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CIRCULATING INSULIN STIMULATES FATTY ACID RETENTION IN WHITE ADIPOSE TISSUE VIA K_{ATP} CHANNEL ACTIVATION IN THE CENTRAL NERVOUS SYSTEM ONLY IN INSULIN-SENSITIVE MICE

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ABSTRACT

Insulin signaling in the central nervous system (CNS) is required for the inhibitory effect of insulin on glucose production. Our aim was to determine whether the CNS is also involved in the stimulatory effect of circulating insulin on the tissue-specific retention of fatty acid (FA) from plasma. In wild-type mice, hyperinsulinemic-euglycemic clamp conditions stimulated the retention of both plasma triglyceride-derived FA and plasma albumin-bound FA in the various white adipose tissues (WAT), but not in other tissues including brown adipose tissue (BAT). Intracerebroventricular (i.c.v.) administration of insulin induced a similar pattern of tissue-specific FA partitioning. This effect of i.c.v. insulin administration was not associated with activation of the insulin signaling pathway in adipose tissue. I.c.v. administration of tolbutamide, a K_{ATP} channel blocker, considerably reduced (during hyperinsulinemic-euglycemic clamp conditions) and even completely blocked (during i.c.v. administration of insulin) WAT-specific retention of FA from plasma. This central effect of insulin was absent in CD36 deficient mice, indicating that CD36 is the predominant FA transporter in insulin-stimulated FA retention by WAT. In diet-induced insulin resistant mice, these stimulating effects of insulin (circulating or i.c.v. administered) on FA retention in WAT were lost. In conclusion, in insulin-sensitive mice, circulating insulin stimulates tissue-specific partitioning of plasma-derived FA in WAT in part through activation of K_{ATP} channels in the CNS. Apparently, circulating insulin stimulates fatty acid uptake in WAT, but not in BAT, directly, and indirectly, through the CNS.

INTRODUCTION

The central nervous system (CNS) is highly sensitive to insulin [1-6]. Insulin in the brain is mostly derived from the circulation, and only a modest amount, if any, is produced locally [6,7]. Circulating insulin can cross the blood-brain barrier [8,9] and exert metabolic effects in peripheral organs via the CNS. Intracerebroventricular (i.c.v.) administration of insulin decreases food intake, resulting in reduced body weight [3,10,11]. In addition, the central action of insulin plays a crucial role in the inhibitory effect of the hormone on hepatic glucose production [4,12].

Recently, a novel regulatory function for the effects of insulin through the CNS with regard to adipose tissue metabolism has been proposed, suggesting that intracellular lipolysis and lipogenesis in WAT is under the neuronal control of central insulin [13,14]. Furthermore, it has been shown that central glucose lowers plasma triglyceride (TG) levels by inhibiting the secretion of TG-rich lipoproteins by the liver [15]. Taken together, these observations imply an important role of the CNS in general and of central effects of insulin in the CNS in particular in the regulation of whole body TG metabolism.

The aim of the present study was to determine whether circulating insulin affects tissue-specific fatty acid (FA) partitioning from plasma through effects in the CNS. Using a dual tracer method, we report here that circulating insulin stimulates the uptake of TG-derived FA as well as albumin-bound FA specifically in WAT during hyperinsulinemic-euglycemic clamp conditions. Short-term (2.5 h) i.c.v. administration of insulin stimulates the retention of FA in WAT in a similar fashion. This effect, which requires the presence of the long chain FA transporter CD36, was prevented by inhibition of central ATP-dependent potassium (K_{ATP}) channels. Moreover, we show that the increase in FA retention in WAT, induced by hyperinsulinemic-euglycemic clamp conditions, is considerably reduced by inhibition of K_{ATP} channels in the CNS, demonstrating that the well-known effect of circulating insulin on insulin-mediated FA retention in WAT is mediated to a considerable extent by the effects of circulating insulin in the CNS. Finally, we show that these stimulating effects of insulin on FA retention through the CNS in WAT are lost in diet-induced obese mice.

MATERIALS AND METHODS

Animals

Male wild-type (WT) and $CD36^{-/-}$ mice (15 weeks old, both on C57Bl/6J background) were housed in a temperature-controlled room on a 12-hour light-dark cycle and were fed a standard mouse chow diet with free access to water. In the diet-induced obesity experiment, mice were fed *ad libitum* high-fat diet for 12 weeks (45 energy% of fat derived from palm oil; Research Diet Services BV, Wijk bij Duurstede, The Netherlands). All animal experiments were performed in accordance with the regulations of Dutch law on animal welfare and the institutional ethics committee for animal procedures from the Leiden University Medical Center, Leiden, The Netherlands approved the protocol.

Surgical procedure

For i.c.v. cannula implantation, the mice were anaesthetized with 0.5 mg/kg BW Medetomidine (Pfizer, Capelle a/d IJssel, The Netherlands), 5 mg/kg BW Midazolam (Roche, Mijdrecht, The Netherlands) and 0.05 mg/kg BW Fentanyl (Janssen-Cilag, Tilburg, The Netherlands) and placed in a stereotactic device (TSE systems, Homburg, Germany). A 25 gauge guide cannula was implanted into the left lateral ventricle using the following coordinates from Bregma: 1.0 mm lateral, 0.46 mm posterior and 2.2 mm ventral. The guide cannula was secured to the skull surface with dental cement (GC Europe N.V., Leuven, Belgium) and the anesthesia was antagonized using 2.5 mg/kg BW Antipamezol (Pfizer, Capelle a/d IJssel, The Netherlands), 0.5 mg/kg BW Flumazenil (Roche, Mijdrecht, The Netherlands) and 1.2 mg/kg BW Naloxon (Orpha, Purkersdorf, Austria). After a recovery period of 1 week, cannula placement was verified. Mice that ate >0.3 g in 1 h in response to i.c.v. injection of 5 μg neuropeptide Y (NPY, Bachem, St. Helens, UK) in 1 μL of artificial cerebrospinal fluid (aCSF) (Harvard Apparatus, Natick, MA, US) were considered to have the cannula correctly placed and were included in the study [16].

Preparation of radiolabeled emulsion particles

Protein-free VLDL-like triglyceride (TG)-rich emulsion particles were prepared from 100 mg total lipid at a weight ratio of triolein (Sigma, St. Louis, MA, US): egg yolk phosphatidylcholine (Lipoid, Ludwigshafen, Germany): lysophosphatidylcholine (Sigma, St. Louis, MA, US): cholesteryl oleate (Janssen, Beersse, Belgium): cholesterol (Sigma, St. Louis, MA, US) of 70: 22.7: 2.3: 3.0: 2.0 in the presence of 200 μCi of glycerol tri[9,10(n)-^{3}H]oleate ([^{3}H]TG) (GE Healthcare, Little Chalfont, UK), as previously described [17]. Lipids were hydrated in 10 mL of 2.4 M sodium chloride, 10 mM Hepes, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4, and sonicated for 30 min at 10 μm output using a Soniprep 150 (MSE Scientific Instruments, UK) equipped with a water bath for temperature (54°C) maintenance. Subsequently, the emulsion particles were divided into fractions with a different average size by density gradient ultracentrifugation. Intermediate sized (80 nm) [^{3}H]TG-labeled particles were mixed with a trace amount of [^{14}C]oleic acid ([^{14}C]FA) (GE Healthcare, Little Chalfont, UK) complexed with bovine serum albumin (BSA) in a ^{3}H:^{14}C ratio of 3:1.

Tissue-specific TG and FA partitioning

Postabsorptive (mice were fasted for 15 h with food withdrawn at 18:00h the day prior to the study), body weight-matched male mice were anaesthetized with 6.25 mg/kg BW Acepromazine (Alfasan, Woerden, The Netherlands), 6.25 mg/kg BW Midazolam (Roche, Mijdrecht, The Netherlands), and 0.31 mg/kg BW Fentanyl (Janssen-Cilag, Tilburg, The Netherlands). In the hyperinsulinemic-euglycemic clamp studies, insulin (Actrapid, Novo Nordisk, Bagsværd, Denmark) was administered i.v. by primed (4.5 mU), continuous (6.8 mU/h for 2.5 h) infusion to attain steady-state circulating insulin levels of ~ 4 ng/mL [16,18,19]. A variable i.v. infusion of a 12.5% *D*-glucose solution was used to maintain euglycemia as determined at 10-min intervals via tail bleeding (<3 μL, Accu-chek, Sensor Comfort; Roche Diagnostics, Mannheim, Germany). Insulin (0.5 mU/h; Actrapid, Novo Nordisk, Bagsværd, Denmark) or the K_{ATP} channel blocker tolbutamide (12 nmol/h [4,20]; Sigma, St. Louis, MA, US) dissolved in aCSF was infused i.c.v. at a constant rate of 2.5 μL/h during the entire experiment using a Harvard infusion pump. The dose of i.c.v. insulin was ascertained in a dose-finding study, to ensure that plasma insulin and glucose levels did not change. Control animals received 5% DMSO in aCSF. Thirty minutes after the start of the i.c.v. infusions or thirty minutes after the start of the hyperinsulinemic-euglycemic clamp experiments (as indicated), an i.v. infusion of the glycerol tri[^{3}H]oleate-labeled emulsion particles (0.33 nmol/h) together with albumin-bound [^{14}C]oleic acid (10.22 nmol/h) was started at a rate of 100 μL/h and maintained for 2 h. Blood samples were taken at baseline and 2 h after starting the i.v. infusion. Subsequently, the mice were sacrificed and organs were quickly harvested and snap-frozen in liquid nitrogen.

