Inhibition of acetyl-CoA carboxylase 2 enhances skeletal muscle fatty acid oxidation and improves whole-body glucose homeostasis in *db/db* mice

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Abstract

Aims/hypothesis Excessive ectopic lipid deposition contributes to impaired insulin action in peripheral tissues and is considered an important link between obesity and type 2 diabetes mellitus. Acetyl-CoA carboxylase 2 (ACC2) is a key regulatory enzyme controlling skeletal muscle mitochondrial fatty acid oxidation; inhibition of ACC2 results in enhanced oxidation of lipids. Several mouse models lacking functional ACC2 have been reported in the literature. However, the phenotypes of the different models are inconclusive with respect to glucose homeostasis and protection from diet-induced obesity.

Methods Here, we studied the effects of pharmacological inhibition of ACC2 using as a selective inhibitor the *S* enantiomer of compound 9c ([*S*]-9c). Selectivity was confirmed in biochemical assays using purified human ACC1 and ACC2.

Results (*S*)-9c significantly increased fatty acid oxidation in isolated extensor digitorum longus muscle from different

mouse models (EC₅₀ 226 nmol/l). Accordingly, short-term treatment of mice with (*S*)-9c decreased malonyl-CoA levels in skeletal muscle and concomitantly reduced intramyocellular lipid levels. Treatment of *db/db* mice for 70 days with (*S*)-9c (10 and 30 mg/kg, by oral gavage) resulted in improved oral glucose tolerance (AUC −36%, *p*<0.05), enhanced skeletal muscle 2-deoxy-2-[¹⁸F]fluoro-D-glucose (FDG) uptake, as well as lowered prandial glucose (−31%, *p*<0.01) and HbA_{1c} (−0.7%, *p*<0.05). Body weight, liver triacylglycerol, plasma insulin and pancreatic insulin content were unaffected by the treatment.

Conclusions/interpretation In conclusion, the ACC2-selective inhibitor (*S*)-9c revealed glucose-lowering effects in a mouse model of diabetes mellitus.

Keywords Diabetes · IMCL · Intramyocellular lipid · Metabolic flexibility · Muscle lipid · Pharmacological inhibitor

Abbreviations

(<i>S</i>)-9c	<i>S</i> Enantiomer of compound 9c
ACC	Acetyl-CoA carboxylase
CT	Computed tomography
EDL	Extensor digitorum longus
FDG	2-Deoxy-2-[¹⁸ F]fluoro-D-glucose
IMCL	Intramyocellular lipid
<i>K</i> _i	Patlak constant
MR	Magnetic resonance
MRS	Magnetic resonance spectroscopy
NMRI	Naval Medical Research Institute
PET	Positron emission tomography
RP	Reverse phase
VOI	Volume of interest
ZDF	Zucker diabetic fatty

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Introduction

A disturbed response of skeletal muscle to insulin stimulation (insulin resistance) contributes to a number of metabolic disorders, including type 2 diabetes mellitus. Obesity-related insulin resistance is associated with reduced lipid oxidation in skeletal muscle and increased intracellular lipid deposition (intramyocellular lipid [IMCL]) [1, 2] and a causal relationship between increased IMCL and insulin resistance has been proposed [3, 4]. Accordingly, an inverse relationship between IMCL and insulin sensitivity has been reported in high-fat-feeding models [5], non-obese people [6] and lean offspring of type 2 diabetic individuals [7], as well as in obese non-diabetic individuals and diabetic individuals receiving a very-low-energy diet [8]. Pharmacological approaches that aim to reduce IMCL levels therefore appear attractive options to improve whole-body glucose homeostasis.

