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Original Paper

Sympathetic Regulation of *Slc2a4* Gene Expression: Participation of a Putative cAMP Responsive Element (CRE) Site in the *Slc2a4* Promoter

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Key Words
Slc2a4 • CRE • CREB • Sympathetic activity

Abstract
Background/Aims: Studies have indicated that sympathetic activity enhances GLUT4 expression (*Slc2a4* gene) by activating beta-adrenergic receptors. This could be mediated by a direct enhancer effect of cyclic AMP-responsive element binding protein (CREB) and family members upon *Slc2a4* gene. However, a cAMP responsive element (CRE) in *Slc2a4* promoter has never been demonstrated. ***Methods:*** *Slc2a4* CRE-site was searched by *in silico* analysis. In skeletal muscles from rats displaying high sympathetic activity (SHR), *Slc2a4* CRE-site was investigated by electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) assay; and *Slc2a4* expression was analyzed by RT-qPCR. Functional activity of the CRE-site was investigated by luciferase assay, 2 hours after 8-br-cAMP stimulation, in 3T3-L1 adipocytes transientely transfected with native and mutated CRE-sites. ***Results:*** *In silico* analysis indicated the -480/-473 segment as a putative CRE-site, with 62.5% of identity to CRE consensus sequence, and highly preserved in mouse, rat and human. CREB/CREM binding in this CRE-site was confirmed to occur *in vitro* (EMSA) and *in vivo* (ChIP assay). Enhancer activity of this segment in *Slc2a4* transcription was confirmed in 3T3-L1 cells. Finally, in extensor digitorum longus muscle from SHR, 80% increase in *Slc2a4* mRNA expression was observed to be accompanied by increased CREB/CREM binding into the CRE-site both *in vitro* and *in vivo*. ***Conclusion:*** This study demonstrates the presence of a functional CRE-site at -480/-473 sequence of the *Slc2a4* gene. This CRE-site has an enhancing activity on *Slc2a4* expression and participates in the *Slc2a4* increased expression observed in glycolytic muscles of rats displaying high sympathetic activity.

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Introduction

Increased *Slc2a4* transcription, causing the solute carrier family 2 facilitated glucose transporter member 4 (GLUT4) protein content to increase in skeletal muscle and adipose tissue, is associated with an improvement in insulin-mediated cellular glucose disposal [1, 2]. The opposite, i.e., a reduction in GLUT4 protein content is associated with insulin resistance and glucose intolerance [1, 2]. Therefore, due to the pivotal role of GLUT4 in determining glycemic homeostasis, it is of great importance to understand the molecular mechanisms regulating *Slc2a4* transcription.

The *Slc2a4* gene promoter contains several responsive elements to transcription factors, which can either activate or repress its transcription [3]. The transcriptional factors myocyte enhancer factor 2 (MEF2), myoblast determination protein 1 (MyoD) and thyroid hormone receptor alpha1 (TRα1) were shown to bind to -502 to -420 promoter region, and interact to synergistically enhance *Slc2a4* transcription [4, 5]. Recently, our group has found that proinflammatory transcriptional factors nuclear factor NF-kappa-B (NFκB) and RELA proto-oncogene NF-KB subunit (RELA), p50 and p65 proteins; respectively, whose activation promotes insulin resistance, inhibit *Slc2a4* transcription by directly binding to -134/-113 and -83/-62 regions of the *Slc2a4* promoter [6].

Previous studies conducted by our group have gathered strong evidence indicating that the sympathetic nervous system plays a role in the regulation of GLUT4 content in skeletal muscle, an effect that seems, at least in part, to be due to the induction of *Slc2a4* expression [7, 8]. More specifically, we have found that the sympathetic nervous system, through activation of β-adrenergic receptors, maintains GLUT4 content in glycolytic skeletal muscle during fasting [7]. Indeed, pharmacological β-adrenergic receptor blockade with propranolol significantly reduced skeletal muscle *Slc2a4* expression in fasted rats [7]. Further spontaneously hypertensive rats (SHR), which display peripheral sympathetic overactivity [9], showed increased *Slc2a4* mRNA levels in both soleus and EDL skeletal muscles during experimental diabetes [8]. β-adrenergic signaling can modulate the transcription of genes that contain a cAMP responsive element (CRE) binding site in their promoter region. Generally, this involves catecholamines binding to β-adrenergic receptor, adenylate cyclase activation, elevation in intracellular cAMP content, protein kinase A (PKA) activation and phosphorylation of transcriptional factors CREB (cyclic AMP-responsive element binding protein) and family members CREM (CRE modulator) and ATF1 (activating transcription factor 1) [10]. Upon phosphorylation, those transcriptional factors, which remain bound to the CRE sequence even in the inactive state, change their conformation and interact with cofactors such as CBP and p300, thus promoting gene transcription [11]. Full consensus CRE consists of a palindromic nucleotide sequence TGACGTCA, but partial CRE sequences (TGACG or CGTCA) and some variants have also been shown to be functionally targeted by CREB [10]. Importantly, CREB, CREM and ATF1, which can bind as homo- or heterodimers in the CRE sequence of target genes [12], have a distinct pattern of expression throughout the body. While CREB and ATF1 are ubiquitously expressed, CREM expression is more restricted to neuroendocrine tissues [13].

CREB transcriptional activity has been pointed out as an important regulator of metabolic processes in different tissues. In skeletal muscle, for example, expression of a dominant negative polypeptide A-CREB was shown to cause dystrophy and extensive fiber necrosis [14]. In hepatocytes, CREB was shown to stimulate a gluconeogenic program by binding to the promoter of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase genes during fasting, whereas, in pancreatic beta cells and adipocytes, it stimulates the IRS2 expression [10].

Considering the above-mentioned implication of α-adrenergic activity in the regulation of glucose homeostasis and GLUT4 content in skeletal muscle, we tested the hypothesis that CREB is a positive regulator of *Slc2a4* transcription by binding to a CRE sequence located in the *Slc2a4* promoter region.

Materials and Methods

Animals

Animal handling and procedures were approved by the Ethical Committee for Animal Research of the Institute of Biomedical Sciences, University of Sao Paulo (Protocol #059/2014). Male Wistar (W) and spontaneously hypertensive rats (SHR) from the Animal Facility of the Institute of Biomedical Sciences, University of Sao Paulo (Sao Paulo, Brazil), were kept on a chow diet (Nuvilab CR1, Nuvital Nutrients S/A, Parana, Brazil) and water ad libitum. Eight-week-old animals were killed by an overdose of sodium thiopental (60 mg/Kg, Cristália®, Itapira, São Paulo, Brazil) for tissue harvesting. Soleus and extensor digitorum longus (EDL) skeletal muscles were collected and processed for further analysis.

