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Metabolic functions of glucocorticoid receptor in skeletal muscle

Taiyi Kuo^{a,b}, Charles A. Harris^{c,d,1}, and Jen-Chywan Wang^{a,b,*}

^aDepartment of Nutritional Science & Toxicology, University of California at Berkeley, Berkeley, CA 94720, United States

^bGraduate Program of Endocrinology, University of California at Berkeley, Berkeley, CA 94720, United States

^cGladstone Institute of Cardiovascular Disease, San Francisco, CA 94158, United States

^dDepartment of Medicine, University of California at San Francisco, San Francisco, CA 94143, United States

Abstract

Glucocorticoids (GCs) exert key metabolic influences on skeletal muscle. GCs increase protein degradation and decrease protein synthesis. The released amino acids are mobilized from skeletal muscle to liver, where they serve as substrates for hepatic gluconeogenesis. This metabolic response is critical for mammals' survival under stressful conditions, such as fasting and starvation. GCs suppress insulin-stimulated glucose uptake and utilization and glycogen synthesis, and play a permissive role for catecholamine-induced glycogenolysis, thus preserving the level of circulating glucose, the major energy source for the brain. However, chronic or excess exposure of GCs can induce muscle atrophy and insulin resistance. GCs convey their signal mainly through the intracellular glucocorticoid receptor (GR). While GR can act through different mechanisms, one of its major actions is to regulate the transcription of its primary target genes through genomic glucocorticoid response elements (GREs) by directly binding to DNA or tethering onto other DNA-binding transcription factors. These GR primary targets trigger physiological and pathological responses of GCs. Much progress has been made to understand how GCs regulate protein and glucose metabolism. In this review, we will discuss how GR primary target genes confer metabolic functions of GCs, and the mechanisms governing the transcriptional regulation of these targets. Comprehending these processes not only contributes to the fundamental understanding of mammalian physiology, but also will provide invaluable insight for improved GC therapeutics.

Keywords

Glucocorticoids; Glucocorticoid receptor; Insulin; Skeletal muscle; Glucose metabolism; Protein metabolism

*Corresponding author. Address: Department of Nutritional Science and Toxicology, 315 Morgan Hall, University of California at Berkeley, Berkeley, CA 94720-3104, United States. Tel.: +1 510 643 1039. ; Email: walwang@berkeley.edu (J.-C. Wang)

¹Present address: Division of Endocrinology, Washington University School of Medicine, St. Louis, MO 63110, United States.

1. Introduction

Glucocorticoids (GCs) are steroid hormones secreted from the adrenal cortex to regulate whole-body metabolic homeostasis. The three major endogenous GC hormones are cortisol (hydrocortisone), corticosterone, and cortisone. In humans, the principal endogenous GC is cortisol, while in rodents it is corticosterone. Cortisone is inactive until converted to cortisol or corticosterone by 11 β hydroxysteroid dehydrogenase type 1 (11 β -HSD1) (Seckl and Walker, 2001; Tomlinson et al., 2004). On the other hand, 11 β -HSD type 2 (11 β -HSD2) converts active cortisol to inactive cortisone (Krozowski et al., 1999). GCs convey their signals mainly through an intracellular glucocorticoid receptor (GR). Cortisol has equal binding affinity for the mineralocorticoid receptor (MR) and GR; therefore, aldosterone-selective tissues, such as kidney, express 11 β -HSD2 to inactivate cortisol. Thus, the levels of 11 β -HSD1 and 11 β -HSD2 in tissues help determine the tissue concentrations of active GCs, thereby modulating these effects. Notably, in some tissues, such as hippocampus and macrophages, GCs act via both GR and MR under normal physiological conditions, in which MR significantly influences cellular responses to GCs.

GCs exert specific metabolic influence on different tissues. Skeletal muscle accounts for approximately 40% of body mass and is a major GC target tissue. Based on myosin heavy-chain isoform expression profile, skeletal muscle fibers are classified into type I, type IIa, type IIx and type IIb. Type I myofibers are also known as slow-twitch fibers, and type II, fast-twitch fibers. Slow-twitch myofibers are slow to fatigue, are rich in mitochondria, and have long contraction times. Fast-twitch myofibers fatigue rapidly and display quick contractions. Type I muscles appear red in color due to the presence of oxygen-binding protein, myoglobin, while type II appear pale. On the basis of the degree of oxidative phosphorylation, type I and IIa fibers exert oxidative metabolism, while type IIx and IIb mainly use glycolytic metabolism to generate ATP. Interestingly, GCs appear to impact type II muscle fibers much more than type I (Dekhuijzen et al., 1995; Falduto et al., 1990; Fournier et al., 2003), though the mechanism of such fiber-specific GC response is unclear.

In skeletal muscle, GCs mainly regulate protein and glucose metabolism (Fig. 1). Under stressful conditions, such as fasting and starvation, circulating GC levels are increased, which in turn decreases the rate of protein synthesis and increases proteolysis to generate amino acids to serve as precursors for hepatic gluconeogenesis. The resulting glucose can then be used by the brain as fuel. Under pathophysiological conditions, having excess endogenous (Cushing's Syndrome) or exogenous (prolonged medical treatment) sustained GC-mediated protein degradation can lead to skeletal muscle atrophy and muscle weakness. Moreover, GCs preserve plasma glucose through inhibiting glucose uptake and utilization in skeletal muscle, and play a permissive role in epinephrine-induced glycogenolysis. This adaptive course maintains adequate circulating glucose to fuel the brain during stress. However, this course becomes maladaptive upon chronic or excess exposure to GCs. Notably, reducing the level of available bioactive GCs has been shown to improve insulin sensitivity in animal models. The approach of inhibiting 11 β -HSD1, thus reducing the level of available bioactive GCs in tissues, is currently under clinical trials for treating type 2 diabetes (Hollis and Huber, 2011; Rosenstock et al., 2010).

Upon binding to GCs, cytosolic GR enters the nucleus and associates with specific genomic sequences called glucocorticoid response elements (GREs). Direct binding of GR to GRE, or negative GRE (nGRE) (Surjit et al., 2011), leads to the recruitment of transcription cofactors to activate, or repress, the transcriptional rate of nearby genes, respectively. Other modes of GR action include tethering (GR binding to other transcription regulators) and squelching (GR binding to and taking away transcription regulator from DNA), which often lead to transcription repression. These genes, defined as GR primary target genes, in turn trigger biological responses of GCs. The goal of this review is to discuss the current understanding of mechanisms governing GC-regulated glucose and protein metabolism, with a main focus on potential GR primary target genes identified in skeletal muscle and mediating the metabolic functions of GCs.

