Life Sciences 229 (2019) 157–165

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Carbenoxolone enhances peripheral insulin sensitivity and GLUT4 expression in skeletal muscle of obese rats: Potential participation of UBC9 protein

Rosana Cristina Mori*, Thaís Poças da Silva, Raquel Saldanha Campello, Ubiratan Fabres Machado

Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, SP, Brazil

ARTICLE INFO

Keywords:
Slc2a4
11βHSD1
SREBP1
Visceral adipose tissue
Subcutaneous adipose tissue
Soleus

ABSTRACT

Aim: This study investigates the insulin sensitizer effect of carbenoxolone (CBX) and potentially involved peripheral mechanisms.

Main methods: Taking glucose transporter 4 (GLUT4) as a marker of glucose disposal, we investigated the CBX effects on whole-body insulin sensitivity and solute carrier 2a4 (*Slc2a4*)/GLUT4 expression in visceral (VAT) and subcutaneous (SAT) adipose tissues and soleus muscle of monosodium glutamate (MSG)-induced obese rats. Sterol regulatory element binding protein (SREBP1), an enhancer of *Slc2a4* expression was analyzed through mRNA content and SREBP1-binding to *Slc2a4* promoter. Finally, the small ubiquitin-modifier conjugating enzyme 9 (UBC9), whose low content indicates accelerated GLUT4 degradation was analyzed in soleus.

Key findings: Hypercorticosteronemia, hyperinsulinemia and low glucose decay rate in the insulin tolerance test of obese rats were restored by CBX ($P < 0.05$). *Slc2a4*/GLUT4 increased in SAT ($P < 0.05$) and decreased in VAT ($P < 0.01$) of obese rats. In soleus, obesity increased *Slc2a4* but decreased GLUT4 ($P < 0.01$), possibly by accelerating GLUT4 degradation, as suggested by decreased UBC9 ($P < 0.01$). CBX restored both UBC9 and GLUT4 contents. SREBP1 did not participate in the *Slc2a4* transcriptional regulation.

Significance: The insulin sensitizer effect of CBX involves the increase of GLUT4 expression in soleus, indicating an increased glucose disposal in skeletal muscle. This observation reinforces the skeletal muscle as the main site of insulin-induced glucose uptake and sheds new light on the metabolic effects of 11βHSD1 inhibitors, since most of the studies so far have focused on its effects on liver and adipose tissues.

1. Introduction

Glucocorticoids (GC) induce insulin resistance [1], which is in the core of a cluster of metabolic diseases such as hypertension, cardiovascular disease, type 2 diabetes and obesity [2]. Although none of these conditions primarily associates to hypercortisolism, GC actions depend not only on circulating levels, but also on the pre-receptor metabolism ruled by the 11β-hydroxysteroid-dehydrogenase (11βHSD) enzymes.

The 11βHSD1 activates GC (cortisol or corticosterone) from its inactive ketometabolite (cortisone or dehydrocorticosterone) in most of the tissues such as liver, adipose, skeletal muscles, endocrine pancreas [3]. It is extensively associated to obesity and insulin resistance [1], and carbenoxolone (CBX), a competitive inhibitor of 11βHSD [4], has attenuated symptoms of the metabolic syndrome in obese mice [5–8], and improved hepatic insulin sensitivity in healthy and diabetic humans [4]. Those studies have focused on hepatic effects, and little is known on peripheral mechanisms affected by CBX.

Tissue glucose disposal varies proportionally to the expression of glucose transporter 4 (GLUT4), which has been recognized as a marker of insulin sensitivity [9]. In fact, GLUT4 content consistently restores together with insulin sensitivity in response to a variety of treatments [10–14], showing GLUT4 content as a reliable indicator of insulin sensitivity. In this context, the monosodium glutamate (MSG)-induced obesity is a very useful tool to observe this relationship: early in MSG-obesity, GLUT4 is preserved in muscles and increased in visceral adipose tissue, increasing adipose insulin sensitivity and favoring fat accumulation in the first stages of obesity. Later, when obesity and insulin

* Corresponding author at: Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo, Av. Prof. Lineu Prestes, 1524, São Paulo, SP 05508-900, Brazil.
E-mail address: morirc-icb@hotmail.com (R.C. Mori).

https://doi.org/10.1016/j.lfs.2019.05.017
Received 13 March 2019; Received in revised form 6 May 2019; Accepted 7 May 2019
Available online 08 May 2019
0024-3205/

resistance are established, GLUT4 expression decreases in all insulin sensitive tissues [15], and restores when insulin sensitivity recovers [10–12,14].

The expression of the GLUT4 encoding gene solute carrier family 2-member 4 (*Slc2a4*) is influenced by several factors, such as sympathetic [16,17] and contractile activities [18], endocannabinoids [12], estradiol [19], insulin [20], T3 [21], unsaturated fatty acids [22], among others. Some of these factors affect transcriptional factors implicated in the *Slc2a4* regulation, including the sterol regulatory element-binding protein 1 (SREBP1) [12,22], which is inhibited by CBX in liver [23]. Since SREBP1 is a *Slc2a4* enhancer [12,22,24], we investigate whether CBX affects *Slc2a4* transcription through SREBP1.

Skeletal muscle accounts for ~80% of the insulin-stimulated glucose uptake [25]. Thus, the chronic GC excess-induced loss of lean mass affects the whole-body insulin sensitivity, especially considering the concomitant loss of GLUT4. Little is known on the degradation of GLUT4, which resides in specialized GLUT4 storage vesicles (GSV) in muscles and adipocytes [26]. Insulin-induced glucose transport depends on GLUT4 turnover and targeting to GSV which among other signs, involve the small ubiquitin like modifier protein (SUMO) conjugating enzyme UBC9, whose absence accelerates GLUT4 degradation [27]. Whether GC level or CBX affect UBC9 has never been investigated.

Circulating cortisol usually does not elevate in human obesity, probably due to elevated cortisol clearance accompanying the higher cortisol secretion [28,29]. However, although hypercortisolism is not biochemically detected, functional hypercortisolism is believed to occur in obesity, due to increased adipose 11βHSD1 [4]. Additionally, obesity is one of the main conditions implicated in the pseudo-Cushing syndrome [30]. Despite that, no study has focused on the effects of elevated GC on GLUT4 expression in obesity.

In this study, we use the hypercorticosteronemic MSG-induced obesity model to investigate the CBX effects on insulin sensitivity and *Slc2a4*/GLUT4 expression, trying to clarify whether the GLUT4 content regulation contributes to the beneficial metabolic effects of the 11βHSD inhibition and which regulatory mechanisms of GLUT4 expression might be involved.

2. Materials and methods

2.1. Animals

Male Wistar rats were rendered obese by neonatal treatment with monosodium glutamate (MSG) as previously described [11]. After weaning, control and obese animals were kept under standard conditions [23 ± 2 °C, 12:12 h light-dark cycle, free access to water and chow (NUVILAB CR-1, Nuvital, Curitiba, Brazil)]. As from 16 weeks of age, half of the obese rats received 50 mg/kg/day of carbenoxolone disodium salt (CBX, C4790, Sigma Aldrich) in the drinking water, for 4 weeks [5], producing three groups as follows: control (C), obese (O) and CBX-treated obese rats (OC). Water intake was monitored to adjust CBX concentration and yield a constant dosage during treatment. Water or food intake, which were measured at every three days, were not affected by CBX. The mean consumptions by the end of each week of treatment is shown in Supplementary Fig. 1. All procedures were in accordance with the relevant guidelines and regulations and were approved by the Ethical Committee for Animal Research, Institute of Biomedical Sciences, University of Sao Paulo (#59/2008).

