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ARTICLE

Acute cannabinoid receptor type 1 (CB1R) modulation influences insulin sensitivity by an effect outside the central nervous system in mice

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Abstract
Aims/hypothesis Modulation of central nervous system (CNS) and extra-CNS cannabinoid receptor type 1 (CB1R) affects metabolic conditions, independently of weight loss. Here we examined the relative contributions of acute CNS and extra-CNS CB1R modulation on insulin sensitivity using pharmacological gain- and loss-of-function of CB1R in mice.
Methods We assessed the effects of acute modulation of CB1R on insulin sensitivity and tissue glucose uptake by administering a CB1R agonist (HU210) and antagonist (AM251) (vs vehicle) i.v. in wild-type mice. In addition, we administered a CB1R agonist (vs vehicle) systemically (i.v.) to *Cb1r* (also known as *Cnr1*) knockout ($Cb1r^{-/-}$) mice or intracerebroventricularly (i.c.v.) in wild-type mice to elucidate the peripheral vs CNS-mediated regulatory effect of CB1R on insulin sensitivity.
Results HU210 induced significant insulin resistance in wild-type mice with a reduction of whole-body glucose disappearance rate and muscle Akt phosphorylation, as well as of glucose uptake by skeletal muscle, but not by adipose tissue, changes that were prevented by pretreatment with AM251. HU210 did not affect insulin sensitivity in $Cb1r^{-/-}$ mice, suggesting that the observed effects were mediated through CB1R. HU210 administered i.c.v. did not induce insulin resistance, suggesting that acute stimulation of CNS CB1R was not required for this effect.
Conclusions/interpretation Skeletal muscle insulin sensitivity is affected by acute CB1R modulation. These changes are mediated by extra-CNS CB1R, probably by the receptors in skeletal muscle tissue.

Keywords Cannabinoid receptor type 1 · Glucose disappearance rate · Insulin resistance · Insulin sensitivity · Tissue glucose uptake

Abbreviations

CB1R	Cannabinoid receptor type 1
$Cb1r^{-/-}$	Whole-body *Cb1r* knockout mice
CNS	Central nervous system
2-DG	2-Deoxy-D-[1-^{14}C]glucose
EGP	Endogenous glucose production
ERK	Extracellular signal-regulated protein kinase
Ginf	Glucose infusion rate
i.c.v.	Introcerebroventricular
R_d	Glucose disappearance rate

Introduction

The prevalence of obesity with its adverse effects on health threatens to undermine recent gains in the prevention of

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atherosclerotic cardiovascular disease, type 2 diabetes and hypertension [1]. Antagonism of the cannabinoid receptor type 1 (CB1R) of the endocannabinoid system represents a novel pharmacological treatment for obesity and the metabolic syndrome. CB1R antagonists have been shown in clinical trials to have highly beneficial effects, inducing weight loss and improving carbohydrate and lipid metabolism in individuals with obesity and related metabolic disorders [2–4]. However, a major limiting factor of CB1R antagonists that cross the blood–brain barrier is that they are associated with serious central nervous system (CNS) side effects such as depression and anxiety, thereby limiting their potential clinical utility [3].

CB1R is highly abundant in the brain [5, 6]. Besides the CNS, CB1Rs are also widely expressed in tissues outside the CNS, such as liver, adipose tissue, skeletal muscle and the pancreas [7–11]. There is evidence that several therapeutic benefits of CB1R antagonists are mediated by their interaction with CB1R in peripheral tissues outside the CNS and that many of their effects are independent of effects on appetite and changes in body weight. For example, in diet-induced obese mice, chronic treatment with rimonabant, a CB1R antagonist, caused a transient reduction in food intake but sustained reduction in body weight [12, 13]. Body weight reductions through CB1R antagonism can only partially be explained by reductions in food intake; moreover, a variety of metabolic benefits of CB1R antagonism occur independently of reductions in energy intake [7, 12–14], suggesting that peripheral tissue CB1R plays a role in inducing weight loss. In diet-induced obesity, chronic CB1R antagonism causes weight loss and improves insulin sensitivity by diverting lipids from storage toward utilisation, a process that occurs independently of food intake [15]. Whole-body *Cb1r* (also known as *Cnr1*) knockout ($Cb1r^{-/-}$) mice are resistant to diet-induced obesity, while wild-type littermates on the same diet with identical energy intake become obese [16]. In addition, there is direct experimental evidence that CB1R antagonism increases energy expenditure in peripheral tissues [14, 17, 18].