Mammalian acetyl CoA carboxylases (ACCs) catalyse the formation of malonyl-CoA from acetyl-CoA [9]. The regulation of ACC activity is complex and includes phosphorylation and dephosphorylation of the ACC protein as well as allosteric regulation [10, 11]. For example, insulin stimulation results in dephosphorylation and activation of ACC [12], while AMP-activated protein kinase (AMPK) phosphorylates and inhibits ACC [13]. Malonyl-CoA—synthesized by ACC1, which is found in the cytosol—is used for de novo synthesis of fatty acids. In contrast, ACC2—localised at the mitochondrial surface—regulates mitochondrial fatty acid oxidation through malonyl-CoA-mediated inhibition of carnitine palmitoyltransferase 1 (CPT1). In particular, the inhibition of ACC2 has been predicted to reduce intracellular lipids by enhancing the use of fat for energy production. Accordingly, genetic deletion of *Acc2* (also known as *Acacb*) has been reported to result in reduced muscle malonyl-CoA levels and enhanced fatty acid oxidation [14, 15], though this has not been consistently demonstrated [16]. However, pharmacological inhibition using isoform-non-selective ACC inhibitors stimulated fat oxidation and reduced plasma triacylglycerol levels in a rodent model of dyslipidaemia [17, 18]; an unresolved question is whether specific inhibition of ACC2 is sufficient to achieve a comparable effect.

Muscle *ACC2* expression is increased in states of obesity and insulin resistance, whereas it decreases with weight loss [2, 19–21]. Genetic deletion or inactivation of *Acc2* in mice gave inconsistent results [14–16]. The first published *Acc2*-knockout mice were protected against diet-induced obesity and diabetes by mechanisms involving enhanced fatty acid oxidation and increased energy expenditure [14, 22–24], data that were not consistently reproduced in two further knockout mice [15, 16]. The reasons for the discrepancy are unresolved, but may relate to the

different mouse strains and knockout strategies used in the studies [15, 16]. To our knowledge, there are no data available investigating the effects of pharmacological inhibition on diet-induced obesity and diabetes using isoform-2-selective ACC inhibitors. The aim of the present study was, therefore, to investigate the effects of an isoform-selective ACC2 inhibitor on glucose homeostasis and body weight in a mouse model of obesity-related diabetes mellitus.

Methods

ACC activity measurement

A human ACC2 construct lacking 128 N-terminal amino acids (nt 385–6966, GenBank entry AJ575592), and full-length human ACC1 (nt 192–7229, GenBank entry NM_198836) were produced in insect cells using a baculoviral expression system and were purified by anion-exchange chromatography. ACC activity was measured using a NADH-linked kinetic method as follows. The assay was carried out in a buffer containing 100 mmol/l Tris (pH 7.5), 10 mmol/l trisodium citrate, 25 mmol/l KHCO₃, 10 mmol/l MgCl₂, 0.5 mg/ml BSA, 3.75 mmol/l reduced L-glutathione, 15 U/ml lactate dehydrogenase, 0.5 mmol/l phosphoenolpyruvate, 15 U/ml pyruvate kinase and compounds at indicated concentrations (final DMSO concentration 1% [vol./vol.]). The enzymatic reaction was started by adding a mixture of (final concentrations) 200 μmol/l NADH, 200 μmol/l acetyl-CoA and 500 μmol/l ATP. The decrease in optical density was determined at 25°C at a wavelength of 340 nm over 15 min. The assay was validated using a radioactive method measuring ACC-mediated incorporation of [¹⁴C]CO₂ into [¹⁴C] malonyl-CoA (data not shown).

Animals

All animals used for this study were obtained from Charles River Laboratories (Kisslegg, Germany) except *db/db* mice which were obtained from Janvier Laboratories (Le-Genest-St-Isle, France). Mice were housed in groups in a temperature- and humidity-controlled environment with a 12 h light–dark cycle. The animals had access to diet and water ad libitum. All animal procedures were approved by the local animal ethics committee and complied with the *Guide for Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996).

Fatty acid oxidation in isolated mouse skeletal muscle

Fatty acid oxidation was measured as previously described [25], with modifications. Briefly, fed mice were killed, and

the extensor digitorum longus (EDL) or soleus muscles were excised and incubated in the presence or absence of (*S*)-9c (final concentration 10 $\mu\text{mol/l}$ unless stated otherwise) or DMSO (final concentration 0.5% [vol./vol.]) at 30°C in vials containing pre-oxygenated (95% O₂, 5% CO₂ [vol./vol.]) incubation buffer (Krebs–Henseleit buffer containing 50 $\mu\text{mol/l}$ palmitate, 5 mmol/l HEPES, 10 mmol/l mannitol, 10 mmol/l glucose, 1.7% BSA and 0.5% DMSO). After 15 min of pre-incubation, insulin was added to a final concentration of 120 nmol/l and the pre-incubation was continued for a further 15 min. Muscles were then transferred to new vials containing fresh incubation buffer with addition of 148 kBq/ml [³H] palmitate and incubated for a further 45 min. Subsequently, fatty acids in the medium were absorbed using activated charcoal and fatty acid oxidation was determined by measuring ³H₂O using a scintillation counter. Fatty acid oxidation is expressed as dpm/100 μg muscle tissue.

