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AMERICAN JOURNAL OF PHYSIOLOGY
ENDOCRINOLOGY AND METABOLISM

Am J Physiol Endocrinol Metab 320: E619–E628, 2021.
First published February 1, 2021; doi:10.1152/ajpendo.00324.2020

RESEARCH ARTICLE

Prolonged β_2-adrenergic agonist treatment improves glucose homeostasis in diet-induced obese UCP1$^{-/-}$ mice

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Abstract

Prolonged supplementation with the β_2-agonist clenbuterol improves glucose homeostasis in diabetic rodents, likely via β_2-adrenoceptor (β_2-AR)-mediated effects in the skeletal muscle and liver. However, since rodents have, in contrast to—especially diabetic—humans, substantial quantities of brown adipose tissue (BAT) and clenbuterol has affinity to β_1- and β_3-ARs, the contribution of BAT to these improvements is unclear. Therefore, we investigated clenbuterol-mediated improvements in glucose homeostasis in uncoupling protein 1-deficient (*UCP1*$^{-/-}$) mice, lacking thermogenic BAT, versus wild-type (WT) mice. Anesthetized WT and *UCP1*$^{-/-}$ C57Bl/6 mice were injected with saline or clenbuterol and whole body oxygen consumption was measured. Furthermore, male WT and *UCP1*$^{-/-}$ C57Bl/6 mice were subjected to 17-wk of chow feeding, high-fat feeding, or high-fat feeding with clenbuterol treatment between *weeks 13* and *17*. Body composition was measured weekly with MRI. Oral glucose tolerance and insulin tolerance tests were performed in *week 15* and *17*, respectively. Clenbuterol increased oxygen consumption approximately twofold in WT mice. This increase was blunted in *UCP1*$^{-/-}$ mice, indicating clenbuterol-mediated activation of BAT thermogenesis. High-fat feeding induced diabetogenic phenotypes in both genotypes. However, low-dose clenbuterol treatment for 2 wk significantly reduced fasting blood glucose by 12.9% in WT and 14.8% in *UCP1*$^{-/-}$ mice. Clenbuterol treatment improved glucose and insulin tolerance in both genotypes compared with HFD controls and normalized to chow-fed control mice independent of body mass and composition alterations. Clenbuterol improved whole body glucose homeostasis independent of UCP1. Given the low human abundancy of BAT, β_2-AR agonist treatment provides a potential novel route for glucose disposal in diabetic humans.

NEW & NOTEWORTHY Improvements in whole body glucose homeostasis of rodents upon prolonged β_2-adrenergic agonist supplementation could potentially be attributed to UCP1-mediated BAT thermogenesis. Indeed, we show that acute injection with the β_2-AR agonist clenbuterol induces BAT activation in mice. However, we also demonstrate that prolonged clenbuterol supplementation robustly improves whole body glucose and insulin tolerance in a similar way in both DIO WT and *UCP1*$^{-/-}$ mice, indicating that β_2-AR agonist supplementation improves whole body glucose homeostasis independent of UCP1-mediated BAT thermogenesis.

β_2-adrenergic agonist; brown adipose tissue; skeletal muscle; type 2 diabetes mellitus; UCP1

INTRODUCTION

Skeletal muscle insulin resistance is a primary factor underlying an impaired postprandial glucose clearance and a major hallmark in the development of type 2 diabetes mellitus (T2DM) (1, 2). The possibility to stimulate skeletal muscle glucose uptake in an insulin-independent manner could therefore significantly contribute to a positive disease outcome. In this context, we have previously shown that stimulation of β_2-adrenergic receptors (β_2-AR) in L6 muscle cells and human primary myotubes enhances glucose uptake via glucose transporter 4 (GLUT4) translocation independent of both the insulin signaling and AMPK pathways, namely, through activation of the mammalian target of rapamycin complex 2 (mTORC2) (3). In line with these findings, prolonged activation of β_2-AR in diet-induced obese (DIO) mice through treatment with the β_2-adrenergic agonist clenbuterol significantly increased in vivo skeletal muscle glucose uptake (4). Furthermore, we and others have shown that prolonged clenbuterol supplementation is associated with robust improvements in both glucose and insulin tolerance of insulin-resistant rodents (3, 5–7), in a dose-dependent manner (4).

Despite the aforementioned effects on skeletal muscle, it cannot be excluded that other tissues also contribute to the favorable effects on whole body glucose homeostasis seen after prolonged β_2-AR stimulation. This is especially of importance for brown adipose tissue (BAT) since clenbuterol has an additional (low) affinity to both β_1- and β_3-adrenergic receptors (8), the primary receptors involved in BAT

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Submitted 23 June 2020 / Revised 7 January 2021 / Accepted 20 January 2021

activation (9). BAT functions as an important thermogenic organ, increasing energy expenditure and the dissipation of heat during cold exposure through uncoupling of mitochondrial oxidative phosphorylation via uncoupling protein 1 (UCP1) (9). Due to its capacity to enhance energy expenditure and to clear large amounts of glucose from the blood (9–11), BAT has been a prominent target for the treatment of T2DM and its therapeutic potential has been highlighted by several rodent studies. Thus, activation of BAT through cold exposure (12, 13) or treatment with the selective β_3-AR agonist CL-316.243 (14, 15) has been shown to robustly improve glucose homeostasis in both mice and rats. In addition, transplantation of BAT into the visceral or dorsal interscapular region in mice has been shown to protect against the detrimental effects of HFD feeding on glucose and insulin tolerance (16, 17), further highlighting the importance of the BAT in the regulation of glucose homeostasis.

Taken together, it can be argued that the previously observed beneficial effects of prolonged β_2-AR stimulation on whole body glucose tolerance could—at least in part—be mediated by BAT thermogenesis, as previously suggested (5). In the current study, we therefore first investigated the ability of the β_2-AR agonist clenbuterol to acutely activate BAT thermogenesis in vivo in mice. Subsequently, we investigated the role of BAT thermogenesis in the metabolic effects of prolonged clenbuterol treatment by studying *UCP1*$^{-/-}$ mice, who are deprived of UCP1-mediated BAT thermogenesis (18, 19). In short, we demonstrate that acute clenbuterol administration activates BAT thermogenesis in vivo in mice. However, prolonged low-dose clenbuterol supplementation robustly improved glucose and insulin tolerance in both WT and *UCP1*$^{-/-}$ mice, indicating that these effects also occur in the absence of the thermogenic function of BAT. We also show here that prolonged β_2-AR stimulation is capable of normalizing glucose and insulin tolerance to a similar level as seen in lean, healthy, chow-fed mice.

MATERIALS AND METHODS

Animals and Ethical Approval

C57Bl/6 WT and *UCP1*$^{-/-}$ mice were bred and housed at the animal facility of Stockholm University. All mice were kept at thermoneutrality (30°C) in a temperature-controlled room with a 12-h light/dark cycle and had access to water and food ad libitum. During the clenbuterol intervention period, all mice were single-caged. All animal experiments were approved by the North Stockholm Ethics Committee for Care and Use of Laboratory Animals.