Cell culture

Rat L6 cells were obtained from ATCC (Rio de Janeiro Cell Bank) and grown as previously indicated [6]. After 80% confluence, cells were maintained in differentiation media for 7 days and used for experiments. Mouse 3T3-L1 preadipocytes obtained from ATCC (Rio de Janeiro Cell Bank) were grown and differentiated to adipocytes as described before [6]. Eight-day differentiated 3T3-L1 cells, treated with 1 mM of 8-br-cAMP (8-bromoadenosine 3', 5'cyclic monophosphate sodium salt, Sigma-Aldrich, St. Louis, MO, USA) for 2 h, or left untreated, were used for *Slc2a4* expression analysis. Those cells were also used for luciferase assay, as described below.

Real time-PCR analyses (qPCR)

Soleus and EDL skeletal muscles and 3T3-L1 cells were subjected to total RNA extraction using Trizol Reagent® (Invitrogen, Carlsbad, CA, USA). Approximately 2 µg of total RNA was reverse-transcribed using the kit ImProm II from Promega (Madison, WI, USA). The qPCR amplification was performed using Taqman® PCR master kit (Applied Biosystems Inc., Foster City, CA, USA) and carried out in the StepOne Plus Instrument (Applied Biosystems Inc.), as previously described [15]. Taqman® ID assays were AI5IQJM186914021_1, Rn00667869_m1, Mm01245502_m1 and Mm99999915_g1, respectively for rat *Slc2a4*, rat *Actb*, mouse *Slc2a4* and mouse *Gapdh*. The reference gene *Actb* was chosen for *Slc2a4* analysis in rat skeletal muscle and L6 cells, and the reference gene *Gapdh* for mouse *Slc2a4* analysis in 3T3L1 adipocytes, was based on the RefFinder software analysis. The method of $2^{-\Delta\Delta Ct}$ was used for mRNA quantification.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear protein extraction and EMSA from skeletal muscles and L6 cells were carried out as previously described [6, 16]. Briefly, double-stranded oligonucleotide containing the putative CRE sequence of the *Slc2a4* promoter gene was end-labeled with T4 polynucleotide kinase (Invitrogen, Carlsbad, CA, USA) and γ^{-32}P ATP (PerkinElmer Life, Waltham, MA, USA). Nuclear proteins were incubated with the labeled oligonucleotide probe in a buffer, for 20 min at room temperature. Competition binding experiments were performed under the same conditions, with the addition of 10-fold molar excess of native and mutant (Mut1 and Mut2) unlabeled oligonucleotides. The oligonucleotides spanned the -489/-467 sequence of the *Slc2a4* gene, and were described in the results. For antibody competition assay, nuclear proteins were incubated for 1.5 h with 10 µg of anti-CREB/CREM antibody (ab5803, Abcam, Cambridge, MA, USA), before adding the probe. DNA/protein complexes were electrophoresed on 4% non-denaturing polyacrylamide gel at 4°C. The gel was dried and exposed to a hyperfilm (Amersham Hyperfilm ECL, GE Healthcare Life Sciences) for 3 days at -80°C. The blots were analyzed by scanner densitometry (ImageScanner III, GE Healthcare, Sweden). Results were expressed as arbitrary units.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed as previously described [6]. Briefly, skeletal muscle was pulverized in buffer (PBS 1X) and incubated in formaldehyde for 10 min. The cross-link reaction was stopped with 0.125 M glycine for 5 min. Samples were then mixed with lysis buffer (1% SDS, EDTA 0.01 M, Tris-HCL 0.05 M, pH 8.1) for 2 h, and subjected to DNA fragmentation by sonication, to obtain fragments of 200-1000 bp. One hundred microliters of the fragmented samples were diluted in buffer (0.1% SDS, 1% Triton X-100, 0.1 M NaCl, 1 mM EDTA, 20 mM Tris, pH 8.0) and pre-cleared with protein-A-sepharose (GE Healthcare, Buckinghamshire, UK) saturated with salmon sperm DNA for 4 h. After that, an aliquot (10 µL) was collected and used as

"input", and the supernatant of the sample was incubated with 10 µg of anti-CREB/CREM antibody (ab5803, Abcam, Cambridge, MA, USA) or without antibody for 16 h. Therefore, 65 µL of protein-A-sepharose (GE Healthcare, Buckinghamshire, UK) saturated with salmon sperm DNA was added for 4 h. The pellet was washed (low salt, high salt, LiCl, TE buffers) and added with elution buffer (1% SDS, 0.1M $NaHCO_3$). The supernatant was treated with NaCl 5 M (to cross-link reversal), RNase and proteinase K. DNA isolation, including the input aliquot, was performed by phenol:chloroform:isoamylalcohol extraction and ethanol precipitation. Ten percent of total sample volume was subjected to DNA amplification, using Platinum™ SYBR™ Green qPCR SuperMix-UDG (ThermoFisher Scientific, CA, USA), and primers sequences (forward, CCAGACACGGTTCTCAGACA; and reverse: GAAACAATGCCCCGAAGTAA) spanning the -670/-461 segment. Results were analyzed by $2^{-(Ct\ Input\ -\ Ct\ IP)}$ x100, and the no antibody-treated sample value was subtracted to obtain the final data. To confirm the efficiency of immunoprecipitation, the PCR product from samples treated or not with anti-CREB/CREM antibody were electrophoresed on 1.5 ethidium bromide-agarose gel, to visualize the amount of the CREB/CREM bound DNA.

Table 1. Sequences of primers used to obtain the -975/-14 segment of the rat *Slc2a4* gene and to acquire the CRE-mutated sequences. In lowercase letters, the sites for restriction enzymes; in dashed line, the deleted nucleotides; and in bold underlined letters, the mutated nucleotides

Primer Name	Sequence
Forward	cgcacgcgtCTTGCACTTCTCTTGCGCTT
Reverse	cgaaagcttCCAAGGCTCTCCGGGATCTA
Mut3 F	AGCTAAAAATAGCCATTCCGGGT------GGGGCATTGTTT
Mut3 R	AAACAATGCCCC------ACCCGGAATGGCTATTTTTAGCT
Mut4 F	AAAAATAGCCATTCCGGG--------GGGCATTGTTTCTGACAC
Mut4 R	GTGTCAGAAACAATGCCC--------CCCGGAATGGCTATTTTT
Mut5 F	GCCATTCCGGG**CC**A**T**T**C**CGGGGCATTGTTTC
Mut5 R	GAAACAATGCCCCG**G**A**A**T**GG**CCCGGAATGGC

Luciferase assay

A fragment of 962 nucleotides, spanning the -975/-14 region of the rat *Slc2a4* gene promoter, was amplified by PCR with primers described in Table 1, and cloned in a directional manner using MluI and HindIII restriction sites upstream the luciferase gene in the pGL3-basic vector. Mutations in the *Slc2a4* promoter CRE site located at position -480/-473 were introduced using the primers described in Table 1 with the help of a mutagenesis kit (QuickChange XL Site-Directed Mutagenesis Kit, Agilent Technologies) following suppliers' protocol. The sequences of all constructs were confirmed by automated DNA sequencing. Plasmids were transformed into DH5α bacteria, which were grown in LB containing ampicillin. Bacteria were harvested by centrifugation and plasmids were purified by maxi-prep (QIAfilter Plasmid Maxi Kit, QIAGEN) following suppliers' recommendations.