2. The regulation of glucose metabolism by glucocorticoids

Skeletal muscle is one the major tissues accountable for glucose homeostasis in mammals. Approximately 80% of glucose utilization occurs in skeletal muscle (DeFronzo and Tripathy, 2009; Ferrannini et al., 1988). Skeletal muscle also serves as a reservoir for glycogen storage. GCs inhibit glucose uptake and utilization and glycogen synthesis, and play a permissive role for catecholamine-stimulated glycogen breakdown in skeletal muscle (Fig. 1). These actions counteract those of insulin, which promotes glucose utilization and glycogen synthesis. Mice treated with GCs have reduced insulin-stimulated glucose uptake, caused by attenuated insulin-induced GLUT4 translocation to the cell membrane in myotubes (Dimitriadis et al., 1997; Morgan et al., 2009; Weinstein et al., 1998). Furthermore, insulin signaling and glycogen synthase activity are suppressed by GCs (Coderre et al., 1991, 1992; Morgan et al., 2009). These metabolic effects are due to, at least in part, the direct effect of GCs on myotubes, as GC treatment of cultured myotubes inhibits insulin-stimulated glucose utilization (Gathercole et al., 2007; Morgan et al., 2009).

One major mechanism by which GCs regulate glucose metabolism is to inhibit insulin signaling (Morgan et al., 2009; Pivonello et al., 2010; Schakman et al., 2008). Insulin binds to the cell-surface insulin receptor (IR), a tyrosine kinase that autophosphorylates and phosphorylates the insulin receptor substrate (IRS) (Lee and White, 2004). Tyrosine-phosphorylated IRS associates with IR and activates downstream signaling pathways (Lee and White, 2004). Mice treated with GCs have reduced levels of tyrosine-phosphorylated IR and total IRS-1 proteins in skeletal muscle (Morgan et al., 2009). The activities of phosphoinositide-3-kinase (PI3K) and Akt, two key signaling molecules downstream of IR and IRS-1, are also decreased (Giorgino et al., 1993; Morgan et al., 2009; Saad et al., 1993). Moreover, the phosphorylation of serine 307 of IRS-1 (pSer307-IRS-1) is increased upon GC treatment (Morgan et al., 2009). This phosphorylation disrupts the association between IR and IRS-1, thus reducing the insulin response (Draznin, 2006; Gual et al., 2005). However, recent studies showed that mice harboring IRS-1 serine 307 mutated to alanine had reduced insulin sensitivity (Copps et al., 2010; Copps and White, 2012). It suggested that serine 307 phosphorylation positively regulates insulin sensitivity in vivo. Downstream of Akt, glycogen synthase kinase-3 (Gsk3) phosphorylates and inhibits glycogen synthase (Cohen and Goedert, 2004; Rayasam et al., 2009), an enzyme involved in converting glucose

to glycogen. While Akt phosphorylates and reduces the activity of Gsk3, GCs, on the other hand, decrease the phosphorylation status of Gsk3 (Buren et al., 2008; Ruzzin et al., 2005).

In parallel with the PI3K/Akt signaling, another mechanism by which insulin promotes glucose uptake is to initiate the TC10 pathway (Leto and Saltiel, 2012). Insulin treatment triggers the phosphorylation of APS (adapter protein with Pleckstrin homology (PH) and Src homology 2 (SH2) domains), which then facilitates the phosphorylation of Cbl (Hu et al., 2003). Cbl-associated protein (CAP, also known as Sorbs1), as its name suggests, associates with Cbl (Baumann et al., 2000). This phosphorylated Cbl-CAP protein complex translocates to the lipid-raft microdomain at the plasma membrane, leading to the recruitment of Rho GTP-binding protein TC10 and the assembly of the exocyst complex, and finally the translocation of Glut4 to cell surface. Whether GCs can modulate this alternative insulin-initiated pathway is unknown. In contrast to insulin resistance induced by excess GCs, reducing GC level improves insulin sensitivity. Circulating GC levels are higher in obese *ob/ob*, *db/db* and lipotrophic *A-ZIP/F-1* mice than normal mice, and adrenalectomy improved insulin-stimulated muscle glucose disposal in these mice (Haluzik et al., 2002; Ohshima et al., 1989). *A-ZIP/F-1* mice expressed a dominant-negative protein, A-ZIP/F, under the control of the adipose-specific aP2 promoter, and had no WAT and markedly reduced brown adipose tissue (Moitra et al., 1998). This A-ZIP/F protein prevents DNA binding of basic leucine zipper (B-ZIP) transcription factor families, such as C/EBP and Jun. Kuo Kondo (KK) mice, derived from selective inbreeding for large body size, develop diabetes and demonstrate neurologic, renal, and retinal complications comparable to those observed in human diabetes (Ikeda, 1994; Reddi and Camerini-Davalos, 1988). Treating C57/B6 or KK mice with an 11 β -HSD1-specific inhibitor decreased fasting blood glucose level and improved insulin sensitivity (Morgan et al., 2009). In skeletal muscle of 11 β -HSD1 inhibitor-treated KK mice, pSer307-IRS-1 decreased and pThr308-Akt, a hallmark of activated Akt, increased (Morgan et al., 2009). While skeletal muscle 11 β -HSD1 mRNA does not correlate with metabolic parameters (Nair et al., 2004), the activity of 11 β -HSD1 in muscle biopsies of type 2 diabetic patients is lower than normal people (Jang et al., 2006, 2007; Whorwood et al., 2001). Also, in the skeletal muscle of diabetic patients, elevated GR mRNA expression correlates with the degree of insulin resistance, and treatments that improve insulin sensitivity normalize GR expression (Vestergaard et al., 2001; Whorwood et al., 2002). These studies indicate that GC signaling could be a target of therapeutic intervention against type 2 diabetes (Alamdari et al., 2010; Morton, 2010).

Overall, how GCs affect glucose metabolism in skeletal muscle is not entirely clear. To date, only a few GR primary target genes, outlined below, have been linked to GC-regulated glucose and glycogen metabolism in in vitro cultured cell models and in vivo animal models.