Recent studies suggest that the endocannabinoid system is involved in modulating carbohydrate metabolism and insulin sensitivity. Chronic antagonism of CB1R leads to weight loss and improved insulin sensitivity in animal models of obesity and in humans [2, 4, 19–21]. However, it is still not clear whether the beneficial effects on insulin sensitivity and metabolic profiles are secondary to reduction in food intake with consequent weight loss or caused by other unrelated mechanisms. Moreover, the tissue sites and contribution of extra-CNS CB1R modulation to whole-body insulin sensitivity, while generally appreciated to play a major role in the metabolic effects of CB1R modulation, are also not well understood.

In the present study, we assessed the effects of acute modulation of CB1R on whole-body insulin sensitivity and tissue glucose uptake, and elucidated the role of the different tissue sites in regulating insulin sensitivity.

Methods

Animals Male wild-type C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA, USA) at the age of 6 to 7 weeks. $Cb1r^{-/-}$ mice were kindly provided by G. Kunos at the National Institutes of Health (Bethesda, MD, USA). $Cb1r^{-/-}$ and $Cb1r^{+/+}$ (littermate) mice were obtained by breeding of heterozygotes that had been backcrossed to a C57BL/6J background [22]. All animals were housed in the Animal Care Facility of the Toronto General Research Institute with a 12-h light/dark cycle and free access to regular chow diet and water. All procedures were approved by the Animal Care Committee of the University Health Network, University of Toronto.

Study groups Wild-type mice were randomly divided into three experimental groups. The first group, Control mice, were injected i.v. with vehicle of the CB1R antagonist AM251 (saline/DMSO/Tween80, 80:10:10, vol./vol.) 1 h before start of the euglycaemic–hyperinsulinaemic clamp, and with vehicle of the CB1R agonist HU210 (saline/ethanol/Tween80, 18:1:1, vol./vol.) 15 min after initiation of the clamp. The second group, the CB1R agonist group (HU210), were injected i.v. with vehicle of AM251 1 h before the start of the clamp and with HU210 (50 ng/g body weight) 15 min after the start of the clamp. The third group, the CB1R antagonist+agonist group (AM251+HU210), were injected i.v. with AM251 (6 μg/g body weight) 1 h preceding start of the clamp, and with HU210 (50 ng/g body weight) 15 min after the start of the clamp. Separate experiments with acute AM251 administration alone showed no effects on clamp variables (electronic supplementary material [ESM] Fig. 1) or insulin-stimulated Akt phosphorylation (ESM Fig. 2). We therefore elected to include only the first of the above groups (Control, vehicle only-treated) as control in further studies. $Cb1r^{-/-}$ mice were randomly assigned to two experimental groups, which received either HU210 (50 ng/g body weight) or vehicle i.v. 15 min after the start of the clamp, without pretreatment with antagonist or its vehicle. Two additional groups of wild-type mice received intracerebroventricular (i.c.v.) injection of HU210 (50 ng/g body weight) or vehicle 15 min after the start of clamp studies. AM251 and HU210 were freshly prepared in concentrations according to 2 μl/g body weight injection volume. In preliminary studies, we had determined that 50 ng/g body weight is an optimal dose to elicit an effect on insulin sensitivity in vivo and is well within the 20–100 ng/g body weight range commonly used by others [11, 21, 23–25]. The dose of AM251 was the

same as reported in chronic studies that elicited metabolic effects on feeding and body weight in mice [26–28]. In the i.c.v. experiments, a total volume of 2 μl HU210 or vehicle (saline/ethanol/Tween80, 18:1:1 vol./vol.) was injected. This volume has been reported in other studies and shown to be safe in mice [29].

Surgical procedures Mice at 8 to 12 weeks of age were catheterised under general anaesthesia (ketamine 100 mg/kg, xylazine 7.5 mg/kg, i.p.). The right jugular vein was cannulated with a microrenathane catheter (MRE 0.25; Braintree Scientific, Braintree, MA, USA), sealed and tunnelled subcutaneously to the back of the neck as previously described [30]. Mice were allowed 3–5 days to recover. Only mice that recovered uneventfully and lost less than 5% of their preoperative body weight were used for subsequent experiments.

In two separate groups of wild-type mice, brain infusion cannulae were stereotaxically placed with their tips in the lateral cerebral ventricle using the following coordinates: 0.7 mm posterior to bregma, 1.0 mm lateral to the midsagittal suture, both to a depth of 2.2 mm, with bregma and lambda at the same vertical dimension, determined from the mice brain atlas of Franklin and Paxinos [31]. The guide cannulae were secured to the skull surface with two screws and dental cement. After 1 week of recovery, the localisation of the cannulae was confirmed by the instant drinking response to an i.c.v. injection of angiotensin II [32]. The right jugular vein was cannulated as above in mice with successful brain ventricle cannulation. Mice were allowed another 3 to 5 days to recover. Again, only mice that recovered uneventfully were used for subsequent clamp studies.