2.2. Tissue and blood sampling

Animals were used for tissue and blood sampling immediately after decapitation (8:00 AM-10:00 AM). Decapitation was performed without anesthesia, within 5–10 s after removing the animals from their cages. Trunk blood was immediately placed on ice and then centrifuged to obtain the plasma. Epididymal fat pad, inguinal subcutaneous fat and soleus muscle were harvested as representatives of visceral adipose tissue (VAT), subcutaneous adipose tissue (SAT) and oxidative skeletal muscle, respectively. All tissues were rapidly weighed, frozen in liquid nitrogen, and stored at −80 °C until assayed.

2.3. Metabolic parameters

Glucose concentration was measured in total blood (Precision QID; MediSense, Sao Paulo, Brazil), immediately after decapitation. Plasma was stored at −20 °C for later analysis of insulin and corticosterone (Coat-a-Count Insulin, TKIN1 and Coat-a-Count Rat Corticosterone, TKRC1, Siemens Medical Solutions Diagnostics, Los Angeles, California, USA) and non-esterified fatty acids [31].

2.4. Intravenous insulin tolerance test (IVITT)

Intravenous insulin tolerance test was performed in fed animals (8:00 AM-10:00 AM), as previously described [11,31].

2.5. Northern blotting

Because of previous data on altered transcript length in obesity [32], we analyzed *Slc2a4* mRNA by Northern Blotting. Total RNA was extracted from VAT, SAT and soleus for *Slc2a4* mRNA analyses [16]. The results were expressed as arbitrary units (AU) after normalization to the corresponding *Actb* mRNA value, considering the mean of control rats as 100.

2.6. Reverse transcription and polymerase chain reaction (RT-PCR)

The RT-PCR analysis of *Srebp1* (NM_001276708.1) in VAT, SAT and soleus was carried out as described [18]. After comparing the expression of *Actb* (NM_031144.3), *Gapdh* (NM_017008.4) and *Rpl9* (NM_001007598.3) in each tissue, *Actb* and *Gapdh* were picked up as internal control for adipose tissues and soleus, respectively. The PCR products were resolved on 1.2% EtBr-agarose gels and the band intensities determined by the Image Quant TL analysis software (GE Healthcare Life Sciences). Results normalized to the respective internal control were expressed as fold change relative to control. Table 1 describes the PCR primers details. The number of cycles were 31 (VAT, soleus) and 37 (SAT) for *Srebp1*; 30 (VAT) and 32 (SAT) for *Actb*; 34 for soleus *Gapdh*.

2.7. Electrophoretic mobility shift assay (EMSA)

Tissues samples were used for nuclear protein extraction, as described [18]. A double-stranded oligonucleotide corresponding to the sequence of rat GLUT4 promoter containing the binding site of SREBP1 (5′- GGCCTTTTGGGGTGTGCGGG-3′) [24] was radio-labeled, incubated with 20 µg protein from nuclear extract, and the DNA–protein complexes were submitted to EMSA, as previously described by Furuya and coworkers [12], who confirmed the specificity of this oligonucleotide

Table 1
RT-PCR characteristics.

Gene	Primer sequence (5′-3′)	Product size (base pairs)	t (°C)
Srebp1c	Forward: GGAGCCATGGATTGCACATT Reverse: AGGAAGGCTTCCAGAGAGGA	191	58.7
Actb	Forward: GAAGTACCCCATTGAACACG Reverse: GAGGCATACAGGGACAACAC	234	55.5
Gapdh	Forward: ACATCATCCCTGCATCCACT Reverse: GGGAGTTGCTGTTGAAGTCA	258	55.7

Srebp1c, sterol regulatory element binding transcription factor 1; *Actb*, actin beta; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase; t, annealing temperature.

binding to SREBP1. Binding activity of control samples was normalized as 100, and results were expressed as arbitrary units.

2.8. Western blotting

Immunoblotting for GLUT4 protein was carried out as described [16]. For UBC9 protein analysis, total protein extract was obtained from soleus homogenized with Polytron (Luzern, Switzerland) in ice-cold lysis buffer [20 mM Tris (pH 7.4), 50 mM NaCl, 50 mM NaF, 5 mM Na pyrophosphate, 250 mM sucrose, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM DTT, 100 μM benzamidine, 1% Triton X-100, 1 μg/mL leupeptin, 1 μg/mL aprotinin]. Homogenates were subjected to centrifugation (4 °C, 12,000 g, 15 min) and supernatant was stored at −80 °C until use. Sixty μg of protein were separated by SDS-PAGE and transferred to nitrocellulose membrane for immunodetection.

Specific antibodies anti-GLUT4 (1:4000, #07-1404, Millipore Corporation, Temacula, CA, USA) and anti-UBC9 (1:1000, ab75854, Abcam, Cambridge, MA, USA) were detected by horseradish peroxidase-linked anti-rabbit immunoglobulin (Amersham Biosciences, Buckinghamshire, UK) and chemiluminescent reagent Luminol (PerkinElmer, Boston, MA). The intensity of the blots generated by chemiluminescence was quantified by Image Quant TL (GE Healthcare Life Sciences) and the mean value of control samples was normalized as 100. The Ponceau-stained nitrocellulose membrane was used as the loading control.

2.9. Rapid amplification of cDNA ends for poly(A) test (RACE-PAT) assay

The poly(A) tail length from GLUT4 mRNA was measured as described, using oligo(dT) anchor (200 ng/L) (5´-GCGAGCTCCGCGGCC GCGT12) and the primer sense for a sequence within GLUT4 cDNA, from bases 2425–2444 (5´-GATAGGGAGCAGAAACCTGG-3′) [17]. PCR products were resolved on 2.5% EtBr-agarose gels and the amplicon sizes were measured by Image Quant TL (GE Healthcare Life Sciences) comparing to a DNA ladder (1 kb Plus; Invitrogen Life Technologies, Carlsbad, California). In each lane, the top of the smear indicated the longest amplified fragment, which represents the *Slc2a4* mRNA poly(A) tail plus 100 bases upstream the tail.

2.10. Data analysis

Data were expressed as mean ± S.E.M. and compared by one-way analysis of variance (ANOVA) and the Student-Newman-Keuls post hoc test, after confirming the normality of the data distribution by Shapiro-Wilk test (GraphPad Prism Software, version 5.00 for Windows, San Diego, CA). The number of animals is indicated in the figure legends and inside the tables, in brackets.

3. Results

3.1. Characteristics of the animals

Table 2 shows that the animals were studied when the features of the MSG-induced obesity had been established: untreated and CBX-treated obese rats (O and OC) had lower body weight and length and lower soleus weight ($P < 0.001$ vs. C), as expected for this obesity model. Obesity is evinced by increased Lee Index and excessive fat accumulation in both VAT and SAT ($P < 0.001$ vs. C). CBX decreased subcutaneous fat ($P < 0.05$ vs. O). Glycaemia was similar among the groups and plasma insulin increased in O rats ($P < 0.05$ vs. C), revealing their insulin resistance, confirmed by the low glucose decay rate (K_{ITT}) in the insulin tolerance test ($P < 0.001$ vs. C). CBX restored both plasma insulin ($P < 0.05$ vs. O) and k_{ITT} ($P < 0.01$ vs O). Corticosteronemia elevated in O rats ($P < 0.001$ vs C) and decreased in OC ($P < 0.05$ vs. O). Circulating NEFA (non-esterified fatty acids) were about 40% elevated (not statistically significant) in O rats, suggesting they are prone to an increased lipid turnover, considering their excessive adipose mass. CBX further increased NEFA ($P < 0.05$ vs. C), despite decreasing circulating corticosterone.

Table 2
Morphometric and metabolic characteristics of the animals.

