Mechanisms of cold-induced improvements in glucose homeostasis

Mechanisms of cold-induced improvements in glucose homeostasis

Sten van Beek

ISBN: 978-94-6458-662-6
Cover design: Cas Wetzels
Lay-out: Publiss | www.publiss.nl
Print: Ridderprint | www.ridderprint.nl

Mechanisms of cold-induced improvements in glucose homeostasis

Dissertation

To obtain the degree of doctor at Maastricht University,
on the authority of the Rector Magnificus,
Prof. Dr. Pamela Habibović,
in accordance with the decision of the Board of Deans,
to be defended in public on
Wednesday November 23rd, 2022 at 16:00 hours

by

Sten Martinus Maria van Beek

Born in Eindhoven, The Netherlands, on August 18th, 1995

Supervisor
Prof. Dr. P. Schrauwen

Co-supervisors
Dr. J. Hoeks
Dr. B. Havekes

Assessment committee
Prof. Dr. E. Blaak (Chair)
Dr. D. Blondin, University of Sherbrooke, Canada
Prof. Dr. B. de Galan
Prof. Dr. D. Jonkers
Dr. E. Kalkhoven, Universitair Medisch Centrum Utrecht

All research described in this thesis was performed within NUTRIM School of Nutrition and Translational Research in Metabolism. The research described was supported by grants of the NUTRIM NWO Graduate Program, ZonMw and the Dutch Diabetes Foundation.

TABLE OF CONTENTS

CHAPTER 1

General introduction

General introduction

With over 650 million adults being classified as obese according to the World Health Organisation in 2016, it is eminently clear that the prevalence of obesity has reached epidemic proportions (1). This poses a major threat to global public health, as obesity is highly associated with the development of various chronic metabolic diseases (2-4), including type 2 diabetes mellitus (T2DM) (5). It is therefore not surprising that the prevalence of T2DM has also increased at an alarming rate, with over 437 million people suffering from the disease in 2019 (6). In fact, T2DM was the ninth leading cause of death worldwide in 2019, with an estimated 1.5 million people succumbing to direct causes of the disease (7).

T2DM is a metabolic disease defined by major perturbations in the glucose homeostasis. These disturbances are the combined result of an impaired secretion of insulin by pancreatic β-cells and a blunted responsiveness of peripheral tissues to insulin, a phenomenon better known as insulin resistance (8). In a healthy condition, insulin is secreted during periods of hyperglycaemia to promote glucose uptake into peripheral tissues (i.e. skeletal muscle and adipose tissue) and inhibit glucose output by the liver, thereby lowering plasma glucose concentrations (9). As such, insulin resistance poses a major risk for the development of chronic hyperglycaemia in the body. To ensure that plasma glucose concentrations are maintained within homeostatic boundaries, the insulin resistance is initially compensated for by an enhanced insulin secretion by pancreatic β-cells. With time, this excessive insulin production causes the pancreatic β-cells to become dysfunctional, thereby reducing their ability for insulin secretion (8). This declining ability for insulin secretion, combined with a continuously exacerbating insulin resistance in peripheral tissues, results in a progressive rise in plasma glucose concentrations due to an unrestrained hepatic glucose production and a blunted glucose uptake into peripheral tissues, ultimately leading to the development of full-blown T2DM.

Although T2DM is a complex, heterogenous disease encompassing various metabolic organs, a central role for the skeletal muscle has long been recognized as it accounts for ~80% of insulin-stimulated glucose disposal (10, 11). Given this remarkable capacity for glucose disposal, a blunted skeletal muscle glucose uptake in response to insulin stimulation (i.e. skeletal muscle insulin resistance) significantly contributes to chronic hyperglycaemia seen in T2DM (10). Hence, stimulation of skeletal muscle glucose uptake could markedly improve whole-body glucose homeostasis in T2DM patients.

Cold-induced improvements in skeletal muscle glucose uptake

Despite the fact that highly effective treatment strategies for T2DM (i.e. dietary- and physical exercise regimes) are readily available, these therapies have proven difficult to sustain in a population that is mostly obese (12, 13). Within this frame of reference, scientists have recently focussed on exposure to low ambient temperatures as a potential novel therapeutic approach to improve glucose homeostasis. Thus, cold exposure has been well-established to significantly improve both skeletal muscle glucose uptake and whole-body glucose homeostasis in various rodent models of diabetes mellitus (14-16). Within our own research group we could show the human relevance of these preclinical findings and demonstrated that prolonged, intermittent, mild cold exposure (14-15 ˚C, 2-6h/day for 10 consecutive days) robustly improved glucose homeostasis of T2DM patients by ~40% (17). These marked effects were primarily attributed to an increased skeletal muscle glucose uptake, albeit that the exact underlying mechanisms remained unknown.

In this thesis, two putative physiological mechanisms were explored potentially underlying the previously observed cold-induced improvements in skeletal muscle glucose uptake and whole-body glucose homeostasis (Figure 1). **Firstly**, upon acute cold exposure, the human body initially defends its core temperature by increasing its energy expenditure via non-shivering thermogenesis. The latter is mediated via the activation of the sympathetic nervous system and the concurrent release of norepinephrine onto various tissues, including skeletal muscle and brown adipose tissue (BAT) (18, 19). Norepinephrine is an agonist of β-adrenergic receptors (β-ARs) and previous *in vitro* studies have demonstrated that activation of skeletal muscle β_2-AR – the most prevalent β-AR in myocytes (20) – enhances glucose uptake (21-25). As such, activation of β_2-ARs during cold exposure could potentially contribute to the improvements in skeletal muscle (and BAT) glucose uptake and whole-body glucose homeostasis. **Secondly**, if the energy generated via non-shivering thermogenesis is insufficient to maintain a stable core temperature, the body induces involuntary muscle contractions for additional heat production, a process better known as shivering thermogenesis (26). These muscle contractions have previously been shown to positively correlate with glucose uptake, meaning that a higher shivering intensity is associated with a higher glucose uptake (27). Furthermore, the prevention of overt shivering during mild cold acclimation (16-17˚C, 2-6h/day, 10 consecutive days) completely abolished the previously reported beneficial effects on glucose homeostasis in T2DM patients (28). These studies combined suggest that cold-induced improvements

in skeletal muscle glucose uptake and whole-body glucose homeostasis could potentially be related to shivering thermogenesis.

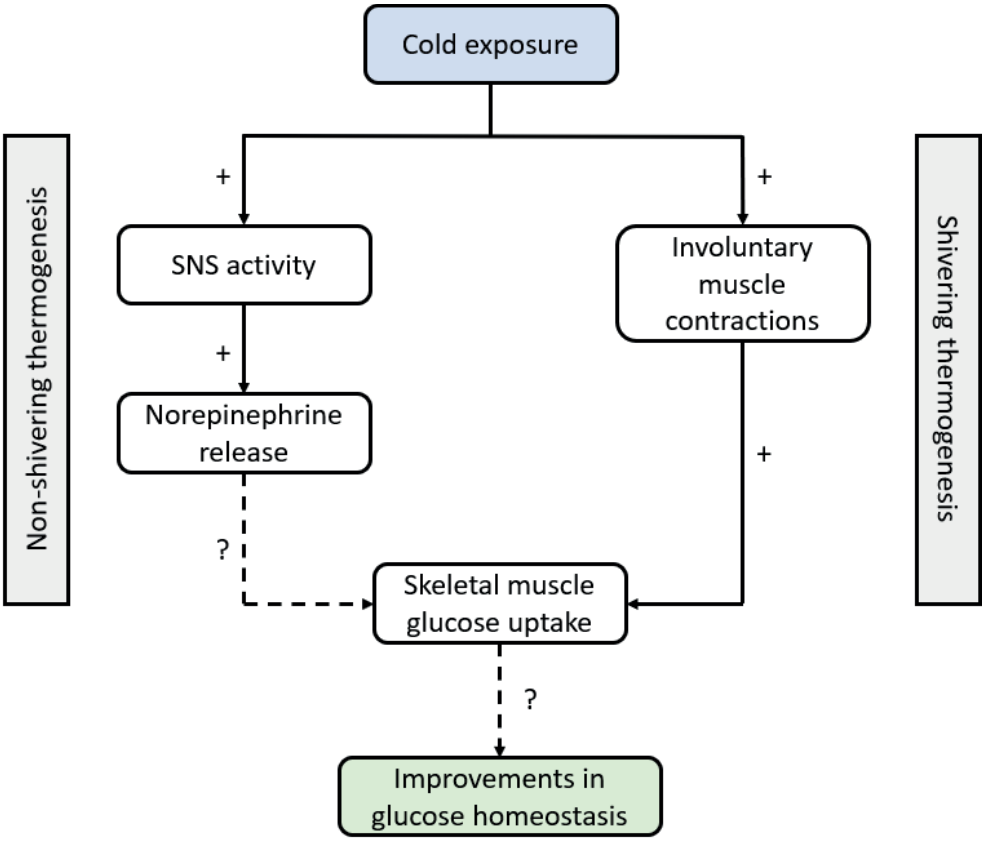


Figure 1. Schematic overview of the potential underlying mechanisms of cold-induced improvements in skeletal muscle glucose uptake and whole-body glucose homeostasis. SNS = sympathetic nervous system.

Thesis outline

This thesis aims to further investigate the effects of **(1)** direct stimulation of the β_2-AR, and **(2)** repeated exposure to cold-induced shivering thermogenesis, on skeletal muscle glucose uptake and whole-body glucose homeostasis.

In **Chapter 2**, the current body of evidence on the potential tissues implicated in mediating cold-induced improvements in glucose homeostasis was reviewed. Although previous studies have primarily focussed on BAT in mediating cold-induced improvements in glucose homeostasis, its role in (especially obese) humans appears

rather limited (17). As such, the potential contribution of the primary organs involved in glucose metabolism (including the pancreas, liver, adipose tissue, and skeletal muscle) to the previously observed improvements in glucose homeostasis were evaluated. In **Chapter 3**, the effects of prolonged stimulation of β_2-ARs, via treatment with the selective β_2-agonist clenbuterol, on skeletal muscle glucose uptake and glucose homeostasis were investigated in insulin resistant, diet-induced obese mice. However, it is important to note that clenbuterol also binds and activates β_1- and β_3-ARs, albeit to a lesser extent than β_2-ARs. Therefore, it cannot be excluded that other tissues are also involved in clenbuterol-mediated improvements in glucose homeostasis. This especially holds true for BAT, which is primarily activated via β_1- and β_3-ARs. In **Chapter 4**, the effects of clenbuterol treatment were therefore investigated in uncoupling protein 1 deficient, diet-induced obese mice, who lack thermogenic BAT. To establish the clinical relevance of these preclinical studies, a randomized, placebo-controlled, double-blinded, cross-over study was performed during which healthy, young, male subjects received a two-week treatment with either the selective β_2-agonist clenbuterol or a placebo, after which the effects on insulin-stimulated skeletal muscle glucose uptake were investigated with the golden standard two-step hyperinsulinemic-euglycemic clamp technique (**Chapter 5**). To address the second aim of this thesis, the effects of repeated cold-induced shivering thermogenesis on whole-body glucose homeostasis were investigated in overweight/obese men and (postmenopausal) women (**Chapter 6**). In this chapter, participants were exposed to shivering (at least 1h/day) for 10 consecutive days. Before and after the intervention, effects on whole-body glucose homeostasis were investigated by means of oral glucose tolerance tests. Finally, in **Chapter 7**, the main results and conclusions of the studies described in this thesis have been highlighted, and their relevance is discussed in a broader perspective.

References

1. World Health Organisation. Factsheet Obesity and Overweight 2021. [Available from https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight].
2. Marchesini G, Moscatiello S, Di Domizio S, Forlani G. Obesity-associated liver disease. J Clin Endocrinol Metab. 2008;93(11 Suppl 1):S74-80.
3. Poirier P, Giles TD, Bray GA, Hong Y, Stern JS, Pi-Sunyer FX, et al. Obesity and cardiovascular disease: pathophysiology, evaluation, and effect of weight loss: an update of the 1997 American Heart Association Scientific Statement on Obesity and Heart Disease from the Obesity Committee of the Council on Nutrition, Physical Activity, and Metabolism. Circulation. 2006;113(6):898-918.
4. Renehan AG, Tyson M, Egger M, Heller RF, Zwahlen M. Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies. Lancet. 2008;371(9612):569-78.
5. Kopelman P. Health risks associated with overweight and obesity. Obes Rev. 2007;8 Suppl 1:13-7.
6. Safiri S, Karamzad N, Kaufman JS, Bell AW, Nejadghaderi SA, Sullman MJM, et al. Prevalence, Deaths and Disability-Adjusted-Life-Years (DALYs) Due to Type 2 Diabetes and Its Attributable Risk Factors in 204 Countries and Territories, 1990-2019: Results From the Global Burden of Disease Study 2019. Front Endocrinol (Lausanne). 2022;13:838027.
7. World Health Organisation. Factsheet Diabetes 2021. [Available from https: https://www.who.int/news-room/fact-sheets/detail/diabetes].
8. Virally M, Blickle JF, Girard J, Halimi S, Simon D, Guillausseau PJ. Type 2 diabetes mellitus: epidemiology, pathophysiology, unmet needs and therapeutical perspectives. Diabetes Metab. 2007;33(4):231-44.
9. Roder PV, Wu B, Liu Y, Han W. Pancreatic regulation of glucose homeostasis. Exp Mol Med. 2016;48:e219.
10. DeFronzo RA, Gunnarsson R, Bjorkman O, Olsson M, Wahren J. Effects of insulin on peripheral and splanchnic glucose metabolism in noninsulin-dependent (type II) diabetes mellitus. J Clin Invest. 1985;76(1):149-55.
11. DeFronzo RA, Jacot E, Jequier E, Maeder E, Wahren J, Felber JP. The effect of insulin on the disposal of intravenous glucose. Results from indirect calorimetry and hepatic and femoral venous catheterization. Diabetes. 1981;30(12):1000-7.
12. Bennett GA, Jones SE. Dropping out of treatment for obesity. J Psychosom Res. 1986;30(5):567-73.
13. Moroshko I, Brennan L, O'Brien P. Predictors of dropout in weight loss interventions: a systematic review of the literature. Obes Rev. 2011;12(11):912-34.
14. Vallerand AL, Lupien J, Bukowiecki LJ. Cold exposure reverses the diabetogenic effects of high-fat feeding. Diabetes. 1986;35(3):329-34.
15. Vallerand AL, Perusse F, Bukowiecki LJ. Cold exposure potentiates the effect of insulin on in vivo glucose uptake. Am J Physiol. 1987;253(2 Pt 1):E179-86.
16. Smith OL, Davidson SB. Shivering thermogenesis and glucose uptake by muscles of normal or diabetic rats. Am J Physiol. 1982;242(1):R109-15.
17. Hanssen MJ, Hoeks J, Brans B, van der Lans AA, Schaart G, van den Driessche JJ, et al. Short-term cold acclimation improves insulin sensitivity in patients with type 2 diabetes mellitus. Nat Med. 2015;21(8):863-5.

18. Dulloo AG, Young JB, Landsberg L. Sympathetic nervous system responses to cold exposure and diet in rat skeletal muscle. Am J Physiol. 1988;255(2 Pt 1):E180-8.
19. Young JB, Saville E, Rothwell NJ, Stock MJ, Landsberg L. Effect of diet and cold exposure on norepinephrine turnover in brown adipose tissue of the rat. J Clin Invest. 1982;69(5):1061-71.
20. Liggett SB, Shah SD, Cryer PE. Characterization of beta-adrenergic receptors of human skeletal muscle obtained by needle biopsy. Am J Physiol. 1988;254(6 Pt 1):E795-8.
21. Sato M, Dehvari N, Oberg AI, Dallner OS, Sandstrom AL, Olsen JM, et al. Improving type 2 diabetes through a distinct adrenergic signaling pathway involving mTORC2 that mediates glucose uptake in skeletal muscle. Diabetes. 2014;63(12):4115-29.
22. Nevzorova J, Bengtsson T, Evans BA, Summers RJ. Characterization of the beta-adrenoceptor subtype involved in mediation of glucose transport in L6 cells. Br J Pharmacol. 2002;137(1):9-18.
23. Nevzorova J, Evans BA, Bengtsson T, Summers RJ. Multiple signalling pathways involved in beta2-adrenoceptor-mediated glucose uptake in rat skeletal muscle cells. Br J Pharmacol. 2006;147(4):446-54.
24. Tanishita T, Shimizu Y, Minokoshi Y, Shimazu T. The beta3-adrenergic agonist BRL37344 increases glucose transport into L6 myocytes through a mechanism different from that of insulin. J Biochem. 1997;122(1):90-5.
25. Mukaida S, Sato M, Oberg AI, Dehvari N, Olsen JM, Kocan M, et al. BRL37344 stimulates GLUT4 translocation and glucose uptake in skeletal muscle via beta2-adrenoceptors without causing classical receptor desensitization. Am J Physiol Regul Integr Comp Physiol. 2019;316(5):R666-R77.
26. Haman F, Blondin DP. Shivering thermogenesis in humans: Origin, contribution and metabolic requirement. Temperature (Austin). 2017;4(3):217-26.
27. Blondin DP, Labbe SM, Phoenix S, Guerin B, Turcotte EE, Richard D, et al. Contributions of white and brown adipose tissues and skeletal muscles to acute cold-induced metabolic responses in healthy men. J Physiol. 2015;593(3):701-14.
28. Remie CME, Moonen MPB, Roumans KHM, Nascimento EBM, Gemmink A, Havekes B, et al. Metabolic responses to mild cold acclimation in type 2 diabetes patients. Nat Commun. 2021;12(1):1516.

CHAPTER 2

Physiological and molecular mechanisms of cold-induced improvements in glucose homeostasis beyond brown adipose tissue

Sten van Beek[1], Tore Bengtsson[2] and Joris Hoeks[1]

[1] Department of Nutrition and Movement Sciences, NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University, Maastricht, the Netherlands.

[2] Department of Molecular Biosciences, The Wenner-Gren Institute, Stockholm University, Stockholm, Sweden.

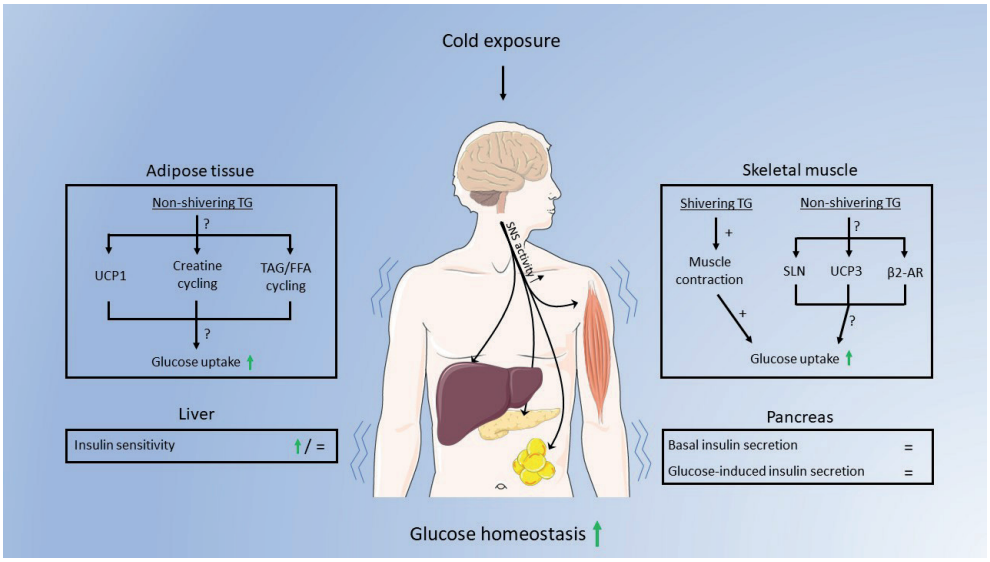
Submitted

Abstract

Exposure to low ambient temperatures has previously been demonstrated to markedly improve glucose homeostasis in both rodents and humans. Although the brown adipose tissue is key in mediating these beneficial effects in rodents, its contribution appears limited in humans. Thus, the exact tissues and underlying mechanisms that do underlie cold-induced improvements in glucose homeostasis in humans remain to be fully established. In this review, we evaluated the response of the main organs involved in glucose metabolism (i.e. pancreas, liver, (white) adipose tissue, and skeletal muscle) to cold exposure and discuss their potential contribution to cold-induced improvements in glucose homeostasis in humans. We here show that cold exposure has widespread effects on metabolic organs involved in glucose regulation. Nevertheless, cold-induced improvements in glucose homeostasis appear primarily mediated via adaptations within skeletal muscle and (presumably) white adipose tissue. Since the underlying mechanisms remain elusive, future studies should be aimed at pinpointing the exact physiological and molecular mechanisms involved. Nonetheless, cold exposure holds great promise as a novel, additive lifestyle approach to improve glucose homeostasis in insulin resistant individuals.

Key words: cold exposure, glucose homeostasis, pancreas, liver, adipose tissue, skeletal muscle.

Graphical abstract



Parts of this graphical abstract were created using (modified) images from Servier Medical Art, licensed under the Creative Commons Attribution 3.0 Unported License.

Abbreviations

AMPK	5'adenosine monophosphate-activated protein kinase
AR	Adrenergic receptor
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
FFA	Free fatty acid
GLUT4	Glucose transporter 4
IVGTT	Intravenous glucose tolerance test
mTOR	Mammalian target of rapamycin
mTORC2	Mammalian target of rapamycin complex 2
OGTT	Oral glucose tolerance test
PET-CT	Positron emission tomography and computed tomography
SERCA	Sarco/endoplasmic reticulum Ca^{2+}-ATPase
SLN	Sarcolipin
SNS	Sympathetic nervous system
T2DM	Type 2 diabetes mellitus
TAG	Triacylglycerol
UCP1	Uncoupling protein 1
UCP3	Uncoupling protein 3
VMH	Ventromedial hypothalamus
WAT	White adipose tissue

1. Introduction

Human physiology dictates that core body temperature is maintained and hypothermia is prevented through peripheral vasoconstriction and the increase in endogenous heat production when exposed to low(er) ambient temperatures. The enhanced heat production can be attributed to the activation of both non-shivering and shivering thermogenic mechanisms, two processes well-known to drastically affect energy expenditure and substrate metabolism (1, 2). Hence, it is not surprising that cold exposure represents a potential novel treatment strategy for obesity-related metabolic perturbations, including insulin resistance and type 2 diabetes mellitus (T2DM). Previous preclinical studies have demonstrated that during cold exposure plasma glucose and insulin concentrations are reduced (3-5), the clearance of plasma glucose is significantly increased (3, 6-9), insulin sensitivity is improved (6, 8), and detrimental metabolic effects of high-fat feeding are reversed (8). Also in (healthy) humans, acute cold exposure (10 ˚C for 3h) significantly increased the clearance of labelled glucose (10) and markedly improved glucose clearance of an intravenous glucose bolus (11). Since continuous exposure of T2DM patients to low ambient temperatures is simply unfeasible, these cold-induced metabolic adaptations should persist upon returning to thermoneutrality in order for cold exposure to be considered as a viable therapy. In this context, a study by Iwen et al. (12) demonstrated that insulin sensitivity was improved by ~20% in healthy young males measured after acute cold exposure (~18 ˚C for 100 minutes). Prolonged, intermittent mild cold exposure (14-15 ˚C, 2-6h/day for 10 consecutive days) also drastically increased insulin sensitivity by ~40% in T2DM patients, assessed ~16h following the last cold exposure (13). A clear understanding of the tissues and underlying mechanisms implicated in mediating these beneficial effects, however, remain elusive to date. Here, we aim to review the current body of literature on the potential tissues involved in mediating cold-induced improvements in glucose homeostasis in humans and set the stage for future research in this field.

2. Beyond brown adipose tissue

When it comes to cold exposure, research has primarily focussed on brown adipose tissue (BAT) as a pivotal organ in mediating cold-induced improvements in glucose homeostasis. BAT is an important organ in the maintenance of body temperature during cold exposure, as it dissipates large amounts of heat via uncoupling protein 1 (UCP1)-mediated uncoupling of mitochondrial oxidative respiration, as expertly reviewed elsewhere (14). This uncoupled mitochondrial respiration is paralleled by a substantial

increase in glucose uptake from the circulation (15, 16), which fostered the believe that BAT could potentially be exploited as a 'metabolic sink' for glucose to improve glucose homeostasis. In line with this hypothesis, stimulation of BAT activity via cold exposure or selective β_3-adrenergic receptor treatment has been associated with significant improvements in glucose homeostasis in rodents (8, 17-19). However, the role of BAT in – especially obese – humans appears rather limited. BAT mass and activity are negatively correlated with body mass index and percentage body fat, meaning that overweight/obese individuals have little to no BAT (20, 21). Furthermore, prolonged, intermittent cold exposure (14-15 ˚C, 2-6h/day for 10 consecutive days) only marginally increased BAT mass and activity in T2DM patients, despite a robust improvement in insulin sensitivity of ~40% (13). Although a large body of evidence thus suggest a substantial role of BAT in cold-induced improvements in glucose homeostasis in rodents, other tissues are likely involved in mediating the beneficial effects in humans.

3. Cold-induced adaptations in pancreatic insulin release

The pancreas is an important regulator of glucose homeostasis due to its ability to tightly control plasma glucose concentrations through secretion of the glucose regulating hormones insulin and glucagon (22). Stressors that drastically influence plasma glucose concentrations, such as a cold stimulus, also markedly affect pancreatic hormone release. Although it is important to note that plasma glucagon concentrations are increased upon cold exposure (23-27), we here primarily focussed on the effects on pancreatic insulin release due to its relevance in the pathophysiology of T2DM. Thus, several preclinical studies have demonstrated a significant reduction in plasma insulin concentrations at baseline and following a glucose bolus during exposure to low ambient temperatures (2-5 ˚C) (3, 5, 27-31). The reduction in basal plasma insulin concentrations during cold exposure has also been reported in humans in some (32, 33), but not all studies (23, 34, 35). Additionally, a reduced insulin concentration following an intravenous glucose bolus was measured in healthy males during acute cold exposure (10 ˚C for 3h) (11). These (pre) clinical findings combined hint towards a reduced responsiveness of pancreatic β-cells to glucose upon cold exposure, an effect likely mediated via an enhanced sympathetic activity (36, 37).

The effects of cold exposure on pancreatic insulin release do not appear to be long-lasting once the cold stimulus ceases. Thus, in rats, the effects of cold exposure (5 ˚C for 28 or 48 hours) on plasma insulin concentrations following a glucose bolus were

completely abolished if rats were re-acclimatized to thermoneutrality for 4 (28) or 15-18 hours (3) prior to the experiments. Also in humans, acute cold exposure (10˚C for 1 hour or 18˚C for 100 minutes, respectively) (12, 35) or cold acclimation (14-17 ˚C, 2-6h/day for 10 consecutive days) (13, 33, 38, 39) did not significantly affect fasting plasma insulin concentrations measured at thermoneutrality after cold exposure. Moreover, plasma insulin concentrations were either not affected (12) or even significantly increased (35) during an IVGTT or OGTT performed at thermoneutrality directly after cold exposure, respectively. These results suggest that the cold-induced effects on pancreatic insulin secretion are part of a highly integrated system of metabolic adaptations to endure cold exposure, which rapidly normalizes upon returning to thermoneutrality.

4. The effect of cold exposure on hepatic insulin sensitivity

Following exposure to a cold stimulus, the liver initiates an array of adaptations to aid in maintaining core body temperature as well as euglycaemia, including increasing endogenous heat production and glucose release, respectively. For the purpose of this review, we will focus on the effects of the cold stimulus on glucose handling and insulin sensitivity. To prevent the development of hypoglycaemia during cold exposure, the liver undergoes major metabolic adaptations to ensure a constant release of glucose into the circulation. Thus, several preclinical studies have reported a significant reduction in hepatic glycogen content (26, 40-42), as well as an increased expression and activity of gluconeogenic enzymes (26, 43, 44) upon cold exposure in rats, thereby supporting an increased hepatic glucose production during cold exposure. Also the liver's ability to respond to an insulin stimulus seems to be greatly affected during cold exposure. Thus, hepatic insulin sensitivity was markedly improved in male Sprague-Dawley rats on the fifth day of cold exposure (4 ˚C), as indicated by a significantly reduced hepatic glucose production during a continuous low-dose insulin infusion (0.6 kg/mg/min) (9). To our knowledge, it remains to be established whether acute cold exposure affects the liver in a similar way in humans. In addition, whether the observed improvements in hepatic insulin sensitivity are conserved upon returning to thermoneutrality is not entirely known and studies about this topic are scarce. Improvements in hepatic insulin sensitivity have been observed in Sprague-Dawley rats following cold exposure (4 ˚C for 3 weeks), even when rats were reacclimatized to ambient room temperature for 30 minutes (45). Prolonged, intermittent mild cold exposure (14-15 ˚C for 10 consecutive days, 2-6h/day) in T2DM patients tended to improve hepatic insulin sensitivity ~16h following the last cold exposure (13), whereas a follow-up study performed at slightly higher ambient

temperatures (16-17 ˚C for 10 consecutive days, 2-6h/day) did not affect hepatic insulin sensitivity (39).

5. Cold exposure and white adipose tissue glucose uptake

Upon exposure to low ambient temperatures, the white adipose tissue (WAT) not only plays an important role in the mobilisation of free fatty acids (FFAs) to heat producing organs, but also contributes to thermogenesis itself. Despite the fact that WAT is often neglected as an energy consuming tissue, it still accounts for ~6% of the variation in human basal metabolic rate (46) and its energy expenditure can be drastically increased upon cold exposure or β_3-agonist treatment (47, 48). In line with these findings, prolonged cold exposure (4-10 ˚C for 48 hours – 3 weeks) or norepinephrine infusion (4 days) is associated with an increased glucose uptake into WAT of rodents in both the basal- and insulin-stimulated state (6, 7, 29, 49, 50) and these effects persisted upon returning to thermoneutrality (7, 51). Although it can be speculated that the increased glucose uptake is used as fuel for the increased energy demand for lipolysis during cold exposure or restoration of the triacylglyceride (TAG) lipid pool following cold exposure, previous studies have also identified several futile thermogenic cycles occurring in WAT upon exposure to low ambient temperatures. Hence, activation of these futile cycles could potentially be related to improvements in whole-body glucose homeostasis.

5.1 White adipose tissue browning and glucose uptake

At thermoneutrality (30˚C), rodent WAT contains relatively large lipid droplets, few mitochondria and is (almost completely) deprived of UCP1 expression (50, 52-54). However, upon cold acclimation (50, 55-58) or prolonged β_3-adrenergic stimulation (55), rodent WAT develops a BAT-like phenotype in a process called WAT browning. More specifically, these brite (brown-in-white) or beige adipocytes develop multilocular lipid droplets, have an increased mitochondrial density, and express UCP1 (52, 55, 58, 59), which is thermogenically functional (56, 57, 60). Given these BAT-like characteristics, beige adipose tissue has been suggested to function as a 'metabolic sink' for glucose, thereby potentially improving whole-body glucose homeostasis. Indeed, beiging of rodent WAT through cold exposure or β_3-adrenergic stimulation is associated with an enhanced WAT glucose uptake (50, 61) and improvements in whole-body glucose homeostasis (62). Despite these promising results in rodents, evidence for WAT beiging and its putative ability to improve glucose homeostasis in humans is scarce and not unequivocal. Thus,

prolonged exposure to mild cold in humans did neither induce WAT beiging markers in subcutaneous WAT biopsies (38) nor improve subcutaneous WAT glucose uptake using PET-CT (13, 33). On the other hand, exposure of the upper thigh region to cold using an ice-pack for 30 minutes/day for 10 consecutive days significantly increased subcutaneous UCP1 expression in both lean and – albeit to a lesser extent – obese individuals (63). In addition, subcutaneous WAT biopsies taken from burn victims – who are characterised by markedly elevated concentrations of circulating norepinephrine – showed markers of WAT browning, including an elevated UCP1 expression, citrate synthase activity and mitochondrial respiration (64). Prolonged treatment (50 mg/day for 12 weeks) of overweight/obese subjects with the selective β_3-adrenergic agonist mirabegron also induced markers of WAT browning and improved insulin sensitivity as assessed by means of an euglycemic clamp (65). The change in WAT UCP1 expression, however, did not correlate with the improvements in glucose infusion rate (65). Taken together, the evidence for functional WAT browning in humans is virtually absent and further studies are required to determine its relevance for humans with respect to whole-body glucose homeostasis.

5.2 Futile TAG/FFA cycling in white adipose tissue

In addition to UCP1-dependent thermogenesis, WAT has also been reported to enhance its energy expenditure through UCP1-independent mechanisms (48, 66, 67), such as the futile creatine and TAG/FFA cycle. Similar to UCP1-dependent thermogenesis, activation of these futile cycles enhances energy expenditure and thereby could potentially affect glucose uptake and homeostasis. The futile creatine cycle takes place within mitochondria and involves the conversion of creatine into phosphocreatine – and vice versa – to induce an enhanced ATP turnover which is accompanied by the dissipation of heat, as reviewed elsewhere (68, 69). In line with this, beige adipose tissue of cold exposed mice (1 week at 4 °C) demonstrated an enhanced activity of mitochondrial creatine kinase activity, as well as an increased expression of genes involved in creatine metabolism, suggesting an increased reliance on this futile cycle for thermogenesis during cold exposure (70). This futile cycle also appears to have marked effects on glucose homeostasis, as adipocyte-specific ablation of key proteins involved in this cycle (such as glycine amidinotransferase or creatine kinase B) is associated with an elevated fasting glucose and a reduced glucose tolerance in rodents (71, 72). To our knowledge, however, whether this futile cycle is activated in human WAT upon cold exposure, as well as its putative role in the regulation of whole-body glucose homeostasis, remains to be established.

Next to the futile creatine cycle, adipose tissue can also induce the futile TAG/FFA cycle to generate heat independent of UCP1. In short, WAT lipolysis of TAGs can induce a futile energy consuming cycle via two pathways: (1) FFAs derived from TAG lipolysis are immediately re-esterified *in situ* into new TAGs (intracellular cycling) or (2) FFAs derived from TAG lipolysis enter the bloodstream and are either stored in hepatocytes or converted into very-low density lipoproteins by the liver, whereafter they are transported back to the WAT (extracellular cycling), as reviewed in more detail elsewhere (68, 73, 74). Isolated adipocytes from subcutaneous WAT biopsies demonstrated a ~40% re-esterification of FFAs, although a large variation between individuals existed (75). Furthermore, it was established in white adipocytes isolated from healthy lean and obese donors that the futile TAG/FFA cycle accounted for ~12% of WAT total energy expenditure and that FFA re-esterification upon isoprenaline stimulation showed a negative correlation with BMI (76). *In vivo* analyses in healthy young males, using infusion of a stable ^{13}C-palmitate and D-5-glycerol tracer, indicated that ~70% of all FFAs are re-esterified (20% intracellular; 50% extracellular) (77), thereby further demonstrating the thermogenic capacity of the TAG/FFA cycle.

Interestingly, the futile TAG/FFA cycle has been shown to be greatly enhanced upon cold exposure or β_3-adrenergic stimulation in rodent WAT (78, 79). Similar findings were reported for human WAT, although differences were observed between studies regarding the relative contribution of the intra- and extracellular TAG/FFA cycle. Thus, 3-hours of cold exposure at 5 ˚C increased the extracellular TAG/FFA cycle, whereas the intracellular cycle remained unaffected (80). In contrast, both mild cold exposure (18 ˚C for 180 minutes) or administration with mirabegron (200 mg) markedly increased the intracellular futile TAG/FFA cycle, with no effects on the extracellular cycling (47). The latter study further demonstrated the thermogenic capacity of the futile TAG/FFA cycle, as it accounted for ~28% and ~55% of the total increase in energy expenditure upon cold exposure or β_3-adrenergic agonist administration, respectively (47). Given the latter, it is tempting to hypothesize that the increase in futile TAG/FFA cycling is inherently associated with an increased glucose uptake. However, it remains to be established whether the TAG/FFA cycle is still fully functional in people with (or at risk for developing) T2DM, and whether it contributes to improving WAT glucose uptake and whole-body glucose homeostasis.

6. The role of skeletal muscle in cold-induced improvements in glucose homeostasis

The skeletal muscle is a metabolic tissue frequently proposed as a pivotal mediator in cold-induced improvements in glucose homeostasis. The latter is not entirely surprising

given the skeletal muscle's extraordinary capacity for substrate oxidation, as well as its prominent regulatory function in glucose homeostasis (81, 82). In fact, an impaired insulin-stimulated skeletal muscle glucose uptake, i.e. skeletal muscle insulin resistance, is a major hallmark for the development of T2DM (81). Several studies have demonstrated robust increases in glucose uptake in a variety of muscles *in vivo* in both rodents and humans upon cold exposure (4, 6, 7, 13, 15, 29, 45, 83, 84), an effect that appears to occur independent of insulin (29, 84). This cold-induced skeletal muscle glucose uptake is therefore likely to be an important driving force behind the positive effects of cold-induced improvements in glucose homeostasis. However, the precise physiological processes and molecular pathways mediating these effects are currently unknown and could potentially be attributed to shivering thermogenesis, non-shivering thermogenesis and/or adrenergic stimulation.

6.1 Shivering thermogenesis and skeletal muscle glucose uptake

Apart from generating physical force, muscle contractions are well-known for the release of substantial amounts of heat – a principle utilized by the body during cold exposure. Thus, cold exposure induces involuntary muscle contractions for the sole purpose of heat production, better known as shivering thermogenesis (1). Shivering is a highly energetic process which is able to maximally increase energy expenditure by ~500% (85). To sustain this demanding process, shivering thermogenesis is fuelled by a combined oxidation of glucose and lipids, as reviewed elsewhere (86). The majority of the oxidized glucose for shivering thermogenesis is obtained from muscle glycogen stores, which accounts for ~75% of total glucose oxidation (87), whereas the remainder (25%) is taken up directly from the circulation (87). In line with the latter, shivering of healthy and diabetic rats during acute cold exposure (4-5 ˚C for 24 or 48h) markedly increases metabolic clearance of labelled plasma glucose, as well as basal skeletal muscle glucose uptake (4, 6, 7, 15). Likewise, Vallerand et al. (10) demonstrated that shivering during acute exposure to cold air (10 ˚C for 3h) robustly increased clearance of labelled plasma glucose in healthy male volunteers. In fact, shivering intensity was shown to positively correlate with glucose uptake, with a higher shivering intensity being associated with a higher total muscle glucose uptake (83).

Although it is thus clear that shivering during cold exposure induces skeletal muscle glucose uptake, the underlying molecular pathways remain elusive and can so far only be speculated upon. In this context, several rodent and human studies have reported shivering-induced skeletal muscle glucose uptake in the absence of changes in plasma

insulin concentrations (3, 4, 32, 83). In fact, cold exposure has been shown to robustly increase skeletal muscle glucose uptake whilst simultaneously decreasing activation of key proteins of the insulin signalling pathway (29, 84), thereby strongly hinting towards insulin-independent mechanisms. Since shivering can be described as a "quasi-exercising" process during which muscle contractions occur but no external work is performed (88), it may stimulate GLUT4-mediated skeletal muscle glucose uptake through contraction-mediated activation of AMP-activated protein kinase (AMPK) (84).

Adding to the effects seen on skeletal muscle glucose uptake, shivering thermogenesis has also been associated with marked effects on glucose homeostasis. Thus, shivering during cold exposure significantly lowers plasma glucose and insulin concentrations (3, 4) and even improves glucose and insulin tolerance in both healthy and diabetic rodents (3, 4, 6, 7). Similarly, acute exposure of healthy males to shivering thermogenesis (10 ˚C for 3h) improved clearance of an intravenously infused glucose bolus during cold exposure, even though oral glucose tolerance remained unaffected (11). Upon returning to thermoneutrality, previous exposure to shivering thermogenesis appears to affect glucose homeostasis in a time- and/or dose-dependent manner. Thus, a recent study by Sellers et al. (35) reported a significant reduction in glucose tolerance of healthy males during an oral glucose tolerance test performed 90 minutes after the cold exposure, an effect that could potentially be related to elevated free fatty acid concentrations, an increased endogenous glucose production or changes in blood flow. In contrast, repeated exposure of T2DM patients to mild cold (14-15 ˚C, 2-6h/day, 10 consecutive days) robustly improved insulin sensitivity by ~40%, ~16 hours following the last cold exposure (13). These results combined suggest that either 1) the beneficial effects of one bout of cold-induced shivering thermogenesis occur after a prolonged period of time upon returning to thermoneutrality or 2) that repeated exposure to shivering thermogenesis is required to achieve beneficial effects on whole-body glucose homeostasis. As such, additional studies investigating the effects of a single bout of shivering thermogenesis after cold exposure, as well as the effects of repeated exposure to shivering thermogenesis, on whole-body glucose homeostasis are warranted.

6.2 Skeletal muscle as a non-shivering thermogenic organ

Before the initiation of shivering thermogenesis, the human body relies on non-shivering thermogenic processes for the production of heat upon cold exposure. As implied by the name, these processes occur independent of muscle contractions and enable the body to maximally increase energy expenditure by ~30% (2). Although the exact tissues

involved in non-shivering thermogenesis in humans is still debated, a substantial role for skeletal muscle has previously been proposed. Thus, upon mild cold exposure (16˚C), overweight/obese individuals - characterized by low BAT activity - demonstrate similar levels of cold-induced thermogenesis as healthy lean individuals (20), suggesting the involvement of other tissues than BAT in non-shivering thermogenesis, such as skeletal muscle. In line with these results, cold acclimation of rats (5 ˚C for several weeks) was shown to significantly increase skeletal muscle glucose uptake independent of shivering thermogenesis (7), hinting towards an increased skeletal muscle energy demand to sustain non-shivering thermogenesis.

The exact impact and origin of skeletal muscle non-shivering thermogenesis is currently unclear, especially in humans. Previous studies have focussed on UCP3 – an UCP1 analogue expressed in skeletal muscle – and its potential role in mitochondrial uncoupling in skeletal muscle during cold exposure (89-92). However, the exact role of UCP3 in skeletal muscle non-shivering thermogenesis remains to be further explored, as discussed in more detail elsewhere (86). In short, several studies have reported an increase in skeletal muscle mitochondrial uncoupling upon acute cold exposure (89-91), albeit that UCP3 protein expression either remained unaffected (90, 92) or tended to increase (89). Furthermore, it appears that UCP3 is primarily involved in the regulation of fatty acid metabolism, thereby further putting into question its role in mitochondrial uncoupling (93).

Apart from UCP3, an emerging body of evidence has suggested a role of futile Ca^{2+} cycling in skeletal muscle non-shivering thermogenesis. Skeletal muscle is characterised by a highly developed sarcoplasmic reticulum for intracellular storage of Ca^{2+}, which is released into the cytoplasm upon stimulation (e.g. depolarisation). The released Ca^{2+} is involved in various processes, including muscle contraction and intracellular signalling, but is afterwards rapidly transported back into the sarcoplasmic reticulum via the SERCA pump at the expense of ATP (94). However, the latter process can be regulated via a small molecular weight protein named sarcolipin (SLN), which uncouples SERCA-mediated Ca^{2+} transport. SLN thereby leaks Ca^{2+} back into the cytoplasm during SERCA-mediated transport into the sarcoplasmic reticulum, resulting in a futile Ca^{2+} cycle that increases ATP utilization and subsequently heat production (95, 96). In line with the latter, mice ablated of intrascapular BAT were able to maintain a stable core temperature upon prolonged cold exposure (4 ˚C for 9 days), independent of shivering, through an upregulation of skeletal muscle SERCA and SLN expression (97). This effect was abolished entirely in UCP1 and SLN double knock-out mice (98) or iBAT-ablated SLN KO mice (99), who were

unable to maintain a stable core temperature during prolonged cold exposure, unless gradually adapted (98). Combined, these results clearly demonstrate a pivotal role of SLN in skeletal muscle non-shivering thermogenesis in rodents. In human skeletal muscle, the expression of SLN is several fold higher as compared to rodents (100), although the role of this protein in human skeletal muscle non-shivering thermogenesis is entirely unknown. Hitherto, only SLN protein expression, but not activity, has been measured and compared between metabolically different individuals. Thus, in T2DM patients it was shown that SERCA2 and SLN expression remained unaltered upon prolonged mild cold acclimation (13). Furthermore, SLN expression was not significantly different between lean and obese subjects and does not correlate with either body fat percentage or resting metabolic rate (101). A role for SLN in human skeletal muscle non-shivering thermogenesis thus remains controversial, but should be investigated in more detail.

Given its apparent role in skeletal muscle non-shivering thermogenesis in rodents, it is not surprising that muscle-specific SLN KO or overexpression has been associated with significant decreases or increases in basal energy expenditure, respectively, both *in vitro* and *in vivo* (99, 102-104). In line with the latter, SLN overexpression also markedly increased whole-body and muscle-specific fatty acid oxidation and transport, as well as mitochondrial density, quality and oxidative capacity (95, 103). Interestingly, these studies also demonstrate robust improvements in fasting glucose concentrations, skeletal muscle glucose uptake, glucose tolerance, and insulin sensitivity upon SLN overexpression, whereas SLN KO displayed the opposite phenotype (95, 99, 103, 104). However, it should be noted that these effects were accompanied by significant changes in body weight and fat mass, thereby limiting robust conclusions of the specific effects of SLN-induced non-shivering thermogenesis on skeletal muscle glucose uptake, and glucose and insulin tolerance (99, 103, 104).

6.3. Skeletal muscle glucose uptake via β-adrenergic receptors

Exposure to cold conditions is well-known for its activation of the sympathetic nervous system (SNS) (105), which has previously been suggested to underlie cold-induced skeletal muscle glucose uptake (7). Activation of the ventromedial hypothalamus (VMH) via either electrical or chemical stimulation has been shown to significantly increase skeletal muscle glucose uptake in rats, independently of changes in plasma insulin levels (106-108). Given that cold-induced SNS activity is highly associated with norepinephrine release by sympathetic nerve endings and – to a lesser extent – secretion of epinephrine into the circulation (109-113), skeletal muscle glucose uptake upon VMH stimulation

is likely attributed to catecholamine secretion. Indeed, inhibition of postganglionic norepinephrine release via guanethidine treatment during VMH stimulation in rats was shown to abolish skeletal muscle glucose uptake, whereas removing the adrenal medulla – which abolishes epinephrine secretion - did not (108). These finding thus indicate that cold-induced skeletal muscle glucose uptake is primarily mediated via local norepinephrine release and is less reliant on circulating epinephrine.

Since norepinephrine binds to adrenergic receptors (ARs) to elicit its metabolic effects, studies have focussed on identifying the ARs involved in skeletal muscle glucose uptake. Although norepinephrine has a higher affinity for β_1- and β_3-ARs (114, 115), the effects on muscle appear primarily mediated via the β_2-AR (116), the major subtype of ARs in myocytes (117). Thus, incubation of L6 muscle cells, C2C12 cells, or human primary myotubes with selective β_2- and/or β_2/β_3-AR agonists was shown to promote glucose uptake through GLUT4 translocation (116, 118-122), whereas selective β_1-AR agonists had no effect (116). Interestingly, these effects are not limited to cell cultures, since incubation of isolated rat soleus or EDL muscle with BRL37344 – a β-agonist specifically designed for β_3-ARs but was recently also shown to bind and activate β_2-ARs – significantly increased glucose uptake through activation of β_2-ARs (123, 124), thereby hinting towards an *in vivo* physiological relevance of (β_2-)adrenergic stimulation on skeletal muscle glucose uptake.

However, investigating the *in vivo* effects of β_2-AR agonists on skeletal muscle glucose uptake is complex. Acute administration of β_2-AR agonists is well-characterised to induce hyperglycaemia and hyperinsulinemia (119, 125-127), thereby inherently affecting skeletal muscle glucose uptake. Thus, acute injection with BRL35135 – which was designed as a β_3-AR agonist but was shown to bind to β_2-ARs – has previously been demonstrated to increase skeletal muscle glucose uptake, an effect that was paralleled by increased plasma insulin concentrations (128). Nevertheless, prolonged treatment (6 days) with the selective β_2-AR agonist clenbuterol markedly increased basal *in vivo* skeletal muscle glucose uptake independent of changes in plasma insulin concentrations (119), demonstrating an insulin-independent mechanism of *in vivo* β_2-AR agonist-mediated glucose uptake. In line with these findings – and in contrast to the acute effects (129) – long-term treatment of diet-induced obese (DIO) mice, as well as diabetic rodents, with a selective β_2-AR agonist has been shown to robustly improve glucose tolerance (118, 119, 130-135), significantly reduce plasma insulin concentrations (119, 130, 132, 133) and improve insulin sensitivity (132, 133, 136).

