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

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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.

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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].

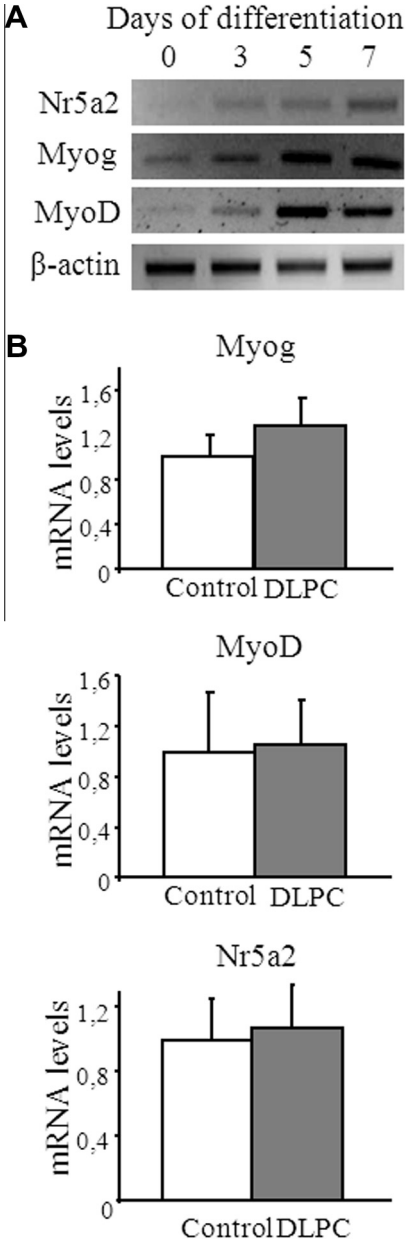

Fig. 1. nr5a2 expression during C2C12 cells differentiation into myotubes. (A) Kinetics of *Nr5a2*, *Myog* and *MyoD* mRNA expression during C2C12 differentiation into myotubes, from undifferentiated (0 days) to different points of the differentiation process (3, 5 and 7 days), β-actin was used as control of loading. (B) Expression analysis of *Nr5a2*, *Myog* and *MyoD* mRNA in myotubes treated for 24 h with either vehicle (Control) or 100 μM DLPC (DLPC). Bars are mean ± S.D. of 4 independent experiments. $^{*}p \leqslant 0.05$; $^{***}p \leqslant 0.,005$.

In Zebrafish, the only system in which the role of the NR5A2-ortologous gene, *ff1a* has been studied in muscle, *ff1a* expression increases during myotube differentiation [21]. To see if a similar pattern is present in C2C12 cells, the expression of NR5A2 was studied during the differentiation process. The mRNA levels of *Myog* or *MyoD*, two myotube differentiation markers, were used as controls of the differentiation process. As shown in Fig. 1A NR5A2 mRNA is almost undetectable in the first day of the differentiation protocol, and its levels grew until they reached a peak at day 7, when the cells are fully differentiated into myotubes. Therefore, all the next series of experiments were done with the cultures in this fully differentiation stage.

Also, to discard any further effect of DLPC treatment on the stage of differentiation of C2C12, cultures of fully differentiated C2C12 cells were treated with 100 μM DLPC. As shown in Fig. 1B, DLPC did not change the mRNA levels of either C2C12 differentiation markers or NR5A2, which is in accordance with previously published results by others for mature liver [2].

3.1. DLPC increases glucose uptake and GLUT4, but not GLUT1 levels

To see whether DLPC treatment was able to alter the ability of muscle cells to remove glucose, DLPC was added, in the concentrations described above, to fully differentiated myotubes. The measure of glucose concentrations at 12 and 24 h indicated that DLPC treated cells had an increased ability to remove glucose from the medium (1.6-fold; Fig. 2A).

Although GLUT4 is their major glucose transporter, differentiated C2C12 myotubes also express significant amounts of GLUT1, another member of the Glut family (lane 7 in Fig. 2B). In order to investigate the contribution of each transporter to the increased entry of glucose, the effect of DLPC on their expression was studied both at the mRNA and the protein levels. DLPC induced a 4- and 2-fold increase in *Glut4* mRNA and protein respectively, but it did not change either *Glut1* mRNA or protein levels (Fig. 2C). When undifferentiated cells, which expressed very low levels of both *Nr5a2* and *Glut4* mRNA, were treated with DLPC they did not show increased levels of *Glut4* (Fig. 2D). This result would be in agreement with a NR5A2-dependent induction of *Glut4* by DLPC.