Differentiated 3T3-L1 adipocytes were transiently transfected (12-well plates) with 0.75 µg of either an empty pGL3-basic (no promoter cloned in), or a wild type or a CRE-mutated *Slc2a4* promoter pGL3-basic, and 0.75 µg of the internal control renilla expressing pRL-CMV vector, using Lipofect-AMINE 3, 000 (ThermoFisher Scientific), according to the manufacturer's protocol. The transfection was carried out overnight (~16 hours), and then, the cells were maintained in differentiation media for 48 more hours. After that, cells were treated or with 8-br-cAMP or left untreated, as described above in cell cultures, and evaluated for luciferase activity using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA), following the instructions of the manufacturer. The results were standardized for renilla luciferase activity.

Data analysis

All values were reported as mean ± SEM. Two means were compared by unpaired two-tailed Student's t-test, and more than two means by one-way ANOVA, with Newman-Keuls posttest. Number of samples were supplied in the legends. The tests were performed using GraphPad Prism version 5.01 for Windows, GraphPad Software, San Diego, California, USA.

Results

High beta-adrenergic activity enhances Slc2a4 gene expression

Slc2a4 mRNA content was measured in skeletal muscle from SHR, a strain that features peripheral hyperactivity of the sympathetic nervous system [9, 17]. As depicted in Fig. 1A and 1B, SHR had higher *Slc2a4* mRNA content in soleus (~35%) and EDL (~80%) skeletal muscles (P<0.001), as compared to Wistar rats. Additionally, in order to confirm the sensitivity of *Slc2a4* gene expression to the intracellular α-adrenergic signaling, 3T3-L1 adipocytes were treated with 8-br-cAMP for 2 h. The *Slc2a4* mRNA increased by 30% (P<0.01), reinforcing a role of α-adrenergic signaling as an enhancer of *Slc2a4* gene expression.

The 5'flanking region of the rat Slc2a4 gene contains one putative CRE site

By in silico analysis, we searched the presence of CRE consensus or homologous sequences [18], using the genome browser Ensembl Release 89 (http://www.ensembl.org/index.html). The -480/-473 sequence of the rat *Slc2a4* gene (positions according to the transcriptional start site) was identified as highly homologous to the previously described CRE-binding sites (Table 2). This segment shows 62.5% of identity to CRE consensus, and 87.5% compared to the CRE described in the phosphoenolpyruvate carboxykinase 1 (*Pck1*) gene and is highly conserved among rat, mouse and human genes (Fig. 2).

Since half CRE sequences (TGACG or CGTCA) were also proposed to be functional in some target genes, we also searched for these sequences in the 5'-flanking region of the mouse *Slc2a4* gene. No half CRE sequences were found in the promoter region; however, a TGACG sequence is present downstream the ATG starting site, at position +30 to +34 of *Slc2a4* exon 1.

Skeletal muscle nuclear proteins bind selectively in the putative Slc2a4 CRE

To demonstrate CREB/CREM binding in this region of the *Slc2a4* promoter, we performed an EMSA using oligonucleotides (-489/-467) containing the putative wild type and mutated CRE sequences (Table 3). EMSA was performed with nuclear proteins from both rat skeletal muscle and L6 muscle cell (Fig. 3). We noticed the formation of 3 complexes of different sizes (lanes

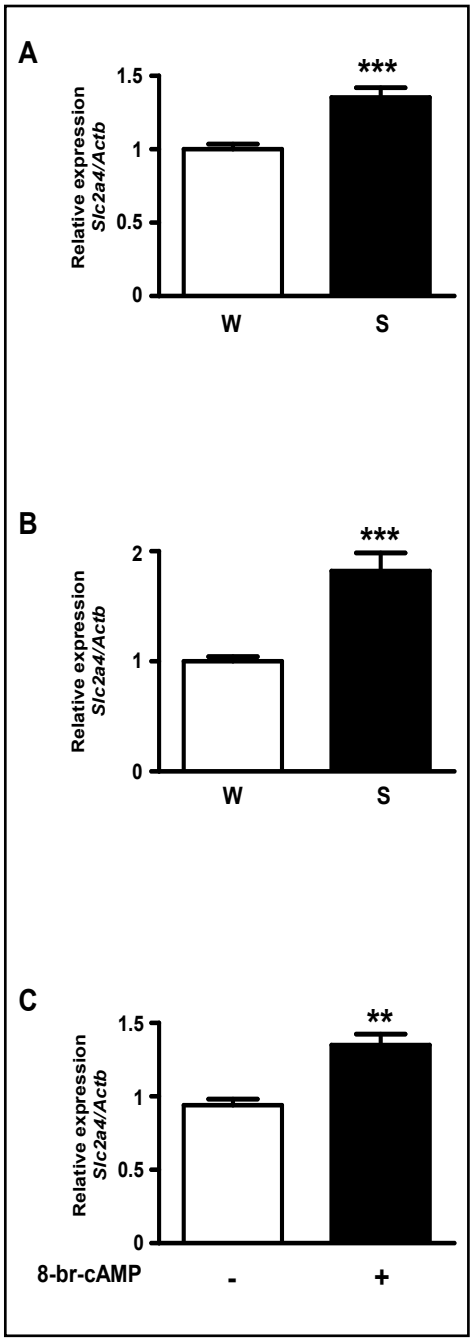

Fig. 1. High beta-adrenergic activity enhances *Slc2a4* gene expression. *Slc2a4* mRNA expression in soleus (A) and extensor digitorum longus (B) muscles of Wistar (W) and spontaneously hypertensive (S) rats, and in 3T3-L1 adipocytes after 2-h treatment with 8-br-cAMP (C). Data are mean ± SEM of 8 to 9 animals in (A) and (B), and of 5 different samples in (C). **P<0.01 and *** P<0.001, unpaired two-tailed Student`s t-test.