Study Designs

Acute effects of clenbuterol injection on BAT thermogenesis.

After 2 wk of acclimatization to 30°C, 13–16-wk-old male WT (n = 5) and *UCP1*$^{-/-}$ mice (n = 8) were anesthetized via an intraperitoneal pentobarbital (70 mg/kg) injection. Mice were placed in metabolic chambers (Promethion, Sable systems) for 20 min to assess the basal metabolic rate. Afterwards, mice were injected intraperitoneally with either saline or clenbuterol (1 mg/kg) in a randomized order and oxygen consumption was measured for 30 min. After a wash-out period of 2 wk, the experiment was repeated in a crossover design. Average baseline and stimulated oxygen consumption were measured over a 10-min stable period and these data were corrected for lean mass. Data of mice that woke up during the procedure were not used.

Effect of prolonged clenbuterol supplementation on the glucose homeostasis in UCP1$^{-/-}$ mice.

At 8 wk of age, 49 male WT and *UCP1*$^{-/-}$ mice (n = 25 and n = 24, respectively) were randomized into two groups receiving a 13-wk run-in diet: a low-fat diet control group (n = 8/genotype) (R70 Lactamin, Sweden) or a high-fat diet (HFD) group (n = 16–17/genotype) (45% kcal from fat, 17% kcal from sucrose, D12451, Research Diets, New Brunswick) to induce obesity and glucose intolerance (Fig. 1). An intraperitoneal glucose tolerance test (IpGTT) and insulin tolerance test (ITT) were performed after 12 and 13 wk of the run-in diet, respectively. After 13 wk, the low-fat control group continued on the chow diet whereas the HFD group of each genotype was further subdivided into two groups that were closely matched for body weight, fat mass, lean mass, and glucose and insulin tolerance: a HFD group (n = 8–9) and a HFD group receiving a 33-day, low-dose (3 mg/L in the drinking water) clenbuterol (clenbuterol hydrochloride, C5423, Sigma Aldrich, ≥95%) treatment (n = 8), as described previously (4).

From the start of the low-dose clenbuterol treatment (t = 0), body composition was measured weekly by means of magnetic resonance imaging (MRI) (EchoMRI-100, Echo Medical Systems). An oral glucose tolerance test (oGTT) and insulin tolerance test (ITT) were performed after 2 and 4 wk of treatment (t = 2 and 4), respectively (Fig. 1). Five days after the ITT, mice were euthanized by means of CO_2, 10 min after an insulin injection (5 U/kg lean mass). The heart was perfused with 20 mL ice-cold phosphate buffer solution (PBS) and gastrocnemius muscle and interscapular BAT were isolated. Tissues were stored at −80°C until further analyses.

Glucose and Insulin Tolerance Tests

Before GTT or ITT, mice were fasted for 5 h during which clenbuterol treatment was temporarily ceased. For the GTT, mice were administered a glucose solution dissolved in saline (2.5 g/kg lean mass) via intraperitoneal injection (IpGTT) or oral gavage (oGTT). During the ITT, mice were injected with 1 U/kg lean mass of human insulin (Actrapid, Novo Nordisk, Denmark). During both GTT and ITT, blood glucose concentrations were measured at t = 0, 15, 30, 60, 90, and 120 min in blood collected from a cut of the tail tip by means of an Accucheck Aviva (Roche Diagnostics Scandinavia AB, Stockholm, Sweden). In addition, blood samples were collected at t = 0, 15, and 30 during the oGTT. Blood plasma was isolated and plasma insulin concentrations were measured by means of an ELISA (Ultra-Sensitive Mouse Insulin ELISA KIT, No. 90080, Crystal Chem) according to the manufacturers' instructions.

Western Blot

Western blotting was performed for quantification of insulin signaling in skeletal muscle and BAT. Gastrocnemius muscle and interscapular BAT samples were homogenized in Bio-Plex Cell Lysis buffer (BioRad Laboratories; Veenendaal, The

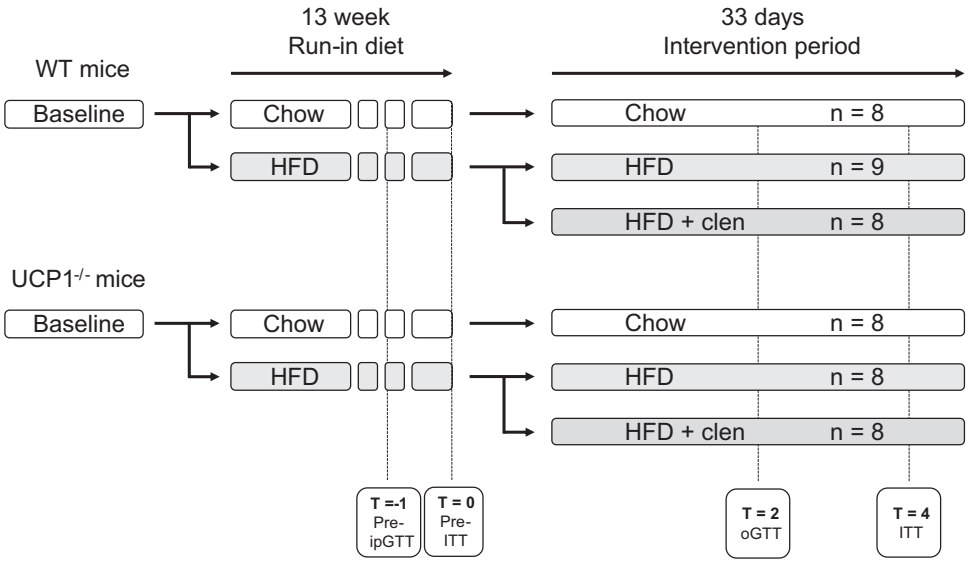

Figure 1. Study design. WT and *UCP1*$^{-/-}$ mice were put on a 13-wk run-in diet consisting of either chow or high-fat diet (HFD). An ipGTT and ITT were performed after 12 and 13 wk of the run-in diet ($t = -1$ and $t = 0$), respectively. Afterwards, the HFD group of each genotype was further subdivided into a HFD control group and a HFD intervention group which received low-dose clenbuterol supplemented water (3 mg/L) for 33 days. At the start of the intervention, HFD groups were matched for body mass, body composition, and glucose and insulin tolerance. An oGTT and ITT were performed in all groups after 2 and 4 wk of treatment ($t = 2$ and $t = 4$), respectively. ipGTT, intraperitoneal glucose tolerance test; ITT, insulin tolerance test; oGTT, oral glucose tolerance test; *UCP1*$^{-/-}$, uncoupling protein 1 deficient; WT, wild-type.

