Ectopic UCP1 overexpression in white adipose tissue improves insulin sensitivity in
Lou/C rats, a model of obesity resistance

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Abstract

Brown adipose tissue (BAT), characterised by the presence of uncoupling protein 1 (UCP1), has been described as metabolically active in humans. Lou/C rats, originating from the Wistar strain, are resistant to obesity. We previously demonstrated that Lou/C animals express UCP1 in beige adipocytes in inguinal WAT (iWAT), suggesting a role of this protein in processes such as the control of body weight and the observed improved insulin sensitivity.

A β3 adrenergic agonist was administered for two weeks in Wistar and Lou/C rats to activate UCP1 and delineate its metabolic impact. The treatment brought about decreases in fat mass and improvements in insulin sensitivity in both groups. In BAT, UCP1 expression increased similarly in response to the treatment in the two groups. However, the intervention induced the appearance of beige cells in iWAT, associated with a marked increase in UCP1 expression, in Lou/C rats only. This increase was correlated with a markedly enhanced glucose uptake measured during euglycemic-hyperinsulinemic clamps, suggesting a role of beige cells in this process.

Activation of UCP1 in ectopic tissues, such as beige cells in iWAT, may be an interesting therapeutic approach to prevent body weight gain, decrease fat mass and improve insulin sensitivity.
Introduction

Obesity has reached epidemic proportions worldwide and has become a major global public health problem in recent decades (1). It represents a considerable risk factor for the development of several co-morbidities, amongst which type 2 diabetes (1). Globally, the key component of the obesity epidemic is long-term dysregulation of energy balance. In view of the relative lack of drugs suppressing appetite, approaches to increase energy expenditure are viewed as potential new therapeutic options to treat obesity and metabolic complications. Along this line, brown adipose tissue (BAT), now known to be present in adult humans (2-4), is the focus of major interest, due to its role in inducing thermogenesis (5). The mitochondria of brown adipocytes are characterized by the presence of uncoupling protein 1 (UCP1), which uncouples oxidative phosphorylation from ATP synthesis, resulting in heat production (6). This process consumes substantial amounts of free fatty acids (FFA) and glucose (7).

In rodents, brown adipocytes are found in discrete areas, such as interscapular, cervical, perirenal and intercostal depots (8), which are referred to as “classical” BAT depots. In white adipose tissue (WAT), brown-like cells, called beige or brite cells, express UCP1 (9). The existence of a specific precursor, different from classical white or brown adipocytes, arising from smooth muscle cells that would differentiate into beige adipocytes in WAT has been proposed (10). Moreover, some studies suggest that, under specific conditions, most or all of white adipocytes transdifferentiate into beige adipocytes (11).

Interestingly, whatever their developmental origin, white, beige and brown adipocytes seem to greatly differ in their function. As mentioned above, BAT is the effector organ of non-shivering thermogenesis that, by utilizing large quantities of glucose and lipids from the circulation, can promote negative energy balance. Moreover, as recently reviewed by Peirce et al (12), the role of BAT activation might be broader than solely promoting negative energy balance. Indeed, such activation was described to exert anti-type 2 diabetic effects (13; 14),
associated with improvements of dyslipidemia (for review, see (15)). These effects are partly interrelated, but can also be dissociated and exerted by different UCP1 expressing-adipocyte types.

The Lou/C rat is a model of age- and diet-induced obesity resistance, which also exhibits a lower body fat mass, increased leptin sensitivity, as well as improved insulin sensitivity compared to Wistar animals (16-18). We also demonstrated that the most striking differences between Lou/C and Wistar rats were the presence, in inguinal WAT (iWAT) of the Lou/C group, of \textit{Ucp1} (16; 17) and of a marked \textit{Adrb3} overexpression (17).

In the present study, we used the Lou/C rat as a model to investigate the impact of UCP1 activation on glucose metabolism. To this end, various groups of Wistar and Lou/C rats were subcutaneously infused for 2 weeks with a beta3 adrenoreceptor agonist (CL-316243) and UCP1 in BAT and WAT depots, as well as the insulin-stimulated glucose utilization rate of different tissues was determined.

\textbf{Materials and Methods}

\textbf{Animals and diets}

Two month-old male Lou/C and Wistar rats were purchased from Harlan UK Limited (Oxon, UK) and Charles River (L’Arbresle, France), respectively. They were housed in pairs under controlled conditions (22°C; light on: 07.00 a.m. - 07.00 p.m.) and were allowed free access to water and diet (RMI, Herstetter, Essex, UK). Osmotic minipumps (Alzet, Cupertino, USA) delivering a beta3 agonist (CL-316243, Tocris, UK, 1 mg/kg/day) or NaCl 0.9% for 14 days were placed subcutaneously at the age of 12 weeks. One week before and during the treatment, body weight and food intake were measured daily (09.00 am). A first colony was submitted to a glucose tolerance test (GTT) at day 9 and a body fat composition analysis by magnetic resonance imaging (Echo-MRI) at day 14. A second colony was submitted to a CT
scan on day 12 and a euglycemic-hyperinsulinemic clamp (EHC) on day 14. A third colony underwent a PET scan coupled to a micro-CT on day 14. A fourth colony was housed in pairs at 22°C or at 30 °C in a climatic chamber (Meditest H 1300L, Froilabo, Meysieu, France). Except for EHC experiments, all rats were sacrificed between 09.00 a.m. and 01.00 p.m., using isoflurane (Halocarbon Laboratories, River Edge, NJ) anesthesia and rapid decapitation. Trunk blood was collected to measure the concentration of various hormones and metabolites. Tissues were freeze-clamped and stored at –80°C for determination of gene and protein expressions. The procedures were approved by the ethic committee of our university and were in accordance with the Swiss guidelines for animal experimentation.