Compounds and dosing

The *S* enantiomer of compound 9c ([*S*]-9c; Fig. 1a) was synthesised according to the procedures of Gu et al [26, 27]. (*R*)-(+)-3-butyn-2-ol served as a chiral building block for the synthesis of the racemic compound 9c. Pioglitazone was purchased from Chemos (Regenstauf, Germany). Compound administration was by oral gavage using 0.5% [wt/vol.] aqueous hydroxyethylcellulose as the vehicle. Compounds were administered once daily in the morning. Control animals received vehicle only.

Analytical procedures

Blood glucose, HbA_{1c}, insulin and triacylglycerol were measured using commercially available assays.

Malonyl-CoA measurement

Animals were killed and liver and muscle samples were immediately snap frozen in liquid N₂. Aliquots were homogenised in 0.5 mol/l perchloric acid and subsequently filtered using Vivaspin Centrifugal Concentrators with a 0.2 μm polyethersulfon membrane (VWR, Darmstadt, Germany). The sample was cleaned online using a weak anion-exchange column (Oasis WAX, Waters, Eschborn, Germany) with 1 mmol/l acetic acid (pH 3.5) as loading buffer and 200 mmol/l NH₃ (pH 11) for elution made up 1:9 with solvent A (5 mmol/l dibutylamine [DBA], 15 mmol/l NH₄OAc pH 7) for transfer onto the reverse-phase (RP) precolumn. Ion-pairing RP-HPLC separation was carried out at 40°C using a precolumn and separating column Zorbax Eclipse XDB-C18 (Agilent, Böblingen, Germany), solvent A and for a gradient solvent B (5 mmol/l DBA, 15 mmol/l NH₄OAc pH 7, in 50% acetonitrile). At 9.5 min, HPLC eluent was directed to an electrospray ionisation MS (Agilent 1946D) and data acquisition was started. Malonyl-CoA was detected at 854 *m/z*.

Determination of in vivo IMCL and liver fat content using magnetic resonance

Magnetic resonance (MR) measurements were performed on a 4.7 T Biospec 47/40 scanner (Bruker BioSpin,

Fig. 1 (*S*)-9c is an isoform-2-selective inhibitor of ACC and increases fatty acid oxidation in isolated mouse skeletal muscle. The structure of (*S*)-9c is shown (a). (*S*)-9c enhances fatty acid oxidation in EDL muscle from NMRI mice in a dose-dependent manner. The dose-response curve and EC₅₀ value are provided; *n*=4 muscles per condition (b). EDL (white bars) and soleus (black bars) were isolated from *db/db* and control C57BL/6 mice. Fatty acid oxidation was determined in the absence and presence of 10 $\mu\text{mol/l}$ (*S*)-9c; *n*=5–6 muscles per condition (c). ****p*<0.001 vs DMSO; †*p*<0.05 and ††*p*<0.01 vs *db/db* (*p*=0.1 for effect of (*S*)-9c in soleus muscle of *db/db* mice)

Ettlingen, Germany) equipped with a BGA12 gradient coil system. To achieve good homogeneity of the static magnetic field, we used standard linear shim routines provided with protocols of the scanner software ParaVision 5.1 (Bruker BioSpin). A bird-cage resonator volume coil was used for signal excitation.