2, 5, 8 and 11). To show which complex depicts the specific binding into the putative CRE site, we performed a competition assay by adding an excess (10-fold molar) of unlabeled wild-type (lanes 3, 6, 9 and 12) or mutant oligonucleotides (lanes 4, 7, 10 and 13). The results show that addition of the unlabeled wild-type, but not unlabeled mutant oligonucleotides, markedly reduced the upper band (bigger DNA/protein complex) only, revealing that that band represents the putative CRE site of the *Slc2a4* promoter.

Skeletal muscle CREB/CREM nuclear proteins selectively bind the Slc2a4 CRE in vitro and in vivo

To confirm the presence of CREB/CREM in the specific DNA/protein complex, *in vitro*, we performed the EMSA analysis with anti-CREB+CREM antibody (Fig. 4A). The presence of the antibody in the reaction almost abolished upper band formation in both skeletal muscle (lanes 2 and 4) and rat L6 cells (lanes 6 and 8). To demonstrate the binding activity of CREB/CREM in the *Slc2a4* promoter *in vivo*, we performed a ChIP assay in skeletal muscle extracted DNA. The fragmented DNA was immunoprecipitated with anti-CREB/CREM antibody, or left untreated, and a CRE-containing segment was amplified by qPCR. PCR from sample immunoprecipitated with anti-CREB/CREM revealed a clear band in ethidium bromide gel, as compared to sample treated without antibody (Fig. 4B). Quantification by qPCR revealed a ~5-fold increase ($P<0.001$) in the *Slc2a4* segment amplification in samples immunoprecipitated with anti-CREB/CREM (Fig. 4C). Together, these results indicate the *in vivo* binding of CREB/CREM into the putative CRE sequence of the *Slc2a4* promoter.

Slc2a4 CRE site participates on the cAMP-stimulated transcriptional activity

To determine whether or not CREB/CREM binding into the CRE site affects the *Slc2a4* transcription, we cloned -975/-14 segments of the *Slc2a4* promoter, containing either a wild-type, or a partially deleted (6 nucleotides), or a completely deleted (8 nucleotides) or a mutated (4 nucleotides) CRE site (Table 4). These constructs were transiently transfected into 3T3-L1 cells, which were evaluated for luciferase activity after 2 h of stimulation with 8-br-cAMP. As depicted in Fig. 5, 3T3-L1 cells transfected wild-type CRE site displayed higher luciferase activity than those transfected with empty promoterless pGLBasic vector. Treatment with 8-br-cAMP significantly increased luciferase activity in cells transfected with the wild-type CRE (~70%), but not in cells transfected with the mutated CRE *Slc2a4* sequences. Taken together, these results support a previously unrecognized direct activation of *Slc2a4* transcription by transcription factors CREB/CREM under α-adrenergic stimulation.

Table 2. In silico analysis identified the -480/-473 sequence of rat *Slc2a4* gene as highly homologous to the consensus CRE and its variants. The CRE consensus sequence was identified in somatostatin (*Sst*), parathyroid hormone (*Pth*), intracesternal A particle-promoted polypeptide (*Ipp*) genes; CRE variant sequences were described in *Pck1*, phosphoenolpyruvate carboxykinase 1; *Fos*, fos proto-oncogene; *Penk*, proenkephalin; *Cxcl8*, c-x-c motif chemokine ligand 8 (former interleukin 8); *G6pc*, glucose-6-phosphatase catalytic subunit genes [18, 22, 23]

Genes	CRE sequence	% of identity
Sst, Pth, Ipp	TGACGTCA	100
Pck1	TTACGTCA	87.5
Fos	TGACGTAG	75.0
Penk	CTGCGTCA	62.5
Cxcl8	TGACATAA	75.0
G6pc (CRE1)	TTGCATCA	62.5
G6pc (CRE2)	TTACGTAA	75.0
Slc2a4	TTACTTCG	62.5

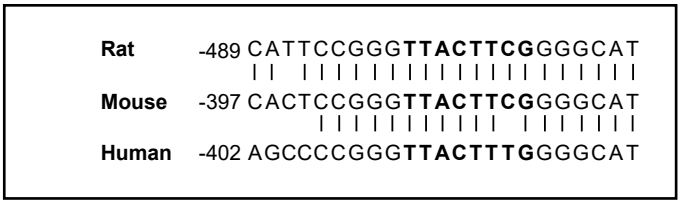

Fig. 2. Putative cAMP responsive element (CRE) in the *Slc2a4* gene. Conservation of the putative *Slc2a4* CRE sequence (in bold) in the *Slc2a4* gene of rat, mouse and human.

In vitro nuclear protein binding activity in the Slc2a4-CRE site increases in extensor digitorum longus (EDL) of spontaneously hypertensive rats (SHR)

To confirm that a classical situation of high sympathetic activity, as in SHR, stimulates the nuclear protein binding activity into the *Slc2a4* CRE site, *in vitro*, an EMSA analysis was performed. As depicted in Fig. 6, binding activity was increased (20%, P=0.0191) in EDL (Fig. 6A), but not in soleus (Fig. 6B) muscles of SHR.

In vivo CREB/CREM binding activity increases in extensor digitorum longus (EDL) of spontaneously hypertensive rats (SHR)

To confirm that nuclear protein binding activity in the *Slc2a4* CRE site also occurs *in vivo*, and involves CREB/CREM transcriptional factors, a ChIP assay was performed. In parallel to EMSA results, the ChIP assay revealed that the spontaneous *in vivo* CREB/CREM binding in the *Slc2a4* CRE site was increased (120%, P=0.0456) in EDL (Fig. 7A), but not in soleus (Fig. 7B) muscle of SHR.

Table 3. Description of wild type (WT), mutants 1 and 2 (Mut1 and Mut2) sequences of the -489/467 sequence of rat *Slc2a4* promoter gene. The putative CRE is underlined, and the lowercase letters indicate the altered nucleotides in the Mut1 and Mut2 oligonucleotides

Oligonucleotide	Rat -489/-467 *Slc2a4* gene sequence
WT	CATTCCGGGTTACTTCGGGGCAT
Mut1	CATTCCGGGccACTcCGGGGCAT
Mut2	CATTCCGGGTTcCTTtaGGGCAT