2.1. Pik3r1 (p85 α)

A GR primary target gene contains GRE(s) near or in its genomic region, and its expression is regulated by GCs. Based on these criteria, a combination of chromatin immunoprecipitation sequencing (ChIPseq) and microarray was conducted to identify primary GR targets in muscle. It is important to note that ChIPseq gives ~300–800 bp DNA

sequences (referred to as GR binding region, or GBR) likely containing GRE(s). We identified 173 genes whose expression was modulated by GC treatment and containing GBRs in mouse C2C12 myotubes (Kuo et al., 2012). Notably, whether these GBRs can confer GC response, thus contain GREs, requires further studies. Therefore, these 173 genes are referred to as “potential” GR primary targets. Interestingly, among these targets, nine genes have been shown to antagonize insulin action: Sorbs1, Grb10 (Holt et al., 2009), Cblb (discussed in a later section) (Nakao et al., 2009), Ptd1 (Wu et al., 2011), Ddit4, Sesn1, Depdc6, Mknk2 and Pik3r1 (Fig. 2). Pik3r1 (a.k.a. p85 α) encodes the regulatory subunit of PI3K. Pik3r1 plays a key role in conferring insulin action. However, excess Pik3r1 monomers can compete with Pik3r1 and PI3KC (catalytic subunit of PI3K, a.k.a. p110) heterodimers to interact with IRS-1 (Barbour et al., 2004; Draznin, 2006), resulting in decreased insulin response. Conversely, reducing the expression of Pik3r1 improves insulin sensitivity (Mauvais-Jarvis et al., 2002). It is important to note that elevated expression of PIK3R1 is found in patients with insulin resistance.

Treating C2C12 myotubes with dexamethasone (Dex), a synthetic GC, reduces the total IRS-1 level, increases the pSer307-IRS-1 level and decreases the level of phosphorylation on serine 473 of Akt (pSer473-Akt), which is required to potentiate its kinase activity. These insulin resistance-associated phenotypes; however, are all compromised when Pik3r1 expression in C2C12 myotubes is reduced specifically by RNA interference (RNAi) (Kuo et al., 2012). Thus, Pik3r1 mediates, at least in part, GC-suppressed insulin signaling. The role of Pik3r1 in GC-induced insulin resistance in vivo is the subject of current research. CAP (Sorbs1), another potential GR primary target gene, is involved in the alternative insulin signaling pathway discussed above. It is essential to learn whether reducing the expressions of genes other than Pik3r1 that are capable of inhibiting insulin signaling compromises GC response.

2.2. Pyruvate dehydrogenase kinase 4 (Pdk4)

Pdk4 inhibits pyruvate dehydrogenase complex and leads to a reduced conversion of pyruvate to acetyl-CoA (Fig. 2) (Sugden and Holness, 2003). Thus, the induction of Pdk4 plays a critical role in GC-decreased glucose utilization (Sugden and Holness, 2003). Recent studies indicated that, in human primary myotubes, GC-repressed glycogen synthesis is diminished with reduced expression of PDK4 (Salehzadeh et al., 2009). GCs upregulate *Pdk4* gene transcription in both liver and skeletal muscle, and GREs of human *PDK4* and rat *Pdk4* have been identified (Connaughton et al., 2010; Kwon et al., 2004). For human *PDK4*, the GRE is located between -824 and -809 (relative to transcription start site, TSS) (Kwon et al., 2004). Interestingly, three binding sites of the FoxO transcription factors, FoxO1 and FoxO3, are located near the GRE (from -738 to -731, -533 to -525, and -361 to -354, respectively). These FoxO binding sites could represent the insulin response element (IRE) that mediates the repressive effect of insulin on *PDK4* gene transcription (Kwon et al., 2004). However, with no insulin present, these FoxO binding sites are required for the maximal induction of *PDK4* gene transcription by GCs. This functional interaction between GR and FoxO proteins have also been reported in hepatic gluconeogenic genes, such as *phosphoenolpyruvate carboxykinase (Pepck)* (Hall et al., 2007) and *glucose-6-phosphatase*

(*G6Pase*) (Vander Kooi et al., 2005), as well as in *MuRF1* gene which is involved in GC-induced protein degradation (see below).

For rat *Pdk4* gene, two GREs are identified between -6634 and -6377 relative to TSS (Connaughton et al., 2010). A FoxO binding site is found between -591 and -338. A mutation at FoxO binding decreases Dex-induced *Pdk4* promoter-containing reporter gene activity. This FoxO binding site is also responsible for insulin repression on *Pdk4* gene (Connaughton et al., 2010). Chromatin immunoprecipitation (ChIP) experiments show that Dex treatment increases GR occupancy at the GREs, whereas FoxO1 is already present at the FoxO binding site even without Dex treatment. Insulin treatment decreases GR recruitment to the GREs and decreases FoxO1 recruitment to the FoxO binding sites (Connaughton et al., 2010). Overall, although GREs of rat *Pdk4* are located further away from its TSS, the players involved in GC and insulin response are similar to human *PDK4* gene.

2.3. Additional potential GR primary targets that may confer glucocorticoid-regulated glucose metabolism

Several studies have shown that GCs increase the expression of FoxO1 and FoxO3 in skeletal muscle (Fig. 2) (Nishimura et al., 2008; Waddell et al., 2008). ChIPseq conducted in C2C12 myotubes and 3T3-L1 adipocytes found several GR binding regions (GBRs) in or near the *FoxO3* genomic region, indicating that FoxO3 is a GR primary target gene (Kuo et al., 2012; Yu et al., 2010). No GBR was identified for FoxO1 gene. However, FoxO1 gene expression is induced by GC in mouse liver, and such induction is markedly reduced in mice expressing a GR mutant that has decreased DNA binding ability (Frijters et al., 2010). It is likely that FoxO1 and/or FoxO3 are involved in GC-regulated glucose oxidation, as demonstrated by FoxO1 playing an important role in the stimulation of *Pdk4* gene transcription. Moreover, transgenic mice overexpressing FoxO1 gene showed impaired glycemic control (Kamei et al., 2004), suggesting that an elevated FoxO activity can cause insulin resistance. Nonetheless, the precise roles of FoxO1 and FoxO3 in GC-regulated glucose metabolism have not yet been examined.

Myostatin (a.k.a. growth differentiation factor 8, Gdf8) is a secreted protein and a member of the transforming growth factor β (TGF β) family. Myostatin binds to activin type 2 receptor located on the membrane of muscle cells, and initiates a cellular signaling cascade that activates transcription factors smad2 and smad3, which in turn regulate the transcription of specific target genes. The function of myostatin in GC-regulated protein metabolism has been studied extensively and will be discussed in a later section. Recent studies suggest that myostatin could also participate in glucose homeostasis (Burgess et al., 2011; Zhang et al., 2011). However, the role of myostatin in GC-regulated glucose metabolism has not been reported.