The analysis of the sequence of a 2 kb sequence of the *Glut4* promoter revealed a putative NR5A2 binding site located at 1750 bp upstream the transcription start site. However, supershift experiments carried out with a fluorescence-labeled oligonucleotide spanning the putative binding regions and a specific anti-NR5A2 antibody, did not show the presence of NR5A2 in the binding complex (not shown). Thus, the possibility of an indirect regulation of *Glut4* was explored. Three genes coding for transcription factors (MEF2C, PGC1α and PPARα) known to regulate *Glut4* expression in muscle were induced by NR5A2 in 2, 2.5 and 3.6-fold respectively (Fig. 2E). MEF2C and PGC1α have been previously associated with the increase of GLUT4 expression [29]. Less clear is the role of PPARα in *Glut4* expression, as in some rodent models PPARα overexpression/activation reduces glucose uptake by repression of *Glut4* expression [30], whereas in C2C12 myotubes, PPARα, in cooperation with GATA6, induces *Glut4* expression and glucose metabolism [31]. Although a regulation via direct binding of NR5A2 to another site within *Glut4* regulatory regions cannot be totally excluded, these results suggest an indirect regulation of *Glut4* by DLPC.

3.2. Activation of NR5A2 increased the expression of several genes of glucose metabolism

In liver cells it has been reported that NR5A2 is a direct regulator of glucokinase expression. Phosphorylated glucose produced by Glucokinase contributes to the increase in glycogen synthesis [13]. In order to further investigate the effect of DLPC treatment in glucose metabolism, the expression of several major genes of glucose metabolism was analyzed in DLPC-treated C2C12 cells. The mRNA levels of *Hk2*, *Pfkm* and *Pfkfb1*, coding for muscle-specific isoform of hexokinase, the muscle phosphofructokinase and 6-phosphofructo-2-kinase respectively, were increased approximately twofold as a result of the DLPC treatment. Twofold increases in the expression of both *Gys1* and *Pygm*, coding for the muscular glycogen synthase, and glycogen phosphorylase respectively were also found (Fig. 3A).

The study of mice with muscle overexpression of GLUT4 and/or HK2 showed that GLUT4, and not HK2, levels limit muscle glucose uptake [18]. Glucose-6-phospate produced by hexoquinase cannot leave the cell and it is directed to either glycolysis or glycogen. In

