Biochemical and Biophysical Research Communications 446 (2014) 614–619

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Activation of nuclear receptor NR5A2 increases Glut4 expression and glucose metabolism in muscle cells

A. Bolado-Carrancio [a], J.A. Riancho [b], J. Sainz [c], J.C. Rodríguez-Rey [a,*]

[a] Department of Molecular Biology, University of Cantabria, IDIVAL, Santander, Spain
[b] Department of Internal Medicine, Hospital U.M. Valdecilla-IDIVAL, University of Cantabria, RETICEF, Santander, Spain
[c] Institute of Biomedicine and Biotechnology of Cantabria (IBBTEC), CSIC-University of Cantabria, Santander, Spain

ARTICLE INFO

Article history:
Received 26 February 2014
Available online 12 March 2014

Keywords:
NR5A2
C2C12
GLUT4
Glucose metabolism
Muscle

ABSTRACT

NR5A2 is a nuclear receptor which regulates the expression of genes involved in cholesterol metabolism, pluripotency maintenance and cell differentiation. It has been recently shown that DLPC, a NR5A2 ligand, prevents liver steatosis and improves insulin sensitivity in mouse models of insulin resistance, an effect that has been associated with changes in glucose and fatty acids metabolism in liver. Because skeletal muscle is a major tissue in clearing glucose from blood, we studied the effect of the activation of NR5A2 on muscle metabolism by using cultures of C2C12, a mouse-derived cell line widely used as a model of skeletal muscle. Treatment of C2C12 with DLPC resulted in increased levels of expression of GLUT4 and also of several genes related to glycolysis and glycogen metabolism. These changes were accompanied by an increased glucose uptake. In addition, the activation of NR5A2 produced a reduction in the oxidation of fatty acids, an effect which disappeared in low-glucose conditions. Our results suggest that NR5A2, mostly by enhancing glucose uptake, switches muscle cells into a state of glucose preference. The increased use of glucose by muscle might constitute another mechanism by which NR5A2 improves blood glucose levels and restores insulin sensitivity.

1. Introduction

NR5A2, also known as liver receptor homolog 1 (LRH-1), is a nuclear receptor belonging to the NR5A/Ftz-F1 subfamily. NR5A2 was initially described as an orphan receptor, but structural and functional studies have shown that dilauroyl phosphatidylcholine (DLPC) and several other natural phospholipids can bind to this receptor, and open the door to the existence of other natural ligands [1,2]. In adult mammals, *NR5A2* is predominantly expressed in endoderm-derived tissues, such as liver, pancreas, and intestine [3,4]. It has been also found in adrenal glands and preadipocytes, where it enhances the transcription of genes coding for enzymes of steroid metabolism [5,6]. Besides, NR5A2 regulates the expression of genes coding for proteins involved in reverse cholesterol transport, such as apolipoprotein AI [7], the scavenger receptor class B type I [8], and CETP [9] and of genes coding for proteins involved in bile acid homeostasis, including CYP7A1 and CYP8B1 [10–12]. Recent results have revealed the importance of NR5A2 in glucose and fatty acid metabolism. NR5A2 binds to Glucokinase gene promoter and induces its expression [13]. Moreover, the activation of NR5A2 with DLPC results in a decreased SREBP-1c expression in mouse liver and in a reduction of hepatic steatosis in two mouse models of insulin resistance. In these models, DLPC treatment also improves blood glucose levels [2].