2.4. Factors that modulate glucocorticoid effects on glucose metabolism

While GCs directly reduce insulin-stimulated glucose uptake and glycogen synthesis in skeletal muscle cells, GCs also affect glucose homeostasis by stimulating lipid mobilization from white adipose tissue to skeletal muscle. Upon GC treatment, increased intramuscular

accumulation of triglyceride (TG) is observed (Gounarides et al., 2008). Also, the level of diacylglycerol (DAG), which can activate protein kinase c (PKC) thus causing insulin resistance (Samuel et al., 2010), is elevated (Gounarides et al., 2008). Treating mice with an 11 β -HSD1 inhibitor reduces the expression of genes involved in lipogenesis and lipid metabolism in skeletal muscle (Morgan et al., 2009). Very likely, GCs positively regulate the expression of these genes. The activation of these genes involved in lipid metabolism by GCs has been well documented in liver and adipose tissues (Amatruda et al., 1983; Cai et al., 2011; Dolinsky et al., 2004; Gathercole et al., 2011; Yu et al., 2010). However, whether GCs can directly affect the transcription of these genes require more studies. Previous studies have shown that treating mice with an inhibitor of lipolysis in white adipose tissue decreased GC-induced insulin resistance (Tappy et al., 1994), seemingly by reducing ectopic lipid accumulation in both liver and skeletal muscle. It is important to note that the accumulation of lipid intermediates, such as DAG and ceramides, rather than neutral lipids, such as triglycerides, is linked to antagonize insulin signaling. In skeletal muscle, when beta oxidative capacity is high, concentrations of ceramide, acyl-CoA and DAG stay low. In contrast, under the condition of low beta oxidative capacity with lipid overload, concentrations of these lipid intermediates of lipid metabolism increase (Hulver et al., 2003). In human muscle, insulin resistance caused by elevated free fatty acid levels during euglycemic-hyperinsulinemic clamping was associated with increased DAG (Itani et al., 2002). Furthermore, elevated intracellular ceramide level in muscle is associated with GC-induced insulin resistance (Holland et al., 2007). In any case, whether GCs modulate the production of lipid intermediates, and how this may affect glucose homeostasis requires more mechanistic studies.

Interestingly, treating mice with anti-oxidants, such as vitamin C and E, attenuates GC-induced insulin resistance in skeletal muscle, liver and white adipose tissue (Williams et al., 2012). The improvement of glucose tolerance is independent of Akt phosphorylation; instead, AMP-activated kinase (AMPK) activity is elevated in skeletal muscle (Williams et al., 2012). GC treatment can repress AMPK activity in skeletal muscle (Christ-Crain et al., 2008; Kola et al., 2008; Lutzner et al., 2012) and increase the level of malonyl-CoA (Nakken et al., 2010), an intermediate of fatty acid synthesis that inhibits fatty acid oxidation. Anti-oxidants counteract such effects of GCs to improve chronic/excess GC-induced insulin resistance. Previous studies done with adipocytes indicated that long-term GC treatment increases the level of reactive oxygen species (ROS), which activates c-Jun N-terminal kinase (JNK) to suppress insulin signaling (Houstis et al., 2006). It has been shown that GC treatment increases ROS levels in L6 myotubes, which leads to an inhibition in protein synthesis, and an elevation in proteolysis and apoptosis (Orzechowski et al., 2003). However, whether oxidative stress could be one of the mechanisms by which GCs repress insulin action on glucose metabolism in skeletal muscle has not been reported.

3. The regulation of protein metabolism by glucocorticoids

It is well documented that the treatment with GCs induces a catabolic response in skeletal muscle. Treating animals with physiological or synthetic GCs causes a decrease in skeletal muscle size (Auclair et al., 1997; Baehr et al., 2011; Hu et al., 2009; Shimizu et al., 2011). In fact, induction of muscle atrophy by several pathological conditions, such as diabetes,

metabolic acidosis and sepsis, all involves GCs. The muscle atrophy phenotype is caused by cell autonomous effects of GR both in vitro and in vivo. Mouse C2C12 and rat L6 myotubes treated with synthetic GCs, such as Dex, have markedly decreased cell diameters (Kuo et al., 2012; Menconi et al., 2008). Furthermore, using RNAi to reduce GR expression in C2C12 myotubes compromises GC-reduced protein degradation (Zhao et al., 2009). In muscle-specific GR knockout (MGRKO) mice, Dex-induced muscle atrophy was completely abolished (Watson et al., 2012). In these mice, muscle atrophy induced by nutritional deprivation, which elevates the circulating corticosterone, is also reduced though not completely blocked (Watson et al., 2012). Similarly, nutritional deprivation-induced muscle atrophy is inhibited after adrenalectomy (Almon and Dubois, 1988). These observations suggest that GR and its primary target genes play a pivotal role in GC-regulated protein metabolism. These are important studies given the previous suggestion that GCs effects on muscle were in part secondary to primary effects on the pituitary and liver from reduced GH and IGF1 secretion. Identifying the primary causative GR target genes responsible for muscle atrophy is important in the hunt for selective GR modulators that retain anti-inflammatory properties of GCs, but have less adverse effects. This issue is particularly relevant in the treatment of critically ill patients who often suffer from “ICU myopathy” (Puthuchery et al., 2010) and in the treatment of inflammatory disease of either muscle or the lungs where decreased muscle function results in decreased pulmonary function (loss of bellows).

3.1. MuRF1

About a decade ago, systematic gene expression analyses identified a list of genes that were upregulated during muscle wasting. These genes are called the atrogenes (Bodine et al., 2001). Well recognized atrogenes include two muscle-specific E3 ubiquitin ligases: muscle RING finer 1 (MuRF1, a.k.a. Trim63) and muscle atrophy F-box (MAFbx, a.k.a. Atrogin-1, Fbxo32) (Bodine et al., 2001; Sandri et al., 2004; Stitt et al., 2004) (Fig. 2). MuRF1 and Atrogin-1 gene expression are increased in most of the muscle wasting scenarios, including denervation, nutritional deprivation, unloading and exogenous GC treatment. For the mouse *MuRF1* gene, the GRE is located between -210 and -196. Interestingly, a binding site for the FoxO family of transcription factors is located adjacent to the *MuRF1* GRE (Waddell et al., 2008). Overexpression of GR and FoxO1 in C2C12 cells synergistically activates a reporter plasmid containing mouse *MuRF1* promoter. Mutation of either the GRE or the FoxO binding site reduces the ability of GCs to induce the activity of the *MuRF1* promoter reporter gene. ChIP experiments demonstrated that GR and FoxO1 occupy the genomic region containing the *MuRF1* GRE. Insulin-like growth factor-1 (IGF-1), which inhibits GC-induced MuRF1 gene transcription, decreases the recruitment of FoxO1 but not GR to the *MuRF1* GRE.