Similar to the effects seen in animals, acute administration of β_2-AR agonists in humans is also highly associated with increases in plasma glucose and insulin levels (137-139).

Thus, acute administration of the selective β_2-AR agonist terbutaline (0.2-0.4 mg) in healthy volunteers significantly increased plasma insulin concentrations as well as leg glucose uptake, although the authors - based on further statistical modelling - concluded the increased glucose clearance to be independent of higher insulin concentrations (137). Similar to the data in rodents, a marked discrepancy also seems to exist between acute and chronic effects of β_2-AR agonist supplementation in humans. Indeed, 10-day supplementation with the selective β_2-/β_3-AR agonist BRL35135 significantly improves glucose tolerance and insulin sensitivity in obese subjects (140). Although the tissues involved in this process were not identified, the authors found that this effect was entirely due to an increased glucose disposal (140). To our knowledge, merely two studies have investigated the effects of prolonged β_2-AR agonist supplementation on skeletal muscle glucose uptake. Thus, oral supplementation with the β_2-AR agonist terbutaline sulphate (3x 5 mg/day for 1 or 2 weeks) significantly increased insulin stimulated glucose disposal by ~29% in healthy young males, an effect that was primarily attributed to an increased non-oxidative glucose disposal of ~45% (141). Similar beneficial effects have been reported in healthy, young males upon 4-week inhalation with the selective β_2-AR agonist terbutaline (4 mg/day), which enhanced glucose infusion rate during a hyperinsulinemic-euglycemic clamp by ~27% (142), albeit that these effects were paralleled by a significant increase in lean mass. These studies combined demonstrate that prolonged β_2-AR treatment markedly affects skeletal muscle glucose uptake and whole-body glucose homeostasis, although this remains to be established in T2DM patients.

In addition to the latter, the molecular pathways involved in mediating skeletal muscle glucose uptake upon β_2-AR stimulation remain mostly elusive. To our knowledge, Sato et al. (118) were the first to investigate these mechanisms, demonstrating that activation of the mammalian target of rapamycin (mTOR) complex 2 (mTORC2) is key for β_2-AR-mediated glucose uptake in both L6 muscle cells and human primary myotubes (118). These stimulatory effects could be abolished by specific mTOR inhibitors or ablation of the fundamental Rictor subunit of mTORC2 via siRNA (118, 122). The role of mTORC2 in β_2-AR-mediated skeletal muscle glucose uptake was recently further highlighted in an elegant study by Meister et al. (134), who demonstrated that clenbuterol-mediated improvements in glucose homeostasis are abolished in skeletal muscle-specific Rictor knock-out mice. However, whether mTORC2 is also involved in mediating cold-induced improvements in (human) skeletal muscle glucose uptake remains to be established.

7. Conclusion

The epidemic rise in the prevalence of T2DM, and the concurrent lack of easily adherable and effective treatment strategies for these patients, have highlighted the urgent need for novel therapeutic approaches to improve glucose homeostasis. In this context, cold exposure has presented itself as a highly attractive treatment strategy for T2DM, with several (pre)clinical studies reporting marked beneficial effects on glucose disposal and insulin sensitivity. These improvements are likely primarily attributed to adaptations within the skeletal muscle, albeit that other metabolic organs (such as the liver and WAT) are also expected to be implicated. To date, the (molecular) pathways underlying the latter improvements remain elusive. Nevertheless, a comprehensive understanding of the mechanisms involved could potentially reveal urgently warranted, novel targets for the treatment of T2DM. It is therefore strongly encouraged that future studies within this – relatively unexplored – field focus on a detailed elaboration of the pathways mediating cold-induced improvements in glucose homeostasis in humans. Until that time, turning down the thermostat, taking a cold(er) shower or even removing a sweater could be a safe, sustainable start to improve your metabolic health.

8. Funding

This research was funded by a grant from the Nutrim NWO graduate program.

References

1. Haman F, Blondin DP. Shivering thermogenesis in humans: Origin, contribution and metabolic requirement. Temperature (Austin). 2017;4(3):217-26.
2. van Marken Lichtenbelt WD, Schrauwen P. Implications of nonshivering thermogenesis for energy balance regulation in humans. Am J Physiol Regul Integr Comp Physiol. 2011;301(2):R285-96.
3. Vallerand AL, Lupien J, Bukowiecki LJ. Interactions of cold exposure and starvation on glucose tolerance and insulin response. Am J Physiol. 1983;245(6):E575-81.
4. Smith OL, Davidson SB. Shivering thermogenesis and glucose uptake by muscles of normal or diabetic rats. Am J Physiol. 1982;242(1):R109-15.
5. Smith OL. Insulin response in rats acutely exposed to cold. Can J Physiol Pharmacol. 1984;62(8):924-7.
6. Vallerand AL, Perusse F, Bukowiecki LJ. Cold exposure potentiates the effect of insulin on in vivo glucose uptake. Am J Physiol. 1987;253(2 Pt 1):E179-86.
7. Vallerand AL, Perusse F, Bukowiecki LJ. Stimulatory effects of cold exposure and cold acclimation on glucose uptake in rat peripheral tissues. Am J Physiol. 1990;259(5 Pt 2):R1043-9.
8. Vallerand AL, Lupien J, Bukowiecki LJ. Cold exposure reverses the diabetogenic effects of high-fat feeding. Diabetes. 1986;35(3):329-34.
9. Cunningham JJ, Gulino MA, Meara PA, Bode HH. Enhanced hepatic insulin sensitivity and peripheral glucose uptake in cold acclimating rats. Endocrinology. 1985;117(4):1585-9.
10. Vallerand AL, Zamecnik J, Jacobs I. Plasma glucose turnover during cold stress in humans. J Appl Physiol (1985). 1995;78(4):1296-302.
11. Vallerand AL, Frim J, Kavanagh MF. Plasma glucose and insulin responses to oral and intravenous glucose in cold-exposed humans. J Appl Physiol (1985). 1988;65(6):2395-9.
12. Iwen KA, Backhaus J, Cassens M, Waltl M, Hedesan OC, Merkel M, et al. Cold-Induced Brown Adipose Tissue Activity Alters Plasma Fatty Acids and Improves Glucose Metabolism in Men. J Clin Endocrinol Metab. 2017;102(11):4226-34.
13. Hanssen MJ, Hoeks J, Brans B, van der Lans AA, Schaart G, van den Driessche JJ, et al. Short-term cold acclimation improves insulin sensitivity in patients with type 2 diabetes mellitus. Nat Med. 2015;21(8):863-5.
14. Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. Physiol Rev. 2004;84(1):277-359.
15. Shibata H, Perusse F, Vallerand A, Bukowiecki LJ. Cold exposure reverses inhibitory effects of fasting on peripheral glucose uptake in rats. Am J Physiol. 1989;257(1 Pt 2):R96-101.
16. Orava J, Nuutila P, Lidell ME, Oikonen V, Noponen T, Viljanen T, et al. Different metabolic responses of human brown adipose tissue to activation by cold and insulin. Cell Metab. 2011;14(2):272-9.
17. Bartelt A, Bruns OT, Reimer R, Hohenberg H, Ittrich H, Peldschus K, et al. Brown adipose tissue activity controls triglyceride clearance. Nat Med. 2011;17(2):200-5.
18. Xiao C, Goldgof M, Gavrilova O, Reitman ML. Anti-obesity and metabolic efficacy of the beta3-adrenergic agonist, CL316243, in mice at thermoneutrality compared to 22 degrees C. Obesity (Silver Spring). 2015;23(7):1450-9.
19. Olsen JM, Csikasz RI, Dehvari N, Lu L, Sandstrom A, Oberg AI, et al. beta3-Adrenergically induced glucose uptake in brown adipose tissue is independent of UCP1 presence or activity: Mediation through the mTOR pathway. Mol Metab. 2017;6(6):611-9.

20. van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts JM, Kemerink GJ, Bouvy ND, et al. Cold-activated brown adipose tissue in healthy men. N Engl J Med. 2009;360(15):1500-8.
21. Brendle C, Werner MK, Schmadl M, la Fougere C, Nikolaou K, Stefan N, et al. Correlation of Brown Adipose Tissue with Other Body Fat Compartments and Patient Characteristics: A Retrospective Analysis in a Large Patient Cohort Using PET/CT. Acad Radiol. 2018;25(1):102-10.
22. Roder PV, Wu B, Liu Y, Han W. Pancreatic regulation of glucose homeostasis. Exp Mol Med. 2016;48:e219.
23. Seitz HJ, Krone W, Wilke H, Tarnowski W. Rapid rise in plasma glucagon induced by acute cold exposure in man and rat. Pflugers Arch. 1981;389(2):115-20.
24. Kuroshima A, Yahata T, Ohno T. Changes in plasma glucagon levels to stressful environmental temperatures. Jpn J Physiol. 1981;31(1):43-52.
25. Kuroshima A, Yahata T, Doi K, Ohno T. Thermal and metabolic responses of temperature-acclimated rats during cold and heat exposures. Jpn J Physiol. 1982;32(4):561-71.
26. Sepa-Kishi DM, Katsnelson G, Bikopoulos G, Iqbal A, Ceddia RB. Cold acclimation reduces hepatic protein Kinase B and AMP-activated protein kinase phosphorylation and increases gluconeogenesis in Rats. Physiol Rep. 2018;6(5).
27. Doi K, Ohno T, Kuroshima A. Role of endocrine pancreas in temperature acclimation. Life Sci. 1982;30(26):2253-9.
28. Morton GJ, Muta K, Kaiyala KJ, Rojas JM, Scarlett JM, Matsen ME, et al. Evidence That the Sympathetic Nervous System Elicits Rapid, Coordinated, and Reciprocal Adjustments of Insulin Secretion and Insulin Sensitivity During Cold Exposure. Diabetes. 2017;66(4):823-34.
29. Gasparetti AL, de Souza CT, Pereira-da-Silva M, Oliveira RL, Saad MJ, Carneiro EM, et al. Cold exposure induces tissue-specific modulation of the insulin-signalling pathway in Rattus norvegicus. J Physiol. 2003;552(Pt 1):149-62.
30. Beck LV, Zaharko DS, Kalser SC. Variation in serum insulin and glucose of rats with chronic cold exposure. Life Sci. 1967;6(14):1501-6.
31. Harada E, Habara Y, Kanno T. Cold acclimation in insulin secretion of isolated perfused pancreas of the rat. Am J Physiol. 1982;242(6):E360-7.
32. Blondin DP, Labbe SM, Noll C, Kunach M, Phoenix S, Guerin B, et al. Selective Impairment of Glucose but Not Fatty Acid or Oxidative Metabolism in Brown Adipose Tissue of Subjects With Type 2 Diabetes. Diabetes. 2015;64(7):2388-97.
33. Hanssen MJ, van der Lans AA, Brans B, Hoeks J, Jardon KM, Schaart G, et al. Short-term Cold Acclimation Recruits Brown Adipose Tissue in Obese Humans. Diabetes. 2016;65(5):1179-89.
34. Chondronikola M, Volpi E, Borsheim E, Porter C, Annamalai P, Enerback S, et al. Brown adipose tissue improves whole-body glucose homeostasis and insulin sensitivity in humans. Diabetes. 2014;63(12):4089-99.
35. Sellers AJ, Pallubinsky H, Rense P, Bijnens W, van de Weijer T, Moonen-Kornips E, et al. The effect of cold exposure with shivering on glucose tolerance in healthy men. J Appl Physiol (1985). 2021;130(1):193-205.
36. Habara Y, Ohno T, Yahata T, Kuroshima A. Effects of adrenal demedullation combined with chemical sympathectomy on cold-induced responses of endocrine pancreas in rats. Experientia. 1983;39(4):399-400.
37. Young JB, Landsberg L. Effect of diet and cold exposure on norepinephrine turnover in pancreas and liver. Am J Physiol. 1979;236(5):E524-33.

38. van der Lans AA, Hoeks J, Brans B, Vijgen GH, Visser MG, Vosselman MJ, et al. Cold acclimation recruits human brown fat and increases nonshivering thermogenesis. J Clin Invest. 2013;123(8):3395-403.
39. Remie CME, Moonen MPB, Roumans KHM, Nascimento EBM, Gemmink A, Havekes B, et al. Metabolic responses to mild cold acclimation in type 2 diabetes patients. Nat Commun. 2021;12(1):1516.
40. Grefhorst A, van den Beukel JC, Dijk W, Steenbergen J, Voortman GJ, Leeuwenburgh S, et al. Multiple effects of cold exposure on livers of male mice. J Endocrinol. 2018;238(2):91-106.
41. Bobbioni-Harsch E, Assimacopoulos-Jeannet F, Jeanrenaud B. Modifications of glucose and lipid metabolism in cold-acclimated lean and genetically obese rats. J Appl Physiol (1985). 1994;76(3):1106-12.
42. Guezennec CY, Nonglaton J, Serrurier B, Merino D, Defer G. Hormonal and metabolic response to physical exercise, fasting and cold exposure in the rat. Effects on ketogenesis in isolated hepatocytes. Eur J Appl Physiol Occup Physiol. 1988;57(1):114-9.
43. Penner PE, Himms-Hagen J. Gluconeogenesis in rats during cold acclimation. Can J Biochem. 1968;46(10):1205-13.
44. Nakagawa H, Nagai K. Cold adaptation. I. Effect of cold-exposure on gluconeogenesis. J Biochem. 1971;69(5):923-34.
45. Smith SA, Young P, Cawthorne MA. Quantification in vivo of the effects of insulin on glucose utilization in individual tissues of warm- and cold-acclimated rats. Biochem J. 1986;237(3):789-95.
46. Johnstone AM, Murison SD, Duncan JS, Rance KA, Speakman JR. Factors influencing variation in basal metabolic rate include fat-free mass, fat mass, age, and circulating thyroxine but not sex, circulating leptin, or triiodothyronine. Am J Clin Nutr. 2005;82(5):941-8.
47. Blondin DP, Nielsen S, Kuipers EN, Severinsen MC, Jensen VH, Miard S, et al. Human Brown Adipocyte Thermogenesis Is Driven by beta2-AR Stimulation. Cell Metab. 2020;32(2):287-300 e7.
48. Granneman JG, Burnazi M, Zhu Z, Schwamb LA. White adipose tissue contributes to UCP1-independent thermogenesis. Am J Physiol Endocrinol Metab. 2003;285(6):E1230-6.
49. Liu X, Perusse F, Bukowiecki LJ. Chronic norepinephrine infusion stimulates glucose uptake in white and brown adipose tissues. Am J Physiol. 1994;266(3 Pt 2):R914-20.
50. Castro E, Vieira TS, Oliveira TE, Ortiz-Silva M, Andrade ML, Tomazelli CA, et al. Adipocyte-specific mTORC2 deficiency impairs BAT and iWAT thermogenic capacity without affecting glucose uptake and energy expenditure in cold-acclimated mice. Am J Physiol Endocrinol Metab. 2021;321(5):E592-E605.
51. Agosto E, Cimmino M, Minaire Y, Geloen A. Short-term cold-exposure does not improve insulin sensitivity in rats. Comp Biochem Physiol A Physiol. 1997;117(2):231-8.
52. Walden TB, Hansen IR, Timmons JA, Cannon B, Nedergaard J. Recruited vs. nonrecruited molecular signatures of brown, "brite," and white adipose tissues. Am J Physiol Endocrinol Metab. 2012;302(1):E19-31.
53. Nedergaard J, Cannon B. The browning of white adipose tissue: some burning issues. Cell Metab. 2014;20(3):396-407.
54. Sepa-Kishi DM, Jani S, Da Eira D, Ceddia RB. Cold acclimation enhances UCP1 content, lipolysis, and triacylglycerol resynthesis, but not mitochondrial uncoupling and fat oxidation, in rat white adipocytes. Am J Physiol Cell Physiol. 2019;316(3):C365-C76.

55. Cousin B, Cinti S, Morroni M, Raimbault S, Ricquier D, Penicaud L, et al. Occurrence of brown adipocytes in rat white adipose tissue: molecular and morphological characterization. J Cell Sci. 1992;103 (Pt 4):931-42.
56. Okamatsu-Ogura Y, Fukano K, Tsubota A, Uozumi A, Terao A, Kimura K, et al. Thermogenic ability of uncoupling protein 1 in beige adipocytes in mice. PLoS One. 2013;8(12):e84229.
57. Shabalina IG, Petrovic N, de Jong JM, Kalinovich AV, Cannon B, Nedergaard J. UCP1 in brite/beige adipose tissue mitochondria is functionally thermogenic. Cell Rep. 2013;5(5):1196-203.
58. Young P, Arch JR, Ashwell M. Brown adipose tissue in the parametrial fat pad of the mouse. FEBS Lett. 1984;167(1):10-4.
59. Wang Z, Ning T, Song A, Rutter J, Wang QA, Jiang L. Chronic cold exposure enhances glucose oxidation in brown adipose tissue. EMBO Rep. 2020;21(11):e50085.
60. Wu J, Bostrom P, Sparks LM, Ye L, Choi JH, Giang AH, et al. Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. Cell. 2012;150(2):366-76.
61. Mossenbock K, Vegiopoulos A, Rose AJ, Sijmonsma TP, Herzig S, Schafmeier T. Browning of white adipose tissue uncouples glucose uptake from insulin signaling. PLoS One. 2014;9(10):e110428.
62. Jia XW, Fang DL, Shi XY, Lu T, Yang C, Gao Y. Inducible beige adipocytes improve impaired glucose metabolism in interscapular BAT-removal mice. Biochim Biophys Acta Mol Cell Biol Lipids. 2021;1866(3):158871.
63. Finlin BS, Memetimin H, Confides AL, Kasza I, Zhu B, Vekaria HJ, et al. Human adipose beiging in response to cold and mirabegron. JCI Insight. 2018;3(15).
64. Sidossis LS, Porter C, Saraf MK, Borsheim E, Radhakrishnan RS, Chao T, et al. Browning of Subcutaneous White Adipose Tissue in Humans after Severe Adrenergic Stress. Cell Metab. 2015;22(2):219-27.
65. Finlin BS, Memetimin H, Zhu B, Confides AL, Vekaria HJ, El Khouli RH, et al. The beta3-adrenergic receptor agonist mirabegron improves glucose homeostasis in obese humans. J Clin Invest. 2020;130(5):2319-31.
66. Meyer CW, Willershauser M, Jastroch M, Rourke BC, Fromme T, Oelkrug R, et al. Adaptive thermogenesis and thermal conductance in wild-type and UCP1-KO mice. Am J Physiol Regul Integr Comp Physiol. 2010;299(5):R1396-406.
67. Ukropec J, Anunciado RP, Ravussin Y, Hulver MW, Kozak LP. UCP1-independent thermogenesis in white adipose tissue of cold-acclimated Ucp1-/- mice. J Biol Chem. 2006;281(42):31894-908.
68. Roesler A, Kazak L. UCP1-independent thermogenesis. Biochem J. 2020;477(3):709-25.
69. Chouchani ET, Kazak L, Spiegelman BM. New Advances in Adaptive Thermogenesis: UCP1 and Beyond. Cell Metab. 2019;29(1):27-37.
70. Kazak L, Chouchani ET, Jedrychowski MP, Erickson BK, Shinoda K, Cohen P, et al. A creatine-driven substrate cycle enhances energy expenditure and thermogenesis in beige fat. Cell. 2015;163(3):643-55.
71. Kazak L, Chouchani ET, Lu GZ, Jedrychowski MP, Bare CJ, Mina AI, et al. Genetic Depletion of Adipocyte Creatine Metabolism Inhibits Diet-Induced Thermogenesis and Drives Obesity. Cell Metab. 2017;26(4):693.
72. Rahbani JF, Roesler A, Hussain MF, Samborska B, Dykstra CB, Tsai L, et al. Creatine kinase B controls futile creatine cycling in thermogenic fat. Nature. 2021;590(7846):480-5.
73. Brownstein AJ, Veliova M, Acin-Perez R, Liesa M, Shirihai OS. ATP-consuming futile cycles as energy dissipating mechanisms to counteract obesity. Rev Endocr Metab Disord. 2022;23(1):121-31.

74. Flachs P, Rossmeisl M, Kuda O, Kopecky J. Stimulation of mitochondrial oxidative capacity in white fat independent of UCP1: a key to lean phenotype. Biochim Biophys Acta. 2013;1831(5):986-1003.
75. Hammond VA, Johnston DG. Substrate cycling between triglyceride and fatty acid in human adipocytes. Metabolism. 1987;36(4):308-13.
76. Bottcher H, Furst P. Decreased white fat cell thermogenesis in obese individuals. Int J Obes Relat Metab Disord. 1997;21(6):439-44.
77. Wolfe RR, Klein S, Carraro F, Weber JM. Role of triglyceride-fatty acid cycle in controlling fat metabolism in humans during and after exercise. Am J Physiol. 1990;258(2 Pt 1):E382-9.
78. Flachs P, Adamcova K, Zouhar P, Marques C, Janovska P, Viegas I, et al. Induction of lipogenesis in white fat during cold exposure in mice: link to lean phenotype. Int J Obes (Lond). 2017;41(6):997.
79. Mottillo EP, Balasubramanian P, Lee YH, Weng C, Kershaw EE, Granneman JG. Coupling of lipolysis and de novo lipogenesis in brown, beige, and white adipose tissues during chronic beta3-adrenergic receptor activation. J Lipid Res. 2014;55(11):2276-86.
80. Vallerand AL, Zamecnik J, Jones PJ, Jacobs I. Cold stress increases lipolysis, FFA Ra and TG/FFA cycling in humans. Aviat Space Environ Med. 1999;70(1):42-50.
81. DeFronzo RA, Gunnarsson R, Bjorkman O, Olsson M, Wahren J. Effects of insulin on peripheral and splanchnic glucose metabolism in noninsulin-dependent (type II) diabetes mellitus. J Clin Invest. 1985;76(1):149-55.
82. DeFronzo RA, Jacot E, Jequier E, Maeder E, Wahren J, Felber JP. The effect of insulin on the disposal of intravenous glucose. Results from indirect calorimetry and hepatic and femoral venous catheterization. Diabetes. 1981;30(12):1000-7.
83. Blondin DP, Labbe SM, Phoenix S, Guerin B, Turcotte EE, Richard D, et al. Contributions of white and brown adipose tissues and skeletal muscles to acute cold-induced metabolic responses in healthy men. J Physiol. 2015;593(3):701-14.
84. Oliveira RL, Ueno M, de Souza CT, Pereira-da-Silva M, Gasparetti AL, Bezzera RM, et al. Cold-induced PGC-1alpha expression modulates muscle glucose uptake through an insulin receptor/Akt-independent, AMPK-dependent pathway. Am J Physiol Endocrinol Metab. 2004;287(4):E686-95.
85. Eyolfson DA, Tikuisis P, Xu X, Weseen G, Giesbrecht GG. Measurement and prediction of peak shivering intensity in humans. Eur J Appl Physiol. 2001;84(1-2):100-6.
86. Blondin DP, Haman F. Shivering and nonshivering thermogenesis in skeletal muscles. Handb Clin Neurol. 2018;156:153-73.
87. Haman F, Peronnet F, Kenny GP, Massicotte D, Lavoie C, Scott C, et al. Effect of cold exposure on fuel utilization in humans: plasma glucose, muscle glycogen, and lipids. J Appl Physiol (1985). 2002;93(1):77-84.
88. Horvath SM. Exercise in a cold environment. Exerc Sport Sci Rev. 1981;9:221-63.
89. Blondin DP, Daoud A, Taylor T, Tingelstad HC, Bezaire V, Richard D, et al. Four-week cold acclimation in adult humans shifts uncoupling thermogenesis from skeletal muscles to brown adipose tissue. J Physiol. 2017;595(6):2099-113.
90. Wijers SL, Schrauwen P, Saris WH, van Marken Lichtenbelt WD. Human skeletal muscle mitochondrial uncoupling is associated with cold induced adaptive thermogenesis. PLoS One. 2008;3(3):e1777.
91. Wijers SL, Schrauwen P, van Baak MA, Saris WH, van Marken Lichtenbelt WD. Beta-adrenergic receptor blockade does not inhibit cold-induced thermogenesis in humans: possible involvement of brown adipose tissue. J Clin Endocrinol Metab. 2011;96(4):E598-605.

92. Schrauwen P, Westerterp-Plantenga MS, Kornips E, Schaart G, van Marken Lichtenbelt WD. The effect of mild cold exposure on UCP3 mRNA expression and UCP3 protein content in humans. Int J Obes Relat Metab Disord. 2002;26(4):450-7.

93. Schrauwen P, Hesselink MK. The role of uncoupling protein 3 in fatty acid metabolism: protection against lipotoxicity? Proc Nutr Soc. 2004;63(2):287-92.

94. Pant M, Bal NC, Periasamy M. Sarcolipin: A Key Thermogenic and Metabolic Regulator in Skeletal Muscle. Trends Endocrinol Metab. 2016;27(12):881-92.

95. Maurya SK, Herrera JL, Sahoo SK, Reis FCG, Vega RB, Kelly DP, et al. Sarcolipin Signaling Promotes Mitochondrial Biogenesis and Oxidative Metabolism in Skeletal Muscle. Cell Rep. 2018;24(11):2919-31.

96. Mall S, Broadbridge R, Harrison SL, Gore MG, Lee AG, East JM. The presence of sarcolipin results in increased heat production by Ca(2+)-ATPase. J Biol Chem. 2006;281(48):36597-602.

97. Bal NC, Maurya SK, Singh S, Wehrens XH, Periasamy M. Increased Reliance on Muscle-based Thermogenesis upon Acute Minimization of Brown Adipose Tissue Function. J Biol Chem. 2016;291(33):17247-57.

98. Rowland LA, Bal NC, Kozak LP, Periasamy M. Uncoupling Protein 1 and Sarcolipin Are Required to Maintain Optimal Thermogenesis, and Loss of Both Systems Compromises Survival of Mice under Cold Stress. J Biol Chem. 2015;290(19):12282-9.

99. Bal NC, Maurya SK, Sopariwala DH, Sahoo SK, Gupta SC, Shaikh SA, et al. Sarcolipin is a newly identified regulator of muscle-based thermogenesis in mammals. Nat Med. 2012;18(10):1575-9.

100. Periasamy M, Herrera JL, Reis FCG. Skeletal Muscle Thermogenesis and Its Role in Whole Body Energy Metabolism. Diabetes Metab J. 2017;41(5):327-36.

101. Morales-Alamo D, Martinez-Canton M, Gelabert-Rebato M, Martin-Rincon M, de Pablos-Velasco P, Holmberg HC, et al. Sarcolipin expression in human skeletal muscle: Influence of energy balance and exercise. Scand J Med Sci Sports. 2020;30(3):408-20.

102. Paran CW, Verkerke AR, Heden TD, Park S, Zou K, Lawson HA, et al. Reduced efficiency of sarcolipin-dependent respiration in myocytes from humans with severe obesity. Obesity (Silver Spring). 2015;23(7):1440-9.

103. Maurya SK, Bal NC, Sopariwala DH, Pant M, Rowland LA, Shaikh SA, et al. Sarcolipin Is a Key Determinant of the Basal Metabolic Rate, and Its Overexpression Enhances Energy Expenditure and Resistance against Diet-induced Obesity. J Biol Chem. 2015;290(17):10840-9.

104. Bombardier E, Smith IC, Vigna C, Fajardo VA, Tupling AR. Ablation of sarcolipin decreases the energy requirements for Ca2+ transport by sarco(endo)plasmic reticulum Ca2+-ATPases in resting skeletal muscle. FEBS Lett. 2013;587(11):1687-92.

105. Dulloo AG, Young JB, Landsberg L. Sympathetic nervous system responses to cold exposure and diet in rat skeletal muscle. Am J Physiol. 1988;255(2 Pt 1):E180-8.

106. Shimazu T, Sudo M, Minokoshi Y, Takahashi A. Role of the hypothalamus in insulin-independent glucose uptake in peripheral tissues. Brain Res Bull. 1991;27(3-4):501-4.

107. Sudo M, Minokoshi Y, Shimazu T. Ventromedial hypothalamic stimulation enhances peripheral glucose uptake in anesthetized rats. Am J Physiol. 1991;261(3 Pt 1):E298-303.

108. Minokoshi Y, Okano Y, Shimazu T. Regulatory mechanism of the ventromedial hypothalamus in enhancing glucose uptake in skeletal muscles. Brain Res. 1994;649(1-2):343-7.

109. Depocas F, Behrens WA. Levels of noradrenaline in plasma during thermogenesis induced by cold-exposure or by noradrenaline infusion in warm- and in cold-acclimated rats. Experientia Suppl. 1978;32:135-46.

110. Picotti GB, Carruba MO, Ravazzani C, Cesura AM, Galva MD, Da Prada M. Plasma catecholamines in rats exposed to cold: effects of ganglionic and adrenoreceptor blockade. Eur J Pharmacol. 1981;69(3):321-9.

111. Cypess AM, Chen YC, Sze C, Wang K, English J, Chan O, et al. Cold but not sympathomimetics activates human brown adipose tissue in vivo. Proc Natl Acad Sci U S A. 2012;109(25):10001-5.

112. Scriven AJ, Brown MJ, Murphy MB, Dollery CT. Changes in blood pressure and plasma catecholamines caused by tyramine and cold exposure. J Cardiovasc Pharmacol. 1984;6(5):954-60.

113. O'Malley BP, Cook N, Richardson A, Barnett DB, Rosenthal FD. Circulating catecholamine, thyrotrophin, thyroid hormone and prolactin responses of normal subjects to acute cold exposure. Clin Endocrinol (Oxf). 1984;21(3):285-91.

114. Molinoff PB. Alpha- and beta-adrenergic receptor subtypes properties, distribution and regulation. Drugs. 1984;28 Suppl 2:1-15.

115. Baker JG. The selectivity of beta-adrenoceptor agonists at human beta1-, beta2- and beta3-adrenoceptors. Br J Pharmacol. 2010;160(5):1048-61.

116. Nevzorova J, Bengtsson T, Evans BA, Summers RJ. Characterization of the beta-adrenoceptor subtype involved in mediation of glucose transport in L6 cells. Br J Pharmacol. 2002;137(1):9-18.

117. Liggett SB, Shah SD, Cryer PE. Characterization of beta-adrenergic receptors of human skeletal muscle obtained by needle biopsy. Am J Physiol. 1988;254(6 Pt 1):E795-8.

118. Sato M, Dehvari N, Oberg AI, Dallner OS, Sandstrom AL, Olsen JM, et al. Improving type 2 diabetes through a distinct adrenergic signaling pathway involving mTORC2 that mediates glucose uptake in skeletal muscle. Diabetes. 2014;63(12):4115-29.

119. Kalinovich A, Dehvari N, Åslund A, van Beek S, Halleskog C, Olsen J, et al. Treatment with a β-2-adrenoceptor agonist stimulates glucose uptake in skeletal muscle and improves glucose homeostasis, insulin resistance and hepatic steatosis in mice with diet-induced obesity. Diabetologia. 2020.

120. Nevzorova J, Evans BA, Bengtsson T, Summers RJ. Multiple signalling pathways involved in beta2-adrenoceptor-mediated glucose uptake in rat skeletal muscle cells. Br J Pharmacol. 2006;147(4):446-54.

121. Tanishita T, Shimizu Y, Minokoshi Y, Shimazu T. The beta3-adrenergic agonist BRL37344 increases glucose transport into L6 myocytes through a mechanism different from that of insulin. J Biochem. 1997;122(1):90-5.

122. Mukaida S, Sato M, Oberg AI, Dehvari N, Olsen JM, Kocan M, et al. BRL37344 stimulates GLUT4 translocation and glucose uptake in skeletal muscle via beta2-adrenoceptors without causing classical receptor desensitization. Am J Physiol Regul Integr Comp Physiol. 2019;316(5):R666-R77.

123. Abe H, Minokoshi Y, Shimazu T. Effect of a beta 3-adrenergic agonist, BRL35135A, on glucose uptake in rat skeletal muscle in vivo and in vitro. J Endocrinol. 1993;139(3):479-86.

124. Liu YL, Cawthorne MA, Stock MJ. Biphasic effects of the beta-adrenoceptor agonist, BRL 37344, on glucose utilization in rat isolated skeletal muscle. Br J Pharmacol. 1996;117(6):1355-61.

125. Philipson LH. beta-Agonists and metabolism. J Allergy Clin Immunol. 2002;110(6 Suppl):S313-7.

126. Guhan AR, Cooper S, Oborne J, Lewis S, Bennett J, Tattersfield AE. Systemic effects of formoterol and salmeterol: a dose-response comparison in healthy subjects. Thorax. 2000;55(8):650-6.

127. Burgess C, Ayson M, Rajasingham S, Crane J, Della Cioppa G, Till MD. The extrapulmonary effects of increasing doses of formoterol in patients with asthma. Eur J Clin Pharmacol. 1998;54(2):141-7.

128. Liu YL, Stock MJ. Acute effects of the beta 3-adrenoceptor agonist, BRL 35135, on tissue glucose utilisation. Br J Pharmacol. 1995;114(4):888-94.

129. Smith SA, Levy AL, Sennitt MV, Simson DL, Cawthorne MA. Effects of BRL 26830, a novel beta-adrenoceptor agonist, on glucose tolerance, insulin sensitivity and glucose turnover in Zucker (fa/fa) rats. Biochem Pharmacol. 1985;34(14):2425-9.

130. Castle A, Yaspelkis BB, 3rd, Kuo CH, Ivy JL. Attenuation of insulin resistance by chronic beta2-adrenergic agonist treatment possible muscle specific contributions. Life Sci. 2001;69(5):599-611.

131. Torgan CE, Brozinick JT, Jr., Banks EA, Cortez MY, Wilcox RE, Ivy JL. Exercise training and clenbuterol reduce insulin resistance of obese Zucker rats. Am J Physiol. 1993;264(3 Pt 1):E373-9.

132. van Beek S, Kalinovich A, Schaart G, Bengtsson T, Hoeks J. Prolonged beta2-adrenergic agonist treatment improves glucose homeostasis in diet-induced obese UCP1(-/-) mice. Am J Physiol Endocrinol Metab. 2021.

133. Pan SJ, Hancock J, Ding Z, Fogt D, Lee M, Ivy JL. Effects of clenbuterol on insulin resistance in conscious obese Zucker rats. Am J Physiol Endocrinol Metab. 2001;280(4):E554-61.

134. Meister J, Bone DBJ, Knudsen JR, Barella LF, Velenosi TJ, Akhmedov D, et al. Clenbuterol exerts antidiabetic activity through metabolic reprogramming of skeletal muscle cells. Nat Commun. 2022;13(1):22.

135. Elayan H, Milic M, Sun P, Gharaibeh M, Ziegler MG. Chronic beta2 adrenergic agonist, but not exercise, improves glucose handling in older type 2 diabetic mice. Cell Mol Neurobiol. 2012;32(5):871-7.

136. Kalinovich A, Dehvari N, Aslund A, van Beek S, Halleskog C, Olsen J, et al. Treatment with a beta-2-adrenoceptor agonist stimulates glucose uptake in skeletal muscle and improves glucose homeostasis, insulin resistance and hepatic steatosis in mice with diet-induced obesity. Diabetologia. 2020;63(8):1603-15.

137. Onslev J, Jensen J, Bangsbo J, Wojtaszewski J, Hostrup M. beta2-Agonist Induces Net Leg Glucose Uptake and Free Fatty Acid Release at Rest but Not During Exercise in Young Men. J Clin Endocrinol Metab. 2019;104(3):647-57.

138. Massara F, Fassio V, Camanni F, Martina V, Molinatti G. Some metabolic and hormonal effects of salbutamol in man. Acta Diabetol Lat. 1976;13(3-4):146-53.

139. Kendall MJ, Dean S, Bradley D, Gibson R, Worthington DJ. Cardiovascular and metabolic effects of terbutaline. J Clin Hosp Pharm. 1982;7(1):31-6.

140. Mitchell TH, Ellis RD, Smith SA, Robb G, Cawthorne MA. Effects of BRL 35135, a beta-adrenoceptor agonist with novel selectivity, on glucose tolerance and insulin sensitivity in obese subjects. Int J Obes. 1989;13(6):757-66.

141. Scheidegger K, Robbins DC, Danforth E, Jr. Effects of chronic beta receptor stimulation on glucose metabolism. Diabetes. 1984;33(12):1144-9.

142. Jessen S, Baasch-Skytte T, Onslev J, Eibye K, Backer V, Bangsbo J, et al. Muscle hypertrophic effect of inhaled beta2 -agonist is associated with augmented insulin-stimulated whole-body glucose disposal in young men. J Physiol. 2022;600(10):2345-57.

CHAPTER 3

Treatment with a β-2-adrenoceptor agonist stimulates glucose uptake in skeletal muscle and improves glucose homeostasis, insulin resistance and hepatic steatosis in mice with diet-induced obesity

Anastasia Kalinovich[1, 2], Nodi Dehvari[1, 2], Alice Åslund[1], Sten van Beek[3], Carina Halleskog[1, 2], Jessica Olsen[1, 2], Elisabete Forsberg[2, 4], Evelyn Zacharewicz[3], Gert Schaart[3], Mia Rinde[1], Anna Sandström[1, 2], Roger Berlin[2], Claes-Göran Östenson[5], Joris Hoeks[3] and Tore Bengtsson[1]

[1]Department of Molecular Biosciences, the Wenner-Gren Institute, Stockholm University, Svante Arrhenius väg 20B, Arrhenius laboratories F3, 10691 Stockholm, Sweden

[2]Atrogi AB, Stockholm, Sweden

[3]Department of Nutrition and Movement Sciences, Maastricht University, Maastricht, the Netherlands

[4]Present address: Takeda AB, Stockholm, Sweden

[5]Department of Molecular Medicine and Surgery, Endocrine and Diabetes Unit, Karolinska Institute, Karolinska University Hospital, Stockholm, Sweden.

Diabetologia. 2020 Aug; 63(8):1603-1615

ABSTRACT

Aims/hypothesis - Chronic stimulation of β_2-adrenoceptors, opposite to acute treatment, was reported to reduce blood glucose levels, as well as to improve glucose and insulin tolerance in rodent models of diabetes by essentially unknown mechanisms. We recently described a novel pathway that mediates glucose uptake in skeletal muscle cells via stimulation of β_2-adrenoceptors. In the current study we further explored the potential therapeutic relevance of β_2-adrenoceptor stimulation to improve glucose homeostasis and the mechanisms responsible for the effect.

Methods - C57Bl/6N mice with diet-induced obesity were treated both acutely and for up to 42 days with a wide range of clenbuterol dosages and treatment durations. Glucose homeostasis was assessed by glucose tolerance test. We also measured *in vivo* glucose uptake in skeletal muscle, insulin sensitivity by insulin tolerance test, plasma insulin levels, hepatic lipids and glycogen.

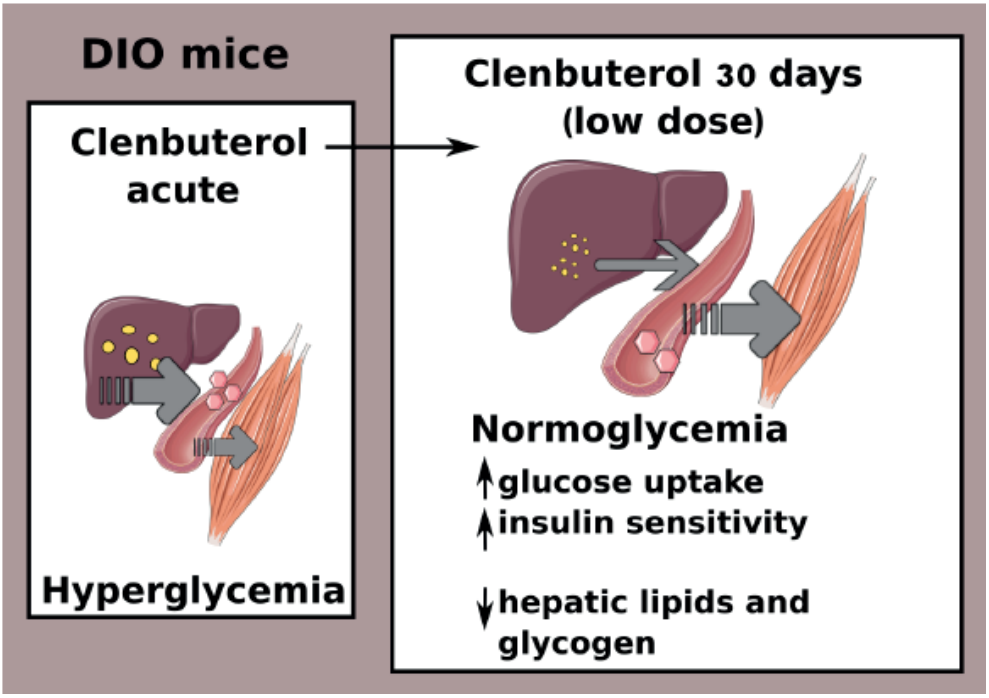
Results - Consistent with previous findings, acute clenbuterol administration increased blood glucose and insulin levels. However, already after 4 days of treatment, beneficial effects of clenbuterol were manifested in glucose homeostasis (32% improvement of glucose tolerance after 4 days of treatment, $p<0.01$) and these effects persisted up to 42 days of treatment. These favourable metabolic effects could be achieved with doses as low as 0.025 mg kg^{-1} day^{-1} (40 times lower than previously studied). Mechanistically, these effects were not due to increased insulin levels, but clenbuterol enhanced glucose uptake in skeletal muscle *in vivo* both acutely in lean mice (by 64%, $p < 0.001$) as well as during chronic treatment in diet-induced obese mice (by 74%, $p < 0.001$). Notably, prolonged treatment with low-dose clenbuterol improved whole-body insulin sensitivity (glucose disposal rate after insulin injection increased up to 1.38 ± 0.31 %/min in comparison with 0.15 ± 0.36 %/min in control mice, $p<0.05$) and drastically reduced hepatic steatosis (by 40 %, $p<0.01$) and glycogen (by 23%, $p<0.05$).

Conclusions/interpretation - Clenbuterol improved glucose tolerance after 4 days of treatment and these effects were maintained for up to 42 days. Effects were achieved with doses in a clinically relevant microgram range. Mechanistically, prolonged treatment with a low dose of clenbuterol improved glucose homeostasis in insulin resistant mice, most likely by stimulating glucose uptake in skeletal muscle and improving whole-body insulin sensitivity as well as by reducing hepatic lipids and glycogen. We conclude that selective β_2-adrenergic agonists might be an attractive potential treatment for type 2 diabetes. This remains to be confirmed in humans.

Keywords

β_2-Adrenergic signalling, Clenbuterol, Insulin resistance, Hepatic steatosis, Skeletal muscle, Type 2 diabetes

Graphical abstract



Abbreviations

AMPK – AMP-activated protein kinase

AR – Adrenergic receptor(s)

DIO mice – Mice with diet-induced obesity

HFD – High-fat diet

mTORC2 – Mammalian target of rapamycin complex 2

Introduction

Type 2 diabetes mellitus is characterised by impaired glucose homeostasis resulting from insulin resistance in peripheral tissues, such as liver and skeletal muscle, as well as from relative impairments in insulin release by pancreatic β-cells. Skeletal muscle is responsible for up to 80% of insulin-stimulated glucose uptake in healthy individuals (1), but glucose uptake in skeletal muscle of diabetic patients is severely impaired owing to insulin resistance. It makes it an attractive target to improve glucose homeostasis. However, there are currently no approved glucose-lowering drugs targeting glucose uptake in skeletal muscles.

We have recently described an entirely novel pathway that stimulates GLUT4-mediated glucose uptake in skeletal muscle cells. This pathway operates independent of the activation of classical regulatory pathways (insulin signalling or AMP-activated protein kinase (AMPK)) but instead involves β_2-adrenergic receptors (β_2-AR) and the activation of the mammalian target of rapamycin complex 2 (mTORC2) (2). We demonstrated that β_2-AR stimulation induced glucose uptake both *in vitro* in myotubes and *in vivo* in skeletal muscle of healthy mice (after acute single injection with a relatively high dose of adrenergic agonists) (2, 3). In addition, we and others have also shown that β_2-AR stimulation, using a relatively high dosage of the β_2-AR agonist clenbuterol, improved glucose tolerance in diet-induced obese (DIO) mice (2), Goto–Kakizaki rats (2) and Zucker fatty rats (4) — well-established rodent models for human type 2 diabetes. In line with these data, β_2-AR-ablation resulted in hyperglycaemia in mice (5, 6).

The beneficial effects described above are not obvious, since it is an accepted fact that β_2-AR agonists administered acutely cause hyperglycaemia (due to enhanced glucose output from the liver) and hyperinsulinaemia in humans and rodents (7-10) and have even been proposed for treatment of hypoglycaemic incidents (11, 12). The mechanism(s) explaining the beneficial effects of prolonged treatment vs deleterious acute effects with β_2-AR agonists *in vivo* have been discussed (13,14), but remain undefined. Thus, we find it important in the present study to highlight and discuss further the difference between acute and chronic treatment with β-AR agonists.

Besides the beneficial effects on glucose tolerance, chronic treatment with clenbuterol or other β-AR agonists improves insulin resistance (13-19). The underlying mechanism by which β-adrenergic agonists beneficially affect insulin sensitivity remains to be elucidated. In the present study we discuss possible contributing factors. While potentiation of insulin-stimulated glucose uptake in muscle was reported for several β-AR agonists,

including clenbuterol (15, 19), basal glucose disposal has not been sufficiently studied, despite its major role in maintaining fasting blood glucose. For this purpose, we studied the effect of β_2-AR agonists on basal glucose uptake (in the fasting state with low insulin levels) during chronic treatment.

Taken together, the current work aimed to further explore the therapeutic relevance of β_2-AR stimulation on glucose homeostasis. To develop our understanding of this matter, we chose clenbuterol as an oral long-lasting selective β_2-AR agonist, since it has been studied most extensively in the field and particularly it has been demonstrated to stimulate glucose uptake in myocytes *in vitro* (2). Clenbuterol has an unfavourable reputation for its misuse in high doses by bodybuilders and fitness enthusiasts because of its hypertrophic effects in muscles and lipolytic effects in adipose tissues. However, clenbuterol in lower doses is generally assumed safe (20-22) and is used in humans for asthma treatment in a number of countries. The current study was performed on a well-established model of type 2 diabetes, DIO mice, which is characterised by obesity, glucose intolerance, insulin resistance and hepatic steatosis. Moreover, DIO mice share the same origin of diabetes with the majority of diabetic patients—obesity-related.

In the current study we first treated DIO mice both acutely and chronically with a wide range of clenbuterol dosages to determine the minimal dose needed to improve glucose tolerance, which has not been studied before. Second, we discussed the mechanistical difference between acute and chronic treatment with β_2-AR agonists. And finally, to explore the mechanisms involved, we treated DIO mice chronically with a relatively low dose of clenbuterol (in comparison with previously used dosages (2, 4, 15) and performed ITT, and measured plasma insulin levels, basal glucose uptake in skeletal muscle as well as hepatic lipids and glycogen.

Methods

For detailed methods, please refer to the ESM.

Animals

C57Bl/6N mice were bred at Stockholm University or purchased from Scanbur (Charles River). To generate diet-induced obesity, mice were fed a high-fat (45% fat) diet (HFD) (D12451, Research Diets) ad libitum for 4-9 months and kept at thermoneutrality (30°C) with a 12 h light/dark cycle and unlimited access to water. Mean body weight of DIO mice was 40-50 g. DIO mice were males unless stated otherwise and 7-10 months old at the

beginning of the treatment. Routinely, mice were housed in individual cages during the treatment. Cages were enriched with wood chips, a cardboard house or a roll, a wooden stick, paper and a piece of cotton. Body composition was measured by EchoMRI-100 (Echo Medical Systems). All procedures were approved by the North Stockholm Ethical Committee for Care and Use of Laboratory Animals.