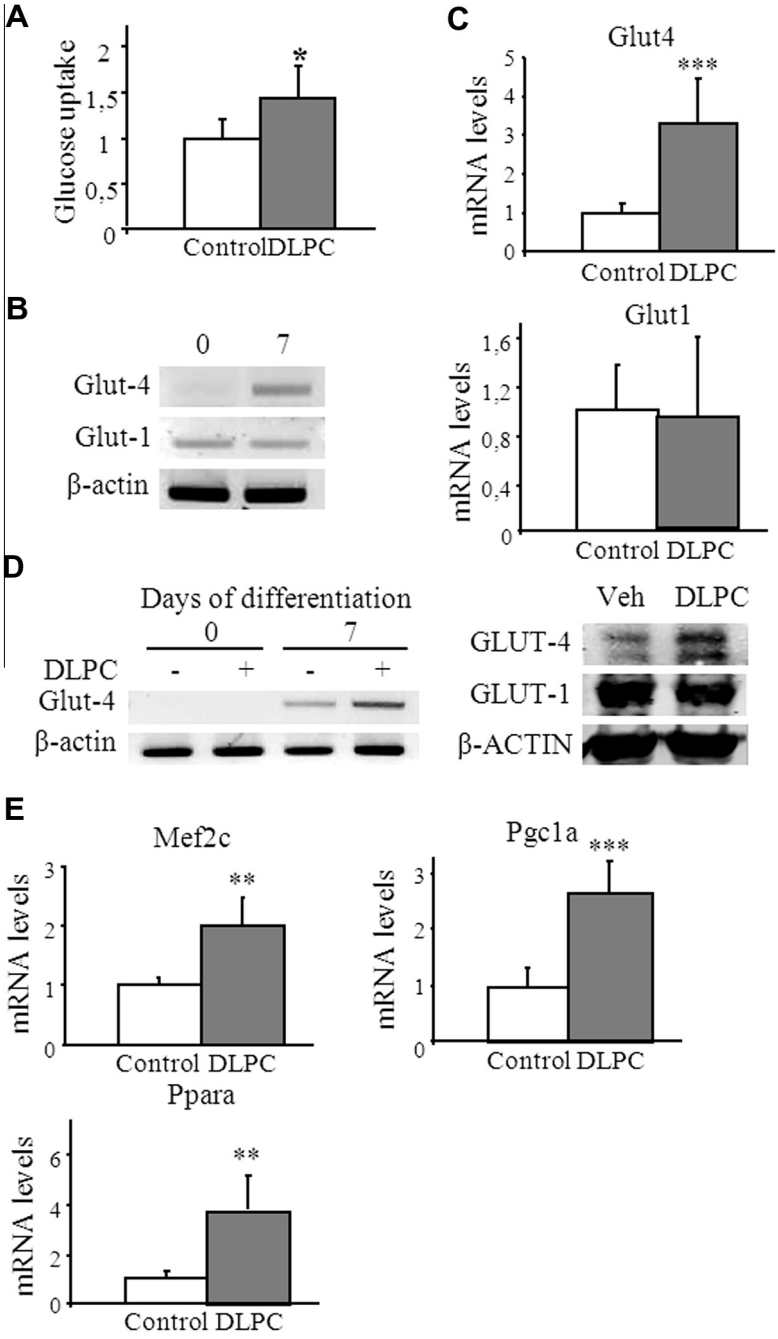

Fig. 2. Effects of DLPC treatment on glucose entrance into C2C12 cells. (A) Relative glucose removal by myotubes treated with vehicle (Control), or 100 μM DLPC (DLPC). (B) Analysis of Glut1 and Glut4 expression levels in undifferentiated (0 days) and fully differentiated (7 days) myotubes. β-Actin was used as control of loading. (C) Expression analysis using qPCR and Western blot of GLUT1 and GLUT4 levels in myotubes treated for 24 h with vehicle (Control) or DLPC 100 μM (DLPC). (D) Expression analysis of Glut4 in response to DLPC treatment in undifferentiated (0) and fully differentiated myotubes (7), β-actin was used as a load control. (D) Western blot of glut4 and glut1 in myotubes treated for 24 h with vehicle (Control) or DLPC 100 μM (DLPC), β-actin was used as control of loading. (E) Expression analysis, by qPCR, of previously described *Glut4* gene regulators. Bars are mean ± S.D. of 4 independent experiments. $^{*}p \leqslant 0.05$. $^{**}p \leqslant 0.01$; $^{***}p \leqslant 0.005$.

part the pathway is selected by the effect of glucose-6-phosphate levels on hexokinase localization [32]. The observed increases in the expression of *Pfkm*, *Pfkfb1* and *Gys1* suggested that NR5A2 could be making muscle cells more capable of metabolizing glucose through either pathway. Experiments that analyzed the changes of [6-14C]-Glucose flux into either glycolysis or glycogen synthesis revealed statistically non-significant increases in glucose flux into either pathway (Fig. 3B), a result which was consistent with the analysis of gene expression and that suggested that the increased glucose entry in the cells mediated by NR5A2 activation did not result in a clear preference for any of the pathways.

3.3. DLPC increases the inhibition of fatty acid consumption by glucose

The activation of liver NR5A2 by DLPC resulted in a decreased expression of SREBP-1c and its lipogenic targets [2]. The effect of NR5A2 activation by DLPC on the expression of five major lipid genes was also analyzed in differentiated C2C12 cells. These included genes coding for proteins participating in fatty acid uptake (*Lpl* and *Vldlr*), intracellular transport (*Fabp3*) and metabolism (*Acc2* and *Cpt1b*). Only *Fapb3* and *Acc2* showed significant changes of expression, 2.4 and 1.7-fold increases respectively (Fig. 4A).