Intramyocellular lipid For signal reception a custom-made Helmholtz coil mouse set-up was used (adapted from Neu-maier et al [28]). The MR spectroscopy (MRS) voxel was positioned in the tibialis anterior muscle using a T_1 -weighted pilot scan with packages of horizontal and axial slices (repetition time (TR) 120 ms, echo time (TE) 4 ms, flip angle 55° , four averages, field-of-view 2.5×2.5 cm, slice thickness 1.0 mm, interslice distance 1.2 mm, matrix 192^2 (zero-filled to 256^2), measuring time 92 s). Single voxel spectra were acquired using a point-resolved spectroscopy (PRESS) sequence: TR 1300 ms, TE 20 ms, voxel size 1.7 mm^3 , 768 averages, measuring time 17 min.

Liver fat content An anatomically adapted volume pick-up coil was used for reception. The MRS voxel was positioned in the left dorsal part of the liver using a T_1 -weighted pilot scan (TR 134 ms, TE 3.5 ms, flip angle 55° , six averages, field-of-view 4.5×4.5 cm, slice thickness 1.75 mm, interslice distance 1.5 mm, matrix 128^2 , measuring time 95 s). MR spectra were acquired using a PRESS sequence: TR 1050 ms, TE 20 ms, voxel size 27 mm^3 , 32 averages, measuring time 34 s.

Analysis of MR spectra MR spectra of muscle and liver lipids were analysed by the LC Model software package (version 6.1) [29–31] using the built-in basis set for analysis of in vivo muscle spectra and liver spectra, respectively. Results of the MRS analysis are given as ratios IMCL/total creatine (muscle) or liver lipid/water (liver).

Positron emission tomography and Patlak data analysis

From day 55 to 60, during subchronic treatment, in vivo glucose metabolism was assessed using positron emission tomography (PET) and the radioactively labelled glucose analogue 2-deoxy-2- $[^{18}\text{F}]$ fluoro-D-glucose (FDG). A Siemens Inveon small-animal PET/computed tomography (CT) system (Siemens Medical Solutions, Molecular Imaging, Knoxville, TN, USA) was used. Animals were anaesthetised using 1.5% isoflurane in a mixture of O_2 and N_2O (30:70 [vol./vol.]) and fixed in a supine position on a warmed animal bed. FDG and insulin were injected through a tail vein catheter. Animals were not fasted prior to the PET/CT measurement.

First, a CT scan was acquired for anatomical reference and attenuation correction. Subsequently, FDG ($16 \pm 3 \text{ MBq}$) was injected and PET data acquisition started. To stimulate glucose

uptake, animals received 2 U of insulin directly before tracer application. Continuous PET data were acquired over 60 min; data were subsequently binned into 17 time frames of increasing length ($4 \times 30 \text{ s}$, $9 \times 120 \text{ s}$, $4 \times 600 \text{ s}$), and reconstructed into PET images using an iterative 2D-ordered-subset expectation maximisation (OSEM) algorithm with scatter correction, decay correction and CT-based attenuation correction.

For the kinetic Patlak analysis of FDG uptake, volumes of interest (VOIs) covering biceps muscle and visceral adipose tissue were placed on both sides of the animal; an average of two VOIs for each tissue was used for further analysis. In addition, one VOI was placed in the central vein to measure radioactive content in the plasma. Plasma and tissue time-activity curves were analysed as described by Patlak [32] to determine the fractional rate of tracer transfer and phosphorylation (Patlak constant $[K_i]$). For all data analyses the software Inveon Research Workplace (Siemens Medical Solutions) was used.

Subchronic treatment of mice

Male *db/db* mice, 9–10 weeks of age, were randomised for HbA_{1c} , triacylglycerol and body weight and treated once daily by oral gavage with vehicle, (*S*)-9c (10 or 30 mg/kg) or pioglitazone (10 mg/kg) for 70 days. Blood glucose measurements in fed animals and an OGTT (treatment day 37) in overnight (16 h)-fasted animals with a glucose challenge of 2 g/kg were performed throughout the study. Blood samples for glucose and HbA_{1c} measurement were obtained by tail bleed. Blood samples for measurement of insulin were drawn from the retrobulbar venous plexus under isoflurane anaesthesia.

Data analysis

Data are expressed as mean $\pm$ SE. Statistical comparisons were conducted by Student's *t* test or one-way ANOVA followed by Bonferroni's post test for group-wise comparisons. A *p* value < 0.05 was considered statistically significant.