**Glucose tolerance test (GTT)**

Wistar and Lou/C rats were food-deprived for 4h (from 09.00 to 01.00pm). A glucose load of 1.5 g/kg was administered i.p.. Blood samples were collected by tail nicking and were used for further analyses of plasma glucose and insulin levels.

**Euglycemic-hyperinsulinemic clamps (EHC)**

Rats were overnight fasted and anesthetized with pentobarbital (i.p., 50 mg/kg; Abbott Laboratories, Chicago, IL). The glucose infusion rate (GIR) required to maintain euglycemia under insulin-stimulated conditions (18 mU/kg/min; Actrapid® HM, NovoNordisk, Bagsvaerd, Denmark) was determined, as previously described (19; 20). At the end of the EHC, the *in vivo* insulin-stimulated glucose utilization index of individual tissues was measured, using 2-deoxy-D-[1,2-³H] glucose (30 μCi/rat; Perkin Elmer NET 328A, Schwerzenbach, Switzerland) (20). Rats were killed by decapitation and tissues stored at –80°C. The 2-deoxy-D-[1,2-³H] glucose specific activity was measured in deproteinized blood samples. Determination of tissue concentration of 2-deoxy-D-[1,2-³H]glucose-6-phosphate
allowed for the calculation of the *in vivo* glucose utilization index of individual tissues, and
was expressed in ng glucose/mg of tissue/min (21; 22).

**Plasma measurements**

Plasma glucose levels were measured by the glucose oxidase method (Glu, Roche Diagnostics GmbH, Rotkreuz, Switzerland). FFA and triglyceride (TG) levels were determined using a Wako Chemicals GmbH (Neuss, Germany) and a Biomérieux (Marcy l’Etoile, France) kit, respectively. Plasma leptin (Linco Research Inc., St Charles, MO) levels were measured using a double antibody radioimmunoassay (RIA) kit. Plasma insulin levels were determined using an ELISA commercial kit (Mercodia 10-1250, Uppsala, Sweden).

**Tissue processing and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

RNA was reverse transcribed (M-MLV-RT, Invitrogen, Basel, Switzerland) and qPCR was performed using the SYBR® green PCR Master Mix (Applied Biosystems, Warrington, UK) on a Stepone Plus machine (Applied Biosystems). Primers (Suppl. Table 1) were designed with the PrimerExpress software (Applied Biosystems). Results were normalized to the expression levels of the housekeeping gene, ribosomal protein S29 (*Rps29*).

**Mitochondrial DNA (mtDNA) copy number**

Quantification of mtDNA copy number was achieved by quantitative PCR. Briefly, DNA was extracted from iWAT using the DNeasy Blood and Tissue kit (Qiagen, Düsseldorf, Germany). Nuclear and mtDNA copy numbers were assessed by real time PCR using primers targeted toward the *Cox1* gene (for mtDNA) and nuclear *RNAseP* (for nuclear DNA).
**Western blotting**

Frozen tissues were homogenized in ice-cold RIPA buffer. Protein levels were quantified using a BCA protein assay (Pierce, Lausanne, Switzerland). Ten and 50 microgram of protein was respectively used for BAT and WAT SDS-PAGE. UCP1 antibody (Abcam, Cambridge, UK) was used at a concentration of 1/10,000 for BAT and 1/1000 for WAT, before the secondary antibody (antirabbit 1/5000) was added. Housekeeping proteins ERM (SantaCruz, Delaware, USA) and actin (Millipore, Massachusetts, USA) were used at 1/2000 and 1/100000, with antigoat (1/10000) or antimouse (1/5000) antibody, respectively. Detection was performed with a chemiluminescence (ECL) detection system (Amersham Biosciences, Amersham, UK). Signals were quantified using the PXi™ and the Genetools™ software from Syngene Laboratories (UK).

**Body composition**

An EchoMRI-700™ quantitative nuclear magnetic resonance analyzer (Echo Medical Systems, Houston, TX) was used to measure body composition (total fat and lean body mass). Rats were also scanned on a multidetector CT scanner (Discovery 750 HD, GE Healthcare, Milwaukee, Wis) to determine the volume of the various fat depots.