Plasma analysis

Blood samples were taken from the tail tip into chilled capillaries coated with paraoxon to prevent ex vivo lipolysis. The tubes were placed on ice and centrifuged at 4°C. Plasma levels of TG, free fatty acids (FFA) and glucose were determined using commercially available kits and standards according to the instructions of the manufacturer (Instruchemie, Delfzijl, The Netherlands). Plasma insulin levels were measured using a mouse-specific insulin ELISA kit (Mercodia AB, Uppsala, Sweden) and plasma leptin levels using a rat/mouse-specific ELISA kit (Millipore, St Charles, USA). Lipids were extracted from plasma according to Bligh and Dyer [21]. The lipid fraction was dried under nitrogen, dissolved into chloroform/methanol (5:1 [v/v]) and subjected to thin layer chromatography (TLC) (LK5D gel 150; Whatman, US) using hexane: diethylether: acetic acid (83:16:1) [v/v/v]) as mobile phase. Standards for TG and FA were included during the TLC procedure to locate spots of these lipids. Spots were scraped, lipids dissolved in hexane and radioactivity measured [22].

Tissue-specific FA retention analysis

Tissues were dissolved in 5 M potassium hydroxide in 50% (v/v) ethanol. After overnight saponification, protein content was determined in the various organs using a bicinchoninic acid (BCA) protein assay kit (BCA Protein Assay Kit, Thermo Scientific Pierce Protein Research Products, Rockford, US). Retention of radioactivity in the saponified tissues was measured per mg protein and corrected for the corresponding plasma specific activities of [^{3}H]TG and [^{14}C]FA [23,24].

Western blot analysis

Tissues were homogenized by Ultra-Turrax (22.000 rpm; 2x5 sec) in a 10:1 (v/w) ratio of ice-cold buffer containing: 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.6), 50 mM sodium fluoride, 50 mM potassium chloride, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid, 5 mM β-glycerophosphate, 1 mM sodium vanadate, 1 mM dithiothreitol, 1% nonyl phenoxypolyethoxylethanol (Tergitol-type NP40) and protease inhibitors cocktail (Complete, Roche, Mijdrecht, The Netherlands). Homogenates were centrifuged (13,200 rpm; 15 min, 4°C) and the protein content of the supernatant was determined using the BCA protein assay kit. Proteins (10-30 μg) were separated by 7-10% SDS-PAGE followed by transfer to a polyvinylidene fluoride transfer membrane. Membranes were blocked for 1 h at room temperature in tris-buffered saline tween-20 buffer with 5% non-fat dry milk followed by an overnight incubation with phospho-specific or total antibodies (all from Cell Signaling Technology, Beverly, US). Blots were then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Bands were visualized by ECL and quantified using Image J (NIH, US).

Statistical analysis

All data are presented as means ± SEM. Most data were analyzed using SPSS. A Kruskall-Wallis test for several independent samples was used, followed by a Mann-Whitney test for independent samples. P-values less than 0.05 were considered statistically significant.

RESULTS

I.v. administration of insulin increases the retention of plasma TG-derived and albumin-bound FA by WAT

To establish to which extent tissue-specific FA uptake from plasma is stimulated by circulating insulin, we compared in various organs the retention of FA derived from both glycerol tri[^{3}H]oleate-labeled VLDL-like emulsion particles and albumin-bound [^{14}C]oleic acid between mice during hyperinsulinemic-euglycemic clamp conditions and mice in basal conditions infused with vehicle (Fig. 1). During the clamp study, insulin levels were significantly higher as compared to basal conditions (4.4 *vs.* 0.3 ng/mL, P<0.01), whereas average plasma glucose levels remained similar (6.1 *vs.* 5.4 mmol/L, not significant). Plasma TG levels were similar (0.3 *vs.* 0.4 mmol/L, not significant), whereas plasma free fatty acids (FFA) levels decreased upon insulin administration (0.3 *vs.* 0.7 mmol/L, P<0.05).

As shown in Figure 1, hyperinsulinemia decreased the retention of plasma TG-derived FA and albumin-bound FA in liver (Fig. 1C), without affecting the retention in brown adipose tissue (Fig. 1B), heart (Fig. 1D) and skeletal muscle (Fig. 1E). Moreover, hyperinsulinemic-euglycemic clamp conditions increased albumin-bound FA retention by 68%, (P<0.05) in visceral fat and TG-derived FA and albumin-bound FA retention by 397% (P<0.05) and 579% (P<0.01), respectively, in subcutaneous fat and by 68% (not significant) and 267% (P<0.01), respectively, in epigonadal fat pads (Fig. 1F-H).

I.c.v. administration of insulin increases the retention of plasma TG-derived and albumin-bound FA by WAT

Subsequently, the dual-tracer method was used to determine tissue-specific FA retention after i.c.v. administration of insulin (0.5 mU/h for 2.5 h) (Fig. 2). Although i.c.v. insulin administration did not alter circulating levels of glucose, TG, FFA, insulin and leptin, it decreased the plasma half-life of [^{3}H]TG by 34% (P<0.05) (Table 1). Central insulin administration did not affect the retention of TG-derived FA and albumin-bound FA in brown adipose tissue, liver, heart and skeletal muscles (Fig. 2B-E). In contrast, i.c.v. insulin administration significantly increased FA retention in the different fat compartments (Fig. 2F-H): i.c.v. insulin increased the retention of TG-derived FA and albumin-bound FA by 145% (P=0.07) and 71% (P<0.05) in visceral fat, by 72% (P=0.06) and 111% (P<0.05) in subcutaneous fat, and by 58% (P<0.05) and 70% (P<0.05) in epigonadal fat, respectively.

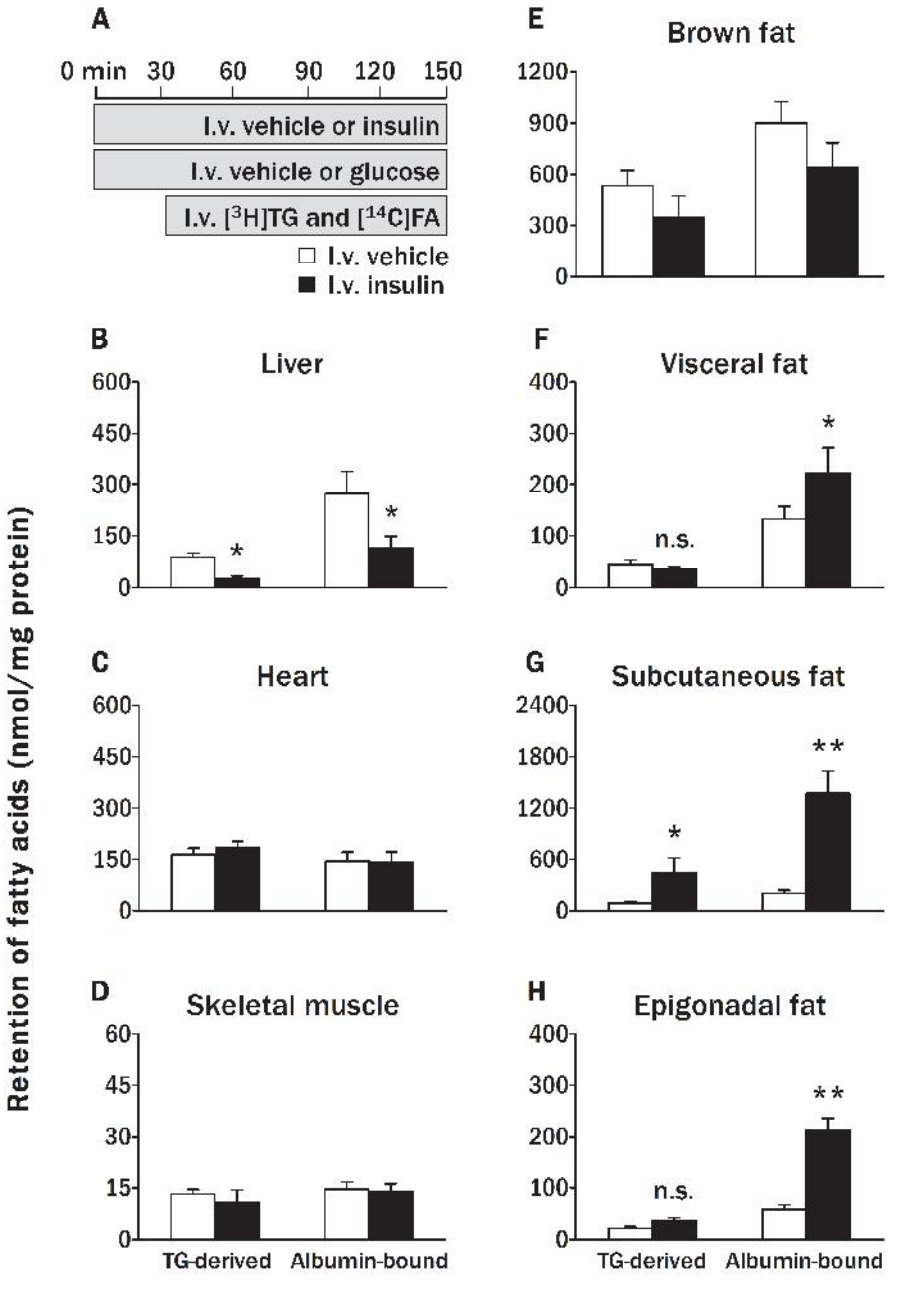

Figure 1. Circulating insulin stimulates FA retention by WAT, while decreasing FA retention by liver. Postabsorptive, body weight-matched WT mice received continuous i.v. infusion of vehicle (white bars) or insulin/glucose (hyperinsulinemic-euglycemic clamp study, black bars) (A). Infusion of glycerol tri[^{3}H]oleate within VLDL-like emulsion particles and albumin-bound [^{14}C]oleic acid was started 30 minutes after the start of i.v. infusion of vehicle or glucose/insulin and maintained for 2 h. Subsequently, the mice were sacrificed and the retention of TG-derived FA and albumin-bound FA was determined in liver (B), heart (C), skeletal muscle (D), brown fat (E), visceral fat (F), subcutaneous fat (G) and epigonadal fat (H). Values are means ± SEM for at least 5 mice per group. * $P<0.05$ *vs.* vehicle, ** $P<0.01$ *vs.* vehicle.