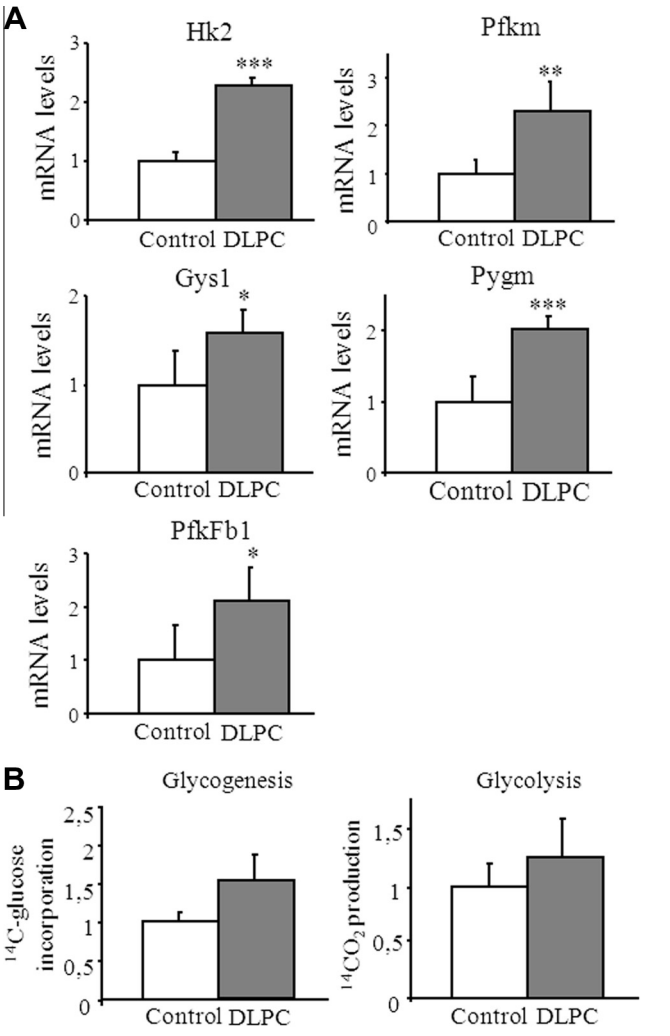

Fig. 3. Relative changes in the mRNA levels of major genes of glucose metabolism induced by DLPC and overall effect on glucose metabolic pathways. (A) qPCR analysis of the expression of several major genes associated with glucose metabolism in myotubes treated for 24 h with vehicle (Control) or 100 μM DLPC (DLPC). (B) Relative glucose fluxes through glycolysis or glycogen synthesis pathways in fully differentiated myotubes treated with the vehicle (Control) or 100 μM DLPC (DLPC). Bars show mean ± S.D. of 4 independent experiments. $^{*}p \leqslant 0.05$; $^{**}p \leqslant 0.01$; $^{***}p \leqslant 0.005$.