Netherlands). Equal amounts of protein were loaded on stain-free 4%–12% TGX gels (Bio-Rad Laboratories) and after electrophoresis transferred to a nitrocellulose membrane (Trans-Blot Turbo Transfer System, Bio-Rad laboratories). Blots were incubated overnight with primary antibodies: phospho-Akt (Ser473) (1:2,500, No. 9271, Cell Signalling, Bioké, Leiden, The Netherlands) or Akt (1:2,500, No. 9272; Cell Signalling). After incubation with the appropriate IRDye-conjugated secondary antibodies (LI-COR, Bad-Homburg, Germany), proteins were detected and quantified using a CLx Odyssey Near Infrared Imager (LI-COR).

Statistical Analyses

All data were statistically analyzed with Graphpad Prism 9.0 (GraphPad Software Inc. La Jolla, CA). All data were assessed for normality by means of Kolmogorov–Smirnov test. Paired data of the acute experiment were analyzed by means of a paired Student's t test in case of normality or a nonparametric Wilcoxon matched-pairs signed rank test in case of nonnormally distributed data. Data acquired during the prolonged clenbuterol supplementation were analyzed by means of different statistical tests. Body weight and composition were analyzed between the three groups (chow, HFD, and HFD + clen) of either WT or *UCP1*$^{-/-}$ mice by means of a two-way ANOVA with a Tukey's post hoc test. Other comparisons between the three groups of each genotype were tested for significance via one-way ANOVA with Tukey's post hoc test if data were normally distributed. In case of nonnormally distributed data, a nonparametric Kruskal–Wallis test was used. Data were considered statistically significant if $P < 0.05$. All data are expressed as means ± SE.

RESULTS

Acute Clenbuterol Injection Activates BAT in Vivo in Mice

To investigate the ability of clenbuterol to activate BAT in vivo in mice, pentobarbital anaesthetized WT and *UCP1*$^{-/-}$ mice were injected acutely with either saline or clenbuterol (1 mg/kg), followed by the measurement of whole body oxygen consumption. As expected, saline injection did not increase whole body oxygen consumption, neither in WT nor in *UCP1*$^{-/-}$ mice (Fig. 2, *A–C*). In contrast, clenbuterol markedly increased whole body oxygen consumption by 88.94% in WT mice ($P = 0.0003$, Fig. 2, *A* and *C*). The oxygen consumption in *UCP1*$^{-/-}$ mice upon clenbuterol injection was blunted, but still significantly increased by 46.52% ($P < 0.0001$, Fig. 2, *B* and *C*). Combined, these results strongly suggest that clenbuterol activates BAT nonshivering thermogenesis in vivo in mice, although the increased oxygen consumption is partly dependent on other metabolic processes as these effects were blunted, but not abolished, in *UCP1*$^{-/-}$ mice.

Prolonged Low-Dose Clenbuterol Supplementation Does Not Affect Body Composition

We next investigated whether prolonged low-dose clenbuterol treatment would improve glucose homeostasis in both WT and *UCP1*$^{-/-}$ DIO mice (Fig. 1). Before this treatment period ($t = 0$), both HFD + clen groups of WT and *UCP1*$^{-/-}$ mice were similar in body weight and body composition as compared with their HFD counterparts (Fig. 3). Obviously, body weight and fat mass were significantly increased in all HFD groups as compared to their respective chow-fed control groups (Fig. 3, *A*, *B*, *E*, and *F*). During the treatment period, the change in body weight was similar between HFD and HFD + clen groups of both WT and *UCP1*$^{-/-}$ mice and body weight averaged 44.36 ± 0.93 g versus 44.36 ± 1.33 g in WT and 44.84 ± 0.88 g versus 44.30 ± 1.86 g in *UCP1*$^{-/-}$ mice after the intervention, respectively (NS for all, Fig. 3, *A* and *B*). Lean and fat mass remained unaffected during the HFD + clenbuterol treatment in both genotypes as compared with HFD alone (Fig. 3, *C–F*).

Clenbuterol Treatment Ameliorates Detrimental Effects of HFD Feeding on Glucose Tolerance in Both WT and UCP1$^{-/-}$ Mice

Fasting glucose was similarly increased in HFD and HFD + clen groups of both genotypes as compared to their respective