Treatment

Prior to treatment, mice in different experimental groups had similar glucose tolerance, body weight and body composition. Clenbuterol (C5423, Sigma Aldrich) was injected i.p. daily (dissolved in saline [154 mmol/l NaCl]) or supplied in drinking water. We defined acute treatment as administration of a single dose and chronic (or prolonged) treatment as multiple dose treatment for 4 days or longer. Mice were treated in random order.

GTT, ITT and pyruvate tolerance test

Clenbuterol was not administered on the day of a test. Mice were fasted for 5 h (prior to GTT and ITT) or 12 h (prior to pyruvate tolerance test). For GTT, glucose 2.5 g/kg lean weight (unless stated otherwise) was either injected i.p. or orally gavaged. For ITT and pyruvate tolerance test, human insulin 1 U/kg body weight (Actrapid, NovoNordisk) or pyruvate 2.5 g/kg lean weight (P8574, Sigma Aldrich) was injected i.p. In OGTTs, IPGTTs and pyruvate tolerance test, the total AUC was calculated with 0 mmol/l blood glucose as a baseline. Please note that the effect on fasting blood glucose contributes to the effect on total AUC. During OGTTs, plasma insulin was measured at time points 0 and 15 min by Elisa kit (90080, CrystalChem). In ITT, the rate of blood glucose disposal (K_{ITT}) was calculated as $0.693\times100/t_{1/2}$, where $t_{1/2}$ is the time necessary to reduce blood glucose by half, calculated from the linear regression analysis of data at 0, 15 and 30 min (23). Mice were accessed in random order.

In vivo glucose uptake

Mice were fasted for 5 h, anesthetised with pentobarbital (60 mg/kg i.p.), injected with clenbuterol (1 mg/kg, i.p.) or saline, and 20 min later injected i.p. with 4.81×10^6 Bq/kg of 2-deoxy[^{3}H]glucose (Perkin Elmer, Waltham MA USA; 2.96×10^{11} Bq/mmol) and euthanised 1 h later (as shown in Fig. 1d). Mice were fasted for 5 h, and 5 h after the last treatment with saline/clenbuterol, mice were anaesthetised with pentobarbital (70 mg/kg i.p.), injected i.p. with 4.81×10^6 Bq/kg of 2-deoxy[^{3}H]glucose and 80 min later euthanised. Serum was collected 20 min after 2-deoxy[^{3}H]glucose injection, and insulin was measured as described above (as shown in Fig. 7a–b). Gastrocnemius muscle was

lysed in NaOH and analysed in a beta-counter. Mice were accessed in mixed order and investigators were blinded to treatment.

Hepatic lipids

Fluorescence dye BODIPY 495/503 (0.1 mg/ml; D3922, Molecular Probes) was applied to frozen liver sections for 90 min at 37°C. Images were obtained using E800-fluorescence microscope (Nikon). Total lipids were extracted by two sequential incubations with 5 ml and 2 ml of methanol:chloroform extraction medium (1:2, vol./vol.) at room temperature for 2 days each time. Extraction media with lipids was collected and evaporated and lipid weight was determined gravimetrically.

Hepatic glycogen

A piece of liver was homogenised in water, boiled, centrifuged for 10 min at 18,000*g* and the supernatant was assayed using a kit (ab65620, Abcam).

Cell cultures

L6 rat myoblasts and L6 myoblasts stably expressing GLUT4-myc were purchased from KeraFast (ESK201 and ESK202), where they were tested for mycoplasma. Normal morphology and growth were always controlled. Cells were grown 90% to confluence and differentiated until formation of myotubes (5-7 days).

In vitro glucose uptake

Differentiated L6 cells were serum-starved for 3.5 h, stimulated for 1.5 h with clenbuterol, washed with glucose-free media, stimulated with clenbuterol/saline for another 20 min, exposed to 50 nmol/l 2-deoxy[^{3}H]glucose (Perkin Elmer, Waltham MA USA; 2.96×10^{11} Bq/mmol) for 10 min, washed in glucose-free medium, lysed with NaOH, mixed with scintillation buffer and assayed in a beta-counter.

In vitro GLUT4 translocation

Differentiated L6 cells stably expressing GLUT4-myc were serum-starved for 3 h, stimulated for 2 h with 1 µmol/l clenbuterol or vehicle, fixed with 2% paraformaldehyde, blocked with glycine and BSA, incubated with primary antibody (rabbit anti-myc, 2278 from Cell Signaling, diluted 1:500 in PBS with 5% BSA) overnight in 4°C, incubated in the dark for 1 h with conjugated Alexa Fluor555 goat anti-rabbit antibody (21429 from Invitrogen, diluted 1:500 in PBS with 1.5% BSA). Fluorescence was detected with a fluorescent confocal microscope

(Zeiss LSM 800). When myc-epitope was probed on the cells by Western blot, it resulted in only one band of a right molecular weight (not shown). Omission of the primary antibody resulted in no staining of the cells, confirming specificity of the secondary antibody.

Statistical analysis

Data are expressed as the mean ± SEM. Each data point is a single mouse or, in cell experiments, a mean of duplicates or triplicates from separate experiments. Criteria for data exclusions were: obvious pippeting errors using insulin ELISA kits, which resulted in almost no signal (one value from each control and glucose groups in Fig. 1f; one control value on Fig. 5a and one treated value on Fig. 5b); improperly injected glucose during IPGTT not resulted in rise of blood glucose (two control values in Fig. 3a,b); water leakage resulted in too high apparent water intake (few days in all groups in Fig. 4a). Data were analysed with unpaired two-tailed Student's *t* test, or one-way or two-way ANOVA with the Dunnett's or Sidak's multiple comparison tests as indicated in figure legends. Statistical analyses were performed using GraphPad Prism 8.2. A significant difference was considered at * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Results

Beneficial effects of clenbuterol on glucose tolerance occur after 4 days of treatment and persist for a prolonged period of time

Previously, we have shown significant increases in glucose uptake in L6 myotubes upon acute incubation with the β_2-adrenergic agonist clenbuterol (2). To confirm and elaborate on these results, we demonstrated that acute clenbuterol-mediated glucose uptake in L6 cells occurs in a dose-dependent manner, with doses as low as 10^{-8} mol/l significantly enhancing glucose uptake by 42% ($p<0.01$, Fig. 1a). We also showed that clenbuterol stimulation significantly enhanced GLUT4 translocation in L6 myotubes (by 298%, $p<0.05$, Fig. 1b,c). Moreover, clenbuterol stimulated glucose uptake *in vivo* in skeletal muscle when administered acutely in healthy mice (64%, $p<0.001$, Fig. 1d).

Next, we examined effects of acute clenbuterol administration on whole-body glucose homeostasis *in vivo*. Acute i.p. injection of clenbuterol (1 mg/kg) in DIO mice increased fasting blood glucose and insulin levels compared with a saline injection, up to similar levels to those observed during IPGTT (Fig. 1e,f). Glucose tolerance was not affected by acute clenbuterol treatment when analysed as total AUC (Fig. 2a,b); however, it was improved if we take into account the higher initial glucose levels (AUC above fasting blood glucose, not shown). Nonetheless, it should be considered that stimulated insulin release could contribute to this acute effect.

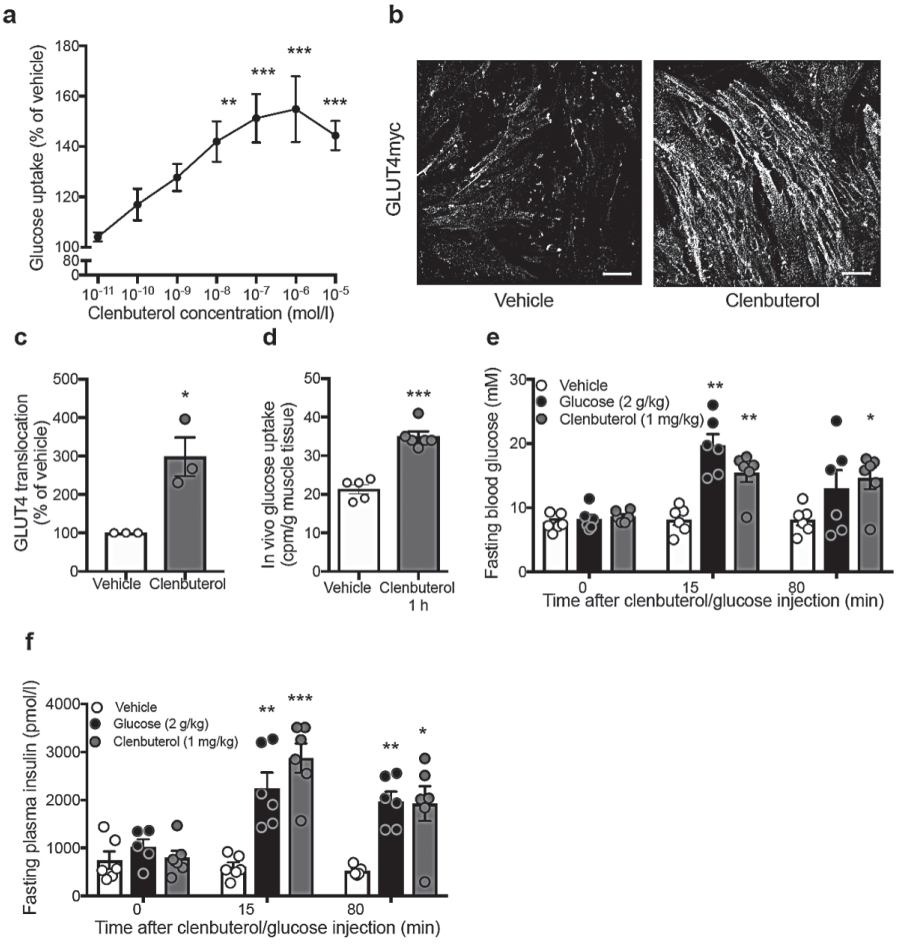


Fig. 1 *In vitro* and acute *in vivo* effects of clenbuterol. (**a**) Clenbuterol stimulation of L6 myotubes induced glucose uptake in a dose-dependent manner; *n*=5–8; data were analysed by one-way ANOVA with Dunnett's multiple comparison test. (**b**, **c**) Stimulation of L6 myotubes with 1 μmol/l clenbuterol induces GLUT4 translocation as quantified in (**c**); *n*=3; data were analysed with unpaired two-tailed Student's *t* test. Scale bars, 50 μm. (**d**) *In vivo* glucose uptake in gastrocnemius muscle of chow-fed mice treated with 1 mg/kg clenbuterol for 1 h; *n*=5–6; data were analysed with unpaired two-tailed Student's *t* test. (**e**, **f**) Acute effects of clenbuterol on blood glucose (**e**) and plasma insulin (**f**); *n*=6 (diet-induced obesity was developed in C57Bl/6N mice maintained at 30°C and on HFD for 7 months; mice were fasted in the morning for 6 h prior to clenbuterol, glucose or saline i.p. injection); data were analysed by two-way ANOVA with Dunnett's multiple comparison test. In all graphs: *p<0.05, **p<0.01, ***p<0.001 vs vehicle (in e and f vs vehicle at the same time point)

Interestingly, and in line with our previous study (2), when the clenbuterol treatment was continued for 4 days, we observed a reduction in fasting blood glucose (by 21%, p<0.05) as well as an improved glucose tolerance (by 32%, p<0.01, Fig. 2c,d, 24 h after last drug administration). Please, note that the effect on fasting blood glucose contributes to the

effect on total AUC. Surprisingly, repetitive daily drug administration did not increase blood glucose even transiently during chronic treatment (ESM Fig. 1). These beneficial effects of clenbuterol persisted for up to 42 days of treatment (Fig. 2e,f). It is important to note here that the beneficial effects of chronic treatment were not attributed to elevated insulin levels, in contrast to the acute effects (see below).

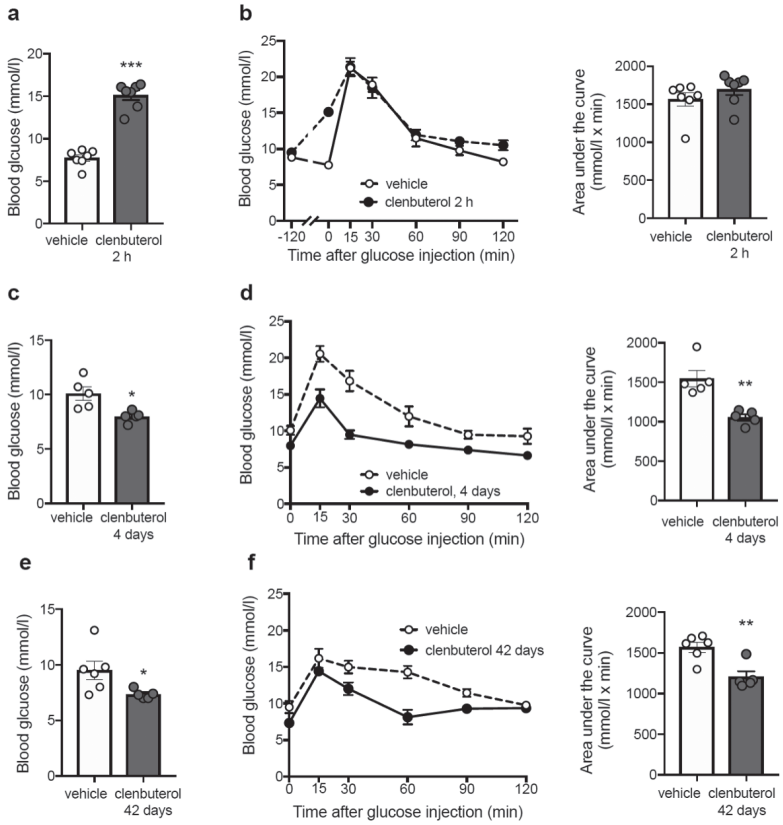


Fig. 2 Prolonged clenbuterol treatment improves glucose tolerance in DIO mice. (**a**, **b**) Effects of acute 2 h pre-treatment with clenbuterol on fasting blood glucose (**a**) and IPGTT (**b**) in DIO mice. Diet-induced obesity was developed in C57Bl/6N mice maintained at 30°C and on HFD for 6 months; mice were fasted for 5 h, clenbuterol (1 mg/kg) was injected i.p. at –120 min relative to blood glucose measurement, glucose (2 g/kg body weight) was injected i.p. at 0 min after blood glucose measurement; *n*=7. (**c**, **d**) Chronic 4 day treatment with clenbuterol decreased fasting blood glucose (**c**) and improved i.p. glucose tolerance (**d**). Diet-induced obesity was developed in C57Bl/6N mice maintained at 30°C and on HFD for 7 months; clenbuterol (1 mg/kg) was injected i.p. every morning for 4 days; on the 5th day mice were fasted for 5 h, glucose (2 g/kg body weight) was injected i.p.; *n*=5. (**e**, **f**) Chronic 42 day treatment with clenbuterol decreased fasting blood glucose (**e**) and improved i.p. glucose tolerance (**f**). Diet-induced obesity was developed in C57Bl/6N mice maintained at 30°C and on HFD for 7 months; clenbuterol (30 mg/l) was administered in drinking water; mean dose received was 2.04 ± 0.1 mg kg^{-1} day^{-1}; mice were fasted for 5 h, glucose (2 g/kg body weight) was injected i.p.; *n*=5–6. AUCs and fasting blood glucose were analysed with unpaired two-tailed Student's *t* test. **p*<0.05, ***p*<0.01, ****p*<0.001 vs vehicle

Chronic treatment with clenbuterol improves glucose tolerance at doses in the microgram range

In a dose–response study, DIO mice were treated daily with different doses of clenbuterol for 4 days, as this duration was shown to be sufficiently long to reach full effects. As seen in Fig. 3a,b, clenbuterol improved glucose tolerance and reduced fasting glucose levels in a dose-dependent manner. A subsequent experiment in a second cohort of mice indicated that a dose as low as 25 µg kg^{-1} day^{-1} (0.025 mg kg^{-1} day^{-1}) still improved glucose tolerance (27%, p<0.01) and reduced fasting blood glucose levels (18%, p<0.05) (Fig. 3c,d). Taken together, clenbuterol improves glucose tolerance in DIO mice already after 4 days of treatment with doses in the microgram range.

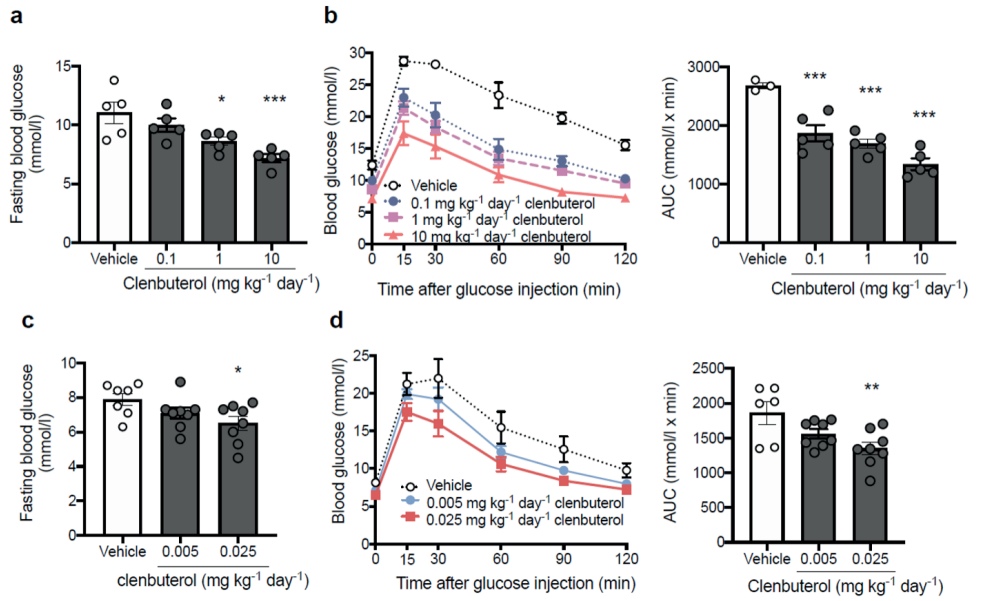


Fig. 3 Clenbuterol improves fasting blood glucose and glucose tolerance in a dose-dependent manner. Two experiments were performed. In one experiment (**a**, **b**) DIO mice were treated with 0.1, 1 or 10 mg/kg of clenbuterol for 4 days; n=3-5. Diet-induced obesity was developed in C57Bl/6N mice maintained at 30°C and on HFD for 9 months; clenbuterol was injected i.p. for 4 days; mice were fasted for 5 h, glucose (2 g/kg body weight) was injected i.p.; (**a**) fasting blood glucose and (**b**) glucose tolerance. In a separate experiment (**c**, **d**) DIO mice were treated with 0.005 or 0.025 mg/kg of clenbuterol for 4 days; n=6–8, both males and females were used. Diet-induced obesity was developed in C57Bl/6N mice maintained at 30°C and on HFD for 4.5 months; clenbuterol was injected i.p. for 4 days; mice were fasted for 5 h, glucose (2 g/kg body weight) was injected i.p.; (**c**) fasting blood glucose and (**d**) glucose tolerance. AUCs and fasting blood glucose were analysed with one-way ANOVA with Dunnett's multiple comparison test. In all graphs: *p<0.05, **p<0.01, ***p<0.001 vs vehicle

Chronic treatment with low-dose clenbuterol does not affect insulin release but improves insulin sensitivity

Since clenbuterol acutely leads to insulin release (Fig. 1f), the positive effects of prolonged clenbuterol treatment on glucose homeostasis could be, in part, due to changes in insulin secretion. To address this question, DIO mice were treated for 32 days with low-dose clenbuterol-supplemented water (3 mg/l). When evaluated by OGTT, glucose tolerance improved after 4 days of low-dose clenbuterol treatment (ESM Fig. 2a) and this effect persisted when the treatment was continued for a prolonged period of time (ESM Fig. 2b), similar to IPGTT in Fig. 2c–f. Treatment with clenbuterol did not significantly affect either water or food intake (Fig. 4a, c). Importantly, body weight, fat mass and lean mass remained unaffected during the treatment period (Fig. 4d-f).

Fasting insulin levels were unchanged after 4 days of clenbuterol treatment (Fig. 5a), whereas they were significantly lowered by 43% after 18 days of treatment ($p<0.01$, Fig. 5b). Fifteen minutes after glucose oral gavage, plasma insulin levels increased to the same extent in control and clenbuterol-treated mice both at 4 and 18 days of treatment (Fig. 5a,b), indicating that improvement of glucose tolerance was not a consequence of higher insulin release. Although clenbuterol administration did not result in a higher insulin release during the OGTTs, it could still potentially improve glucose tolerance by increasing insulin sensitivity. To explore this possibility, we performed an ITT after 24 days of low-dose clenbuterol treatment. As expected, blood glucose of DIO mice in the control group did not respond to insulin injection, indicating insulin resistance. Remarkably, clenbuterol-treated DIO mice showed a significant improvement of insulin sensitivity**.** Rates of glucose disposal (K_{ITT}) were markedly increased from 0.15 ± 0.36 to 1.38 ± 0.31 %/min in control vs treated groups, respectively, $p<0.05$ (Fig. 5c–e)**.** In fact, clenbuterol fully abolished insulin resistance caused by high-fat diet, since clenbuterol-treated mice responded to insulin similarly to chow-fed mice, which were, however, tested at a separate occasion (ESM Fig. 3). Together, these data indicate that the beneficial glucose-lowering effect of clenbuterol manifested on glucose tolerance is not due to increased insulin secretion but involves increases in insulin sensitivity.

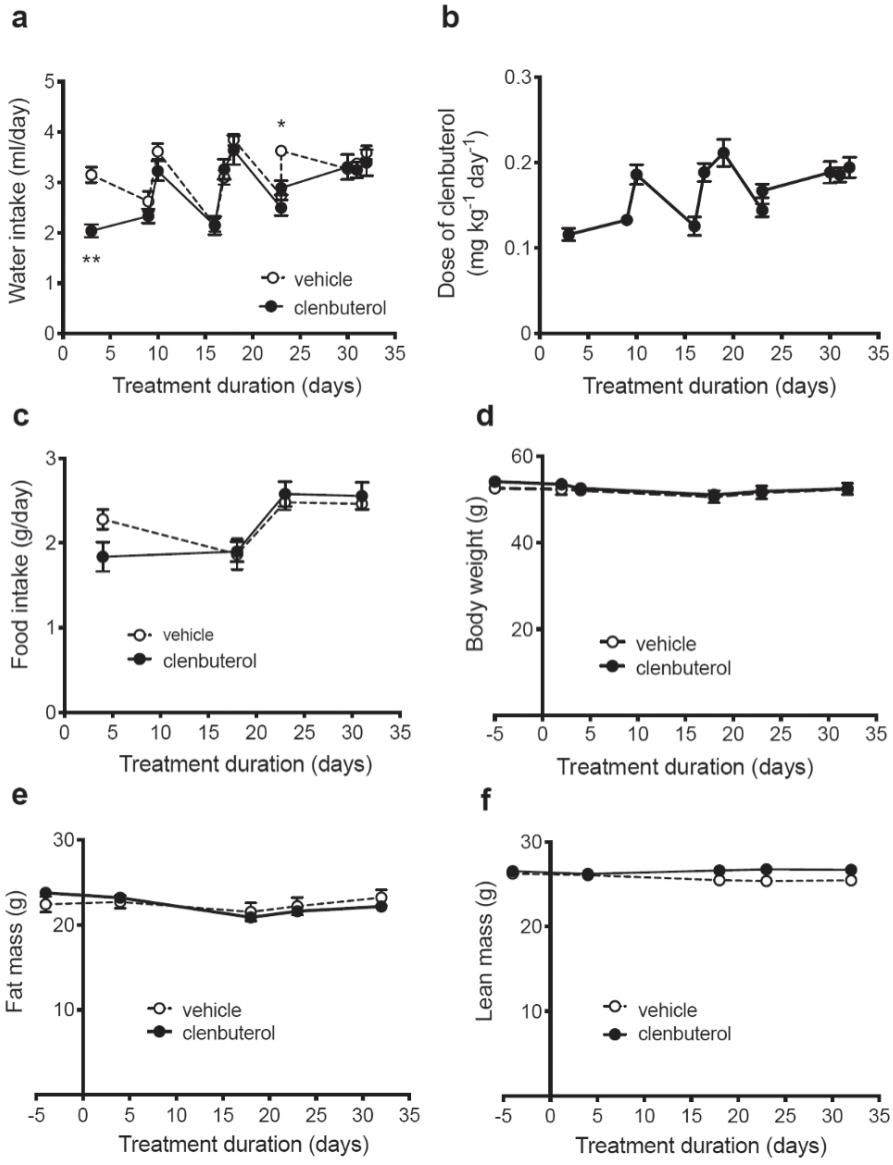


Fig. 4 Effects of prolonged treatment with a low dose of clenbuterol on food and water intake, body weight and body composition. DIO mice were treated with low dose of clenbuterol for 32 days; *n*=6–7. Diet-induced obesity was developed in C57Bl/6N mice maintained at 30°C and on HFD for 5 months; 3 mg/l clenbuterol was administered in drinking water. (**a**) water intake, (**b**) dose of clenbuterol calculated based on water intake, (**c**) food intake, (**d**) body weight, (**e**) body fat mass, (**f**) body lean mass. In (**a**) the data were analysed by mixed-effects analysis (since a few random data points were missing owing to water bottle leakage) with Sidak's multiple comparison test; in (**c–f**) the data were analysed by two-way ANOVA with Sidak's multiple comparison test; in all graphs the experimental group was not significantly different from the vehicle group.

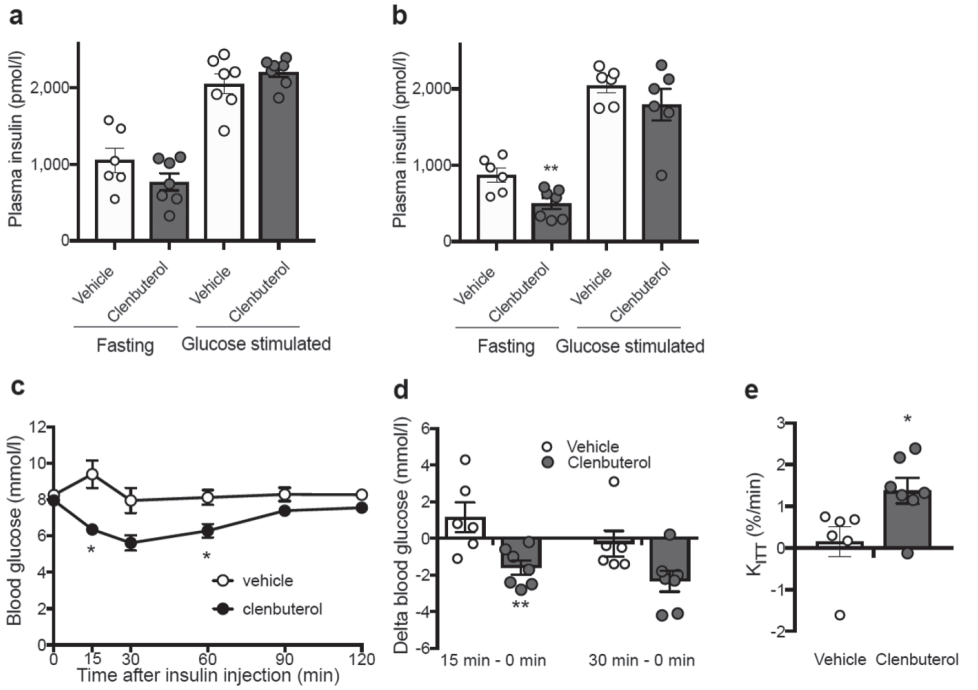


Fig. 5 Prolonged treatment with a low dose of clenbuterol does not increase insulin levels (OGTT) but improves insulin sensitivity. The data are from the same experiment as in Fig. 4 and ESM Fig. 2. Mice were fasted for 5 h. Plasma insulin was measured before and 15 min after glucose gavage in mice pre-treated with clenbuterol for 4 (**a**) and 18 (**b**) days; *n*=6–7. The data were analysed with unpaired two-tailed Student's *t* test separately for fasting and glucose stimulated conditions. (**c**) ITT performed on 25th day of treatment with clenbuterol. After 5 h of fasting, insulin (1 U/kg body weight) was injected i.p. and blood glucose was measured after 15, 30, 60, 90 and 120 min; *n*=6–7. Insulin response from (**c**) is presented as change of blood glucose (15 min minus 0 min) and (30 min minus 0 min, p=0.056) (**d**), and rate of blood glucose disposal (K_{ITT}) (**e**). The data in (**c**, **d**) were analysed by two-way ANOVA with Sidak's multiple comparison test, and in (**e**) with unpaired two-tailed Student's *t* test. In all graphs: *p<0.05, **p<0.01 vs vehicle

Adaptations in liver and skeletal muscle underlie the clenbuterol-mediated improvements in glucose homeostasis

To further elaborate on the mechanisms that could underlie the improvements in glucose tolerance and insulin sensitivity upon clenbuterol treatment, we focused on the liver and skeletal muscle. As hepatic lipid accumulation is closely associated with insulin resistance (24-27), we first assessed hepatic lipid content by histological analysis. The area of hepatic lipid droplets was markedly reduced by ~ 40% (p<0.01) after prolonged (32 days) low-dose clenbuterol treatment. This reduction in hepatic steatosis was entirely due to a decrease in lipid droplet size (49%, p<0.05), since number of lipid droplets remained unaffected (Fig. 6a–d). A gravimetric assay for total amount of extracted lipids generated similar results (ESM Fig. 4). Apart from lipids, prolonged clenbuterol treatment decreased

liver glycogen by 23% (p<0.05, Fig. 6e) but did not affect gluconeogenesis, as addressed by pyruvate tolerance test (Fig. 6f).

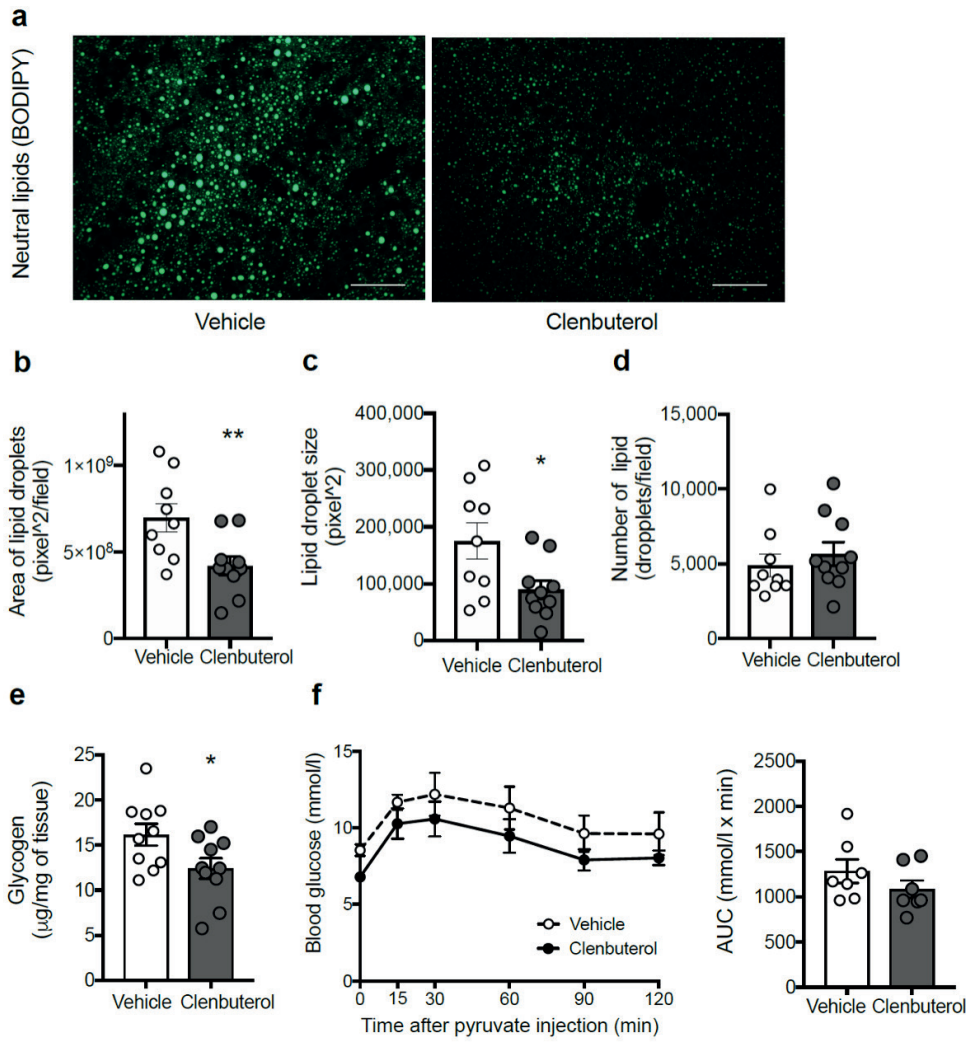


Fig. 6 A low dose of clenbuterol reduces hepatic steatosis and hepatic glycogen. (**a–e**) DIO mice were treated with clenbuterol for 31 days; n=9–10, both males and females were used. Diet-induced obesity was developed in C57Bl/6N mice maintained at 30°C and on HFD for 7 months; 3 mg/l clenbuterol was administered in drinking water; mean actual dose of clenbuterol calculated based on water intake was 0.16 mg kg^{-1} day^{-1}; mice were fasted for 5 h in the morning before dissections. (**a**) Representative fluorescent images of liver lipid droplets stained with BODIPY (green, lipid); scale bars, 50 µm. Quantification of images in (**a**) using ImageJ, in terms of total area of lipid droplets (**b**), size (**c**) and number of lipid droplets (**d**). (**e**) liver glycogen. (**f**) Pyruvate tolerance test was performed after 9 days of treatment during the same experiment as in Figs 4, 5 and ESM Fig. 2. The data were analysed with unpaired two-tailed Student's t test in (b-e) and AUC in (f). In all graphs: *p<0.05, **p<0.01 vs vehicle.

To test whether basal glucose uptake in skeletal muscle was stimulated upon chronic low-dose clenbuterol treatment in DIO mice, we performed an *in vivo* glucose uptake assay after 6 days of treatment. Low doses of clenbuterol stimulated glucose uptake in skeletal muscle by 74% even 5 h after the last treatment ($p<0.001$, Fig. 7a). Importantly, stimulated glucose uptake was not attributed to increased insulin levels. In fact, serum insulin was reduced in clenbuterol-treated mice during the experiment (by 38%, $p<0.05$, Fig. 7b, similarly to Fig. 5b).

Combined, these data suggest that adaptations in both the liver and skeletal muscle as well as an increase in insulin sensitivity contribute to the clenbuterol-mediated improvements in glucose homeostasis.

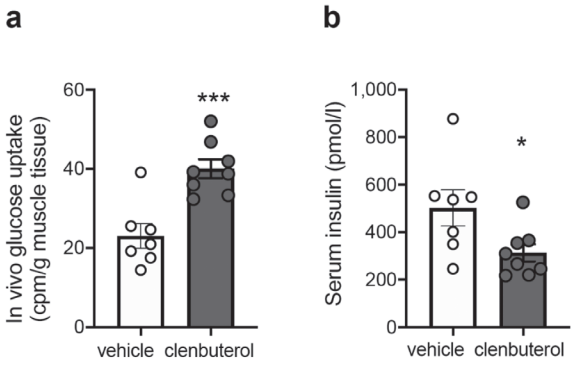


Fig. 7 A low dose of clenbuterol stimulates *in vivo* glucose uptake in gastrocnemius muscle of DIO mice during chronic treatment. (**a**, **b**) DIO was developed in C57Bl/6N mice maintained at 30°C and on HFD for 8 months. DIO mice were treated with saline (vehicle; $n=7$) or clenbuterol ($n=8$) for 6 days. After 4 days of treatment, IPGTT was performed (not shown). After 6 days of treatment *in vivo* glucose uptake was measured in gastrocnemius muscle, 5 h after the last treatment and after 5 h of fasting (**a**). Insulin was measured in serum collected during the test (**b**). The data were analysed with unpaired two-tailed Student's *t* test: $*p<0.05$, $***p<0.001$ vs vehicle

Discussion

In the present study, we investigated the therapeutic relevance of β_2-AR stimulation on glucose homeostasis in DIO mice. We demonstrated that treatment with the β_2-adrenergic agonist clenbuterol improves glucose tolerance already after 4 days of treatment. These effects persisted at least up to 42 days of treatment and could also be achieved with doses in the microgram range, which are about 40 times lower than previously studied (2, 4, 15, 28). Moreover, low-dose clenbuterol stimulated basal *in vivo* glucose uptake in skeletal muscle and improved whole-body insulin sensitivity as well as reduced hepatic steatosis under chronic stimulation in DIO mice.

In the present study, in line with previous findings, acute clenbuterol administration increased blood glucose and insulin levels (7-10). However, we could also show that, after 4 days of treatment, clenbuterol reduced fasting blood glucose and improved glucose tolerance (24 h after last administration). And these effects were maintained at least up to 42 days. On the basis of these combined results we hypothesised that treatment with β_2-adrenergic agonists induces a dynamic response over time (Fig. 8). Thus, clenbuterol (acutely or chronically) stimulates glucose uptake in skeletal muscles. However, in the acute setting, clenbuterol augments hepatic glucose output, which is not fully compensated by the enhanced glucose uptake in peripheral tissues resulting in hyperglycaemia. Upon prolonged treatment with clenbuterol, peripheral glucose clearance appears to exceed the hepatic glucose output, resulting in a favourable metabolic profile. Hepatic glucose output is likely to be diminished under prolonged clenbuterol treatment at least in part due to improved insulin sensitivity and reduced glycogen levels.

Regarding the underlying mechanism, we first investigated how a low dose of clenbuterol affects body composition. Clenbuterol is well known for its capacity to increase lean mass and decrease fat mass (29, 30). In turn, improvements in glucose homeostasis could be attributed to any of these two effects (31, 32). However, clenbuterol did not affect either lean or fat mass in the current study, presumably owing to the low dose of clenbuterol applied. We also excluded enhanced insulin secretion as a contributing factor since fasting plasma insulin concentrations were significantly reduced upon prolonged low-dose clenbuterol treatment and since plasma insulin levels rose to a similar extent in treated vs non-treated mice upon glucose administration during OGTTs.

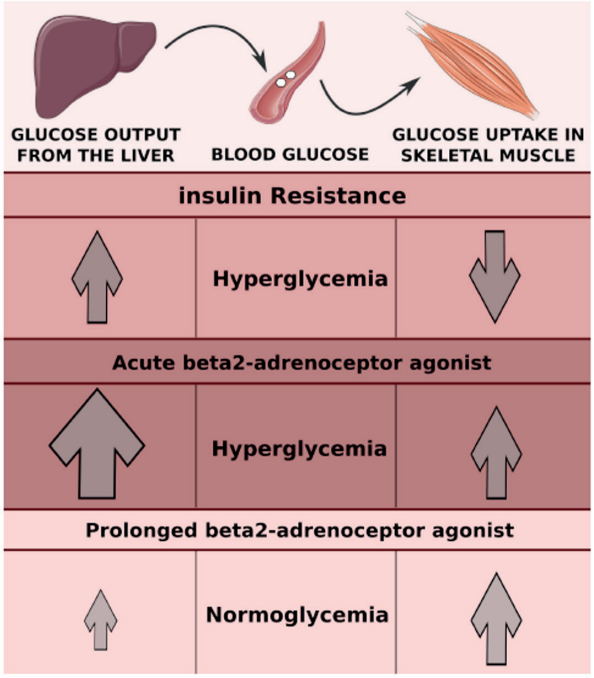


Fig. 8 Working hypothesis of clenbuterol actions acutely and chronically. Prediabetic and diabetic people and rodents are characterised by insulin resistance, which leads to increased glucose output from the liver and reduced glucose uptake in peripheral tissues, including muscle, and which results in hyperglycaemia and glucose intolerance. Treatment with a β_2-AR agonist such as clenbuterol (acutely or chronically) stimulates glucose uptake in skeletal muscles. However, acutely clenbuterol augments hepatic glucose output, which is not fully compensated by enhanced glucose uptake in peripheral tissues and results in hyperglycaemia as a net effect. However, under prolonged treatment with clenbuterol, peripheral glucose clearance matches hepatic glucose output, resulting in a favourable metabolic profile. Hepatic glucose output is likely to be reduced under prolonged clenbuterol treatment at least in part because of normalised insulin sensitivity and reduced liver glycogen. This working hypothesis is elaborated in the discussion section. Images were taken from Servier Medical Art (smart.servier.com)

In the absence of changes in insulin secretion, we hypothesised that the improvements in glucose tolerance were linked to an increased insulin sensitivity. Indeed, whole-body insulin sensitivity improved remarkably upon prolonged low-dose clenbuterol treatment, as assessed by ITT. These results confirm previously published data, which, however, were obtained with a relatively high dose of clenbuterol (15). It is a remarkable effect considering that insulin resistance is a characteristic manifestation of type 2 diabetes, it is closely associated with several other conditions including cardiovascular diseases and it remains poorly treated to date (33). Here we show that selective β_2-adrenergic agonists present an attractive avenue for treatment of insulin resistance.

The underlying mechanism by which β_2-adrenergic agonists beneficially affect insulin sensitivity remains to be elucidated. Although these effects have previously been related to tissue redistribution (4, 15), we excluded this factor as was discussed above. Instead, the insulin-sensitising effects of clenbuterol could be, in part, mediated by reduction in hepatic lipid accumulation (34-36). Remarkably, we could show that total liver lipid content was drastically reduced upon clenbuterol treatment. These results contradict previous data on isoprenaline (isoproterenol), which stimulated hepatic lipid accumulation in mice (37); the difference however may be accounted for the fact that isoprenaline is a non-selective β-AR agonist, which moreover was administered at a very high dose. Another possible mechanism for how clenbuterol could improve insulin sensitivity, particularly in the liver, is by reducing liver glycogen levels. An experimentally-increased amount of glycogen in the liver by itself induced hepatic insulin resistance and inhibited new glycogen synthesis in healthy dogs (38). Interestingly, novel classes of glucose-lowering drugs, SGLT2 inhibitors and dual GLP1/glucagon agonists, are also known to reduce liver glycogen storage (39-41). In addition, clenbuterol could also affect insulin sensitivity through a reduction in inflammation, since these two conditions are closely associated (reviewed in (42, 43)). In fact, other β_2-adrenergic agonists, salbutamol and terbutaline, exhibited anti-inflammatory effects in diabetic rats and DIO mice (44, 45). Ultimately, corrected hyperglycaemia can also improve insulin sensitivity, since glucotoxicity by itself can lead to insulin resistance in healthy men and mice (46, 47).

Most importantly, one consequence of improved insulin sensitivity is reduced (better controlled) hepatic glucose output, which in turn is an important contributor to normal glucose homeostasis (see working hypothesis in Fig. 8). Moreover, lower hepatic glycogen levels may reduce glucose output from the liver, since the rate of glycogenolysis is known to depend on the amount of glycogen (48). Collectively, higher insulin sensitivity and lower glycogen in the liver of clenbuterol-treated mice may contribute to decreased (normalised) glucose output from the liver and, together with stimulated glucose disposal in muscles, lead to restored glucose homeostasis (Fig. 8).

Besides the liver, we also studied skeletal muscle for its contribution to the clenbuterol-induced improvements in glucose homeostasis. We clearly showed that chronic treatment with clenbuterol stimulated basal glucose uptake in skeletal muscle *in vivo* independently of insulin, indicating an intrinsic ability of adrenergic stimulation to induce glucose uptake in skeletal muscles *in vivo* (probably through activation of the β_2-mTORC2 pathway (2)). Interestingly, stimulation of basal glucose uptake in muscles was able to improve glucose homeostasis in mice overexpressing GLUT1 in skeletal muscle (49).

Thus, our findings support that prolonged treatment with the β_2-adrenergic agonist clenbuterol stimulates basal glucose uptake in skeletal muscle, which, together with the previously established effect on insulin-stimulated glucose uptake, is likely to contribute to the observed improvements in whole-body glucose homeostasis.

Human relevance of the β_2-adrenergic pathway was indicated in our previous study, in which mild cold exposure stimulated glucose uptake in skeletal muscle and improved insulin sensitivity in diabetic people without activation of insulin- or AMPK-signalling (50). In another study on healthy men, insulin sensitivity was improved under chronic treatment with β_2-AR agonist terbutaline (15 mg/day for 1-2 weeks) (18). In the present study we demonstrated that clenbuterol improved glucose tolerance in DIO mice at doses in the microgram range, which are generally assumed safe for treatment of asthma (20-22). Demonstration of effectiveness of clenbuterol at much lower doses than was previously studied is important, since reducing the dose of the drug may diminish potential side-effects. As such, the true clinical relevance of this novel, insulin-independent pathway to improve whole-body glucose homeostasis (namely, three conditions: glucose tolerance, insulin resistance and hepatic steatosis) could be readily tested in humans.

Our study has its limitations. DIO mice are characterised by insulin resistance but only mild hyperglycaemia. Thus, they can be interpreted as a model for only early stages of type 2 diabetes. However, clenbuterol has been shown to improve glucose homeostasis in two other models (2, 4, 15). Second, it would be of clinical relevance to extend our study to other β_2-AR agonists, which are currently in wider clinical use for asthma. To date there are only few studies addressing these objectives (16-18). Third, our study was almost entirely limited to male mice, partly due to relative resistance of female C57Bl/6 mice to developing obesity, glucose intolerance and insulin resistance.

In summary, prolonged supplementation of a β_2-AR agonist robustly improved glucose homeostasis in DIO mice already after 4 days of treatment. Remarkably, these effects could be maintained for up to 42 days and could be achieved with very low doses. These effects on glucose homeostasis were most likely to be mediated both by an enhanced glucose uptake in skeletal muscle and an improved whole-body insulin sensitivity. In addition, prolonged treatment with clenbuterol reduced hepatic glycogen and profoundly improved hepatic steatosis.

Acknowledgements

We are thankful to all members of our laboratory at Stockholm University for fruitful and critical discussions. Images for Figure 8 and the graphical abstract were taken from Servier Medical Art (smart.servier.com). Some of the data were presented as an abstract at the 55th EASD Annual Meeting in 2019.

Data availability

The datasets generated during the current study are available from the corresponding author on reasonable request.

Funding

The study was supported by Vetenskapsrådet-Medicin (VR-M) from the Swedish Research Council, Stiftelsen Svenska Diabetesförbundets Forskningsfond, the Magnus Bergvall Foundation, the Carl Tryggers Foundation and Olle Engqvist byggmastare foundation.

Authors' relationships and activities

TB and RB own stocks in Atrogi AB. AK, ND, CH, JO, EF and AS became employees in Atrogi AB in the late stages of the study. EF is currently an employee in Takeda AB. The authors declare that there are no other relationships or activities that might bias, or be perceived to bias, their work.

Contribution statement

AK, ND and TB conceived and designed research. AK, ND, AÅ, CH, JO, EF, EZ, GS, MR, AS performed experiments. All authors analysed data. AK and ND prepared figures. AK and SvB drafted manuscript. All authors edited and revised manuscript. All authors approved the final version of the manuscript to be published. TB is the guarantor of this work.