Euglycaemic–hyperinsulinaemic clamp procedure and determination of tissue glucose uptake Euglycaemic–hyperinsulinaemic clamps were conducted in fasted, conscious, tail-retained mice as previously described [30] with minor modifications. After baseline blood sampling from the tail vein, a primed (1.85×10^5 Bq bolus) constant (1.85×10^3 Bq/min) infusion of [^{3}H]glucose (New England Nuclear, Boston, MA, USA) was initiated and continued for 100 min. Mice were pretreated with AM251 (6 μg/g body weight) or vehicle 1 h before the start of the clamp, i.e. 40 min after start of the basal period. During the last 20 min of the basal period, blood samples were taken every 10 min for determination of basal glucose-specific activity, glucose concentrations and plasma hormones. Subsequently, the clamp period was started with infusion of insulin (50 mU/kg body weight bolus+1.5 mU kg^{-1} min^{-1} infusion) and [^{3}H]glucose (1.85×10^3 Bq/min). Blood samples were collected every 10 min and glucose levels measured with a glucometer (Sure Step, One Touch; Lifescan, Milpitas, CA, USA). A 25% (wt/vol.) dextrose solution was infused at a variable rate to maintain blood glucose at the basal level. Additional [^{3}H]glucose was added to the dextrose infusate to minimise the decline in glucose-specific activity during the clamp. Steady-state of the clamp was achieved between 90 and 120 min, during which time blood samples were collected every 10 min for determination of glucose-specific activity, glucose concentrations and plasma insulin levels. CB1R agonist HU210 (50 ng/g body weight) or vehicle was administered i.v. or i.c.v. 15 min after the start of the clamp period. An i.v. bolus of 2-deoxy-D-[1-^{14}C]glucose (2-DG) (3.7×10^5 Bq; NEN Life Science, Boston, MA, USA) was administered 80 min before the end of the clamp. At the end of the clamp, mice were killed with sodium pentobarbital. Within 5 min of death, three pieces of muscle tissue (quadriceps, soleus and gastrocnemius from both hindlimbs) and abdominal visceral adipose tissue were rapidly excised, weighed, immediately frozen in liquid nitrogen and stored at −70°C for later analysis.

Assays Plasma insulin was measured using a radioimmunoassay kit (Millipore, Billerica, MA, USA). For determination of plasma [^{3}H]glucose and 2-DG specific activities, plasma was deproteinised with zinc sulphate and barium hydroxide, dried to remove ^3H_2O and counted in scintillation fluid on dual channels for separation of ^{3}H and ^{14}C (Beckman Coulter, Fullerton, CA, USA). For determination of tissue 2-DG-6-P content, muscle and adipose tissue samples were homogenised and centrifuged (1,000×*g*, 5 min), and the supernatant fractions subjected to an ion-exchange column to separate 2-DG-6-P from 2-DG, as previously described [33].

Calculations Endogenous glucose production rate (EGP) during clamps was determined by subtracting the glucose infusion rate (Ginf) from whole-body glucose uptake. Insulin-mediated glucose disappearance was the glucose disappearance rate (R_d) measured with [^{3}H]glucose during the clamp minus mean baseline R_d values [34, 35]. Data were smoothed with the optimal segments routine, using the optimal error algorithm [36]. Glucose uptake in individual skeletal muscle and adipose tissues was calculated from plasma 2-DG specific activity and tissue 2-DG-6-P content as previously described [37].

Acute in vivo insulin stimulation and analysis ex vivo of skeletal muscle Akt phosphorylation Wild-type mice of 9 weeks of age were fasted overnight before i.v. injection of vehicle, HU210 or AM251+HU210, exactly as in the clamp experiments described above. At the end of the treatments, i.e. time corresponding to the end of the clamp, muscle tissue were collected as described [38]. Briefly, under

anaesthesia with pentobarbital, the abdominal cavity was opened and portal vein exposed. Gastrocnemius muscle from one hindlimb was rapidly collected and frozen immediately in liquid nitrogen. A maximal bolus of insulin (10 U/kg body weight) was then injected into the portal vein. At 5 min after insulin injection, gastrocnemius muscle from the opposite hindlimb was collected and immediately frozen. Tissue protein preparation and western blotting were done as previously described [39]. Whole-tissue protein from lysates (approximately 30 μg protein) was separated by 10% SDS-PAGE, transferred to nitrocellulose membranes and incubated with antibodies against Akt, phosphorylated Akt Ser473 and Thr308 (Cell Signaling Technology, Beverly, MA, USA). Detection of blotted proteins was performed by enhanced chemiluminescence (Thermo Fisher Scientific, Rockford, IL, USA) according to the manufacturer's instructions.

Statistical analyses Values are reported as mean±SEM. For body weight, glucose, insulin and variables of euglycaemic–hyperinsulinaemic clamps, one-way ANOVA was used to compare the control, CB1R agonist and CB1R antagonist+agonist experimental groups. Differences between the groups were assessed by post-hoc analysis using Tukey's test. A value of $p<0.05$ was considered to be statistically significant.