Results

In vitro characterisation of the ACC2-selective inhibitor (*S*)-9c

Biochemical characterisation (*S*)-9c was tested in biochemical assays using recombinant human ACC1 and ACC2. These assays showed that (*S*)-9c was selective for ACC2 over ACC1 with IC_{50} values of 0.07 and $> 30 \text{ } \mu\text{mol/l}$, respectively. This is in line with the values reported for the racemic compound 9c (5c in Gu et al [27]).

Studies in skeletal muscle ex vivo Several studies demonstrate that loss of ACC2 or pharmacological isoform-non-

selective inhibition of ACC increases fatty acid oxidation in skeletal muscle [14, 15, 17]. We therefore measured (*S*)-9c-mediated palmitate oxidation in isolated extensor digitorum longus muscle from Naval Medical Research Institute (NMRI) mice. (*S*)-9c significantly increased fatty acid oxidation in both the absence (data not shown) and the presence of insulin. Insulin inhibits fatty acid oxidation, at least in part, through activation of ACC [12], which can be reversed by ACC2 inhibition. The EC₅₀ of (*S*)-9c under the applied conditions was 226 nmol/l (Fig. 1b). IMCL levels have been shown to be significantly elevated in muscle from obese *db/db* mice (Fig. 2b) [33]. Therefore, isolated EDL and soleus muscles from *db/db* and control C57BL/6 mice were used to study the effect of adiposity on ex vivo fatty acid oxidation. We found no difference in basal palmitate oxidation in muscle from *db/db* mice compared with control mice in the absence of insulin in the incubation media (data not shown). However, in the presence of insulin, palmitate oxidation in muscle from insulin-resistant *db/db* mice was elevated compared with control mice. The effect reached statistical significance only for EDL muscle (Fig. 1c). Independent of the presence or absence of insulin in the incubation media, (*S*)-9c significantly enhanced palmitate oxidation in EDL and soleus muscle from control mice as well as in EDL muscle from *db/db* mice (Fig. 1c). We observed somewhat higher variability in palmitate oxidation measurements in soleus compared with EDL muscle, which may explain why the effect of (*S*)-9c did not reach statistical significance in soleus muscle from *db/db* mice ($p=0.10$, Fig. 1c).

In vivo effects of (*S*)-9c

Short-term treatment Because (*S*)-9c inhibited ACC2 in a biochemical system and was effective in increasing fatty acid oxidation in isolated mouse skeletal muscle, we

Fig. 2 Short-term treatment with (*S*)-9c decreases muscle malonyl-CoA levels and reduces muscle IMCL concentrations. *db/db* mice were treated with vehicle (white bars) or 30 mg/kg (*S*)-9c (black bars) either once or for 3–4 days. (a) In the single-dose group, muscles were excised 2 h after treatment and malonyl-CoA levels were determined. (b) In the multiple-dose group, IMCL levels were measured by NMR after 3–4 days. For comparison, the IMCL level of wild-type C57BL/6 mice is shown (grey bar). Data are presented as IMCL-to-creatinine ratios. ** $p<0.01$ and *** $p<0.001$ vs vehicle treatment; $n=6$ –7 mice per group

addressed the question of whether the compound is also effective in an acute in vivo situation. To do so, *db/db* mice were treated with vehicle or 30 mg/kg (*S*)-9c. Quadriceps muscle was isolated and malonyl-CoA levels were measured 2 h after compound administration. In line with previous reports for genetic loss of *Acc2* [14, 15], pharmacological treatment with (*S*)-9c resulted in decreased malonyl-CoA levels in muscle (Fig. 2a), indicating that (*S*)-9c inhibited ACC2 in skeletal muscle in vivo.

In a second study, we then investigated whether inhibition of ACC2 in vivo for several days would be efficacious in reducing skeletal muscle IMCL levels. Animals were treated with 30 mg/kg (*S*)-9c for 3–4 days and IMCL was measured in vivo in tibialis anterior muscle by ¹H-MRS. As shown in Fig. 2b, IMCL levels were significantly reduced after this short-term treatment with (*S*)-9c. Similar effects were also observed with other ACC2-selective inhibitors (data not shown).