	C	O	OC
Body weight (g)	350 ± 10.2 (12)	321 ± 5.2** (14)	309 ± 6.2*** (14)
Body length (cm)	23.1 ± 0.25 (12)	21.3 ± 0.18*** (14)	21.2 ± 0.16*** (14)
Lee Index ($\sqrt[3]{}$g/cm)	30.1 ± 0.18 (12)	32.4 ± 0.31*** (14)	32.4 ± 0.13*** (14)
VAT weight (g)	3.98 ± 0.26 (8)	7.88 ± 0.36*** (10)	7.26 ± 0.44*** (11)
SAT weight (g)	5.12 ± 0.46 (8)	15.4 ± 0.95*** (10)	12.8 ± 0.52***,# (11)
Soleus weight (g)	0.134 ± 0.003 (10)	0.096 ± 0.002*** (10)	0.100 ± 0.002*** (10)
Glycaemia (mg/dL)	119 ± 3.50 (9)	114 ± 4 40 (11)	113 ± 2.80 (11)
Plasma insulin (μU/mL)	40.1 ± 5.50 (8)	61.4 ± 4.60* (9)	46.5 ± 4.30# (11)
k_{ITT} (%/min)	5.06 ± 0.30 (9)	3.42 ± 0.30*** (11)	4.68 ± 0.21## (12)
Plasma corticosterone (ng/mL)	113 ± 21.4 (7)	229 ± 14.6*** (7)	175 ± 15.4*,# (8)
NEFA (mM)	0.365 ± 0.093 (5)	0.524 ± 0.080 (5)	0.853 ± 0.119*,# (6)

C, control rats; O, obese rats; OC, obese rats treated with 50 mg/Kg/day of carbenoxolone for four weeks; Lee Index, [$\sqrt[3]{}$ body weight (g) ÷ body length (cm)] X 100;VAT, visceral adipose tissue; SAT, subcutaneous adipose tissue; k_{ITT}, constant of glucose decay rate during the insulin tolerance test; NEFA, non-esterified fatty acids. Data are mean ± S.E.M., compared by 1-way ANOVA/Student Newman-Keuls test.

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$ vs. C.
\# $P < 0.05$ vs. O.
\#\# P < 0.01 vs. O.

3.2. CBX has no effect on Slc2a4 expression, but increases GLUT4 protein content in soleus

In visceral (VAT) and subcutaneous (SAT) adipose tissues of obese rats, *Slc2a4* mRNA content had opposite changes, decreasing in VAT (Fig. 1A) and increasing in SAT (Fig. 1B). CBX mildly increased the *Slc2a4* mRNA only in SAT. GLUT4 protein expression varied accordingly, decreasing in VAT and increasing in SAT (Fig. 1D and E) of obese rats, with no significant changes after CBX treatment. Thus, apparently, *Slc2a4*/GLUT4 expression is regulated at the transcriptional level in adipose tissues.

In soleus of obese rats, *Slc2a4* mRNA increased, and did not change after CBX (Fig. 1C). However, the GLUT4 content decreased significantly, and CBX restored it (Fig. 1F). Thus, there was no parallel variation between mRNA and protein changes, suggesting the occurrence of posttranscriptional regulations in *Slc2a4*/GLUT4 expression in soleus of obese rats.

3.3. SREBP1 does not determine the Slc2a4 expression in obesity or CBX treatment

In obese rats, *Srebp1* mRNA expression (Fig. 2A–C) varied in parallel with *Slc2a4* mRNA in all tissues evaluated, decreasing in VAT and increasing in SAT and soleus. CBX restored *Srebp1* mRNA to control levels in VAT and soleus.

SREBP1 binding activity (Fig. 2D–F) did not change in any tissues of obese rats, suggesting obesity affected the *Slc2a4* expression by mechanisms other than SREBP1 transcriptional activity. Moreover, CBX

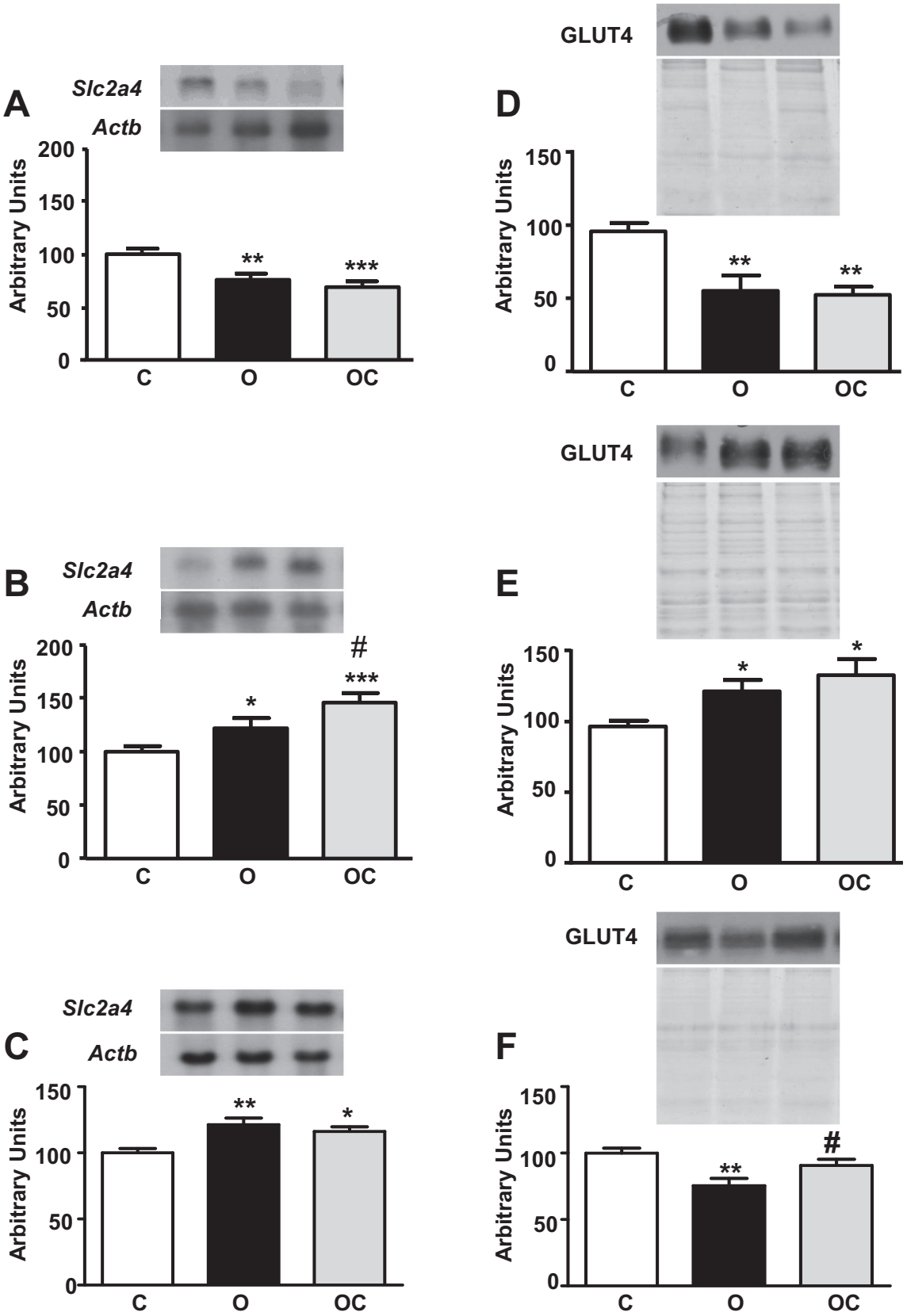

Fig. 1. *Slc2a4* mRNA (A–C) and GLUT4 protein (D–F) in visceral adipose tissue (A and D), subcutaneous adipose tissue (B and E) and soleus muscle (C and F) of control rats (C), untreated obese rats (O) and obese rats treated with 50 mg/Kg/day of carbenoxolone (OC) in the drinking water for four weeks. Representative autoradiograms are shown on top of each graph. Respective *Actb* autoradiograms (A–C) and respective loading control in the Ponceau-stained nitrocellulose membranes (D–F) are shown in the lower panels. Results are mean ± S.E.M. of 7 to10 animals, compared by one-way analysis of variance, followed by the Student Newman-Keuls post-test. * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$ vs. C; # $P < 0.05$ vs. O.