Results

General characteristics of animals Body weights, baseline fasting blood glucose and plasma insulin concentrations prior to euglycaemic–hyperinsulinaemic clamp studies were not significantly different between experimental groups for wild-type and *Cb1r*$^{-/-}$ mice (Table 1). Fasting insulin levels in *Cb1r*$^{-/-}$ mice tended to be lower than in wild-type mice. Although the difference did not reach statistical significance, possibly due to the younger age of mice in our study, the trend towards lower insulin levels in *Cb1r*$^{-/-}$ than in wild-type mice is consistent with findings of others [16].

Effect of acute CB1R modulation on insulin sensitivity in wild-type and Cb1r$^{-/-}$ *mice* Glucose and insulin concentrations during the last 30 min of the clamp were not significantly different between experimental groups for wild-type mice (Table 1). Plasma glucose-specific activity was maintained close to baseline (within 20% of basal specific activity) and within a similar range during the last 30 min of the clamp in all experimental groups. In wild-type mice, acute administration of HU210 significantly lowered the Ginf that was required to maintain euglycaemia during the last 30 min of the clamp ($p<0.01$; Fig. 1a), indicating that CB1R agonism acutely induced insulin resistance in treated mice. The acute effect of HU210 on induction of insulin resistance was rapid, since the reduction of Ginf occurred as early as 40 min after clamp initiation. Pretreatment of mice with the selective CB1R antagonist AM251 completely prevented the decrease of Ginf (i.e. the insulin resistance) induced by HU210 in wild-type mice (Fig. 1a). In contrast, HU210 administration had no effect on Ginf (i.e. whole-body insulin sensitivity) in *Cb1r*$^{-/-}$ mice (Fig. 2a).

Effect of acute CB1R modulation on EGP and R_d in wild-type and Cb1r$^{-/-}$ *mice* To determine whether the above changes in whole-body insulin sensitivity were due to changes in R_d, EGP or both, we calculated these variables by modelling changes in [^{3}H]glucose-specific activity as described above. R_d was significantly reduced in wild-type mice treated with HU210 (Fig. 1b), indicating that the CB1R agonist acutely reduced whole-body glucose uptake. Pretreatment with AM251 completely prevented the reduction in R_d induced by HU210 in wild-type mice. However, insulin-induced suppression of EGP during the last 30 min of the clamp did not differ between experimental groups of wild-type mice (Fig. 1c, d). Antagonist alone did not elicit similar insulin-desensitising effects, as EGP and R_d levels in AM251 group were not statistically significantly different from those in vehicle-treated animals (ESM Fig. 1). In *Cb1r*$^{-/-}$ mice, insulin-induced stimulation of R_d and suppression of EGP were not significantly different between HU210- and vehicle-treated groups (Fig. 2b–d).

Effect of acute CB1R modulation on insulin-stimulated glucose uptake in muscle and adipose tissues In wild-type mice, HU210 significantly reduced glucose uptake in soleus (mainly type I fibre), quadriceps (mainly type II fibre) and gastrocnemius (mixture, and type II and type I fibres) muscles, as compared with vehicle-treated group (Table 2). This effect was abolished by pretreatment with AM251. Glucose uptake was similar in adipose tissue in all experimental groups (Table 2).

Effect of acute CB1R modulation in vivo on Akt phosphorylation in muscle tissues To further investigate the mechanisms of CB1R acute modulation of insulin signalling in skeletal muscle, we examined Akt phosphorylation in vivo under acute insulin stimulation after treatment of mice with CB1R agonist and/or antagonist. Insulin-stimulated Akt phosphorylation at Ser473 and Thr308 was significantly impaired by HU210, this impairment being prevented by pre-treatment with AM251 (Fig. 3). AM251 alone did not significantly affect Akt phosphorylation (ESM Fig. 2).

Effect of acute i.c.v. CB1R agonist administration on insulin sensitivity in wild-type mice Ginf (Fig. 4a) during

Table 1 Body weight, fasting basal and clamped plasma glucose and insulin in wild-type and *Cb1r*$^{-/-}$ mice

Variables per mouse type	Experimental groups		
	Vehicle	HU210	AM251+ HU210
Wild-type			
Body weight (g)	25.4±0.6	25.2±0.2	24.5±0.6
Basal blood glucose (mmol/l)	7.2±0.3	7.0±0.3	6.8±0.3
Clamped blood glucose (mmol/l)	7.7±0.3	7.3±0.3	7.2±0.2
Basal plasma insulin (pmol/l)	75.1±8.6	76.6±5.2	81.9±1.2
Clamped plasma insulin (pmol/l)	224.2±46.7	241.7±13.2	277.2±32.8
Cb1r$^{-/-}$			
Body weight (g)	23.1±0.9	22.5±1.1	
Basal blood glucose (mmol/l)	6.8±0.5	6.5±0.8	
Clamped blood glucose (mmol/l)	7.1±0.3	6.6±0.4	
Basal plasma insulin (pmol/l)	62.3±4.8	70.6±11.7	
Clamped plasma insulin (pmol/l)	201.9±21.3	211±26.7	