Subchronic treatment Increased IMCL have been linked to impaired glucose tolerance [34]. Because IMCL was reduced by (*S*)-9c treatment after only 3–4 days of treatment, translation into improved glycaemic control after subchronic treatment was assumed. We therefore treated *db/db* mice with either 10 or 30 mg/kg (*S*)-9c for 70 days. Pioglitazone at 10 mg/kg served as a positive control and vehicle treatment as negative control. As expected, pioglitazone treatment enhanced body weight gain [35], while (*S*)-9c treatment was weight neutral (Fig. 3a). Plasma triacylglycerol levels were reduced by 30 mg/kg (*S*)-9c ($p<0.01$) (Fig. 3b). Independent of dosage, (*S*)-9c treatment significantly reduced HbA_{1c} (−0.7% [−7.7 mmol/mol]; $p<0.05$) as well as prandial glucose levels ($p<0.01$) over the treatment period (Fig. 3c, d). The reduction was smaller than that with pioglitazone treatment. Oral glucose tolerance measured on day 37 was improved by (*S*)-9c ($p<0.05$ and $p<0.01$ for AUCs of 10 and 30 mg/kg (*S*)-9c, respectively, vs control [Fig. 4a, b]). In vivo insulin-stimulated uptake of the glucose analogue FDG into skeletal muscle was enhanced by treatment with (*S*)-9c, but not by pioglitazone treatment. In contrast, pioglitazone, but not (*S*)-9c treatment, resulted in enhanced FDG uptake into adipose tissue (Fig. 4c, d). No changes in liver triacylglycerol levels were detected after (*S*)-9c treatment. In contrast, pioglitazone treatment resulted in liver steatosis ($p<0.001$ on day 70 [Fig. 5a, b]). Neither (*S*)-9c nor pioglitazone had a significant effect on plasma insulin levels or pancreatic insulin content (Fig. 5c, d).

Discussion

The aim of the present study was to investigate the effects of pharmacological inhibition of ACC2 in a mouse model of

Fig. 3 Subchronic treatment of *db/db* mice with (*S*)-9c improves glucose homeostasis. *db/db* mice were treated for 70 days with vehicle (white circles), 10 mg/kg (black triangles) or 30 mg/kg (inverted black triangles) (*S*)-9c or 10 mg/kg pioglitazone (white squares). Body weight was measured throughout the study (a). On days 0, 16, 43 and 70 plasma triacylglycerol (b), HbA_{1c} (c) and prandial glucose (d) were determined. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs control group, $n = 7$ –10 mice per group. TG, triacylglycerol

Fig. 4 Subchronic treatment of *db/db* mice with (*S*)-9c improves glucose tolerance. *db/db* mice were treated for 70 days with vehicle (white circles), 10 mg/kg (black triangles) or 30 mg/kg (inverted black triangles) (*S*)-9c or 10 mg/kg pioglitazone (white squares). OGTT was performed on day 37 and the glucose AUC calculated (a, b). Transfer constants K_i for the uptake of FDG into skeletal muscle (c) and adipose tissue (d) were determined by Patlak analysis. * $p < 0.05$ and ** $p < 0.01$ vs control group, $n = 7$ –10 (a, b) and $n = 6$ –8 (c, d) mice per group

Fig. 5 Subchronic treatment of *db/db* mice with (*S*)-9c does not induce liver steatosis and has no effect on plasma insulin levels or pancreatic insulin content. Liver fat content was measured by NMR on day 23 (**a**). On day 70, liver triacylglycerol (**b**), plasma insulin levels (**c**) and pancreatic insulin content (**d**) were determined. *** $p < 0.001$ vs control group, $n = 7$ –10 mice per group. TG, triacylglycerol

diabetes mellitus. We demonstrate that the isoform-2-selective ACC inhibitor, (*S*)-9c [27], has glucose-lowering properties. (*S*)-9c potently inhibits recombinant ACC2, but not ACC1. (*S*)-9c enhances fatty acid oxidation in mouse skeletal muscle ex vivo. Short-term treatment of *db/db* mice with (*S*)-9c decreases muscle malonyl-CoA levels and lowers IMCL. Finally, we provide evidence that subchronic treatment of *db/db* mice for 70 days with (*S*)-9c improves oral glucose tolerance, enhances insulin-stimulated FDG uptake into skeletal muscle, lowers HbA_{1c} and prandial glucose and reduces plasma triacylglycerol levels.