References

1. Defronzo RA, Jacot E, Jequier E, Maeder E, Wahren J, Felber JP (1981) The Effect of Insulin on the Disposal of Intravenous Glucose. Results from Indirect Calorimetry and Hepatic and Femoral Venous Catheterization. Diabetes 30:1000–1007
2. Sato M, Dehvari N, Öberg AI, et al (2014) Improving type 2 diabetes through a distinct adrenergic signaling pathway involving mTORC2 that mediates glucose uptake in skeletal muscle. Diabetes 63(12):4115–4129. https://doi.org/10.2337/db13-1860
3. Mukaida, S; Sato, M; Öberg, A I et al (2019) BRL37344 stimulates GLUT4 translocation and glucose uptake in skeletal muscle via b-2 adrenoceptors without causing classical receptor desensitization. Am J Physiol Integr Comp Physiol 46(0). https://doi.org/.1037//0033-2909.I26.1.78
4. Castle A, Yaspelkis BB, Kuo CH, Ivy JL (2001) Attenuation of insulin resistance by chronic β2-adrenergic agonist treatment possible muscle specific contributions. Life Sci 69(5):599–611. https://doi.org/10.1016/S0024-3205(01)01149-3
5. Fernandes GW, Ueta CB, Fonseca TL, et al (2014) Inactivation of the adrenergic receptor β2 disrupts glucose homeostasis in mice. J Endocrinol 221(3):381–390. https://doi.org/10.1530/JOE-13-0526
6. Cipolletta E, Del Giudice C, Santulli G, Trimarco B, Iaccarino G (2017) Opposite effects of β2-adrenoceptor gene deletion on insulin signaling in liver and skeletal muscle. Nutr Metab Cardiovasc Dis 27(7):615–623. https://doi.org/10.1016/j.numecd.2017.05.011
7. Guhan AR, Cooper S, Oborne J, Lewis S, Bennett J, Tattersfield AE (2000) Systemic effects of formoterol and salmeterol: a dose-response comparison in healthy subjects. Thorax 55(8):650–656. https://doi.org/10.1136/thorax.55.8.650
8. Burgess C, Ayson M, Rajasingham S, Crane J, Della Cioppa G, Till MD (1998) The extrapulmonary effects of increasing doses of formoterol in patients with asthma. Eur J Clin Pharmacol 54(2):141–147. https://doi.org/10.1007/s002280050435
9. Philipson LH (2002) b-Agonists and metabolism. J Allergy Clin Immunol 110(6):S313–S317
10. Nonogaki K (2000) New insights into sympathetic regulation of glucose and fat metabolism. Diabetologia 43(5):533–549
11. Belfort-Deaguiar RD, Naik S, Hwang J, Szepietowska B, Sherwin RS (2015) Inhaled formoterol diminishes insulin-Induced hypoglycemia in type 1 diabetes. Diabetes Care 38(9):1736–1741. https://doi.org/10.2337/dc14-2472
12. Szepietowska B, Zhu W, Sherwin RS (2013) β2-Adrenergic receptor agonist administration promotes counter-regulatory responses and recovery from hypoglycaemia in rats. Diabetologia 56(11):2517–2523. https://doi.org/10.1007/s00125-013-3009-7
13. Smith SA, Levy AL, Sennitt M V., Simson DL, Cawthorne MA (1985) Effects of BRL 26830, a novel β-adrenoceptor agonist, on glucose tolerance, insulin sensitivity and glucose turnover in Zucker (fa/fa) rats. Biochem Pharmacol 34(14):2425–2429. https://doi.org/10.1016/0006-2952(85)90521-0
14. Lupien JR, Hirshman MF, Horton ES (1990) Effects of norepinephrine infusion on in vivo insulin sensitivity and responsiveness. Am J Physiol Metab 259(2):E210–E215
15. Pan SJ, Hancock J, Ding Z, Fogt D, Lee M, Ivy JL (2001) Effects of clenbuterol on insulin resistance in conscious obese Zucker rats. Am J Physiol Metab 280(4):E554–E561. https://doi.org/10.1152/ajpendo.2001.280.4.e554

16. Elayan H, Milic M, Sun P, Gharaibeh M, Ziegler MG (2012) Chronic β_2 adrenergic agonist, but not exercise, improves glucose handling in older type 2 diabetic mice. Cell Mol Neurobiol 32(5):871–877. https://doi.org/10.1007/s10571-012-9819-1
17. Saleh N, Elayan HE, Zihlif M (2018) The effect of salbutamol on PGC-1 α and GLUT4 mRNA expression in the liver and muscle of elderly diabetic mice. Acta Endocrinol (Copenh) 14(2):184–191. https://doi.org/10.4183/aeb.2018.184
18. Scheidegger K, Robbins DC, Danforth E (1984) Effects of chronic beta receptor stimulation on glucose metabolism. Diabetes 33(12):1144–1149. https://doi.org/10.2337/diab.33.12.1144
19. Jacob S, Fogt D, Dietze G, Henriksen EJ (1999) The b-adrenergic modulator celiprolol reduces insulin resistance in obese Zucker rats. Life Sci 64(22):2071–2079
20. Jiang G-L, Gu Y-D, Zhang L-Y, Shen L-Y, Yu C, Xu J-G (2011) Randomized, Double-Blind, and Placebo-Controlled Trial of Clenbuterol in Denervated Muscle Atrophy. ISRN Pharm 2011: 98125. https://doi.org/10.5402/2011/981254
21. Brusasco V, Crid E, Mangini S, Vibelli C (2011) A clinical trial of oral clenbuterol (NAB 365) in chronic airways obstruction. Curr Med Res Opin 6(7):449–455. https://doi.org/10.1185/03007998009109466
22. Anderson G, Wilkins E (1977) A trial of clenbuterol in bronchial asthma. Thorax 32(6):717–719. https://doi.org/10.1136/thx.32.6.717
23. Bonora E, Moghetti P, Zancanaro C, et al (2009) Estimates of in vivo insulin action in man: comparison of insulin tolerance tests with euglycemic and hyperglycemic glucose clamp studies. J Clin Endocrinol Metab 68(2):374–378. https://doi.org/10.1210/jcem-68-2-374
24. Seppälä-Lindroos A, Vehkavaara S, Häkkinen A-M, et al (2002) Fat Accumulation in the Liver Is Associated with Defects in Insulin Suppression of Glucose Production and Serum Free Fatty Acids Independent of Obesity in Normal Men. J Clin Endocrinol Metab 87(7):3023–3028. https://doi.org/10.1210/jcem.87.7.8638
25. Matsuzaka T, Shimano H (2018) Molecular mechanisms involved in hepatic steatosis and insulin resistance. J Diabetes Investig 2(3):170–175
26. Kato K, Takeshita Y, Misu H, Zen Y, Kaneko S, Takamura T (2015) Liver steatosis is associated with insulin resistance in skeletal muscle rather than in the liver in Japanese patients with non-alcoholic fatty liver disease. J Diabetes Investig 6(2):158–163. https://doi.org/10.1111/jdi.12271
27. Farese RV Jr; Zechner R, Newgard CB, Walther TC (2012) The problem of establishing relationships between hepatic steatosis and hepatic insulin resistance. Cell Metab 15(5):570–573. https://doi.org/10.1161/ATVBAHA.114.303112.ApoA-I
28. Torgan CE, Brozinick JT, Banks EA, Cortez MY, Wilcox RE, Ivy JL (1993) Exercise training and clenbuterol reduce insulin resistance of obese Zucker rats. Am J Physiol 264(3 Pt 1):E373-E379. https://doi.org/10.1152/ajpendo.1993.264.3.E373
29. Kamalakkannan G, Petrilli CM, George I, et al (2008) Clenbuterol Increases Lean Muscle Mass but Not Endurance in Patients With Chronic Heart Failure. J Hear Lung Transplant 27(4):457–461. https://doi.org/10.1016/j.healun.2008.01.013
30. Reeds PJ, Hay SM, Dorward PM, Palmer RM (1988) The effect of β-agonists and antagonists on muscle growth and body composition of young rats (Rattus sp.). Comp Biochem Physiol Part C, Comp 89(2):337–341. https://doi.org/10.1016/0742-8413(88)90234-4
31. Schenk S, Harber MP, Shrivastava CR, Burant CF, Horowitz JF (2009) Improved insulin sensitivity after weight loss and exercise training is mediated by a reduction in plasma fatty acid mobilization, not enhanced oxidative capacity. J Physiol 587(20):4949–4961. https://doi.org/10.1113/jphysiol.2009.175489

32. Lombardo GE, Arcidiacono B, De Rose RF, et al (2016) Normocaloric diet restores weight gain and insulin sensitivity in obese mice. Front Endocrinol (Lausanne) 7(MAY):1–7. https://doi.org/10.3389/fendo.2016.00049

33. Di Pino A, DeFronzo RA (2019) Insulin Resistance and Atherosclerosis: Implications for Insulin Sensitizing Agents. Endocr Rev (May):1447–1467. https://doi.org/10.1210/er.2018-00141

34. Marchesini G, Brizi M, Morselli-Labate AM, et al (1999) Association of nonalcoholic fatty liver disease with insulin resistance. Am J Med 107(5):450–455. https://doi.org/10.1016/S0002-9343(99)00271-5

35. Petersen KF, Dufour S, Feng J, et al (2006) Increased prevalence of insulin resistance and nonalcoholic fatty liver disease in Asian-Indian men. Proc Natl Acad Sci 103(48):18273–18277. https://doi.org/10.1073/pnas.0608537103

36. Samuel VT, Shulman GI (2012) Mechanisms for insulin resistance: Common threads and missing links. Cell 148(5):852–871. https://doi.org/10.1016/j.cell.2012.02.017

37. Ghosh PM, Shu Z-J, Zhu B, et al (2012) Role of β-adrenergic receptors in regulation of hepatic fat accumulation during aging. J Endocrinol 213(3):251–261. https://doi.org/10.1530/joe-11-0406

38. Winnick JJ, An Z, Kraft G, et al (2013) Liver glycogen loading dampens glycogen synthesis seen in response to either hyperinsulinemia or intraportal glucose infusion. Diabetes 62(1):96–101. https://doi.org/10.2337/db11-1773

39. Sawada Y, Izumida Y, Takeuchi Y, et al (2017) Effect of sodium - glucose cotransporter 2 (SGLT2) inhibition on weight loss is partly mediated by liver - brain - adipose neurocircuitry. Biochem Biophys Res Commun 493(1):40–45. https://doi.org/10.1016/j.bbrc.2017.09.081

40. Atageldiyeva K, Fujita Y, Yanagimachi T, et al (2016) Sodium - glucose cotransporter 2 inhibitor and a low carbohydrate diet affect gluconeogenesis and glycogen content differently in the kidney and the liver of non-diabetic mice. PLoS One 11(6):1–17. https://doi.org/10.1371/journal.pone.0157672

41. Elvert R, Herling AW, Bossart M, et al (2018) Running on mixed fuel - dual agonistic approach of GLP-1 and GCG receptors leads to beneficial impact on body weight and blood glucose control: A comparative study between mice and non-human primates. Diabetes, Obes Metab 20(8):1836–1851. https://doi.org/10.1111/dom.13212

42. Catrysse L, van Loo G (2017) Inflammation and the Metabolic Syndrome: The Tissue-Specific Functions of NF-kB. Trends Cell Biol xx(6):1–13. https://doi.org/10.1016/j.tcb.2017.01.006

43. Tilg H, Moschen AR (2008) Insulin resistance, inflammation, and non-alcoholic fatty liver disease. Trends Endocrinol Metab 19(10):371–379. https://doi.org/10.1016/j.tem.2008.08.005

44. Noh H, Yu MR, Kim HJ, et al (2017) b-2-adrenergic receptor agonists are novel regulators of macrophage activation in diabetic renal and cardiovascular complications. Kidney Int 92(1):101–113

45. Gálvez I, Martín-Cordero L, Hinchado MD, Álvarez- Barrientos A, Ortega E (2019) Anti-inflammatory effect of β_2 adrenergic stimulation on circulating monocytes with a pro-inflammatory state in high-fat diet-induced obesity. Brain Behav Immun. https://doi.org/10.1016/j.jclepro.2018.03.006

46. Tripathy D, Merovci A, Basu R, Abdul-Ghani M, Defronzo RA (2019) Mild Physiologic Hyperglycemia Induces Hepatic Insulin Resistance in Healthy Normal Glucose-Tolerant Participants. J Clin Endocrinol Metab 104(7):2842–2850. https://doi.org/10.1210/jc.2018-02304

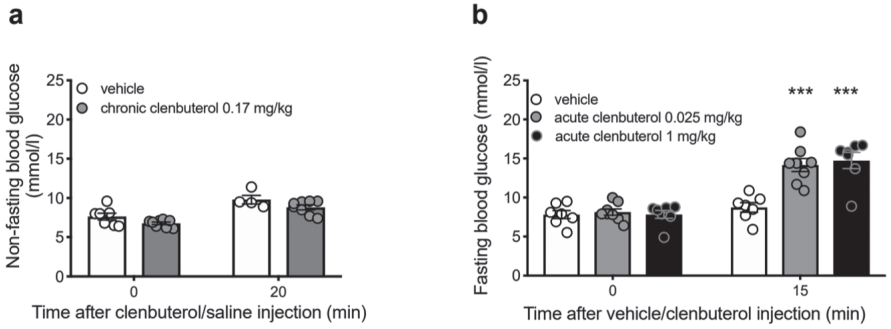
47. Rossetti L, Smith D, Shulman GI, Papachristou D, DeFronzo RA (1987) Correction of hyperglycemia with phlorizin normalizes tissue sensitivity to insulin in diabetic rats. J Clin Invest 79(5):1510–1515. https://doi.org/10.1172/JCI112981

48. König M, Bulik S, Holzhütter HG (2012) Quantifying the contribution of the liver to glucose homeostasis: A detailed kinetic model of human hepatic glucose metabolism. PLoS Comput Biol 8(6). https://doi.org/10.1371/journal.pcbi.1002577

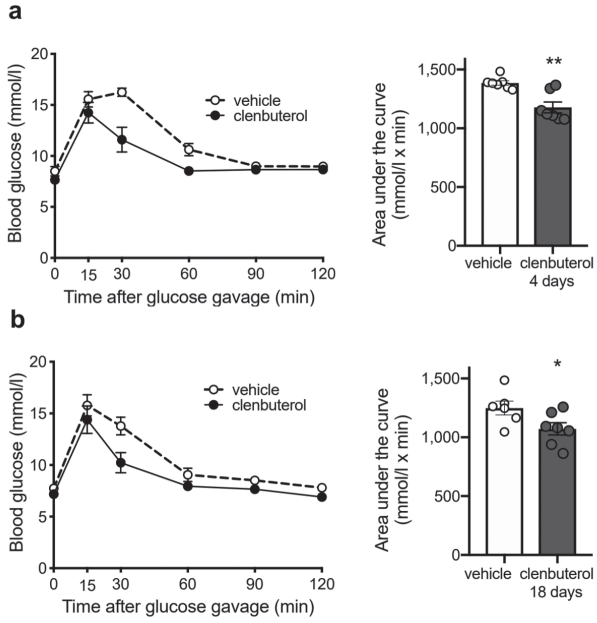
49. Marshall BA, Ren JM, Johnson DW, et al (1993) Germline manipulation of glucose homeostasis via alteration of glucose transporter levels in skeletal muscle. J Biol Chem 268(25):18442–18445

50. Hanssen MJW, Hoeks J, Brans B, et al (2015) Short-term cold acclimation improves insulin sensitivity in patients with type 2 diabetes mellitus. Nat Med 21(8):863–865. https://doi.org/10.1038/nm.3891

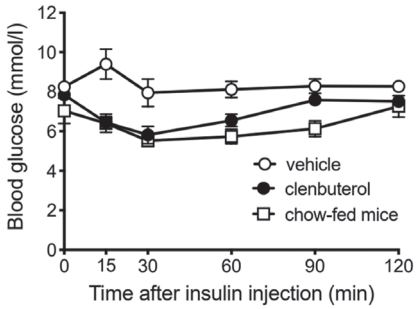
Supplementary figures



ESM Fig. 1. Repetitive daily administration of clenbuterol did not increase blood glucose. **(a)** DIO was developed in C57Bl/6N mice maintained at 30°C and on HFD for 8 months. DIO mice were injected i.p. with saline (n=7) or clenbuterol (0.17 mg kg^{-1} day^{-1}, n=8) for 7 days. On the last day of the treatment, non-fasting blood glucose was measured before and 20 min after drug administration. Data were analyzed by mixed-effects analysis with Dunnett's multiple comparison test. **(b)** acutely, single injection with clenbuterol elevates fasting blood glucose even at the lowest dose of the drug (0.025 mg/kg). Data were analyzed by two-way ANOVA with Dunnett's multiple comparison test. *** $p < 0.001$.

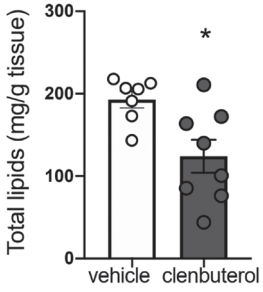


ESM Fig. 2. Prolonged treatment with low-dose clenbuterol improves oral glucose tolerance. The data are from the same experiment as in figures 4, 5 and 6f. DIO mice were treated with clenbuterol (3mg/l in drinking water) for 32 days. Oral GTT was performed after 4 **(A)** and 18 **(B)** days of the treatment. After 5 hours of fasting, glucose (2,5 mg/kg lean mass) was administrated by oral gavage and blood glucose was measured in 15, 30, 60, 90 and 120 minutes. AUC were analyzed with unpaired two-tailed Student's T-test. * $p < 0.05$, ** $p < 0.01$.



ESM Fig. 3. Prolonged treatment with low dose of clenbuterol normalizes insulin sensitivity to the level of young chow-fed mice kept at room temperature.

Data for vehicle and clenbuterol are the same as in figure 5C. Please note, that chow-fed mice are from a separate experiment, performed at a different occasion (mice were not treated). Data in clenbuterol and chow-fed groups were not statistically different at time points 0, 15 and 30 minutes, as analyzed by two-way ANOVA with Tukey's multiple comparison test.



ESM Fig. 4. Prolonged treatment with low dose of clenbuterol reduces hepatic steatosis.

Diet-induced obesity was developed in C57Bl/6N mice maintained at 21°C or 30°C and on HFD for 4 months; they were treated with clenbuterol for 40 days, 3 mg/l clenbuterol was administered in the drinking water. Mice were 10 months old in the beginning of the treatment. Hepatic lipids were extracted and measured gravimetrically. Data were analyzed by unpaired two-tailed Student's T-test. * $p < 0.05$.

CHAPTER 4

Prolonged β2-adrenergic agonist treatment improves glucose homeostasis in diet-induced obese UCP1-/- mice

Sten M.M. van Beek[1], Anastasia Kalinovich[2], Gert Schaart[1], Tore Bengtsson[2] and Joris Hoeks[1]

[1]Department of Nutrition and Movement Sciences, NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University, Maastricht, the Netherlands.

[2]Department of Molecular Biosciences, The Wenner-Gren Institute, Stockholm University, Stockholm, Sweden.

Am J Physiol Endocrinol Metab. 2021 Mar 1;320(3):E619-E628.

Abstract

Objectives - 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, vs. wild-type (WT) mice.

Methods - Anaesthetized 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-weeks of chow feeding, high-fat feeding or high-fat feeding with clenbuterol treatment between week 13-17. Body composition was measured weekly with MRI. Oral glucose tolerance and insulin tolerance tests were performed in week 15 and 17, respectively.

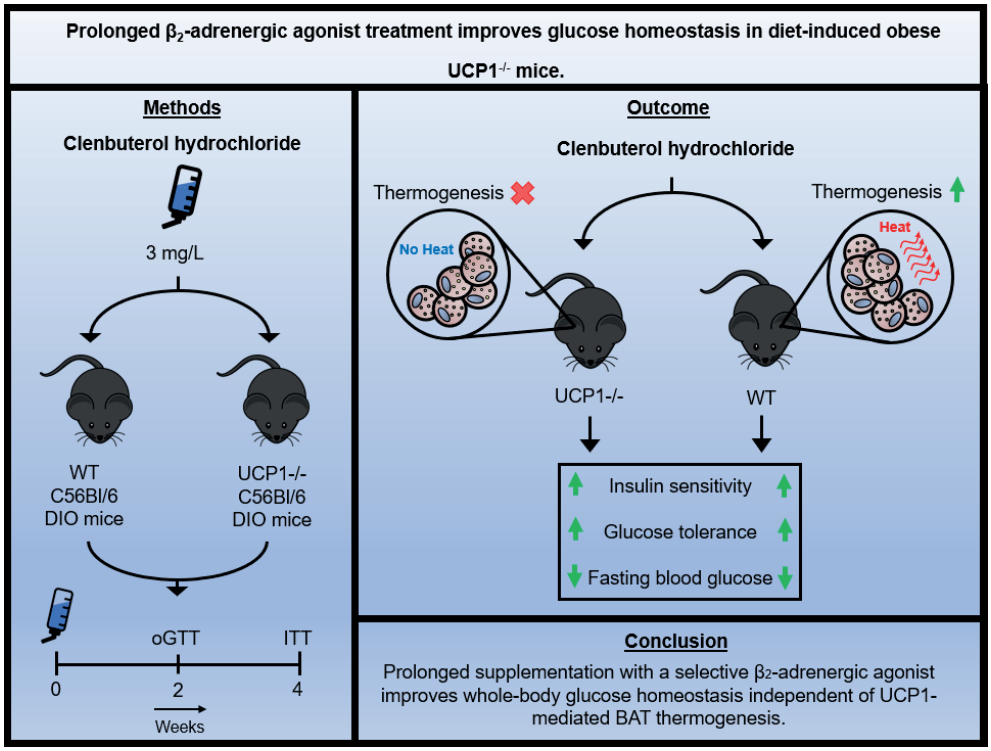
Results - Clenbuterol increased oxygen consumption ~2-fold in WT mice. This increase was blunted in UCP1$^{-/-}$ mice, indicating clenbuterol-mediated activation of BAT thermogenesis. High-fat feeding induced diabetogenic phenotypes in both genotypes. However, 2-weeks low-dose clenbuterol treatment significantly 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 to HFD controls and normalized to chow-fed control mice independent of body mass and -composition alterations.

Conclusions - Clenbuterol improved whole-body glucose homeostasis independent of UCP1. Given the low human abundancy of BAT, β_2-agonist treatment provides a potential novel route for glucose disposal in diabetic humans.

New and 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-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-agonist supplementation improves whole-body glucose homeostasis independent of UCP1-mediated BAT thermogenesis.

Key words: skeletal muscle, brown adipose tissue, UCP1, β_2-adrenergic agonist, type 2 diabetes mellitus

Graphical abstract



1. 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) (8, 9). 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) (37). 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 (16). 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 (7, 33, 37, 40), in a dose-dependent manner (16).

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 (1), the primary receptors involved in BAT activation (6). 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) (6). Due to its capacity to enhance energy expenditure and to clear large amounts of glucose from the blood (6, 32, 38), 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 (3, 43) or treatment with the selective β_3-AR agonist CL-316.243 (31, 45) 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 (19, 39), 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 (7). 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 (10, 23). 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.

2. Material and methods

2.1 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-hour 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.

2.2 Study designs

Acute effects of clenbuterol injection on BAT thermogenesis

After 2 weeks of acclimatization to 30°C, 13-16 weeks old male WT (n = 5) and UCP1$^{-/-}$ mice (n = 8) were anaesthetized via an intraperitoneal pentobarbital (70 mg/kg) injection. Mice were placed in metabolic chambers (Promethion, Sable systems) for 20 minutes to assess 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 minutes. After a wash-out period of 2-weeks, the experiment was repeated in a cross-over 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 was not used.

Effect of prolonged clenbuterol supplementation on the glucose homeostasis in UCP1-/- mice

At 8-weeks of age, 49 male WT and UCP1$^{-/-}$ mice (n = 25 and n = 24, respectively) were randomized into two groups receiving a 13-week 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, USA) to induce obesity and glucose intolerance (**Figure 1**). An intraperitoneal glucose tolerance test (IpGTT) and insulin tolerance test (ITT) were performed after 12 and 13-weeks of the run-in diet, respectively. After 13 weeks, 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 (16).

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, USA). An oral glucose tolerance test (oGTT) and insulin tolerance test (ITT) were performed after 2 and 4 weeks of treatment (t = 2 and 4), respectively (Figure 1). Five days after the ITT, mice were euthanised by means of CO_2, 10 minutes 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.

2.3 Glucose and insulin tolerance tests

Prior to a GTT or ITT, mice were fasted for 5 hours 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 the GTT and the ITT, blood glucose concentrations were measured at t = 0, 15, 30, 60, 90 and 120 minutes 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, #90080, Crystal Chem) according to the manufacturers' instructions.

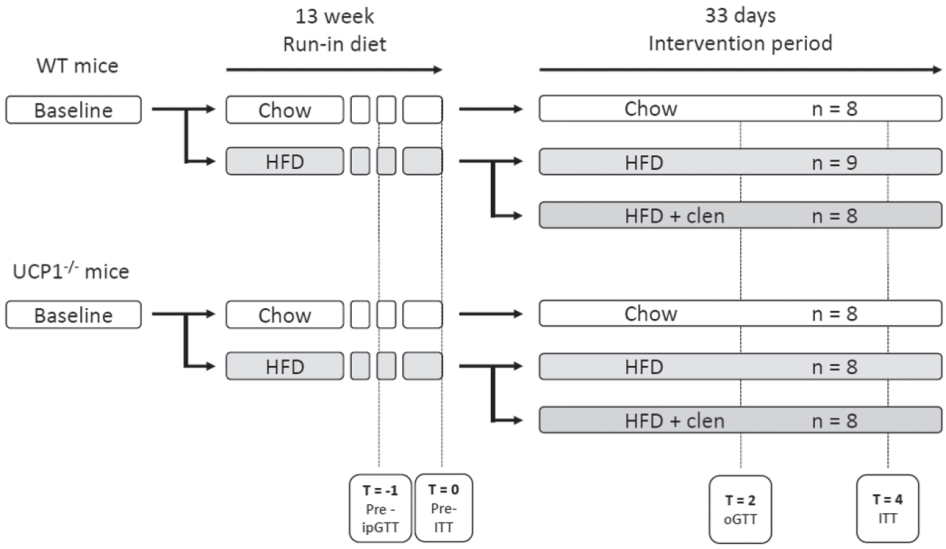


Figure 1. Study design. WT and UCP1$^{-/-}$ mice were put on a 13-week run-in diet consisting of either chow or high-fat diet (HFD). An ipGTT and ITT were performed after 12 and 13 weeks of the run-in diet (t = -1 and t = 0), respectively. Afterwards, the HFD diet group of each genotype was further subdivided 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 weeks of treatment (t = 2 and t = 4), respectively.

2.4 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 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, #9271, Cell Signalling, Bioké, Leiden, The Netherlands) or Akt (1:2,500, #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).

2.5 Statistical analyses

All data were statistically analysed with GraphPad Prism 9.0 (GraphPad Software Inc. La Jolla, CA, USA). All data were assessed for normality by means of an Kolmogorov-Smirnov

test. Paired data of the acute experiment were analysed by means of a Paired Student's T-test in case of normality or a non-parametric Wilcoxon matched-pairs signed rank test in case of non-normally distributed data. Data acquired during the prolonged clenbuterol supplementation was analysed by means of different statistical tests. Body weight and composition were analysed 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 a one-way ANOVA with Tukey's post hoc test if data were normally distributed. In case of non-normally distributed data, a non-parametric Kruskal-Wallis test was used. Data were considered statistically significant if $p < 0.05$. All data are expressed as mean ± SE.

3. Results

3.1 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 (**Figure 2A-C)**. In contrast, clenbuterol markedly increased whole-body oxygen consumption by 88.94% in WT mice ($p = 0.0003$, **Figure 2A and C**). The oxygen consumption in UCP1$^{-/-}$ mice upon clenbuterol injection was blunted, but still significantly increased by 46.52% ($p < 0.0001$, **Figure 2B and C**). Combined, these results strongly suggest that clenbuterol activates BAT non-shivering 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.

3.2 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 (**Figure 1**). Prior to this treatment period (t = 0), both HFD + clen groups of WT and UCP1$^{-/-}$ mice were similar in body weight and body composition as compared to their HFD counterparts (**Figure 3**). Obviously, body weight and fat mass were significantly increased in all HFD groups as compared to their respective chow-fed control groups (**Figure 3A-B and 3E-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 vs. 44.36 ± 1.33 grams in WT and 44.84 ± 0.88 vs. 44.30 ± 1.86 grams in UCP1$^{-/-}$ mice after the intervention, respectively (NS for all, **Figure 3A-B**). Lean and fat mass remained unaffected during the HFD + clenbuterol treatment in both genotypes as compared to HFD alone (**Figure 3C-F**).

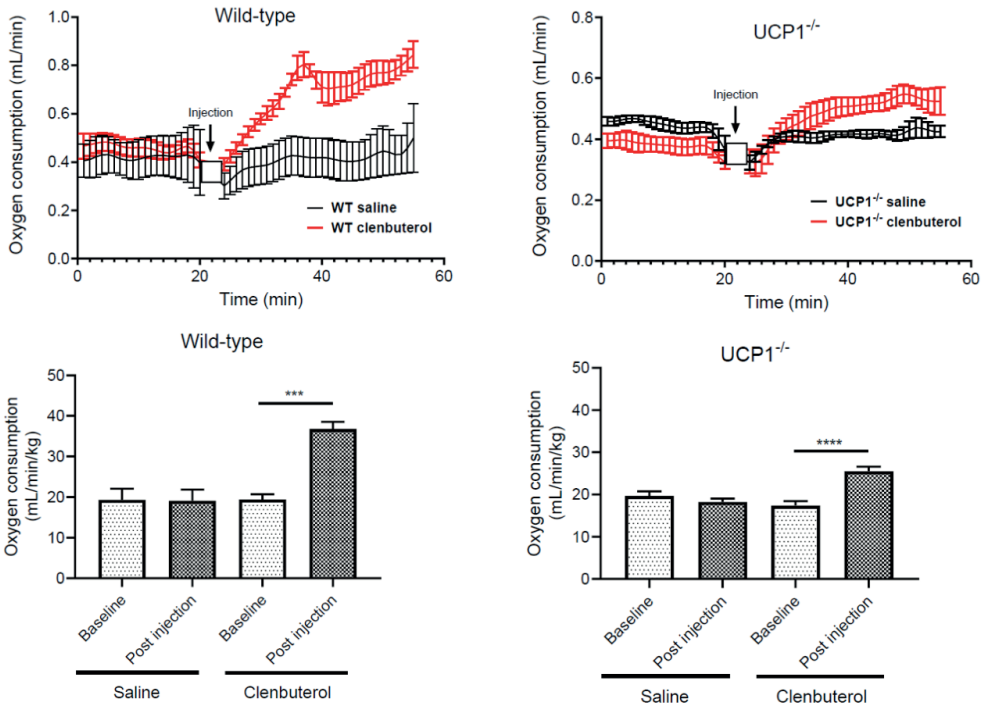


Figure 2. Clenbuterol activates brown adipose tissue *in vivo* in mice. A. Oxygen consumption in male WT mice upon saline (n = 4) and clenbuterol injection (n = 5). B. Oxygen consumption in male UCP1$^{-/-}$ mice upon saline (n = 6) and clenbuterol injection (n = 6). C. Average oxygen consumption in basal and stimulated state of WT mice. D. average oxygen consumption in basal and stimulated state of UCP1$^{-/-}$ mice. Data were analyzed by means of a Student's Paired T-test or non-parametrical Wilcoxon matched-pairs signed ranked test. ***p < 0.001, ****p < 0.0001.

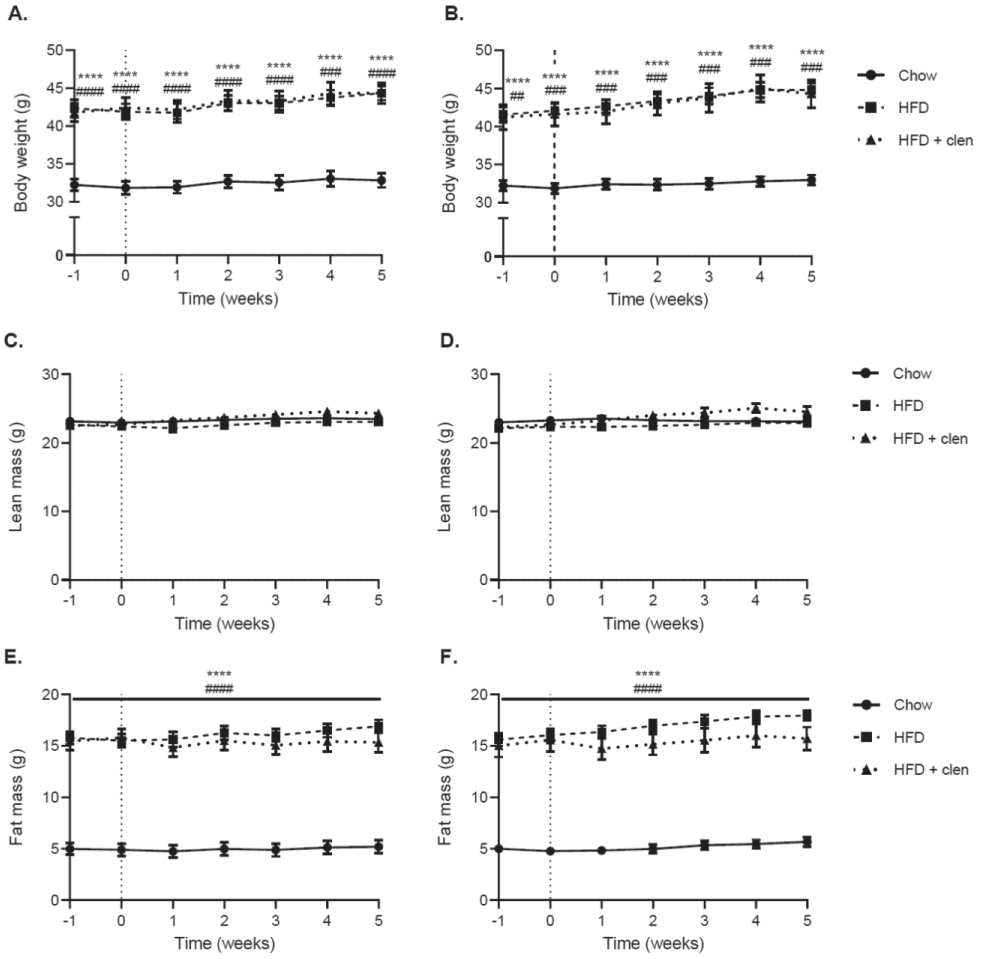


Figure 3. Body weight and composition are not affected upon prolonged low-dose clenbuterol supplementation. A. Body weight of WT mice, B. Body weight of UCP1$^{-/-}$ mice. C. Lean mass of WT mice, D. Lean mass of UCP1$^{-/-}$ mice, E. Fat mass of WT mice, F. Fat mass of UCP1$^{-/-}$ mice. Dotted lines indicates 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 analyzed by means of a two-way ANOVA with Tukey's post hoc test. ****p < 0.0001, $^{\#\#}$p < 0.01, $^{\#\#\#}$p < 0.001, $^{\#\#\#\#}$p < 0.0001.

3.3 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 chow-fed control groups prior to the start of clenbuterol treatment (**Suppl. Figure A1**). Following 2 weeks of clenbuterol treatment, fasting glucose

was significantly lowered by 12.9% and 14.8% for WT and UCP1$^{-/-}$ treated mice as compared to their respective HFD control group ($p = 0.0115$ and $p = 0.0001$), respectively (**Figure 4A**). For both genotypes, fasting glucose in HFD + clen groups remained significantly higher as compared to their chow-fed control groups ($p = 0.023$ and $p = 0.034$ for WT and UCP1$^{-/-}$, respectively, **Figure 4A**).

Prior to clenbuterol treatment, glucose tolerance was comparable between the HFD and HFD + clen groups of WT as well as UCP1$^{-/-}$ mice (**Suppl. Figure A1**) and significantly reduced in comparison with the respective chow-fed control group (**Suppl. Figure A1**). Upon 2 weeks 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, **Figure 4B-D**). Remarkably, clenbuterol treatment normalized glucose clearance from the blood to similar values as lean, healthy chow-fed control mice (AUC WT: 1194 ± 44.9 vs. 1192 ± 29.7 and UCP1$^{-/-}$: 1192 ± 32.5 vs. 1109 ± 26.0, NS for all, respectively, **Figure 4B**).

As acute β_2-AR administration is associated with increased insulin release (16, 34), 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 to HFD alone, neither in the fasting state nor during the oGTT (**Figure 4E**). In contrast, plasma insulin levels were significantly reduced in UCP1$^{-/-}$ mice upon HFD + clenbuterol treatment as compared to HFD, both in the fasting state and 30 minutes following the glucose bolus ($p = 0.0102$ and $p = 0.0103$, respectively) (**Figure 4F**).

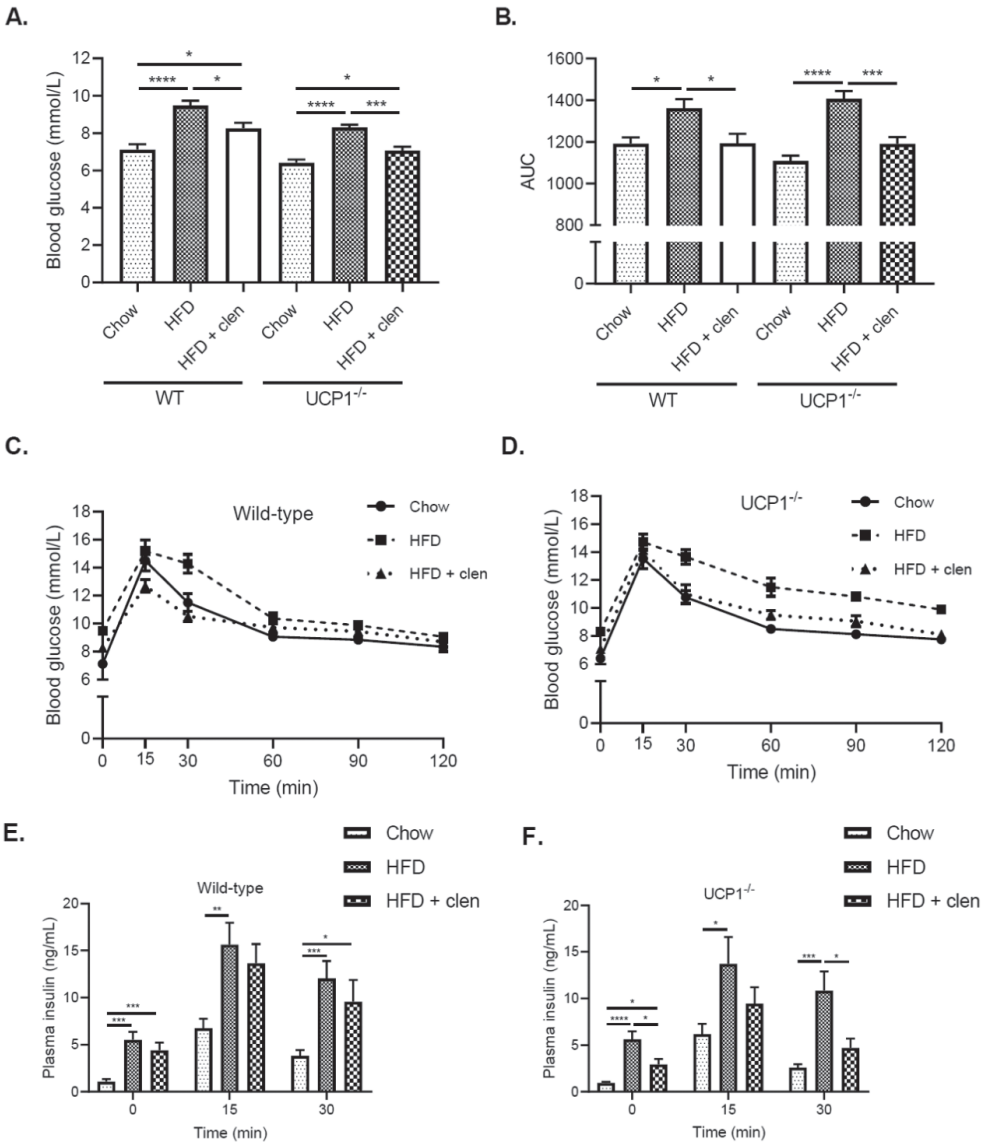


Figure 4. Clenbuterol supplementation improves oral glucose tolerance of WT and UCP1$^{-/-}$ mice after 2 weeks of treatment. A. Fasting glucose of WT and UCP1$^{-/-}$ mice, B. Area under the curve calculated from 0 of both WT and UCP1$^{-/-}$ groups, C. oGTT of WT mice, D. oGTT of UCP1$^{-/-}$ mice. E. Insulin concentrations of WT mice during the oral glucose tolerance test. F. Insulin concentrations of UCP1$^{-/-}$ mice during the glucose tolerance test. Data analyzed by means of a one-way ANOVA with a Tukey's post hoc test or a non-parametric Kruskall-Wallis test. *p < 0.05, ** p < 0.01, ***p < 0.001, ****p < 0.0001.

3.4 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 signalling.

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

Insulin tolerance was similar between HFD + clen groups and their respective HFD control group of both genotypes preceding the clenbuterol intervention period (**Suppl. Figure A2**). After 17-weeks 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 (**Figure 5B-E**). Remarkably, both WT and UCP1$^{-/-}$ HFD mice treated with clenbuterol for 4 weeks showed a major decrease in blood glucose concentrations as compared to the respective HFD control groups, both at 15 and 30 minutes after the insulin injection (WT: p = 0.0011 and p = 0.0014 and UCP1$^{-/-}$: p = 0.0020 and p = 0.0073, respectively) (**Figure 5B-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 normalisation of insulin sensitivity in clenbuterol treated mice (**Figure 5B-E**).

To further investigate the underlying mechanisms by which clenbuterol improves whole-body insulin sensitivity, mice were euthanised 10 minutes 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 signalling pathway. HFD feeding significantly reduced p-AKT/totalAKT ratio in gastrocnemius muscle as compared to chow fed diet in both WT and UCP1$^{-/-}$ mice by 50.4% and 45.7%, respectively (p = 0.022 and p = 0.006) (**Figure 6A**). However, clenbuterol treatment did not improve p-AKT/totalAKT ratio in gastrocnemius muscle as compared to HFD feeding alone in both genotypes (p = 0.968 and p = 0.989, respectively) (**Figure 6A**) 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, **Figure 6B**). 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, **Figure 6B**).

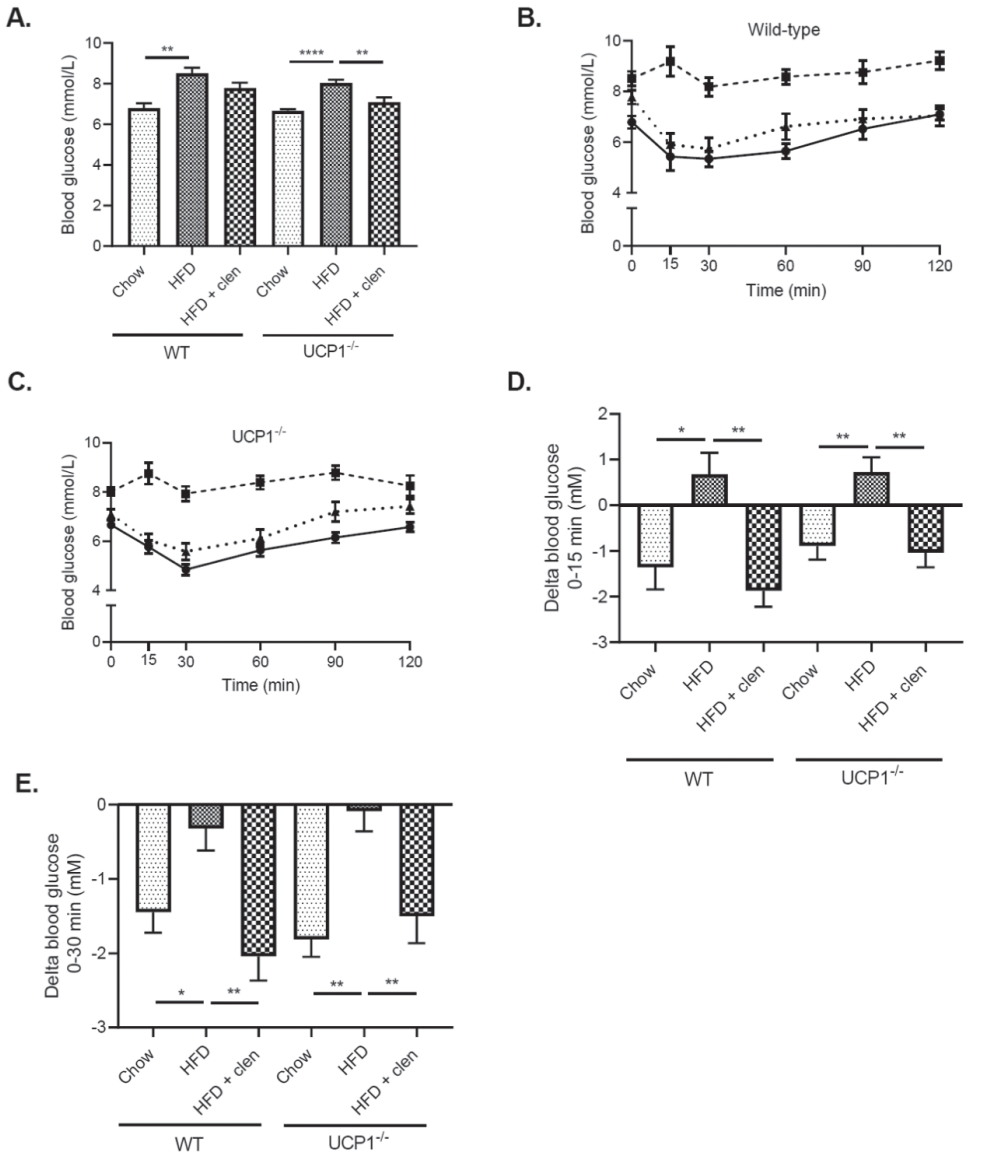


Figure 5. Clenbuterol improves insulin tolerance after 4 weeks in WT and UCP1$^{-/-}$. A. Fasting blood glucose of WT and UCP1$^{-/-}$ mice. B. Time-response curve of blood glucose following an insulin injection (1 U/kg lean mass) in WT mice, C. Time-response curve of blood glucose following an insulin injection (1 U/kg lean mass) in UCP1$^{-/-}$ mice. D. Delta blood glucose between t = 0 and 15 minutes for WT and UCP1$^{-/-}$ mice, E. Delta blood glucose between t = 0 and 30 minutes for WT and UCP1$^{-/-}$ mice. WT: Chow n = 7, HFD = 9, HFD + clen = 8; UCP1$^{-/-}$: chow = 8, HFD = 8, HFD + clen = 8. Data was analyzed by means of a one-way ANOVA with Tukey's post hoc test or a non-parametric Kruskall-Wallis test. *p < 0.05, **p < 0.01, ****p < 0.0001.

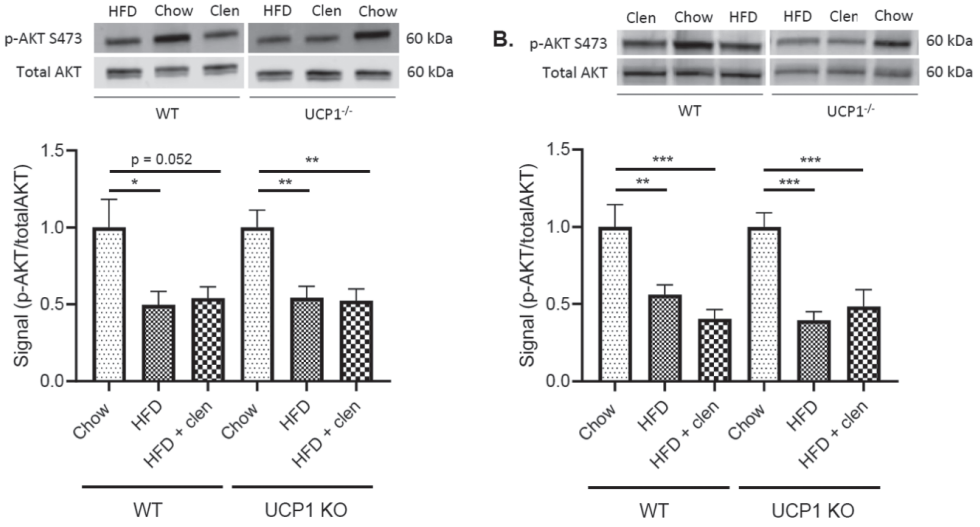


Figure 6. Clenbuterol supplementation does not affect insulin signaling in gastrocnemius muscle and interscapular BAT. A. Western blot of p-AKTS473/total-AKT in gastrocnemius muscle (WT: Chow n = 8, HFD = 9, Clen = 7; UCP1$^{-/-}$: Chow = 8, HFD = 7, Clen = 7). B. Western blot of p-AKTS473/total-AKT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

4. Discussion

Recently, we have demonstrated robust improvements in glucose homeostasis of DIO mice upon prolonged supplementation with the β_2-agonist clenbuterol and highlighted the potential role of the liver and especially skeletal muscle therein (16, 37). 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 (15, 44), 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 glucose and insulin tolerance, 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 independently of UCP1-mediated BAT thermogenesis.