Mice lacking *MuRF1* gene were generated to examine its role in GC-induced muscle atrophy in vivo (Baehr et al., 2011). Following 14 days of Dex treatment, wild type (WT) mice have markedly decreased tricep surae (TS) and tibialis anterior (TA) muscle weights. In *MuRF1* null mice, Dex treatment still decreased the weight of TS and TA muscles; however, these decreases were significantly less than those of WT mice. Also, *MuRF1* null mice showed a significant sparing of fiber cross-section area and tension output of gastrocnemius

muscles after Dex treatment. These results clearly confirm the important role of MuRF1 in GC-induced muscle atrophy. Interestingly, while Dex-reduced protein synthesis was compromised in *MuRF1* null mice, Dex-induced protein degradation was not affected in these mice, although the basal protein degradation rate is lower in *MuRF1* null mice (Baehr et al., 2011). Previous studies have reported that MuRF1 targets several myofibrillar proteins for degradation (Clarke et al., 2007; Cohen et al., 2009; Polge et al., 2011), and these results suggest that MuRF1 targets more than myofibrillar proteins when conferring GC response. Interestingly, transgenic mice overexpressing MuRF1 in skeletal muscle have altered whole-body energy and glucose homeostasis (Hirner et al., 2008; Koyama et al., 2008). Eight-week old transgenic mice do not show a muscle atrophy phenotype. Their plasma insulin levels are two-fold higher than those of WT mice, and they have lower hepatic glycogen levels. Pyruvate dehydrogenase (Pdh) levels are lower in these mice, and yeast two-hybrid showed that MuRF1 interacts with Pdh, Pdk2, and Pdk4, key regulators of glycolysis (Hirner et al., 2008). Overall, MuRF1 could be a multi-functional protein that participates in both protein and glucose metabolism. It is important to note that nutritional deprivation-induced muscle atrophy is not spared in *MuRF1* null mice (Baehr et al., 2011), suggesting either GC-independent pathways act during nutritional deprivation to mediate atrophy or other GR primary targets are involved in glucocorticoid-regulated protein metabolism during nutritional deprivation.

3.2. MAFbx

MAFbx encodes an ubiquitin E3 ligase, and its GRE, if any, has not been identified. It is possible that *MAFbx* is not a primary but a secondary target gene of GR. *MAFbx* gene transcription is activated by transcription factors FoxO1, FoxO3 and Klf15 (Sandri et al., 2004; Shimizu et al., 2011; Stitt et al., 2004). These three transcription factors are potential GR primary target genes in skeletal muscle (see below). Following 14 days of Dex treatment, WT mice and mice lacking *MAFbx* gene show similar extent of muscle atrophy (Baehr et al., 2011). Surprisingly, there is no muscle sparing in *MAFbx* null mice under this experimental condition. While we cannot rule out the potential role of MAFbx in GC-regulated protein metabolism in other conditions, these results clearly indicate that if MAFbx has a role in GC-regulated protein metabolism, it must be distinct from that of MuRF1. MAFbx has been shown to target and degrade MyoD, myogenin, and Eif3f (Csibi et al., 2009; Jogo et al., 2009; Lagirand-Cantaloube et al., 2008; Tintignac et al., 2005), and degradation of any of these proteins reduces myotube diameters. Interestingly, treatment of GCs has been reported to increase N-terminal ubiquitination of MyoD (Sun et al., 2008).

3.3. Myostatin

Myostatin negatively regulates muscle growth and differentiation (Walsh and Celeste, 2005). Myostatin has been shown to inhibit Akt (Amirouche et al., 2009; Morissette et al., 2006; Trendelenburg et al., 2009), which is activated by two major protein-synthesis-promoting hormones, insulin and insulin-like growth factor-1 (IGF-1). GCs can increase the expression of myostatin (Ma et al., 2001, 2003) (Fig. 2). GCs also increase the activity of a reporter gene containing 3.3 kb of the human *MYOSTATIN* promoter. However, the exact location of the *MYOSTATIN*GRE is not yet identified, and the occupancy of GR on this promoter region has not been addressed (Ma et al., 2001). The *MYOSTATIN* promoter contains two

binding sites for CCAAT/enhancer binding protein family of transcription factors (C/EBP). A *MYOSTATIN* promoter-embedded reporter is activated by the overexpression of CCAAT/enhancer binding protein α , β and δ (C/EBP α , β , and δ , respectively), with C/EBP δ overexpression demonstrating the strongest induction (Allen et al., 2010a,b). In C2C12 myotubes, GCs elevate the expression of C/EBP δ and C/EBP β (Gonnella et al., 2011), and mutating C/EBP binding sites in *MYOSTATIN* promoter reporter compromised GC response, suggesting that myostatin could be a secondary GR target (Allen et al., 2010a,b). It is unclear whether C/EBP δ is a GR primary target gene, since no GRE has been identified. C/EBP β is discussed in detail in a later section.

Naturally occurring myostatin nulls have been found in human and cattles, and their muscles are enlarged significantly (Allen et al., 2010a,b). Mice lacking the *myostatin* gene were created to elucidate its role in GC-regulated muscle atrophy. Ten-days of Dex treatment (1 mg/kg body weight, BW) caused muscle atrophy in WT mice and this was greatly attenuated in *myostatin* null mice (Gilson et al., 2007). When WT mice were treated with a higher dose of Dex (5 mg/kg BW) for 4 days, the expression of *MuRF1*, *Atrogin-1* and *Cathepsin L* and chymotrypsin-like proteasomal activity were increased. However, the induction of these genes was reduced in *myostatin* null mice (Gilson et al., 2007). Therefore, myostatin likely participates in GC-induced protein degradation. Another study that measured the rate of phenylalanine tracer incorporation into myofibrillar proteins in *myostatin* null mice; however, showed that muscle hypertrophy seen in *myostatin* null mice is a result of increased protein synthesis, rather than decreased proteolysis, per muscle fiber (Welle et al., 2011). This phenotype could be due to myostatin's ability to inhibit Akt, since activated Akt leads to protein synthesis and the phosphorylation of FoxO1 and FoxO3 to exclude them from the nucleus. Notably, myostatin expression is induced upon food deprivation and its function is required for food deprivation-induced muscle atrophy (Allen et al., 2010a,b).

