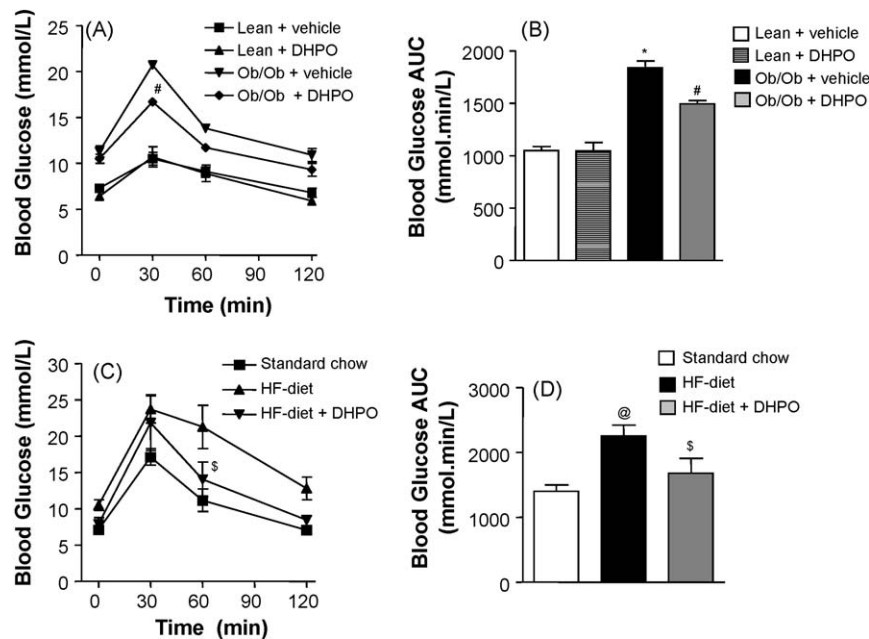

Fig. 1. Effect of DHPO on whole-body glucose-uptake in genetic and dietary mouse models of diabetes. Mice received DPHO (20 mg/kg/day) intraperitoneally for a period of 3-weeks following which blood glucose levels in lean and ob/ob mice (A) and in standard chow and high-fat-diet-fed mice (C) at different time points following intraperitoneal glucose challenge (1 g/kg) were assessed. Panels B and D illustrate the corresponding incremental AUC_{0-120}. Data are represented as means ± SEM, and [*]$p < 0.05$ versus lean control, [#]$p < 0.001$ versus ob/ob control, [@]$p < 0.05$ versus standard chow, [$]$p < 0.05$ versus high-fat-fed mice.
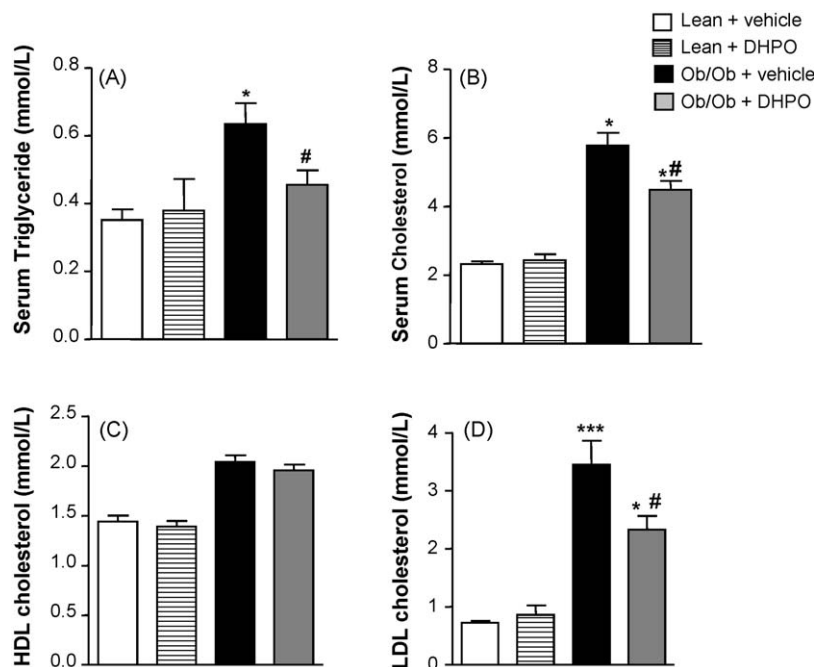
Fig. 2. Effect of DHPO on serum triglyceride (A), cholesterol (B), high-density lipoprotein (C) and low-density-lipoprotein (D) in ob/ob and lean mice following intraperitoneal administration of DPHO (20 mg/kg/day intraperitoneal for 3 weeks). Data are represented as means ± SEM, and *$p < 0.05$ compared to lean control, ***$p < 0.001$ versus lean control, #$p < 0.05$ versus ob/ob control.