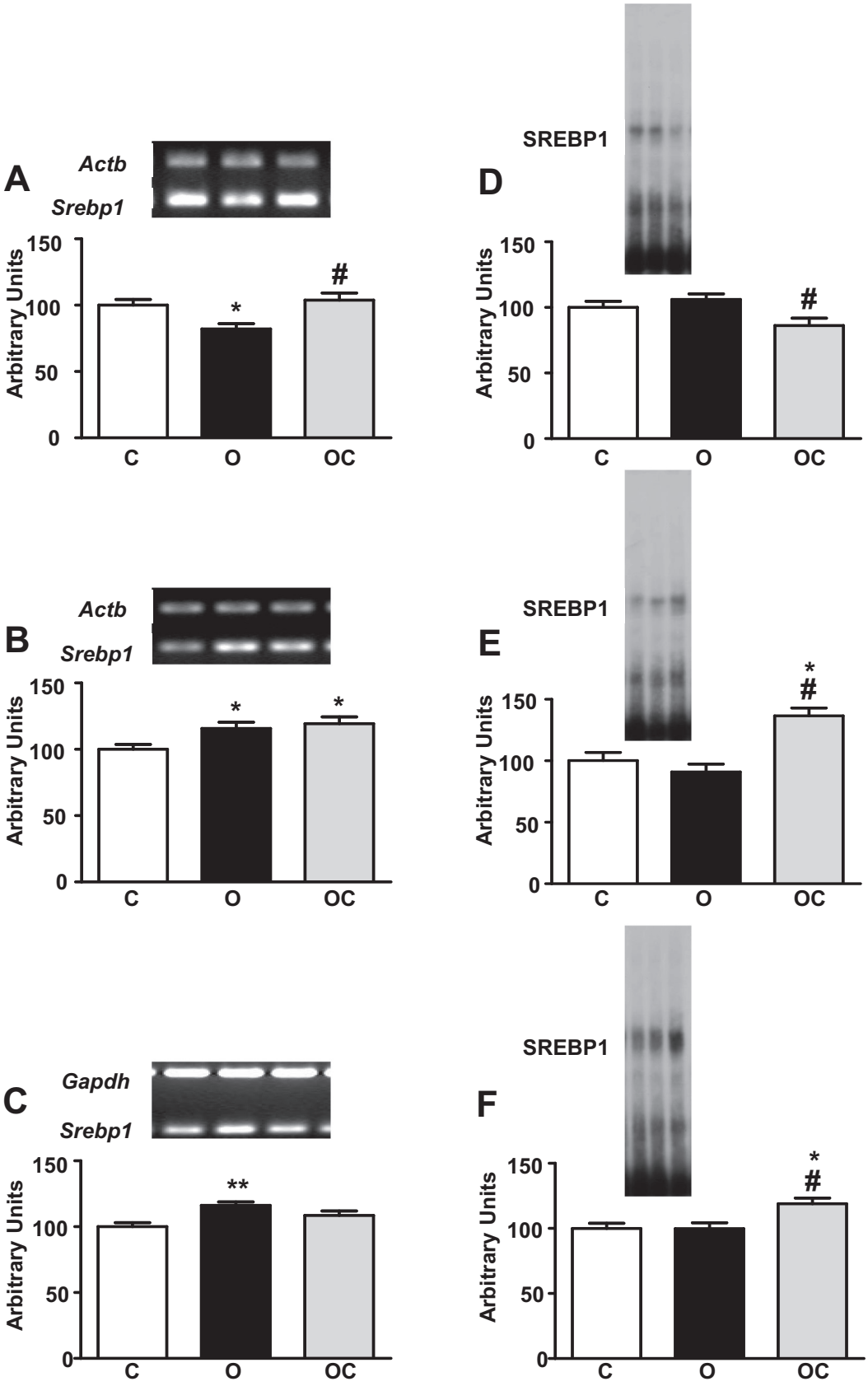

Fig. 2. *Srebp1* mRNA expression (A–C) and SREBP1 protein bound to the oligonucleotide representing its binding site in *Slc2a4* gene promoter (D–F). Total RNA and nuclear protein extracts were obtained from visceral adipose tissue (A and D), subcutaneous adipose tissue (B and E) and soleus muscle (C and F) of control (C) and obese rats, untreated (O) or treated with 50 mg/Kg/day of carbenoxolone (OC) in the drinking water for four weeks. Representative autoradiograms are shown on top of each graph. Results are mean ± SEM of 5 to 8 animals, compared by one-way analysis of variance, followed by the Student Newman-Keuls post-test. *$P < 0.05$ vs. C; ** $P < 0.01$ vs. C; #$P < 0.05$ vs. O.