In the insulin-resistant state, liver shows an impaired response to insulin and it is unable to block gluconeogenesis [14]. The effect is mostly due to the increment of the deposits of fat within liver cells. The activation of NR5A2 by DLPC inhibits liver SREBP-1c, thus decreasing fatty acid synthesis and liver fat accumulation. This ef-

Abbreviations: NR5A2, nuclear receptor subfamily 5, group A, member; GLUT4, glucose transporter 4; *Acc2*, acetyl-CoA carboxylase 2; DLPC, dilauroyl phosphatidylcholine; CEPT, Cholesteryl ester transfer protein; CYP7A1, cholesterol 7 alpha-hydroxylase; CYP8B1, sterol 12-alpha-hydroxylase; T2DM, type 2 diabetes mellitus; DMEM, Dulbecco's modified Eagle's medium; *Myog*, myogenin; *MyoD*, myogenic differentiation 1; *Hk2*, hexokinase 2; *Pkfm*, muscle phosphofructokinase; *PfkFb1*, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1; *Gys1*, muscle glycogen synthase; *Pygm*, muscle glycogen phosphorylase; *Mef2c*, myocyte-specific enhancer factor 2C; *Pgc1α*, peroxisome proliferator activated receptor gamma coactivator 1 alpha; *Pparα*, peroxisome proliferator activated receptor alpha; *Cpt1b*, muscular carnitine palmitoyltransferase 1; *Vldlr*, very low density lipoprotein receptor; *Lpl*, lipoprotein lipase; *Fabp3*, muscular fatty acid binding protein; S.D., standard deviation.

* Corresponding author. Address: Department of Molecular Biology, Avda Cardenal H Oria s/n, 39011 Santander, Spain. Fax: +34 942201945.
E-mail address: rodriguj@unican.es (J.C. Rodríguez-Rey).

http://dx.doi.org/10.1016/j.bbrc.2014.03.010
0006-291X/

fect of DLPC provides an explanation for the improvement of insulin resistance in the liver of DLPC-treated mice.

Skeletal muscle is another major tissue involved in insulin sensitivity, being responsible for about 80% of the insulin-induced glucose removal from blood. Accordingly, the impairment of blood glucose removal has been associated with T2DM [15,16]. On the other hand exercise, which increases the consumption of glucose by skeletal muscle, improves insulin resistance [17]. Insulin induces the entry of glucose into skeletal muscle cells through the translocation of the muscle glucose transporter, GLUT4, to the membrane. Glucose entry in skeletal muscle cells is limited by the number of GLUT4 receptors on the cell surface [18]. Consequently, an altered expression of GLUT4 has been associated with insulin resistance and diabetes [19,20].

The study of the effect of NR5A2 activation on metabolism has been mostly focused on liver [2,13]. Because of the importance of skeletal muscle in the development of insulin resistance we investigated the role of NR5A2 in muscle metabolism. The role of NR5A2 in skeletal muscle had been only studied in *Zebrafish*, where the NR5A2 orthologous gene *ff1a* is involved in muscle differentiation and organization [21]. For this study we used C2C12, a mesoderm cell line often used as a model of skeletal muscle. The results presented here indicate that NR5A2 plays a major role in skeletal muscle glucose metabolism.

2. Materials and methods

2.1. Products

Dilauroyl phosphatidylcholine (DPLC) was purchased from Sigma Aldrich (St Louis, MO, USA) and used at 100 μM, as described in [2].

2.2. Cell cultures

C2C12 cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 50 u/ml penicillin, and 50 μg/ml streptomycin in a 5% CO_2 atmosphere at 37 °C. When the cells reached 80–90% confluence, the growth medium was changed to differentiation medium (Dulbecco's modified Eagle's medium, supplemented with 2% horse serum, 50 u/ml penicillin, and 50 μg/ml streptomycin), until the cells were fully differentiated.

2.3. RNA preparation and qPCR analysis

RNA samples were prepared with TRIzol® (Life technologies, Carlsbad, California, USA) according to the manufacturer's instruction. RNA quality was checked by electrophoreses in 1% TAE formaldehyde/agarose gel. Total RNA (1 μg) was used for cDNA synthesis (cDNA synthesis kit, Bio-Rad Laboratories Inc, Hercules, CA, USA). The primers used for qPCR are listed in the Supplementary Table 1. qPCRs were performed in the qPCR thermal cycler Bio-Rad IQ5® analysis system.