post-challenge blood glucose concentrations. Treatment with DHPO facilitated glucose clearance in obese mice with lower post-challenge blood glucose concentrations at all time points compared to the vehicle-treated mice. The total incremental area under the curve (AUC) for blood glucose was significantly higher in the obese mice compared to the lean mice whereas the AUC was significantly attenuated in obese mice that received DHPO (Fig. 1B). Similarly, high-fat-fed mice exhibited sever hyperglycemia and impaired glucose tolerance upon administration of glucose as evidenced by the high post-challenge blood glucose levels (Fig. 1C). As was the case with obese mice, DPHO-treated mice showed lower blood glucose levels. Whereas DHPO showed maximum glucose lowering effect at the 30 min time point following glucose challenge in obese mice, in the high-fat-fed mice the maximum glucose lowering effect was observed at 60 min post-glucose challenge. The total AUC for post-challenge blood glucose was significantly higher in the high-fat-fed mice compared to the mice that received normal chow, which was significantly attenuated by DHPO (Fig. 1D).

3.4. Effect of DHPO on serum lipids

Obese mice exhibited severe dyslipidemia as evidenced by the elevated serum cholesterol, triglycerides (TG), and low-density lipoproteins (LDL)-cholesterol mice compared to lean mice. Interestingly, mice that received DHPO had all these elevated lipid parameters reverted back significantly (Fig. 2A–D). In contrast, serum high-density lipoprotein (HDL) levels were unaltered in all the groups. DHPO-treatment had qualitatively similar effects on serum lipid levels in high-fat-fed animals (Online Fig. 2A-D).

3.5. Effect of in vivo treatment with DHPO on skeletal muscle glucose-uptake

Since skeletal muscle is the major peripheral tissue which plays an important role in energy balance, glucose-uptake and disposal, we next sought to investigate the effect of DHPO-treatment on glucose-uptake in the gastrocnemius muscles isolated from lean and

obese mice. As anticipated, basal glucose-uptake was significantly blunted in the gastrocnemius muscles of obese mice compared to lean mice (Fig. 3). Interestingly, intraperitoneal injections with DHPO for 14 days reversed significantly the effects of obesity on skeletal muscle glucose-uptake as evidenced by higher levels of glucose-uptake compared to the vehicle-treated mice. A trend towards increased muscle glucose-uptake was also observed in the lean mice treated with DHPO but it failed to reach significance.
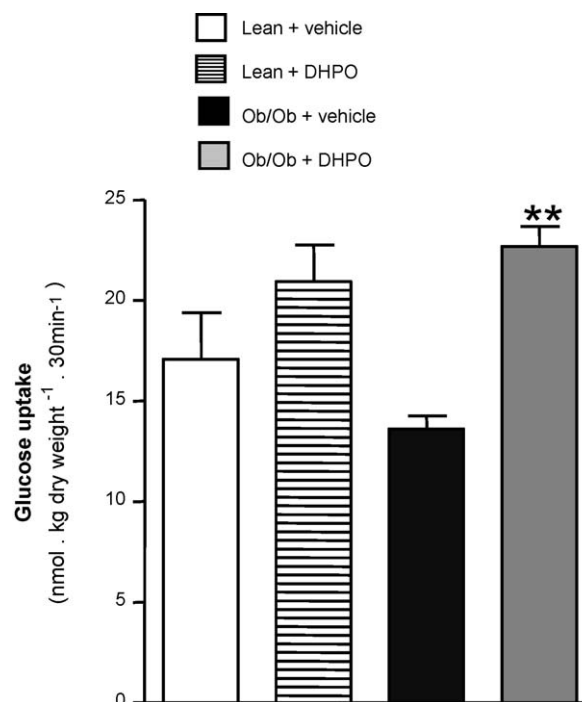

Fig. 3. Effect of DHPO on glucose-uptake in isolated gastrocnemius muscle. Glucose-uptake in gastrocnemius muscle isolated from ob/ob and lean mice treated with DPHO (20 mg/kg/day, intraperitoneal for 3 weeks). Data are represented as means ± SEM, **$p < 0.01$ versus ob/ob control.
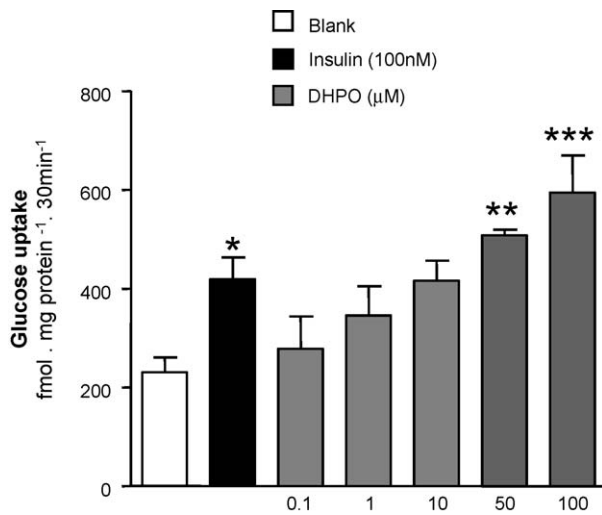
Fig. 4. Effect of DHPO on basal glucose-uptake in cultured C2C12 myoblasts. Values are means ± SEM (n = 3–4 independent experiments). $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus blank. Insulin (100 nM) was used as a positive control.