Several mouse models of ACC2 deficiency are described in the scientific literature. Whole-body *Acc2*-knockout mice, initially reported by Abu-Elheiga and co-workers, presented with enhanced whole-body fatty acid oxidation, increased energy expenditure, reduced fat mass and protected from diet-induced obesity and insulin resistance [14, 22, 23]. While these (and other) findings rendered ACC2 an attractive drug target against diet-induced obesity and insulin resistance, two recent studies using independently generated ACC2 mutant mice observed markedly diverse phenotypes [15, 16]. Therefore, as an alternative approach to studying the consequences of diminished ACC2 activity, we intended to inhibit ACC2 pharmacologically. (*S*)-9c was selected because of its highly selective inhibition of ACC2 over

ACC1 [27]. The reported selectivity for ACC2 was tested and confirmed in our own biochemical assays using recombinant human ACC1 and ACC2. As expected from the molecular mechanism, we found (*S*)-9c to increase muscle fatty acid oxidation ex vivo. The EC₅₀ of the effect was 226 nmol/l, an expected approximate fourfold shift compared with the biochemical IC₅₀. Genetic loss of *Acc2* was also found to enhance ex vivo skeletal muscle fatty acid oxidation [15, 22], concomitant with reduced levels of muscle malonyl-CoA levels [14, 15]. In contrast, others did not observe these effects in their ACC2 mutant mouse model. The reasons for the discrepancy are unknown, but may be due to compensatory downregulation of malonyl-CoA decarboxylase activity [16]. However, in the acute setting, as in the present study, our findings are in line with the concept that diminishing ACC2 activity results in enhanced fatty acid oxidation in peripheral tissues.

Obesity-related insulin resistance is associated with reduced lipid oxidation in skeletal muscle and increased intracellular lipid deposition in humans [1, 2]. To see whether (*S*)-9c would also be active in the setting of obesity-induced muscle insulin resistance, EDL and soleus muscles from *db/db* mice and wild-type control mice were used. Ex vivo muscle fatty acid oxidation was similar between *db/db* mice and control mice in the absence of insulin. However, when insulin was added to the incubation media, fatty acid oxidation was higher in muscle from *db/db* mice compared with control muscle, probably because of the lack of insulin-mediated inhibition of fatty acid oxidation in insulin-resistant *db/db* muscle. These data are in line with findings of a study measuring indirect calorimetry across the leg in human lean and obese volunteers [36], in which fat oxidation in the human leg tissues was suppressed in lean, but not in obese, individuals during insulin infusion.

It has been suggested that in type 2 diabetes and obesity, skeletal muscle is inflexible in the transition between the use of lipid and carbohydrate as fuel during fasting or in response to insulin, such as in postprandial conditions. As a result, partitioning of lipid between oxidation and storage is altered [37]. Thus, our observations in muscle from *db/db* mice likely reflect the metabolic inflexibility of the skeletal muscle. Metabolic inflexibility has been linked to the accumulation of IMCL and is considered an important aspect of insulin resistance of skeletal muscle in obesity and type 2 diabetes mellitus. (*S*)-9c significantly enhanced fatty acid oxidation in EDL muscle from *db/db* mice, indicating that ACC2 inhibition is also functional in enhancing fatty acid oxidation in skeletal muscle from insulin-resistant *db/db* mice. We have not measured glycogen content in the present study. However, based on published data from *Acc2*-knockout mouse heart muscle and our own data obtained in Zucker Diabetic Fatty (ZDF) rats using another isoform-

specific ACC2 inhibitor (data not shown) no effect on muscle glycogen content is expected.

