Expression of Glucocorticoid Receptors in the Regenerating Human Skeletal Muscle

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Running title: Human skeletal muscle glucocorticoid receptor expression

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Summary

Many stress conditions are accompanied by skeletal muscle dysfunction and regeneration, which is essentially a recapitulation of the embryonic development. However, regeneration usually occurs under conditions of hypothalamus-pituitary-adrenal gland axis activation and therefore increased glucocorticoid (GC) levels. Glucocorticoid receptor (GR), the main determinant of cellular responsiveness to GCs, exists in two isoforms (GRα and GRβ) in humans. While the role of GRα is well characterized, GRβ remains an elusive player in GC signalling. To elucidate basic characteristics of GC signaling in the regenerating human skeletal muscle we assessed GRα and GRβ expression pattern in cultured human myoblasts and myotubes and their response to 24-hour dexamethasone (DEX) treatment. There was no difference in GRα mRNA and protein expression or DEX-mediated GRα down-regulation in myoblasts and myotubes. GRβ mRNA level was very low in myoblasts and remained unaffected by differentiation and/or DEX. GRβ protein could not be detected. These results indicate that response to GCs is established very early during human skeletal muscle regeneration and that it remains practically unchanged before innervation is established. Very low GRβ mRNA expression and inability to detect GRβ protein suggests that GRβ is not a major player in the early stages of human skeletal muscle regeneration.

Key words: Myoblasts Myotubes Glucocorticoid receptors Stress

Introduction

Skeletal muscle is a prominent target for glucocorticoids (GCs) in health and disease. Although GCs constitute an essential part of systemic stress response, this often comes at the expense of the skeletal muscle. Chronically increased levels of (endogenous or exogenous) GCs can result in proteolysis, muscle wasting and overt myopathy, while shorter exposures to high GC concentrations in the intensive care setting have been implicated in the development of critical illness myopathy (Schweickert and Hall 2007, Menconi *et al*. 2007, Glass 2010). GCs may also adversely affect skeletal muscle during recovery from injury, which could be at least partly due to their antimyogenic effects in skeletal muscle precursors that participate in regeneration (Beiner *et al*. 1999, Almekinders *et al*. 1999, Ferrando 2000, Prelovšek *et al*. 2006, Dekelbab *et al*. 2007, Betters *et al*. 2008, Bruscoli *et al*. 2010). Perhaps paradoxically, GCs are currently the only effective pharmacological treatment for Duschenne muscular dystrophy, where degeneration-regeneration cycle is a prominent feature, and they were even reported to enhance differentiation in skeletal muscle precursors from mdx mice (Passaquin *et al*. 1993, Bushby *et al*. 2010, Bach *et al*. 2010, Matthews *et al*. 2010, Ciciliot and Schiaffino 2010).

Despite its importance, data on GC signalling during human skeletal muscle regeneration, which is characterized by satellite cell activation, myoblast proliferation, fusion into myotubes and later by (re)innervation and maturation into fully developed muscle fibers (Chargé and Rudnicki 2004, Ciciliot and Schiaffino 2010), remain incomplete. GC responsiveness of skeletal muscle precursors and adult fibers depends on the expression of the glucocorticoid receptor (GR), which has two splice variants (GRα and GRβ) in humans. Although the physiological role of the ligand-binding isoform GRα is very well established and characterized, the role of GRβ, which does not bind GCs and probably acts as a dominant

negative regulator of GRα, remains elusive and its expression in skeletal muscle has not been unequivocally confirmed (Hollenberg *et al*. 1985, Bamberger *et al*. 1995, Bamberger *et al*. 1996, Oakley *et al*. 1996, De Castro *et al*. 1996, Oakley *et al*. 1997, Oakley *et al.* 1999, Pujols *et al*. 2002). Different levels of GR expression in myoblasts and myotubes could lead to their differential GC responsiveness with potentially important functional consequences. By exerting their effects primarily on myoblasts, which are actively proliferating cells, GCs could primarily affect the number of myogenic cells formed in response to injury and/or their fusion into myotubes. On the other hand, in case of greater responsiveness in postmitotic myotubes, GCs could primarily disrupt protein synthesis and maturation into fully developed muscle fibers. In fact, myoblasts and myotubes were already reported to display differential responsiveness to tumour necrosis factor α and lipopolysaccharide (Prelovsek *et al*. 2006).

Our aim was to therefore to assess GRα and GRβ mRNA and protein expression pattern in skeletal muscle precursors during regeneration and to evaluate how GCs affect their expression level. Tissue response to GCs depends also on 11β-hydroxysteroid dehydrogenase type 1 and type 2, which under prevailing cellular conditions activate or inactivate, respectively, glucocorticoids (cortisol in humans and corticosterone in rodents) (Morton 2010). Type 1 11β-hydroxysteroid dehydrogenase seems to play an important role in skeletal muscle, but skeletal muscle expression of type 2 11β-hydroxysteroid dehydrogenase, whose main role is to protect mineralocorticoid target tissues from GC action, has been subject to conflicting reports (Whorlwood *et al*. 2002, Abdallah *et al*. 2005, Jang *et al*. 2007, Pirkmajer *et al*. 2010, Morton *et al*. 2010). In this study we focused on GRs since they are the main determinants of GC responsiveness (Burgeois and Newby 1979, Gehring *et al*. 1984).