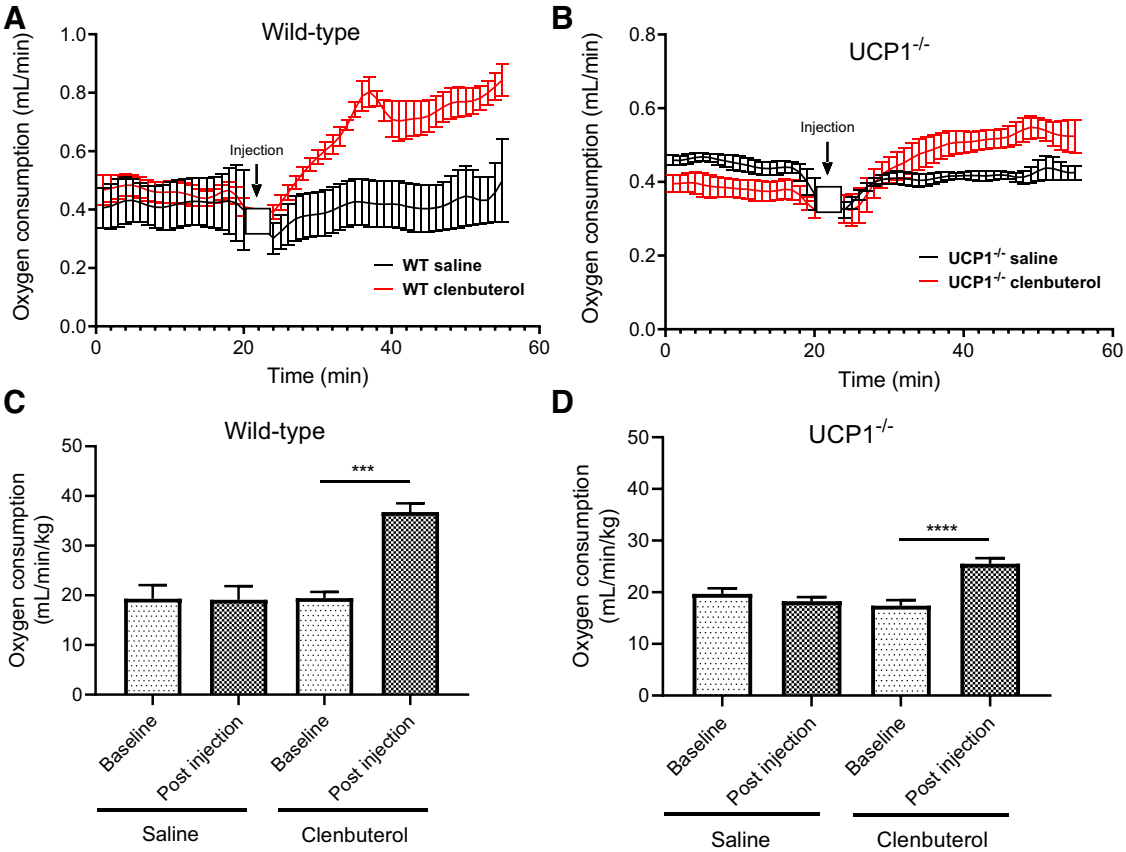

Figure 2. Clenbuterol activates brown adipose tissue *in vivo* in mice. *A*: oxygen consumption in male WT mice upon saline ($n = 4$) and clenbuterol injection ($n = 5$). *B*: oxygen consumption in male *UCP1*$^{-/-}$ mice upon saline ($n = 6$) and clenbuterol injection ($n = 6$). *C*: average oxygen consumption in basal and stimulated state of WT mice. *D*: average oxygen consumption in the basal and stimulated state of *UCP1*$^{-/-}$ mice. Data were analyzed by means of a Student's paired *t* test or nonparametrical Wilcoxon matched-pairs signed-ranked test. ***$P < 0.001$, ****$P < 0.0001$. *UCP1*$^{-/-}$, uncoupling protein 1 deficient; WT, wild-type.

chow-fed control groups before the start of clenbuterol treatment (Supplemental Fig. S1; all Supplemental material is available at https://doi.org/10.6084/m9.figshare.13526363). Following 2 wk of clenbuterol treatment, fasting glucose was significantly lowered by 12.9% and 14.8% for WT- and *UCP1*$^{-/-}$ treated mice as compared with their respective HFD control group ($P = 0.0115$ and $P = 0.0001$), respectively (Fig. 4*A*). For both genotypes, fasting glucose in HFD + clen groups remained significantly higher than chow-fed control groups ($P = 0.023$ and $P = 0.034$ for WT and *UCP1*$^{-/-}$, respectively, Fig. 4*A*).

Before clenbuterol treatment, glucose tolerance was comparable between the HFD and HFD + clen groups of WT as well as *UCP1*$^{-/-}$ mice (Supplemental Fig. S1) and significantly reduced in comparison with the respective chow-fed control group (Supplemental Fig. S1). Upon 2 wk of clenbuterol treatment, glucose tolerance was significantly improved in both WT and *UCP1*$^{-/-}$ mice as compared to their HFD controls (AUC: $P = 0.0166$ and $P = 0.0003$, respectively, Fig. 4, *B–D*). Remarkably, clenbuterol treatment normalized glucose clearance from the blood to similar values as lean, healthy chow-fed control mice (AUC WT: 1,194 ± 44.9 vs. 1,192 ± 29.7 and *UCP1*$^{-/-}$: 1,192 ± 32.5 vs. 1,109 ± 26.0, NS for all, respectively, Fig. 4*B*).

As acute β_2-AR administration is associated with increased insulin release (4, 20), we next investigated if these improvements in glucose tolerance were paralleled by elevated plasma insulin concentrations. Clenbuterol treatment did however not affect plasma insulin levels of HFD + clen WT mice as compared with HFD alone, neither in the fasting state nor during the oGTT (Fig. 4*E*). In contrast, plasma insulin levels were significantly reduced in *UCP1*$^{-/-}$ mice upon HFD + clenbuterol treatment as compared with HFD, both in the fasting state and 30 min following the glucose bolus ($P = 0.0102$ and $P = 0.0103$, respectively) (Fig. 4*F*).

Insulin Tolerance Normalizes to Similar Values as Healthy Chow-Fed Mice upon Clenbuterol Treatment in Both WT and UCP1$^{-/-}$ DIO Mice Independent of Skeletal Muscle and BAT Insulin Signaling

After 4 wk of treatment, fasting blood glucose levels of HFD + clen *UCP1*$^{-/-}$ mice remained significantly lowered by 11.7% as compared with their HFD controls ($P = 0.002$, Fig. 5*A*) and were similar to the fasting glucose of their chow-fed control group (7.09 ± 0.23 mM vs. 6.66 ± 0.07 mM, $P = 0.198$, Fig. 5*A*). However, 4-wk HFD + clenbuterol treatment in WT mice did not result in a lowered fasting glucose as compared with HFD alone ($P = 0.235$, Fig. 5*A*).