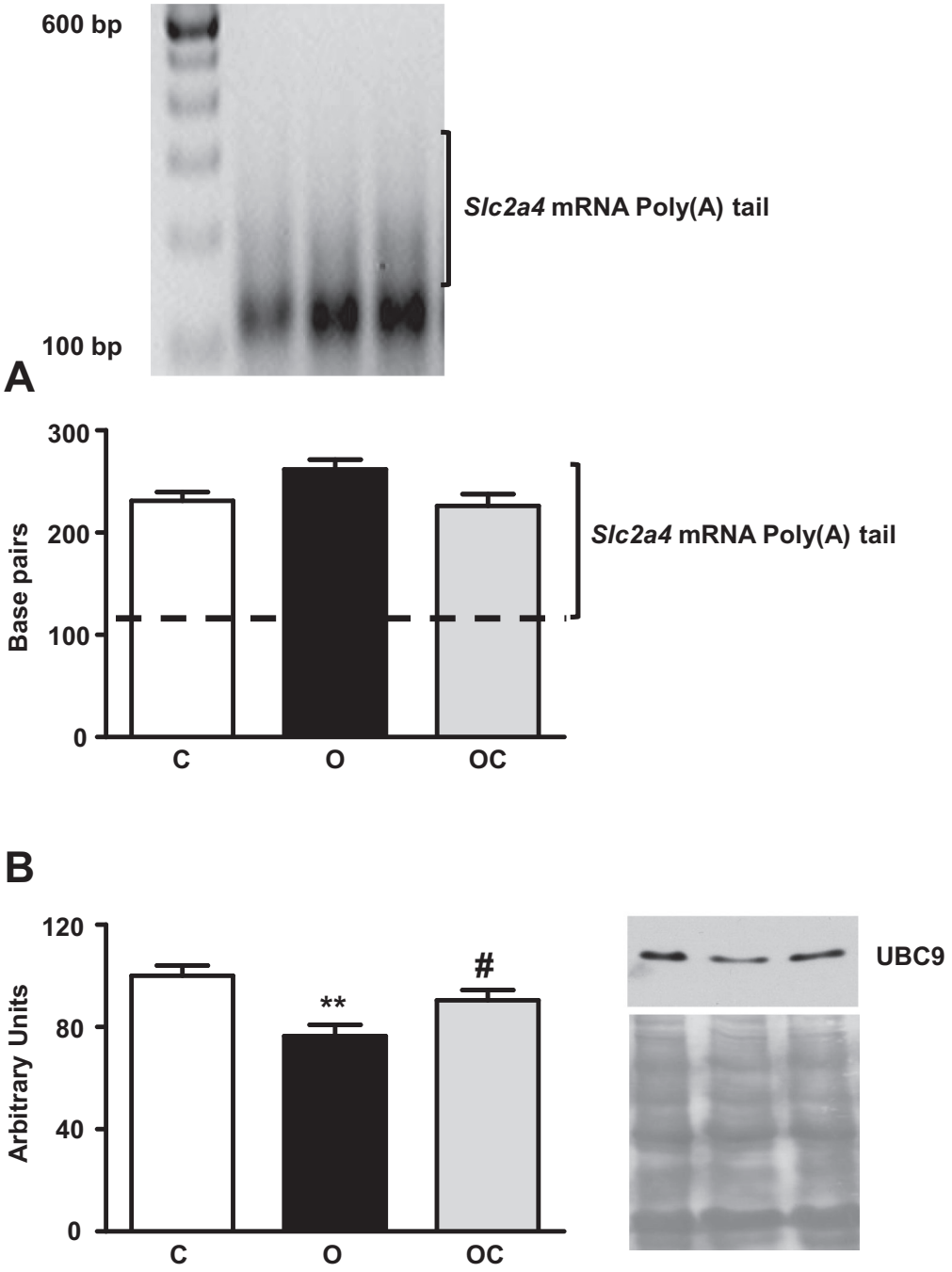

Fig. 3. Possible mechanisms of post-transcriptional regulation of the *Slc2a4*/GLUT4 expression were investigated by measuring the poly(A) tail length (A) and the UBC9 protein content (B). Total mRNA and total protein extract were obtained from soleus muscle of control (C) and obese rats, untreated (O) or treated with 50 mg/Kg/day of carbenoxolone (OC) in the drinking water for four weeks. Representative blots are shown on top (A) and right (B). Results are mean ± SEM of 5 to 7 animals, compared by one-way analysis of variance, followed by the Student Newman-Keuls post-test. $^{**}P < 0.01$ vs. C; $\#P < 0.05$ vs. O.

decreased the SREBP1 binding activity in VAT and increased it in SAT and soleus, changes that do not correlate with the observed *Slc2a4* mRNA content in these tissues.

3.4. Increased GLUT4 degradation can explain the Slc2a4/GLUT4 discrepancy in soleus

In adipose tissues, GLUT4 protein varied in accordance with the observed changes in *Slc2a4* mRNA. Thus, in these tissues, GLUT4 content seems to be regulated at the transcriptional level. However, in soleus of obese rats, despite the high *Slc2a4* mRNA content, GLUT4 protein decreased. CBX restored (increased) GLUT4 content to control level but had no effect on *Slc2a4* mRNA. These results suggested a posttranscriptional regulation for this gene in soleus.

The stability of mRNA molecule conferred by the poly(A) tail length is a well-known posttranscriptional mechanism regulating the protein translation. In the present study, neither obesity nor CBX affected the poly(A) tail length (Fig. 3A), as depicted by the smear representing the sizes of poly(A) in the autoradiographic image. Thus, this post-transcriptional regulatory mechanism does not determine the discrepant variation in *Slc2a4*/GLUT4 content found in soleus.

To test for the possibility of altered GLUT4 protein degradation, we examined the UBC9 protein content, which was decreased in soleus of obese rats ($P < 0.01$ vs. C, Fig. 3B), suggesting an accelerated

degradation of GLUT4 protein in this group. CBX restored the UBC9 content ($P < 0.05$ vs O), what may account for the recovery of GLUT4 content in OC group.

4. Discussion

MSG-induced obesity has been known since the seventies [33]. It is induced by subcutaneous injections of MSG in the neonatal period when blood-brain barrier is not established yet, leading to brain damage, especially neurons of the arcuate nucleus [34], and resulting in some neuroendocrine disfunctions in the adulthood. MSG-obese animals display clear insulin resistant condition, with hyperinsulinemia and hypercorticosteronemia, making it a good experimental model for the study of the possible insulin sensitizer effect of CBX.

In this study, we demonstrate that the 11βHSD inhibitor CBX improves peripheral insulin sensitivity and increases GLUT4 expression in oxidative muscle of obese rats. Data revealed that the *Slc2a4*/GLUT4 changes does not involve the SREBP1 binding activity, and that post-transcriptional mechanisms, such as UBC9-related GLUT4 degradation, may contribute to determine the final GLUT4 content in soleus.

CBX enhanced insulin sensitivity (restored insulinemia and k_{ITT}) and effectively inhibited 11βHSD1 (decreased plasma corticosterone) of obese rats. Previous studies have found increased insulin and unaltered NEFA and corticosterone after 10–16 days of CBX treatment [7,35], differences that may relate to specific features of the obesity models or to the dose/period of treatments. Indeed, longer CBX treatments (4 weeks) have reversed hyperinsulinemia [5,8], in agreement with our findings, but decreased circulating NEFA in the LDL-receptor deficient obese mice [5].

Those differences in terms of obesity models and treatment protocols arise an interesting point on the use of CBX to treat metabolic syndrome in humans, what has been discouraged because of mild effects on insulin sensitivity [4]. The point is that for ethical reasons, studies in humans used low doses (300 mg/day) for short periods (2 weeks), possibly insufficient to produce expressive metabolic results. In fact, the inhibition of the enzymatic activity of 11βHSD1 also seems to occur differently depending on the rat strain, the route of administration and duration of treatment, since one study shows that CBX induces the reduction of 11βHSD1 activity only in liver [35], while other authors show that it significantly inhibits 11βHSD1 activity in both liver and adipose tissue [8].

Interestingly, back in the 1970´s, CBX was used to treat peptic ulcer, and mineralocorticoid-like side effects such as sodium and water retention occurred in some patients receiving excessive doses, but those undesirable effects were fully controlled by ordinary anti-hypertensive treatment [36]. Thus, if peripheral insulin sensitizer effects of CBX come to be unraveled, maybe we could carefully reconsider it (conjugated to spironolactone) as an adjuvant in the treatment of metabolic syndrome and T2D.

Concerning the regulation by glucocorticoids (GC), increased or preserved GLUT4 expression in skeletal muscles (depending on the fiber type) have been reported [37], indicating a tissue specific GC effect. By the beginning of the 2000´s, the GC-induced peripheral insulin resistance had been associated to impaired GLUT4 translocation with no changes in its expression [38]. However, GC was also shown to induce a progressive degree of insulin resistance, acutely impairing only GLUT4 translocation, but depleting cellular GLUT4 content after chronic exposure [39], what agrees with the current knowledge that chronic resistance to insulin-stimulated glucose uptake eventually involves decreased GLUT4 expression [9].

Regarding regional differences between VAT and SAT, the antilipolytic action of insulin is higher in SAT while the lipolytic effect of catecholamines and GC as well as the 11βHSD1 expression are higher in VAT [40]. However, the lipolytic effect of GC has been described to be blunted at high GC concentrations [41]. Thus, considering that CBX reduced corticosteronemia of our obese rats, it may have also increased lipolysis as suggested by their increased NEFA. Finally, since circulating NEFA reflects especially the lipolysis in SAT [40], it is reasonable to assume that CBX-treated obese rats had increased lipolysis rate in SAT, which indeed presented significant decrease in weight.