**Light microscopy**

Tissues were fixed in formalin (10%) after dissection. They were washed in PBS, dehydrated, cleared, and finally embedded in paraffin blocks, which were cut at 7µm and then stained with hematoxylin eosin (Sigma, Buchs, Switzerland). For immunohistochemistry, the same UCP1 antibody as that used for Western blots was employed at a concentration of 1/100, before the secondary antirabbit Cy3 antibody (1/250) (Jackson ImmunoResearch).
**18-FDG glucose uptake measurement in tissues**

Rats were overnight fasted and anesthetized using isoflurane. Glycemia was measured before the experiment. Animals received a 3.5mg/kg i.p. injection of furosemide (Lasix®, Sanofi, France) to empty their bladder. $[^{18}\text{F}]$-fluoro-deoxyglucose ($[^{18}\text{F}]$-FDG) was injected via the pudendal vein (20 MBq/rat). Glucose uptake was measured with a microPET/CT (Triumph, Trifoil, CA, USA). A micro-CT analysis was simultaneously performed to localize the $[^{18}\text{F}]$-FDG emission in different structures. Data were analysed by the Osirix software (Pixmeo, Geneva, Switzerland).

**Data analyses**

Results are expressed as mean ± SEM. Comparison between the four groups was performed by Graph Pad Prism (San Diego, CA, USA), using the two-way ANOVA analysis followed by a Tukey’s post hoc test. Correlation between the Ucp1 mRNA expression and the glucose uptake index in WAT was calculated using the Pearson correlation coefficient. Statistical significance was established at p<0.05.

**Results**

As previously reported (16; 17), Lou/C had a lower body weight than Wistar rats. The 2-week CL-316243 treatment had no impact on body weight gain in Wistar and Lou/C animals (Fig 1A). Total food intake of Lou/C was lower than that of Wistar rats, but it was unaffected by the treatment in both groups (Fig 1A). Interestingly, food efficiency was similar in Wistar and Lou/C rats, whether treated or not (Fig. 1A). Analysis of body composition by magnetic resonance imaging (EchoMRI) further showed that the β3 agonist treatment decreased the
total fat mass and consequently increased the lean body mass in both Wistar and Lou/C animals (Fig. 1B). In addition, CT-scan analysis showed a significant treatment-induced decrease in the mass of all fat depots (interscapular BAT, subcutaneous WAT, intra-abdominal WAT) in the two animal groups, except for subcutaneous WAT of Lou/C rats (Fig. 1C). This was in keeping with a significant or with a trend toward a significant decrease in leptinemia in β3 agonist-treated Wistar and Lou/C rats, respectively (Table 1). Qualitatively similar results were obtained for plasma glucose, insulin and TG levels, while plasma FFA concentrations were unaffected by the treatment in both groups (Table 1).

To evaluate the impact of the β3 agonist treatment on glucose metabolism, a glucose tolerance test (GTT) was performed at day 9 of the study (Fig. 2A). The treatment had no impact on basal glycemia in both groups (data not shown). As indicated by the changes in glycemia from baseline values, as well as the areas under the curves during the whole tests (AUC), glucose tolerance of Wistar and Lou/C rats was improved by the treatment (Fig. 2A). With regard to insulin, the treatment effect on the AUC during the GTT almost reached statistical significance (p = 0.057), this effect being clearly more marked in the Lou/C than in the Wistar group (Fig. 2B).

Peripheral insulin sensitivity was then evaluated by performing EHC. Basal and steady-state (clamp) values of glycemia and insulinemia are provided in Suppl. Table 2. As previously reported (16; 17), the GIR of Lou/C rats was higher than that of Wistar animals, indicating improved insulin sensitivity (Fig. 2C). In both the Wistar and the Lou/C group, the β3 agonist treatment increased the GIR (Fig. 2C). As clearly depicted by the dynamic GIR changes, the values reached in Wistar-treated rats were similar to those of the untreated Lou/C group, while the β3 agonist treatment further increased the GIR in Lou/C animals (Fig. 2C). Glucose uptake by individual insulin-sensitive tissues was then assessed, using the 2-deoxy glucose technique (20-22). The treatment had no impact on skeletal muscle glucose uptake in both
groups (Fig. 2D). It resulted in similar two to three fold increases in glucose uptake in BAT of Wistar and Lou/C rats (Fig. 2D). Interestingly, glucose uptake by different WAT depots was not significantly modified in Wistar, but was markedly increased in Lou/C animals. This was observed in abdominal WAT (epididymal WAT (eWAT) and retroperitoneal WAT (rpWAT)), as well as in the inguinal subcutaneous (iWAT) fat depot (Fig. 2D).

To try understanding the mechanisms underlying the β3 agonist treatment-induced increases in BAT and WAT glucose uptake, and in view of the well-known effect of CL-316243 on UCP1 (23), the expression of this protein was measured in these tissues. Considering BAT, hematoxylin eosin staining revealed less lipid inclusions, and therefore activated tissue in the Wistar- and the Lou/C-treated groups (Fig. 3A). Accordingly, Ucp1 mRNA expression was increased by four fold by the treatment in the two groups (Fig. 3B). These results were confirmed by Western blot analysis (Fig. 3C). The treatment also had similar effects in Wistar and Lou/C rats regarding the expression of the Adrb3, which was markedly inhibited (Fig. 3B).