Values are mean±SEM

There were no differences in any variables between experimental groups (n=6 in each experimental group)

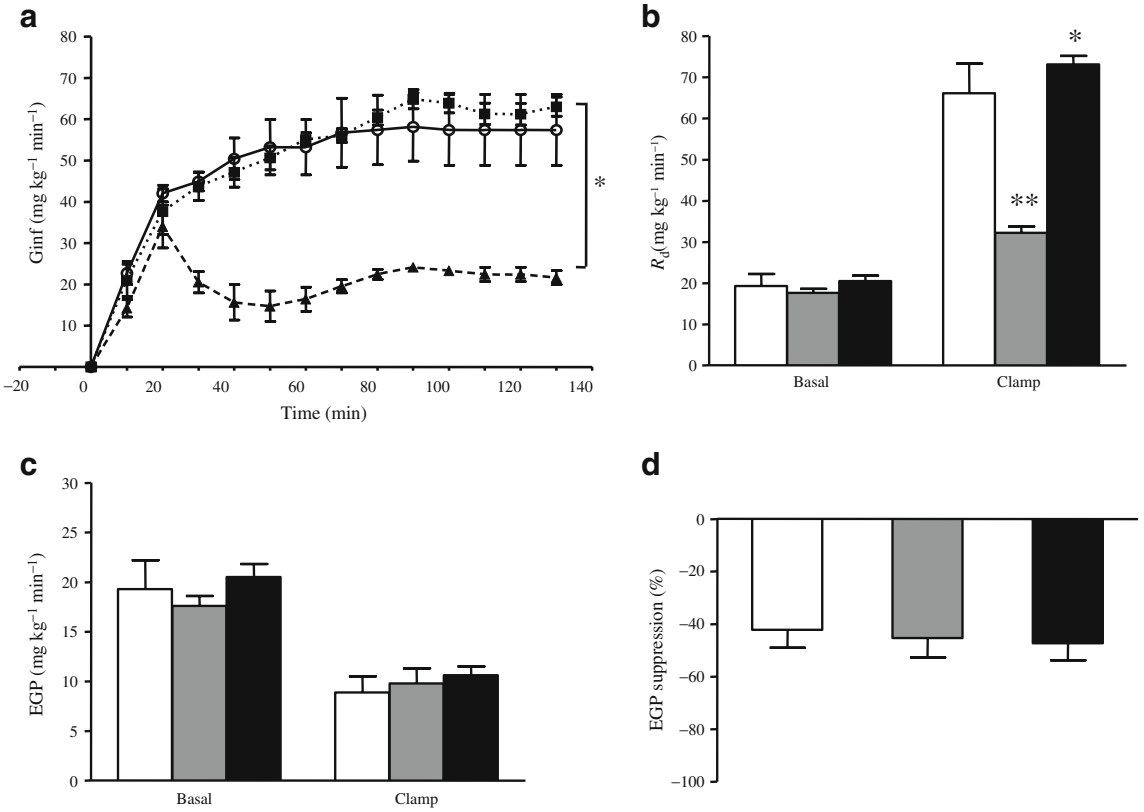

Fig. 1 In wild-type C56BL/6 mice (8–12 weeks of age), CB1R agonist (HU210, 50 ng/g body weight, i.v., 15 min after start of clamp at time 0 min) (black triangles, grey bars) induced whole-body insulin resistance, as evidenced by a 61% lower Ginf required to maintain euglycaemia during the last 30 min of a 120 min euglycaemic–hyperinsulinaemic clamp vs vehicle (white circles, white bars). Pretreatment of the mice with a selective CB1R antagonist AM251 (black squares, black bars) (6 μg/g body weight, i.v., 2 h before clamp) prevented the development of insulin resistance induced by HU210, as indicated by a similar Ginf to that in vehicle-treated mice (**a**). The profound impairment of insulin sensitivity induced by HU210 was due to a significant reduction of R_d (**b**), rather than to diminished insulin suppression of EGP (**c, d**). Pretreatment of the mice with AM251 prevented the reduction (**b**) of R_d induced by HU210. *p< 0.05 vs HU210; **p<0.01 vs vehicle and AM251+HU210