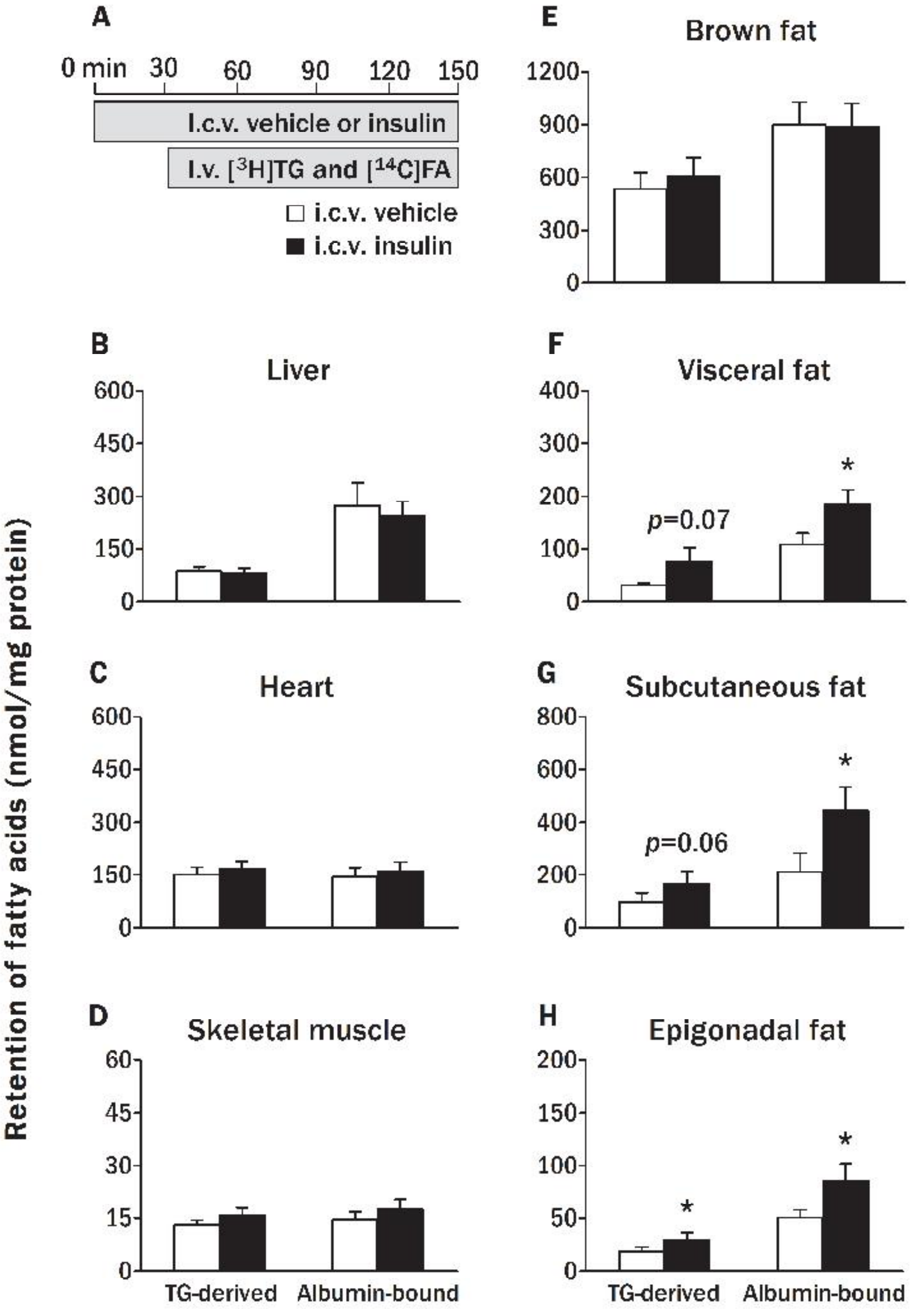

Figure 2. I.c.v. insulin administration stimulates FA retention by WAT. Postabsorptive, body weight-matched WT mice received continuous i.c.v. infusion of vehicle (white bars) or insulin (black bars, 0.5 mU/h) (A). Thirty minutes after starting the i.c.v. infusion, the mice were infused for 2 h with glycerol tri[^{3}H]oleate within VLDL-like emulsion particles and albumin-bound [^{14}C]oleic acid. Subsequently, the mice were sacrificed and the retention of TG-derived FA and albumin-bound FA was determined in liver (B), heart (C), skeletal muscle (D), brown fat (E), visceral fat (F), subcutaneous fat (G) and epigonadal fat (H). Values are means ± SEM for at least 7 mice per group. * $P<0.05$ *vs.* vehicle.

The effect of i.c.v. insulin administration on FA retention in WAT is independent of modulation of insulin, leptin or cAMP-dependent signaling pathways in WAT

To investigate the molecular mechanism(s) underlying the i.c.v. insulin-induced FA retention in WAT, we studied various signaling pathways involved in the regulation of FA metabolism in both epigonadal and visceral fat. In agreement with the absence of any effect on plasma insulin levels (Table 1), i.c.v. insulin administration did not affect peripheral insulin signaling pathways, as phosphorylation of PKB on Ser473 and Thr308 was not increased in WAT (Fig. 3), nor of FOXO1 on Ser256 (data not shown). In addition, i.c.v. insulin administration did not induce any changes in STAT3 Tyr705 or CREB Ser133 phosphorylation in WAT, indicating that neither leptin nor PKA signaling pathways are modified by i.c.v. insulin administration.

Table 1. I.c.v. insulin administration does not affect plasma parameters, except for [^{3}H]TG half-life. Plasma parameters of mice that received i.c.v. infusion of vehicle or insulin obtained at baseline and 2 h after starting an i.v. infusion of glycerol tri[^{3}H]oleate-labeled VLDL-like emulsion particles and albumin-bound [^{14}C]oleic acid. Values are means ± SEM for at least 9 mice per group. TG, triglycerides; (F)FA, (free) fatty acids. * P<0.05 *vs.* vehicle.

	Baseline		2 hours	
	I.c.v. vehicle	I.c.v. tolbutamide	I.c.v. vehicle	I.c.v. tolbutamide
Glucose (mmol/L)	4.9 ± 0.2	5.5 ± 0.3	4.7 ± 0.2	4.8 ± 0.4
TG (mmol/L)	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
FFA (mmol/L)	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
Insulin (ng/mL)	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
Leptin (ng/mL)	1.0 ± 0.1	1.3 ± 0.4	1.3 ± 0.1	1.4 ± 0.3
Half-life [^{14}C]FA (min)	-	-	0.6 ± 0.2	0.6 ± 0.1
Half-life [^{3}H]TG (min)	-	-	3.5 ± 0.1	2.3 ± 0.1 *

The effect of i.c.v. insulin administration on FA retention by WAT is dependent on the activation of central K_{ATP} channels

Part of the central effects of insulin on the regulation of food intake and hepatic glucose production are dependent on activation of hypothalamic K_{ATP} channels [25]. Therefore, we investigated whether the stimulatory effect of i.c.v. insulin administration on FA retention in WAT could be blocked by i.c.v. co-administration of the K_{ATP} channel blocker tolbutamide (Fig. 4). I.c.v. administration of insulin (0.5 mU/h) did not affect plasma levels of glucose, TG, FFA and insulin, irrespective of co-administration of tolbutamide (12 nmol/h) (Table 2). In accordance with the previous experiments, the plasma half-life of [^{3}H]TG decreased by 29%, (P<0.05) upon i.c.v. insulin administration compared to vehicle. However i.c.v. insulin, administered concurrently with i.c.v. tolbutamide, did not alter plasma TG and FA kinetics. I.c.v. administration of tolbutamide alone did not affect tissue-specific FA partitioning. Comparable to the previous study, FA retention in liver, heart and skeletal muscles was unaltered upon i.c.v. insulin administration and remained unaltered upon co-administration of tolbutamide (Fig. 4B-D). Interestingly, tolbutamide completely blocked the stimulation of both plasma TG-derived FA and albumin-bound FA retention in WAT induced by i.c.v. insulin administration (Fig. 4E-G).