Subsequently, BAT activity was evaluated by PET scan analysis, using $^{18}$F-FDG. Fig. 3D shows micro-CT, PET scan and merged micro-CT and PET scan images of one representative animal per group. PET scan allowed for the detection of three main BAT depots: at the basis of the brain (cBAT), in the interscapular region (iBAT) and along the vertebral column (pvBAT). As clearly revealed following quantification of the signals in all the animals studied, the β3 agonist treatment increased glucose uptake to a similar extent in each of these three depots in Wistar and Lou/C rats, whereas the signals were absent under standard conditions (Fig. 3D).

In line with our previous observation of measurable UCP1 expression in iWAT of Lou/C, but not of Wistar rats (16; 17), we looked at the potential presence of UCP1-positive beige adipocytes in this tissue, following the β3 agonist treatment. Examination of hematoxylin
eosin staining revealed the presence of multilocular adipocytes in the β3 agonist treated-
Lou/C group only (Fig. 4A). Immunofluorescence analysis further showed that these
multilocular adipocytes expressed UCP1, demonstrating the presence of beige cells in the
iWAT of Lou/C-treated rats (Fig. 4A). In keeping with these results, Ucp1 mRNA expression
was increased a hundred fold in iWAT of Lou/C rats in response to the treatment. It was also
induced in iWAT of Wistar-treated animals, reaching values measured in the untreated Lou/C
group (Fig. 4B). Western blot analysis clearly showed that UCP1 protein was expressed in the
iWAT of the Lou/C-treated group only (Fig. 4C). In line with the results obtained for Ucp1,
the expression of Pgc1α (Fig. 4B), of Tbx1 (Suppl. Fig 1) and of various markers of
mitochondrial biogenesis (Suppl. Fig. 1), as well as mitochondrial DNA (Fig 4E) were
markedly enhanced by the treatment in Lou/C rats (600% increase in the mtDNA copy
number). Next, we measured the activity of iWAT by PET scan coupled with micro-CT and
observed a higher glucose uptake in treated Lou/C rats compared to the three other groups
(Fig. 4D). To substantiate the existence of the previously proposed link between glucose
uptake/insulin sensitivity and Ucp1 expression in adipose tissue (24), we looked at the
correlation between these two parameters, considering all the animals studied. As shown on
Fig. 4F, the data fitted a hyperbolic regression with a positive and significant correlation,
suggesting that UCP1-positive beige cells in iWAT may be involved in the improvement in
insulin sensitivity in this tissue. With regard to the expression of the Adrb3, it was markedly
overexpressed in iWAT of Lou/C compared to Wistar rats, in which it was barely detectable.
The β3 agonist treatment downregulated the expression of the Adrb3 in the Lou/C, but not in
the Wistar group (Fig. 4B).

Given that the insulin-induced glucose uptake was increased by the β3 agonist, not only in
iWAT, but also in the eWAT, we examined the potential presence of beige adipocytes in this
depot. Multilocularisation of adipocytes was observed in Lou/C-treated rats only (Suppl. Fig.
2A), in keeping with the induction of a marked *Ucp1* gene expression (by 160 fold) (Suppl. Fig. 2B). The mRNA expression of the *Adrb3* was low in both Wistar and Lou/C rats, with no inter-group difference and no impact of the β3 agonist treatment (Suppl. Fig. 2B).

It is well known that thermoneutrality in rodents is reached at around 28-30°C (24°C for rats and 30°C for mice) and that, although it is the usual temperature used in animal quarters, 22°C represents a cold stimulus increasing UCP1 expression and activity (25). It was therefore possible that the presence of UCP1-positive beige cells in WAT observed in the Lou/C group without any treatment was a consequence of a better temperature sensing than in Wistar rats. To address this issue, groups of Wistar and Lou/C rats were maintained at 22°C or at 30°C for 4 weeks. At 30°C, both food intake and body weight gain were reduced in Wistar, as well as in Lou/C rats (Fig. 5A). This resulted in similar food efficiencies in response to the treatment under the two experimental conditions, with lower values in the Lou/C than in the Wistar groups (Fig. 5B). Regarding BAT *Ucp1* expression, it was lower in Lou/C than in Wistar animals, and it was reduced by the exposure at 30°C in the Wistar group (Fig. 5D). Such conditions also brought about a decrease in BAT *Adrb3* expression in Wistar and Lou/C rats (Fig. 5D). In iWAT, *Ucp1* expression was doubled in Lou/C compared to Wistar at 22°C, although this did not reach statistical significance, and it remained at the same values at 30°C (Fig. 5E). In this tissue, expression of the *Adrb3* was higher in Lou/C than in Wistar rats, with no effect of the temperature (Fig. 5E). Similar observations were made for the GIR measured during EHC (Fig. 5C), indicating that the improved insulin sensitivity of the Lou/C group is maintained at 30°C, and is therefore not linked to cold exposure, even of minor magnitude.