Exposure to the ACC non-selective inhibitor CP-640186 has been reported to stimulate rat whole-body fatty acid oxidation and cause reductions in liver, adipose and skeletal muscle fat content and whole-body fat mass, and to improve insulin sensitivity [17]. The ACC non-selective inhibitor 4m stimulated fat oxidation as shown by lowering of the respiratory quotient and reducing plasma triacylglycerol levels in a rodent model of dyslipidaemia [18]. Soraphen, another non-selective inhibitor of ACC, was demonstrated to improve peripheral insulin sensitivity in high-fat-fed mice [38]. Because of our ex vivo observations we wanted to determine whether similar in vivo effects could be observed with selective inhibition of ACC2. To this end, *db/db* mice were treated with (S)-9c. As expected, muscle malonyl-CoA levels were already significantly reduced 2 h after treatment. This is in line with reduced muscle malonyl-CoA levels reported from ACC2 mutant mouse models [14, 15]. IMCL concentrations were already significantly reduced after 3–4 days of treatment with (S)-9c. This indicates that selective inhibition of ACC2 reduces IMCL levels through enhancement of fatty acid oxidation.

To investigate whether (S)-9c has anti-obesity and glucose-lowering effects in vivo, *db/db* mice were treated for up to 70 days with 10 or 30 mg/kg (S)-9c. Vehicle and pioglitazone served as control treatments. While pioglitazone treatment led to an expected increase in body weight, there was no effect of (S)-9c on body weight development. This finding is in line with recently reported studies using ACC2 mouse models [15, 16], but contrasts with the reduced body weight of ACC2 mutant mice reported earlier [22]. Despite the lack of effect on body weight, plasma triacylglycerol levels were dose-dependently lowered by (S)-9c treatment. Importantly, treatment with (S)-9c had glucose-lowering effects, significantly reducing HbA_{1c} and prandial glucose concentrations and improving glucose tolerance. Determination of skeletal muscle GLUT4 protein and *Glut4* (also known as *Slc2a4*) mRNA content revealed no significant differences between the treatment groups (data not shown).

We assessed a potential insulin sensitisation by (S)-9c treatment. As an indicator of insulin-stimulated glucose removal into tissues, we measured uptake of the glucose analogue FDG into adipose tissue and skeletal muscle, the main target tissues of pioglitazone and (S)-9c, respectively, after 56–60 days of treatment. Pioglitazone significantly increased FDG uptake into adipose tissue only. In contrast, (S)-9c treatment resulted in increased uptake of the FDG tracer into skeletal muscle only, suggesting an enhanced insulin effect in this tissue. In support of these data, we found significantly increased insulin sensitivity as measured in insulin tolerance tests in ZDF rats following 18 days of treatment with another selective ACC2

inhibitor (data not shown). Taken together, these data indicate insulin sensitisation by treatment with ACC2-selective inhibitors.

Relative to the effects of pioglitazone treatment, the effects of (S)-9c treatment on plasma triacylglycerol and HbA_{1c} as well as prandial glucose were comparably smaller. In contrast to pioglitazone treatment, however, treatment with (S)-9c did not affect liver triacylglycerol and did not result in increased body weight. Considering the effects observed in muscle ex vivo, our data support the hypothesis that enhanced fatty acid oxidation through inhibition of ACC2 in muscle tissues improves whole-body glucose homeostasis. Whether this effect is solely a consequence of ACC2 inhibition in skeletal muscle or whether it requires a contribution from other tissues needs further investigation.

In our study we did not observe any obvious side effects of (S)-9c, even though the racemic compound 9c (referred to as 5c in Gu et al [27]), as well as several derivatives, have been reported to cause ACC2-independent central and cardiac effects in a rat model. The lack of side effects of (S)-9c in our study is in line with the published observation that off-target activity occurred at plasma levels of greater >72 µg/ml [27], which is clearly above the levels observed in the present study. Recent publications have demonstrated that, in comparison to mice, ACC2 is expressed more widely in humans, e.g. in adipose tissue [19, 39]. We can only speculate on the consequences of ACC2 inhibition in human adipose tissue, and this area requires further investigation.

Taking the results together, this study demonstrates that (S)-9c, an isoform-2-selective ACC inhibitor, improves glucose homeostasis in *db/db* mice. This occurs concomitantly with reduced muscle malonyl-CoA levels, enhanced muscle fatty acid oxidation and reduced IMCL concentrations.

We conclude that ACC2-selective inhibition improves glucose homeostasis in vivo in a mouse model of diabetes mellitus, providing further evidence for ACC2 as a drug target for treating type 2 diabetes.

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