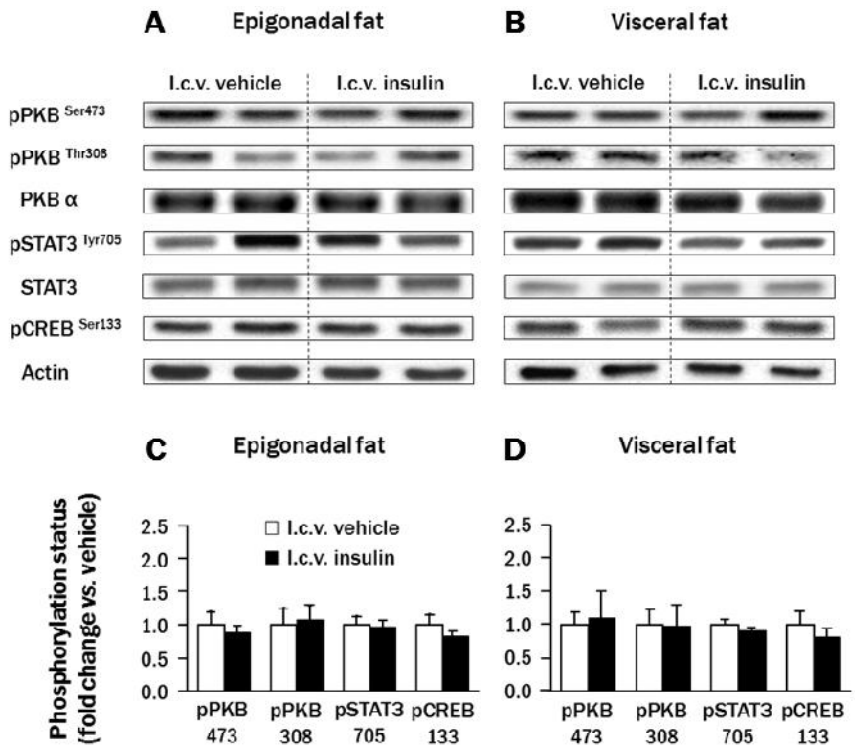

Figure 3. I.c.v. insulin administration does not affect insulin, leptin and cAMP signaling pathways in WAT. The phosphorylation state of PKB, STAT3 and CREB was analyzed by western blot in both epigonadal fat (A) and visceral fat (B) from mice that received i.c.v. infusion of vehicle or insulin for 2.5 h. The corresponding quantification of the western blot data was normalized for total protein or actin and expressed as fold change compared to vehicle (C-D). Values represent means ± SEM for at least 5 mice per group.

The effect of circulating insulin on FA retention by WAT is mediated through K_{ATP} channel activation in the CNS

In order to investigate the contribution of the CNS to the effect of circulating insulin on FA retention in WAT, we examined whether i.c.v. administration of the K_{ATP} channel blocker tolbutamide inhibits the insulin-stimulated FA retention in WAT during hyperinsulinemic-euglycemic clamp conditions (Fig. 5). In steady-state clamp conditions, plasma glucose, TG and insulin concentrations were similar in both groups, as shown in Table 2. Hyperinsulinemia suppressed FFA levels to a similar extent in i.c.v. tolbutamide- and i.c.v. vehicle-infused mice. The glucose infusion rates required to maintain euglycemia, however, were 22% lower in mice that received i.c.v. tolbutamide compared to i.c.v. vehicle ($P<0.05$). This is consistent with previous findings showing that i.c.v. administration of the K_{ATP} channel blocker tolbutamide impair the inhibition of hepatic glucose production in response to hyperinsulinemia [4,26]. The plasma half-life of [^{3}H]TG decreased by 60% ($P<0.05$) during hyperinsulinemic conditions, but was partly restored by i.c.v. tolbutamide (Table 3). FA retention in liver, heart and skeletal muscles was unaffected by i.c.v. tolbutamide (Fig. 5B-D). Remarkably, i.c.v. tolbutamide considerably decreased the stimulatory effect of circulating insulin during clamp conditions on FA retention in WAT (Fig. 5E-G).

Table 2. I.c.v. tolbutamide administration does not affect plasma parameters. Plasma parameters of mice that received i.c.v. infusion of vehicle or tolbutamide at baseline or hyperinsulinemic conditions. Values are means ± SEM for at least 6 mice per group. TG, triglycerides; (F)FA, (free) fatty acids. * $P<0.05$ *vs.* vehicle.

	Baseline				2 hours			
	Vehicle	Insulin	Tobutamide	Insulin + Tolbutamide	Vehicle	Insulin	Tobutamide	Insulin + Tolbutamide
Glucose (mmol/L)	6.4 ± 0.3	6.7 ± 0.1	6.1 ± 0.8	7.0 ± 0.7	5.5 ± 0.3	5.5 ± 0.1	5.6 ± 1.1	5.7 ± 1.6
TG (mmol/L)	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
FFA (mmol/L)	1.2 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.8 ± 0.1
Insulin (ng/mL)	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
Half-life [^{14}C]FA (min)	-	-	-	-	0.6 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.7 ± 0.1
Half-life [^{3}H]TG (min)	-	-	-	-	2.8 ± 0.5	2.0 ± 0.2*	2.7 ± 0.3	2.8 ± 0.1

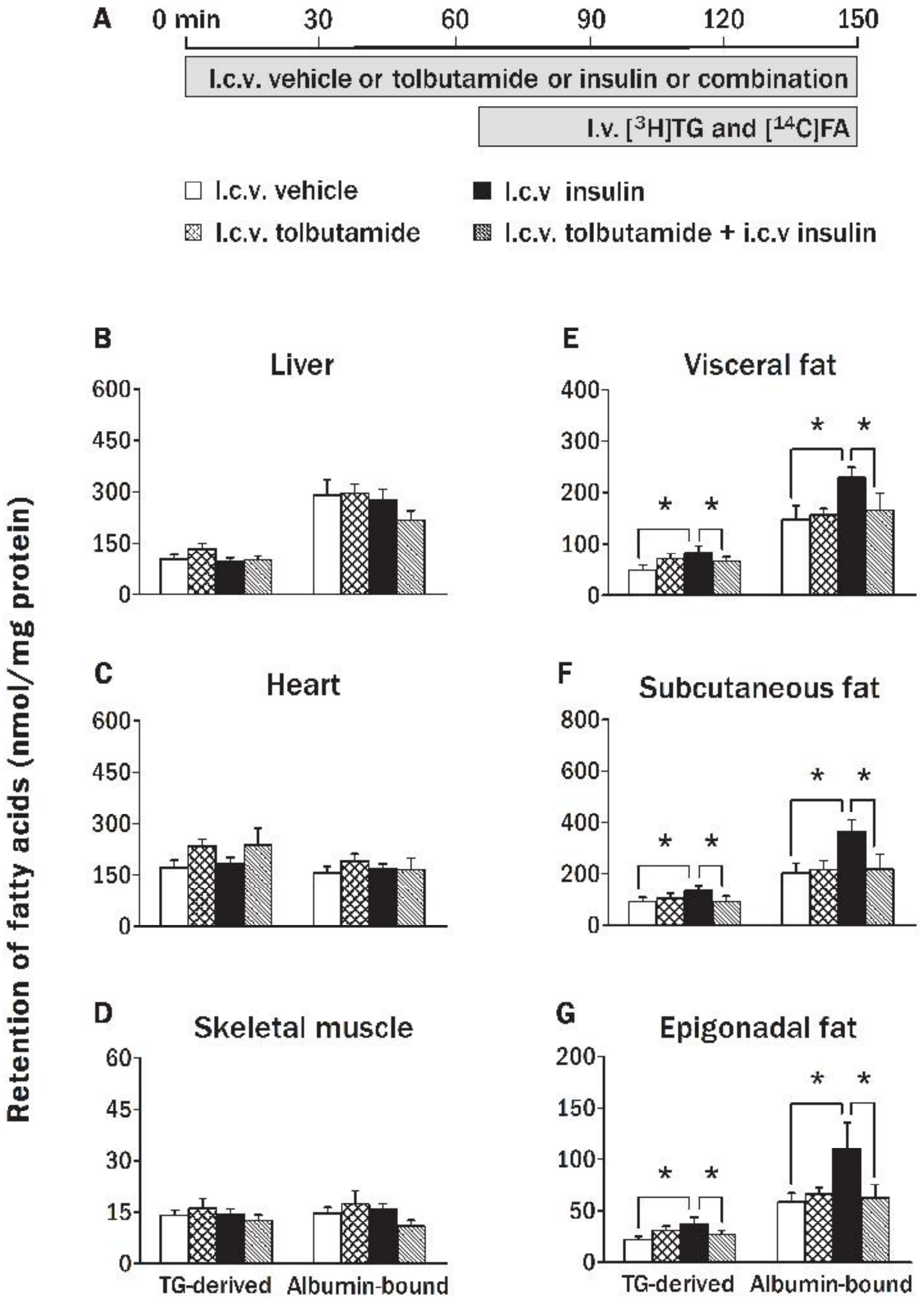

Figure 4. I.c.v. co-administration of the K_{ATP} channel blocker tolbutamide blocks the stimulation of FA retention in WAT by i.c.v. insulin. Postabsorptive, body weight-matched WT mice received continuous i.c.v. infusion of vehicle (white bars), tolbutamide (cross-hatched bars, 12 nmol/h), insulin (black bars) or insulin in combination with tolbutamide (hatched bars) (A). Thirty minutes after starting the i.c.v. infusion, the mice were infused for 2 h with glycerol tri[^{3}H]oleate within VLDL-like emulsion particles and albumin-bound [^{14}C]oleic acid. Subsequently, the mice were sacrificed and the retention of TG-derived FA and albumin-bound FA was determined in liver (B), heart (C), skeletal muscle (D), visceral fat (E), subcutaneous fat (F) and epigonadal fat (G). Values are means ± SEM for at least 8 mice per group. * $P<0.05$ *vs.* vehicle.