3.4. Pik3r1 (p85 α)

Overexpression of Pik3r1 in C2C12 myotubes resulted in a marked decrease in cell diameters (Kuo et al., 2012). In contrast, Dex-decreased myotube diameters and Dex-increased protein synthesis are compromised when reducing Pik3r1 expression in C2C12 myotubes (Kuo et al., 2012). These observations suggest that Pik3r1 is involved in GC-inhibited protein synthesis, leading to decreased myotube diameters (Fig. 2). Interestingly, Dex-induced expression of *MuRF1*, *Atrogin-1* and *FoxO3* are all compromised in Pik3r1 knockdown cells (Kuo et al., 2012), implying that Pik3r1 participates in Dex-induced protein degradation in vitro, though the precise role of Pik3r1 in GC-regulated protein metabolism needs to be confirmed in *Pik3r1* null mice.

Administration of a physiological dose of GCs to adrenalectomized and acutely diabetic (induced by injecting streptozotocin, STZ) mice demonstrated decreased IRS-1-associated PI3K activity in muscle and resulted in progressive muscle atrophy (Hu et al., 2009). These responses are related to increased association of PI3K with GR proteins. In muscle-specific GRKO mice, acute diabetes minimally reduces IRS-1-associated PI3K activity in skeletal muscle, and these mice are resistant to muscle atrophy. However, when a physiological dose of GCs is given to muscle-specific insulin receptor knockout mice, muscle protein

degradation is elevated (Hu et al., 2009). Fluorescence resonance energy transfer and an in vitro binding assay showed that hormone-bound GR interacts with Pik3r1 protein thus reducing the association of PI3K with IRS-1. In this case, GCs exert non-genomic action to regulate protein metabolism by antagonizing insulin/IGF-1 responses. The interaction between GR and Pik3r1 proteins has been reported in other cell types (Arancibia et al., 2011; Hafezi-Moghadam et al., 2002; Leis et al., 2004). Identifying GR mutants that do not interact with Pik3r1 or Pik3r1 mutants which do not associate with GR will help dissect the mechanism of GC-regulated protein metabolism.

Grb10 is another potential GR primary target capable of negatively regulating insulin action. Mice lacking Grb10 exhibited increased muscle weight and hypermuscularity due to increased myofiber size (Holt et al., 2012) compared to WT mice. The role of Grb10 in GC-regulated protein metabolism is unclear. It is likely that additional GR primary targets inhibiting insulin signaling also participate in GC-regulated protein metabolism.

3.5. C/EBP β

In L6 myotubes, reducing C/EBP β expression significantly compromises Dex-induced *MuRF1* and *MAFbx* gene expression and attenuates Dex-decreased cell diameters though overexpressing C/EBP β did not affect *MuRF1* and *MAFbx* gene expression (Gonnella et al., 2011). Since the *MuRF1* gene contains a GRE, C/EBP β could cooperate with GR to induce *MuRF1* gene expression. For *MAFbx*, C/EBP β may act with FoxO1, FoxO3, and/or Klf15 to activate its expression. The C/EBP β gene expression is induced by Dex in both L6 and C2C12 myotubes (Gonnella et al., 2011). ChIPseq identified a GR binding region (GBR) located at approximately 9.5 kb upstream from the C/EBP β TSS (Kuo et al., 2012). Whether this GBR mediates GC-stimulated C/EBP β gene transcription has not been tested.

C/EBP β interacts with a histone acetyltransferase (HAT), p300, whose expression is increased by GCs in L6 myotubes and skeletal muscles of rat (Fig. 2) (Yang et al., 2005). p300 serves as a coactivator for C/EBP β to activate genes involved in protein metabolism. In L6 myotubes, knocking down p300 with RNAi reduces Dex-decreased cell diameters and Dex-increased protein degradation (Yang et al., 2007). Overexpressing p300 lacking HAT activity (Yang et al., 2007) or p300 inhibitor Cited2 reduces these GC responses (Tobimatsu et al., 2009). It is important to note that p300 also acts as a coactivator for GR to stimulate the transcription of GR primary target genes (Amat et al., 2007; Shipp et al., 2010). This function of p300 may contribute to GC-induced muscle atrophy. Interestingly, inducing sepsis in rats increases p300 expression and decreases histone deacetylase HDAC3 and HDAC6 expression in skeletal muscle (Alamdari et al., 2010). These effects are attenuated when treating septic rats with RU486, a partial GR antagonist. Therefore, GCs are required for sepsis-induced p300 expression. Interestingly, in L6 myotubes, RU486 did not block sepsis-induced p300 expression, instead, it induces p300 expression, suggesting that different mechanisms are adapted by Dex to regulate p300 expression in vitro and in vivo.

3.6. FoxO1 and FoxO3

The roles of FoxO1 and FoxO3 in the regulation of protein metabolism have been described in many review articles (Gross et al., 2008; Schakman et al., 2008). Briefly, they activate

genes involved in protein degradation, such as *MuRF1* and *MAFbx* (Fig. 2). In addition, they stimulate genes encoding proteins that inhibit protein synthesis, such as 4EBP1. Furthermore, in myotubes, FoxO3 can activate the expression of genes involved in autophagy, such as *Bnip3*, *Lc3* and *Atg14*, and trigger protein degradation through lysosomal and ubiquitin-proteosomal pathway (Mammucari et al., 2007; Masiero et al., 2009). In C2C12 myotubes, overexpressing a dominant negative form of FoxO3 protein blocked Dex-decreased cell diameters (Sandri et al., 2004) and Dex-induced *MuRF1* and *MAFbx* expression (Sandri et al., 2004; Stitt et al., 2004). In another pathway, FoxO3 is required for GC-inducing insulin receptor substrate 2 (IRS-2), which in turn activates Mek and Erk kinases. Erk then phosphorylates Sp1, a transcription factor that upregulates the expression of ubiquitin C (Ubc), contributing to protein degradation (Zheng et al., 2010). Overall, FoxO proteins can confer GC response and act with GR to regulate specific gene transcription, such as MuRF-1.