Finally, the expression of genes encoding for enzymes known to be involved in lipid metabolism was measured in BAT and iWAT. Acetyl CoA carboxylase (ACC1) represents the rate-limiting step in fatty acid synthesis. Carnitine palmitoyl transferase-1 (CPT1α) is the
rate-limiting step of the fatty acid oxidation pathway, mediating fatty acid transport from the cytosol to the mitochondria. Medium and large chain acyl-CoA dehydrogenases (MCAD and LCAD) are involved in fatty acid oxidation. Phosphoenolpyruvate carboxykinase (PEPCK) is allowing for the *in situ* production of glycerol-3-phosphate (glyceroneogenesis) and therefore for the formation of TG. Hormone-sensitive lipase (HSL) is one of the main enzymes involved in lipolysis. In iWAT, *Acc1* expression was twofold higher in Lou/C than in Wistar rats in the absence of β3 agonist treatment. Furthermore, it was more than doubled in the Lou/C group in response to the treatment, while it remained unchanged in Wistar rats (Fig. 6A). As expected from its inhibitory regulation by malonyl CoA, *Cpt1a* expression was lower in Lou/C than in Wistar animals, with a trend toward a further decrease in the β3 agonist-treated group (Fig. 6A). On the contrary, the β3 agonist treatment resulted in an enhancement of *Hsl, Lcad* and *Mcad* expression in Lou/C rats, without any change in the Wistar group (Fig. 6A). Similar results were obtained for the expression of *Pepck* (Fig. 6A).

In BAT, *Acc1* expression was similar in Wistar and Lou/C untreated rats and it was reduced by the treatment in the Lou/C group only (Fig. 6B). The expression of *Cpt1a* was under the detection limit, as a likely consequence of elevated *Acc1* expression in this tissue. There was no difference in *Hsl* expression in Wistar and Lou/C rats, whether treated or not (data not shown). In control animals, *Pepck* was less express in the Lou/C compared to the Wistar group, but it was increased by the treatment in Lou/C rats to reach the expression level measured in Wistar rats (Fig 6B).

**Discussion**

This study was undertaken to determine the impact of recruiting UCP1-expressing adipocytes in a model of obesity resistance characterized by the presence of beige cells in iWAT (16; 17). For this purpose, Lou/C and Wistar rats were treated for 2 weeks with a β3 agonist (CL-316243). The treatment had no effect on food intake and body weight gain, while it decreased
the fat mass, as well as the weight of interscapular BAT and of various WAT depots in the two groups.

In terms of its effects on UCP1 expression, different types of results were obtained in BAT and WAT, when comparing Wistar and Lou/C rats. In BAT, we and others previously reported that *Ucp1* mRNA and/or protein levels were lower in Lou/C than in Wistar rats at 22°C or at 25°C (17; 26), whereas, in the present study, this was observed for UCP1 protein at 22°C, but not for *Ucp1* gene at 22°C or 30°C. Regarding the effects of the β3 agonist treatment, it increased *Ucp1* and decreased *Adrb3* expression in BAT of both groups to a similar extent. These results, confirmed by the measurement of UCP1 protein, closely correlated with the observation of similar increases in BAT glucose uptake in Wistar and Lou/C rats, as measured during EHC and by [18F]-FDG-PET scan analysis.

UCP1-expressing beige cells are known to be present in various WAT depots (27; 28). Chronic cold exposure was shown to recruit beige adipocytes in WAT, resulting in WAT “browning” (29; 30). Interestingly, resistance to diet-induced obesity in rats and mice has been suggested to depend on the induction of beige adipocyte recruitment in WAT (16; 31). Moreover, such increased recruitment of beige cells has been shown to compensate for decreased BAT thermogenesis, and the specific loss of beige adipocytes due to adipose tissue-specific deletion of a transcriptional factor involved in *Ucp1* expression (32), PRDM16, was shown to cause obesity (32; 33). Beige adipocytes are also strikingly involved in the regulation of glucose metabolism, as demonstrated by the beneficial effects on glucose tolerance and insulin sensitivity observed in transgenic mice overexpressing PRDM16 in iWAT (32).

In the present study, we showed that the β3 agonist treatment affected the Lou/C group only, markedly increasing UCP1 gene and protein expression, mitochondrial biogenesis, as well as the insulin-stimulated glucose uptake measured during EHC (subcutaneous and intra-
abdominal fat depots) or using \[^{18}\text{F}]\text{-FDG-PET}\) scan analysis (subcutaneous fat depot), as previously reported (34). Furthermore, in iWAT from Lou/C- treated rats, a hyperbolic correlation between glucose uptake and \(Ucp1\) mRNA expression could be observed. Although this does not allow concluding about cause-effect relationships, these data suggest that the amount of beige adipocytes in WAT depots can determine the overall tissue glucose utilization rate. Such conclusion is supported by the appearance of multilocular beige cells in iWAT of CL-316243-treated Lou/C rats, as determined by histological means. Whether these beige cells result from activation of existing adipocytes or from transdifferentiation of white into brown cells is an important issue that will need to be addressed in future work.