The effect of i.c.v. insulin administration on FA retention in WAT requires the presence of CD36

As CD36 is one of the main long-chain FA transporters and insulin can upregulate translocation and protein expression of CD36 [27,28], we studied to which extent CD36 is involved in the i.c.v. insulin-stimulated FA retention by WAT. Therefore, we investigated the effects of i.c.v. insulin on tissue-specific FA retention in $CD36^{-/-}$ *vs.* WT mice (Fig. 6). In accordance with previous observations [29], basal plasma TG and FFA levels were increased in $CD36^{-/-}$ mice compared to WT mice by 28% and 26%, respectively ($P<0.05$), but they remained unaltered, as did circulating glucose and insulin levels, following i.c.v. administration of insulin (data not shown). I.c.v. insulin decreased the half-lives of plasma $[^{3}H]$TG and $[^{14}C]$FA in WT mice, but not in $CD36^{-/-}$ mice (data not shown). Again, we confirmed in WT mice that i.c.v. insulin stimulated the retention of TG-derived FA and albumin-bound FA by 100% ($P<0.05$) and 107% ($P<0.05$) in visceral fat, by 53% ($P=0.09$) and 85% ($P<0.05$) in subcutaneous fat and by 77% ($P=0.06$) and 104% ($P<0.05$) in epigonadal fat pads, respectively. In basal conditions, *i.e.* i.c.v. administration of vehicle, the retention of both FA by WAT was dramatically decreased in $CD36^{-/-}$ mice compared to WT mice (Fig. 6B-D). More importantly, i.c.v. insulin administration did not stimulate FA retention from plasma by WAT in $CD36^{-/-}$ mice. I.c.v. insulin administration did not affect FA retention in other organs of $CD36^{-/-}$ mice, in accordance with the observations made in WT mice (see above). Since i.c.v. insulin administration was unable to stimulate FA retention by WAT in $CD36^{-/-}$ mice, these data suggest that CD36 is the predominant FA transporter mediating i.c.v. insulin-stimulated FA retention by WAT.

Table 3. I.c.v. administration of tolbutamide decreases glucose infusion rate during hyperinsulinemic-euglycemic clamp conditions. Plasma parameters of mice that received i.c.v. infusion of vehicle or tolbutamide at baseline and during hyperinsulinemic conditions. Values are means ± SEM for at least 9 mice per group. TG, triglycerides; (F)FA, (free) fatty acids; GIR, glucose infusion rate. * $P<0.05$ *vs.* vehicle.

	Baseline		Clamp conditions	
	I.c.v. vehicle	I.c.v. tolbutamide	I.c.v. vehicle	I.c.v. tolbutamide
Glucose (mmol/L)	7.3 ± 1.3	7.3 ± 1.0	6.1 ± 0.4	6.7 ± 1.3
TG (mmol/L)	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
FFA (mmol/L)	0.9 ± 0.1	0.8 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
Insulin (ng/mL)	0.3 ± 0.1	0.2 ± 0.1	4.4 ± 1.5	4.1 ± 0.8
GIR (umol/min/kg)	-	-	114 ± 5.7	89 ± 3.2 *
Hematocrit (%)	43 ± 2	42 ± 2	42 ± 2	41 ± 2
Half-life $[^{14}C]$FA (min)	-	-	0.5 ± 0.1	0.7 ± 0.1
Half-life $[^{3}H]$TG (min)	-	-	1.4 ± 0.1	1.7 ± 0.1

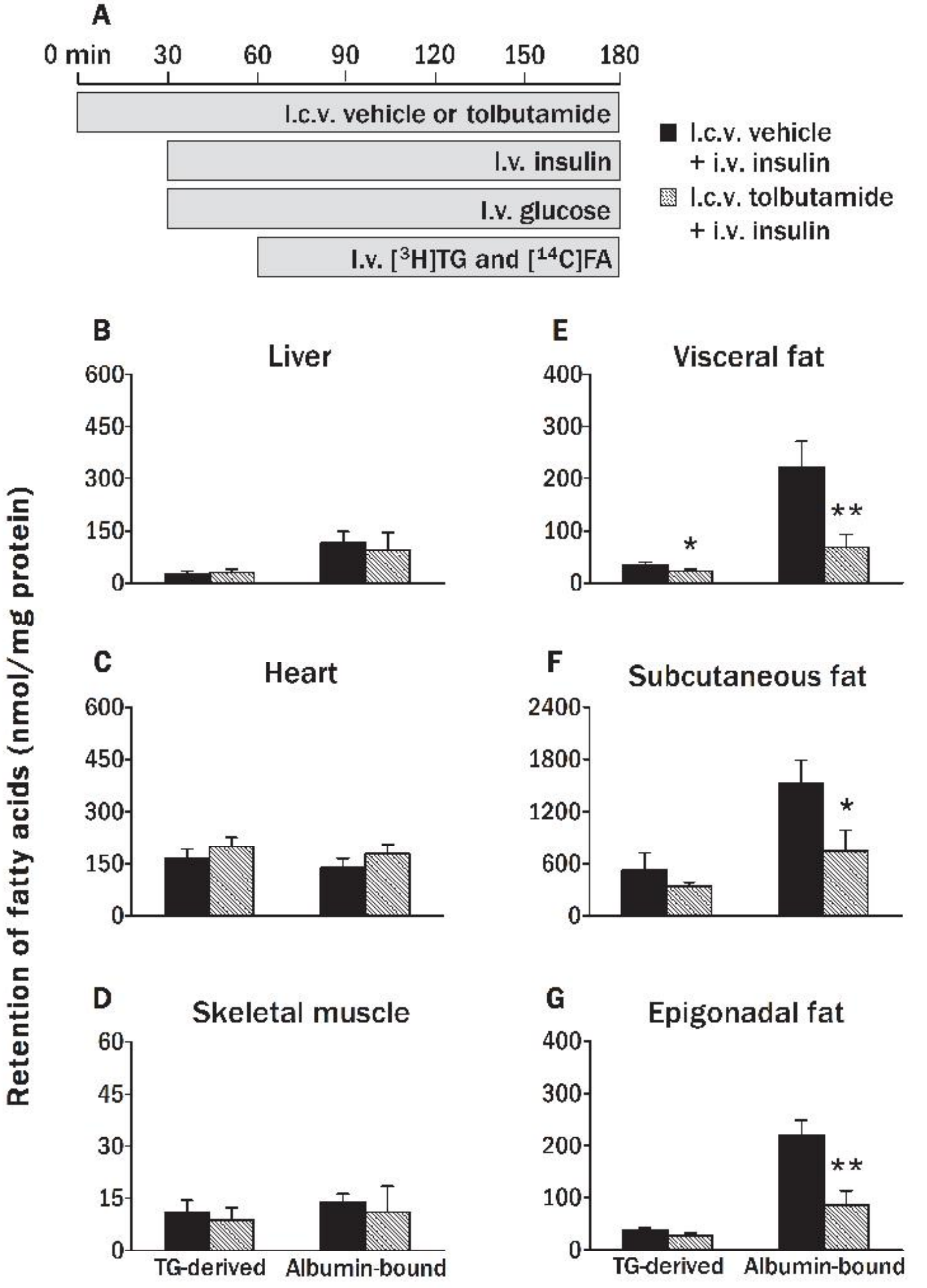

Figure 5. I.c.v. administration of tolbutamide impairs the stimulation of FA retention in WAT induced by hyperinsulinemic-euglycemic clamp conditions. Postabsorptive, body weight-matched mice received continuous i.c.v. infusion of vehicle (black bars) or tolbutamide (12 nmol/h, hatched bars) 30 minutes before the start of hyperinsulinemic-euglycemic clamp study (A). Infusion of glycerol tri[^{3}H]oleate within VLDL-like emulsion particles and albumin-bound [^{14}C]oleic acid was started 30 minutes after the start of the hyperinsulinemic-euglycemic clamp study and maintained for 2 h. Subsequently, the mice were sacrificed and the retention of TG-derived FA and albumin-bound FA was determined in liver (B), heart (C), skeletal muscle (D), visceral fat (E), subcutaneous fat (F) and epigonadal fat (G). Values are means ± SEM for at least 5 mice per group. * $P<0.05$ *vs.* vehicle, ** $P<0.01$ *vs.* vehicle.

The effect of i.c.v. insulin on FA retention in WAT is lost in diet-induced obesity

Subsequently, we examined the effect of i.c.v. insulin administration on FA retention in high-fat fed mice (Fig. 7). Body weight of the diet-induced obese mice was 40% higher compared to chow fed mice (P<0.01). I.c.v. insulin administration did not alter glucose, TG, FFA and insulin levels (Table 4). In contrast to chow fed mice, i.c.v. administration of insulin did not decrease the plasma half-life of [^{3}H]TG in these diet-induced obese mice. I.c.v. insulin administration did not alter FA retention in liver, heart or skeletal muscles, but surprisingly also not in WAT (Fig. 7B-G).

Table 4. I.c.v. insulin administration in diet-induced obese mice does not affect plasma parameters. Parameters of diet-induced obese mice that received i.c.v. infusion of vehicle or insulin obtained at baseline and 2 h after starting an i.v. infusion of glycerol tri[^{3}H]oleate-labeled VLDL-like emulsion particles and albumin-bound [^{14}C]oleic acid. Values are means ± SEM for at least 9 mice per group. TG, triglycerides; (F)FA, (free) fatty acids.