3.6. Effect of DHPO on glucose-uptake in cultured, differentiated myotubes

To investigate whether DHPO directly acts on the skeletal muscles to cause glucose-uptake, we next studied the effect of DHPO on glucose-uptake in cultured myotubes. DHPO-treatment resulted in a concentration-dependent increase in glucose-uptake in cultured myotubes with a 2-fold increase over basal glucose-uptake was observed at a concentration of 100 μM of DHPO

(Fig. 4). Interestingly, the glucose-uptake stimulated by a 10 μM concentration of DHPO was comparable to that observed with 100 nM insulin. DHPO at the concentrations used in the experiments did not cause any overt cellular damage (data not shown).

3.7. Effect of DHPO on insulin and AMPK-signaling cascade

To understand the mechanisms of DHPO-stimulated glucose-uptake we investigated the effect of DHPO-treatment on key cellular mediators of insulin signaling. To this end, we treated cultured myotubes with DHPO and assessed the phosphorylation levels of Akt, a key downstream effector of insulin signaling. As illustrated in Fig. 5A, treatment with DHPO failed to induce phosphorylation of Akt (ser-473) at the concentrations used whereas treatment with insulin resulted in a pronounced phosphorylation of Akt. Neither did treatment of DHPO cause phosphorylation of the insulin receptor beta (data not shown).

Since muscle cells can uptake glucose independent of insulin signaling, via the activation of AMPK, we determined the phosphorylation (hence activity) of AMPK at the thr-172 residue in cultured myotubes following DHPO-treatment. Interestingly, in treatment with DHPO resulted in a small but significant phosphorylation of AMPK thr-172 as shown in Fig. 5B. In addition to AMPK phosphorylation, DHPO-treatment also resulted in the phosphorylation of acetyl co-A-carboxylase (ACC) a downstream substrate of AMPK at the Ser-79 residue. The phosphorylation of ACC was observed at lower concentrations of DHPO compared to that required to cause AMPK phosphorylation.

We next used pharmacological inhibitors of the PI3-kinase pathway and the AMPK-pathway Wortmannin and compound C respectively in an effort to elucidate the mechanism of DHPO. As expected, Wortmannin at concentration of 1 μM completely
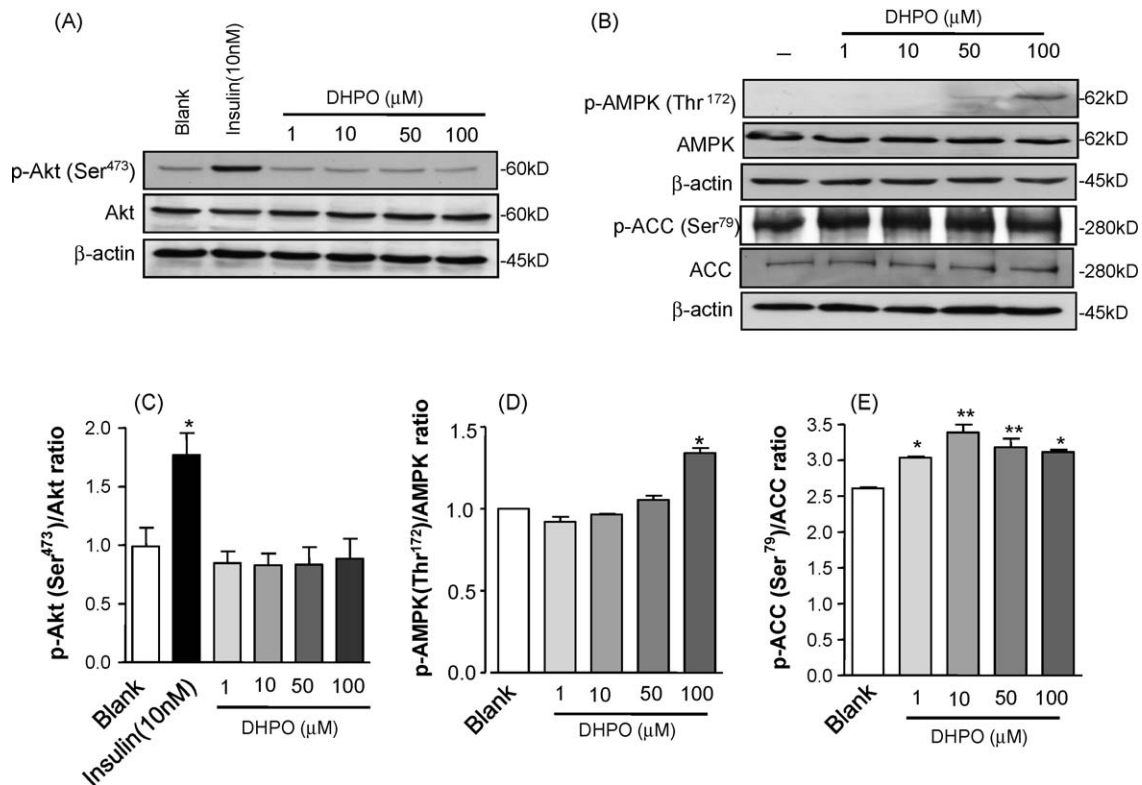