As schematized by Fig. 7, the molecular mechanisms underlying the \(\beta_3\) agonist-induced increase in glucose metabolism in iWAT of Lou/C rats seem to involve inductions of increased FA synthesis (\(\text{de novo}\) lipogenesis, DNL) and lipolysis (increased \(Acc1\) and \(Hsl\), respectively), as well as of enhanced FA beta oxidation (increased \(Mcad\) and \(Lcad\)). Additionally, the marked increase in the expression of \(Pepck\) in iWAT of Lou/C-treated rats suggests the presence of increased glyceroneogenesis. Similar data of gene expression responsible for enzymes involved in lipid metabolism in adipose tissues were reported in normal mice treated with CL 316 243 for 7 days (35).

With regard to the overall insulin-stimulated glucose utilization, as reflected by the glucose infusion rate (GIR) during EHC (under our experimental conditions of suppressed hepatic glucose production), we observed that it was similarly increased by the treatment in Wistar and Lou/C rats. Given that skeletal muscle glucose utilization was unaltered by the treatment in the two groups, only BAT and WAT glucose uptake could be held responsible for the treatment-induced increase in GIR. By considering the total mass of BAT and WAT, as well as the mean glucose uptake in these tissues, it was possible to approximate the percent contribution of each of these two tissues to the increased GIR. Thus, in Wistar rats, both BAT
(around 30%) and WAT (around 60%) contributed to the GIR enhancement in response to the treatment. In contrast, in Lou/C rats, the treatment doubled BAT glucose uptake, but decreased the BAT mass by twofold. Therefore, in these animals, the stimulatory effect of the treatment on GIR could only be attributed to increased glucose uptake in WAT depots containing beige cells. It should however be mentioned that these calculations may underestimate BAT glucose uptake as the reduction in tissue weight may reflect loss of lipids due to increased thermogenic activity induced by the β3 agonist treatment. These data are in keeping with previously reported results showing that chronic stimulation of the sympathetic nervous system stimulates glucose uptake in white and brown adipose tissues, without any effect in skeletal muscles (36). The predominant role of beige cells in WAT glucose uptake is further supported by the previous observation that the increased glucose utilization under sympathetic stimulation depends from UCP1 activation (37; 38).

Altogether, the results of the present study extend other data showing that chronic cold exposure or β3 agonist treatment exerts beneficial effects on glucose metabolism in high fat diet-induced obese mice (for rev, see (12)) and normalize hyperglycemia and hypertriglyceridermia in mouse models of diabetes and dyslipidemia (39-41). They also extend previous data (42), supporting the notion of interdependency between glucose uptake, lipid metabolism and thermogenesis. As recently further discussed (43), a particularly important role for the futile cycling between fatty acid synthesis and oxidation linked to thermogenesis and glucose disposal is played by de novo lipogenesis (DNL). This may be particularly relevant in beige cells of white adipose tissue depots, in keeping with the observation that WAT is required for the full thermogenic response to CL 316243 (44). To go a step further in the understanding of the mechanisms involved in the effects of β3 agonists, several data indicate that DNL is required for the synthesis of several signaling lipids with systemic effects ("lipokines") supporting metabolic homeostasis. The presence of these molecules in our
different experimental groups could therefore be of interest and will be investigated in the future.

To conclude, induction of DNL coupled with a simultaneous increase in fatty acid oxidation as occurs during browning of white adipose tissue (CL-treated normal mice, (35); CL-treated Lou/C rats, present study) appear to be important for the regulation of glucose homeostasis and may therefore be of clinical relevance for the treatment of obesity, as well as type 2 diabetes in humans.

Acknowledgments:

Authors' contributions

ALP carried out the animal, the qPCR, Western blot and immunofluorescence studies, and drafted the manuscript; CVD, ALP and FRJ conceived the study, participated in its design and coordination and drafted the manuscript; CVD, JA, JL and AC helped with the animal treatments; JL participated in qPCR experiments; JA carried out biostatistical analysis and drafted the manuscript; XM performed CT scan and its analysis; DJC performed $^{18}$F-FDG. All authors read and approved the final manuscript.

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Figure 1: CL-316 243 decreases fat mass in both Wistar and Lou/C rats.

(A) Total body weight gain of Wistar and Lou/C rats measured after 14 days of CL-316 243 treatment 1mg/kg/day (CL) or saline solution (NaCl); Total food intake measured after 9 days; Food efficiency calculated from delta body weight gain divided by total food intake at 9 days. (B) Determination of the percent lean and fat mass repartition, using magnetic resonance imaging. (C) Determination of the volume of various fat depots by CT scan.