Inverse variations of GLUT4 and 11βHSD1 were found in VAT and SAT of nonobese humans [42] and VAT of obese rats [43]. Those data suggested an inhibitory effect of GC on GLUT4 expression, depending on the GC bioavailability determined by the 11βHSD1. Accordingly, in our obese rats, *Slc2a4*/GLUT4 expression decreased in VAT and increased in SAT, while 11βHSD1 increased and decreased, respectively (Supplementary Fig. 2). Differently, in MSG-obese mice, decreased GLUT4 was observed in SAT [44], which may be related to the fact that MSG-obesity has been observed to be much more severe in mice than rats [15,45]. Besides, the regulation of the11βHSD1 expression is unknown in tissues of MSG-mice. Finally, we cannot discard the possibility that GLUT4 would reduce in SAT of MSG-obese rats later.

Since there was no CBX effect upon *Slc2a4*/GLUT4 expression in adipose tissues, we assume the CBX-induced improvement of insulin sensitivity was not related to glucose clearance by these territories. Differently, CBX increased GLUT4 in soleus muscle, indicating that an increased glucose disposal in oxidative fibers could contribute to the improvement of whole-body insulin sensitivity.

In the search of potential approaches to control peripheral insulin sensitivity, it is important to investigate molecular mechanisms regulating the *Slc2a4*/GLUT4 expression. Modulations of SREBP1 expression and/or binding to target genes have been described in several conditions related to insulin resistance and, more than that, SREBP1 has been pointed as an enhancer of *Slc2a4* expression in adipose and muscle cells [12,22,24].

Our data revealed tissue-specific expression of *Srebp1* mRNA in untreated and CBX-treated obese rats, which could determine changes in the cellular SREBP1 content and, eventually, participate in the transcriptional regulation of target genes. However, by analyzing the binding activity of nuclear proteins into the *Slc2a4* SREBP1 binding site (EMSA), our data revealed that obesity did not alter the SREBP1 binding.

It is important to highlight that one gene expression regulation by a specific transcriptional factor can be investigated either in vitro by EMSA or in vivo by ChIP (chromatin immunoprecipitation) assay. Regarding that, we cannot discard the possibility of different results for SREBP1 binding into *Slc2a4* in a ChIP assay, which we could not perform due to restricted experimental animals and sample amount. Thus, what we can infer from our data is that the SREBP1 binding to the *Slc2a4* promoter did not match the *Slc2a4* mRNA variation, indicating SREBP1 is not a fundamental transcriptional factor in the present experimental conditions.

In VAT and SAT, changes in GLUT4 content agree with *Slc2a4* mRNA variations, indicating the *Slc2a4*/GLUT4 regulation occurs at the transcriptional level in these tissues. Since the SREBP1 is not a modulator of *Slc2a4* in the present condition, other transcriptional factors must be involved, such as the nuclear factor kappa B, whose signaling pathway has been reported to be activated in MSG-obese mice [46].

In soleus of obese rats, GLUT4 content decreased, and that was reversed by CBX. This regulation is in line with the whole-body insulin sensitivity observed in untreated or CBX-treated obese rats. However, GLUT4 regulation did not match the *Slc2a4* mRNA regulation, revealing the occurrence of some posttranscriptional regulation in soleus. In the insulin resistance of age-related obesity and 48 h-fasting, shortening of poly(A) tail has been considered the responsible for the discrepancy between increased *Slc2a4* mRNA and unchanged GLUT4 protein [17,32], pointing out that the shorter the poly(A) tail, the less efficient the protein translation. That was not the case in the present study, since the poly(A) tail length was unaltered among the groups.

Recently, epigenetic regulation by microRNAs (miRNA) has been shown as important posttranscriptional regulators, by destabilizing the mRNA and/or inhibiting their translation. Some miRNAs have been

predicted to regulate GLUT4 expression and, in skeletal muscle of diabetic rats, increased miR-29b-3p and miR-29c-3p correlates with decreased GLUT4 content [47]. Thus, the involvement of miRNAs in the GLUT4 regulation in soleus of obese rats cannot be excluded and deserves future studies.

Finally, considering the catabolic action of GC upon skeletal muscles, the discrepant *Slc2a4*/GLUT4 regulation may involve accelerated GLUT4 degradation. The UBC9, a structural homologue of the E2 ubiquitin-conjugating enzymes, inhibits GLUT4 degradation [27]. This UBC9 protein has been directly related to the GLUT4 content in adipocytes, muscular cells and muscle of T2D patients [27,48,49]. In this study, obesity decreased UBC9 in soleus, and CBX reversed that, which is in accordance with the observed GLUT4 regulations.

5. Conclusion

This study shows that CBX improved the whole-body insulin sensitivity of obese rats. This effect involved no changes in *Slc2a4*/GLUT4 expression in adipose tissues, but increased GLUT4 protein in skeletal muscle. Furthermore, GLUT4 regulation in muscle was not explained by transcriptional regulation of *Slc2a4* gene; but it was related to UBC9-mediated degradation of GLUT4 protein. These data not only agree with the knowledge that skeletal muscle is the main site of insulin-induced glucose clearance, but also reveal muscular GLUT4 plays a role in the insulin sensitizer effect of CBX, shedding new light on the metabolic effects of 11βHSD1 inhibitors, since most of the previous studies have focused on its effects on liver and adipose tissues.

Funding

This research was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) grant 2007/59722-4 and 2016/15603-0. RCM is a postdoctoral fellow from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

Declaration of Competing Interest

The authors have no competing interest to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.05.017.

References

[1] N.M. Morton, Obesity and corticosteroids: 11beta-hydroxysteroid type 1 as a cause and therapeutic target in metabolic disease, Mol. Cell. Endocrinol. 316 (2) (2010) 154–164, https://doi.org/10.1016/j.mce.2009.09.024 Mar 25.

[2] R.A. DeFronzo, E. Ferrannini, Insulin resistance. A multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease, Diabetes Care 14 (3) (1991 Mar) 173–194 (Review. PMID: 2044434).

[3] K. Chapman, M. Holmes, J. Seckl, 11β-hydroxysteroid dehydrogenases: intracellular gate-keepers of tissue glucocorticoid action, Physiol. Rev. 93 (3) (2013) 1139–1206 Jul. Review https://doi.org/10.1152/physrev.00020.2012.

[4] A. Stomby, R. Andrew, B.R. Walker, T. Olsson, Tissue-specific dysregulation of cortisol regeneration by 11βHSD1 in obesity: has it promised too much? Diabetologia 57 (6) (2014) 1100–1110, https://doi.org/10.1007/s00125-014-3228-6 Jun.

[5] A.M. Nuotio-Antar, D.L. Hachey, A.H. Hasty, Carbenoxolone treatment attenuates symptoms of metabolic syndrome and atherogenesis in obese, hyperlipidemic mice, Am. J. Physiol. Endocrinol. Metab. 293 (6) (2007) E1517–E1528, https://doi.org/10.1126/science.1066285 Dec.

[6] Y. Liu, Y. Nakagawa, Y. Wang, L. Liu, H. Du, W. Wang, X. Ren, K. Lutfy, T.C. Friedman, Reduction of hepatic glucocorticoid receptor and hexose-6-phosphate dehydrogenase expression ameliorates diet-induced obesity and insulin resistance in mice, J. Mol. Endocrinol. 41 (2) (2008) 53–64, https://doi.org/10.1677/JME-08-0004 Aug.

[7] N. Dhanesha, A. Joharapurkar, G. Shah, S. Kshirsagar, V. Dhote, A. Sharma, M. Jain, Inhibition of 11β-hydroxysteroid dehydrogenase 1 by carbenoxolone affects glucose homeostasis and obesity in db/db mice, Clin. Exp. Pharmacol. Physiol. 39 (1) (2012) 69–77, https://doi.org/10.1111/j.1440-1681.2011. 05640.x Jan.