Fig. 5. Effect of DHPO on Akt, AMPK, and ACC phosphorylation in differentiated C2C12 myoblast. C2C12 myotubes were stimulated with the indicated doses of DHPO and were Western blotted against antibodies that recognize phospho-Akt, Akt, beta-actin phospho-AMPK, phospho-ACC, AMPK, and ACC. Panel A is a representative Western blot for p-Akt, Akt and beta-actin and pane B is a representative Western blot for p-AMPK, AMPK, p-ACC, ACC and beta-actin. Relative densities of respective phosphorylated to total protein and/or beta-actin are shown in lower panels (C–E). Values are represented as mean ± SEM, n = 4–6. $^*p < 0.05$, $^{**}p < 0.01$ versus control.
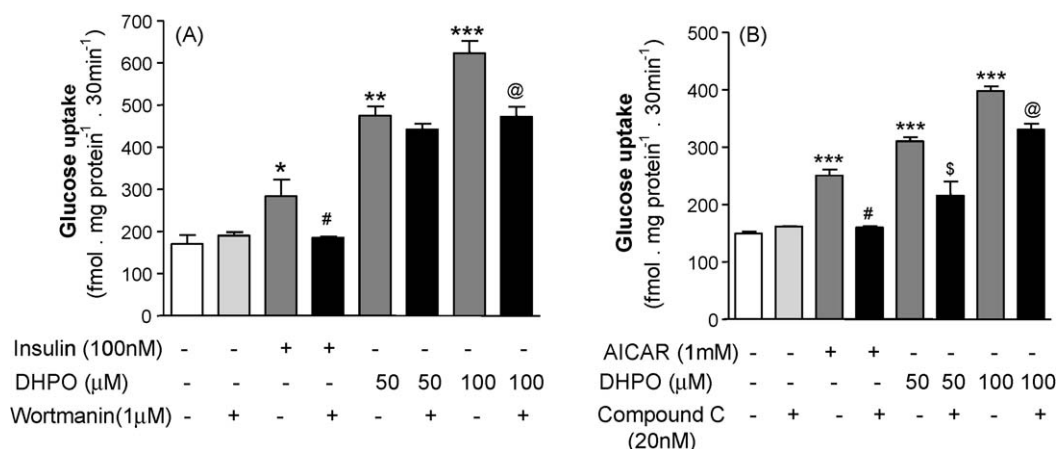
Fig. 6. Effect of Wortmanin and compound C on DHPO-stimulated basal glucose-uptake in cultured C2C12 myoblasts. A. Wortmanin (1 mM) significantly ($p < 0.01$) reduced DHPO (100 mM)-stimulated glucose-uptake. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus basal; $^\#p < 0.01$ versus insulin 100 nM; $^@p < 0.05$ versus DHPO 100 μM. (B) Compound C (20 mM) significantly reduced DHPO-induced glucose-uptake. $^{***}p < 0.001$ versus basal; $^\#p < 0.01$ versus AICAR (1 mM); $^\$p < 0.01$ versus DHPO (50 μM); $^@p < 0.05$ versus DHPO (100 μM). Values are represented as mean $\pm$ SEM, $n = 3$–4 independent experiments.

inhibited insulin (100 nM)-stimulated glucose-uptake in myo-tubes (Fig. 6A) whereas it failed to inhibit DHPO-stimulated glucose-uptake. When the concentration of DHPO was doubled to 100 μM a partial, but significant inhibition of DHPO-stimulated glucose-uptake was observed. In contrast, treatment with com-pound C caused a 58% inhibition of DHPO (50 μM)-stimulated glucose-uptake (Fig. 6B). When the DHPO concentration was raised to 100 μM, the inhibition caused by compound C reduced to 41% indicating competition kinetics. As expected compound C, completely blocked the glucose-uptake stimulated by 5-aminoi-midazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), a phar-macological activator of AMPK.