	Baseline		2 hours	
	I.c.v. vehicle	I.c.v. insulin	I.c.v. vehicle	I.c.v. insulin
Body mass (g)	32.5 ± 0.6	32.0 ± 0.4	32.5 ± 0.6	32.0 ± 0.4
Glucose (mmol/L)	7.7 ± 0.3	7.7 ± 0.2	6.0 ± 0.3	5.6 ± 0.2
TG (mmol/L)	1.0 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
FFA (mmol/L)	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
Insulin (ng/mL)	3.8 ± 0.3	4.0 ± 0.4	4.0 ± 0.4	3.8 ± 0.3
Half-life [^{14}C]FA (min)	-	-	1.1 ± 0.1	1.2 ± 0.1
Half-life [^{3}H]TG (min)	-	-	9.2 ± 1.1	9.4 ± 1.6

K_{ATP} channel blockage does not affect circulating insulin-stimulated FA retention by WAT in diet-induced obese mice

Finally, we examined whether i.c.v. administration of the K_{ATP} channel blocker tolbutamide would still inhibit the insulin-stimulated FA retention in WAT during hyperinsulinemic-euglycemic clamp conditions in high-fat fed mice (Fig. 8). Therefore, we determined FA retention between mice during hyperinsulinemic-euglycemic clamp conditions infused i.c.v. either with tolbutamide or vehicle and mice in basal conditions infused i.c.v. with vehicle. Body weight of the high-fat fed mice was 65% higher compared to chow fed mice (P<0.01). In steady-state clamp conditions, plasma glucose and TG concentrations were similar between tolbutamide- and vehicle-treated mice, as shown in Table 5. During hyperinsulinemic-euglycemic clamp conditions, circulating insulin levels were fourfold higher in both groups as compared to basal conditions, resulting in a decrease of ~50% in FFA levels. The rate of glucose infusion necessary to maintain euglycemia, was not different between tolbutamide- and vehicle-treated animals. The plasma half-life of [^{3}H]TG was similar in basal conditions as in hyperinsulinemic conditions, irrespective of i.c.v. tolbutamide administration. Similar to chow fed mice, hyperinsulinemia decreased the retention of plasma TG-derived FA and albumin-bound FA in liver, which was unaltered by i.c.v. tolbutamide administration (Fig. 8B). FA retention in liver, heart en skeletal muscles was similar in all groups (Fig. 8B-D). Unlike in chow fed mice, hyperinsulinemia did not stimulate FA retention in WAT and i.c.v. tolbutamide did not decrease FA retention in WAT (Fig. 8E-G).

DISCUSSION

This study addressed the effects of circulating insulin on tissue-specific TG-derived FA and albumin-bound FA retention, and the role of central insulin action in these effects. Circulating insulin, which activated insulin signaling in the brain, stimulated retention of both TG-derived FA and albumin-bound FA in WAT. Centrally administered insulin stimulated retention of both FA sources in a similar manner, but without activating insulin signaling in WAT. Tolbutamide, a K_{ATP} channel blocker, decreased this insulin-stimulated FA partitioning to WAT during peripheral insulin infusion, and even abolished this effect during i.c.v. co-administration with insulin. Taken together, we show that the central effects of circulating insulin contribute on average ~30% to TG-derived FA uptake in WAT and ~66% to albumin-derived FA uptake in WAT to the total effects of circulating insulin during hyperinsulinemic clamp conditions. In contrast, in diet-induced obese mice, centrally administered insulin was unable to stimulate FA retention in WAT. Furthermore, inhibition of central action of i.c.v. administered insulin or circulating insulin by tolbutamide did not affect FA retention in WAT. Collectively, these data indicate that circulating insulin stimulates FA partitioning from plasma to WAT to a considerable extent through indirect effects on central neural pathways and that insulin-stimulated FA partitioning through these central neural pathways is absent in mice with insulin resistance after 12 weeks of high-fat feeding.

First, we determined tissue-specific FA uptake during hyperinsulinemic-euglycemic clamp conditions compared to basal conditions. In agreement with the rise in plasma insulin levels, hypothalamic insulin signaling was activated during hyperinsulinemic-euglycemic clamp conditions as phosphorylation of PKB on Thr308 (1.24 ± 0.05 *vs.*. 1.00 ± 0.10, P=0.08) and its downstream target PRAS40 on Thr246 (1.51 ± 0.11 *vs.*. 1.00 ± 0.06, P<0.05) were increased compared to basal conditions (unpublished data). We observed that peripheral as well as i.c.v. insulin administration promotes FA storage specifically in WAT. By employing the dual tracer methods described by Teusink *et al.* [23], we made a distinction between FA derived from plasma TG and FA derived from plasma albumin. Both peripheral and i.c.v. administration of insulin stimulated the retention of both sources of plasma FA in WAT. This insulin-stimulated FA retention by WAT was accompanied by a decreased half-life of TG, reflecting increased turnover

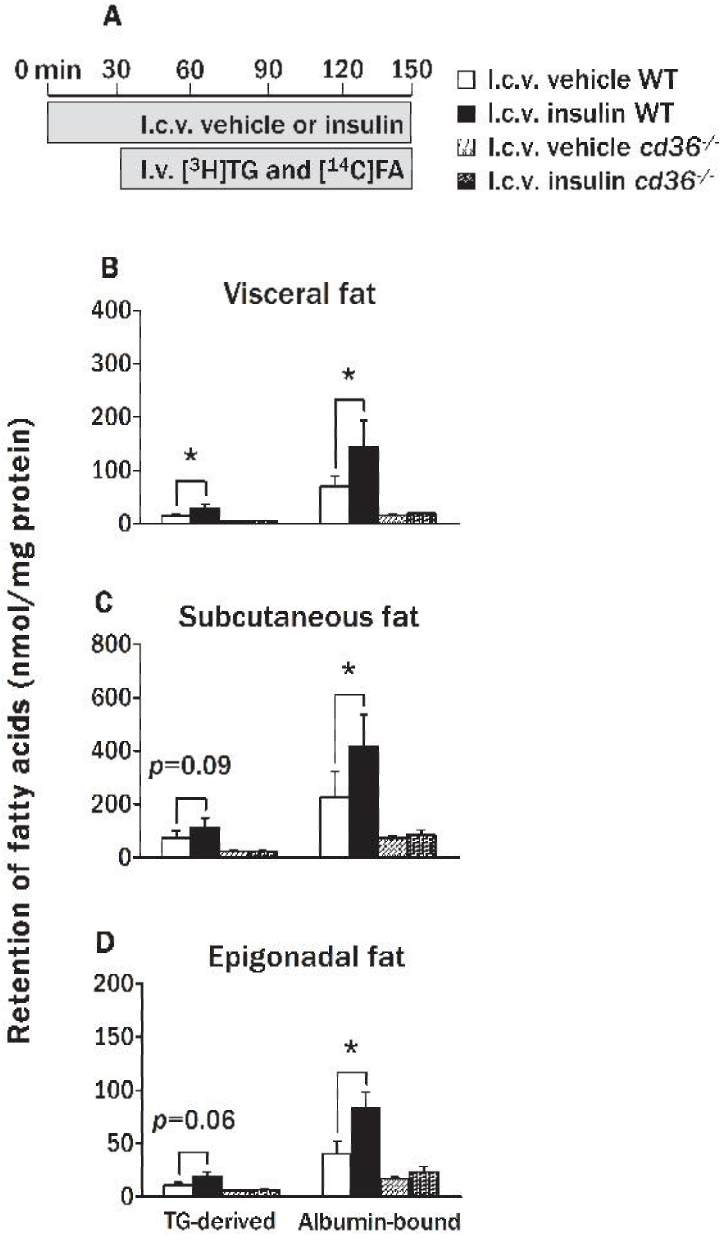

Figure 6. The stimulation of FA retention in WAT by i.c.v. insulin administration is abrogated in CD36-deficient mice. Postabsorptive, body weight-matched WT and CD36-deficient mice received continuous i.c.v. infusion of vehicle (white and hatched bars) or insulin (black and cross-hatched bars) (A). Thirty minutes after starting the i.c.v. infusion, the mice were infused for 2 h with glycerol tri[^{3}H] oleate within VLDL-like emulsion particles and albumin-bound [^{14}C]oleic acid. Subsequently, the mice were sacrificed and the retention of TG-derived FA and albumin-bound FA was determined in visceral fat (B), subcutaneous fat (C) and epigonadal fat (D). Values are means ± SEM for at least 6 mice per group. * $P<0.05$ *vs.* vehicle.