[8] S.S. Prasad Sakamuri, M. Sukapaka, V.K. Prathipati, H. Nemani, U.K. Putcha, S. Pothana, S.R. Koppala, L.R. Ponday, V. Acharya, G.N. Veetill, V. Ayyalasomayajula, Carbenoxolone treatment ameliorated metabolic syndrome in WNIN/Ob obese rats, but induced severe fat loss and glucose intolerance in lean rats, PLoS One 7 (12) (2012), https://doi.org/10.1371/journal.pone.0050216.

[9] M.L. Corrêa-Giannella, U.F. Machado, SLC2A4gene: a promising target for pharmacogenomics of insulin resistance, Pharmacogenomics 14 (8) (2013) 847–850, https://doi.org/10.2217/pgs.13.45 Jun.

[10] P.C. Papa, P.M. Seraphim, U.F. Machado, Loss of weight restores GLUT4 content in insulin-sensitive tissues of monosodium glutamate-treated obese mice, Int. J. Obes. Relat. Metab. Disord. 21 (11) (1997) 1065–1070 Nov. (PMID: 9368832).

[11] R.C. Mori, S.M. Hirabara, A.E. Hirata, M.M. Okamoto, U.F. Machado, Glimepiride as insulin sensitizer: increased liver and muscle responses to insulin, Diabetes Obes. Metab. 10 (7) (2008) 596–600, https://doi.org/10.1111/j.1463-1326.2008.00870.x Jul.

[12] D.T. Furuya, A.C. Poletto, H.S. Freitas, U.F. Machado, Inhibition of cannabinoid CB1 receptor upregulates *Slc2a4* expression via nuclear factor-κB and sterol regulatory element-binding protein-1 in adipocytes, J. Mol. Endocrinol. 49 (2) (2012) 97–106, https://doi.org/10.1530/JME-12-0037 Jul 25.

[13] J.Z. Shen, L.N. Ma, Y. Han, J.X. Liu, W.Q. Yang, L. Chen, Y. Liu, Y. Hu, M.W. Jin, Pentamethylquercetin generates beneficial effects in monosodium glutamate-induced obese mice and C2C12 myotubes by activating AMP-activated protein kinase, Diabetologia 55 (6) (2012) 1836–1846, https://doi.org/10.1007/s00125-012-2519-z Jun.

[14] C.Y. Yonamine, E. Pinheiro-Machado, M.L. Michalani, A.B. Alves-Wagner, J.V. Esteves, H.S. Freitas, U.F. Machado, Resveratrol improves glycemic control in type 2 diabetic obese mice by regulating glucose transporter expression in skeletal muscle and liver, Molecules 22 (7) (2017), https://doi.org/10.3390/molecules22071180 pii: E1180. Jul 14.

[15] P. de Carvalho Papa, A.M. Vargas, J.L. da Silva, M.T. Nunes, U.F. Machado, GLUT4 protein is differently modulated during development of obesity in monosodium glutamate-treated mice, Life Sci. 71 (16) (2002 Sep 6) 1917–1928.

[16] M.M. Zanquetta, M.E. Nascimento, R.C. Mori, B. D'Agord Schaan, M.E. Young, U.F. Machado, Participation of beta-adrenergic activity in modulation of GLUT4 expression during fasting and refeeding in rats, Metabolism 55 (11) (2006) 1538–1545, https://doi.org/10.1016/j.metabol.2006.06.026 Nov.

[17] A.B. Alves-Wagner, H.S. De Freitas, P.B. De Souza, P.M. Seraphim, R.C. Mori, U.F. Machado, Beta-adrenergic activity preserves GLUT4 protein in glycolytic fibers in fasting, Muscle Nerve 40 (5) (2009) 847–854, https://doi.org/10.1002/mus.21359 Nov.

[18] J.L. Silva, G. Giannocco, D.T. Furuya, G.A. Lima, P.A. Moraes, S. Nachef, S. Bordin, L.R. Britto, M.T. Nunes, U.F. Machado, NF-kappaB, MEF2A, MEF2D and HIF1-a involvement on insulin- and contraction-induced regulation of GLUT4 gene expression in soleus muscle, Mol. Cell. Endocrinol. 240 (1–2) (2005) 82–93, https://doi.org/10.1016/j.mce.2005.05.006 Aug 30.

[19] R.S. Campello, A.B. Alves-Wagner, T.F. Lucas, R.C. Mori, D.T. Furuya, C.S. Porto, U.F. Machado, Estrogen receptor 1 agonist PPT stimulates Slc2a4 gene expression and improves insulin-induced glucose uptake in adipocytes, Curr. Top. Med. Chem. 12 (19) (2012) 2059–2069, https://doi.org/10.2174/1568026611212190004.

[20] P.A. Moraes, C.Y. Yonamine, D.C. Pinto Junior, J.V. Esteves, U.F. Machado, R.C. Mori, Insulin acutely triggers transcription of Slc2a4 gene: participation of the AT-rich, E-box and NFKB-binding sites, Life Sci. 114 (1) (2014 Sep 26) 36–44, https://doi.org/10.1016/j.lfs.2014.07.040.

[21] M.M. Zanquetta, A.B. Alves-Wagner, R.C. Mori, R.S. Campello, U.F. Machado, Recovery of insulin sensitivity and Slc2a4 mRNA expression depend on T3 hormone during refeeding, Metabolism 63 (3) (2014) 328–334, https://doi.org/10.1016/j.metabol.2013.11.001 Mar.

[22] A.C. Poletto, D.T. Furuya, A. David-Silva, P. Ebersbach-Silva, C.L. Santos, M.L. Corrêa-Giannella, M. Passarelli, U.F. Machado, Oleic and linoleic fatty acids downregulate *Slc2a4*/GLUT4 expression via NFKB and SREBP1 in skeletal muscle cells, Mol. Cell. Endocrinol. 401 (2015 Feb 5) 65–72, https://doi.org/10.1016/j.mce.2014.12.001.

[23] S.D. Rhee, C.H. Kim, J.S. Park, W.H. Jung, S.B. Park, H.Y. Kim, G.H. Bae, T.J. Kim, K.Y. Kim, Carbenoxolone prevents the development of fatty liver in C57BL/6-Lep ob/ob mice via the inhibition of sterol regulatory element binding protein-1c activity and apoptosis, Eur. J. Pharmacol. 691 (1–3) (2012) 9–18, https://doi.org/10.1016/j.ejphar.2012.06.021 Sep 15.

[24] S.S. Im, S.K. Kwon, S.Y. Kang, T.H. Kim, H.I. Kim, M.W. Hur, K.S. Kim, Y.H. Ahn, Regulation of GLUT4 gene expression by SREBP-1c in adipocytes, Biochem. J. 399 (1) (2006) 131–139, https://doi.org/10.1042/BJ20060696 Oct 1.

[25] R.A. DeFronzo, E. Jacot, E. Jequier, E. Maeder, J. Wahren, J.P. Felber, The effect of insulin on the disposal of intravenous glucose. Results from indirect calorimetry and hepatic and femoral venous catheterization, Diabetes 30 (12) (1981) 1000–1007 Dec.

[26] N.J. Bryant, R. Govers, D.E. James, Regulated transport of the glucose transporter GLUT4, Nat. Rev. Mol. Cell Biol. 3 (2002) 267–277, https://doi.org/10.1038/nrm782.

[27] L.B. Liu, W. Omata, I. Kojima, H. Shibata, The SUMO conjugating enzyme Ubc9 is a regulator of GLUT4 turnover and targeting to the insulin-responsive storage compartment in 3T3-L1 adipocytes, Diabetes 56 (2007) 1977–1985, https://doi.org/10.1016/j.devcel.2005.04.004.