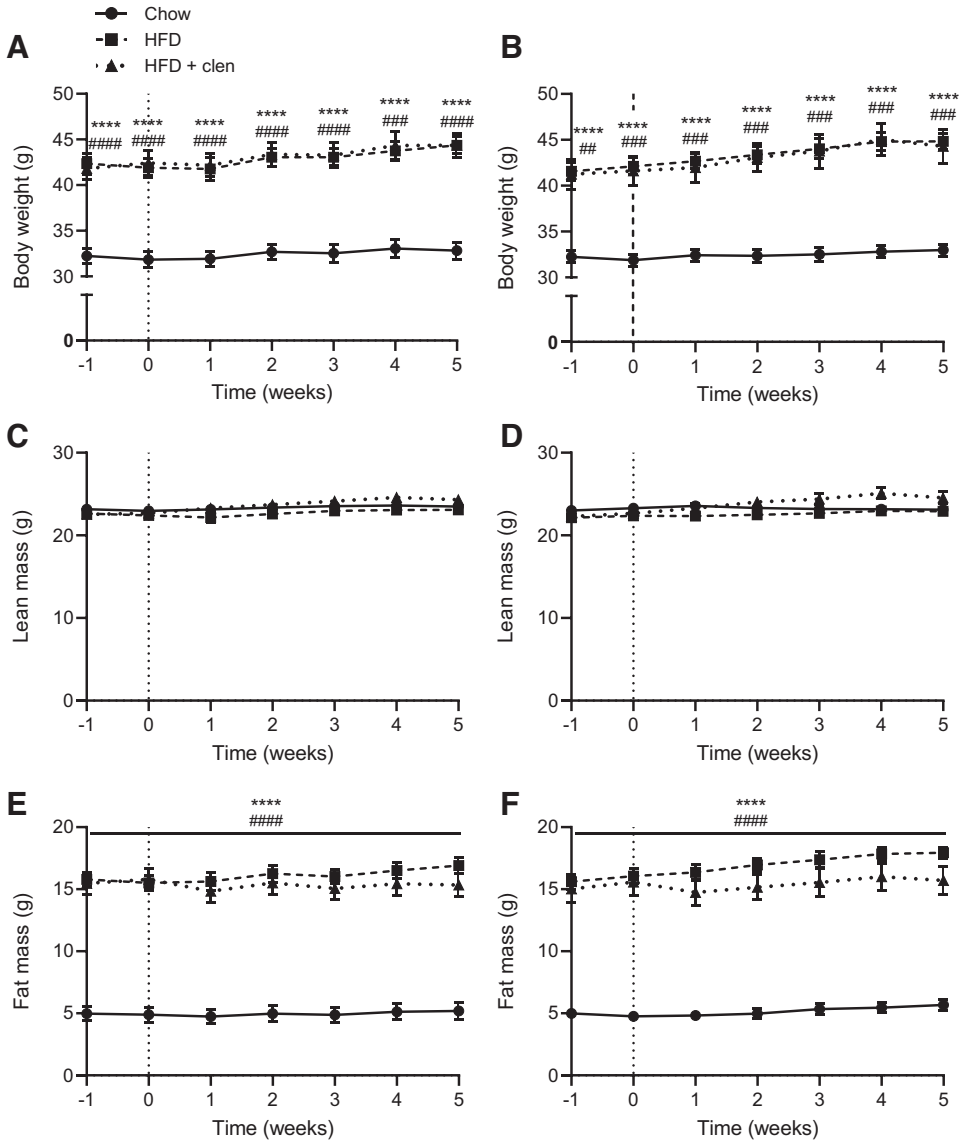

Figure 3. Body weight and composition are not affected upon prolonged low-dose clenbuterol supplementation. *A*: body weight of WT mice. *B*: body weight of *UCP1*$^{-/-}$ mice. *C*: lean mass of WT mice. *D*: lean mass of *UCP1*$^{-/-}$ mice. *E*: fat mass of WT mice. *F*: fat mass of *UCP1*$^{-/-}$ mice. Dotted lines indicate start of clenbuterol treatment. *Significantly different between chow and HFD, #significantly different between chow and HFD + clen. WT: chow n = 8, HFD = 9, HFD + clen = 7–8. *UCP1*$^{-/-}$: chow = 8, HFD = 8, HFD + clen = 8. Data were analyzed by means of two-way ANOVA with Tukey's post hoc test. ****P < 0.0001, ##P < 0.01, ###P < 0.001, ####P < 0.0001. HFD, high fat diet; *UCP1*$^{-/-}$, uncoupling protein 1 deficient; WT, wild-type.

Insulin tolerance was similar between HFD + clen groups and their respective HFD control group of both genotypes preceding the clenbuterol intervention period (Supplemental Fig. S2). After 17 wk of HFD feeding, blood glucose levels of HFD-fed control mice of both genotypes did not respond to an insulin injection, indicating the development of insulin resistance (Fig. 5, *B–E*). Remarkably, both WT and *UCP1*$^{-/-}$ HFD mice treated with clenbuterol for 4 wk showed a major decrease in blood glucose concentrations as compared with the respective HFD control groups, both at 15 and 30 min after the insulin injection (WT: P = 0.0011 and P = 0.0014 and *UCP1*$^{-/-}$: P = 0.0020 and P = 0.0073, respectively) (Fig. 5, *B–E*). In fact, the decreases in blood glucose concentrations upon insulin injection in HFD + clen mice were similar to the insulin response of their chow-fed control group, indicating a normalization of insulin sensitivity in clenbuterol-treated mice (Fig. 5, *B–E*).

To further investigate the underlying mechanisms by which clenbuterol improves whole body insulin sensitivity, mice were euthanized 10 min following an insulin injection (5 U/kg lean). Isolated gastrocnemius muscle and interscapular BAT were then assessed for the ratio of p-AKTS473/totalAKT, a key protein in the insulin signaling pathway. HFD feeding significantly reduced p-AKT/totalAKT ratio in gastrocnemius muscle as compared with chow-fed diet in both WT and *UCP1*$^{-/-}$ mice by 50.4% and 45.7%, respectively (P = 0.022 and P = 0.006) (Fig. 6*A*). However, clenbuterol treatment did not improve p-AKT/totalAKT ratio in gastrocnemius muscle as compared with HFD feeding alone in both genotypes (P = 0.968 and P = 0.989, respectively) (Fig. 6*A*). Similar to gastrocnemius muscle, the p-AKT/totalAKT ratio in interscapular BAT was significantly reduced upon HFD feeding by 44.0% and 60.5% in WT and *UCP1*$^{-/-}$ mice, respectively (P = 0.009 and P = 0.0003, Fig. 6*B*). However, clenbuterol treatment did not improve the p-AKT/totalAKT ratio in interscapular BAT of both genotypes as compared to HFD feeding alone (P = 0.485 and P = 0.759, respectively, Fig. 6*B*).