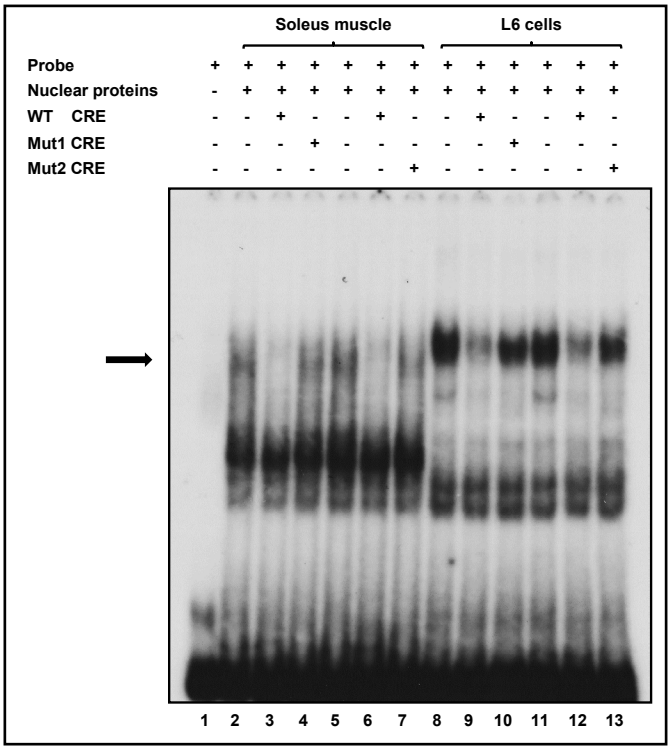

Fig. 3. Skeletal muscle nuclear proteins bind selectively in the putative *Slc2a4* CRE. Nuclear proteins extracted from rat soleus muscle and L6 muscle cells were subjected to electrophoretic mobility shift assay to determine the binding activity into the putative CRE site of the rat *Slc2a4* promoter. Nuclear protein extracts (20μg) were incubated with the radiolabeled wild type (WT) *Slc2a4* CRE containing domain (probe); specific binding competitions were performed using 10-fold molar excess of unlabeled WT CRE, mutant 1 CRE (Mut1) and mutant 2 CRE (Mut 2). Representative image of the EMSA; the arrow indicates the specific protein/DNA band.

Discussion

In this study, we investigated the presence of a functional cAMP responsive element (CRE) in the *Slc2a4* gene promoter, as well as whether α-adrenergic activity regulates or not *Slc2a4* gene transcription in a CREB/CREM-mediated way. Our main findings indicate that the -480/-473 region of the *Slc2a4* promoter is a CRE binding site, to which CREB/CREM proteins bind and enhance the *Slc2a4* transcriptional activity. Furthermore, we confirmed that this CRE site is involved in the increase of *Slc2a4* mRNA expression, acutely induced by a cAMP analog *in vitro*, and chronically induced in glycolytic skeletal muscle of rats displaying high sympathetic activity.

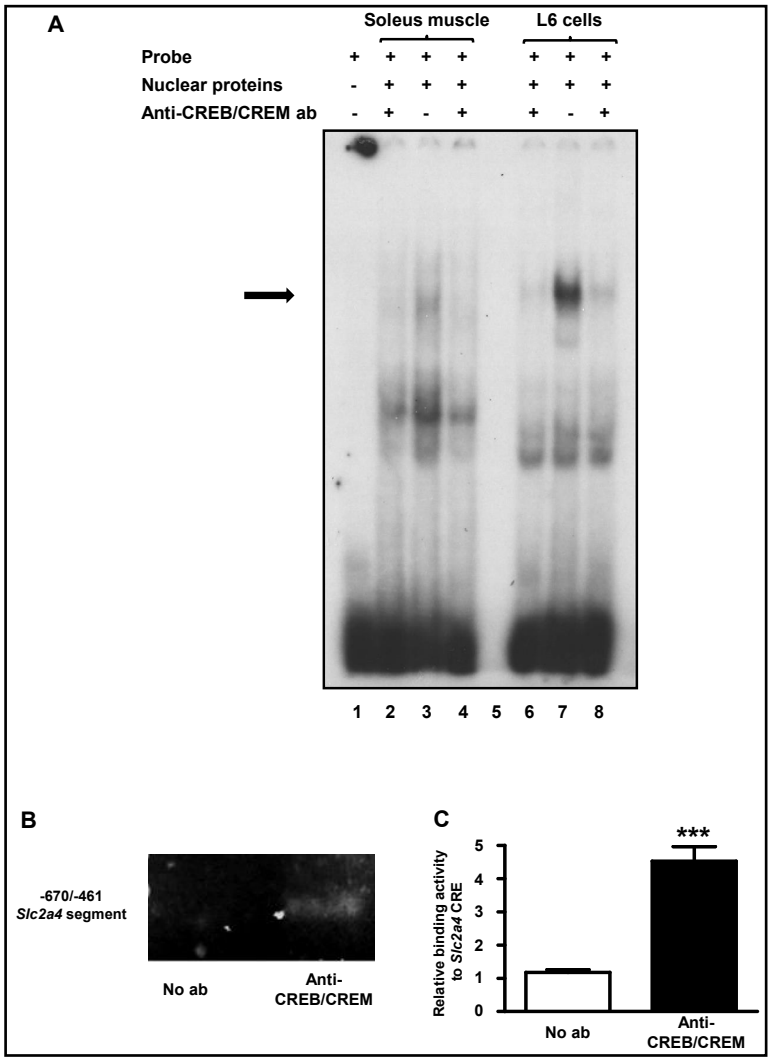

Fig. 4. Skeletal muscle CREB/CREM nuclear proteins bind selectively in the *Slc2a4* CRE *in vitro* and *in vivo*. *In vitro* binding activity (A) of nuclear proteins from rat soleus and L6 muscle cells was analyzed by electrophoretic mobility shift assay, using the wild type CRE binding site of the rat *Slc2a4* promoter as probe, and competitions were performed by adding 10μg of anti-CREB/CREM antibody (CREB, cAMP responsive element binding protein; CREM, CRE modulator protein). *In vivo* CREB/CREM binding activity (B and C) into the CRE binding site of the rat *Slc2a4* promoter gene was analyzed by chromatin immunoprecipitation (ChIP) assay. DNA was extracted from soleus muscle, fragmented, immunoprecipitated with anti-CREB/CREM antibody or not (No ab), and the -670/-461 sequence of *Slc2a4* gene was amplified by qPCR. To confirm the efficiency of immunoprecipitation, the PCR products were electrophoresed on ethidium bromide-agarose gel (B) or quantified in the qPCR (C). A and B, representative images; C, mean ± SEM of samples from 3 animals, treated or not with antibody. ***P<0.001, unpaired two-tailed Student`s t-test.

It is well-accepted that CREB and CREM are effective enhancers of most of their target genes [10], as we are now reporting for *Slc2a4* gene. Montminy and colleagues, through in silico analysis, have identified 10, 447 full CRE consensus sequences (TGACGTCA) in the human genome [19]. However, despite the observation that unmethylated functional CREB-binding sites can be found in ~5, 000 genes, exposure to cAMP stimulates the expression of only about 100 CREB target genes [10]. Nevertheless, some studies have already proposed a CREB/ATF3 repressor effect upon *Slc2a4* gene [20, 21], as it will be discussed below.