Results are means ± SEM of 8 experiments per group. Statistical significance assessed by two-way ANOVA: # strain effect, @ treatment effect, $ interaction between strain and treatment. *significant difference by post hoc pairwise comparison between different conditions. Single and triple symbols imply p<0.05 and p<0.001, respectively.

Figure 2: Improvement in insulin sensitivity in Lou/C rats is due to an increase in glucose uptake by WAT.

(A) Delta glycemia during a glucose tolerance test (GTT, 1.5 g/kg i.p.) after 4 hours of fasting; areas under the glucose curves (AUC) at 120 min. (B) Plasma insulin levels during the GTT; insulin AUC after 60 min. (C) Glucose infusion rate during (right panel) and at the end (left panel) of euglycemic-hyperinsulinemic clamps in Wistar NaCl, Wistar-treated with CL (Wistar CL), Lou/C NaCl and Lou/C-treated with CL (Lou/C CL) rats. (D) Tissue-specific insulin-stimulated glucose uptake. Quadriceps red (Qr), quadriceps white (Qw); interscapular brown adipose tissue (BAT); inguinal white adipose tissue (iWAT), epididymal white adipose tissue (eWAT), retroperitoneal white adipose tissue (rpWAT). Results are means ± SEM of 6-9 experiments. Statistical significance assessed by two-way ANOVA: # strain effect, @ treatment effect, $ interaction between strain and treatment. *significant difference by post
hoc pairwise comparison between different conditions. Single, double, and triple symbols imply p<0.05, p<0.01, and p<0.001, respectively.

For the dynamic changes of delta glucose and insulinemia during the GTT (A and B, left panels), as well as of GIR as a function of time (C, right panel): *: Wistar NaCl vs Lou/C NaCl; †: Wistar NaCl vs Wistar CL; ‡: Lou/C NaCl vs Lou/C CL; §: Wistar CL vs Lou/C CL.

Figure 3: UCP1 is equally overexpressed in BAT in both Wistar and Lou/C rats by CL-316 243.

(A) Representative hematoxylin and eosin (H&E) staining of BAT (7µm), scale bars = 50 µm. 
(B) Ucp1 and β3 adrenoreceptor (Adrb3) mRNA expression in BAT. (C) Protein expression by Western blot with relative quantification in BAT. (D) Representative sagittal cuts of [18F]-FDG uptake measured by microPET; microCT was performed to localize glucose uptake in the tissues; quantification of glucose uptake in interscapular BAT (29), cervical BAT (cBAT), and paravertebral BAT (pvBAT) in control and CL-treated Wistar and Lou/C rats.

Results are means ± SEM of 4-8 experiments. Statistical significance assessed by two-way ANOVA: @ treatment effect. *significant difference by post hoc pairwise comparison between different conditions. Double and triple symbols imply p<0.01 and p<0.001, respectively.

Figure 4: UCP1 is overexpressed in the inguinal subcutaneous white adipose tissue (iWAT) depot in Lou/C rats by CL-316 243.

(A) Representative H&E staining in iWAT (7µm), scale bars = 50 µm. Inguinal WAT sections were stained with an anti-UCP1 antibody. Hematoxylin staining was used to
recognize structures. The sections were examined by fluorescence microscopy, red is for UCP1. (B) *Ucp1, Adrb3* and *Pgc1α* mRNA expression in iWAT. (C) UCP1 protein expression measured by Western blot with relative quantification in iWAT. (D) Representative axial cuts of [18F]-FDG uptake measured by microPET; microCT was performed to localize glucose uptake in the tissues; quantification of glucose uptake in iWAT of control and CL-treated Wistar and Lou/C rats. (E) Mitochondrial DNA copy number in iWAT. DNA copies of mitochondrial complex I (COX1, mtDNA) was normalized to the DNA levels of nuclear RNaseP (nDNA). (F) Nonlinear regression with hyperbolic correlation between 2-DG glucose uptake and *Ucp1* expression in iWAT.

Results are means ± SEM of 8 experiments per group. Statistical significance assessed by two-way ANOVA: # strain effect, @ treatment effect, $ interaction between strain and treatment. *significant difference by post hoc pairwise comparison between different conditions. Single and triple symbols imply p<0.05 and p<0.001, respectively.

Figure 5: Differences between Wistar and Lou/C rats are not due to a difference in thermoneutrality.

(A) Delta body weight gain of Wistar and Lou/C rats measured between days 4 and 30 of 22°C (control) and 30°C exposure; Total food intake measured between days 4 and 30. (B) Food efficiency calculated from delta body weight gain divided by total food intake between days 4 and 30. (C) GIR measured at the end of euglycemic-hyperinsulinemic clamps in Wistar and Lou/C rats housed at 22°C and at 30°C. (D) *Ucp1* and *Adrb3* mRNA expression in BAT. (E) *Ucp1* and *Adrb3* mRNA expression in iWAT.