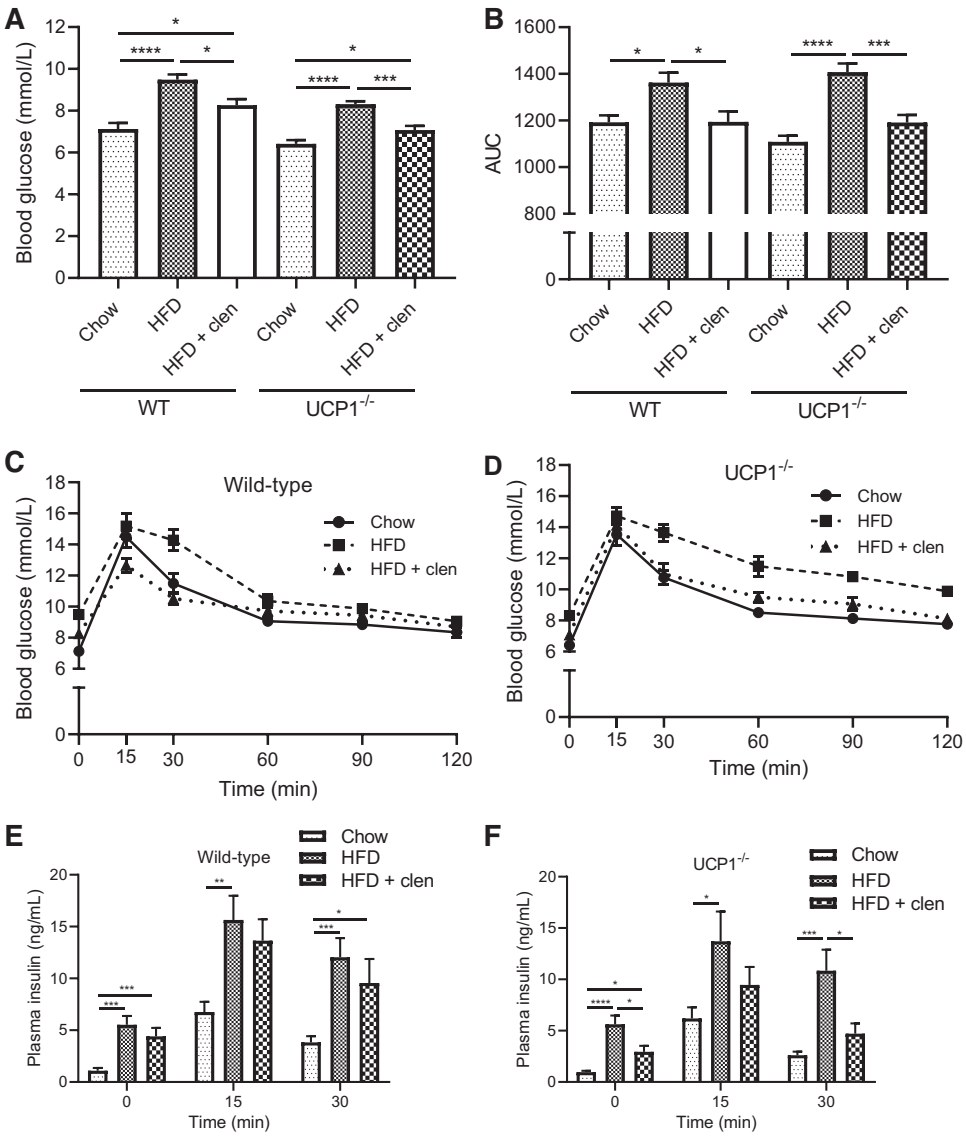

Figure 4. Clenbuterol supplementation improves oral glucose tolerance of WT and *UCP1*$^{-/-}$ mice after 2 wk of treatment. *A*: fasting glucose of WT and *UCP1*$^{-/-}$ mice. *B*: area under the curve calculated from 0 of both WT and *UCP1*$^{-/-}$ groups. *C*: oGTT of WT mice. *D*: oGTT of *UCP1*$^{-/-}$ mice. *E*: insulin concentrations of WT mice during the oral glucose tolerance test. *F*: insulin concentrations of *UCP1*$^{-/-}$ mice during the glucose tolerance test. *A–D* WT: chow n = 8, HFD = 9, HFD + clen = 8; *UCP1*$^{-/-}$: chow = 8, HFD = 8, HFD + clen = 8. E-F WT: chow = 8, HFD = 9, HFD + clen = 7–8; *UCP1*$^{-/-}$: chow = 8, HFD = 6–8, HFD + clen = 8. Data were analyzed by means of one-way ANOVA with a Tukey's post hoc test or a nonparametric Kruskall–Wallis test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. HFD, high fat diet; oGTT, oral glucose tolerance test; *UCP1*$^{-/-}$, uncoupling protein 1 deficient; WT, wild-type.

DISCUSSION

Recently, we have demonstrated robust improvements in glucose homeostasis of DIO mice upon prolonged supplementation with the β_2-AR agonist clenbuterol and highlighted the potential role of the liver and especially skeletal muscle therein (3, 4). However, our previous studies did not investigate a potential contribution of BAT thermogenesis in the clenbuterol-mediated improvements in whole body glucose regulation. Given that rodents are, in contrast to—especially diabetic—humans (21, 22), characterized by high quantities of BAT, the contribution of BAT thermogenesis to these improvements in whole body glucose tolerance needs to be considered when extrapolating data from rodent models to humans. We established that acute clenbuterol injection indeed activates BAT thermogenesis in vivo in mice. However, prolonged low-dose clenbuterol supplementation robustly improved tolerance to glucose and insulin, both in WT and *UCP1*$^{-/-}$ mice, which lack the thermogenic BAT response. Based on the latter findings, we conclude that these beneficial effects of low-dose clenbuterol supplementation occur independent of UCP1-mediated BAT thermogenesis.

In the current study, we demonstrated approximately twofold increase in oxygen consumption upon acute clenbuterol injection in WT mice. Previous studies have also demonstrated significant increases in whole body oxygen consumption upon acute clenbuterol injection in rats (23) but also showed that norepinephrine, the most commonly used agonist for the assessment of BAT activation, appears to be a more potent activation of BAT in mice (24). Taken together, these results indicate that acute clenbuterol stimulation leads to an activation of BAT thermogenesis, which is likely mediated directly via nonspecific binding of clenbuterol to β_1- and/or β_3-ARs (8), the primary receptors involved in the activation BAT thermogenesis in rodents (9), or indirectly via an increased clenbuterol-induced BAT tissue blood flow (25).

Notably, the rise in whole body oxygen consumption upon acute clenbuterol injection was severely blunted—but not abolished—in *UCP1*$^{-/-}$ mice, which lack the key protein