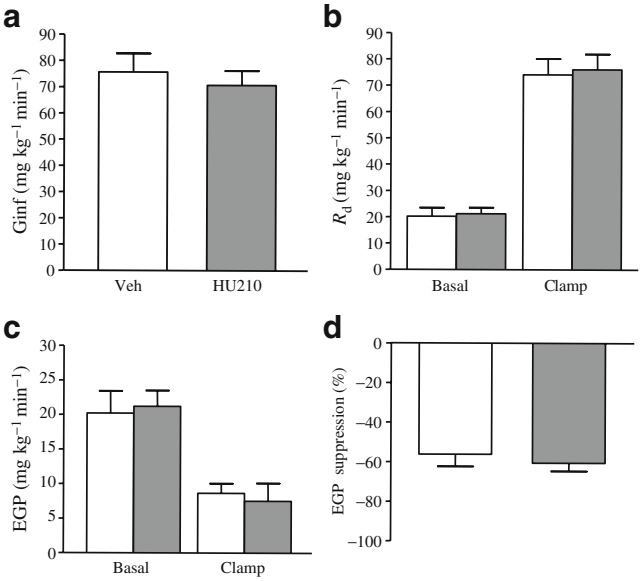

Fig. 2 In *Cb1r*$^{-/-}$ mice (8–12 weeks of age), Ginf (**a**), R_d (**b**) and EGP (**c, d**) were not significantly different between HU210-treated group (grey bars) (50 ng/g body weight, i.v., 15 min after start of clamp) and the vehicle-treated group (white bars), indicating resistance of *Cb1r*$^{-/-}$ mice to the acute insulin desensitising effect of HU210; n=6

the last 30 min of the clamp were not significantly different between i.c.v. HU210-treated group and i.c.v. vehicle-treated group in wild-type mice. EGP and R_d were similar between groups (Fig. 4b–d).

Discussion

Increasing evidence indicates that the endocannabinoid system is involved in regulation of insulin sensitivity. However, most changes in insulin sensitivity have been shown to accompany weight loss or resistance to diet-induced obesity. For example, plasma insulin levels are low in *Cb1r*$^{-/-}$ mice compared with their wild-type littermates; moreover, the former are resistant to diet-induced obesity and insulin resistance [16]. Plasma insulin levels were reduced in diet-induced obese mice treated with a specific CB1R antagonist SR141716 [13, 40]. In rats after chronic treatment with a CB1R antagonist, insulin sensitivity and skeletal muscle glucose uptake were also enhanced, whereas hepatic glucose production was diminished [20]. Chronic, peripheral CB1R antagonism decreases hepatic glucose production, promotes lipid mobilisation independently of food intake and increases glucose utilisation in rats [20]. Recent in vitro data also suggest that adipocytes may secrete CB1R-activating factors and that CB1Rs in human skeletal muscle participate in the negative crosstalk between fat and muscle [41], with muscle CB1R playing a major role in the development of insulin resistance. *Cb1r*$^{-/-}$ mice do not develop hyperinsulinaemia, hyperleptinaemia and dyslipidaemic profile, even after chronic feeding with high-fat diet [11, 21]. In clinical studies, insulin sensitivity was significantly improved after 1 or 2 years of treatment with the CB1R antagonist rimonabant [2, 4]. Although these combined data strongly suggest an important role of CB1R in modulating insulin sensitivity and glucose metabolism, it was not possible to separate insulin sensitisation secondary to weight loss from the direct effects of the drug in these studies. Neither the direct role of CB1R in modulating insulin sensitivity in vivo, nor the major tissue sites and mechanisms of action of CB1R agonists and antagonists in this process are currently well understood. In addition, understanding of CB1R's acute in vivo effects, independent of that on weight loss, in these processes is limited.

To examine the role of CB1R in modulating insulin sensitivity in vivo, we first assessed the effect of acute gain- and loss-of-function of whole-body CB1R on insulin sensitivity using the euglycaemic–hyperinsulinaemic clamp in conscious mice. We showed that, in wild-type mice, administration of the CB1R agonist HU210 acutely induced whole-body insulin resistance, as revealed by the significant 50% to 60% reduction of Ginf during the last 30 min of a 120 min euglycaemic–hyperinsulinaemic clamp. This effect was abolished when the animals were pretreated with a selective CB1R antagonist, AM251. The insulin resistance induced by acute CB1R agonism was accounted for by decreased glucose disposal, which is mainly by skeletal muscle, whereas we did not detect an acute effect on insulin's ability to suppress EGP, which is mainly hepatic (discussed further below). Since EGP was not altered by CB1R agonist HU210, hepatic CB1R does not seem to mediate acute changes in insulin sensitivity. Our findings that the effect of HU210 was completely absent in *Cb1r*$^{-/-}$ mice (which lack CB1R in the CNS and outside the CNS), as well as in wild-type mice treated with the CB1R antagonist AM251, prove that it was indeed stimulation of CB1R that was responsible for the acute reduction in insulin sensitivity, and not an off-target drug effect.