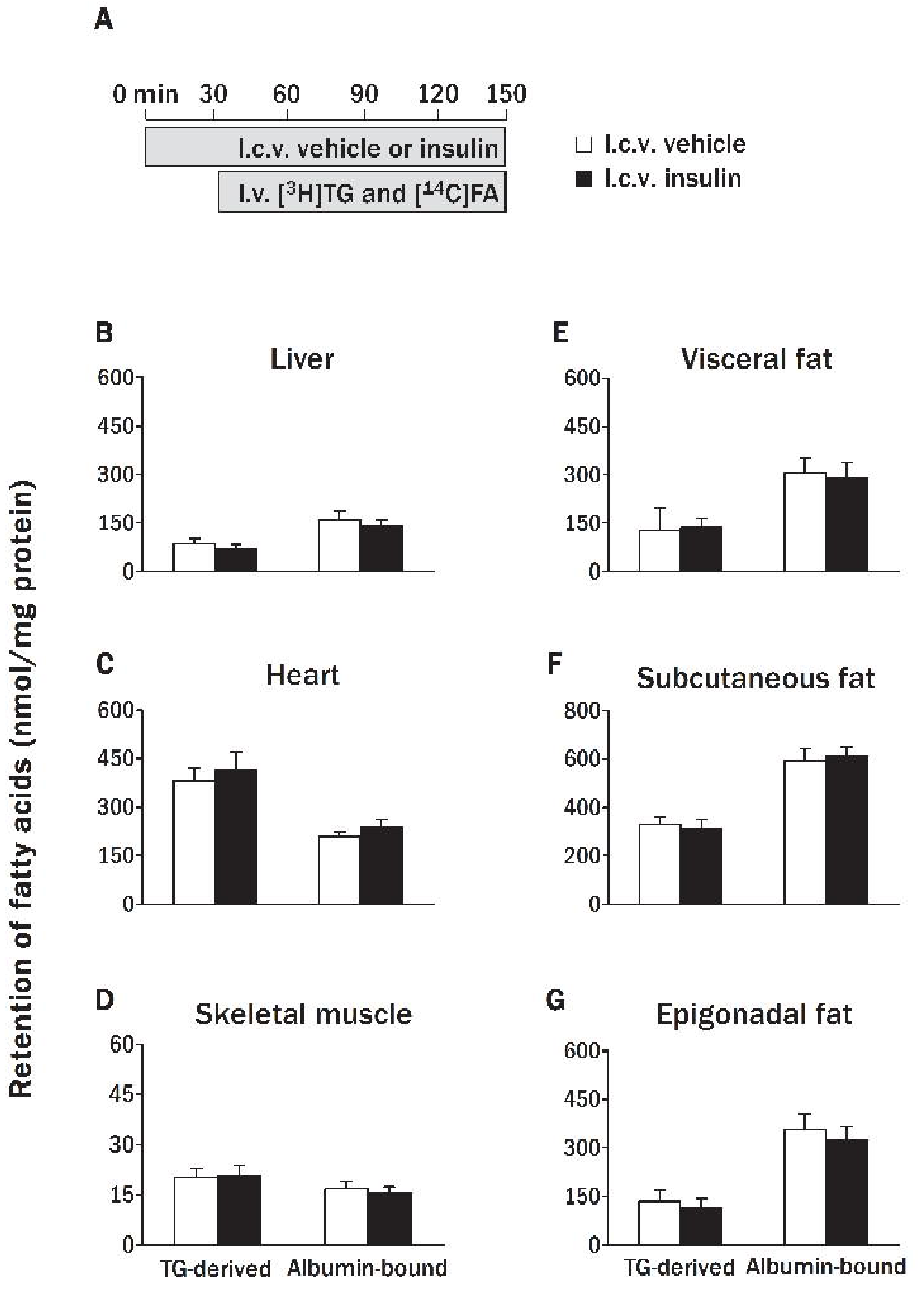

Figure 7. I.c.v. insulin administration in diet-induced obese mice does not stimulate FA retention by WAT. Postabsorptive, body weight-matched WT mice received continuous i.c.v. infusion of vehicle (white bars) or insulin (black bars, 0.5 mU/h) (A). Thirty minutes after starting the i.c.v. infusion, the mice were infused for 2 h with glycerol tri[^{3}H]oleate within VLDL-like emulsion particles and albumin-bound [^{14}C]oleic acid. Subsequently, the mice were sacrificed and the retention of TG-derived FA and albumin-bound FA was determined in liver (B), heart (C), skeletal muscle (D), visceral fat (E), subcutaneous fat (F) and epigonadal fat (G). Values are means ± SEM for at least 9 mice per group.

Table 5. I.c.v. administration of tolbutamide in diet-induced obese mice during hyperinsulinemic-euglycemic clamp conditions does not affect plasma parameters. Parameters of diet-induced obese mice that received i.c.v. infusion of vehicle or tolbutamide at baseline and during basal infusions or hyperinsulinemic conditions. Values are means ± SEM for at least 5 mice per group. TG, triglycerides; (F)FA, (free) fatty acids; GIR, glucose infusion rate.

	Baseline		Basal infusion	Clamp conditions	
	I.c.v. vehicle	I.c.v. tolbutamide	I.c.v. vehicle	I.c.v. vehicle	I.c.v. tolbutamide
Body mass (g)	38.6 ± 0.8	38.6 ± 0.4	38.6 ± 0.8	39.6 ± 0.3	38.6 ± 0.4
Glucose (mmol/L)	6.6 ± 0.2	6.0 ± 0.2	5.7 ± 0.2	7.0 ± 0.2	7.5 ± 0.2
TG (mmol/L)	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.1
FFA (mmol/L)	0.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.5 ± 0.1	0.4 ± 0.1
Insulin (ng/mL)	1.8 ± 0.3	1.4 ± 0.2	2.2 ± 0.3	7.7 ± 0.4	6.9 ± 0.1
GIR (μmol/kg.min)	-	-	-	52.4 ± 1.5	53.3 ± 2.7
Hematocrit (%)	44± 2	44 ± 1	42 ± 2	41 ± 2	41 ± 2
Half-life [^{14}C]FA (min)	-	-	1.2 ± 0.1	1.5 ± 0.2	1.2 ± 0.1
Half-life [^{3}H]TG (min)	-	-	14.6 ± 1.5	16.1 ± 0.5	17.0 ± 1.2

of plasma TG. Interestingly, these acute effects of i.c.v. insulin administration on plasma TG and FA fluxes towards WAT, together with recent data showing that brain insulin suppresses intracellular lipolysis and stimulates lipogenesis in adipocytes [14], provide an explanation for the finding that chronic i.c.v. insulin administration increases fat mass [13].

In addition to stimulating FA retention by WAT, peripheral insulin infusion decreased FA storage by the liver. However, i.c.v. administration of insulin or blockage of central K_{ATP} channels did not affect FA retention by the liver. This suggests that the effect of peripheral insulin on liver FA retention is not mediated by the CNS, but, rather, seems to be a direct effect on the liver caused by an associated reduction in the availability of plasma FFAs.

Recently, Bartelt *et al.* described the fundamental role of BAT for TG and FA clearance [30]. Exposure of mice to cold accelerated TG uptake by BAT, in a lipoprotein lipase (LPL) and CD36-dependent manner. In the current study, we have also determined the effects of insulin on FA retention by BAT. However, peripheral nor central insulin administration increased FA retention by BAT, indicating that insulin, in contrast to cold exposure, is not a major stimulator of TG and FA uptake by BAT.

The question arises how insulin exerts its indirect effects through the central nervous system on FA uptake in WAT. We used an i.c.v. dose of insulin, ascertained in a dose-finding study, which did not affect plasma insulin and glucose levels. Nonetheless, this i.c.v. dose of insulin stimulated FA uptake by WAT. Therefore, the stimulatory effect of i.c.v. insulin on retention of FA by WAT is not caused by i.c.v. insulin-induced changes in plasma levels of glucose and insulin. We can not exclude the possibility of other neuroendocrine, insulin-potentiating effects induced by the effects of insulin on the central nervous system, like a decrease in plasma levels of the insulin antagonist epinephrine or corticosterone. Furthermore, it is possible that the indirect effects of insulin through the CNS involve alterations in the activity of the autonomic nerves projecting towards WAT, *i.e.* in (para)sympathetic activity. In accordance with this concept, we documented in a model of suprarenal fat pads in rats that selective vagotomy (*i.e.* parasympathetic denervation of the suprarenal fat pads) reduced FA uptake in WAT by 36% during hyperinsulinemic clamp conditions [31]. An increase in parasympathetic activity towards WAT might be involved in the indirect effects of circulating insulin through the CNS, although the existence of parasympathetic innervation of other fat compartments is at present uncertain. Alternatively, it might be hypothesized that a change in the activity of the sympathetic activity towards WAT may be involved in the indirect effects of insulin on FA uptake in WAT *in vivo*, as it has been shown that insulin administered in the mediabasal hypothalamus dampens the sympathetic activity [14].

We determined to which extent the stimulatory effects of circulating insulin on FA retention in WAT were mediated through the CNS by i.c.v. administration of tolbutamide. Tolbutamide, which belongs to the sulfonylurea family, inhibits K_{ATP} channels in the hypothalamus when administered i.c.v. [4]. Sulfonylureas bind to the K_{ATP} channels on the cell membrane of certain hypothalamic neurons, where they inhibit the hyperpolarizing efflux of potassium [32]. Insulin acts in central neurons by opening/activating K_{ATP} channels, and, consequently, i.c.v. administration of tolbutamide blocks central K_{ATP} channel-mediated insulin signaling [4]. Our study shows that the rapid stimulatory effect of i.c.v. insulin administration on FA uptake in WAT is blocked by i.c.v. co-administration of tolbutamide. This indicates that tissue-specific stimulation of FA uptake by WAT induced by i.c.v. insulin administration is mediated by activation of K_{ATP} channels in the brain. By blocking central insulin signaling by i.c.v. administration of tolbutamide during hyperinsulinemic-euglycemic clamp conditions, we showed that insulin stimulates FA retention in WAT to a considerable extent via action in the CNS.

We also assessed the effects of high-fat diet on the central effects of circulating insulin on FA retention in WAT. The high-fat diet abolished the stimulating effect of i.c.v. administration of insulin on FA retention in WAT. Furthermore, peripheral administered insulin did not result in FA retention in WAT and blocking central insulin signaling by i.c.v. administration of tolbutamide did not affect the FA retention in WAT. The absence of insulin effects on FA retention in these diet-induced obese mice is probably the result of both high-fat-diet induced insulin resistance of WAT and central insulin resistance associated with blunted activation of hypothalamic K_{ATP} channels [25,33-36]. This present study indicates that in high-fat fed conditions, FA partitioning is no longer influenced by (central acting) insulin.