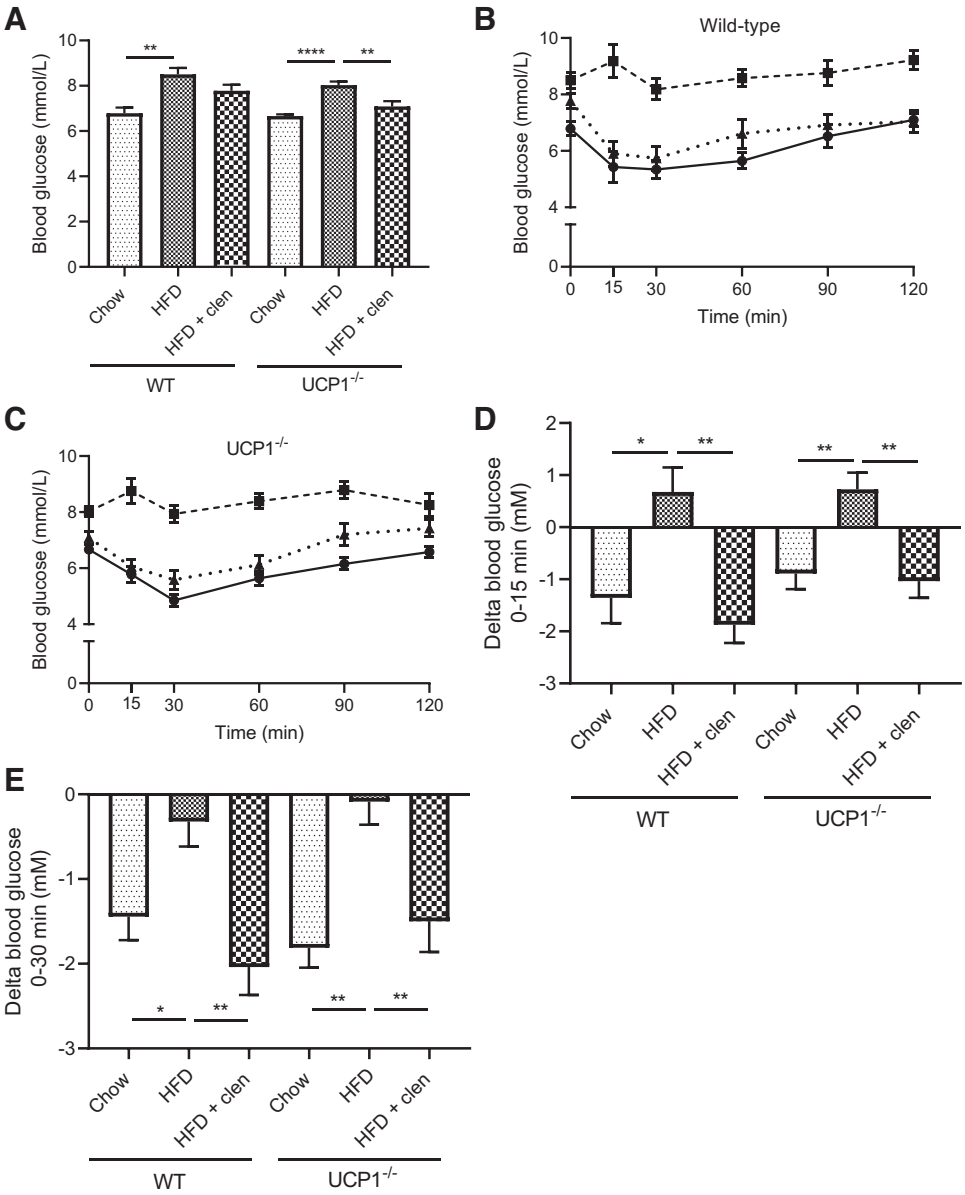

Figure 5. Clenbuterol improves insulin tolerance after 4 wk in WT and *UCP1*$^{-/-}$. *A*: fasting blood glucose of WT and *UCP1*$^{-/-}$ mice. *B*: time-response curve of blood glucose following an insulin injection (1 U/kg lean mass) in WT mice. *C*: time-response curve of blood glucose following an insulin injection (1 U/kg lean mass) in *UCP1*$^{-/-}$ mice. *D*: delta blood glucose between $t = 0$ and 15 min for WT and *UCP1*$^{-/-}$ mice. *E*: delta blood glucose between $t = 0$ and 30 min for WT and *UCP1*$^{-/-}$ mice. WT: chow n = 7, HFD = 9, HFD + clen = 8; *UCP1*$^{-/-}$: chow = 8, HFD = 8, HFD + clen = 8. Data were analyzed by means of one-way ANOVA with Tukey's post hoc test or a nonparametric Kruskall–Wallis test. $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$. *UCP1*$^{-/-}$, uncoupling protein 1 deficient; WT, wild-type.

for BAT thermogenesis (18, 19). This finding was in line with previous studies showing increases in oxygen consumption in *UCP1*$^{-/-}$ mice upon norepinephrine injection (26–28). These results thus indicate that next to UCP1-dependent thermogenesis, other metabolic processes also contribute to the increase in whole body oxygen consumption upon acute β-AR agonist administration. Although the exact underlying mechanisms remain to be elucidated, these effects could potentially be related to the activation of recently proposed UCP1-independent thermogenic processes, including the futile lipid, creatine, or Ca^{2+} cycle (29–31). Nonetheless, these results clearly show that clenbuterol is capable of activating BAT thermogenesis in vivo in mice and that this phenomenon could potentially contribute to the previously observed beneficial effects of clenbuterol on whole body glucose tolerance (3–7).

Therefore, we next investigated if a prolonged, low-dose clenbuterol treatment would result in similar beneficial effects on metabolic health in *UCP1*$^{-/-}$ mice, who—in our view—mimic the human T2DM phenotype more closely, since patients with T2DM are characterized by very low amounts of BAT (21). In line with our previous studies (3, 4), we here showed that prolonged (low-dose) clenbuterol treatment of WT DIO mice reduced fasting glucose by 12.9%, robustly improved glucose clearance during an oral glucose tolerance test as compared to HFD controls, and even induced a normalization of glucose tolerance to the values of their healthy chow-fed counterparts. Interestingly, we also observed a significant reduction in fasting blood glucose of 14.8% in *UCP1*$^{-/-}$ mice upon prolonged clenbuterol treatment, which was accompanied by a marked improvement in glucose tolerance as compared to the respective HFD control group. Much like the WT mice, clenbuterol treated *UCP1*$^{-/-}$ mice showed a normalization of glucose tolerance as compared to their respective healthy chow-fed control group.

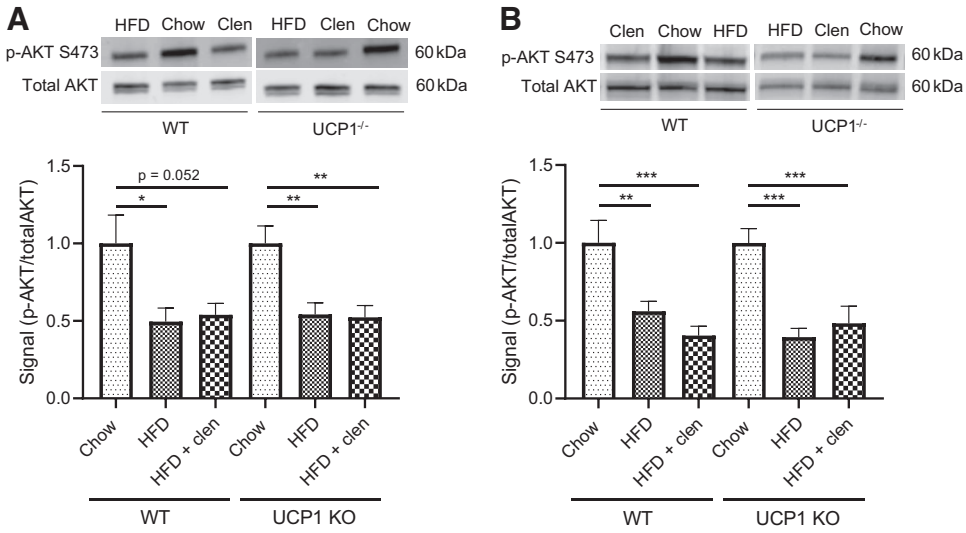

Figure 6. Clenbuterol supplementation does not affect insulin signaling in gastrocnemius muscle and interscapular BAT. *A*: Western blot of p-AKTS473/total-AKT in gastrocnemius muscle (WT: Chow $n = 8$, HFD = 9, Clen = 7; *UCP1*$^{-/-}$: Chow = 8, HFD = 7, Clen = 7). *B*: Western blot of p-AKTS473/total-AKT of interscapular BAT (WT: Chow $n = 8$, HFD = 9, Clen = 8; *UCP1*$^{-/-}$: Chow = 8, HFD = 8, Clen =8). Chow groups were normalized to 1 and data were analyzed by means of one-way ANOVA with Tukey's post hoc test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. BAT, brown adipose tissue; HFD, high-fat diet; *UCP1*$^{-/-}$, uncoupling protein 1 deficient; WT, wild-type.

Altogether, these data strongly suggest that these beneficial effects are not mediated by UCP1-dependent BAT thermogenesis.