Table 4. Description of wild type (WT) and mutant (Mut3, Mut4 and Mut5) CRE domains of *Slc2a4* promoter transfected in 3T3-L1 adipocytes. Wild type (WT), 6 nucleotides deleted (Mut3), 8 nucleotides deleted (Mut4) and 4 nucleotides mutated (Mut5) CRE sequences (underlined) of the transfected *Slc2a4* (dashes indicate deleted nucleotides and lowercase letters indicate mutated nucleotides)

Primer name	CRE domains of *Slc2a4* promoter transfected for luciferase assay
WT	GGGTTACTTCGGGG
Mut3	GGGT - - - - - - GGGG
Mut4	GGG - - - - - - - - GGG
Mut5	GGG ccAtTcCG GGG

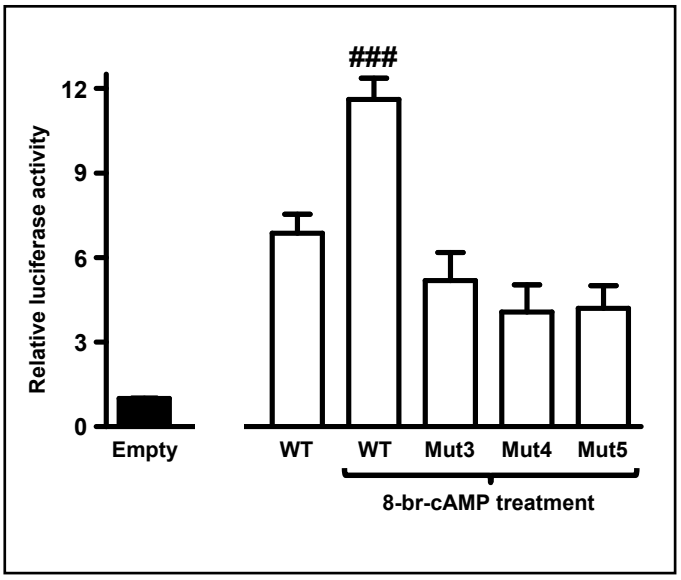

Fig. 5. *Slc2a4* CRE site participates in the cAMP-stimulate transcriptional activity. Luciferase activity of 3T3-L1 adipocytes transfected with *Slc2a4* promoter sequence containing WT, Mut3, Mut4 and Mut5 CRE domains, and stimulated with 8-br-cAPM for 2 h; basal transcriptional activity of cells transfected with the empty vector is shown on the left. Data are means ± SEM of 3 different experiments. ###P<0.001 vs all groups, one-way ANOVA, Student Newman Keuls post hoc test.

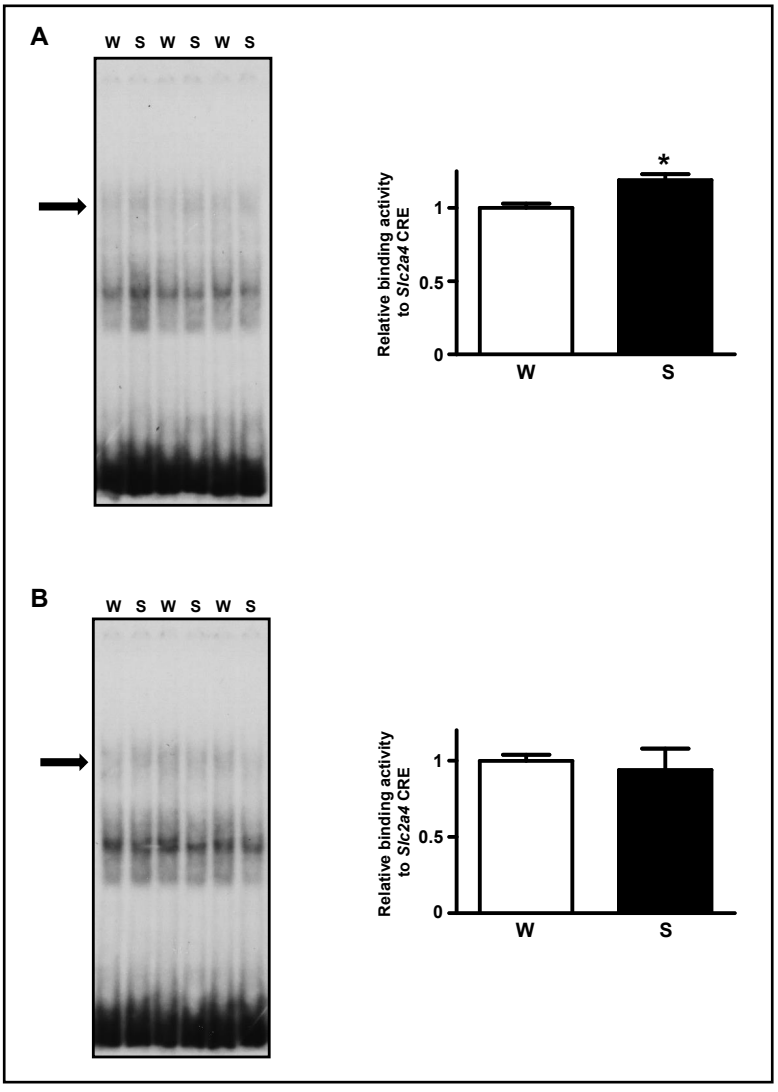

Fig. 6. *In vitro* nuclear protein binding activity into the *Slc2a4* CRE increases in extensor digitorum longus (EDL) of spontaneously hypertensive rats. Nuclear proteins from extensor digitorum longus (A) and soleus (B) muscles of Wistar (W) and spontaneously hypertensive (S) rats were subjected to electrophoretic mobility shift assay (EMSA), using a *Slc2a4*-CRE containing oligonucleotide as probe. In each panel, on the left, an EMSA image, on the right, quantification of the relative binding activity. Data are mean ± SEM of 3 animals. *P<0.05, unpaired two-tailed Student`s t-test.