Results are means ± SEM of 8-12 experiments. Statistical significance assessed by two-way ANOVA: # strain effect, @ treatment effect, $ interaction between strain and treatment.
*significant difference by post hoc pairwise comparison between different conditions. Double and triple symbols imply p<0.01 and p<0.001, respectively.

Figure 6: The higher glucose uptake in inguinal subcutaneous adipose tissue (iWAT) is explained by an increase in fatty acid synthesis in CL-treated Lou/C rats. (A) Acc1, Cpt1a, Hsl, Pepck, Lcad and Mcad mRNA expression in iWAT. (B) Acc1 and Pepck mRNA expression in BAT. Results are means ± SEM of 8 experiments per group. Statistical significance assessed by two-way ANOVA: # strain effect, @ treatment effect. *significant difference by post hoc pairwise comparison between different conditions. Single, double and triple symbols imply p<0.05, p<0.01 and p<0.001, respectively.

Figure 7:
Schematic representation of a beige adipocyte in iWAT of β3 agonist-treated Lou/C rats, showing the occurrence of futile cycling between lipolysis and lipogenesis. Higher glucose uptake is converted to malonyl-CoA by ACC1, which is markedly increased. Together with HSL, this increases the amount of FA within the cell. These FA are used by the mitochondria, as suggested by the increase in the expression of enzymes involved in β- oxidation (LCAD and MCAD), despite a slight decrease in CPT1 (likely due to an increase in the production of malonyl-CoA). Enhanced FA utilization induces the production of acetyl-CoA, as well as of a protons’ gradient between the two mitochondrial membranes. Such a gradient is dissipated by UCP1, increasing heat production and decreasing ATP synthesis. Part of mitochondrial acetyl-CoA can be transformed to oxaloacetate in the cytosol (following its transformation to citrate), where it can be converted G3P via PEPCK (glyceroneogenesis), ultimately increasing lipogenesis.
Yellow arrows represent lipogenic, whereas green ones represent lipolytic pathways.

Supplementary Figure 1: The expression of mitochondrial and beiging markers is increased by the CL-316243 treatment in Lou/C rats.

Expression of *Tfam*, *Cox4i*, *Pparg1* and *Pparg2* for mitochondrial markers in iWAT.

Expression of *Tbx1* as a beiging marker in iWAT.

Supplementary Figure 2: UCP1 is overexpressed in eWAT of Lou/C rats by CL-316 2423.

(A) Representative H&E staining in eWAT (7µm), scale bars = 50 µm. (B) *Ucp1* and *Adrb3* mRNA expression in eWAT.

Results are means ± SEM of 8 experiments per group. Statistical significance assessed by two-way ANOVA: $ interaction between strain and treatment. *significant difference by post hoc pairwise comparison between different conditions. Single symbols imply p<0.05.

Table1:

Plasma was collected from rats receiving NaCl or CL-316243 treatment for 14 days starting at the age of 12 weeks.

Results are means ± SEM of 8 experiments per group. Statistical analyses were performed using a two-way ANOVA (Tukey’s post-test) with ††<0.01, †††<0.001: Wistar NaCl vs Wistar CL; ‡<0.05 Lou/C NaCl vs Lou/C CL.

Suppl Table 2:

Euglycemic hyperinsulinemic clamps were performed in Wistar and Lou/C rats receiving NaCl or CL-316243 treatment for 14 days. Basal and clamp plasma glucose and insulin levels. Results are means ± SEM of 6-9 experiments. Statistical analyses were performed
using a two-way ANOVA (Tukey's post-test), with no statistically significant difference amongst the groups.
Table 1: CL316243 improves metabolic parameters

<table>
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<tr>
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<th>Wistar NaCl</th>
<th>Wistar CL</th>
<th>Lou/C NaCl</th>
<th>Lou/C CL</th>
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</thead>
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<tr>
<td>Glycemia (mmol/L)</td>
<td>7.6 ±0.3</td>
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<tr>
<td>Insulin (ng/mL)</td>
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## Supplementary Table 1: Primers

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<td>Adb3</td>
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<td>Mcad</td>
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<td>Lcad</td>
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<td><code>Pparg1</code></td>
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Supplementary Table 2: Glycemia and insulinemia values before and at the end of euglycemic-hyperinsulinemic clamps

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<th>Lou/C</th>
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<td></td>
<td>NaCl</td>
<td>CL</td>
<td>NaCl</td>
<td>CL</td>
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<tr>
<td>Basal glycemia (mmol/L)</td>
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<tr>
<td>Steady state glycemia (mmol/L)</td>
<td>5.7±0.2</td>
<td>5.4±0.1</td>
<td>5.7±0.1</td>
<td>5.7±0.1</td>
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<tr>
<td>Basal insulin (ng/mL)</td>
<td>3.62±0.70</td>
<td>2.94±1.28</td>
<td>2.66±0.75</td>
<td>1.57±0.16</td>
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<tr>
<td>Steady state insulin (ng/mL)</td>
<td>34.31±2.14</td>
<td>26.74±2.48</td>
<td>28.21±1.20</td>
<td>24.41±2.21</td>
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