Table 2 2-DG uptake in skeletal muscles and visceral adipose of wild-type mice

Tissue	Vehicle	HU210	AM251+HU210
Soleus	226±15	75±17†	171±30*
Quadriceps	195±18	68±8†	168±24*
Gastrocnemius	202±21	80±12†	185±14*
Visceral adipose	20.6±5.1	16.1±3.9	15.7±5.2

Values are expressed as mean±SEM, with units in μmol kg^{-1} min^{-1}

† p<0.05 vs Vehicle and AM251+HU210; * p<0.05 vs HU210

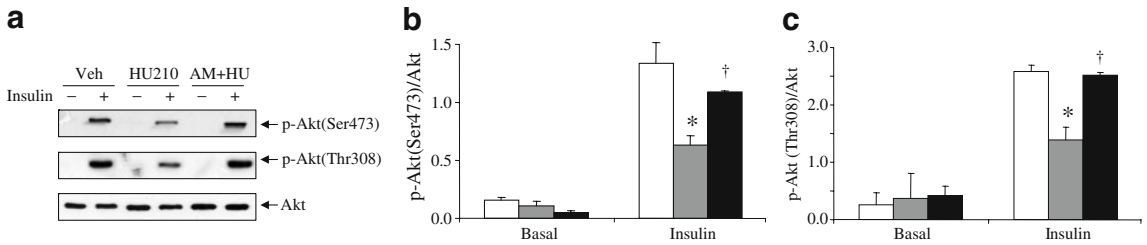

Fig. 3 Insulin-stimulated Akt phosphorylation, as assessed by western blot (**a**) and quantified for phosphorylated (p)-Akt Ser473 (**b**) and Thr308 (**c**), in muscle of wild-type mice was reduced by acute i.v. injection of CB1R agonist HU210 (50 ng/g body weight, 105 min before muscle collection) (grey bars) compared with vehicle-treated animals (white bars), which was prevented by pre-treatment with CB1R antagonist AM251 (6 μg/g body weight, 180 min before muscle collection) (black bars). *$p<0.05$ vs vehicle (Veh); †$p<0.05$ vs HU210

The profound impairment of peripheral glucose utilisation with CB1R acute agonism in the absence of a significant reduction in insulin-mediated suppression of EGP suggests that the effect of CB1R modulation on insulin sensitivity occurred in skeletal muscle and/or adipose tissue, rather than in liver. This finding was further supported by measurement of peripheral tissue 2-DG uptake, which confirmed that HU210 acutely reduced insulin-stimulated glucose uptake in skeletal muscle, with significantly lower 2-DG uptake in quadriceps (−65.1%), gastrocnemius (−60%) and soleus (−66.4%). CB1R antagonism with AM251 prevented the subsequent HU210-mediated reduction of 2-DG uptake in skeletal muscles of wild-type mice. Interestingly, HU210 had no effect on 2-DG uptake in adipose tissue in these experiments, indicating that adipose tissue CB1R plays a negligible role in mediating the acute changes of insulin sensitivity with CB1R agonism/antagonism. Altered glucose and lipid metabolism, and insulin sensitivity have been shown with chronic modulation of CB1R in peripheral tissues, including the liver [18, 20]. Our study is complementary to the anticipated long-term effects of CB1R modulation on insulin sensitivity, in that we demonstrate an acute prominent effect on insulin action in muscle.

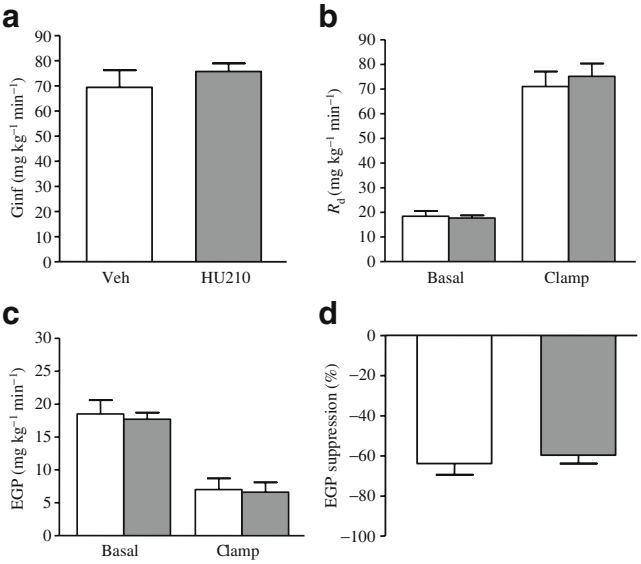

Fig. 4 In wild-type C56BL/6 mice (8–12 weeks of age), Ginf (**a**), R_d (**b**) and EGP (**c, d**) were not significantly different between HU210-treated group (grey bars) (i.c.v., 50 ng/g body weight, 15 min after start of clamp) and the vehicle treated group (white bars) ($n=6$), indicating that i.c.v. administration of HU210 failed to reproduce the acute insulin desensitisation that was observed when the identical dose was administered systemically (i.v.)