2.4. Western blot experiments

80 μg of proteins extracts, in 2× Laemmli buffer, were separated in a 10% SDS–PAGE and transferred to a nitrocellulose membrane in 25 mM Tris, 192 mM glycine, and 20% methanol using a Trans Blot® SD semi dry transfer cell (BioRad), for 20 min at 15 V. Following transfer, the membrane was blocked in 3% nonfat milk for 30 min. at room temperature. Membranes were then incubated for 1 h with primary and secondary antibodies at a concentration of 1:500 (*Glut4*), 1:200 (Glut1) or 1:1000 (Actin) respectively in TTBS, and supplemented with BSA 1% (p/v). Blots were developed with secondary antibodies conjugated to IRDye680 or IRDye800 (Li-Cor Biosciences) at 1:10,000 and visualized in an Odyssey scanner. The primary antibodies were anti-GLUT4 (N-20), anti-GLUT1 (H-43) and anti-ACTIN (I-19) from Santa Cruz Biotech.

2.5. Growth curves and glucose-consumption assays

Glucose consumption assays were performed as described [22]. Glucose concentration was determined in the culture medium at several time points up to 24 h. Cell cultures were trypsinized, cells counted and the glucose consumption calculated as micrograms of glucose uptaked by 10^4 cells.

2.6. Fatty acid oxidation assays

[1-14C]-Oleyl-CoA (Perkin Elmer, Waltham, Massachusetts, USA) was used to determine fatty acid oxidation rate. Experiments were performed as described [23]. Total protein was estimated to normalize samples. Fatty acid oxidation rates were expressed as the CPM of the precipitated $^{14}CO_2$ per microgram of protein.

2.7. Glucose oxidation assays

[6-14C]-Glucose (Perkin Elmer, Waltham, Massachusetts, USA) was used to determine the glucose oxydation rate. C2C12 myotubes were treated with 0.1 μCi/ml and incubated for 2 h. Glucose oxidation rates were determined by measuring the release of $^{14}CO_2$ using the same protocol as in [23]. Glucose oxidation was expressed as CPM of the precipitated $^{14}CO_2$ per microgram of protein.

2.8. Glycogen synthesis assays

[6-14C]-Glucose was used to determine the glycogen synthesis rate. C2C12 myotubes were treated with 0.1 μCi/ml and incubated for 2 h. Glycogen was extracted as described in [24]. Pellets were dissolved in 1 ml of water and radioactivity was measured in a scintillation counter. Total protein was used to normalize samples. Glycogen synthesis was measured as CPM per microgram of protein.

2.9. Electroforetic mobility assays (EMSA)

For the EMSA experiments were used double strand oligonucleotides with the putative binding site for NR5A2 or mutated (AGGCTGGCCTTGGGGTTAGAG and AGGCTGGACACGGGGTTAGAG respectively), labelled in 5′ with IRDye680®. Nuclear extracts from C2C12 were extracted as described in [25]. EMSAs were carried out as described in [26]. The anti-NR5A2 antibody used for the supershift experiments was anti-NR5A2 (H-75), from Santa Cruz Biotech.

3. Results and discussion

One of the characteristics of insulin resistance is the inability of insulin to suppress the expression of gluconeogenesis genes [14]. It has been recently reported that the treatment of mouse models of insulin resistance with DLPC improves insulin sensitivity. Accordingly, DLPC-treated insulin-resistant mice show a lower expression of *PEPCK* and less hepatic glucose production [2]. Insulin resistance also encompasses a defect in insulin-dependent uptake into muscle [27], but the effect of DLPC treatment on glucose uptake by muscle cells had not been studied.

To address the effect of NR5A2 activation on glucose uptake by muscle we used a mouse cell line, C2C12, able to differentiate into myotubes and which has been previously used for the study of glucose transport by the insulin-dependent transporter [28].