3.8. Effect of DHPO on skeletal muscle glucose-uptake in transgenic, AMPK kinase-dead mice

To ascertain the role of AMPK in DHPO-induced glucose-uptake, we next investigated the effects of DHPO on glucose-uptake in the gastrocnemius muscles of wild-type mice and transgenic mice with muscle-specific over-expression of a kinase-dead (KD) from of the catalytic α-2 subunit of AMPK [11]. To ascertain the KD-phenotype, we used AICAR to stimulate muscle glucose-uptake in the isolated gastrocnemius muscle of these transgenic mice. Data presented in Fig. 7 demonstrate that incubation with AICAR failed to stimulate muscle glucose-uptake in ex vivo preparations of gastrocnemius muscle from the transgenic AMPK-KD mice. Interestingly, similar to AICAR, incubation with DHPO failed to stimulate muscle glucose-uptake in the KD mice, while it stimulated glucose-uptake in the muscles from the wild-type, C57/BL6 mice.

3.9. Effect of DHPO treatment on skeletal muscle AMPK-signaling and GLUT-4 translocation

Because we found changes in augmentation of AMPK-signaling in cultured cells treated with DHPO we were interested in investigating the in vivo effects of DHPO on these pathways. As shown in Fig. 8 the skeletal muscles of obese mice had lower levels of phosphorylated-AMPK compared to lean mice. Obese mice receiving DHPO had higher levels of phospho-AMPK which did not differ significantly from that observed in lean mice. However, no significant changes were seen in the phospho-ACC levels in any of the groups. We also measured the extent of Glut-4 translocation to the membrane in the skeletal muscle in both the obese and lean

mice. Glut-4 levels were significantly lower in the obese mice compared to lean mice. DHPO-treated obese mice had elevated membrane Glut-4 levels compared to vehicle-treated obese mice.

3.10. Effect of DHPO-treatment on liver insulin signaling and AMPK-pathways

We also assessed the effect of DHPO-treatment on insulin and AMPK-signaling in the liver of lean and obese mice following treatment with DHPO. As shown in Fig. 9, obesity markedly reduced protein levels of IRβ, phospho-IRS-1 and phospho (thr-308)-Akt in the liver of obese mice compared to lean controls. No changes were seen in Akt (Ser-473), phospho-AMPK or phospho-ACC. DHPO-treatment did not alter the phosphorylation status of any of these markers in the livers or either obese or lean mice. Interestingly, DHPO-treated lean mice reduced IRS-phosphoryla-tion levels in the lean mice without altering it in the obese mice.

3.11. Effect of DHPO on AMPK kinase activity

Next we sought to investigate whether DHPO is a direct activator of AMPK in an in vitro assay by incubating DHPO with recombinant AMPK and estimating the fluorescence of an AMPK-peptide substrate. We first prepared standard curves to ascertain concentration- and time-dependent phosphorylation of the pep-tide substrate by AMPK (data not shown). Based on these standard curves, 5 ng of AMPK was chosen and the incubation period was set for 60 min. As shown in online Fig. 3, DHPO had no effect on AMPK-mediated phosphorylation of the substrate peptide at the concentration range of 0–100 μM. Neither did preincubating DHPO with AMPK for different time periods (0–60 min) affect the phosphorylation of the peptide substrate (data not shown).

4. Discussion

The major findings of our study are that a novel, synthetic small molecule, DHPO alleviates glucose intolerance in genetic (leptin deficient ob/ob) and dietary (high-fat-fed) mouse models of insulin resistance. DHPO-treatment markedly improved the impaired glucose-uptake in the gastrocnemius muscles in obese mice, suggesting that DPHO-treatment augments peripheral glucose utilization. Interestingly, DHPO-treatment was also associated with a reduction in the gain in body mass in the high-fat-fed mice. In addition to improving carbohydrate metabolism, DHPO-treatment
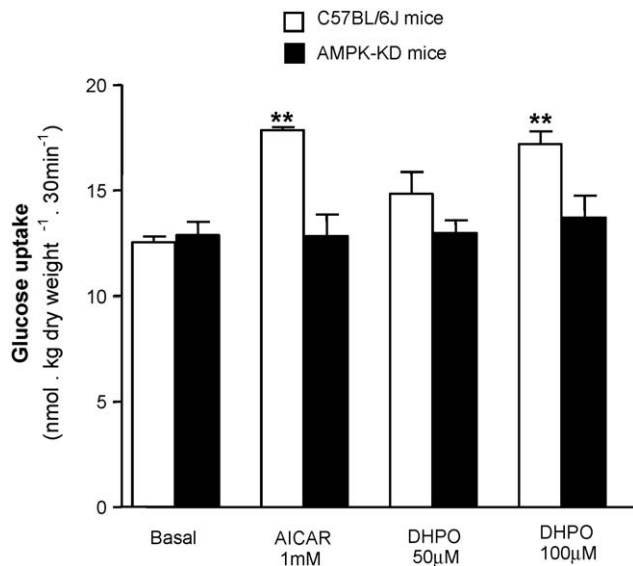
Fig. 7. Effect of DHPO on glucose-uptake in isolated gastrocnemius muscle in AMPK-KD mice. DHPO-stimulated glucose-uptake in gastrocnemius muscle from C57/BL6 mice, but failed to show similar effect in muscle preparations from AMPK-KD mice. Values are represented as mean ± SEM, $n = 4$–5 mice in each group. $**p < 0.01$ versus basal glucose-uptake.