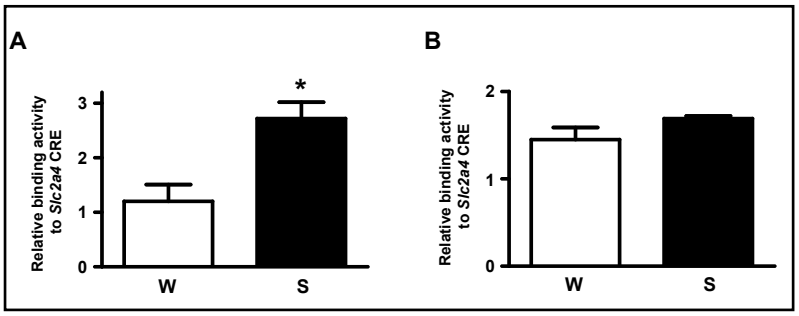

Fig. 7. *In vivo* CREB/CREM binding activity into the *Slc2a4* CRE increases in extensor digitorum longus (EDL) of spontaneously hypertensive rats. CREB/CREM binding activity into de *Slc2a4*-CRE binding site was evaluated by chromatin immunoprecipitation (ChIP) assay in EDL (A) and soleus (B) muscles from Wistar (W) and spontaneously hypertensive (S) rats. Muscle DNA was extracted, fragmented, immunoprecipitated with anti-CREB/CREM antibody or not, and the -670/-480 sequence of *Slc2a4* gene was amplified by qPCR. Results of antibody-treated samples were subtracted for calculation of the final relative binding activity. Data are mean ± SEM of 3 animals. *P<0.05, unpaired two-tailed Student`s t-test.

Although *Slc2a4* CRE-binding sites have already been proposed, their sequence, location and functional activity have never been extensively investigated. The CRE consensus sequence TGACGTCA is not present in the *Slc2a4* promoter. However, we identified and investigated a putative variant of CRE (TTACTTCG), located at the -480/-473 segment of the rat *Slc2a4* gene promoter. This non-canonical CRE sequence displays 62.5% homology with the consensus CRE sequence, and is well-conserved among species such as rat, mouse and human.

Other CRE variant sequences have been reported as functional, most of them showing 62.5 to 87.5% homology to the consensus sequence [18, 22, 23]. Regarding the *Slc2a4* gene, a putative CRE variant sequence (TTAAGTCA) was proposed, by in silico analysis, in the *Slc2a4* gene of the teleost fish Fugu [24]; however, this sequence has never been investigated either as target for CREB/CREM proteins, as sensitive to cAMP, or as a regulator of *Slc2a4* transcription. Furthermore, an allusion to the presence of an ATF3 binding site, between -555/-508 region of the *Slc2a4* gene, was made [20]; however, once more there was no demonstration that this domain binds the ATF3 or displays *Slc2a4* transcriptional regulation. In fact, there is neither CRE consensus sequence nor highly homologous variant sequence in that segment referred to.

Half CRE sequences (TGACG or CGTCA) have also been proposed as potential sites for CREB/CREM regulation of target genes [10], but in silico analysis of rat *Slc2a4* promoter did not reveal any half CRE. That highlights that every CREB/CREM-mediated regulation of *Slc2a4* gene must be related to the CRE site we are reporting.

We started the characterization of that putative CRE-binding site by EMSA. We found that nuclear proteins from soleus muscle tissue or L6 muscle cells specifically bind an oligonucleotide containing the *Slc2a4*-CRE *in vitro*. Three DNA/protein complexes were observed, and by using oligonucleotides carrying mutations in the core of the putative CRE (TTAAGTCA), it was observed that the heavier complex must be related to CREB/CREM binding. Indeed, the addition of anti CREB/CREM-antibody drastically decreased the intensity of this upper band, in both muscle tissue and cells, proving that this complex contains CREB/CREM proteins.

The classic result of antibody competition in EMSA is the supershift band, for which the antibody binds to the transcriptional factor, without interfering with the protein/DNA binding, thus increasing the molecular weight of the complex. However, it has become more evident that the addition of the antibody in the reaction can compete with the DNA, shifting the binding, and thus weakening the band. This type of result has already been observed for other transcriptional factors that regulate the *Slc2a4* gene such as the myocyte enhancer factor 2D (MEF2D), hypoxia inducible factor 1A (HIF1A) and nuclear factors kappa B p50 and p65 [6, 25].

Subsequently, to confirm that CREB and CREM bind the *Slc2a4* promoter *in vivo*, we performed a ChIP assay in soleus muscle samples. By immunoprecipitating CREB/CREM cross-linked to the chromatin, we confirmed that these transcriptional factors bind endogenously to the *Slc2a4* CRE site. Increased forskolin-induced content of *Slc2a4* promoter bound to ATF3, analyzed by ChIP assay, was observed in HEK393T cells [20]. However, except for one ethidium bromide stained image, no additional information is provided to the reader concerning this result and its respective methodological details [20].

Finally, for functional characterization of the *Slc2a4* gene CRE site, luciferase activity was evaluated in the target cell 3T3L1 adipocyte, transfected with the -975/-14 *Slc2a4* segment, mutated at the CRE site. Two-hour stimulation with 8-br-cAMP increased the luciferase activity of the wild type promoter; however, all CRE-mutated promoters did not respond to the cAMP analogue. Those results clearly show a rapid CRE-mediated enhancer effect on *Slc2a4* gene transcription, pointing out that the effect observed was induced by the native cellular mediators of cAMP activity, and in a classical target cell such as the adipocyte.

Interestingly, the CRE site we characterized is located in the -500/-400 *Slc2a4* promoter region which includes the binding sites of several other enchancers such as MEF2, MyoD, TRα1, LXR [5, 26, 27], and that makes probable cooperative interactions among these transcription factors. Particularly, the TRE and LXRE sites [5, 26] overlap the CRE site we are characterizing, and competitivity and/or cooperativity among these transcription factors deserves future studies.

Curiously, opposing regulation of CREB/ATF3 upon *Slc2a4* gene transcription (inhibition) has already been proposed. However, this suggestion was based mainly on GLUT4 protein increase in adipose tissue of a high-fat-fed transgenic mice expressing a dominant negative CREB inhibitor protein (ACREB) in adipose tissue, or in ATF3$^{-/-}$ mice [20]. In those transgenic animals, several intermediary modulators of *Slc2a4* expression can be involved; and thus, these observations should be carefully analyzed, before the proposal of a direct CRE-mediated effect. Unexpectedly, the ACREB protein-induced increase in GLUT4 was shown only in high fat-fed mice [20], a condition that classically is known to repress GLUT4 expression [28, 29]. Additionally, this study [20] clearly demonstrated that in ACREB mice the high fat-induced inflammatory activity in adipose tissue was substantially reduced, a condition well-known to enhance *Slc2a4* gene expression, since the inflammatory mediator nuclear factor kappa B is a powerful inhibitor of the *Slc2a4* gene transcription [6]. Furthermore, transfected HEK293T cells, supposedly overexpressing A-CREB or ATF3 and a *Slc2a4*-luciferase reporter, subjected to 4-hour treatment with forskolin, were also used to propose the CRE-mediated repressor effect [20], but that could be an indirect effect.