In the current study, we demonstrated a ~2-fold 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 but also showed that norepinephrine, the most commonly used agonist for the assessment of BAT activation, appears to be a more potent activator 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 non-specific binding of clenbuterol to β_1- and/or β_3-ARs (1), the primary receptors involved in the activation BAT thermogenesis in rodents (6), or indirectly via an increased clenbuterol-induced BAT tissue blood flood flow (36).

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 for BAT thermogenesis (10, 23). This finding was in line with previous studies showing increases in oxygen consumption in UCP1$^{-/-}$ mice upon norepinephrine injection (11, 14, 21). These results thus indicate that next to UCP1-dependent thermogenesis, also other metabolic processes contribute to the increase in whole-body oxygen consumption upon acute β-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 (2, 5, 18). 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 (7, 16, 33, 37, 40).

Therefore, we next investigated if a prolonged, low-dose clenbuterol treatment would result in similar beneficial effects on metabolic health effect in UCP1$^{-/-}$ mice, who – in our view - mimic the human T2DM phenotype more closely, since T2DM patients are characterized by very low amounts of BAT (15). In line with our previous studies (16, 37), 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. Combined, 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 whereas reducing fat mass (7, 17, 33, 40-42), which has previously been considered a primary factor in mediating improvements in glucose tolerance (33). 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 (16) - a time-frame 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-agonists are well-known to acutely induce the secretion of insulin by pancreatic β-cells (16, 34). 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 (7, 16, 33, 40). 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 (4, 13, 22, 35), 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 and BAT.

Although BAT glucose uptake can also be facilitated in an UCP1-independent manner (31), 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) (31). 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, 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 (3, 12, 25). 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 (16) and skeletal muscle (16, 37). 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 (20). This improved liver insulin sensitivity may relate to a diminished hepatic lipid accumulation upon clenbuterol treatment, as observed previously (16). Additionally, we and others have previously demonstrated significant increases in *in vitro* muscle cell glucose uptake upon selective β_2-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 (16, 26-30, 37). 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 (16).

5. 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 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 (pre)diabetic patients, our findings in this mouse model suggest that selective β_2-agonist treatment may provide a novel molecular route to improve glucose disposal in the insulin resistant state.

Acknowledgement

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

Disclosures

TB owns stocks in Atrogi AB. AK is an employee within Atrogi AB. The authors declare no other conflicts of interest.

Funding

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

Contribution statement

All authors contributed to the design of the study. Experiments were performed by SvB, AK and GS. Data was analyzed and figures were drafted by SvB,AK and GS.

SvB drafted the manuscript and AK, GS, TB and JH reviewed and edited the manuscript. All authors read and approved the final version of the manuscript. JH is the guarantor of the study.

Data availability statement

All data are available upon request.

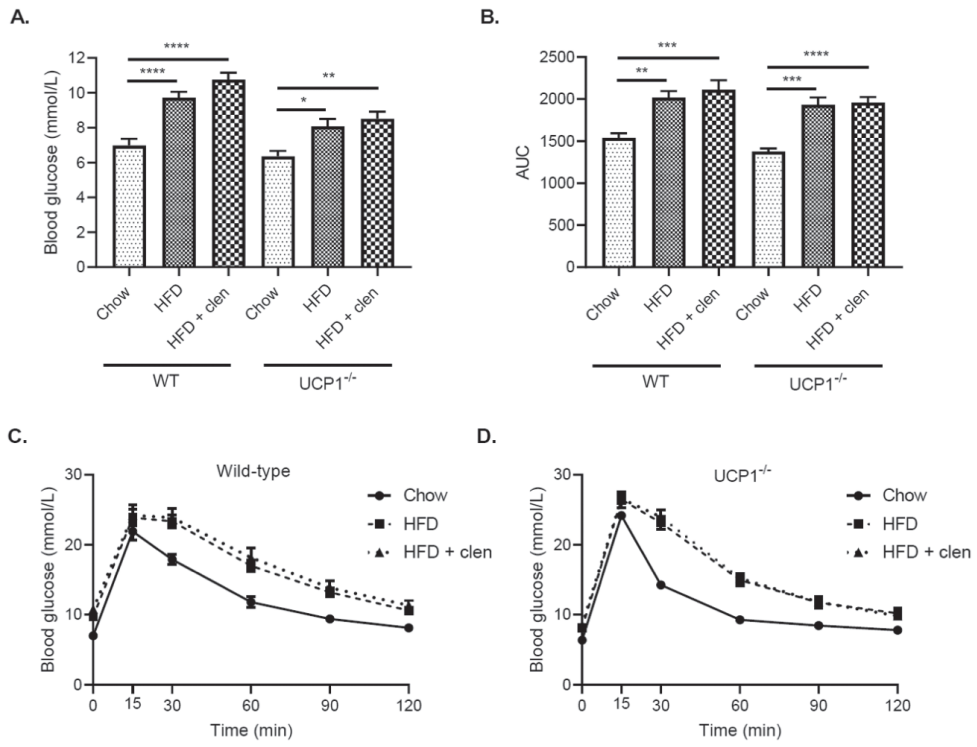
References

1. Baker JG. The selectivity of beta-adrenoceptor agonists at human beta1-, beta2- and beta3-adrenoceptors. *Br J Pharmacol* 160: 1048-1061, 2010.
2. Bal NC, Maurya SK, Sopariwala DH, Sahoo SK, Gupta SC, Shaikh SA, Pant M, Rowland LA, Bombardier E, Goonasekera SA, Tupling AR, Molkentin JD, and Periasamy M. Sarcolipin is a newly identified regulator of muscle-based thermogenesis in mammals. *Nat Med* 18: 1575-1579, 2012.
3. Bartelt A, Bruns OT, Reimer R, Hohenberg H, Ittrich H, Peldschus K, Kaul MG, Tromsdorf UI, Weller H, Waurisch C, Eychmuller A, Gordts PL, Rinninger F, Bruegelmann K, Freund B, Nielsen P, Merkel M, and Heeren J. Brown adipose tissue activity controls triglyceride clearance. *Nat Med* 17: 200-205, 2011.
4. Beck Jorgensen S, O'Neill HM, Hewitt K, Kemp BE, and Steinberg GR. Reduced AMP-activated protein kinase activity in mouse skeletal muscle does not exacerbate the development of insulin resistance with obesity. *Diabetologia* 52: 2395-2404, 2009.
5. Blondin DP, Nielsen S, Kuipers EN, Severinsen MC, Jensen VH, Miard S, Jespersen NZ, Kooijman S, Boon MR, Fortin M, Phoenix S, Frisch F, Guerin B, Turcotte EE, Haman F, Richard D, Picard F, Rensen PCN, Scheele C, and Carpentier AC. Human Brown Adipocyte Thermogenesis Is Driven by beta2-AR Stimulation. *Cell Metab* 32: 287-300 e287, 2020.
6. Cannon B, and Nedergaard J. Brown adipose tissue: function and physiological significance. *Physiol Rev* 84: 277-359, 2004.
7. Castle A, Yaspelkis BB, 3rd, Kuo CH, and Ivy JL. Attenuation of insulin resistance by chronic beta2-adrenergic agonist treatment possible muscle specific contributions. *Life Sci* 69: 599-611, 2001.
8. Czech MP. Insulin action and resistance in obesity and type 2 diabetes. *Nat Med* 23: 804-814, 2017.
9. DeFronzo RA, Gunnarsson R, Bjorkman O, Olsson M, and Wahren J. Effects of insulin on peripheral and splanchnic glucose metabolism in noninsulin-dependent (type II) diabetes mellitus. *J Clin Invest* 76: 149-155, 1985.
10. Enerback S, Jacobsson A, Simpson EM, Guerra C, Yamashita H, Harper ME, and Kozak LP. Mice lacking mitochondrial uncoupling protein are cold-sensitive but not obese. *Nature* 387: 90-94, 1997.
11. Feldmann HM, Golozoubova V, Cannon B, and Nedergaard J. UCP1 ablation induces obesity and abolishes diet-induced thermogenesis in mice exempt from thermal stress by living at thermoneutrality. *Cell Metab* 9: 203-209, 2009.
12. Fischer AW, Behrens J, Sass F, Schlein C, Heine M, Pertzborn P, Scheja L, and Heeren J. Brown adipose tissue lipoprotein and glucose disposal is not determined by thermogenesis in uncoupling protein 1-deficient mice. *J Lipid Res* 61: 1377-1389, 2020.
13. Frosig C, Jensen TE, Jeppesen J, Pehmoller C, Treebak JT, Maarbjerg SJ, Kristensen JM, Sylow L, Alsted TJ, Schjerling P, Kiens B, Wojtaszewski JF, and Richter EA. AMPK and insulin action--responses to ageing and high fat diet. *PLoS One* 8: e62338, 2013.
14. Golozoubova V, Cannon B, and Nedergaard J. UCP1 is essential for adaptive adrenergic nonshivering thermogenesis. *Am J Physiol Endocrinol Metab* 291: E350-357, 2006.
15. Hanssen MJ, Hoeks J, Brans B, van der Lans AA, Schaart G, van den Driessche JJ, Jorgensen JA, Boekschoten MV, Hesselink MK, Havekes B, Kersten S, Mottaghy FM, van Marken Lichtenbelt WD, and Schrauwen P. Short-term cold acclimation improves insulin sensitivity in patients with type 2 diabetes mellitus. *Nat Med* 21: 863-865, 2015.

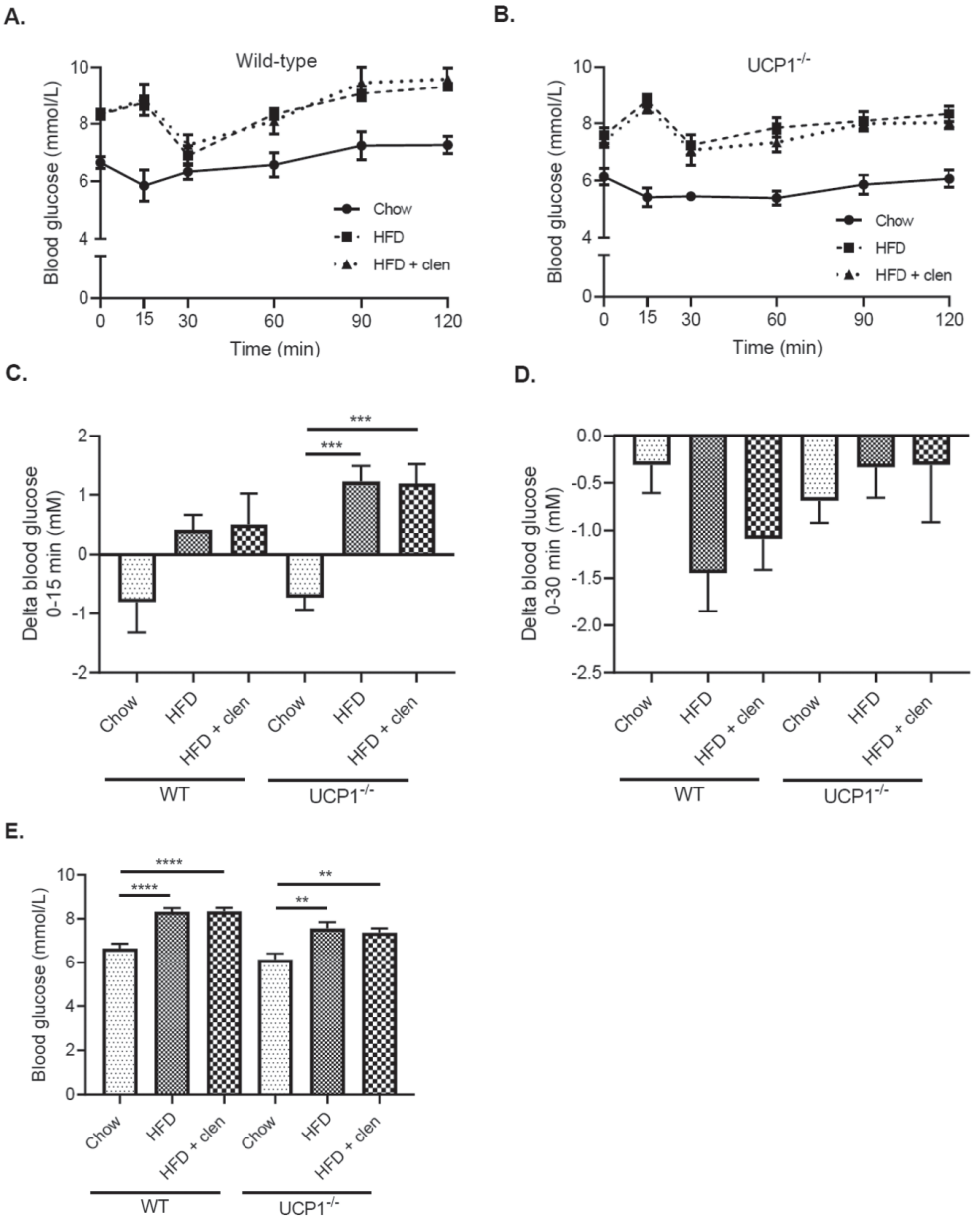
16. Kalinovich A, Dehvari N, Aslund A, van Beek S, Halleskog C, Olsen J, Forsberg E, Zacharewicz E, Schaart G, Rinde M, Sandstrom A, Berlin R, Ostenson CG, Hoeks J, and Bengtsson T. Treatment with a beta-2-adrenoceptor agonist stimulates glucose uptake in skeletal muscle and improves glucose homeostasis, insulin resistance and hepatic steatosis in mice with diet-induced obesity. *Diabetologia* 63: 1603-1615, 2020.
17. Kamalakkannan G, Petrilli CM, George I, LaManca J, McLaughlin BT, Shane E, Mancini DM, and Maybaum S. Clenbuterol increases lean muscle mass but not endurance in patients with chronic heart failure. *J Heart Lung Transplant* 27: 457-461, 2008.
18. Kazak L, Chouchani ET, Jedrychowski MP, Erickson BK, Shinoda K, Cohen P, Vetrivelan R, Lu GZ, Laznik-Bogoslavski D, Hasenfuss SC, Kajimura S, Gygi SP, and Spiegelman BM. A creatine-driven substrate cycle enhances energy expenditure and thermogenesis in beige fat. *Cell* 163: 643-655, 2015.
19. Liu X, Wang S, You Y, Meng M, Zheng Z, Dong M, Lin J, Zhao Q, Zhang C, Yuan X, Hu T, Liu L, Huang Y, Zhang L, Wang D, Zhan J, Jong Lee H, Speakman JR, and Jin W. Brown Adipose Tissue Transplantation Reverses Obesity in Ob/Ob Mice. *Endocrinology* 156: 2461-2469, 2015.
20. Loria P, Lonardo A, and Anania F. Liver and diabetes. A vicious circle. *Hepatol Res* 43: 51-64, 2013.
21. Luijten IHN, Brooks K, Boulet N, Shabalina IG, Jaiprakash A, Carlsson B, Fischer AW, Cannon B, and Nedergaard J. Glucocorticoid-Induced Obesity Develops Independently of UCP1. *Cell Rep* 27: 1686-1698 e1685, 2019.
22. Martins AR, Crisma AR, Masi LN, Amaral CL, Marzuca-Nassr GN, Bomfim LHM, Teodoro BG, Queiroz AL, Serdan TDA, Torres RP, Mancini-Filho J, Rodrigues AC, Alba-Loureiro TC, Pithon-Curi TC, Gorjao R, Silveira LR, Curi R, Newsholme P, and Hirabara SM. Attenuation of obesity and insulin resistance by fish oil supplementation is associated with improved skeletal muscle mitochondrial function in mice fed a high-fat diet. *J Nutr Biochem* 55: 76-88, 2018.
23. Matthias A, Ohlson KB, Fredriksson JM, Jacobsson A, Nedergaard J, and Cannon B. Thermogenic responses in brown fat cells are fully UCP1-dependent. UCP2 or UCP3 do not substitute for UCP1 in adrenergically or fatty scid-induced thermogenesis. *J Biol Chem* 275: 25073-25081, 2000.
24. Mattsson CL, Csikasz RI, Shabalina IG, Nedergaard J, and Cannon B. Caveolin-1-ablated mice survive in cold by nonshivering thermogenesis despite desensitized adrenergic responsiveness. *Am J Physiol Endocrinol Metab* 299: E374-383, 2010.
25. Mottillo EP, Balasubramanian P, Lee YH, Weng C, Kershaw EE, and Granneman JG. Coupling of lipolysis and de novo lipogenesis in brown, beige, and white adipose tissues during chronic beta3-adrenergic receptor activation. *J Lipid Res* 55: 2276-2286, 2014.
26. Mukaida S, Sato M, Oberg AI, Dehvari N, Olsen JM, Kocan M, Halls ML, Merlin J, Sandstrom AL, Csikasz RI, Evans BA, Summers RJ, Hutchinson DS, and Bengtsson T. BRL37344 stimulates GLUT4 translocation and glucose uptake in skeletal muscle via beta2-adrenoceptors without causing classical receptor desensitization. *Am J Physiol Regul Integr Comp Physiol* 316: R666-R677, 2019.
27. Nevzorova J, Bengtsson T, Evans BA, and Summers RJ. Characterization of the beta-adrenoceptor subtype involved in mediation of glucose transport in L6 cells. *Br J Pharmacol* 137: 9-18, 2002.
28. Nevzorova J, Evans BA, Bengtsson T, and Summers RJ. Multiple signalling pathways involved in beta2-adrenoceptor-mediated glucose uptake in rat skeletal muscle cells. *Br J Pharmacol* 147: 446-454, 2006.
29. Ngala RA, O'Dowd J, Wang SJ, Agarwal A, Stocker C, Cawthorne MA, and Arch JR. Metabolic responses to BRL37344 and clenbuterol in soleus muscle and C2C12 cells via different atypical pharmacologies and beta2-adrenoceptor mechanisms. *Br J Pharmacol* 155: 395-406, 2008.

30. Ngala RA, O'Dowd J, Wang SJ, Stocker C, Cawthorne MA, and Arch JR. Beta2-adrenoceptors and non-beta-adrenoceptors mediate effects of BRL37344 and clenbuterol on glucose uptake in soleus muscle: studies using knockout mice. *Br J Pharmacol* 158: 1676-1682, 2009.
31. Olsen JM, Csikasz RI, Dehvari N, Lu L, Sandstrom A, Oberg AI, Nedergaard J, Stone-Elander S, and Bengtsson T. beta3-Adrenergically induced glucose uptake in brown adipose tissue is independent of UCP1 presence or activity: Mediation through the mTOR pathway. *Mol Metab* 6: 611-619, 2017.
32. Orava J, Nuutila P, Lidell ME, Oikonen V, Noponen T, Viljanen T, Scheinin M, Taittonen M, Niemi T, Enerback S, and Virtanen KA. Different metabolic responses of human brown adipose tissue to activation by cold and insulin. *Cell Metab* 14: 272-279, 2011.
33. Pan SJ, Hancock J, Ding Z, Fogt D, Lee M, and Ivy JL. Effects of clenbuterol on insulin resistance in conscious obese Zucker rats. *Am J Physiol Endocrinol Metab* 280: E554-561, 2001.
34. Philipson LH. beta-Agonists and metabolism. *J Allergy Clin Immunol* 110: S313-317, 2002.
35. Roberts-Toler C, O'Neill BT, and Cypess AM. Diet-induced obesity causes insulin resistance in mouse brown adipose tissue. *Obesity (Silver Spring)* 23: 1765-1770, 2015.
36. Rothwell NJ, Stock MJ, and Sudera DK. Changes in tissue blood flow and beta-receptor density of skeletal muscle in rats treated with the beta2-adrenoceptor agonist clenbuterol. *Br J Pharmacol* 90: 601-607, 1987.
37. Sato M, Dehvari N, Oberg AI, Dallner OS, Sandstrom AL, Olsen JM, Csikasz RI, Summers RJ, Hutchinson DS, and Bengtsson T. Improving type 2 diabetes through a distinct adrenergic signaling pathway involving mTORC2 that mediates glucose uptake in skeletal muscle. *Diabetes* 63: 4115-4129, 2014.
38. Shibata H, Perusse F, Vallerand A, and Bukowiecki LJ. Cold exposure reverses inhibitory effects of fasting on peripheral glucose uptake in rats. *Am J Physiol* 257: R96-101, 1989.
39. Stanford KI, Middelbeek RJ, Townsend KL, An D, Nygaard EB, Hitchcox KM, Markan KR, Nakano K, Hirshman MF, Tseng YH, and Goodyear LJ. Brown adipose tissue regulates glucose homeostasis and insulin sensitivity. *J Clin Invest* 123: 215-223, 2013.
40. Torgan CE, Brozinick JT, Jr., Banks EA, Cortez MY, Wilcox RE, and Ivy JL. Exercise training and clenbuterol reduce insulin resistance of obese Zucker rats. *Am J Physiol* 264: E373-379, 1993.
41. Torgan CE, Etgen GJ, Jr., Brozinick JT, Jr., Wilcox RE, and Ivy JL. Interaction of aerobic exercise training and clenbuterol: effects on insulin-resistant muscle. *J Appl Physiol (1985)* 75: 1471-1476, 1993.
42. Torgan CE, Etgen GJ, Jr., Kang HY, and Ivy JL. Fiber type-specific effects of clenbuterol and exercise training on insulin-resistant muscle. *J Appl Physiol (1985)* 79: 163-167, 1995.
43. Vallerand AL, Lupien J, and Bukowiecki LJ. Cold exposure reverses the diabetogenic effects of high-fat feeding. *Diabetes* 35: 329-334, 1986.
44. van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts JM, Kemerink GJ, Bouvy ND, Schrauwen P, and Teule GJ. Cold-activated brown adipose tissue in healthy men. *N Engl J Med* 360: 1500-1508, 2009.
45. Xiao C, Goldgof M, Gavrilova O, and Reitman ML. Anti-obesity and metabolic efficacy of the beta3-adrenergic agonist, CL316243, in mice at thermoneutrality compared to 22 degrees C. *Obesity (Silver Spring)* 23: 1450-1459, 2015.

Supplementary figures



Supplemental Figure 1. Glucose tolerance was comparable between high-fat diet groups prior to clenbuterol treatment. A. Fasting glucose at t = 12 weeks. B. Area under the curve calculated from 0 of both WT and UCP1$^{-/-}$ groups. C. IpGTT of WT mice. D. IpGTT of UCP1$^{-/-}$ mice. Data was analysed by means of a one-way ANOVA with Tukey's post hoc test or non-parametric Kruskall-Wallis test. **p < 0.01, ***p < 0.001, ****p < 0.0001.



Supplemental Figure 2. Insulin tolerance test prior to the start of the intervention period. A. Time-curve of blood glucose concentrations during the insulin tolerance test of WT mice. B. Time-curve of blood glucose concentrations during the insulin tolerance test of UCP1$^{-/-}$ mice. C. Delta blood glucose concentrations between t = 0 and 15 minutes. D. Delta blood glucose concentrations between t = 0 and 30 minutes. E. Fasting blood glucose concentrations. Data was analysed by means of a one-way ANOVA with Tukey's post hoc test or non-parametric Kruskall-Wallis test. **p < 0.01, ***p < 0.001, ****p < 0.0001.

CHAPTER 5

β_2-agonist treatment promotes insulin-stimulated skeletal muscle glucose uptake in healthy males in a randomised placebo-controlled trial

Sten M.M. van Beek[1], Yvonne M.H. Bruls[2], Froukje Vanweert[1], Ciarán E. Fealy[1], Niels J. Connell[1], Gert Schaart[1], Esther Kornips[1], Johanna A. Jörgensen[1], Frédéric M. Vaz[4,5,6], Ellen T.H.C. Smeets[1], Peter J. Joris[1], Anne Gemmink[1], Riekelt H. Houtkooper[4,5,7], Matthijs K.C. Hesselink[1], Tore Bengtsson[3], Bas Havekes[1,8], Patrick Schrauwen[1] and Joris Hoeks[1]

[1] Department of Nutrition and Movement Sciences, NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University, Maastricht, the Netherlands.

[2] Department of Radiology and Nuclear Medicine, NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University Medical Center +, Maastricht, the Netherlands

[3] Department of Molecular Biosciences, The Wenner-Gren Institute, Stockholm University, Stockholm, Sweden.

[4] Laboratory Genetic Metabolic Diseases, Amsterdam UMC location University of Amsterdam, Meibergdreef 9, Amsterdam, Netherlands

[5] Amsterdam Gastroenterology, Endocrinology, and Metabolism, Amsterdam, The Netherlands.

[6] Core Facility Metabolomics, Amsterdam UMC location University of Amsterdam, Amsterdam, The Netherlands

[7] Amsterdam Cardiovascular Sciences, Amsterdam, The Netherlands.

[8] Department of Internal Medicine, Division of Endocrinology and Metabolic Disease, NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University Medical Center, Maastricht, The Netherlands.

Submitted

Abstract

β_2-agonist treatment improves skeletal muscle glucose uptake and whole-body glucose homeostasis in rodents, likely via mTORC2-mediated signalling. However, human data on this topic is virtually absent. We here investigate the effects of two-weeks treatment with the β_2-agonist clenbuterol (40 µg/day) on glucose control as well as energy- and substrate metabolism in eleven healthy young males (age: 18-30 years, BMI: 20-25 kg/m^2) in a randomised, placebo-controlled, double-blinded, cross-over study (ClinicalTrials.gov-identifier: NCT03800290). The primary and secondary outcome were peripheral insulin-stimulated glucose disposal and skeletal muscle GLUT4 translocation, respectively. No serious adverse events were reported. The study was performed at Maastricht University, Maastricht, The Netherlands, between August 2019 and April 2021. Clenbuterol treatment improved insulin-stimulated glucose uptake by 13% (46.6±3.5 versus 41.2±2.7 µmol/kg/min, p=0.020) and non-oxidative glucose disposal by 18%, without changes in body mass- and composition. GLUT4 translocation and mTORC2 activation assessed in overnight fasted muscle biopsies were unaffected. These results highlight the potential of β_2-agonist treatment in improving skeletal muscle glucose uptake and underscore the therapeutic value of this pathway for the treatment of type 2 diabetes. However, given the well-known (cardiovascular) side-effects of systemic β_2-agonist treatment, further exploration on the underlying mechanisms is needed to identify viable therapeutic targets.

Key words: Type 2 diabetes mellitus, skeletal muscle glucose uptake, β_2-agonist, GLUT4, mTORC2

Abbreviations

AMPK	Adenosine monophosphate activated protein kinase
BCAA	Branched-chain amino acids
ECG	Electrocardiogram
EGP	Endogenous glucose production
FFA	Free fatty acid
FMD	Flow-mediated dilation
GLUT4	Glucose transporter 4
LPL	Lipoprotein lipase
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2
NOGD	Non-oxidative glucose disposal
OxPhos	Oxidative phosphorylation
pFMDv	Flow-mediated dilation corrected for peak velocity
Ra	Rate of appearance
Rd	Rate of disposal
SMR	Sleeping metabolic rate
TOMM20	Translocase of outer mitochondrial membrane 20
T2DM	Type 2 diabetes mellitus
VDAC	Voltage-dependent anion channel
β_2-AR	β_2-adrenergic receptor

Introduction

Reduced skeletal muscle glucose uptake in response to insulin is central to the development of type 2 diabetes mellitus (T2DM) (1). However, antidiabetic drugs aimed at improving skeletal muscle glucose uptake in T2DM patients are currently non-existent, underscoring the need for novel targets. In this context, β_2-adrenergic receptor (β_2-AR) agonists were shown to have profound effects on skeletal muscle glucose uptake in preclinical models. Indeed, stimulation of the β_2-AR – the most abundant type of β-AR in skeletal muscle (2) – with either the selective β_2-AR agonists zinterol or clenbuterol markedly increased glucose uptake in L6 muscle cells (3-6). In line with these findings, *in vivo* glucose uptake in gastrocnemius muscle was enhanced by ~74% in C56Bl/6N mice upon 6 days of treatment with clenbuterol (6). More prolonged treatment (4 days up to 5 weeks) with either low- or high doses of clenbuterol also robustly improved both glucose and insulin tolerance in various rodent models of insulin resistance and type 2 diabetes (5, 7-11).

Despite the mounting evidence that β_2-AR agonists improve skeletal muscle glucose uptake and whole-body glucose homeostasis, the exact underlying (molecular) mechanisms remain elusive. However, given the well-known (cardiovascular) side-effects of systemic β_2-AR agonists, this information is essential to uncover novel potential targets for the treatment of insulin resistance and T2DM. In this context, previous *in vitro* data by Sato et al. (5) has revealed a role for the mammalian target of rapamycin complex 2 (mTORC2) in β_2-AR agonist-stimulated skeletal muscle glucose uptake, an effect that was mediated via glucose transporter 4 (GLUT4) translocation to the myocyte membrane. In accordance with these findings, it was recently demonstrated in an elegant series of experiments that the antidiabetic effects of clenbuterol appeared largely dependent on mTORC2, as improvements in glucose homeostasis upon clenbuterol treatment were drastically reduced in mice ablated of skeletal muscle-specific Rictor – a key component of mTORC2 (11).

Given these promising preclinical findings, we here investigated in a double-blinded, randomized, placebo-controlled, cross-over study whether clenbuterol treatment (2x 20 µg/day for 14 days) could improve insulin-stimulated skeletal muscle glucose disposal in healthy, lean males, as assessed by the gold standard, two-step hyperinsulinemic-euglycemic clamp technique. To assess putative underlying physiological and molecular mechanisms, we performed in-depth metabolic phenotyping after clenbuterol treatment using state-of-the-art techniques. Thus, we determined body composition, assessed (sleeping) energy metabolism by whole-room calorimetry, femoral artery blood flow

velocity using Doppler ultrasonography whereas muscle biopsies were taken in the overnight fasted state to test if clenbuterol treatment was associated with enhanced skeletal muscle mTORC2 activation and GLUT4 translocation.

Here, we show that clenbuterol treatment significantly increases insulin-stimulated peripheral glucose uptake and non-oxidative glucose disposal in healthy young males by ~13 and ~18%, respectively. However, these effects occurred independent of changes in skeletal muscle GLUT4 translocation or mTORC2 activation. These results highlight the potential therapeutic value of targeting the β_2-adrenergic receptor to improve skeletal muscle glucose uptake, although the underlying mechanisms remain elusive.

Results

Subject characteristics

In total, eleven healthy young male subjects (age: 24.9 ± 3.7 years, BMI: 23.1 ± 1.9 kg/m^2) received a two-week treatment with clenbuterol (20µg twice daily) versus placebo in a randomised, double-blinded, cross-over study design. Baseline subject characteristics are displayed in Table 1. Compliance – measured as the number of capsules returned by subjects divided by the total capsules dispensed – was >95% for both treatment periods. Side-effects were reported in five out of eleven subjects and included tremors in the hands (n = 5), muscle ache, tense muscles or muscle cramps (n = 4), feeling anxious and/or restless (n = 2) and headache (n = 2). All side-effects disappeared upon withdrawal of the drug. No serious adverse events were reported during the study.

Clenbuterol acutely increases energy expenditure and fat metabolism

On day 1 of each treatment arm, we investigated the acute effects of clenbuterol (20 µg) or placebo administration on blood pressure, heart rate, energy metabolism, and plasma substrates over the course of 4 hours following initial capsule intake. Systolic- and diastolic blood pressure remained unaffected during the 4 hours following acute clenbuterol administration (20 µg) as compared to placebo (treatment effect: p = 0.129 and p = 0.606 for systolic and diastolic blood pressure, respectively, Fig. 2A-B). Heart rate showed a decrease over time in the placebo arm – an effect likely caused by the subjects' prolonged supine position – which appeared to be blunted following clenbuterol administration. However, the treatment effect did not show statistical significance (p = 0.215, Fig. 2C). Energy expenditure was significantly increased upon acute clenbuterol administration

as compared to placebo (treatment effect: p = 0.006, Fig. 3A). This increase was primarily attributed to an enhanced fat oxidation (treatment effect: p = 0.068, Fig. 3C), whereas carbohydrate oxidation was similar between conditions (treatment effect: p = 0.505, Fig. 3B). The increased fat oxidation was accompanied by significantly elevated plasma FFA concentrations upon acute clenbuterol administration (treatment effect: p = 0.002, Fig. 3G), whereas plasma glucose and triglyceride concentrations remained unaltered (Fig. 3E and 3H). Plasma insulin levels were significantly different between the placebo and clenbuterol arms; however, this appeared mainly due to an unexpected difference in baseline insulin levels, which were determined prior to clenbuterol/placebo intake (Fig. 3F).

Table 1 – Participant characteristics at screening.

Participant characteristics	**Mean + SD**
Subjects (n)	11
Age (years)	24.9 (3.7)
Height (cm)	176.3 (4.4)
Weight (kg)	72.2 (6.6)
BMI (kg/m^2)	23.1 (1.9)
Blood pressure	
Systolic pressure (mmHg)	120 (9.9)
Diastolic pressure (mmHg)	72 (6.7)
Heart rate (beats/min)	58 (6.4)
Blood parameters	
ALAT (U/L)	25 (11)
ASAT (U/L)	23 (7)
γ-GT (U/L)	19 (6)
Creatinine (μmol/L)	82 (7)
eGRF CKD-EPI (mL/min/1.73m)	112.2 (9.1)
Haemoglobin (mmol/L)	9.6 (0.5)
Potassium (mmol/L)	4.25 (0.22)
TSH (mU/L)	2.2 (1.0)

Data is presented as mean (standard deviation) for n = 11. BMI = body mass index, ALAT = alanine transaminase, ASAT = aspartate aminotransferase, γ-GT = gamma-glutamyl transferase, eGRF = estimated glomerular filtration rate, TSH = thyroid-stimulating hormone.

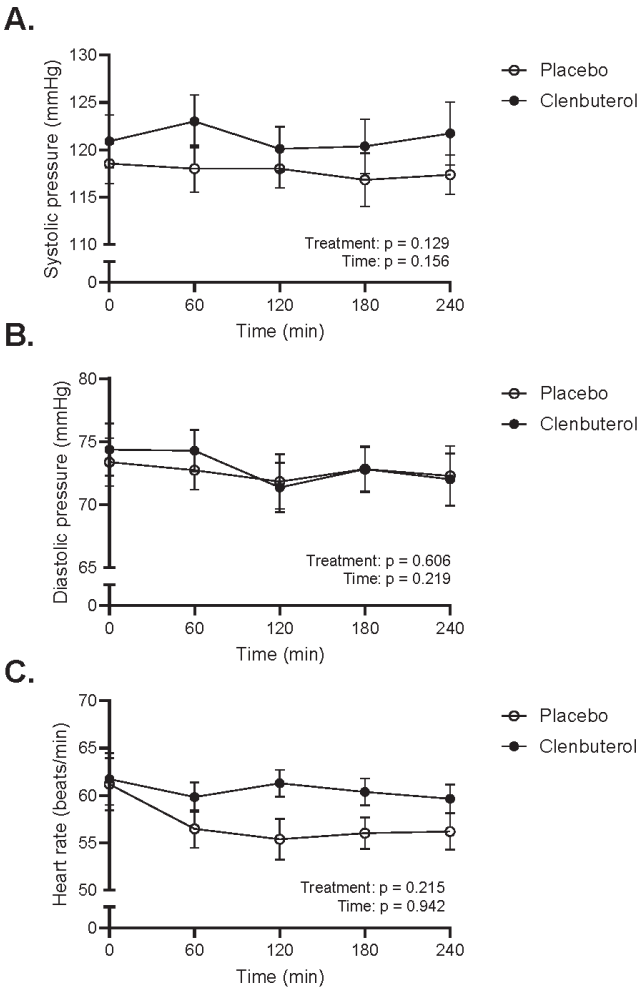


Figure 2. Acute clenbuterol treatment (20 µg) does not affect blood pressure and heart rate. A. Systolic blood pressure (mmHg). B. Diastolic blood pressure (mmHg). C. Heart rate (beats/min). All data were analysed by means of a linear mixed model analysis. Clenbuterol: n = 11. Placebo: n = 11.

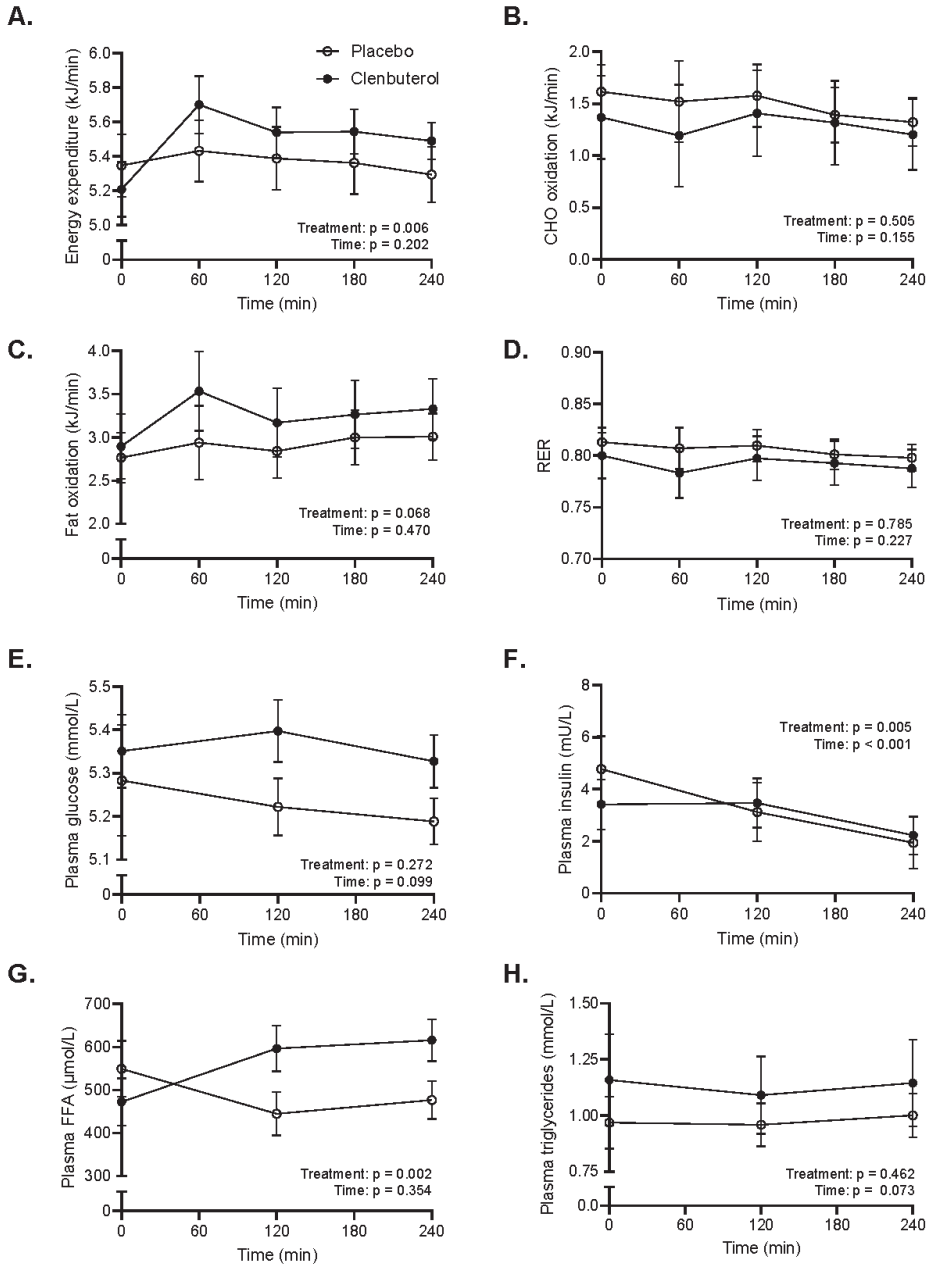


Figure 3. Acute clenbuterol administration (20 µg) enhances energy expenditure and plasma free fatty acid concentrations. A. Energy expenditure (kJ/min). B. Carbohydrate oxidation (kJ/min). C. Fat oxidation (kJ/min). D. Respiratory exchange ratio. E. Plasma glucose concentrations (mmol/L). F. Plasma insulin concentrations (mU/L). G. Plasma free fatty acid concentrations (µmol/L). H. Plasma triglyceride concentrations (mmol/L). N = 10 for indirect calorimetry data and n = 11 for plasma analyses. FFA = free fatty acids. All data were analysed by means of a linear mixed model analysis.

Clenbuterol treatment increases femoral artery blood flow velocity

Next, we investigated longer-term effects (14-days) of clenbuterol treatment and first focused on haemodynamic outcome parameters. Thus, two weeks of clenbuterol administration tended to significantly increase systolic blood pressure by ~3 mmHg ($p = 0.072$, Fig. 4A), whereas it increased heart rate by ~11 beats/min ($p = 0.003$, Fig. 4C). Despite these changes, both systolic blood pressure and heart rate remained within normal clinical values. Next, we determined blood flow responses and endothelial function using flow-mediated vasodilation (FMD) measurements of the femoral artery. Baseline femoral blood flow velocity was 16.7% higher upon clenbuterol treatment as compared to placebo ($p = 0.010$, Fig. 4D), whereas baseline femoral artery diameters were significantly increased by ~6% ($p = 0.005$, Fig. 4E). However, we did not detect any changes in endothelial function upon clenbuterol treatment, i.e. the FMD responses were not affected (Fig. 4F-G).

Body composition is not affected by clenbuterol treatment

As high doses of clenbuterol are well-known for their repartitioning effects on body composition (i.e. increasing lean mass and reducing fat mass) (7-9, 12) and changes in protein metabolism (11, 13), we first investigated the effects of two-weeks clenbuterol treatment on body weight and -composition. Body weight remained unaffected ($p = 0.677$, Fig. 5A) and no effects on body composition were observed, as both fat- and lean mass were similar between the clenbuterol and placebo group (Fig. 5B-E). Since two weeks of clenbuterol treatment may be too short to expect changes in net protein balance and body composition, we also measured the phosphorylation of mTOR S2448 (mTORC1), a marker of protein synthesis, as well as plasma amino acid levels. In line with the non-significant effects on lean mass, mTORC1 activation was unaffected upon clenbuterol treatment (Supplementary Fig. 2A). However, two-weeks of clenbuterol treatment did significantly reduce fasting plasma concentrations of 12 amino acids, including all three branched chain amino acids (Supplementary Fig. 2B).

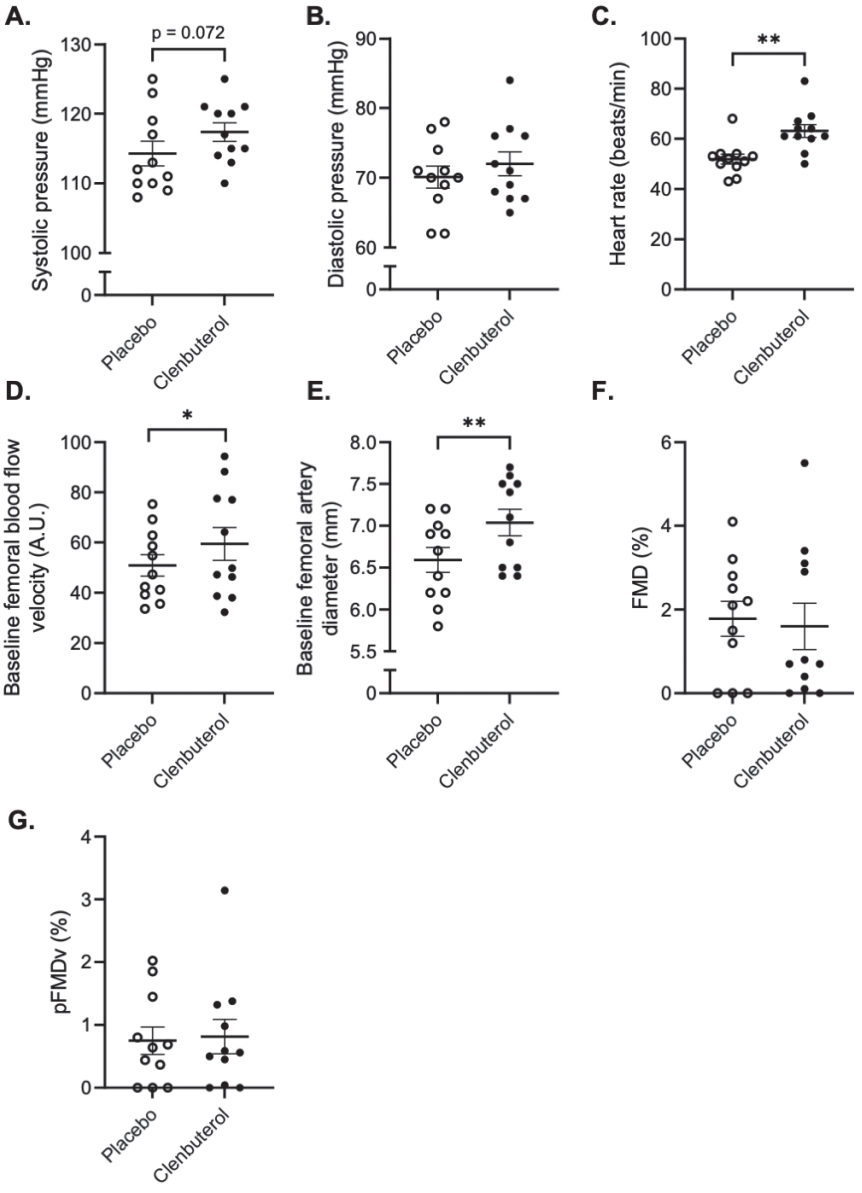


Figure 4. 14-days clenbuterol treatment minimally affects blood pressure and heart rate but increases femoral artery blood flow velocity and diameters. A. Systolic pressure (mmHg). B. Diastolic pressure (mmHg). C. Heart rate (beats/min). D. Baseline femoral artery blood flow velocity (A.U). E. Baseline femoral artery diameters (mm). F. Flow-mediated vasodilation (%). G. Flow-mediated vasodilation corrected for peak velocity flow stimulus (%). Heart rate, baseline femoral artery diameters, flow-mediated vasodilation, and flow-mediated vasodilation corrected for peak velocity flow stimulus were analysed by means of a Wilcoxon signed rank test. All other data were analysed by means of a Paired Student's T-test. Placebo: n = 11, Clenbuterol: n = 11. * $p < 0.05$, ** $p < 0.01$.

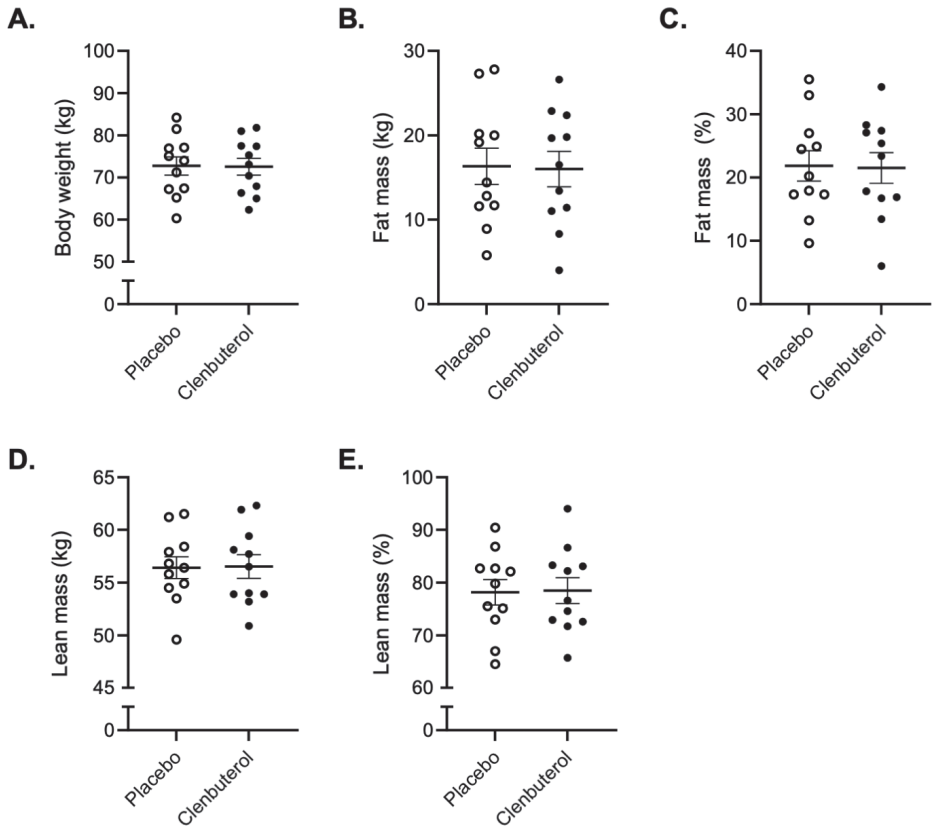


Figure 5. Prolonged clenbuterol administration does not affect body weight or composition. A. Body weight (kg). B. Fat mass (kg). C. Fat mass as percentage of body weight (%). D. Lean mass (kg). E. Lean mass as percentage of body weight (%). Data was analyzed by means of a Student's paired sample T-test. Clenbuterol: n = 11. Placebo: n = 11.