corrected the lipid abnormalities associated with obesity and high-fat diet. Skeletal muscles from obese mice showed significantly attenuated glucose-uptake compared to lean mice which was reversed in mice that were treated with DHPO. Of great interest are the experiments that demonstrate the lack of effect of DHPO on glucose-uptake in AMPK-KD mice. These results strongly suggest that DHPO may be mediating its effects by modulating the AMPK-signaling pathway.

To further ascertain the role of AMPK and insulin signaling pathways in DHPO-mediated augmentation of glucose-uptake we

investigated the effects of DHPO on glucose transport in cultured myotubes in the presence of compound C and Wortmannin. As expected, in cultured myotubes DHPO augmented glucose-uptake in a concentration-dependent manner. There was however a discrepancy in the concentrations mediating the in vivo and in vitro activity. Whereas in vivo serum concentrations of DHPO ranged in the nanomolar levels, micromolar concentrations were necessary for in vitro activity. Although we are unable to reconcile this difference a potential explanation may be the accumulation of the drug on chronic treatment and/or the presence of active metabolites of DHPO that may be responsible for the activity. In addition to augmenting glucose-uptake, DHPO caused an increase in phosphorylation of AMPK and its downstream substrate ACC. However, contrary to our expectations both compound C and Wortmannin only modestly inhibited DHPO's effect, while these compounds completely blocked the effects of insulin and AICAR. In addition, DHPO failed to alter the activity of AMPK in an in vitro kinase assay. Taken together, these results seem to suggest that DHPO may not be mediating its activity by directly activating AMPK, although it is likely that AMPK is necessary for its actions. It is also plausible that other unknown mechanisms in addition to augmenting the AMPK-signaling may contribute to the observed biological effects of DHPO.

Insulin mediates its action by binding to the e insulin receptor (IR), a disulfide-bonded heterotetrameric membrane protein [12]. Binding of insulin to the alpha-subunit of IR causes conformational changes that lead to autophosphorylation of the membrane-spanning beta-subunit and activation of the receptor's intrinsic tyrosine kinase activity [13]. Thus activated insulin receptor transphosphorylates several downstream substrates (on Tyr residues) including insulin receptor substrate (IRS) proteins, causing the activation of Akt and subsequent translocation of Glut-4 glucose transporter vesicles to the cell-surface resulting in glucose-uptake. In our studies DHPO failed to augment any of these pathways suggesting that DHPO may not affect the insulin signaling pathway. Interestingly however, we did observe an
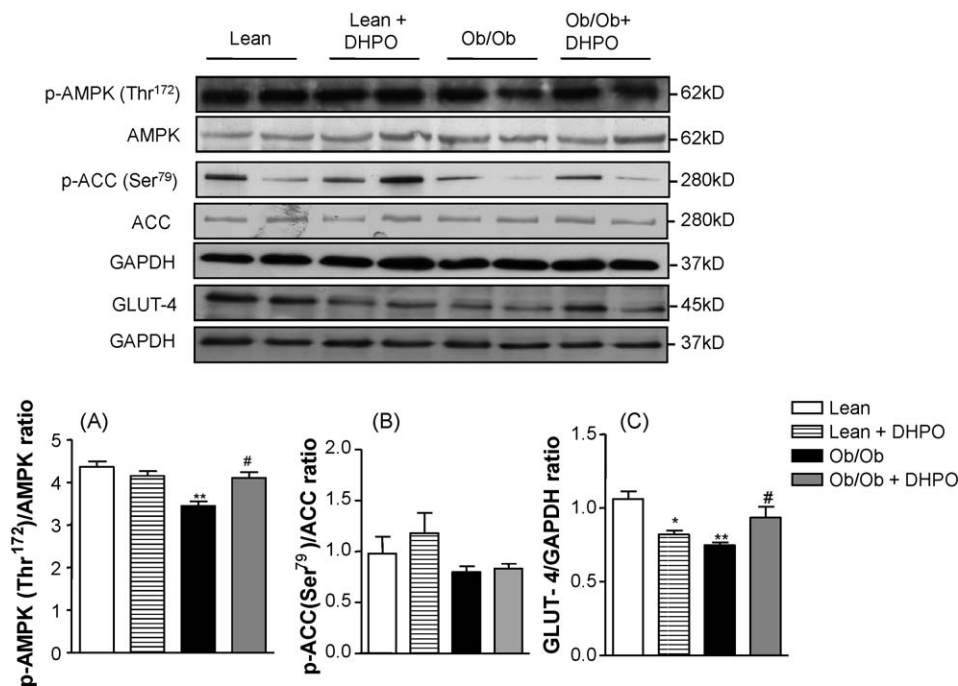