High doses of clenbuterol are well-known for its repartitioning effect on the body, i.e., increasing muscle mass while reducing fat mass (5–7, 32–34), which has previously been considered a primary factor in mediating improvements in glucose tolerance (6). During this study however, we were unable to detect significant changes in body composition upon prolonged low-dose clenbuterol treatment as assessed by MRI. This discrepancy could potentially be attributed to hypertrophic and glucose-lowering effects of clenbuterol enrolling distinct pathways with different sensitivities to the agonist, with low doses of clenbuterol being insufficient to stimulate the hypertrophic pathway of the receptor. We previously also demonstrated that clenbuterol already improves the glucose homeostasis after 4 days of treatment (4) – a timeframe likely too brief to enhance skeletal muscle mass. Taken together, our findings demonstrate that the clenbuterol-mediated effects on glucose metabolism can occur independent of increases in lean body mass.

These favorable effects on glucose tolerance could potentially be explained by chronically elevated plasma insulin levels, since β_2-AR agonists are well-known to acutely induce the secretion of insulin by pancreatic β-cells (4, 20). However, we and others have previously demonstrated that prolonged clenbuterol treatment significantly reduced – and even normalized – both fasting and glucose-induced plasma insulin concentrations in WT rodents (4–7). In the present study, *UCP1*$^{-/-}$ mice also demonstrated robust decreases in both fasting and glucose-induced plasma insulin concentrations during the OGTT upon prolonged clenbuterol treatment, whereas these parameters remained unaltered in WT mice. Although the mechanism for this discrepancy between *UCP1*$^{-/-}$ and WT mice remains unresolved, it could potentially be related to a higher adrenergic sensitivity/sympathetic tonus in *UCP1*$^{-/-}$ mice. Despite these apparent differences between genotypes, these results strongly suggest that the positive effects of clenbuterol treatment on glucose tolerance are not mediated by higher insulin levels, but could potentially be attributed to improvements in insulin sensitivity. Indeed, we also demonstrate that prolonged clenbuterol treatment robustly improved insulin tolerance in both DIO WT and *UCP1*$^{-/-}$ mice as compared to their respective HFD control groups. To further elaborate on the improvement in whole body insulin sensitivity, we also measured Akt phosphorylation in gastrocnemius muscle and interscapular BAT as a marker for insulin signaling. As observed previously (35–38), HFD feeding significantly lowered the phosphorylation of Akt S473 in gastrocnemius muscle and interscapular BAT. However, HFD + clenbuterol treatment did not affect Akt S473 phosphorylation as compared to HFD alone in both tissues, indicating that the increase in whole body insulin sensitivity occurred without improvements in the activation of the key insulin signaling protein Akt in skeletal muscle or BAT.

Although BAT glucose uptake can also be facilitated in an UCP1-independent manner (14), we have previously demonstrated that UCP1 is essential for the amelioration of BAT-mediated improvements in glucose tolerance of DIO mice. More specifically, acute injection of the β_3-AR agonist CL-316,243 robustly enhanced BAT glucose uptake in WT and *UCP1*$^{-/-}$ mice but, in contrast to WT mice, did not result in significant improvements in glucose tolerance in *UCP1*$^{-/-}$ mice upon prolonged treatment (1 mg/kg for 4 days) (14). Although the present study thus excludes UCP1 and BAT thermogenesis as a mediator of clenbuterol-induced improvements in whole body glucose homeostasis in DIO mice at the dose used in this study, it cannot conclude on the exact mechanisms that do underlie these improvements. It is also important to note that BAT is not merely involved in thermogenesis but also plays a role in various other metabolic processes which are mediated independently of UCP1, including de novo lipogenesis and lipid turnover (12, 39, 40). Thus, we cannot exclude that these UCP1-independent mechanisms may still contribute to the beneficial effects seen upon clenbuterol supplementation. In addition, other metabolic tissues that are likely involved are the liver (4) and skeletal muscle (3, 4). Thus, the significant reductions in fasting glucose concentrations upon prolonged clenbuterol

treatment observed in the present study hint towards a decreased hepatic glucose production and an improved hepatic insulin sensitivity (41). This improved liver insulin sensitivity may relate to a diminished hepatic lipid accumulation upon clenbuterol treatment, as observed previously (4). In addition, we and others have previously demonstrated significant increases in in vitro muscle cell glucose uptake upon selective β_2-AR agonist incubation independent of both the insulin and AMPK-signaling pathways, namely through the activation of mammalian target of rapamycin complex 2 (mTORC2) and subsequently GLUT4 translocation (3, 4, 42–46). Activation of this pathway through clenbuterol supplementation could therefore stimulate skeletal muscle glucose uptake in vivo and potentially underlie improvements in glucose tolerance independent of insulin. In fact, this hypothesis is further supported by the fact that 6 days of clenbuterol supplementation has previously been shown to robustly increase in vivo skeletal muscle glucose uptake (4).

CONCLUSIONS

In conclusion, we here demonstrate that clenbuterol – at the dose currently used during this study – improves whole body glucose homeostasis independent of UCP1-dependent thermogenesis, since similar improvements were observed in both WT and *UCP1*$^{-/-}$ mice, lacking thermogenic BAT. Hence, these metabolic effects are most likely mediated by adaptations in the liver and/or skeletal muscle. However, the exact underlying mechanisms underlying these improvements should be investigated in future studies. Given that *UCP1*$^{-/-}$ mice may—in our view—better reflect the human situation due to low amounts of BAT in prediabetic patients, our findings in this mouse model suggest that selective β_2-AR agonist treatment may provide a novel molecular route to improve glucose disposal in the insulin resistant state.

ACKNOWLEDGMENTS

The authors thank all members of our laboratory at Stockholm University for assistance and discussions during this study.

GRANTS

This study was supported by a Grant from the NUTRIM NWO Graduate Program, the Vetenskapsrådet-Medicin (VR-M) from the Swedish Research Council, the Stiftelsen Svenska Diabetesförbundets Forskningsfond, the Magnus Bergvall Foundation, and the Carl Tryggers Foundation.

DISCLOSURES

T. Bengtsson owns stocks in Atrogi AB. A. Kalinovich is an employee within Atrogi AB. None of the other authors has any conflicts of interest, financial or otherwise, to disclose.

AUTHOR CONTRIBUTIONS

S.M.M.v.B., A.K., T.B., and J.H. conceived and designed research; S.M.M.v.B., A.K., and G.S. performed experiments; S.M.M.v.B., A.K., and G.S. analyzed data; S.M.M.v.B., A.K., T.B., and J.H. interpreted results of experiments; S.M.M.v.B. and G.S. prepared figures; S.M.M.v.B. drafted manuscript; S.M.M.v.B., A.K., T.B., and J.H. edited and revised manuscript; S.M.M.v.B., A.K., T.B., and J.H. approved final version of manuscript.

DATA AVAILABILITY

All data are available upon request.

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