Another attempt to demonstrate an ATF3/CRE-mediated repressor effect upon *Slc2a4* expression was conducted in 3T3-L1 adipocytes, in which overexpression or silencing of the inducible cAMP early repressor (ICER) was accompanied by increased and decreased *Slc2a4* mRNA expression [21], respectively. However, in that study, no investigation concerning a direct ATF3/CRE-mediated repressor effect upon *Slc2a4* transcription was conducted, and conclusions were based on the indirect ICER-mediated changes in intracellular cAMP content.

Considering the discussion above, reports of CREB/ATF3-CRE-mediated repressor effect upon *Slc2a4* expression could be an indirect effect triggered in highly manipulated biological systems, instead of a direct CRE-mediated regulation of *Slc2a4* gene, as we are reporting here in wild target cell.

Over the years, we have gathered evidence indicating that the sympathetic nervous system, by regulating *Slc2a4* expression, plays an important role in controlling GLUT4 protein content in skeletal muscle. Indeed, we have shown that the sympathetic nervous system, through the activation of β-adrenergic receptors, stimulates *Slc2a4* mRNA expression in glycolytic and oxidative skeletal muscles during fasting [7], and that the spontaneously hypertensive rat (SHR), which displays peripheral sympathetic overactivity [9], also shows increased expression of *Slc2a4* in muscle [8]. In the present study, by RT-qPCR, we confirmed that SHR have increased *Slc2a4* expression in both oxidative and glycolytic muscles. Besides,

the participation of the cAMP in this regulation was reinforced by the demonstration that 3T3-L1 cells treated with 8-br-cAMP for 2 hours increase the *Slc2a4* mRNA expression. These results demonstrate that high sympathetic activity increases *Slc2a4* expression, evincing this regulation as a cAMP-mediated effect.

Curiously, in contrast to our results, it has been previously reported that prolonged *in vitro* treatments (24 to 48 h) of adipose or muscle cells with cAMP inhibit rather than enhancing the *Slc2a4* expression [30, 31]. However, experimental conditions of quantitative analysis of mRNA expression were quite inaccurate in this era, results were restricted to 3 samples only, without endogenous control, and none of those studies investigated whether this response was due to a direct action of CREB upon a CRE site in the *Slc2a4* gene. Indeed, long-term treatment of 3T3-L1 adipocytes with 8-br-cAMP was reported to repress the *Slc2a4* gene expression by a mechanism involving the nuclear factor 1B (NF1B), which binds into the -706/-676 5'-flanking region of the murine *Slc2a4* gene [32]; a region which does not match the CRE region we are reporting now. So, we propose that the repressor effect on *Slc2a4* expression described earlier, and apparently related to cAMP, does not represent a direct CREB/CREM-mediated regulation in a CRE site; but a specific *in vitro* effect observed in response to long-term treatment with 8-br-cAMP, involving NF1B activation.

In order to demonstrate that high sympathetic activity enhances *Slc2a4* expression in skeletal muscles of SHR by a CRE-mediated mechanism we measured the CREB/CREM binding activity in the *Slc2a4* promoter. In EDL muscle of SHR, a higher CREB/CREM binding activity in the *Slc2a4* CRE was observed, both *in vitro* (EMSA) and *in vivo* (ChIP), and this definitively reveals the participation of CRE activity in the *Slc2a4* gene expression observed under this condition. However, increased *Slc2a4* mRNA expression was also detected in soleus muscle of SHR, despite the fact that CRE binding activity was unaltered, suggesting that the CREB/CREM regulation of *Slc2a4* gene in this muscle is not as important as in the EDL muscle.

Comparing soleus and EDL, distinct regulations of *Slc2a4* expression have been described under conditions in which sympathetic activity is altered such as fasting [7], diabetic SHR [8] and insulin-treated diabetic Wistar rats [17]. Glycolytic muscles, such as the EDL, obtain energy primarily by glycolysis, using glucose as the main fuel, whereas oxidative muscles, such as soleus, have high capacity to also use fatty acids to generate ATP, reducing their need for glucose [33, 34]. It is reasonable to understand that after prolonged fasting, GLUT4 expression is decreased in adipose tissue and red muscle, contributing to maintaining glycemia; but it is preserved in white muscle [35]. Maintenance of GLUT4 expression in glycolytic muscles during fasting was described as related to the beta-adrenergic activity [7]. This regulation highlights the relevance of GLUT4 to guarantee glucose-dependent glycolytic activity of fast-twitch fibers, which is fundamental to "fight-or-flight" responses controlled by the SNS, and likely played an important role in the evolution of humans. Regarding that, it is noticible that in evolutionary terms, the glycolytic fiber seems to appear after the oxidative fibers, as it does in the vertebrate's ontology, and this is a process related to motoneuron control [36].

In addition, although oxidative muscle displays a greater *in vivo* sensitivity to catecholamines [37], the effect of beta-adrenergic antagonists is higher in glycolytic fiber [38, 39], reinforcing that CREB/CREM-mediated regulation of *Slc2a4* may be most important in glycolytic fibers. However, although CREB/CREM binding into *Slc2a4* is unaltered in soleus muscle, we cannot discard CREB/CREM-induced indirect regulation of the *Slc2a4* gene. For instance, CREB/CREM was reported to increase the transcriptional activity of the myocyte enhancer factor 2 (MEF2) [14], which is a powerful enhancer of *Slc2a4* gene expression in soleus muscle [5, 16, 25].

We might also analyze the possibility of some CRE-mediated alpha-adrenergic regulation of *Slc2a4* expression, since alpha 2-AR is a Gi-protein-coupled receptor, and it decreases cAMP generation. However, it is known that beta1/2/3-adrenergic activities are predominant in muscle and adipose cells [40, 41], and some alpha-adrenergic effects may

occur in muscle and adipose tissues just by altering blood flows, since alpha-ARs are highly expressed in the arteries [42].

Conclusion

This study demonstrates the previously unrecognized presence of a CRE site TTACTTCG at the -480/-473 region of the *Slc2a4* promoter gene. This CRE site is functional, displaying an enhancer role upon *Slc2a4* gene transcription. This study demonstrates that the high sympathetic activity found in glycolytic muscle of SHR increases *Slc2a4* transcription, and this regulation is associated with increased CREB/CREM binding into the *Slc2a4* CRE site measured both under *in vitro* and *in vivo* conditions. Together, these results highlight the important role of cAMP-mediated increase in *Slc2a4* expression in glycolytic muscles, which is fundamental to ensure metabolic support during a contractile response in stressful situations, such as hunting or escaping.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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