Fig. 8. Effect of DHPO on skeletal muscle AMPK-signaling and membrane associated GLUT-4 in ob/ob and lean mice following intraperitoneal administration of DPHO (20 mg/kg/day intraperitoneal for 3 weeks). Upper panel is a representative Western blot and lower panels are relative densities of phosphorylated to total AMPK, phosphorylated ACC to total ACC and membrane associated GLUT-4. Glyceraldehyde 3-phosphate dehydrogenase was used as a loading control. Values are represented as mean ± SEM, $n = 4$–6. $*p < 0.05$, $**p < 0.01$ versus Lean and $\#p < 0.05$ versus ob/ob.
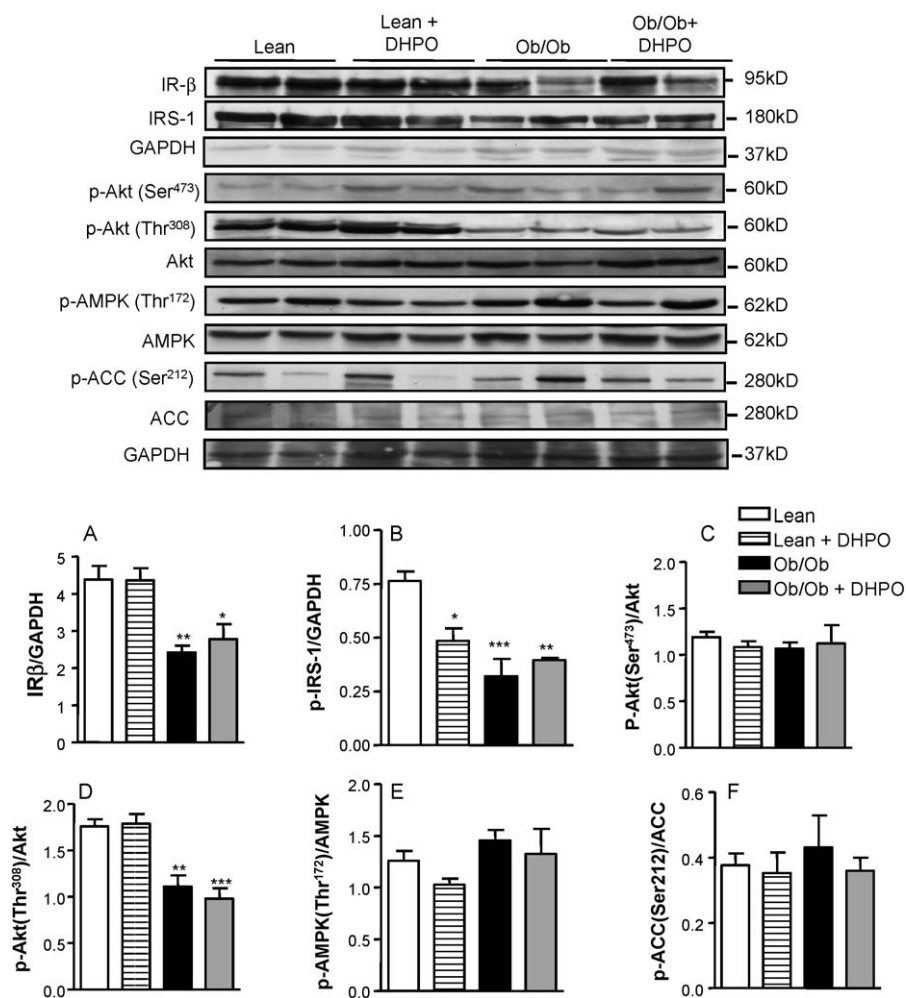
Fig. 9. Effect of DHPO on liver insulin and AMPK-signaling in ob/ob and lean mice following intraperitoneal administration of DPHO (20 mg/kg/day intraperitoneal for 3 weeks). Upper panel is a representative Western blot and lower panels are relative densities of IR-β, phospho-IRS-1, phosphorylated Ser[473] and Thr[308] to total-Akt, phosphorylated to total AMPK and phosphorylated ACC to total ACC. Glyceraldehyde 3-phosphate dehydrogenase was used as a loading control. Values are represented as mean ± SEM, n = 4–6. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.01$ versus lean.

augmentation of Glut-4 translocation in response to DHPO-treatment.