Clenbuterol treatment increases basal- and sleeping metabolic rate

Next, we investigated if 14-days of clenbuterol treatment increased energy expenditure and altered substrate utilization. For this, we did not only measure basal metabolic rate, but also determined sleeping metabolic rate (SMR) using whole-room calorimetry, as it is known that SMR is the most sensitive measure to detect changes in energy needs. We found that sleeping- and basal metabolic rate increased by 10.2% and 5.6% ($p = 0.002$ and $p = 0.006$), respectively (Fig. 6A and Supplementary Fig. 3A). These changes in sleeping and basal energy expenditure were accompanied by modest increases in both fat and carbohydrate oxidation, albeit that these effects did not reach statistical significance (Fig. 6B-D and Supplementary Fig. 3B-C). In addition to these physiological

data reflecting *in vivo* substrate metabolism, we also determined acylcarnitine profiles in plasma as a reflection of fat metabolism. In line with the indirect calorimetry data, fasting acylcarnitine profiles remained largely unaffected upon clenbuterol treatment versus clenbuterol (Supplementary Fig. 4). Plasma glucose, insulin, and free fatty acids concentrations remained unaltered upon prolonged clenbuterol treatment (Fig. 7A, B and D), whereas plasma triglyceride concentrations were significantly lowered by 16.4% as compared to placebo (p = 0.016, Fig. 7C).

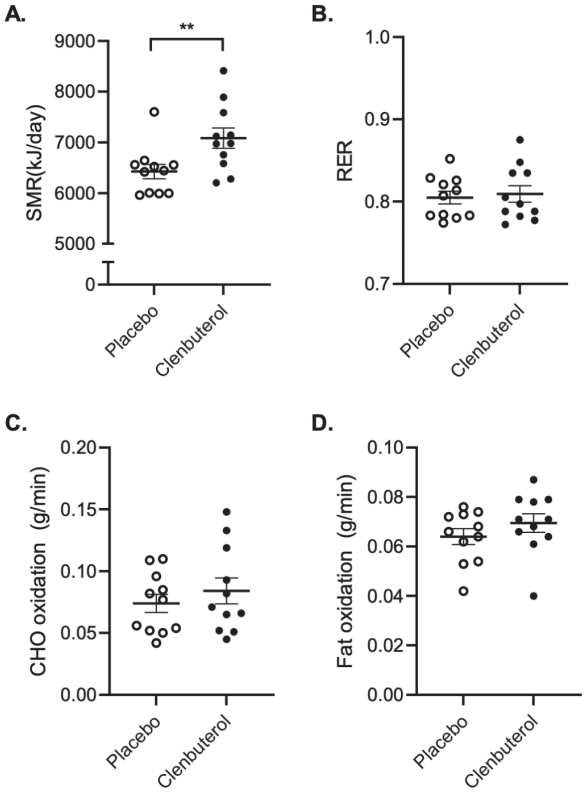


Figure 6. Prolonged clenbuterol treatment enhances sleeping metabolic rate independent of selective increases in substrate oxidation. A. Sleeping metabolic rate (kJ/day). B. Respiratory exchange ratio. C. Carbohydrate oxidation during the night (g/min). D. Fat oxidation during the night (g/min). SMR = sleeping metabolic rate. RER = respiratory exchange ratio. CHO = carbohydrate. SMR data was analysed by means of a Wilcoxon signed rank test. All other data was analysed by means of a Paired Student's T-test. Clenbuterol: n = 11, Placebo: n = 11. ** p < 0.01.

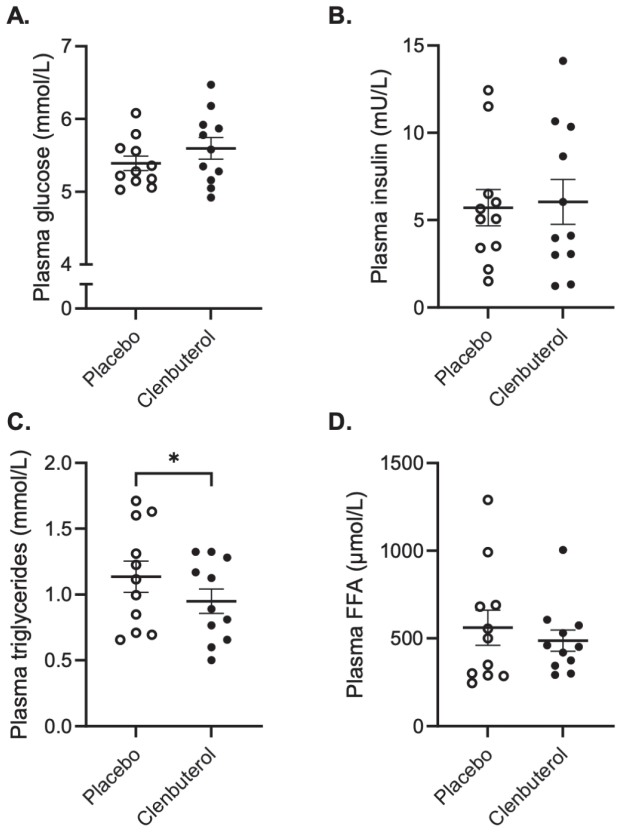


Figure 7. The effect of prolonged clenbuterol treatment on fasting plasma substrate concentrations. A. Plasma glucose concentrations (mmol/L). B. Plasma insulin concentrations (mU/L). C. Plasma triglyceride concentrations (mmol/L). D. Plasma free fatty acid concentrations (µmol/L). FFA = free fatty acid. Plasma FFA data were analysed by means of a Wilcoxon rank signed test. All other data were analysed by means of a Paired Student's T-test. Clenbuterol: n = 11, Placebo: n = 11. * $p < 0.05$.

Insulin-stimulated glucose uptake in peripheral tissues is augmented by clenbuterol

To investigate the effect of 14-days of clenbuterol treatment on insulin sensitivity, a two-step hyperinsulinemic-euglycemic clamp using a stable isotope-labelled glucose was performed. Baseline endogenous glucose production (EGP) and rate of glucose disposal (Rd) were not different between clenbuterol and placebo ($p = 0.891$ and $p = 0.199$, respectively, Fig. 8A and C). Similarly, hepatic insulin sensitivity – expressed as the percentage EGP suppression during the low-insulin phase of the clamp – was unaffected by clenbuterol treatment ($p = 0.505$, Fig. 8B). Additionally, the percentual suppression of plasma FFA during the low- and high-insulin phases of the clamp, which reflects adipocyte

insulin sensitivity, remained unaffected upon clenbuterol treatment (Supplementary Fig. 5). However, the change in insulin-stimulated glucose disposal in the high-insulin phase over baseline (ΔRd) was ~13% higher upon clenbuterol treatment ($p = 0.020$, Fig. 8D), indicating an augmented glucose uptake into the peripheral tissues, primarily skeletal muscle. This increased glucose disposal was primarily accounted for by a ~18% higher non-oxidative glucose disposal (ΔNOGD) ($p = 0.035$, Fig. 8E-F), whereas carbohydrate oxidation during the high-insulin phase of the clamp remained unaffected ($p = 0.352$, Supplementary Fig. 3B).

No GLUT4 translocation or mTORC2 activation upon clenbuterol treatment

To gain further insights into the putative underlying molecular mechanisms through which clenbuterol could stimulate peripheral glucose uptake and energy metabolism, skeletal muscle biopsies were taken in the overnight fasted state after 14 days of clenbuterol treatment and were examined for GLUT4 translocation, mTORC2 activation, and markers of mitochondrial capacity. Total GLUT4 content as well as the fraction of total GLUT4 detected at the cellular membrane, reflecting GLUT4 translocation, remained unaffected by clenbuterol treatment ($p = 0.963$ and $p = 0.865$, respectively, Fig. 9A-D). In addition, we were unable to detect differences in the phosphorylation of mTOR S2481, a marker for mTORC2 activation, between clenbuterol and placebo ($p = 0.416$, Fig. 9E). Similarly, no changes were observed in the protein content of structural components of the different OxPhos complexes, or in the outer membrane proteins VDAC and TOMM20, all markers of mitochondrial capacity (Supplementary Fig. 6).

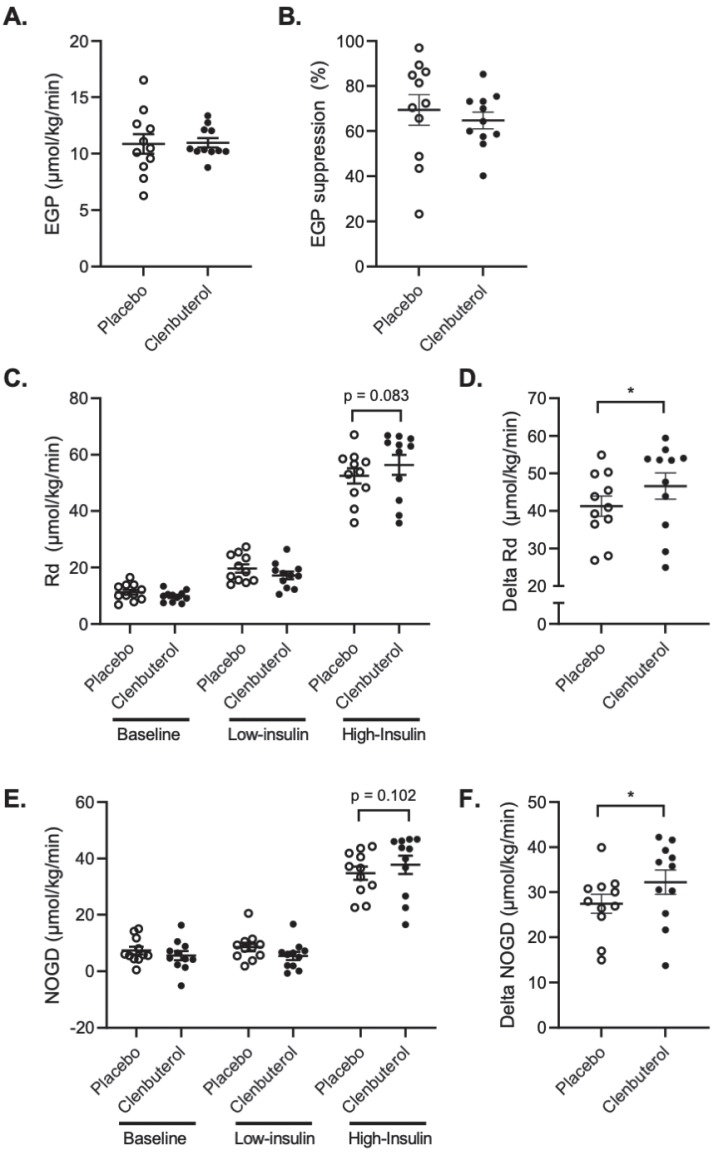


Figure 8. Clenbuterol treatment enhances insulin-stimulated glucose uptake and non-oxidative glucose disposal. A. Baseline endogenous glucose production (µmol/kg/min). B. Endogenous glucose production suppression during the low-insulin phase of the hyperinsulinemic-euglycemic clamp (%). C. Rate of glucose disposal (Rd) during the hyperinsulinemic-euglycemic clamp at baseline, low- and high-insulin phases (µmol/kg/min). D. Rate of glucose disposal during the high-insulin phase corrected for baseline (µmol/kg/min). E. Non-oxidative glucose disposal (NOGD) during the hyperinsulinemic-euglycemic clamp at baseline, low- and high-insulin phases (µmol/kg/min). F. Non-oxidative glucose disposal during the high-insulin phase corrected for baseline (µmol/kg/min). Rd and NOGD in the high-insulin phase were analysed by means of a Wilcoxon signed rank test. All other data were analysed with a Paired Student's T-test. Placebo: n = 11, Clenbuterol: n = 11. * $p < 0.05$.

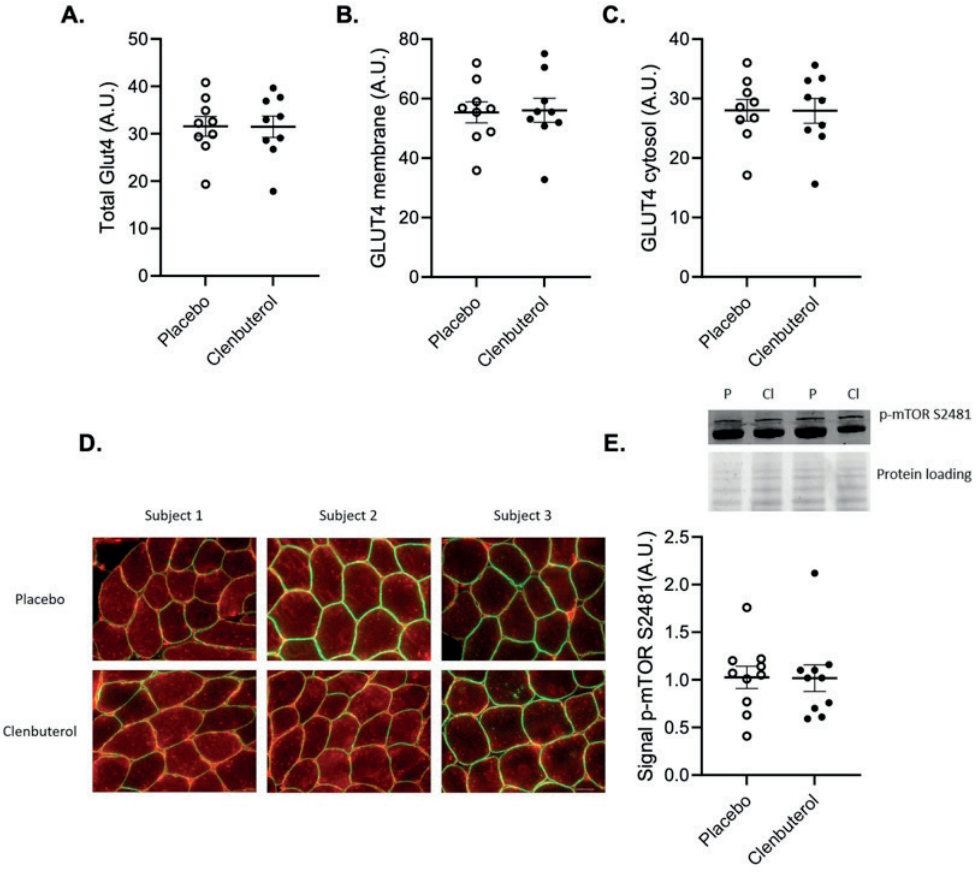


Figure 9. Prolonged clenbuterol treatment does not affect skeletal muscle GLUT4 translocation or mTORC2 activation. A. Total GLUT4 content (A.U.). B. GLUT4 content at the myocellular cell membrane (A.U.). C. GLUT4 content in the myocellular cytosol (A.U.). D. Representative images of GLUT4 immunohistochemistry staining for 3 subjects, scale bar 50 µm. E. Phosphorylation of mTORS2481 as a marker of mTORC2 activation (A.U.). Phosphorylation of mTOR S2481 was analysed by means of a Wilcoxon signed Rank test. All other data are analysed by means of a Student's paired sample T-test. A-D: n = 9 per group, E: n = 10 per group. P = placebo, Cl = clenbuterol

Discussion

Preclinical studies have shown that treatment with a selective β_2-AR agonist can robustly improve skeletal muscle glucose uptake and whole-body glucose homeostasis in rodents, independent of changes in insulin concentrations (5-11). However, the therapeutic potential of these data is largely unknown, as information on the effects of longer-term, systemic β_2-AR agonist treatment on skeletal muscle glucose uptake and whole-body metabolism in humans is limited. Therefore, we here investigated the effect of prolonged clenbuterol treatment (40 µg/day for 14 days) on insulin-stimulated skeletal

muscle glucose uptake in healthy young males, as assessed by means of a two-step hyperinsulinemic-euglycemic clamp. To gain further insights into the putative underlying mechanisms involved in β_2-AR agonist-mediated skeletal muscle glucose uptake, we used state-of-the-art techniques for detailed metabolic phenotyping of the study participants. We demonstrate that 14-days of clenbuterol treatment enhances peripheral insulin-stimulated rate of glucose disposal by ~13%, an effect primarily accounted for by an increased non-oxidative glucose disposal of ~18%. In addition, basal- and sleeping metabolic rate were significantly elevated upon clenbuterol treatment, whereas body weight- and composition remained unaltered. Lastly, clenbuterol treatment induced a marked increase in both femoral artery blood flow velocity and diameters whereas GLUT4 translocation and mTORC2 activation remained unaffected in overnight fasted muscle biopsies.

In the current study, an acute dose of clenbuterol (20 µg), as well as prolonged clenbuterol treatment, led to a significant increase in resting energy expenditure in comparison with placebo. Although the exact mechanisms through which selective β_2-AR agonists exert their thermogenic effects remain elusive, it is likely that these effects are mediated via various energy-consuming processes in potentially skeletal muscle (i.e. through stimulation of the futile Ca^{2+} cycle or Na^+/K^+-ATPase pumps (14)) or brown adipose tissue (15). In line with previous studies (16-19), the increase in energy expenditure upon acute clenbuterol administration was primarily fuelled by an enhanced fat oxidation and was accompanied by a higher availability of plasma FFAs, likely mediated via β_2-AR-stimulated adipose tissue lipolysis. In contrast, the increased energy expenditure upon two weeks of clenbuterol treatment occurred independently of selective changes in fat oxidation or plasma FFA concentrations. In addition to these findings, plasma acylcarnitine concentrations were largely similar between clenbuterol and placebo treatments, thereby indicating that fat oxidation was not significantly altered.

Acute administration of β_2-AR agonists is also described as 'pro-diabetogenic' due to initial elevations in plasma glucose and insulin concentrations (6, 20, 21). However, these detrimental effects of acute β_2-AR agonists administration appear to diminish over time, as longer-term β_2-AR agonists treatment is not associated with increased plasma glucose and insulin concentrations (22-24). In the current study, neither acute (20 µg) nor more prolonged clenbuterol administration significantly affected plasma glucose and insulin levels in healthy young males, likely due to the relatively low dose of clenbuterol applied. Two weeks of clenbuterol treatment did markedly affect lipid metabolism, as fasting plasma triglyceride content was significantly reduced by 16.4%. These effects are

presumably caused by an enhanced lipoprotein lipase (LPL) activity within skeletal muscle and/or brown adipose tissue, as previous rodent studies have demonstrated significant increases in LPL activity in these tissues upon clenbuterol or β-AR agonist treatment (25-27).

Previously, several *in vitro* and pre-clinical studies have highlighted the ability of β_2-AR agonists to enhance skeletal muscle glucose uptake and to improve glucose tolerance (3-11). In line with these findings, two previous studies in humans (28, 29) reported a ~2.0- and ~1.84-fold increase in leg glucose uptake at rest upon administration with the selective β_2-AR agonists salbutamol (24 mg orally) or terbutaline sulphate (0.2-0.4 mg infusion), respectively. However, these studies involved an acute β_2-AR agonist stimulation and, importantly, the observed effects were paralleled by significant increases in plasma insulin concentrations (28), thereby inherently affecting skeletal muscle glucose uptake. Information on the effects of more prolonged, systemic β_2-AR agonist stimulation on skeletal muscle glucose uptake and glucose homeostasis in humans is currently scarce. Next to the present study, merely one other study previously reported on prolonged (1-2 weeks), systemic treatment with a β_2-AR agonist (terbutaline sulphate; 5 mg, 3x/day orally) and showed an increased glucose disposal during insulin infusion as well as insulin-stimulated non-oxidative glucose disposal in healthy males by ~29 and ~45%, respectively, independent of changes in insulin concentrations (22). The beneficial effects of prolonged clenbuterol treatment on glucose homeostasis observed in the present study are in line with the latter study and also occurred without differences in plasma insulin levels. Although direct comparison between these two studies is difficult due to the different β_2-AR agonist used, it is important to highlight that the beneficial effects of β_2-AR agonist treatment reported here were already achieved at lower therapeutic doses (40 μg/day orally, recommended dose clenbuterol: 40-80 μg/day), whereas Scheidegger et al. (22) used a relatively high dose of terbutaline sulphate (15 mg/day orally, recommended dose: 7.5-15 mg/day). Nevertheless, combined these studies clearly indicate the potential of β_2-AR agonists in stimulating human skeletal muscle glucose uptake.

Given that clenbuterol is infamous for its repartitioning effects upon high-doses (i.e. increasing lean mass whilst reducing fat mass) (7-9, 12), the increased insulin-stimulated peripheral glucose uptake could potentially be attributed to a higher skeletal muscle mass, as previously suggested (7, 8). Nevertheless, in this study we were unable to attribute the effects of clenbuterol on glucose uptake to an increased lean mass, as body weight- and composition remained unaffected. In line with these findings, previous studies have reported similar beneficial effects of clenbuterol treatment on glucose homeostasis in

rodents without changes in body composition (6, 10, 11), thereby further supporting the notion that β_2-AR agonist treatment can improve glucose homeostasis independent of alterations in lean mass. To study more subtle changes in protein metabolism, we also determined mTORC1 phosphorylation, a marker of protein synthesis, as well as fasting levels of plasma amino acids. Whereas mTORC1 activation remained unaffected, two-weeks of clenbuterol treatment significantly lowered fasting plasma concentrations of 12 amino acids, including all three branched chain amino acids (BCAAs), which may indicate higher amino acid uptake and turnover. In that context, a previous study in mice indeed reported significant increases in various amino acids in skeletal muscle upon clenbuterol treatment, albeit following a glucose bolus (11). Combined, these results suggest that clenbuterol treatment alters skeletal muscle protein metabolism, although it remains to be established whether these amino acids are used for oxidation or protein synthesis. Nevertheless, elevated plasma concentrations of BCAAs in overweight/obese and T2DM subjects correlate with insulin resistance (30-32) and pharmaceutical stimulation of BCAA oxidation via sodium phenylbutyrate treatment significantly increases insulin sensitivity in T2DM patients (33). Based on these data, our observed improvements in insulin-stimulated glucose disposal may – at least in part – be attributable to changes in BCAA metabolism.

An alternative mechanism by which clenbuterol could affect skeletal muscle glucose uptake is through an increased tissue perfusion. More specifically, selective β_2-AR agonists are well-known for their ability to stimulate (peripheral) tissue blood flow (28, 34, 35), an important rate-limiting step in skeletal muscle glucose uptake (36). Although femoral artery blood flow – here assessed by means of Doppler ultrasonography – likely does not accurately reflect peripheral tissue capillary perfusion, it is tempting to hypothesize that our reported ~17% increase femoral artery blood flow is associated with an enhanced capillary perfusion and thereby potentially glucose uptake. Although a previous study has been unable to establish an apparent link between acute β_2-AR agonists administration, increases in blood flow and skeletal muscle glucose uptake (28), it remains possible that improvements in glucose uptake upon prolonged β_2-AR agonist treatment are – at least in part – mediated via an enhanced tissue perfusion.

Although the exact underlying molecular mechanisms remain elusive, the improvements in glucose homeostasis upon selective β_2-AR agonists in preclinical models appear to be largely dependent on mTORC2 signalling within the skeletal muscle (5, 11). Thus, a previous study uncovered a novel pathway stimulating GLUT4-mediated skeletal muscle glucose uptake via activation of the β_2-AR independent of the insulin and AMPK pathways,

namely via mTORC2 (5). In line with these findings, a recent study demonstrated that clenbuterol-mediated improvements in glucose homeostasis are markedly reduced in mice ablated of skeletal muscle-specific Rictor, a key subunit of mTORC2 (11). Unbiased transcriptomics and metabolomics performed in the latter study further highlighted the central role of mTORC2 in clenbuterol-induced improvements in glucose homeostasis (11). To investigate if mTORC2 signalling and GLUT4 translocation were involved in the increase in β_2-AR agonist-mediated glucose uptake that we observed, we here measured skeletal muscle GLUT4 translocation as well as the phosphorylation of mTOR S2481, a marker for the activation of mTORC2, in muscle biopsies collected in the overnight fasted state. However, we were unable to detect differences in both GLUT4 translocation as well as mTORC2 activation, upon prolonged clenbuterol treatment.

Several limitations can be identified in our study. Firstly, only male subjects were included in our study. It is therefore important that future studies within this field are performed with both men and women to investigate if similar effects occur in both sexes. Secondly, we did not measure urinary nitrogen to accurately assess whole-body protein oxidation, but for the calculation of substrate metabolism instead assumed protein oxidation to be a fixed percentage of energy expenditure. However, this assumption might not hold true in situations of an enhanced energy expenditure (i.e. clenbuterol treatment), during which variable protein oxidation can occur. Important differences in substrate oxidation could therefore be missed. Thirdly, we recognize that the sample size in this study was limited, which could potentially explain the lack of findings with respect to GLUT4 translocation or mTORC2 activation. Lastly, our muscle biopsies were taken in the overnight fasted, unstimulated state, whereas glucose uptake was elevated upon insulin stimulation. In other words, analysis of insulin-stimulated skeletal muscle material upon β_2-AR agonist treatment may be imperative to address the mechanisms underlying the observed improvement in peripheral insulin sensitivity.

Conclusion

In conclusion, we here demonstrate that a two-week treatment with the selective β_2-AR agonist clenbuterol enhances insulin sensitivity, mainly via increased insulin-stimulated non-oxidative glucose disposal, in healthy young males. These beneficial effects were accompanied by increases in sleeping metabolic rate, improvements in plasma triglyceride concentrations, reductions in plasma amino acid concentrations, and increases in arterial blood flow velocity. However, skeletal muscle mTORC2 activation and GLUT4 translocation, assessed in biopsies taken in the overnight fasted state, remained

unaffected upon two-week clenbuterol treatment. Given these effects in young, healthy volunteers, it is tempting to postulate that prolonged treatment with a selective β_2-AR agonist also has the potential to have to marked beneficial effects on insulin sensitivity in volunteers with disturbed glucose homeostasis, such as in T2DM patients. However, given the well-known (cardiovascular) side-effects associated with prolonged systemic β_2-AR agonist treatment, the development of novel, highly selective β_2-AR agonists is crucial for further translation into the clinic. Otherwise, further exploration on the underlying (molecular) mechanisms involved could lead to the identification of viable therapeutic targets and the development of a novel class of therapeutic compounds for the treatment of T2DM.

Methods

Ethical approval

Data was collected at Maastricht University, Maastricht, The Netherlands between the 13th of August 2019 and the 23rd of April 2021. The study was temporarily halted from the 16th of March until the 1st of July 2020 due to the national COVID-19 lockdown in The Netherlands. Based on a paired samples T-test power calculation, an expected standard deviation of 9.7 µmol/kg/min (37), and an expected mean difference of 25%, we calculated that 11 subjects would be required to complete the study in order to reject the null hypothesis with a power of 80% and a type 1 error probability of 0.05. In total, 12 subjects were enrolled in the study, with 1 subject dropping out due to the COVID-19 pandemic (see **Supplementary Fig. 1** for an inclusion flow chart). The study was performed in accordance with the declaration of Helsinki and was reviewed and approved by the Ethics Committee of the Maastricht University Medical Centre+ (NL67646.068.18). The study was registered at ClinicalTrials.gov under the identifier NCT03800290. All subjects provided their written informed consent prior to screening.

Participants

Healthy, young, male subjects were included in the study. Subjects were of Caucasian origin, aged between 18-30 years with a BMI between 20-25 kg/m^2 and did not participate in organized or structured physical exercise. Subjects were recruited in Maastricht and its direct surroundings by means of flyers and advertisements on the internet. All subjects were screened for eligibility and were excluded if one of the following conditions were met: cardiovascular diseases (as determined by ECG, blood pressure measurements, and medical questionnaires); respiratory diseases; unstable body weight (weight gain

or loss >5 kg in the last three months); intention to lose or gain body weight (through caloric restriction or exercise); excessive alcohol and/or drug abuse; hypokalaemia; hyperthyroidism; anaemia; epilepsy; smoking; renal and/or liver insufficiency; medication use known to hamper the subject's safety during the study procedures.

Experimental design

In a randomised, placebo-controlled, double-blinded, cross-over design subjects received either the selective β_2-AR agonist clenbuterol hydrochloride (2x 20 µg/day) (Spiropent, Hikma Pharmaceuticals, Portugal) or a placebo for two weeks with a four week wash-out period (Fig. 1). Due to the national COVID-19 lockdown in the Netherlands, two subjects had an extended wash-out of 25 weeks each. Subjects were randomly allocated to a study arm by means of controlled randomisation and the allocation sequence was generated in blocks of four by an independent researcher. Due to several drop-outs between inclusion of subjects and the start of the intervention period, the final allocation ratio was 7 (period 1: clenbuterol, period 2: placebo) to 4 (period 1: placebo, period 2: clenbuterol) (see Supplementary Fig. 1). Clenbuterol hydrochloride and placebo capsules were prepared by Radboud University Pharmacy, Nijmegen, The Netherlands. For this, clenbuterol hydrochloride tablets (20 µg) were encapsulated and filled with the inactive compound lactose. Clenbuterol hydrochloride capsules contained 20 µg clenbuterol hydrochloride/capsule. Placebo capsules only contained the inactive compound lactose. Containers were sequentially numbered to conceal the allocation sequence. Subjects were instructed to consume two capsules of clenbuterol (2x 20 µg = 40 µg/day) or placebo daily with breakfast and dinner. All unused capsules were returned by the subject. During the study period, subjects were asked to maintain their normal eating and physical activity habits. Prior to all visits, subjects were asked to refrain from alcohol and physical activity other than their normal daily routine for 72 hours.

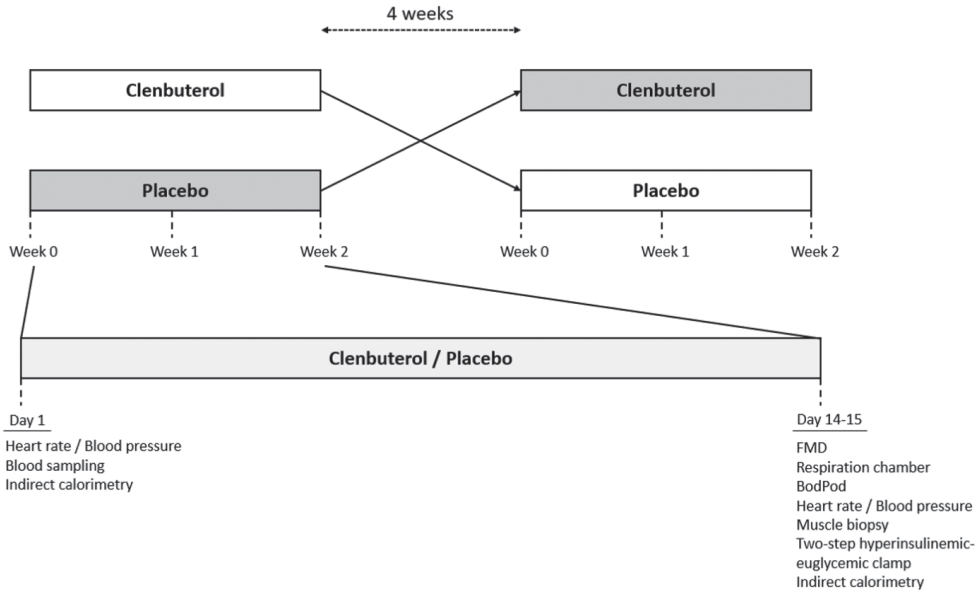


Figure 10. Study design. FMD = flow mediated vasodilation

Acute effects of clenbuterol on heart rate, blood pressure, energy expenditure and plasma substrates

At the start of each study arm (day 1), subjects arrived at the research unit in an overnight fasted state (i.e. no food consumption after 22:00 the night before). Body weight, heart rate and blood pressure were measured followed by the collection of a fasting blood sample. Subsequently, baseline energy expenditure was measured for 30 minutes using indirect calorimetry (T = -30 till T = 0). Afterwards, subjects consumed their first capsule of the study arm (i.e. 20 µg of clenbuterol hydrochloride or placebo) under supervision of the researcher (T = 0). The following 4 hours after initial drug intake, blood samples were collected every two hours (T = 120 and 240), whereas heart rate and blood pressure were measured every hour (T = 60, 120, 180 and 240). In addition, energy expenditure and substrate oxidation were measured for 30 minutes every hour (T = 60-90, 120-150, 180-210 and 240-270).

Half-way safety check-up

After one week of treatment (day 8), a half-way safety check-up was performed. During this visit, subjects were asked about potential emerged side-effects and their overall well-being.

Effects of two-week clenbuterol treatment on glucose homeostasis

Following two weeks of treatment (day 14), subjects arrived at the research unit at 17:00 to first determine endothelial function and blood flow responses using flow-mediated vasodilation of the femoral artery in the leg. Afterwards (~18:00), subjects received a standardized meal during which the last capsule of the respective study period was consumed. Subjects then stayed overnight in a respiration chamber to determine sleeping metabolic rate and substrate oxidation. The following morning, body composition was determined by means of air displacement plethysmography (BodPod). Afterwards, blood pressure and heart rate were measured, and a muscle biopsy was taken in the overnight fasted state. Subsequently, a two-step hyperinsulinemic-euglycemic clamp with indirect calorimetry was performed for the determination of hepatic and peripheral insulin-stimulated glucose disposal.

Primary and secondary research outcomes

The primary research outcome of this study was peripheral insulin sensitivity upon 2 weeks of clenbuterol versus placebo treatment, expressed as the change in insulin-stimulated glucose disposal in the high-insulin phase over baseline (ΔRd) during the hyperinsulinemic-euglycemic clamp. The secondary research outcome was skeletal muscle GLUT4 translocation, as assessed by means of immunohistochemistry and confocal microscopy.

Indirect calorimetry

An automated respiratory gas analyser and ventilated hood system (Omnical, IDEE, Maastricht, The Netherlands) were used to measure whole-body oxygen consumption and carbon dioxide production of subjects in a supine position over a 30-minute period. For the acute effects of clenbuterol, indirect calorimetry data for one subject was excluded due to a technical error. Total energy expenditure and carbohydrate- and fat oxidation was calculated according to Brouwers' equation (38), and protein oxidation was set at 12.4% of basal energy requirements as determined by Weir's equation (39). For the determination of sleeping metabolic rate (SMR) and substrate oxidation, subjects spent the night from day 14 to 15 in a respiration chamber (Omnical, IDEE, Maastricht, The Netherlands). SMR was defined as the lowest energy expenditure for 3-consecutive hours as calculated according to Weir's equation (39). Total energy expenditure and substrate oxidation during the night were calculated as described above.

Heart rate and blood pressure

Prior to the measurement of heart rate and blood pressure, subjects were rested for 10 minutes in a supine position. Heart rate and blood pressure were measured 3 times in a row by means of an automatic inflatable cuff (Omron Healthcare, Hamburg, Germany) on day 1 and 15.

Femoral artery flow-mediated vasodilation

On day 14, femoral artery flow-mediated vasodilation was assessed by Doppler ultrasonography (MyLab™-Gamma, Esaote, Maastricht, The Netherlands) by using a 13-4 MHz linear transducer. Measurements were performed in B-mode with Doppler to assess continuous artery diameter and blood flow velocity profiles. After recording a 3-minute baseline reference period, a pneumatic cuff placed around the subject's right leg was inflated at 200 mmHg for 5 minutes. Following this hypoxic period, the cuff was released and images were recorded for another 5 minutes. The echo images were analysed offline to determine diameter and velocity profiles over the entire measurement period with a custom-written MatLab program (MyFMD 2015, Prof. A.P.G. Hoeks, Department of Biomedical Engineering, Maastricht University, Maastricht, the Netherlands). The FMD response was quantified as the maximal percentage change in post occlusion arterial diameter relative to baseline diameter. The FMD corrected for peak velocity (pFMDv) was calculated by dividing the FMD by the percentage change in post-occlusion peak velocity flow.

Body composition

Body weight- and composition were determined at ~06:00 on day 15 following an overnight fast (from ~18:00 the previous day) using air displacement plethysmography (BodPod®, COSMED, Inc., Rome, Italy).

Plasma substrate analyses

Blood samples were collected in EDTA, NaF and serum tubes. After collection, EDTA and NaF tubes were stored on ice and spun down at 4 ˚C for 10 minutes at 1300 RCF. Serum tubes were stored at room temperature for at least 30 minutes to allow coagulation and were thereafter spun down at 21 ˚C for 10 minutes at 1300 RCF. Blood plasma and serum was collected and stored at -80 ˚C until further analyses. Plasma glucose (Horiba, Montpellier, France), free fatty acids (WAKO, Neuss, Germany) and triglycerides (Sigma,

St Louis, USA) concentrations were measured by means of a colorimetric analysis using a Cobas Pentra C400 analyzer (Horiba, Montpellier, France). Plasma insulin concentrations were measured by means of an ELISA (Crystal Chem, Elk Grove Village, USA).

Plasma metabolomics

Plasma acylcarnitines and amino acids were determined by flow injection MS/MS and LC-MS/MS, respectively, as described previously (40, 41).

Two-step hyperinsulinemic-euglycemic clamp

A two-step hyperinsulinemic-euglycemic clamp with primed continuous co-infusion of D-[6,6-2H2]-glucose (0.04 mg/kg/min) was performed to assess hepatic and whole-body insulin sensitivity (42). Suppression of endogenous glucose production (EGP) during low-insulin infusion (10 mU/m^2/min) was used as a marker for hepatic insulin sensitivity. Peripheral glucose disposal (Rd) was measured during low- and high-insulin infusion (10 mU/m^2/min and 40 mU/m^2/min, respectively). Glucose appearance (Ra) and glucose disposal (Rd) were calculated according to Steele's single pool non-steady state equations (43). Delta Rd (ΔRd) was determined in the low- and high-insulin phases by calculating the Rd $insulin_{low/high}$ minus Rd basal. The volume of distribution of glucose was assumed to be 0.160 L/kg. Energy expenditure and substrate oxidation were measured over a 30-minute period with indirect calorimetry during baseline, low-insulin and high-insulin phases of the clamp. Non-oxidative glucose disposal (NOGD) was defined as Rd minus carbohydrate oxidation. Delta NOGD (ΔNOGD) in the low- and high insulin phases was determined by calculating NOGD $insulin_{low/high}$ minus NOGD basal. The percentual suppression of plasma FFAs during the low- and high-insulin phases of the clamp as compared to fasting plasma FFA values was used as a marker of adipocyte insulin sensitivity.

Skeletal muscle biopsy

On day 15 of each study period, a skeletal muscle biopsy was obtained from the *m.* vastus lateralis under local anaesthesia (1% lidocaine without adrenaline) according to the Bergström technique (44). Part of the muscle biopsy was embedded into Tissue-Tek and frozen in melting isopentane for immunohistochemical analysis. The remainder was immediately frozen in melting isopentane. For one subject, the muscle biopsy could not be obtained due to technical reasons. Muscle tissue from another subject was of inadequate quality for immuno-histochemical analyses. All samples were stored at -80 ˚C until further analyses.

Immunohistochemistry

For GLUT4 imaging, immunofluorescence assays were performed on 5 µm thick sections, cut from the skeletal muscle biopsies (n=9). After fixation with acetone, sections were incubated with a polyclonal rabbit antibody directed to GLUT4 (ab33780; Abcam) and a mouse monoclonal antibody directed to caveolin (610421; BD Biosciences), and the appropriate Alexa Fluor 555– and Alexa Fluor 488–conjugated secondary antibodies. Images were acquired on a Nikon E800 fluorescence microscope (Nikon Europe BV, Amsterdam, the Netherlands) coupled to Nikon DS-Fi1c colour CCD camera (Nikon) using NIS-Elements imaging software (Nikon). Images were captured with identical exposure time and gain settings in paired ("before-and-after") samples. Without any adjustments with respect to colour intensity, brightness, or contrast, 8-bit images were quantified using ImageJ (NIH, Bethesda, USA). Cell membranes were thresholded and selected. Based on the thresholded cell membranes the muscle fibers were selected and were shrunken with 12 pixels to have a clean separation of the cytosol and the cell membrane. Subsequently the mean intensity of GLUT4 fluorescence was measured on the cell membranes and in the cytosol.

Western blot

Analyses of protein expression in skeletal muscle biopsies (n = 10) was performed by means of Western blotting. Muscle tissue (10-15 mg) was homogenised in Bio-Plex Cell Lysis buffer (Bio-Rad Laboratories, Hercules, CA, USA). Stain-free gradient (4-15%) TGX gels (Bio-Rad Laboratories) or Invitrogen Bolt 4-12% Bis-Tris Protein gels (ThermoFisher Scientific, Waltham, Massachusetts, USA) were loaded with equal quantities of protein (5 or 10 µg/well) and after electrophoresis transferred to a nitrocellulose membrane (Trans-Blot Turbo Transfer System, Bio-Rad laboratories, Hercules, CA, USA). Blots were incubated overnight with the primary p-mTORC2 S2481 (1:1000, #2974, Cell Signalling, Danvers, MA, USA), p-mTORC1 S2448 (1:1000, #2971, Cell Signalling, Danvers, MA, USA), VDAC (1:1000, sc-390336, Santa Cruz Biotechnology, Dallas, Texas, USA), TOMM20 (1:10.000, ab18734, Abcam, Cambridge, UK), or an OxPhos antibody cocktail (1:1000, ab110411, Abcam, Cambridge, UK). The following morning, blots were incubated with the appropriate IRDye800-conjugated secondary antibody (LI-COR, Lincoln, Nebraska, USA). Proteins were visualized and quantified by means of a CLx Odyssey Near Infrared Imager (LI-COR, Lincoln, Nebraska, USA).

Statistical analyses

Data were statistically analysed with SPSS IBM version 27 and graphs were created in GraphPad Prism version 9.3.1. Linear mixed model analyses with random intercept were

performed to analyse changes over time as compared to baseline upon clenbuterol versus placebo administration. For this, timepoints were normalised for baseline values and a linear mixed model analyses was performed on the time points T = 60, 120, 180, and 240. Time, treatment and the time*treatment interaction term were used as fixed factors, whereas participant was used as random factor. If the interaction term was not statistically significant, it was omitted from the model and only the main effects (i.e., treatment and time) were reported. An unstructured covariance structure was used for these analyses. Paired data were evaluated for normality by means of a Shapiro-Wilk normality test. In case of normally distributed data, data was statistically analysed by means of a two-sided Paired Student's T-test, whereas non-normally distributed data was analysed with a non-parametric Wilcoxon signed-rank test. Statistical analyses were considered significant if $p < 0.05$. All data are presented as mean ± SEM unless stated otherwise.

Acknowledgement

This study was supported by a grant from ZonMW and the Dutch Diabetes Research Foundation (J.H) and the Nutrim NWO graduate program (S.v.B). The authors would like to thank Jorg Sander and Martin Vervaart for their assistance during this study.

Author contribution

S.v.B., T.B., M.H., B.H., P.S., J.H were involved in designing the study. Experiments were performed by S.v.B., Y.B., F.V. C.F., N.C., G.S., E.K., F.V., J.J., E.S., P.J., and A.G. Data was analysed by S.v.B, F.V., G.S., E.K., J.J., E.S., P.J., A.G., R.H., P.S., and J.H. S.v.B., B.H., P.S., and J.H. drafted the manuscript. The manuscript was reviewed and edited by R.H., B.H., P.S., and J.H. All authors read and approved the final version of the manuscript before submission. J.H. is the guarantor of the study.

Data availability

Source data for all tables and figures can be found in the Source Data file, which is available following publication. The dataset generated during and/or analysed during the current study are not publicly available but are available from the corresponding author upon reasonable request.

Conflict of interest

T.B. owns stock in Atrogi AB. None of the other authors has any conflict of interest to disclose.

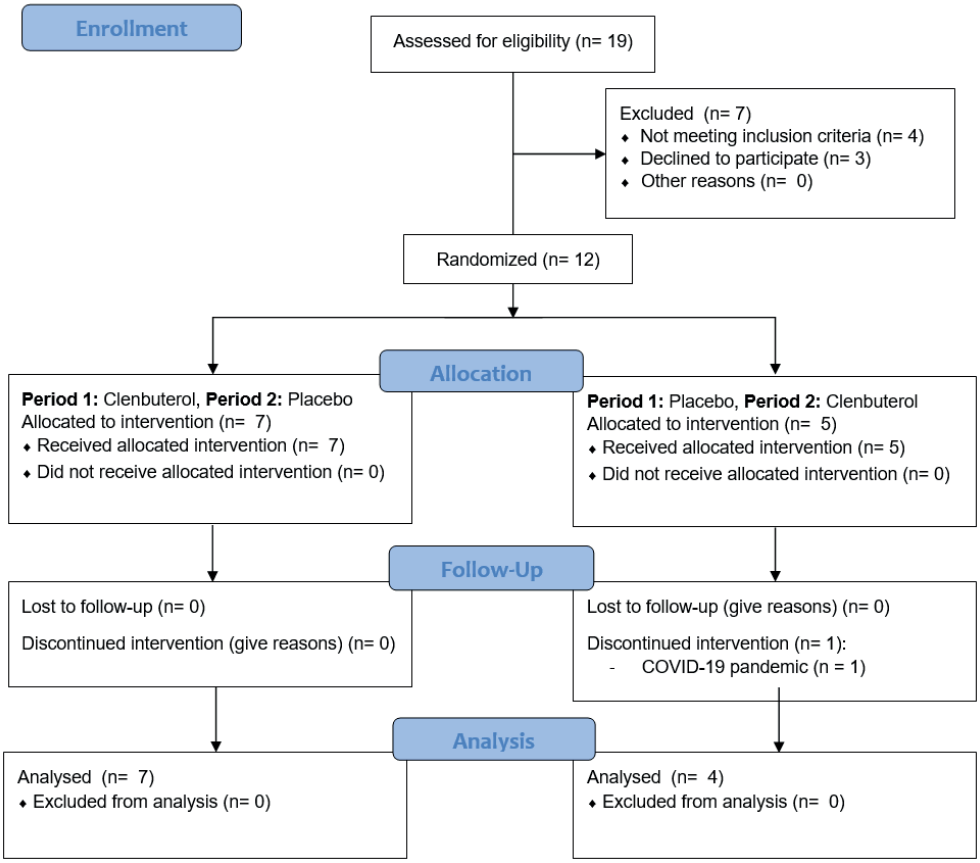
References

1. DeFronzo RA, Gunnarsson R, Bjorkman O, Olsson M, Wahren J. Effects of insulin on peripheral and splanchnic glucose metabolism in noninsulin-dependent (type II) diabetes mellitus. J Clin Invest. 1985;76(1):149-55.
2. Liggett SB, Shah SD, Cryer PE. Characterization of beta-adrenergic receptors of human skeletal muscle obtained by needle biopsy. Am J Physiol. 1988;254(6 Pt 1):E795-8.
3. Nevzorova J, Bengtsson T, Evans BA, Summers RJ. Characterization of the beta-adrenoceptor subtype involved in mediation of glucose transport in L6 cells. Br J Pharmacol. 2002;137(1):9-18.
4. Nevzorova J, Evans BA, Bengtsson T, Summers RJ. Multiple signalling pathways involved in beta2-adrenoceptor-mediated glucose uptake in rat skeletal muscle cells. Br J Pharmacol. 2006;147(4):446-54.
5. Sato M, Dehvari N, Oberg AI, Dallner OS, Sandstrom AL, Olsen JM, et al. Improving type 2 diabetes through a distinct adrenergic signaling pathway involving mTORC2 that mediates glucose uptake in skeletal muscle. Diabetes. 2014;63(12):4115-29.
6. Kalinovich A, Dehvari N, Åslund A, van Beek S, Halleskog C, Olsen J, et al. Treatment with a β-2-adrenoceptor agonist stimulates glucose uptake in skeletal muscle and improves glucose homeostasis, insulin resistance and hepatic steatosis in mice with diet-induced obesity. Diabetologia. 2020.
7. Castle A, Yaspelkis BB, 3rd, Kuo CH, Ivy JL. Attenuation of insulin resistance by chronic beta2-adrenergic agonist treatment possible muscle specific contributions. Life Sci. 2001;69(5):599-611.
8. Pan SJ, Hancock J, Ding Z, Fogt D, Lee M, Ivy JL. Effects of clenbuterol on insulin resistance in conscious obese Zucker rats. Am J Physiol Endocrinol Metab. 2001;280(4):E554-61.
9. Torgan CE, Brozinick JT, Jr., Banks EA, Cortez MY, Wilcox RE, Ivy JL. Exercise training and clenbuterol reduce insulin resistance of obese Zucker rats. Am J Physiol. 1993;264(3 Pt 1):E373-9.
10. van Beek S, Kalinovich A, Schaart G, Bengtsson T, Hoeks J. Prolonged beta2-adrenergic agonist treatment improves glucose homeostasis in diet-induced obese UCP1(-/-) mice. Am J Physiol Endocrinol Metab. 2021.
11. Meister J, Bone DBJ, Knudsen JR, Barella LF, Velenosi TJ, Akhmedov D, et al. Clenbuterol exerts antidiabetic activity through metabolic reprogramming of skeletal muscle cells. Nat Commun. 2022;13(1):22.
12. Kamalakkannan G, Petrilli CM, George I, LaManca J, McLaughlin BT, Shane E, et al. Clenbuterol increases lean muscle mass but not endurance in patients with chronic heart failure. J Heart Lung Transplant. 2008;27(4):457-61.
13. Goncalves DA, Silveira WA, Lira EC, Graca FA, Paula-Gomes S, Zanon NM, et al. Clenbuterol suppresses proteasomal and lysosomal proteolysis and atrophy-related genes in denervated rat soleus muscles independently of Akt. Am J Physiol Endocrinol Metab. 2012;302(1):E123-33.
14. Hostrup M, Jacobson GA, Jessen S, Lemminger AK. Anabolic and lipolytic actions of beta2 -agonists in humans and antidoping challenges. Drug Test Anal. 2020;12(5):597-609.
15. Blondin DP, Nielsen S, Kuipers EN, Severinsen MC, Jensen VH, Miard S, et al. Human Brown Adipocyte Thermogenesis Is Driven by beta2-AR Stimulation. Cell Metab. 2020;32(2):287-300 e7.
16. Hoeks J, van Baak MA, Hesselink MK, Hul GB, Vidal H, Saris WH, et al. Effect of beta1- and beta2-adrenergic stimulation on energy expenditure, substrate oxidation, and UCP3 expression in humans. Am J Physiol Endocrinol Metab. 2003;285(4):E775-82.