3.7. Genes encoding proteins in the mTOR pathway

GCs reduce the activity of mammalian target of rapamycin (mTOR), a protein kinase involved in the activation of protein synthesis that is downstream of Akt and upstream of p70 S6 kinase (p70S6K) (Shah et al., 2000; Wang et al., 2006). Several potential GR primary targets identified from ChIPseq in C2C12 myotubes can inhibit mTOR signaling. These genes include Sestrin 1 (*Sesn1*) (Budanov and Karin, 2008), *Depdc6* (a.k.a. deceptor) (Peterson et al., 2009), *Ddit4* (a.k.a. *Redd1*, *Rtp801*) (Brugarolas et al., 2004) and *Mknk2* (Hu et al., 2012) (Fig. 2). Recent studies also suggest that *Mknk2* associates with mTOR and inhibits the phosphorylation and activation of p70S6 kinase (Hu et al., 2012). GR binding regions of these genes can mediate GC response when inserted upstream of a TATA box in a reporter plasmid. The role of *Sesn1*, *Depdc6* and *Mknk2* in GC-suppressed protein synthesis has not been examined. In L6 myotubes, reducing the expression of *Ddit4* significantly attenuates Dex-suppressed protein synthesis. Although several potential GR primary targets are identified as inhibitors of the insulin/IGF-1-PI3K-Akt-mTOR signaling, reducing the expression of either *Pik3r1* or *Ddit4* already showed compromised phenotypes on GC-reduced protein synthesis in vitro (Wang et al., 2006). These studies demonstrated that these potential GR primary targets likely do not play a redundant role. Instead, each might confer specific GC responses, and in vivo studies are required to confirm their roles. If deleting one of these genes could spare GC-induced muscle atrophy in vivo, it would have invaluable implications in GC therapeutics with reduced side effects.

Notably, the activation of mTOR signaling appears to antagonize GR activity (Shimizu et al., 2011). L6 myoblasts treated with branched-chain amino acids (BCAAs), which activates a GTPase called Rheb that positively regulates mTORC1, resulted in a reduced GC response of a reporter gene with tandem repeats of perfect palindromic GREs (AGAACAGGATGTTCT). Mice treated with BCAA have compromised Dex response on the induction of known GR primary targets. Intriguingly, the recruitment of GR to its cognate GRE of these target genes is also diminished, suggesting that mTOR signaling affects GR and DNA interaction. In addition, BCAA treatment in rats compromises Dex-inhibited mTOR activity and Dex-induced muscle atrophy. Overall, the balance between

GCs and mTOR signaling can determine the catabolic and anabolic nature of skeletal muscle.

3.8. Klf-15

Branched-chain amino acid transaminase 2 (*Bcat2*) encodes a mitochondrial enzyme catalyzing the first reaction in the catabolism of BCAA to promote BCAA degradation and alanine production in skeletal muscle. In skeletal muscle, *Bcat2* expression is activated by a transcription factor called Klf15, which is a GR primary target gene (Fig. 2) (Shimizu et al., 2011). With ChIP and reporter assay, two rat *Klf15* GREs were identified in the intron region between +479 and +494, and between +1014 and +1028. In addition to activating *Bcat2* transcription to reduce mTOR activity, Klf15 also elevates *MuRF1* and *MAFbx* transcription, with Klf15 binding sites identified in rat *Bcat2*, *MuRF1* and *MAFbx*. Adenoviral mediated overexpression of Klf15 in skeletal muscle ex vivo caused muscle atrophy, which confirmed the involvement of Klf15 in protein metabolism. Interestingly, Klf15 null mice have abnormal lipid and energy flux, excessive reliance on carbohydrate fuels, exaggerated muscle fatigue, and impaired endurance exercise capacity (Haldar et al., 2012). However, whether Dex-induced muscle atrophy is spared in *Klf15* null mice has not been reported.

3.9. Cblb

Cblb encodes an ubiquitin E3 ligase that stimulates the ubiquitination and degradation of IRS-1 (Fig. 2). In C2C12 myotubes, overexpressing Cblb attenuates IGF-1's ability to antagonize Dex-reduced myotube diameters and Dex-induced *MAFbx* gene expression, possibly due to Cblb's role in degrading IRS-1 thus suppressing IGF-1 action (Nakao et al., 2009). However, it is unclear whether reducing the expression of Cblb would compromise GC effects on myotube diameters and atrogene expression. Unloading-induced muscle atrophy is protected in *Cblb* null mice (Nakao et al., 2009). Whether *Cblb* null mice are protected from GC-induced muscle atrophy has not been reported. Notably, overexpressing Cblb did not affect myotube diameters or the expression of *MAFbx* gene. These results indicate that, unlike *Pik3r1* or *Ddit4*, the induction of *Cblb* alone is not sufficient to induce muscle atrophy.

3.10. Factors that modulate glucocorticoid effects on protein metabolism

In skeletal muscle, several signaling pathways are capable of suppressing the catabolic effects of GCs. It is not surprising that IGF-1 is one of them, as IGF-1 is the main anabolic signal in skeletal muscle. In fact, factors that modulate insulin/IGF-1 signaling pathway have potential to regulate the catabolic effects of GCs. For example, reducing the expression of Gsk3 β in C2C12 myotubes compromised Dex-reduced myofibrillar protein levels and *MAFbx* gene expression (Verhees et al., 2011). Androgens are another category of anabolic hormones that antagonize GC-induced muscle atrophy. Testosterone suppresses Dex-induced expression of *MuRF1*, *MAFbx*, *Pik3r1*, *Ddit4*, *4Ebp1* and *FoxO1* in vivo (Wu et al., 2010; Yin et al., 2009). Testosterone treatment alone reduces FoxO1 expression but exerts no effect on *Pik3r1* expression (Wu et al., 2010). Testosterone deprivation by castration in rats resulted in an increased expression of *MuRF1* and *MAFbx* in testosterone-sensitive fast-twitch pelvic levator ani muscle (Pires-Oliveira et al., 2010). Administration of testosterone

in these castrated rats then reduces the expression of these two genes. Androgen receptor (AR) binds to androgen response element (ARE) to modulate the transcription of its target genes. The consensus DNA sequence of ARE and GRE are very similar. The observations of these two transcription factors (AR and GR) that appear to be able to recognize the same DNA sequence yet exhibit opposite effects on protein metabolism in the same tissue (skeletal muscle) have puzzled endocrinologists for years. Notably, most endogenous AREs and GREs are composite elements that require the participation of other cis-acting elements and their trans-acting factors to carry complete hormonal responses. However, AR and GR are structurally distinct and likely interact with overlapping but different sets of proteins. Thus, a GRE in a specific genomic context may not be responsive to AR, because AR cannot interact with DNA-binding transcription factors occupying the cis-acting elements adjacent to this GRE. It would be intriguing to learn whether cis-acting elements co-localize with AREs and GREs are indeed distinct in myotubes. This question can be answered with ChIPseq assays.