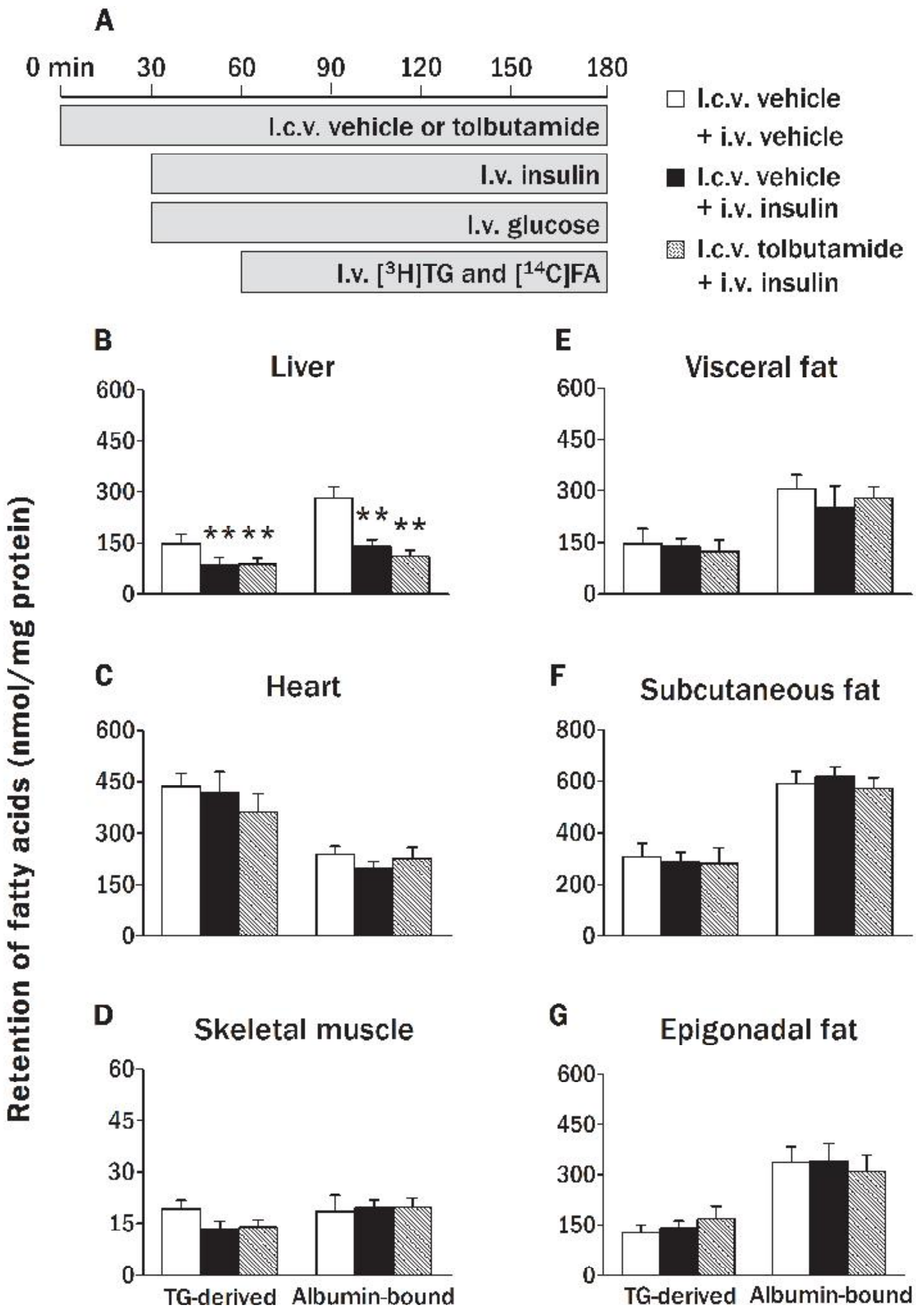

Figure 8. I.v. insulin administration in diet-induced obese mice does not stimulate FA retention by WAT. Postabsorptive, body weight-matched mice received continuous i.c.v. infusion of vehicle (white and black bars) or tolbutamide (12 nmol/h, hatched bars) in basal state (i.v. infusion of vehicle; white bars) or hyperinsulinemic-euglycemic state (black and hatched bars) (A). Infusion of glycerol tri[^{3}H]oleate within VLDL-like emulsion particles and albumin-bound [^{14}C]oleic acid was started 30 minutes after initiating the i.v. infusion of vehicle or insulin/glucose, and maintained for 2 h. Subsequently, the mice were sacrificed and the retention of TG-derived FA and albumin-bound FA was determined in liver (B), heart (C), skeletal muscle (D), visceral fat (E), subcutaneous fat (F) and epigonadal fat (G). Values are means ± SEM for at least 5 mice per group. ** $P<0.01$ *vs.* vehicle.

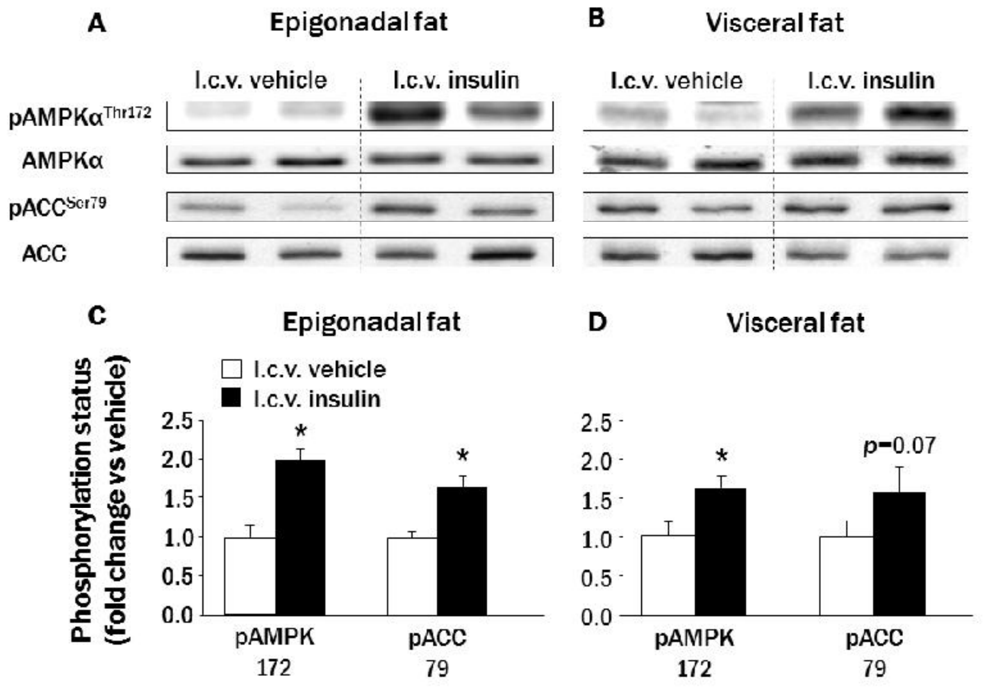

Figure 9. I.c.v. insulin administration activates AMPK in WAT. The phosphorylation state of AMPK and its downstream target ACC was analyzed by Western blot in both epigonadal fat (A) and visceral fat (B) in mice that received i.c.v infusion of vehicle or insulin for 2.5 h. The corresponding quantification of the Western blot data was normalized for total protein and expressed as fold change compared to vehicle (C-D). Values represent means ± SEM for at least 5 mice per group. * $P<0.05$ *vs.* vehicle.

The present results showed that insulin stimulates FA retention in WAT to a considerable extent through action in the CNS. Since insulin stimulates FA transport by upregulating and translocating the long chain FA transporter CD36 in isolated skeletal muscles and cardiac myocytes [27,28], we hypothesize that the stimulating effect of centrally acting insulin on FA retention in WAT can be mediated by CD36 translocation. When administered in CD36-deficient mice, i.c.v. insulin was unable to stimulate FA retention in WAT, suggesting a role of this FA transporter in i.c.v. insulin-stimulated FA retention in WAT. Accordingly, AMP-activated protein kinase (AMPK), which can stimulate FA retention by promoting translocation of CD36 from intracellular pool to the plasma membrane [37], was activated in WAT upon i.c.v. insulin administration (Fig. 9). These preliminary data suggest that central insulin action can lead to CD36 translocation following AMPK activation, thereby resulting in FA retention in WAT.

Recent observations have challenged the traditional notion that adipose tissue acquires FA from TG-rich lipoproteins mediated by the LPL system. Shadid *et al*. documented direct uptake of plasma FFA by adipose tissue in humans in the postabsorptive state [38]. In accordance, we previously demonstrated considerable uptake of plasma FFA in adipose tissue in both fed and fasted mice [23]. In older experiments the uptake of FA in adipose tissue from plasma FFA and plasma TG was assessed by quantification of the uptake as a percentage of the total administered radioactive dose [39]. In the present study, we assessed tissue FA uptake from plasma FFA and plasma TG per mg protein after correction for the corresponding plasma specific activities of [^{14}C]FA and [^{3}H]TG. This correction for precursor pool enrichment enabled to assess the absolute rate of FA uptake from the two plasma sources as compared to the assessment of the relative uptake of FA radio-isotopes in relation to the total administered radioactive doses in the older experiments. In addition, there are other methodological differences including labeling procedures of plasma TG, the use of the postabsorptive state *vs.* hyperinsulinemic-euglycemic clamp procedure, that limit a simple comparison of the results of our and those previous experiments.

I.c.v. administration of insulin did not stimulate FA retention by WAT to the same extent as peripherally administered insulin. However, the i.c.v. dose of insulin used in the i.c.v. experiments and the i.v. dose used in the hyperinsulinemic euglycemic clamp experiments cannot easily be compared. The i.c.v. dose of insulin used in the current study was ascertained in a dose-finding study to exclude hormone-sensitive lipase inactivation by i.c.v. insulin (as shown recently by Scherer *et al*. [14]). Therefore, the change in FA retention by WAT upon i.c.v. insulin is the result of a net influx of FA, whereas FA retention upon peripheral insulin administration is the result of suppression of lipolysis and FA uptake, explaining the difference in the amount of FA retention by WAT between centrally and peripherally administered insulin.

In conclusion, we show that circulating insulin stimulates tissue-specific FA retention by WAT to a considerable extent by indirect pathways, involving activation of K_{ATP} channels in the brain, which is lost in diet-induced obese mice. These observations highlight a paradigm that circulating hormones can act on target tissues directly, as well as indirectly through the CNS and indicate that such a central pathway is disturbed in insulin resistance of WAT in diet-induced obesity.

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