Recent studies have shown that in skeletal muscle, glucose-uptake can be induced in an insulin-independent manner *via* the activation of AMPK [14], which plays a pivotal role in the regulation of carbohydrate and fat metabolism [15,16]. Consequently, AMPK has emerged as a novel and attractive therapeutic target to treat or prevent diseases and symptoms associated with impaired carbohydrate and fat metabolism. Muscle-specific expression of a kinase-dead form of the catalytic α-2 subunit of AMPK in mice results in an insulin resistant phenotype whereas, over-expression of this subunit results in lowering of blood glucose and increased fatty acid oxidation [17]. At the cellular level, AMPK is thought to mediate cellular glucose-uptake by increasing glut-4 translocation to the cell-surface [18]. Interestingly, insulin sensitizing activity of the antidiabetic drugs such as metformin and thiazolidinediones has been attributed, at least in part, to their stimulation of AMPK activity [19]. The ability of DHPO to cause Glut-4 translocation independent of insulin and apparent lack of glucose-uptake in skeletal muscle of AMPK-KO mice suggest that DHPO may be mediating its effects, at least in part, by activating the AMPK-pathway.

AMPK is activated by known antidiabetic drugs such as metformin and rosiglitazone [19] and by a variety of structurally unrelated molecules that include, AICAR [20], A-769662 [21], resveratrol [22,23] and caffeic acid phenylethyl ester [24]. Cool and

coworkers have characterized novel thienopyridones as selective activators of AMPK, and have demonstrated that these molecules alleviate metabolic symptoms and dyslipidemia in mice [21,25]. More recently, Pang and coworkers have characterized a novel small molecule activator of AMPK called PT1 capable of docking with AMPK near the auto-inhibitory domain and directly relieve the auto-inhibition [26]. Activation of AMPK requires phosphorylation of thr-172 in the catalytic α-subunit activation T-loop which is regulated by several upstream kinases and downstream phosphatases [27]. The diversity in the chemical structure of compounds that have been reported thus far to cause specific activation of AMPK suggests that these molecules may be working by different mechanisms or at multiple targets in the AMP-signaling cascade. Both compound C (to a greater extent) and Wortmannin (to a lesser extent) suppressed glucose-uptake induced by DHPO in myotubes although both compounds failed to completely negate the effects of DHPO suggesting that DHPO may be mediating its effect by targeting multiple pathways. On the other hand, the increase in phosphorylation of thr-172 and lack of phosphorylation of Akt, in cells treated with DHPO suggest that AMPK-pathway may be playing a predominant role in mediating the effects of DHPO, although contributions from other unknown pathways cannot be ruled out.

In the present study we also found that treatment with DHPO results in a robust attenuation of serum LDL and triglyceride levels which are elevated in obese mice. The role of AMPK–ACC pathway

in lipid metabolisms has been well documented [28,29]. ACC catalyzes the biosynthesis of malonyl-CoA, the primary substrate for fatty acid biosynthesis as well as a potent inhibitor of carnitine palmitoyltransferase I, the rate-limiting step for mitochondrial fatty acid oxidation. Previous studies have demonstrated that the lipid-lowering properties of leptin and metformin may be mediated *via* the activation of AMPK [30,31]. However, hepatic p-ACC levels did not differ significantly (although a trend towards reduction was observed) between obese mice treated with vehicle and DHPO suggesting that the reduction in LDL-levels caused by DHPO may be mediated by other factors beyond ACC phosphorylation that affect fatty acid synthesis or oxidation, such as sterol regulatory element-binding proteins. Interestingly, there were no changes in serum HDL-levels following treatment with DHPO in both the ob/ob and high-fat-fed mice. *ob/ob* mice used in this study may not be an ideal model to study HDL owing to the fact that the basal HDL-cholesterol levels are significantly higher in these animals compared to the lean controls [32]. It is however interesting that high-fat feeding did not show a significant reduction of HDL in our experiments. It is likely that the short duration of feeding may be the reason for this lack of change in serum HDL levels. Further extensive studies, with long duration feeding of high-fat diet will be needed to understand the effects of DHPO on HDL levels and to reveal the critical mediators of the lipid-lowering effect of DHPO.

Although DHPO altered AMPK-pathway in the peripheral tissues, treatment with DHPO failed to change insulin signaling pathway or AMPK-signaling pathway in the liver—the phosphorylation status of AMPK, ACC and IRβ remained unchanged following DHPO treatment in the hepatic tissues of obese and lean mice. This suggests that DHPO may not be affecting insulin/AMPK-signaling in the liver and may be mediating its effect on glucose-uptake by acting predominantly in the periphery. A discrepancy however is the IRS-1 phosphorylation in the liver tissue which was significantly lowered by DHPO in lean mice compared to vehicle treatment. This attenuation of IRS-1 phosphorylation did not alter the phosphorylation status of its downstream effector Akt and therefore the significance of the reduced IRS-1 phosphorylation in response to DHPO-treatment is difficult to explain.

In summary, these results suggest that DHPO may be used as a template to develop newer, more potent and selective pharmacological agents to treat or control insulin resistance observed with obesity and type-2 diabetes. It also supports the view that activation of AMPK may represent a potential therapeutic option in the treatment of diabetes and its complications.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.09.018.

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