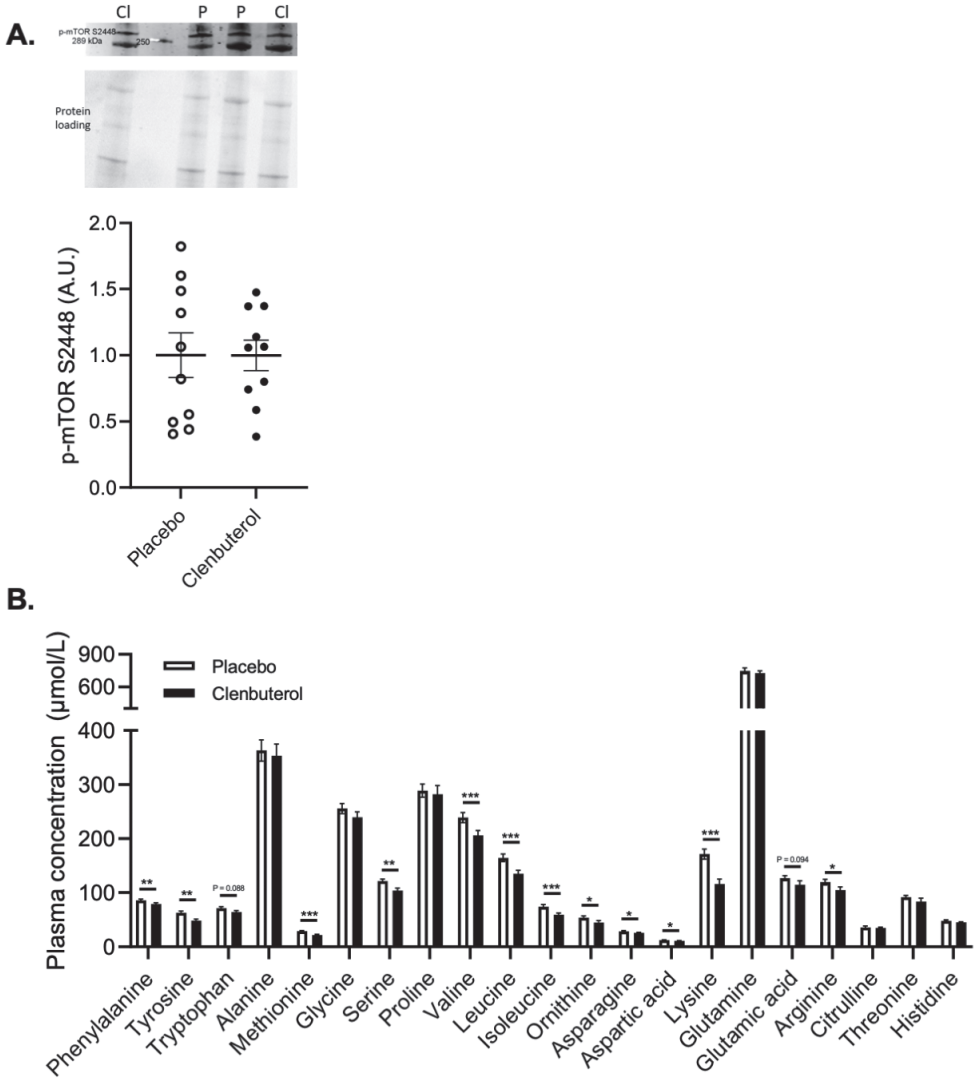
17. Onslev J, Jacobson G, Narkowicz C, Backer V, Kalsen A, Kreiberg M, et al. Beta2-adrenergic stimulation increases energy expenditure at rest, but not during submaximal exercise in active overweight men. Eur J Appl Physiol. 2017;117(9):1907-15.
18. Jessen S, Solheim SA, Jacobson GA, Eibye K, Bangsbo J, Nordsborg NB, et al. Beta2 -adrenergic agonist clenbuterol increases energy expenditure and fat oxidation, and induces mTOR phosphorylation in skeletal muscle of young healthy men. Drug Test Anal. 2020;12(5):610-8.
19. Haffner CA, Kendall MJ, Maxwell S, Hughes B. The lipolytic effect of beta 1- and beta 2-adrenoceptor activation in healthy human volunteers. Br J Clin Pharmacol. 1993;35(1):35-9.
20. Bremner P, Woodman K, Burgess C, Crane J, Purdie G, Pearce N, et al. A comparison of the cardiovascular and metabolic effects of formoterol, salbutamol and fenoterol. Eur Respir J. 1993;6(2):204-10.
21. Guhan AR, Cooper S, Oborne J, Lewis S, Bennett J, Tattersfield AE. Systemic effects of formoterol and salmeterol: a dose-response comparison in healthy subjects. Thorax. 2000;55(8):650-6.
22. Scheidegger K, Robbins DC, Danforth E, Jr. Effects of chronic beta receptor stimulation on glucose metabolism. Diabetes. 1984;33(12):1144-9.
23. Anstead MI, Hunt TA, McConnell JW, Jr., Burki NK. Effects of therapeutic doses of albuterol on beta2-adrenergic receptor density and metabolic changes. J Asthma. 2001;38(1):59-64.
24. Formgren H. Experiences of longterm treatment of asthma with terbutaline. A study on frequency of side effects and therapeutic effect. Acta Allergol. 1971;26(2):81-9.
25. Belahsen R, Deshaies Y. Modulation of lipoprotein lipase activity in the rat by the beta 2-adrenergic agonist clenbuterol. Can J Physiol Pharmacol. 1992;70(12):1555-62.
26. Deshaies Y, Geloen A, Paulin A, Marette A, Bukowiecki LJ. Tissue-specific alterations in lipoprotein lipase activity in the rat after chronic infusion of isoproterenol. Horm Metab Res. 1993;25(1):13-6.
27. Eadara JK, Dalrymple RH, DeLay RL, Ricks CA, Romsos DR. Effects of cimaterol, a beta-adrenergic agonist, on lipid metabolism in rats. Metabolism. 1989;38(6):522-9.
28. Onslev J, Jensen J, Bangsbo J, Wojtaszewski J, Hostrup M. beta2-Agonist Induces Net Leg Glucose Uptake and Free Fatty Acid Release at Rest but Not During Exercise in Young Men. J Clin Endocrinol Metab. 2019;104(3):647-57.
29. Onslev J, Thomassen M, Wojtaszewski J, Bangsbo J, Hostrup M. Salbutamol increases leg glucose uptake and metabolic rate but not muscle glycogen resynthesis in recovery from exercise. J Clin Endocrinol Metab. 2021.
30. Newgard CB, An J, Bain JR, Muehlbauer MJ, Stevens RD, Lien LF, et al. A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. Cell Metab. 2009;9(4):311-26.
31. Wurtz P, Soininen P, Kangas AJ, Ronnemaa T, Lehtimaki T, Kahonen M, et al. Branched-chain and aromatic amino acids are predictors of insulin resistance in young adults. Diabetes Care. 2013;36(3):648-55.
32. Vanweert F, de Ligt M, Hoeks J, Hesselink MKC, Schrauwen P, Phielix E. Elevated Plasma Branched-Chain Amino Acid Levels Correlate With Type 2 Diabetes-Related Metabolic Disturbances. J Clin Endocrinol Metab. 2021;106(4):e1827-e36.
33. Vanweert F, Neinast M, Tapia EE, van de Weijer T, Hoeks J, Schrauwen-Hinderling VB, et al. A randomized placebo-controlled clinical trial for pharmacological activation of BCAA catabolism in patients with type 2 diabetes. Nat Commun. 2022;13(1):3508.
34. Rolett EL, Strange S, Sjogaard G, Kiens B, Saltin B. Beta 2-adrenergic stimulation does not prevent potassium loss from exercising quadriceps muscle. Am J Physiol. 1990;258(5 Pt 2):R1192-200.

35. Hagstrom-Toft E, Enoksson S, Moberg E, Bolinder J, Arner P. beta-Adrenergic regulation of lipolysis and blood flow in human skeletal muscle in vivo. Am J Physiol. 1998;275(6):E909-16.
36. Baron AD, Steinberg H, Brechtel G, Johnson A. Skeletal muscle blood flow independently modulates insulin-mediated glucose uptake. Am J Physiol. 1994;266(2 Pt 1):E248-53.
37. Hoeks J, van Herpen NA, Mensink M, Moonen-Kornips E, van Beurden D, Hesselink MK, et al. Prolonged fasting identifies skeletal muscle mitochondrial dysfunction as consequence rather than cause of human insulin resistance. Diabetes. 2010;59(9):2117-25.
38. Brouwer E. On simple formulae for calculating the heat expenditure and the quantities of carbohydrate and fat oxidized in metabolism of men and animals, from gaseous exchange (Oxygen intake and carbonic acid output) and urine-N. Acta Physiol Pharmacol Neerl. 1957;6:795-802.
39. Weir JB. New methods for calculating metabolic rate with special reference to protein metabolism. J Physiol. 1949;109(1-2):1-9.
40. Vreken P, van Lint AE, Bootsma AH, Overmars H, Wanders RJ, van Gennip AH. Quantitative plasma acylcarnitine analysis using electrospray tandem mass spectrometry for the diagnosis of organic acidaemias and fatty acid oxidation defects. J Inherit Metab Dis. 1999;22(3):302-6.
41. Casetta B, Tagliacozzi D, Shushan B, Federici G. Development of a method for rapid quantitation of amino acids by liquid chromatography-tandem mass spectrometry (LC-MSMS) in plasma. Clin Chem Lab Med. 2000;38(5):391-401.
42. DeFronzo RA, Tobin JD, Andres R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. Am J Physiol. 1979;237(3):E214-23.
43. Steele R. Influences of glucose loading and of injected insulin on hepatic glucose output. Ann N Y Acad Sci. 1959;82:420-30.
44. Bergstrom J, Hermansen L, Hultman E, Saltin B. Diet, muscle glycogen and physical performance. Acta Physiol Scand. 1967;71(2):140-50.

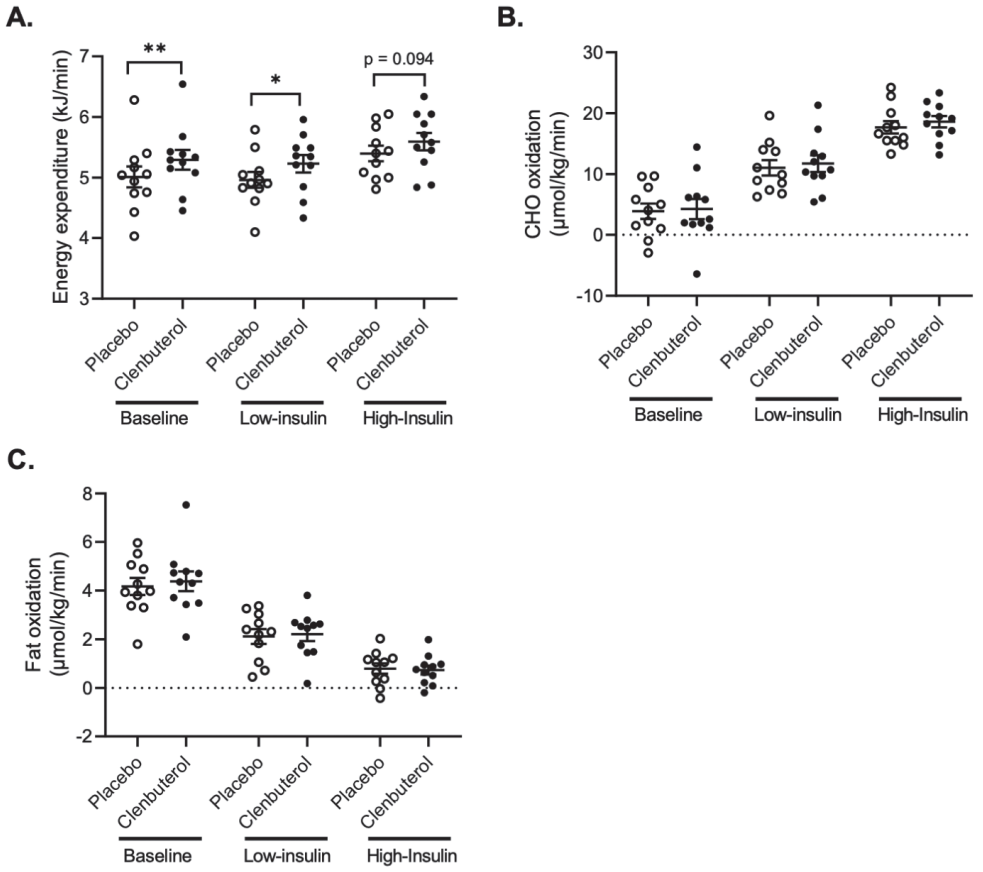
Supplementary figures



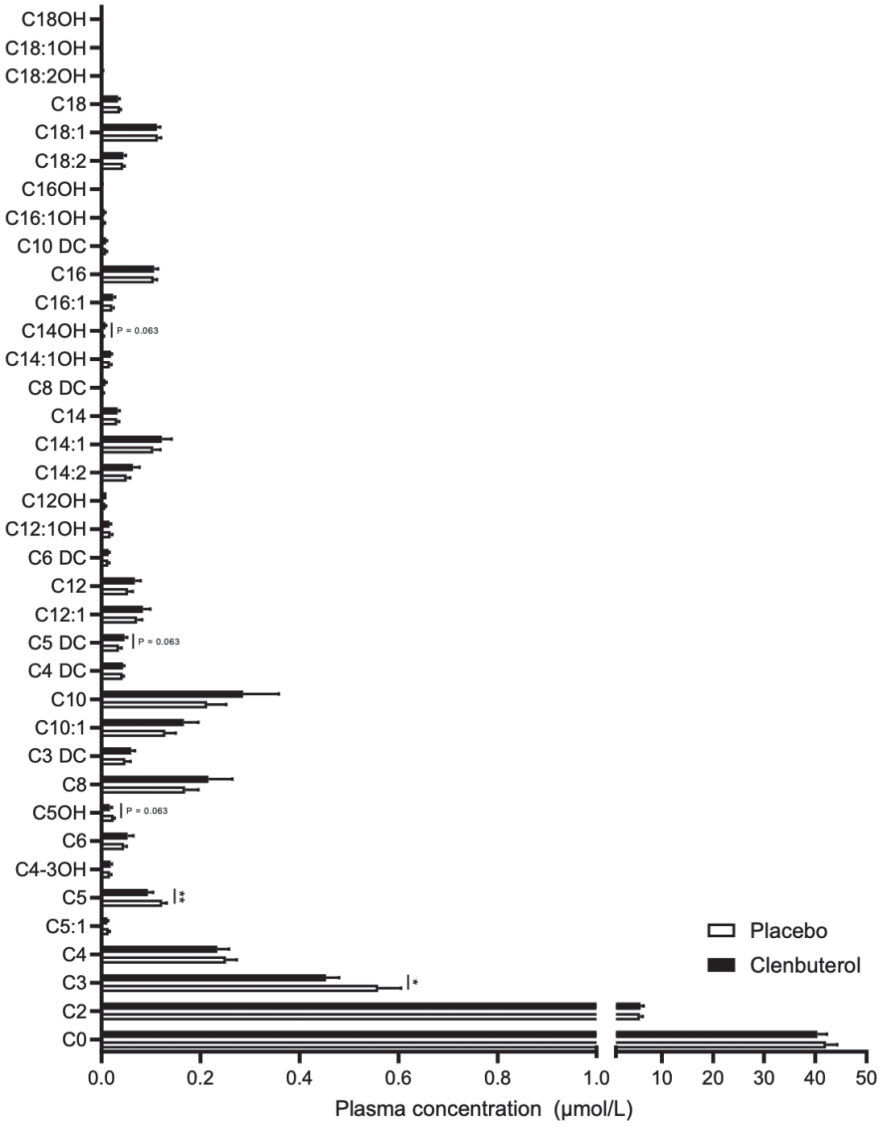
Supplemental Figure 1. Inclusion flow-chart of the study. P1 = period 1, P2 = period 2.



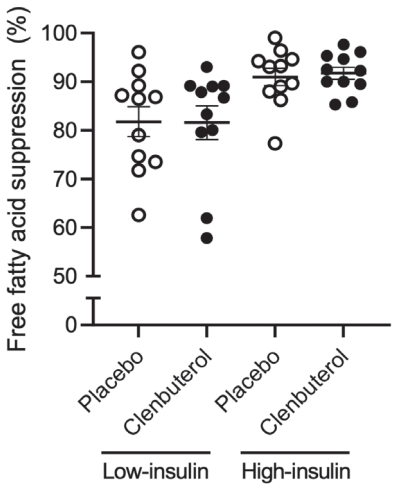
Supplementary Figure 2. Effect of two-weeks clenbuterol treatment on the activation of mTORC1 and plasma amino acids. A. phosphorylation of mTOR S2448 (mTORC1), B. plasma amino acid concentrations. Plasma amino acid concentrations of tyrosine, valine, leucine, ornithine, lysine, arginine, and citrulline were analysed by means of a Wilcoxon Rank-Signed test. All other data was analysed by means of a Student's Paired Sample T-test. P = placebo, Cl = clenbuterol. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



Supplemental Figure 3. Effect of prolonged clenbuterol treatment on basal metabolic rate and substrate oxidation. A. Energy expenditure during baseline, low- and high-insulin infusion (kJ/min). B. Carbohydrate oxidation during baseline, low- and high-insulin infusion (µmol/kg/min). C. Fat oxidation during baseline, low- and high-insulin infusion (µmol/kg/min). All data were analysed by means of a Paired Student's T-test. Placebo: n = 11 per group, Clenbuterol: n = 11 per group.



Supplementary Figure 4. The effect of clenbuterol treatment on fasting plasma acylcarnitine profiles. Data of C0, C2, C3, C12:1, C16:1, and C16 acylcarnitines were analysed by means of a Student's Paired samples T-test. All other data were analysed by means of a Wilcoxon Signed-Rank Test. * $p < 0.05$, ** $p < 0.01$.



Supplemental Figure 5. Effect of prolonged clenbuterol treatment on adipose tissue sensitivity expressed as the percentage free fatty acid suppression during both the low- and high- insulin phases of the two-step hyperinsulinemic-euglycemic clamp as compared to baseline. Free fatty acid suppression in the low-insulin phase was compared with a Wilcoxon rank signed test and FFA suppression in the high-insulin phase by means of a Paired Student's T-test. Placebo: n = 11 per group, Clenbuterol: n = 11 per group.

CHAPTER 6

Repeated cold-induced shivering improves glucose tolerance and components of the metabolic syndrome in overweight and obese adults

Sten M.M. van Beek[1*], Adam J. Sellers[1*], Dzhansel Hashim[1], Hanneke Pakbiersky[1], Esther Moonen-Kornips[1], Gert Schaart[1], Anne Grimmink[1], Tineke van de Weijer[2], Matthijs K.C. Hesselink[1], Patrick Schrauwen[1], Joris Hoeks[1#], Wouter van Marken Lichtenbelt[1#]

[1] Department of Nutrition and Movement Sciences, School of Nutrition and Translational Research in Metabolism, Maastricht University, Maastricht, The Netherlands
[2] Department of Radiology and Nuclear Medicine, School of Nutrition and Translational Research in Metabolism, Maastricht University Medical Centre, Maastricht, the Netherlands

[*, #] These authors contributed equally to this work

Manuscript in preparation

CHAPTER 7

General discussion and conclusion

General discussion

Type 2 diabetes mellitus (T2DM) has evolved into a major global health issue and its prevalence continues to rise at an alarming pace (1), with an estimated 600 million people suffering from the disease by 2035 (2). As T2DM is highly associated with the development of severe comorbidities (3-5) and greater mortality (1), a drastic negative impact on the global quality of life is imminent unless effective treatment strategies are promptly implemented. At the moment, common lifestyle interventions such as dietary and exercise regimes have repeatedly been proven to be highly successful in treating T2DM patients (6, 7). Despite their effectiveness, long-term adherence to these interventions by T2DM patients is rather poor, underscoring the need for novel therapies to improve glucose homeostasis.

As T2DM is a metabolic disease characterized by an energy excess in the body, alternative methods to enhance energy expenditure could result in a positive disease outcome. In this context, the 'rediscovery' of brown adipose tissue (BAT) in adult humans in 2009 (8-10) has sparked a scientific interest in the activation of BAT as a potential new therapeutic approach to improve glucose homeostasis in T2DM patients. BAT is a highly specialised thermogenic tissue, capable of drastically increasing heat production when exposed to its main (physiological) activator, namely cold (11). This enhanced dissipation of heat is accompanied by a substantial increase in the uptake of circulating plasma glucose (12, 13), hence making BAT a prime candidate to counter metabolic derangements associated with T2DM, including glucose intolerance. In line with this notion, activation of rodent BAT via cold exposure or pharmacological stimulation (i.e. β_3-adrenergic receptor agonists) has previously been associated with marked beneficial effects on glucose homeostasis (14-17), thereby hinting towards a potential translational value for humans as well.

In humans, cold exposure has proven to be a potent stimulator of BAT (8-10, 18) and BAT mass and/or activity can be significantly enhanced with repeated exposure to cold conditions, i.e. cold acclimation (19-22). Despite this increase in BAT, however, the amount of recruitable BAT in human appears to be rather limited. Thus, where rodents are characterised by high quantities of BAT relative to their body weight, adults humans have relatively small BAT depots, ranging from a few millilitres to a couple of hundred (23). Furthermore, the amount of BAT in humans inversely correlates with BMI and percentage body fat (8, 24), indicating that the overall quantity of BAT in overweight/obese patients with T2DM is potentially even lower. These data therefore cast doubt on the therapeutic value of BAT activation in improving glucose homeostasis in T2DM. This scepticism is further supported by studies demonstrating that BAT only accounted for

~1% of systemic glucose turnover upon cold exposure in healthy males (25), suggesting that glucose clearance by BAT in overweight/obese patients upon cold exposure is even lower. Furthermore, mild cold acclimation (14-15 ˚C, 6h/day, 10 consecutive days) only led to minor increases in BAT glucose uptake in T2DM patients (26). Altogether, it seems highly unlikely that activation of BAT alone is sufficient to drastically impact glucose homeostasis in overweight/obese T2DM patients.

Although the effect of cold-induced BAT activation on glucose homeostasis appears to be limited in humans, cold exposure itself has still been reported to markedly improve glucose homeostasis (27, 28), even in T2DM patients (26). Cold exposure therefore remains a promising, alternative strategy to improve glucose homeostasis, with other tissues than BAT likely responsible for mediating these beneficial effects. To gain a deeper understanding of the potential tissues involved, a comprehensive literature study was performed to investigate the effects of cold exposure on the primary organs involved in the regulation of glucose homeostasis, including the pancreas, liver, (white) adipose tissue and skeletal muscle, which can be found in **Chapter 2** of this thesis. Although human studies on this topic are scarce, it was established that a highly prominent role of skeletal muscle in these cold-induced improvements in glucose homeostasis is evident. The latter comes as little surprise, as the skeletal muscle has an extraordinary ability to take up plasma glucose (29, 30) and additionally has an unprecedented capacity for substrate oxidation and heat production. In line with this notion, several rodent studies have previously reported significant increases in basal- and insulin-stimulated skeletal muscle glucose uptake upon exposure to low ambient temperatures (13, 31-36), which was associated with robust improvements in glucose homeostasis. These effects are not limited to rodent models, as an increase in skeletal muscle glucose uptake has also been reported in humans *during* cold exposure (37). In fact, our research group has previously demonstrated that the beneficial effects of cold acclimation persist upon returning to thermoneutrality, with a marked increase in glucose transporter 4 (GLUT4)-mediated skeletal muscle glucose uptake in T2DM patients the day after mild cold acclimation, an effect that was associated with an ~43% increase in insulin sensitivity (26). Combined, these studies suggest that an increased skeletal muscle glucose uptake is – at least to a large extent – involved in mediating cold-induced improvements in glucose homeostasis.

To this day, however, the exact underlying mechanisms through which cold induces skeletal muscle glucose uptake is not fully known. Nevertheless, cold-induced stimulation of glucose uptake appeared to occur independent of the conventional insulin- and AMPK-signalling pathways (26), indicating that other pathways may be at play. Given the

pronounced beneficial effects of cold exposure on glucose homeostasis, further insight into these underlying mechanisms could potentially reveal novel therapeutic avenues for the treatment of T2DM. Therefore, the aim of this thesis was to further explore two putative (physiological) mechanisms of cold-induced improvements in skeletal muscle glucose uptake and, subsequently, whole-body glucose homeostasis. These mechanisms include **(1)** direct stimulation of skeletal muscle β_2-adrenergic receptors (β_2-ARs) and **(2)** shivering thermogenesis, which will be further discussed below.

Could activation of β2-adrenergic receptors underlie cold-induced improvements in skeletal muscle glucose uptake?

Initiation of thermogenesis upon exposure to low(er) ambient temperatures is mediated via activation of the sympathetic nervous system (SNS), which is characterised by postganglionic release of norepinephrine onto various tissues to stimulate energy expenditure. This activation of the SNS has previously been hypothesized to regulate skeletal muscle glucose uptake upon exposure to cold (38). Indeed, activation of the ventromedial hypothalamus, and subsequent release of norepinephrine, has been demonstrated to significantly enhance glucose uptake in skeletal muscle in rodents (39-41). In line with these findings, various *in vitro* studies have shown a significant increase in skeletal muscle glucose uptake upon activation of β_2-ARs (42-45), the most abundant adrenoceptor in skeletal muscle (46), for which norepinephrine has affinity as well. Combined, these results suggest that activation of β_2-ARs may contribute to the beneficial effects of cold exposure on skeletal muscle glucose uptake. However, information about the physiological and clinical relevance of this pathway is virtually absent.

In **Chapter 3** of this thesis, it was therefore investigated if treatment with the selective β_2-agonist clenbuterol could boost *in vivo* skeletal muscle glucose uptake in diet-induced obese (DIO) mice, a well-known rodent model of insulin resistance. Interestingly, merely 6 days of clenbuterol treatment significantly enhanced basal skeletal muscle glucose uptake by ~74%, thereby confirming a physiological relevance of β_2-ARs in mediating skeletal muscle glucose uptake *in vivo*. These effects were accompanied by significant improvements in both whole-body glucose tolerance and insulin sensitivity, which occurred independent of changes in plasma insulin concentrations or body composition. These results are in agreement with previous studies investigating the effects of β_2-agonist treatment on whole-body glucose homeostasis in rodents (47-49), albeit that these results demonstrated that these beneficial effects could be achieved upon doses ~40 times lower than studied before. In addition, this study is the first to show that

β_2-agonist-induced improvements in glucose homeostasis are likely attributed to an increased skeletal muscle glucose uptake.

Nonetheless, it is important to note that β-ARs are widely expressed throughout the body and that clenbuterol also has an (albeit low) affinity towards β_1- and β_3-ARs (50), thereby indicating that other tissues besides the skeletal muscle could potentially contribute to the beneficial effects of clenbuterol treatment on whole-body glucose homeostasis. In rodents, this especially applies to the BAT, which is primarily activated via β_1- and β_3-ARs (11) and – as described above – can significantly impact glucose homeostasis (14, 16, 17). In **Chapter 4** of this thesis, acute clenbuterol injection indeed markedly enhanced whole-body oxygen consumption and this effect was severely blunted in uncoupling protein 1 (UCP1) deficient mice, which lack thermogenic BAT. These findings indicate that clenbuterol is indeed able to enhance BAT activity *in vivo* in mice, which in turn may contribute to the effects on whole-body glucose homeostasis. To test this possibility, the effects of clenbuterol treatment were investigated in UCP1$^{-/-}$ DIO mice, which – in our opinion – also better reflect the human T2DM phenotype as overweight/obese individuals have low quantities of BAT (8). Interestingly, clenbuterol treatment markedly improved glucose tolerance and insulin sensitivity, even in the absence of thermogenically functional BAT. In fact, glucose tolerance and insulin sensitivity of both UCP1$^{-/-}$ and wild-type mice were normalised to similar levels of those of healthy, chow-fed mice, thereby further demonstrating the efficacy of β_2-agonist treatment in improving glucose homeostasis *in vivo*.

Based on the outcomes of **Chapter 3 and 4**, it can be concluded that β_2-ARs represent an interesting target to improve skeletal muscle glucose uptake and whole-body glucose homeostasis in T2DM patients. However, whether prolonged β_2-agonist treatment also affects skeletal muscle glucose uptake in humans is largely unknown. To investigate the latter, a proof-of-principle study was performed in healthy young males, described in **Chapter 5** of this thesis. Thus, in a randomised, placebo-controlled, double-blinded, cross-over study, subjects received a treatment with either clenbuterol (20 µg twice daily) or a placebo for two weeks with a 4-week wash-out period in between. Following these two weeks of treatment, the golden standard two-step hyperinsulinemic-euglycemic clamp technique was performed to assess insulin-stimulated skeletal muscle glucose uptake. In line with the results from the animal models described in **Chapter 3 and 4**, it was established that, also in humans, clenbuterol treatment significantly increased insulin-stimulated skeletal muscle glucose uptake by ~13%, an effect that was primarily attributed to an increased non-oxidative glucose disposal of ~18%.

To our knowledge, merely two studies have previously investigated the effects of prolonged β_2-agonist treatment on glucose homeostasis in humans. Thus, 1-2 weeks treatment with the selective β_2-agonist terbutaline sulphate (15 mg/day orally) has been shown to markedly improve insulin-stimulated glucose uptake and non-oxidative glucose disposal by ~29% and ~45%, respectively, in healthy young males (51). Along similar lines, 4-weeks of terbutaline treatment (4 mg/day inhalation) significantly increased glucose infusion rate during a hyperinsulinemic-euglycemic clamp in healthy young males by 27% (52), although this was primarily attributed to an increase in lean muscle mass. Combined with our findings, these studies strongly indicate that selective β_2-agonist treatment increases skeletal muscle glucose uptake and improves whole-body glucose homeostasis in humans. It is especially important to highlight that all studies involved healthy, young, male subjects, who are not characterised by disturbances in their glucose homeostasis. The window of opportunity for β_2-agonist treatment may therefore be even larger in individuals with an impaired glucose homeostasis. As such, it is strongly recommended that follow-up studies are performed to investigate the effects of (even more prolonged) β_2-agonists treatment in individuals with pre-diabetes and T2DM patients.

Despite the beneficial effects of β_2-adrenergic stimulation on glucose homeostasis, long-term (systemic) treatment with currently existing β_2-agonists is associated with significant (cardiovascular) side-effects (53), complicating direct translation into the clinic. Given these side effects, exact knowledge of the underlying mechanisms mediating β_2-agonist-induced improvements in skeletal muscle glucose uptake could prove helpful in identifying novel therapeutic targets for targeting this pathway without the negative side effects. However, the physiological and/or molecular pathways involved in β_2-stimulated glucose uptake are largely unknown to this day. In this context, this study is the first to perform an in-depth metabolic profiling on the effects of clenbuterol treatment to assess the putative mechanisms implicated in mediating the improvements in skeletal muscle glucose uptake (**Chapter 5**). In short, it was demonstrated that the beneficial effects of clenbuterol treatment could not be attributed to an enhanced lean muscle mass, changes in plasma insulin concentrations, or alterations in substrate oxidation, despite a significant increase in resting and sleeping metabolic rate. Interestingly, a significant increase in femoral artery blood flow velocity was observed upon clenbuterol treatment, which hints towards a higher peripheral tissue perfusion and thereby (potentially) an increased skeletal muscle glucose uptake (54). However, the role of an enhanced tissue perfusion upon clenbuterol treatment in mediating skeletal muscle glucose uptake needs to be investigated in more detail.

Besides these physiological measurements, the molecular mechanisms involved were also investigated by means of detailed analyses of skeletal muscle biopsies, which were acquired in the overnight, fasted state following clenbuterol treatment. In this context, the effects of β_2-agonists on skeletal muscle glucose uptake in rodents appear to primarily depend on the activation of the mammalian target of rapamycin complex 2 (mTORC2) and, subsequently, GLUT4 translocation (44). More specifically, (muscle-specific) ablation of the mTORC2-subunit Rictor or the use of specific mTORC2 inhibitors severely blunts the effects of β_2-agonist on skeletal muscle glucose uptake in rodents (44, 55). Despite the apparent central role of mTORC2, clenbuterol-mediated improvements in glucose homeostasis in mice were also shown to occur independent of changes in skeletal muscle GLUT4 translocation (55). In our human study described in **Chapter 5**, GLUT4 translocation and mTORC2 activation were therefore assessed following 2 weeks of clenbuterol treatment. However, no differences in GLUT4 translocation or mTORC2 activation were observed. This discrepancy could potentially be attributed to the relatively small sample size of our study. In addition, it could be speculated that alternative measurement techniques (such as metabolomics) are required to identify a role of mTORC2 (55). In light of these findings, however, it is also important to mention that other (currently unknown) molecular mechanisms could be involved in clenbuterol-mediated improvements in skeletal muscle glucose uptake in humans. In this context, marked reductions in plasma triglyceride concentrations were observed following clenbuterol treatment, suggesting an enhanced lipid turnover by (potentially) skeletal muscle. As lipid intermediates (such as ceramides and diacylglycerol) are known to interfere with insulin signalling (56), a higher lipid turnover within skeletal muscle could potentially underlie our clenbuterol-mediated improvements in insulin-stimulated glucose disposal. However, no direct evidence for this was obtained during this study.

In addition to skeletal muscle, other metabolic tissues could also contribute to the beneficial effects seen upon prolonged β_2-agonist treatment. In this context, a particular tissue of interest is BAT, as it has recently been demonstrated that human BAT is primarily activated via the β_2-AR (57). Although overweight/obese individuals are characterized by very low BAT quantities, lean, young individuals are known to have somewhat larger BAT depots (8), and BAT is able to take up glucose from the circulation upon its activation (12, 13, 25). Therefore, a potential role for BAT in mediating the β_2-agonist-mediated improvements in insulin-stimulated glucose disposal observed in **Chapter 5** cannot be excluded. Hence, it would be interesting for follow-up studies to assess the effects of β_2-agonist treatment on BAT glucose uptake by means of PET-MRI or PET-CT in healthy

young, but also overweight/obese individuals. Besides BAT, β_2-agonists are also well-known to stimulate lipolysis and energy expenditure in white adipose tissue (WAT) (57, 58), which could potentially be associated with improvements in glucose uptake. Although no significant changes in insulin-mediated suppression of FFAs were identified during the two-step hyperinsulinemic-euglycemic clamp – reflecting WAT insulin sensitivity – upon clenbuterol treatment, future studies could include subcutaneous adipose tissue biopsies to investigate the metabolic effects of β_2-agonists on WAT.

When combining the results of **Chapter 3, 4, and 5**, evidence is provided that activation of β_2-ARs could be a potential explanation for our previously observed effects of mild cold acclimation on skeletal muscle glucose uptake and whole-body glucose homeostasis in humans (26). Nevertheless, additional (physiological) mechanisms besides β_2-ARs stimulation could still be at play in mediating cold-induced improvements in skeletal muscle glucose uptake. In this context, cold-induced shivering of skeletal muscle is a likely candidate, which will be discussed in more detail below.

Is cold-induced shivering thermogenesis related to improvements in glucose homeostasis?

Upon substantial bodily heat loss, the human body - among other things - prevents the development of hypothermia by means of shivering thermogenesis (59), which is capable of increasing energy expenditure up to 5x resting metabolic rate (60). The latter is sustained by both an enhanced carbohydrate and fat oxidation, albeit that the relative contribution of these substrates is dependent on several factors, including the shivering intensity (61), with a higher shivering intensity being associated with a higher carbohydrate oxidation. The glucose consumed to fuel this carbohydrate oxidation is derived from both skeletal muscle glycogen stores and glucose from the circulation, and both glycogen breakdown and plasma glucose uptake are known to correlate positively with shivering intensity (37, 62). Based on the latter, it has previously been hypothesized that shivering could potentially improve whole-body glucose homeostasis. Indeed, a previous study demonstrated that the clearance of an intravenously infused glucose bolus in healthy, young males is markedly improved *during* exposure to shivering thermogenesis (3h at 10˚C), although oral glucose tolerance assessed under the same conditions was not affected (27).

In our previous study, mild cold acclimation (14-15 ˚C, 6h/day, 10 consecutive days) improved insulin-stimulated glucose uptake by 43%, as assessed at thermoneutrality the

day after the last cold exposure (26). Although the study aimed to maximally stimulate non-shivering thermogenesis, the study participants self-reported the presence of low-intensity shivering (i.e. tense muscles) during the mild cold exposure sessions, which was apparent even after cold acclimation (26). Our research group therefore performed a follow-up study with a comparable study design, albeit with a slightly higher ambient temperature (16-17 ˚C) to avoid shivering during the mild cold exposure sessions (63). Surprisingly, this study was unable to reproduce the cold-induced improvements in insulin-stimulated glucose disposal in T2DM patients, thereby hinting towards a role for shivering in mediating the beneficial effects of cold acclimation. This notion was further supported by skeletal muscle gene expression data of both mild cold acclimation studies, which showed an upregulation of genes associated with shivering in the initial study (26), but not in the follow-up (63).

To directly test the effects of shivering on skeletal muscle glucose uptake and whole-body glucose homeostasis, a single-arm intervention study was performed during which overweight/obese men and (postmenopausal) women were repeatedly exposed to at least 1 hour of shivering per day for 10 days (**Chapter 6**). Subsequently, oral glucose tolerance tests (OGTTs) were performed at thermoneutrality before, the day after a single cold exposure with shivering, and the day after a 10-day cold acclimation with shivering. Despite the fact that a single cold exposure with shivering was unable to affect oral glucose tolerance, cold acclimation significantly reduced the glucose area under the curve during the OGTT, as well as fasting glucose concentrations, indicative of an improved whole-body glucose homeostasis. These combined results therefore strongly hint towards a role of shivering in mediating the effects of (mild) cold acclimation on whole-body glucose homeostasis. It could be argued, however, that our observed improvements in glucose area under the curve (-6%) are smaller than our previously reported improvements in insulin sensitivity upon mild cold acclimation (+43%) (26). This discrepancy could potentially be explained by differences between studies with respect to their studied population (healthy overweight/obese versus T2DM patients) and assessment method for glucose homeostasis (OGTT vs. clamp). Thus, T2DM patients likely have a greater window of opportunity to improve glucose homeostasis in comparison to healthy, overweight/obese individuals. Future studies should therefore focus on individuals with pre-diabetes or patients with T2DM to allow decent comparison with our previous study (26) and to assess the true potential of cold acclimation with shivering. Furthermore, future studies that assess the effects of cold acclimation with shivering on glucose homeostasis by means of a hyperinsulinemic-euglycemic clamp

will allow further comparison between these studies. Finally, follow-up studies should additionally investigate the optimal frequency, duration, and intensity of cold exposure with shivering to improve glucose homeostasis, as daily exposure to 1 hour of shivering is unrealistic within a clinical setting.

Contrary to our previous study (26), the effects of shivering on glucose homeostasis could not be attributed to an increased skeletal muscle GLUT4 translocation. It is important to note, however, that these skeletal muscle biopsies were acquired in the overnight, fasted state, whereas our improvements in glucose tolerance were observed in the insulin-stimulated state. As such, the possibility that GLUT4 translocation was enhanced upon insulin stimulation, i.e. *during* the OGTT, cannot be excluded. Nonetheless, besides changes in skeletal muscle GLUT4 translocation, other mechanisms could also be at play in mediating the observed increase in glucose tolerance. In this context, plasma FFA concentrations, both in the fasted state and during the OGTT, were robustly decreased following cold acclimation with shivering. Given the association between increased levels of FFAs and a blunted insulin-stimulated glucose disposal in peripheral tissues (64), our observed improvements in whole-body glucose homeostasis could potentially be mediated via a reduction in plasma FFAs. In addition, as shivering is well-known to depend on skeletal muscle glycogen stores (62) and previous studies have indicated that a reduced muscle glycogen content can stimulate glucose uptake (65, 66), decreased glycogen concentrations following cold exposure could be associated with an enhanced skeletal muscle glucose uptake. Given the latter, it would be highly interesting for follow-up studies to determine skeletal muscle glycogen content following cold acclimation with shivering by means of, for example, magnetic resonance spectroscopy.

Besides the skeletal muscle, it is important to highlight that other tissues could also be involved in mediating cold-induced improvements in whole-body glucose homeostasis. Indeed, our improvements in fasting glucose concentrations following cold acclimation with shivering could also be related to a reduced endogenous hepatic glucose production, indicative of an improved hepatic insulin sensitivity (67). This notion is supported by our previous mild cold acclimation study, which tended to improve hepatic insulin sensitivity in T2DM patients (26), an effect that could not be replicated if overt shivering was prevented (63). In addition to the liver, previous rodent studies have demonstrated a significant increase in basal- and insulin-stimulated glucose uptake in white adipose tissue (WAT) upon prolonged cold exposure (33, 34, 38), although mild cold acclimation has been unable to induce similar effects in humans (22, 26). Nevertheless, as the intensity of cold exposure in the current study (**Chapter 6**) is higher as compared to our

previous mild cold acclimation studies, a potential improvement in WAT glucose uptake cannot be excluded.

The results presented in **Chapter 6** provide strong evidence that shivering thermogenesis may underlie our previously observed improvements in whole-body glucose homeostasis upon mild cold acclimation, although the molecular pathways mediating these improvements remain to be established. Besides the beneficial effects on whole-body glucose homeostasis, it was also demonstrated that acclimation to cold with shivering markedly improved both blood pressure and fasting lipid profiles in overweight/obese subjects, two well-known parameters of the metabolic syndrome. These findings combined therefore suggest that cold acclimation with shivering could be an interesting strategy to not only improve glucose homeostasis, but also other components of the metabolic syndrome in T2DM patients.

Concluding remarks

The main aim of this thesis was to further investigate two potential mechanisms (i.e. β_2-ARs stimulation and shivering thermogenesis) through which cold exposure could enhance skeletal muscle glucose uptake and, thereby, improve glucose homeostasis. Based on the data presented in this thesis, it can be concluded that both direct stimulation of β_2-ARs, as well as exposure to shivering thermogenesis, may underlie our previously observed (mild) cold-induced improvements in skeletal muscle glucose uptake and whole-body glucose homeostasis. To what extent either mechanism is responsible for the cold-induced improvements in glucose homeostasis is impossible to delineate at this point. It could even be hypothesized that both mechanisms additively or synergistically enhance skeletal muscle glucose uptake and/or improve glucose homeostasis. As such, the relative contribution of each mechanism in cold-induced improvements in skeletal muscle glucose uptake and whole-body glucose homeostasis remains to be established. Nonetheless, the research described in this thesis provides some pieces of the puzzle in our search for alternative treatment strategies for patients with T2DM, although implementation into the clinic is still a long way ahead.

References

1. World Health Organisation. Factsheet Diabetes 2021. [Available from https: https://www.who.int/news-room/fact-sheets/detail/diabetes].
2. Guariguata L, Whiting DR, Hambleton I, Beagley J, Linnenkamp U, Shaw JE. Global estimates of diabetes prevalence for 2013 and projections for 2035. Diabetes Res Clin Pract. 2014;103(2):137-49.
3. Emerging Risk Factors C, Sarwar N, Gao P, Seshasai SR, Gobin R, Kaptoge S, et al. Diabetes mellitus, fasting blood glucose concentration, and risk of vascular disease: a collaborative meta-analysis of 102 prospective studies. Lancet. 2010;375(9733):2215-22.
4. Vigneri P, Frasca F, Sciacca L, Pandini G, Vigneri R. Diabetes and cancer. Endocr Relat Cancer. 2009;16(4):1103-23.
5. Garcia-Compean D, Jaquez-Quintana JO, Gonzalez-Gonzalez JA, Maldonado-Garza H. Liver cirrhosis and diabetes: risk factors, pathophysiology, clinical implications and management. World J Gastroenterol. 2009;15(3):280-8.
6. Meex RC, Schrauwen-Hinderling VB, Moonen-Kornips E, Schaart G, Mensink M, Phielix E, et al. Restoration of muscle mitochondrial function and metabolic flexibility in type 2 diabetes by exercise training is paralleled by increased myocellular fat storage and improved insulin sensitivity. Diabetes. 2010;59(3):572-9.
7. Lara-Castro C, Newcomer BR, Rowell J, Wallace P, Shaughnessy SM, Munoz AJ, et al. Effects of short-term very low-calorie diet on intramyocellular lipid and insulin sensitivity in nondiabetic and type 2 diabetic subjects. Metabolism. 2008;57(1):1-8.
8. van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts JM, Kemerink GJ, Bouvy ND, et al. Cold-activated brown adipose tissue in healthy men. N Engl J Med. 2009;360(15):1500-8.
9. Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, et al. Identification and importance of brown adipose tissue in adult humans. N Engl J Med. 2009;360(15):1509-17.
10. Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, Niemi T, et al. Functional brown adipose tissue in healthy adults. N Engl J Med. 2009;360(15):1518-25.
11. Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. Physiol Rev. 2004;84(1):277-359.
12. Orava J, Nuutila P, Lidell ME, Oikonen V, Noponen T, Viljanen T, et al. Different metabolic responses of human brown adipose tissue to activation by cold and insulin. Cell Metab. 2011;14(2):272-9.
13. Shibata H, Perusse F, Vallerand A, Bukowiecki LJ. Cold exposure reverses inhibitory effects of fasting on peripheral glucose uptake in rats. Am J Physiol. 1989;257(1 Pt 2):R96-101.
14. Bartelt A, Bruns OT, Reimer R, Hohenberg H, Ittrich H, Peldschus K, et al. Brown adipose tissue activity controls triglyceride clearance. Nat Med. 2011;17(2):200-5.
15. Vallerand AL, Lupien J, Bukowiecki LJ. Cold exposure reverses the diabetogenic effects of high-fat feeding. Diabetes. 1986;35(3):329-34.
16. Olsen JM, Csikasz RI, Dehvari N, Lu L, Sandstrom A, Oberg AI, et al. beta3-Adrenergically induced glucose uptake in brown adipose tissue is independent of UCP1 presence or activity: Mediation through the mTOR pathway. Mol Metab. 2017;6(6):611-9.
17. Xiao C, Goldgof M, Gavrilova O, Reitman ML. Anti-obesity and metabolic efficacy of the beta3-adrenergic agonist, CL316243, in mice at thermoneutrality compared to 22 degrees C. Obesity (Silver Spring). 2015;23(7):1450-9.