4. Future directions

In the last two decades, much progress has been made to decipher how GCs regulate glucose and protein metabolism. Animal studies have confirmed the role of GR primary target genes, such as *MuRF1*, and a potential GR secondary target gene, *Myostatin*, in GC-induced muscle atrophy. It is clear that additional GR primary targets are involved in GC-regulated protein metabolism, as neither *MuRF1* nor *Myostatin* null mice are completely spared from effects of GCs. In fact, GC-induced protein degradation is not affected in *MuRF1* null mice. In addition to identifying additional GR primary targets involved in protein metabolism and mediate GC response, it is important to dissect in more detail how these identified GR primary target genes, such as *MuRF1*, mediate the physiological changes induced by GCs. There is still a sizable gap in our understanding linking known changes in gene expression to observed changes in glucose metabolism caused by GCs. To our knowledge, no GR primary target in skeletal muscle has been shown to confer GC-induced insulin resistance in vivo. Last but not least, whether GC-altered lipid metabolism results in increased levels of certain lipid intermediates to cause insulin resistance is another crucial issue awaiting future studies.

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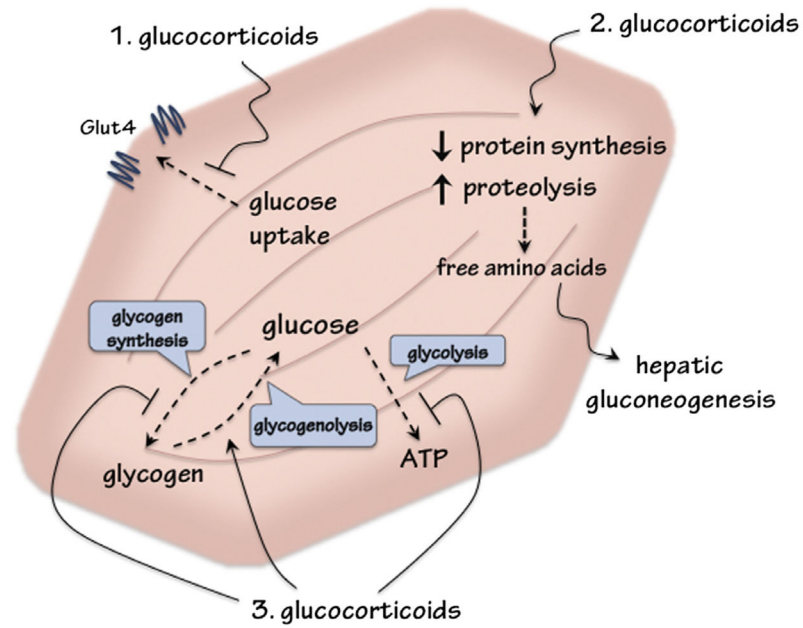


Fig. 1. Metabolic influences of glucocorticoids (GCs) in skeletal muscle to regulate glucose homeostasis. (1) GCs inhibit insulin-stimulated glucose uptake. (2) GCs decrease protein synthesis and increase proteolysis to release amino acids for hepatic gluconeogenesis. (3) GCs downregulate glucose utilization by inhibiting glycolysis. GCs also suppress glycogen synthesis, and act with catecholamine to upregulate glycogenolysis.

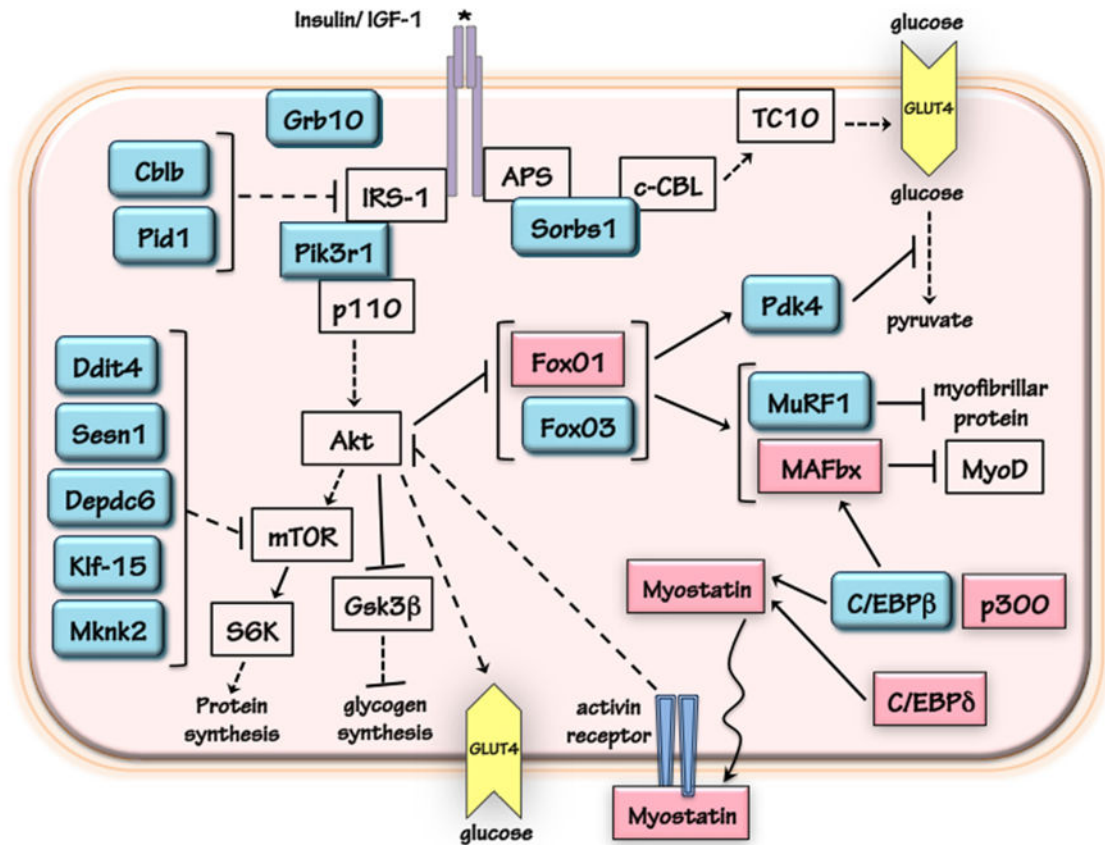


Fig. 2. The regulation of protein and glucose metabolism by glucocorticoid receptor (GR) in skeletal muscle cells. The genes in teal are GR primary target genes, and the expression of genes in magenta are upregulated by glucocorticoids with GRE not yet defined, if any. Dashed line represents the involvement of multiple steps. Arrow shows activation while bar indicates inhibition.