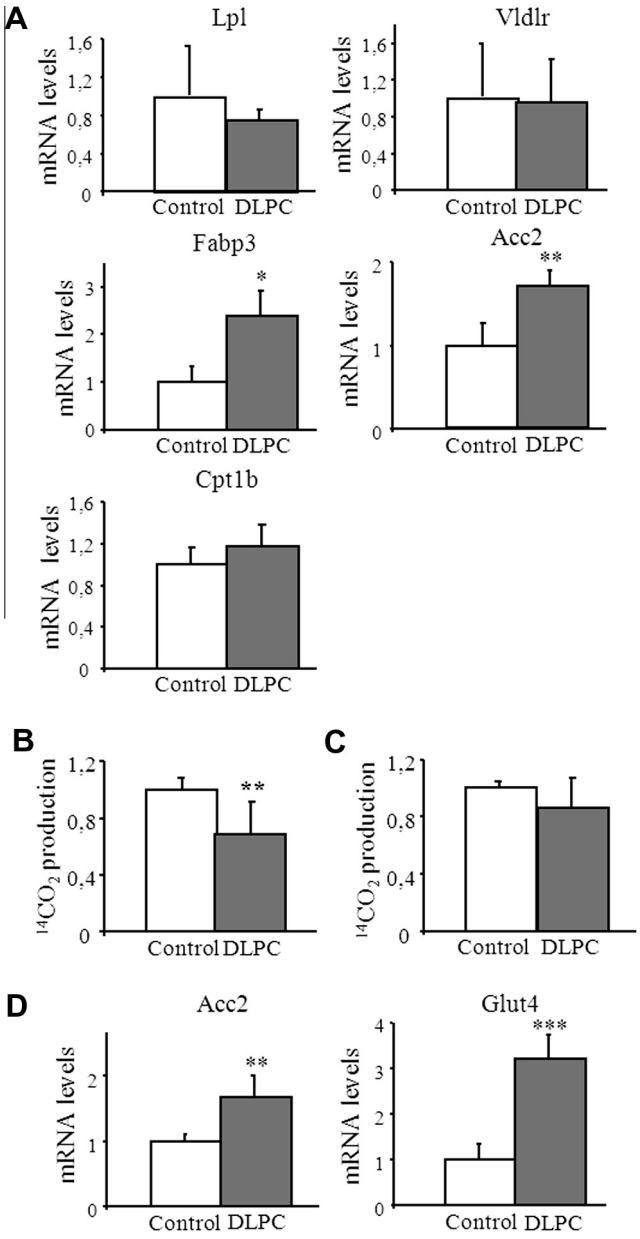

Fig. 4. DLPC effect on fatty acid oxidation by differentiated C2C12 cells. (A) Relative expression analysis of several genes associated with fatty acid metabolism in myotubes, after 24 h of treatment with either vehicle (Control) or 100 μM DLPC (DLPC). (B) Relative fatty acid oxidation, as measured by $^{14}CO_2$ production, by C2C12 myotubes treated with vehicle (Control) or 100 μM DLPC (DLPC). (C) The same experiment as in B but in low glucose conditions (3125 mM glucose). (D) Relative expression of *Acc2* and *Glut4* in C2C12 myotubes treated for 24 h with either vehicle (Control) or 100 μM DLPC (DLPC) in cells cultured in low glucose conditions. Bars show the mean ± S.D. of 4 independent experiments. $^{**}p \leqslant 0.01$; $^{***}p \leqslant 0.005$.

Acc2 codes for the mitochondrial membrane isoform of acetyl-CoA carboxylase, the enzyme that converts acetyl-CoA into malonyl-CoA. This metabolite is the major allosteric inhibitor of CTP1b, which in turn is the major regulatory enzyme of fatty acid transport through mitochondrial membrane. The change in *Acc2* expression seen in DLPC treated muscle cells is in contrast with the DLPC effect on liver cells, where a decreased *Acc2* expression has been reported [2]. Whether this difference arises from a different regulation of *Acc2* gene in muscle and in liver is an interesting issue that deserves further research. It is worth noting that the synthesis of malonyl-CoA also requires high levels of glucose. Thus the combination of an increased glucose uptake and an increased *Acc2* expression might allow the cell to switch faster from fatty acid to glucose oxidation without compromising the overall ability of the cell to oxidize fatty acids. This is also supported by the increased expression of *Fabp3*, which although potentially associated with insulin-resistant states, could also contribute to metabolic flexibility of the skeletal muscle [33] by allowing the cell to switch from the use of glucose to fatty acids, an effect which could be also potentiated by the increase in *Acc2*.

The degradation of fatty acids was analyzed by measuring the liberation of $^{14}CO_2$ from [1-14C]-oleyl-CoA. Our results showed that DLPC-treated cells oxidized fatty acids at about 0.65-fold the rate of vehicle-treated cells (Fig. 4B). A reduced fatty acid oxidation might be explained by the combination of a higher *Acc2* expression and the allosteric effect of an increased glucose entrance via GLUT4 [34]. As discussed above, malonyl CoA synthesis requires high glucose levels. In its absence, the increase in *Acc2* expression would not be enough for switch off the transport of fatty acids for degradation. This was supported by the results of the analysis of both gene expression and fatty acid degradation carried out in

low glucose conditions. As shown in Fig. 4C, DLPC still induced both *Glut4* and *Acc2* expression and therefore it was unlikely that the effect of DLPC on either *Glut4* or *Acc2* expression was consequence of the increased glucose uptake. However fatty acid degradation was not affected suggesting that, in low glucose conditions, the levels of Krebs cycle intermediates might not be enough to inhibit fatty acid oxidation via ACC2 [35].

In conclusion, the activation of NR5A2 by DLPC, induced an increase in glucose uptake by C2C12 myotubes and both glycolysis and glycogen synthesis are increased as a result. This is accompanied by a decrease in fatty acid degradation. Thus NR5A2 could be acting as a regulator that switches the cell from fatty acids to glucose oxidation. Further experiments using *in vivo* models will be needed to elucidate the systemic consequences of the activation of muscle NR5A2 which could constitute a potential target for the treatment of glucose homeostasis-related diseases.

Conflict of interest

The authors have declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.010.

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