18. Cypess AM, Chen YC, Sze C, Wang K, English J, Chan O, et al. Cold but not sympathomimetics activates human brown adipose tissue in vivo. Proc Natl Acad Sci U S A. 2012;109(25):10001-5.
19. van der Lans AA, Hoeks J, Brans B, Vijgen GH, Visser MG, Vosselman MJ, et al. Cold acclimation recruits human brown fat and increases nonshivering thermogenesis. J Clin Invest. 2013;123(8):3395-403.
20. Yoneshiro T, Aita S, Matsushita M, Kayahara T, Kameya T, Kawai Y, et al. Recruited brown adipose tissue as an antiobesity agent in humans. J Clin Invest. 2013;123(8):3404-8.
21. Blondin DP, Labbe SM, Tingelstad HC, Noll C, Kunach M, Phoenix S, et al. Increased brown adipose tissue oxidative capacity in cold-acclimated humans. J Clin Endocrinol Metab. 2014;99(3):E438-46.
22. Hanssen MJ, van der Lans AA, Brans B, Hoeks J, Jardon KM, Schaart G, et al. Short-term Cold Acclimation Recruits Brown Adipose Tissue in Obese Humans. Diabetes. 2016;65(5):1179-89.
23. Blondin DP, Labbe SM, Turcotte EE, Haman F, Richard D, Carpentier AC. A critical appraisal of brown adipose tissue metabolism in humans. Clin lipidol. 2015;10:259–80. 10.2217/clp.15.14.
24. Brendle C, Werner MK, Schmadl M, la Fougere C, Nikolaou K, Stefan N, et al. Correlation of Brown Adipose Tissue with Other Body Fat Compartments and Patient Characteristics: A Retrospective Analysis in a Large Patient Cohort Using PET/CT. Acad Radiol. 2018;25(1):102-10.
25. Blondin DP, Labbe SM, Phoenix S, Guerin B, Turcotte EE, Richard D, et al. Contributions of white and brown adipose tissues and skeletal muscles to acute cold-induced metabolic responses in healthy men. J Physiol. 2015;593(3):701-14.
26. Hanssen MJ, Hoeks J, Brans B, van der Lans AA, Schaart G, van den Driessche JJ, et al. Short-term cold acclimation improves insulin sensitivity in patients with type 2 diabetes mellitus. Nat Med. 2015;21(8):863-5.
27. Vallerand AL, Frim J, Kavanagh MF. Plasma glucose and insulin responses to oral and intravenous glucose in cold-exposed humans. J Appl Physiol (1985). 1988;65(6):2395-9.
28. Iwen KA, Backhaus J, Cassens M, Waltl M, Hedesan OC, Merkel M, et al. Cold-Induced Brown Adipose Tissue Activity Alters Plasma Fatty Acids and Improves Glucose Metabolism in Men. J Clin Endocrinol Metab. 2017;102(11):4226-34.
29. DeFronzo RA, Gunnarsson R, Bjorkman O, Olsson M, Wahren J. Effects of insulin on peripheral and splanchnic glucose metabolism in noninsulin-dependent (type II) diabetes mellitus. J Clin Invest. 1985;76(1):149-55.
30. DeFronzo RA, Jacot E, Jequier E, Maeder E, Wahren J, Felber JP. The effect of insulin on the disposal of intravenous glucose. Results from indirect calorimetry and hepatic and femoral venous catheterization. Diabetes. 1981;30(12):1000-7.
31. Smith OL. Insulin response in rats acutely exposed to cold. Can J Physiol Pharmacol. 1984;62(8):924-7.
32. Smith OL, Davidson SB. Shivering thermogenesis and glucose uptake by muscles of normal or diabetic rats. Am J Physiol. 1982;242(1):R109-15.
33. Vallerand AL, Perusse F, Bukowiecki LJ. Cold exposure potentiates the effect of insulin on in vivo glucose uptake. Am J Physiol. 1987;253(2 Pt 1):E179-86.
34. Gasparetti AL, de Souza CT, Pereira-da-Silva M, Oliveira RL, Saad MJ, Carneiro EM, et al. Cold exposure induces tissue-specific modulation of the insulin-signalling pathway in Rattus norvegicus. J Physiol. 2003;552(Pt 1):149-62.
35. Smith SA, Young P, Cawthorne MA. Quantification in vivo of the effects of insulin on glucose utilization in individual tissues of warm- and cold-acclimated rats. Biochem J. 1986;237(3):789-95.

36. Oliveira RL, Ueno M, de Souza CT, Pereira-da-Silva M, Gasparetti AL, Bezzera RM, et al. Cold-induced PGC-1alpha expression modulates muscle glucose uptake through an insulin receptor/Akt-independent, AMPK-dependent pathway. Am J Physiol Endocrinol Metab. 2004;287(4):E686-95.
37. Blondin DP, Labbe SM, Noll C, Kunach M, Phoenix S, Guerin B, et al. Selective Impairment of Glucose but Not Fatty Acid or Oxidative Metabolism in Brown Adipose Tissue of Subjects With Type 2 Diabetes. Diabetes. 2015;64(7):2388-97.
38. Vallerand AL, Perusse F, Bukowiecki LJ. Stimulatory effects of cold exposure and cold acclimation on glucose uptake in rat peripheral tissues. Am J Physiol. 1990;259(5 Pt 2):R1043-9.
39. Minokoshi Y, Okano Y, Shimazu T. Regulatory mechanism of the ventromedial hypothalamus in enhancing glucose uptake in skeletal muscles. Brain Res. 1994;649(1-2):343-7.
40. Shimazu T, Sudo M, Minokoshi Y, Takahashi A. Role of the hypothalamus in insulin-independent glucose uptake in peripheral tissues. Brain Res Bull. 1991;27(3-4):501-4.
41. Sudo M, Minokoshi Y, Shimazu T. Ventromedial hypothalamic stimulation enhances peripheral glucose uptake in anesthetized rats. Am J Physiol. 1991;261(3 Pt 1):E298-303.
42. Nevzorova J, Bengtsson T, Evans BA, Summers RJ. Characterization of the beta-adrenoceptor subtype involved in mediation of glucose transport in L6 cells. Br J Pharmacol. 2002;137(1):9-18.
43. Nevzorova J, Evans BA, Bengtsson T, Summers RJ. Multiple signalling pathways involved in beta2-adrenoceptor-mediated glucose uptake in rat skeletal muscle cells. Br J Pharmacol. 2006;147(4):446-54.
44. Sato M, Dehvari N, Oberg AI, Dallner OS, Sandstrom AL, Olsen JM, et al. Improving type 2 diabetes through a distinct adrenergic signaling pathway involving mTORC2 that mediates glucose uptake in skeletal muscle. Diabetes. 2014;63(12):4115-29.
45. Kalinovich A, Dehvari N, Åslund A, van Beek S, Halleskog C, Olsen J, et al. Treatment with a β-2-adrenoceptor agonist stimulates glucose uptake in skeletal muscle and improves glucose homeostasis, insulin resistance and hepatic steatosis in mice with diet-induced obesity. Diabetologia. 2020.
46. Liggett SB, Shah SD, Cryer PE. Characterization of beta-adrenergic receptors of human skeletal muscle obtained by needle biopsy. Am J Physiol. 1988;254(6 Pt 1):E795-8.
47. Castle A, Yaspelkis BB, 3rd, Kuo CH, Ivy JL. Attenuation of insulin resistance by chronic beta2-adrenergic agonist treatment possible muscle specific contributions. Life Sci. 2001;69(5):599-611.
48. Pan SJ, Hancock J, Ding Z, Fogt D, Lee M, Ivy JL. Effects of clenbuterol on insulin resistance in conscious obese Zucker rats. Am J Physiol Endocrinol Metab. 2001;280(4):E554-61.
49. Torgan CE, Brozinick JT, Jr., Banks EA, Cortez MY, Wilcox RE, Ivy JL. Exercise training and clenbuterol reduce insulin resistance of obese Zucker rats. Am J Physiol. 1993;264(3 Pt 1):E373-9.
50. Baker JG. The selectivity of beta-adrenoceptor agonists at human beta1-, beta2- and beta3-adrenoceptors. Br J Pharmacol. 2010;160(5):1048-61.
51. Scheidegger K, Robbins DC, Danforth E, Jr. Effects of chronic beta receptor stimulation on glucose metabolism. Diabetes. 1984;33(12):1144-9.
52. Jessen S, Baasch-Skytte T, Onslev J, Eibye K, Backer V, Bangsbo J, et al. Muscle hypertrophic effect of inhaled beta2 -agonist is associated with augmented insulin-stimulated whole-body glucose disposal in young men. J Physiol. 2022;600(10):2345-57.
53. Kamalakkannan G, Petrilli CM, George I, LaManca J, McLaughlin BT, Shane E, et al. Clenbuterol increases lean muscle mass but not endurance in patients with chronic heart failure. J Heart Lung Transplant. 2008;27(4):457-61.

54. Baron AD, Steinberg H, Brechtel G, Johnson A. Skeletal muscle blood flow independently modulates insulin-mediated glucose uptake. Am J Physiol. 1994;266(2 Pt 1):E248-53.

55. Meister J, Bone DBJ, Knudsen JR, Barella LF, Velenosi TJ, Akhmedov D, et al. Clenbuterol exerts antidiabetic activity through metabolic reprogramming of skeletal muscle cells. Nat Commun. 2022;13(1):22.

56. Kitessa SM, Abeywardena MY. Lipid-Induced Insulin Resistance in Skeletal Muscle: The Chase for the Culprit Goes from Total Intramuscular Fat to Lipid Intermediates, and Finally to Species of Lipid Intermediates. Nutrients. 2016;8(8).

57. Blondin DP, Nielsen S, Kuipers EN, Severinsen MC, Jensen VH, Miard S, et al. Human Brown Adipocyte Thermogenesis Is Driven by beta2-AR Stimulation. Cell Metab. 2020;32(2):287-300 e7.

58. Schiffelers SL, Saris WH, Boomsma F, van Baak MA. beta(1)- and beta(2)-Adrenoceptor-mediated thermogenesis and lipid utilization in obese and lean men. J Clin Endocrinol Metab. 2001;86(5):2191-9.

59. Haman F, Peronnet F, Kenny GP, Massicotte D, Lavoie C, Scott C, et al. Effect of cold exposure on fuel utilization in humans: plasma glucose, muscle glycogen, and lipids. J Appl Physiol (1985). 2002;93(1):77-84.

60. Eyolfson DA, Tikuisis P, Xu X, Weseen G, Giesbrecht GG. Measurement and prediction of peak shivering intensity in humans. Eur J Appl Physiol. 2001;84(1-2):100-6.

61. Blondin DP, Haman F. Shivering and nonshivering thermogenesis in skeletal muscles. Handb Clin Neurol. 2018;156:153-73.

62. Haman F, Peronnet F, Kenny GP, Massicotte D, Lavoie C, Weber JM. Partitioning oxidative fuels during cold exposure in humans: muscle glycogen becomes dominant as shivering intensifies. J Physiol. 2005;566(Pt 1):247-56.

63. Remie CME, Moonen MPB, Roumans KHM, Nascimento EBM, Gemmink A, Havekes B, et al. Metabolic responses to mild cold acclimation in type 2 diabetes patients. Nat Commun. 2021;12(1):1516.

64. Ferrannini E, Barrett EJ, Bevilacqua S, DeFronzo RA. Effect of fatty acids on glucose production and utilization in man. J Clin Invest. 1983;72(5):1737-47.

65. Jensen J, Jebens E, Brennesvik EO, Ruzzin J, Soos MA, Engebretsen EM, et al. Muscle glycogen inharmoniously regulates glycogen synthase activity, glucose uptake, and proximal insulin signaling. Am J Physiol Endocrinol Metab. 2006;290(1):E154-E62.

66. Jensen J, Aslesen R, Ivy JL, Brors O. Role of glycogen concentration and epinephrine on glucose uptake in rat epitrochlearis muscle. Am J Physiol. 1997;272(4 Pt 1):E649-55.

67. Loria P, Lonardo A, Anania F. Liver and diabetes. A vicious circle. Hepatol Res. 2013;43(1):51-64.

ADDENDUM

A

Impact

What is the main aim of the research described in the thesis and what are the most important results and conclusions?

Repeated cold exposure (or cold acclimation as it is called in science) has presented itself as a new way to help patients with type 2 diabetes mellitus (T2DM) maintain their blood glucose levels within normal boundaries. This glucose homeostasis appears to be primarily caused by an increased glucose uptake from the blood by skeletal muscle, as was shown by (clinical) studies performed by our own research group, as well as by others (**Chapter 2**). To this day, it is unknown how cold exposure enhances glucose uptake by skeletal muscle. The main aim of this thesis was to investigate two potential mechanisms how repeated exposure to cold enhances skeletal muscle glucose uptake and improves glucose homeostasis.

One potential mechanism through which cold could have its beneficial metabolic effects, is via activation of a specific part of the nervous system named the sympathetic nervous system, which also controls the well-known fight-or-flight response. Cold exposure is known to stimulate this part of the nervous system, in order to defend core body temperature. Activation of the sympathetic nervous system causes the release of the hormone norepinephrine onto skeletal muscle cells, which can stimulate specific receptors better known as the β_2-adrenergic receptors (β_2-ARs). In cultured muscle cells, it has previously been shown that compounds with the ability to activate these receptors (so-called β_2-agonists), can stimulate glucose uptake. However, it was unknown whether this effect could also occur *in vivo*. In **Chapter 3**, we therefore treated obese, insulin-resistant mice, with the β_2-agonist clenbuterol and investigated skeletal muscle glucose uptake, as well as whole-body glucose homeostasis. Interestingly, we found that clenbuterol treatment improved skeletal muscle glucose uptake and also whole-body glucose homeostasis in these mice.

Although clenbuterol mainly activates β_2-ARs, it also has some affinity for β_1- and β_3-ARs. As these receptors are present on several other tissues influencing blood glucose levels, the possibility exists that other tissues than muscle contribute to the improvements in whole-body glucose homeostasis described in **Chapter 3**. An important tissue in this context is the brown adipose tissue (BAT), which is capable of taking up large amounts of glucose from the blood once it is activated upon cold, via β_1- and β_3-ARs. In contrast to rodents however, it is known that (especially obese) humans have low amounts of BAT. It is therefore important to investigate if the results described in **Chapter 3** are dependent on BAT, as otherwise similar effects might not be achieved in humans. In **Chapter 4**, we therefore investigated the effects of clenbuterol treatment in obese mice that have non-

functional BAT. Also in these mice, clenbuterol was able to improve glucose homeostasis, suggesting that BAT is not essential for these beneficial effects.

After these studies in mice, we next investigated if activation of the β_2-AR could also improve skeletal muscle glucose uptake in humans (**Chapter 5**). For this study, we treated healthy, young, male subjects with clenbuterol for two weeks and subsequently measured insulin-stimulated skeletal muscle glucose uptake. In line with the results found in mice (**Chapter 3** and **Chapter 4**), clenbuterol could also enhance insulin-stimulated skeletal muscle glucose uptake in humans, although it remains unknown how this exactly works inside the skeletal muscle. Combined, the animal and human studies described in this thesis demonstrate that activation of β_2-AR could potentially be used as a therapy to improve glucose homeostasis in patients diagnosed with T2DM. However, future studies in T2DM patients are required to further explore the therapeutic potential of β_2-agonist treatment.

The second potential mechanism for the improved glucose regulation upon cold exposure is the occurrence of shivering. When the body loses a lot of heat during cold exposure, it will start to produce extra heat via involuntary muscle contractions, a phenomenon known as shivering. Muscle contractions are known to enhance skeletal muscle glucose uptake and hence, some level of (mild) shivering or muscle activity may be involved in improving glucose homeostasis upon repeated cold exposure. However, it has never been directly tested whether cold exposure with shivering can improve glucose homeostasis in humans. In **Chapter 6** of this thesis, we therefore exposed overweight/obese individuals to shivering via cold exposure for 10 days, with at least 1 hour of shivering per day. Interestingly, the 10 days of cold exposure with shivering not only led to improvements in glucose tolerance, but also in blood lipid content and blood pressure. However, the exact mechanisms responsible for these effects are still not known. Nevertheless, the results of this study indicate that shivering could potentially be a way by which cold exposure improves glucose homeostasis.

What is the contribution of the results from this research to science and social challenges?

The number of people diagnosed with T2DM has increased tremendously over the past few decades. As T2DM is well-known to increase the risk for the development of other diseases, the latter creates an extraordinary economic burden for health care funds and society itself, as well as reduces the quality of life of millions of people. Finding new ways to treat patients with T2DM is therefore highly important to improve the global quality of life, alleviate the immense pressure currently experienced by the health care system, and help lower ever-increasing costs of health care.

This thesis contributes to this search for new ways to improve glucose homeostasis and highlights an alternative lifestyle strategy for people at risk for, or patients with, T2DM. It is however important for future research to determine the frequency and intensity by which patients should be exposed to cold to maximally improve glucose homeostasis. The outcomes of the research described in this thesis also shed some light on the potential physiological mechanisms through which cold exposure improves skeletal muscle glucose uptake and glucose homeostasis in humans. Since not all relevant target groups could be studied in the context of this thesis and since not all findings could be mechanistically explained, this thesis also provides a foundation for other researchers to further investigate the effects of cold exposure or activation of β_2-ARs on glucose homeostasis.

To whom are the research results interesting and/or relevant?

The outcomes of the studies described in this thesis are primarily interesting for other researchers within the field of metabolism and/or diabetes, as this newly generated knowledge provides additional information with respect to the effects of cold exposure on glucose homeostasis. The results described in this thesis will be published in scientific journals in order for this information to become available to these researchers. In addition, the research results will be highlighted at various national and international congresses within the field of metabolism, which are visited by researchers all over the globe. Individuals that participated in these clinical studies will also be informed about the results by means of special participant events, as well as internet and social media.

Besides other researchers, these results could also be interesting for the pharmaceutical industry, as future research within this field could potentially identify new targets for drug development. The development of these drugs could provide T2DM patients with a whole new class of medication to improve their disease state. In addition, the outcomes of the studies described in this thesis could potentially lead to the foundation of new companies providing cold exposure therapy to T2DM patients. The results are also interesting for individuals with prediabetes or T2DM patients, as it could provide them with additional knowledge on how to improve their disease state. The outcomes of the studies described in this thesis could also potentially contribute to new lifestyle advices for T2DM patients, thereby also making these results interesting for general practitioners or lifestyle coaches. Finally, these results may also be of interest for policy makers, as it may indicate that lowering ambient temperature (e.g. in offices) may not only be favourable to minimize energy consumption, but may also have beneficial health effects.

Summary

The high prevalence of obesity has led to a global escalation in the number of people diagnosed with type 2 diabetes mellitus (T2DM). Non-medical treatment options to improve glucose homeostasis in these patients mainly consist of lifestyle interventions, such as exercise- and dietary regimes. Nevertheless, these forms of interventions are not widely enthused by most humans, as evidenced by the low long-term adherence rates. Therefore, there is the need for new therapies to improve glucose homeostasis in patients with T2DM.

In search for alternative strategies to improve the disease state of T2DM patients, our research group has previously shown that repeated exposure to mild cold (i.e. mild cold acclimation) improves insulin-stimulated glucose disposal by ~43% in T2DM patients. Further elaboration on the mechanisms underlying these improvements revealed that these effects were primarily attributed to an increased uptake of glucose by skeletal muscle, although the exact intracellular pathways involved remained unknown. Nevertheless, given the pronounced effects of cold exposure on glucose homeostasis, identification of the underlying mechanisms mediating skeletal muscle glucose uptake upon cold exposure could reveal new therapeutic avenues for the treatment of T2DM. In this thesis, it was therefore investigated whether activation of β_2-adrenergic receptors (β_2-ARs) or the occurrence of shivering could underlie these observed improvements in skeletal muscle glucose uptake and glucose homeostasis following cold acclimation.

This thesis primarily focusses on potential mechanisms by which cold enhances skeletal muscle glucose uptake. However, it is also possible that other metabolic tissues contribute to cold-induced improvements in glucose homeostasis. To gain more insights into the contribution of different tissues to cold-induced effects on glucose homeostasis, a comprehensive literature study was performed in **Chapter 2**. In this chapter, emphasis was put on the effects of cold exposure on the pancreas, liver, (white) adipose tissue, and skeletal muscle – the primary organs regulating glucose homeostasis – whereas only limited attention was paid to brown adipose tissue (BAT), as the abundance of this tissue is relatively low in overweight/obese adult humans. Based on the studies reviewed in this chapter, it was concluded that adaptations within skeletal muscle, resulting in an increased glucose uptake, are key for the effects of cold exposure on glucose homeostasis, although a potential role for the liver and white adipose tissue should not be overlooked.

The first mechanism by which cold exposure could enhance skeletal muscle glucose uptake is via activation of the sympathetic nervous system and subsequent activation of the highly expressed β_2-AR located on skeletal muscle cells. Activation of β_2-ARs has previously been associated with an increased glucose uptake *in vitro*. However, whether activation of β_2-ARs also stimulates skeletal muscle glucose uptake *in vivo* was entirely unknown. In **Chapter 3**, diet-induced obese, insulin resistant mice were treated with the selective β_2-agonist clenbuterol. It was found that clenbuterol could stimulate glucose uptake by skeletal muscle *in vivo*, thereby highlighting a physiological relevance of this receptor in mediating skeletal muscle glucose uptake. Furthermore, treatment with clenbuterol markedly improved both glucose and insulin tolerance in these mice, indicating a potential therapeutical relevance as well.

Although the findings in **Chapter 3** are highly promising, it could be hypothesized that these effects are partly mediated via activation of BAT, as clenbuterol has a low affinity towards the primary receptors involved in BAT activation (i.e. β_1- and β_3-AR), and rodents are characterised by large volumes of this metabolically active tissue. In terms of a potential therapeutical relevance for T2DM patients, it is highly important to investigate the contribution of BAT in these β_2-agonist-mediated improvements in glucose homeostasis, as overweight/obese individuals generally have small BAT depots. In **Chapter 4**, the effects of clenbuterol treatment on whole-body glucose homeostasis were therefore investigated in diet-induced obese, insulin resistant mice ablated of UCP1, the key protein mediating BAT thermogenesis. Remarkably, clenbuterol treatment significantly improved glucose homeostasis even in the absence of thermogenically functional BAT, indicating that BAT is not indispensable for β_2-agonist-mediated improvements in glucose homeostasis. These findings suggest that other tissues than BAT are responsible for β_2-adrenergic stimulated improvements in glucose homeostasis, and therefore suggests that such an approach may also be effective in humans who are characterized by low amounts of BAT.

In **Chapter 5**, the findings of these rodent studies were translated into a clinical setting to investigate whether treatment with a β_2-agonist could boost skeletal muscle glucose uptake in humans. Thus, healthy, young, male subjects were treated with clenbuterol or a placebo for 2-weeks in a randomised, placebo-controlled, double-blinded cross-over study, after which insulin-stimulated skeletal muscle glucose uptake was determined with the golden standard hyperinsulinemic-euglycemic clamp technique. Interestingly, even in these healthy subjects that do not have impairments in their glucose homeostasis,

clenbuterol treatment could significantly enhance insulin-stimulated skeletal muscle glucose uptake. Unfortunately, the molecular mechanisms involved in these effects could not be identified. The findings in **Chapter 5** are nevertheless highly important, as they potentially indicate that similar beneficial effects could occur in individuals with an impaired glucose homeostasis, such as T2DM patients.

Besides activation of β_2-ARs, shivering during cold exposure could also explain the previously observed improvements in skeletal muscle glucose uptake and glucose homeostasis upon cold acclimation. Thus, in our previous mild cold acclimation study, subjects self-reported the occurrence of tense muscles (i.e. low-grade shivering), and a follow-up study with measures to prevent shivering was unable to reproduce the positive effects of mild cold acclimation in T2DM patients, hinting towards an important role for shivering. However, studies investigating the effects of repeated cold-induced shivering on glucose homeostasis are largely lacking. In **Chapter 6**, overweight/obese subjects were therefore exposed to cold-induced shivering for 10 days (with at least 1 hour of shivering per day) and effects on glucose homeostasis were investigated before, the day after a single cold exposure, and after cold acclimation with shivering by means of oral glucose tolerance tests (OGTT). It was found that cold acclimation with shivering improved glucose clearance during an OGTT in these overweight/obese subjects, suggesting an improvement in glucose homeostasis. In addition, profound reductions were observed in fasting glucose, free-fatty acids, and triglyceride concentrations, whereas also robust improvements in blood pressure were reported. Combined, these results indicate that repeated cold-induced shivering could potentially be an alternative strategy to not only improve glucose homeostasis in T2DM patients, but also components of the metabolic syndrome associated with overweight/obesity.

When combining the results described in **Chapters 3-6**, it can be concluded that both activation of β_2-ARs and the occurrence of shivering could explain our previously observed improvements in skeletal muscle glucose uptake and whole-body glucose homeostasis following mild cold acclimation in T2DM patients. In addition, the results of these studies strongly suggest that both these mechanisms could potentially be exploited as an alternative treatment strategy to improve the disease state of individuals with prediabetes or T2DM patients. However, further studies are needed to investigate the clinical relevance of these therapeutic avenues.

Samenvatting

De hoge prevalentie van obesitas heeft geleid tot een wereldwijde escalatie van het aantal mensen die zijn gediagnostiseerd met type 2 diabetes mellitus (T2DM). Niet-medische behandelopties om de glucosehomeostase van deze patiënten te verbeteren bestaan voornamelijk uit leefstijlinterventies, zoals bewegings- en dieetregimes. Toch zijn deze vormen van therapie niet erg populair bij de meeste mensen, zoals blijkt uit de lage therapietrouwheid. Om deze reden is er dringend behoefte aan nieuwe therapieën om de glucosehomeostase van mensen met T2DM te verbeteren.

In de zoektocht naar alternatieve behandelstrategieën heeft onze onderzoeksgroep eerder aangetoond dat herhaalde blootstelling aan milde kou (oftewel koude acclimatisatie) de glucoseopname door insuline met ~43% kon verbeteren in patiënten met T2DM. Deze effecten werden voornamelijk veroorzaakt door een verhoogde glucoseopname door de skeletspieren, alhoewel de precieze intracellulaire mechanismen onbekend bleven. Aanvullend onderzoek naar de onderliggende mechanismen zou daarom nieuwe therapieën kunnen onthullen voor de behandeling van T2DM. In dit proefschrift werd daarom onderzocht of activering van de β_2-adrenerge receptoren (β_2-AR) of het rillen van de spieren verantwoordelijk zouden kunnen zijn voor de verbeteringen in glucoseopname door de spier en de glucosehomeostase tijdens koude acclimatisatie.

Dit proefschrift richt zich voornamelijk op potentiële mechanismen waarmee kou de glucoseopname door de spier kan verbeteren. Het is echter ook mogelijk dat andere metabole weefsels bijdragen aan de verbeteringen in glucosehomeostase na blootstelling aan kou. Om meer inzicht te krijgen in de bijdrage van verschillende weefsels aan de effecten van kou op de glucosehomeostase, werd in **Hoofdstuk 2** een uitgebreide literatuurstudie uitgevoerd. In dit hoofdstuk werd de nadruk gelegd op de effecten van kou op de alvleesklier, lever, (wit) vetweefsel en skeletspieren – de primaire organen die de glucosehomeostase reguleren. Daarentegen werd slechts beperkte aandacht besteed aan het bruin vetweefsel (BAT), omdat dit weefsel relatief weinig voorkomt bij volwassenen met overgewicht en obesitas. Uit dit literatuuronderzoek werd geconcludeerd dat adaptaties in de skeletspieren, die resulteren in een verhoogde glucoseopname, zeer belangrijk zijn voor de effecten van kou op de glucosehomeostase. Een mogelijke rol voor het wit vetweefsel en de lever mag echter niet over het hoofd worden gezien.

Het eerste mechanisme waarmee kou de glucoseopname door de spier kan verbeteren is via activatie van het sympathische zenuwstelsel en de daaropvolgende activatie van β_2-AR die zich bevinden op spiercellen. Activering van β_2-AR is eerder in verband gebracht

met een verhoogde glucoseopname in spiercellen *in vitro*. Het was echter geheel onbekend of activering van β_2-AR ook de glucoseopname in spieren kan verhogen *in vivo*. In **Hoofdstuk 3** werden daarom muizen met obesitas en insulineresistentie behandeld met de selectieve β_2-agonist clenbuterol. De resultaten van deze studie lieten zien dat clenbuterol de glucoseopname door de spieren in deze muizen kon stimuleren. Daarnaast verbeterde de behandeling met clenbuterol zowel de glucose- als insulinetolerantie van deze muizen, duidend op een mogelijke therapeutische relevantie.

Alhoewel de bevindingen in **Hoofdstuk 3** veelbelovend zijn, is het mogelijk dat deze effecten gedeeltelijk worden veroorzaakt door activering van het BAT. Clenbuterol heeft namelijk een lage affiniteit voor de primaire receptoren die betrokken zijn bij de activering van het BAT (namelijk de β_1- en β_3-AR) en muizen worden gekenmerkt door een grote hoeveelheid van dit metabool actief weefsel. Aangezien mensen met overgewicht/obesitas relatief weinig BAT hebben, is het van groot belang de bijdrage van het BAT aan de effecten van clenbuterol op de glucosehomeostase te onderzoeken. In **Hoofdstuk 4** werden daarom de effecten van clenbuterol onderzocht in muizen met obesitas en insulineresistentie die daarnaast het belangrijkste eiwit miste voor BAT thermogenese, namelijk UCP1. In lijn met de resultaten van onze vorige studie verbeterde clenbuterol de glucosehomeostase aanzienlijk, zelfs in de muizen die geen functioneel BAT hadden. Deze bevindingen suggereren dat de positieve effecten van β_2-agonisten op de glucosehomeostase niet afhankelijk zijn van het BAT en dat andere weefsels hiervoor verantwoordelijk zijn. Een dergelijke behandeling met β_2-agonisten zou daarom ook effectief kunnen zijn voor het verbeteren van de glucosehomeostase bij mensen met een lage hoeveelheid BAT, zoals patiënten met T2DM.

In **Hoofdstuk 5** werden de bevindingen van deze muizenstudies vertaald naar een klinische setting om te onderzoeken of behandeling met een β_2-agonist de glucoseopname door de spieren ook kan verhogen in mensen. In dit hoofdstuk werden gezonde, jonge, mannelijke proefpersonen gedurende twee weken behandeld met clenbuterol of een placebo in een gerandomiseerde, placebogecontroleerde, dubbelblind, cross-over studie. Na deze behandeling werd de glucoseopname in de spier tijdens insuline stimulatie onderzocht. Zelfs bij deze volledig gezonde proefpersonen kon clenbuterol de glucoseopname door de spier tijdens insuline stimulatie sterk verbeteren. Echter konden de moleculaire mechanismen die hierbij betrokken zijn niet worden geïdentificeerd. Desalniettemin zijn de bevindingen in **Hoofdstuk 5** van groot belang, aangezien ze aangeven dat soortgelijke effecten mogelijk kunnen optreden bij mensen met een verstoorde glucosehomeostase, zoals patiënten met T2DM.

Naast activering van de β_2-AR, zou het rillen van de spieren tijdens blootstelling aan kou ook verantwoordelijk kunnen zijn voor de verbeteringen in glucoseopname door de spier en de glucosehomeostase. In onze vorige studie met acclimatisatie aan milde kou rapporteerde de proefpersonen namelijk zelf het optreden van gespannen spieren (d.w.z. lichte rillingen). Daarnaast was een vervolgstudie met extra maatregelen om rillen te voorkomen niet in staat de gunstige effecten van koude acclimatisatie te repliceren, duidend op een belangrijke rol voor rillen. Echter zijn er tot op heden geen studies die de effecten van herhaalde blootstelling aan kou met rillen op de glucosehomeostase hebben onderzocht. In **Hoofdstuk 6** werden proefpersonen met overgewicht/obesitas daarom gedurende 10 dagen blootgesteld aan rillen door kou (met tenminste 1 uur rillen per dag). De effecten op de glucosehomeostase werden onderzocht vóór, een dag na eenmalige blootstelling aan kou met rillen, en na koude acclimatisatie met rillen door middel van orale glucosetolerantie testen (OGTT). Koude acclimatisatie met rillen verbeterde de glucoseklaring tijdens een OGTT, duidend op een verbetering van de glucosehomeostase. Daarnaast werden sterke verlagingen waargenomen in nuchtere glucose, vrije vetzuren, en triglyceridenconcentraties, terwijl ook grote verbeteringen in bloeddruk werden gevonden. Samen duiden deze resultaten erop dat herhaalde blootstelling aan rillen door kou een alternatieve strategie zou kunnen zijn om zowel de glucosehomeostase alsmede overige componenten van het metabool syndroom te verbeteren in patiënten met T2DM.

Wanneer de resultaten van **Hoofdstuk 3-6** worden gecombineerd, kan worden geconcludeerd dat zowel activering van β_2-AR alsmede het rillen van de spieren tijdens blootstelling aan kou verantwoordelijk kunnen zijn voor een verhoogde glucoseopname door de spier en verbeteringen in glucosehomeostase. Bovendien suggereren de resultaten van deze onderzoeken dat beide mechanismen mogelijk kunnen worden gebruikt als alternatieve behandelstrategieën om de ziektetoestand van mensen met prediabetes of patiënten met T2DM te verbeteren. Echter zijn vervolgstudies nodig om de klinische relevantie van deze therapieën te onderzoeken.

About the author

Sten van Beek was born on August 18th 1995 in Eindhoven, the Netherlands. After completing high school at Pleincollege Bisschop Bekkers in 2013, he enrolled in the bachelor program Biomedical Sciences at Maastricht University, which he completed with distinction (cum laude) in 2016. After obtaining his bachelor's degree, he started the master program Biomedical Sciences at Maastricht University with a specialisation in Nutrition and Metabolism. During his senior practical internship, he visited the laboratory of Prof. Dr. Tore Bengtsson at the Department of Molecular Biosciences at Stockholm University, Stockholm, Sweden, where he investigated the effects of β_2-adrenergic receptor stimulation on skeletal muscle glucose uptake and whole-body glucose homeostasis in diet-induced obese, insulin-resistant mice.

After obtaining his master's degree with distinction (cum laude) in 2018, he applied for the NUTRIM NWO Graduate Program grant to finance (part of) his own PhD, which was honoured in the summer of 2018. From September 2018 onwards, he was a PhD candidate within the Department of Nutrition and Movement Sciences under supervision of Prof. Dr. Patrick Schrauwen, Dr. Joris Hoeks and Dr. Bas Havekes, with a special focus on the mechanisms of cold-induced improvements in glucose homeostasis.

List of publications

1. **van Beek S**, Bengtsson T, Hoeks J. Physiological and molecular mechanisms of cold-induced improvements in glucose homeostasis beyond brown adipose tissue. *Submitted*.

2. Kalinovich A, Dehvari N, Åslund A, **van Beek S**, Halleskog C, Olsen J, et al. Treatment with a β-2-adrenoceptor agonist stimulates glucose uptake in skeletal muscle and improves glucose homeostasis, insulin resistance and hepatic steatosis in mice with diet-induced obesity. *Diabetologia*. 2020.

3. **van Beek S**, Kalinovich A, Schaart G, Bengtsson T, Hoeks J. Prolonged beta2-adrenergic agonist treatment improves glucose homeostasis in diet-induced obese UCP1(-/-) mice. *Am J Physiol Endocrinol Metab*. 2021.

4. **van Beek S**, Bruls Y, Vanweert F, Fealy C, Connell N, Schaart G, et al. Beta-2 agonist treatment promotes insulin-stimulated skeletal muscle glucose uptake in healthy males in a randomised placebo-controlled trial. *Submitted*.

5. **van Beek S**[*], Sellers A[*], Hashim D, Pallubinsky H, Kornips E, Schaart G, et al. Repeated cold-induced shivering improves glucose tolerance and components of the metabolic syndrome in overweight and obese adults. *Manuscript in preparation*.

[*] These authors contributed equally

Dankwoord

Dan is toch ein-de-lijk het moment daar dat ik mijn dankwoord mag schrijven! Ik ben stiekem toch wel trots op het boekje dat jij momenteel aan het lezen bent, maar het voelt verkeerd dat alleen mijn naam op de voorkant staat, want dit boekje was er nooit geweest zonder een heleboel mensen die mij immens hebben geholpen en gesteund, zowel op het werk als daarbuiten. Deze mensen hebben er dan ook enorm aan bijgedragen dat mijn PhD een onvergetelijke tijd is geworden en daarom zou ik ze heel graag willen bedanken!

Als eerste wil ik graag mijn promotieteam bedanken. **Joris**, wat ben ik blij dat jij mijn tutor was in mijn tweede jaar van mijn bachelor Biomedische Wetenschappen en dat jij mij de kans hebt gegeven om bij jou in de groep mijn stages en PhD te doen. We hebben er inmiddels al heel wat jaren opzitten (ik denk inmiddels al 8?), maar ik wil je heel erg bedanken voor alles wat je mij hebt geleerd en het vertrouwen dat je mij hebt gegeven met het uitvoeren van onderzoek. Hoewel ik in het begin nogal schrok van de hoeveelheid rode tekst die ik terug kreeg van je, heb ik ook enorm veel geleerd van hoe jij schrijft en dat bewonder ik tot op de dag van vandaag. Het was daarnaast heel fijn dat ik altijd even snel bij je binnen kon lopen voor een (meestal niet zo) kort praatje, zelfs als het ging over niet serieuze zaken. Dat ga ik zeker missen! **Patrick**, ik wil je enorm bedanken voor de kans om binnen jouw onderzoeksgroep mijn promotietraject te doen. Jouw manier van denken en schrijven vind ik enorm bewonderingswaardig en ik heb daar enorm van geleerd en genoten! **Bas**, dankjewel voor alle (medische) hulp de afgelopen jaren. Ik kan me voorstellen dat je enigszins je twijfels had toen een nieuwe PhD student een of ander Bulgaars middel wilde toedienen bij mensen, maar zonder jouw hulp was het nooit gelukt dit onderzoek af te ronden. Laten we er snel nog eens eentje op drinken!

Daarnaast wil ik ook de overige leden van de DMRG board graag bedanken voor alle hulp gedurende mijn PhD: **Matthijs, Mijke, Vera en Esther P**. Esther P., een speciaal bedankje voor jou voor alle hulp tijdens de afwezigheid van Joris en daarnaast voor het feit dat je altijd een luisterend oor had, zelfs als het ging om zaken buiten werk!

I would like to thank all members of the assessment committee: **Prof. Dr. Ellen Blaak, Prof. Dr. Bastiaan de Galan, Prof. Dr. Daisy Jonkers, Dr. Denis Blondin, and Dr. Erik Kalkhoven** for their approval of my thesis and presence during my PhD defence.

Ik wil ook alle proefpersonen die hebben meegedaan aan mijn onderzoeken enorm bedanken! Zonder jullie hulp is wetenschappelijk onderzoek onmogelijk en was dit

boekje nooit tot stand gekomen. Daarnaast hebben jullie er ook aan bijgedragen dat de testdagen altijd een plezier waren, met veel ruimte voor lachen en leuke gesprekken.

I would also like to thank all the (ex) members of the DMRG group: **Yvonne, Kay, Anne, Anna, Charlotte, Evi, Froukje, Frieder, Jeremy, Julian, Marit, Rodrigo, Ivo, Dzhansel, Pip, Alex, Emmani, Bas, Daniel, Edmundo, Frederieke, Elena, Maaike, Ciarán, Stephanie, Yvo, Manon, Nynke, Lotte, Pandi, Jeroen, Tineke, Marlies, Niels, Carlijn, Vera and Evelyn.** Thank you for all the help during my PhD and for all the laughs during work! It made my time as a PhD unforgettable. Also a big thank you to all the interns that have helped me during my research: **Tilly, Dlan, Brenda, and Dzhansel.**

Daarnaast ook een speciale dank aan **Gert, Esther K., en Johanna** voor alle hulp met de analyses die zijn uitgevoerd voor mijn studies. Ik heb veel van jullie geleerd en zonder jullie hulp was dit boekje er niet geweest!

Natuurlijk ook een speciale dank aan **Yolanda** en **Désirée** voor alle ondersteuning de afgelopen jaren. Het was enorm fijn dat jullie altijd bereid waren om te helpen!

Marlies, ik ben echt enorm blij dat jij mijn paranimf bent! Maar ik ben nog veel blijer dat je zo'n goede vriendin van me bent geworden over de afgelopen jaren. Tijdens de start van mijn PhD heb ik ontzettend veel van jou geleerd, maar gelukkig was er ook altijd ruimte voor lachen en (nogal) vreemde gesprekken. Ik denk niet dat ik de volgende woorden ook maar enigszins hoef uit te leggen en je snapt de context al: dumpster, zoon, blauwe bakken, how low can you go. En dit zijn er maar een paar. Ook buiten de PhD is het altijd gezellig tijdens de spelletjesavonden en de kopjes koffie en ik ben je enorm dankbaar voor alle steun en hulp de afgelopen jaren, vooral ook buiten het werk!

Niels, ik ben trots dat jij mijn paranimf bent en dat je straks naast me staat in de aula! Ik sta er altijd van versteld hoeveel jij weet en jouw specialisatie in pranks is legendarisch (ook al ben ik zelf regelmatig het slachtoffer geweest). Ik heb enorm veel van je geleerd en ik ben je dankbaar voor alle hulp tijdens de testdagen, maar vooral voor al het lachen tijdens de werkdagen. Het heeft een meerdere moeilijke/zware dagen een heel stuk aangenamer gemaakt. Daarnaast ben ik heel blij dat je ook naast het werk altijd in bent voor een kopje koffie om gezellig bij te kletsen of om advies/steun te geven, zelfs als jouw schema al volledig vol is. Daar wil ik je enorm voor bedanken!

Adam, I want to thank you for the amazing collaboration and all the help during the shivering study. I still find it remarkable that we have been able to pull off a grand total of 210 test days within a year, which would have been completely impossible without your

help. I could not have imagined a better colleague to have done this study with. All the early mornings and weekends of work have been worth it! I admire your eye for detail and I will always remember our great discussions about science. I wish you all the best in your future endeavours and I can't wait to be your paranymph for your defence!

A massive thank you is also in place for **Dzhansel**. Dzhansel, thank you for being able to help Adam and me with the shivering study, even when this meant starting four months early with your internship. Without your help, we would not have been able to finish this study as fast as we did, and you did it without a single complaint (even when you had to work at 07:30 on Saturdays and Sundays for weeks in a row). I wish you good luck with your PhD, but I am 100% sure you will do great! Keep me up to date about the results of your own shivering study, as I am very curious!

Een speciale dank aan de overige leden van de TherMU groep voor alle hulp en steun de afgelopen jaren: **Wouter, Hannah, Pascal, en Wei**. Ik heb veel van jullie geleerd!

Tore and **Anastasia**, thank you for welcoming me with open arms in your group at Stockholm University during my senior master internship. I had an amazing time in Sweden and I learned a lot from both of you. I am also incredibly grateful that we continued our collaboration during my PhD. Also a massive thank you to all the other colleagues at Stockholm University for helping me during my internship!

Yvonne, dankjewel voor de leuke gesprekken op het kantoor en de hulp tijdens alle testdagen. Het was fijn om na al die maanden alleen op het kantoor, weer een kamergenoot te hebben om gezellig mee te kunnen kletsen!

Ellen en **Peter**, dank jullie wel voor alle hulp met de clenbuterol studie! Zonder jullie hulp was het niet mogelijk geweest om de FMD metingen te doen en was de paper niet zo mooi geweest als dat hij nu is.

Maikel, ik wil je heel erg bedanken voor al het samen sporten, lachen en de eindeloze steun/adviezen de afgelopen jaren. Jouw steun en advies heeft me enorm geholpen, zeker de afgelopen paar maanden! Ik vind het prachtig om te zien hoe jij in het leven staat en vooral hoe Marlies en jij samen kleine Tobias opvoeden. Hopelijk mag ik dit nog vele jaren van dichtbij bewonderen. Ik ga je wel waarschuwen, ik ga je nog inhalen op de bench!

Emmani and **Axel,** thank you for being such great friends! I always love hanging out with you as it puts a big smile on my face. You are both amazing and warm people, and I am

incredibly grateful for that. Thank you for being there for me throughout the past couple of years, also in the tough times. I am super excited that you will both be in Maastricht now and I am looking forward to many more game nights and shopping trips together. Let's plan something again soon!

Jan and **Sophia**, even though you are all the way in Munich, I want to thank you for being great friends! We have known each other for quite some years now, but I am very happy that we are still in touch and we visit each other regularly. Thank you for all the smiles and adventures, hopefully I will be able to come visit again soon!

Andreas and **Marissa**, who would have thought that the corona pandemic would actually bring about some good things as well! Andreas, I am incredibly grateful to have met you and to be able to call you one of my best friends even though we haven't known each other for that long. Yes, you read it correctly, *one of* my best friends. You can fight the other ones at the defence. I love every second of our constant bantering and I look forward to many more boys nights, holidays and gaming sessions! Marissa, thank you for everything the past couple of years and I am super excited for many more barbecues, game nights and holidays!

Iris, broooooootje! We kennen elkaar inmiddels al een flink aantal jaren, maar ik ben ontzettend blij dat we nog steeds regelmatig afspreken! Met jou is geen enkele avond saai en is het altijd veel lachen. Laten we snel nog eens afspreken om bij te kletsen!

Alex, thank you for all the laughs and dank memes over the last couple of years! It was incredibly fun to have you as my supervisor during my bachelor internship and it's a shame we only got to work together for 2 months when I started my PhD. Nevertheless, I am happy that we are still in touch and let's plan something again soon to catch up!

Bart, ik vind het prachtig om te zien dat wij al sinds groep 5 van de basisschool beste vrienden zijn! Ook al missen we regelmatig nog elkaars telefoontje (volgens mij had ik je al 2 weken geleden moeten bellen op het moment dat ik dit schrijf), maar onze vriendschap blijft altijd hetzelfde. Ik wil je bedanken voor alle memorabele momenten (we need more ships, el snorro, SIR put the gun down, talking to the moon) en de onvergetelijke vakanties samen. Zonder jou was ik niet de persoon die ik vandaag de dag ben, en daar ben ik je enorm dankbaar voor. Hopelijk kunnen we snel weer eens afspreken en een nieuwe vakantie inplannen!

Niki, sista! Wat ben ik ontzettend trots om jou als mijn grote zus te hebben. Je bent al heel mijn leven een enorme inspiratiebron voor me en ik ben zo trots op hoe jij je de

afgelopen jaren hebt ontwikkeld en momenteel in het leven staat. Je doet het fantastisch en ik ben heel erg blij om te zien dat je zo gelukkig bent met Dion. Ik kan niet wachten om snel weer eens een broer-zus dag te doen!

Harry en **Wilma,** lieve papa en mama, ik denk niet dat woorden genoeg zijn om jullie te vertellen hoe erg ik jullie wil bedanken voor alles. Jullie zijn er altijd voor me geweest, in de goede en slechte momenten, en ik ben zo ontzettend trots om jullie als mijn ouders te hebben. Ik vind het zo fijn dat jullie ons hebben geleerd hoe belangrijk familie is en dat er altijd een thuis is waar je op terug kunt vallen als iets misgaat. Zonder al jullie steun was dit boekje er nooit geweest. Ik hou van jullie!