[28] S.A. Lottenberg, D. Giannella-Neto, H. Derendorf, M. Rocha, A. Bosco, S.V. Carvalho, A.E. Moretti, A.C. Lerário, B.L. Wachenberg, Effect of fat distribution

on the pharmacokinetics of cortisol in obesity, Int. J. Clin. Pharmacol. Ther. 36 (9) (1998) 501–505 Sep. (PMID: 9760012).
[29] M. Moro, P. Putignano, M. Losa, C. Invitti, C. Maraschini, F. Cavagnini, The desmopressin test in the differential diagnosis between Cushing's disease and pseudo-Cushing states, J. Clin. Endocrinol. Metab. 85 (10) (2000) 3569–3574, https://doi.org/10.1210/jcem.85.10.6862 Oct.
[30] O. Chabre, The difficulties of pseudo-Cushing's syndrome (or "non-neoplastic hypercortisolism"), Ann. Endocrinol. (Paris) 79 (3) (2018) 138–145, https://doi.org/10.1016/j.ando.2018.04.017 Jun.
[31] R.S. Campello, A.B. Alves-Wagner, F. Abdulkader, R.C. Mori, U.F. Machado, Carbohydrate- and lipid-enriched meals acutely disrupt glycemic homeostasis by inducing transient insulin resistance in rats, Can. J. Physiol. Pharmacol. 90 (5) (2012) 537–545, https://doi.org/10.1139/y2012-056 May.
[32] P.M. Seraphim, M.T. Nunes, G. Giannocco, U.F. Machado, Age related obesity-induced shortening of GLUT4 mRNA poly(A) tail length in rat gastrocnemius skeletal muscle, Mol. Cell. Endocrinol. 276 (1–2) (2007) 80–87, https://doi.org/10.1016/j.mce.2007.07.004 Sep 30.
[33] A. Djazayery, D.S. Miller, The use of gold-thioglucose and monosodium glutamate to induce obesity in mice, Proc. Nutr. Soc. 32 (1) (1973) 30A–31A May.
[34] Nemeroff CB, Grant LD, Bissette G, Ervin GN, Harrell LE, Prange AJ Jr. Growth, endocrinological and behavioral deficits after monosodium L-glutamate in the neonatal rat: possible involvement of arcuate dopamine neuron damage. Psychoneuroendocrinology. 1977;2(2):179–96.
[35] D.E. Livingstone, B.R. Walker, Is 11beta-hydroxysteroid dehydrogenase type 1 a therapeutic target? Effects of carbenoxolone in lean and obese Zucker rats, J. Pharmacol. Exp. Ther. 305 (1) (2003) 167–172, https://doi.org/10.1124/jpet.102.044842 Apr.
[36] R.M. Pinder, R.N. Brogden, P.R. Sawyer, T.M. Speight, R. Spencer, G.S. Avery, Carbenoxolone: a review of its pharmacological properties and therapeutic efficacy in peptic ulcer disease, Drugs 11 (4) (1976) 245–307.
[37] R.S. Haber, S.P. Weinstein, Role of glucose transporters in glucocorticoid-induced insulin resistance. GLUT4 isoform in rat skeletal muscle is not decreased by dexamethasone, Diabetes 41 (6) (1992) 728–735, https://doi.org/10.2337/diab.41.6.728 Jun.
[38] H. Sakoda, T. Ogihara, M. Anai, M. Funaki, K. Inukai, H. Katagiri, Y. Fukushima, Y. Onishi, H. Ono, M. Fujishiro, M. Kikuchi, Y. Oka, T. Asano, Dexamethasone-induced insulin resistance in 3T3-L1 adipocytes is due to inhibition of glucose transport rather than insulin signal transduction, Diabetes 49 (10) (2000) 1700–1708, https://doi.org/10.2337/diabetes.49.10.1700 Oct.
[39] W.T. Garvey, T.P. Huecksteadt, R. Monzon, S. Marshall, Dexamethasone regulates the glucose transport system in primary cultured adipocytes: different mechanisms of insulin resistance after acute and chronic exposure, Endocrinology 124 (5) (1989 May) 2063–2073, https://doi.org/10.1210/endo-124-5-2063.
[40] M.J. Lee, Y. Wu, S.K. Fried, Adipose tissue heterogeneity: implication of depot differences in adipose tissue for obesity complications, Mol. Asp. Med. 34 (1) (2013) 1–11, https://doi.org/10.1016/j.mam.2012.10.001 Feb.
[41] J.E. Campbell, A.J. Peckett, A.M. D'souza, T.J. Hawke, M.C. Riddell, Adipogenic and lipolytic effects of chronic glucocorticoid exposure, Am. J. Physiol. Cell Physiol. 300 (1) (2011) C198–C209, https://doi.org/10.1152/ajpcell.00045.2010 Jan.
[42] Walker GE, Verti B, Marzullo P, Savia G, Mencarelli M, Zurleni F, Liuzzi A, Di Blasio AM. Deep subcutaneous adipose tissue: a distinct abdominal adipose depot. Obesity (Silver Spring). 2007 Aug;15(8):1933–43, doi: https://doi.org/10.1038/oby.2007.231 https://doi.org/10.1038/oby.2007.231.
[43] S. Shao, X. Zhang, M. Zhang, Inhibition of 11β-hydroxysteroid dehydrogenase type 1 ameliorates obesity-related insulin resistance, Biochem. Biophys. Res. Commun. 478 (1) (2016) 474–480, https://doi.org/10.1016/j.bbrc.2016.06.015 Sep 9.
[44] A.C. Poletto, A. David-Silva, A.P. Yamamoto, U.F. Machado, D.T. Furuya, Reduced *Slc2a4*/GLUT4 expression in subcutaneous adipose tissue of monosodium glutamate obese mice is recovered after atorvastatin treatment, Diabetol. Metab. Syndr. 7 (2015) 18, https://doi.org/10.1186/s13098-015-0015-6 Mar 14.
[45] U.F. Machado, I. Shimizu, M. Saito, Reduced content and preserved translocation of glucose transporter (GLUT 4) in white adipose tissue of obese mice, Physiol. Behav. 55 (4) (1994) 621–625. Apr.
[46] D.T. Furuya, A.C. Poletto, R.R. Favaro, J.O. Martins, T.M. Zorn, U.F. Machado, Anti-inflammatory effect of atorvastatin ameliorates insulin resistance in monosodium glutamate-treated obese mice, Metabolism 59 (3) (2010) 395–399, https://doi.org/10.1016/j.metabol.2009.08.011 Mar.
[47] J.V. Esteves, C.Y. Yonamine, D.C. Pinto-Junior, F. Gerlinger-Romero, F.J. Enguita, U.F. Machado, Diabetes modulates MicroRNAs 29b-3p, 29c-3p, 199a-5p and 532-3p expression in muscle: possible role in GLUT4 and HK2 repression, Front. Endocrinol. (Lausanne) 9 (2018 Sep 12) 536, https://doi.org/10.3389/fendo.2018.00536.
[48] F. Giorgino, O. de Robertis, L. Laviola, C. Montrone, S. Perrini, K.C. McCowen, R.J. Smith, The sentrin conjugating enzyme mUbc9 interacts with GLUT4 and GLUT1 glucose transporters and regulates transporter levels in skeletal muscle cells, Proc. Natl. Acad. Sci. U. S. A. 97 (3) (2000 Feb 1) 1125–1130 (PMCID:PMC15543).
[49] U. Kampmann, B. Christensen, T.S. Nielsen, S.B. Pedersen, L. Ørskov, S. Lund, N. Møller, N. Jessen, GLUT4 and UBC9 protein expression is reduced in muscle from type 2 diabetic patients with severe insulin resistance, PLoS One 6 (11) (2011) e27854, , https://doi.org/10.1371/journal.pone.0027854.