Although our results indicate that peripheral tissues such as skeletal muscle are major tissue sites responsible for the change in insulin sensitivity with acute CB1R modulation, the change of R_d induced by HU210 in wild-type mice could still have been secondary to CNS regulation of CB1R activity. Over the past decade, there has been mounting evidence that activation of the neural circuits in the hypothalamus can control hepatic and extra-hepatic carbohydrate metabolism [42]. To rule out this possibility, we administered HU210 or vehicle into the right brain ventricle in a separate group of wild-type mice during a euglycaemic–hyperinsulinaemic clamp. We found that i.c.v. injection of HU210 did not induce insulin resistance as seen when the same dose of HU210 was administered peripherally in wild-type mice. We deliberately chose to administer the same dose of HU210 i.c.v. as that we had administered systemically to demonstrate an acute insulin desensitising effect of HU210, reasoning that the i.c.v. CNS drug concentration with this dose would exceed the CNS concentration achieved with systemic administration of the drug. It is possible that this relatively high dose administered i.c.v. could have leaked out of the CNS into the systemic circulation, thereby exerting systemic effects on insulin sensitivity. However, the total absence of effect on insulin sensitivity with i.c.v. administration rules out this possibility in this experiment. These findings suggest that CB1Rs in the brain are not involved in the acute change of insulin sensitivity induced by systemic HU210 administration. Thus the insulin-desensitising effect of CB1R agonism in peripheral tissues are not likely to have

been mediated by CNS factors in our experiments. Our findings are consistent with accumulating evidence suggesting that part of the metabolic effect of chronic CB1R antagonists is independent of reduction in energy intake, pointing to a role for peripheral tissue CB1R in induction of weight loss. In humans [43] and animal models [10, 12–16], changes in body weight during CB1R agonist or antagonist treatments can be independent of food intake, suggesting that CB1R plays a role in directly mediating metabolism. In accordance with our finding that CB1R mediates muscle insulin sensitivity, the role of CB1R in mediating energy expenditure has previously been shown to occur in peripheral tissues, by which mechanism a non-brain-penetrant CB1R antagonist reduced body weight and improved metabolism in mice [44].

The potential mechanisms involved in changes in insulin sensitivity following CB1R modulation remain unclear. It has been shown in vitro that insulin signalling may be affected by short- to long-term modulation of CB1R activity in muscle cells through differing mechanisms [41, 45]. In L6 cells, 24 h activation of CB1R with *N*-(2-chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (ACEA) decreased signalling mediated by extracellular signal-regulated protein kinase (ERK)1/2, but not signalling mediated by Akt, while antagonism with SR141716 increased Akt and ERK1/2 activation [45]. Our finding that acute agonism of CB1R impaired insulin-stimulated Akt phosphorylation is in agreement with several in vitro studies that have shown rapid effects of CB1R modulation on insulin signalling. For example, Esposito et al. demonstrated that SR141716 stimulated 2-DG uptake in L6 cells through phosphatidylinositol 3-kinase and downstream proteins, including Akt, an effect observed as early as 30 min post treatment [46]. In human skeletal muscle cells, treatment with the CB1R agonist anandamide for 1 h increased ERK1/2 and p38 mitogen-activated protein kinase, and impaired insulin-stimulated phosphorylation of Akt (Ser473), but not of Akt (Thr308) [41]. These studies indicate that tonic modulation of CB1R can lead to changes in insulin action in muscle, possibly through the cross-talk between endocannabinoid system and insulin signalling pathways. On the other hand, it is possible that the effects on muscle insulin sensitivity are secondary to systemic factors in the in vivo setting. For instance, CB1R modulation affects adipose tissue production and secretion of adiponectin [10, 47], which is closely associated with insulin sensitivity [48]. Although changes in insulin sensitivity by CB1R modulation may be linked to adiponectin in chronic conditions [49, 50], acute modulation of CB1R is not expected to significantly modify adiponectin production and secretion.

We conclude that extrahepatic insulin sensitivity is impaired after acute stimulation of non-CNS CB1R, most likely of CB1Rs residing in skeletal muscle. Our studies did not address the clinical question of whether treatment of insulin-resistant humans with a CB1R antagonist exerts its beneficial metabolic effect via CB1Rs outside the CNR. However, we speculate from our findings of acute modulation of CB1R that this may indeed be an important mechanism of action of this promising class of therapeutic agents. Our findings support the development and application of non-brain-penetrant CB1R antagonists that improve insulin sensitivity and ameliorate metabolic abnormalities accompanying insulin-resistant states.

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