Experiments were carried out on cultured human myoblasts and myotubes, which were treated for 24 hours with a synthetic GC dexamethasone (DEX). Human skeletal muscle culture is an established *in vitro* model of skeletal muscle regeneration, which can be used to

study either early stages (myoblast proliferation and myotube formation) in aneural culture or later stages if nerve-muscle co-culture is used (Askanas *et al*. 1987, Grubic *et al*. 1995, Mars *et al*. 2003, Prelovsek *et al*. 2006). The use of human tissue makes such studies even more relevant in clinical context, since characteristics of human and animal cultures differ in several important aspects (Askanas *et al*. 1987, Gajsek *et al*. 2008).

Methods

Human skeletal muscle cell culture

All the studies reported here were approved by the Ethical Commission at the Ministry of Health of the Republic of Slovenia (permit No: 63/01/99) muscle. Cultures were prepared as described in detail before (Askanas *et al*. 1987, Grubic *et al*. 1995, Mars *et al*. 2003, Prelovsek *et al*. 2006). Briefly, myoblast cultures were prepared from muscle tissue routinely discarded at orthopaedic operations from donors without neuromuscular disease. Muscle tissue was cleaned of connective and adipose tissue, cut to small pieces and trypsinised at 37°C to release muscle satellite cells. Isolated cells were grown in 100 mm Petri dishes (BD Falcon, Franklin Lakes, NJ) in growth medium (Advanced MEM supplemented with 10% (v/v) fetal bovine serum, 0.3% (v/v) Fungizone, 0.15% (v/v) Gentamicin (all obtained from Invitrogen, Paisley, UK)) at 37˚C in 5% CO_2-enriched air at saturation humidity. Myoblast colonies were selectively trypsinised just prior to fusion, transferred to 75 cm^2 cell culture flasks and were grown for 2-3 more passages before they were transferred into 6-well plates and then either used for experiments or differentiated into myotubes. Myogenic differentiation was induced by switching subconfluent myoblast cultures from growth medium to differentiation medium (Advanced MEM supplemented with 2% fetal bovine serum). In a few days myoblasts started to fuse and polynuclear myotubes were formed. Myotubes were used

for experiments after 3 weeks of differentiation. All experiments were carried out in polystyrene-treated 6-well plates (BD Falcon).

Dexamethasone treatment

Dexamethasone-phosphate (DEX) (Krka, Novo Mesto, Slovenia) was used to avoid solubility problems of dexamethasone. Myoblasts and myotubes were incubated with either 0.2 μM or 1 μM DEX for 24 hours. Control cultures were grown in parallel with treated cultures and were harvested at the same time.

Real-time PCR

Total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse transcribed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was performed on ABI PRISM SDS 7500 (Applied Biosystems, Foster City, CA, USA), in 96-well format. GRα and GRβ mRNA were determined with gene expression assays based on TaqMan chemistry. Assay that was used to detect GRα contained 800 nM forward (5′-GAAGGAAACTCCAGCCAGAA-3′) and reverse (5′-CAGCTAACATCTCGGGGAAT-3′) primers and 200 nM sense probe (6FAM-5′-GCTTCCAAACATTTTTGGATAAGACCAT-3′-TAMRA) (Melo *et al,* 2004). Gene expression assay Hs00354508_m1 (Applied Biosystems) was used to detect GRβ. Relative (ΔΔCt) quantification method was employed to assess expression levels of target genes and 18S rRNA (Applied Biosystems) was used as the endogenous control (see below).

Endogenous controls for real-time PCR

The ideal endogenous control should have a constant expression level regardless of experimental conditions. Although no such universal housekeeping gene exits, it is still

extremely important to find endogenous control that displays the lowest degree of variation in experimental system under consideration. We intended to study human skeletal muscle cells in two developmental stages (i.e. non-differentiated mononuclear myoblasts and differentiated myotubes) and their responses to DEX. Since myogenic differentiation and DEX both affect expression of many different housekeeping genes (Nishimura *et al*. 2008), it was therefore really vital to avoid artifacts due to changes in the endogenous control. We tested several commonly used endogenous controls (TaqMan Human Endogenous Control Plate, Applied Biosystems) and some of them showed profound differentiation-dependent or DEX-dependent changes (data not shown). 18S rRNA was chosen out of 11 commonly used housekeeping genes (acidic ribosomal protein (huPO), beta-actin (huBA), b2-microglobulin (hub2m), cyclophilin (huCYC), glyceraldehyde-3-phosphate-dehydrogenase (huGAPDH), b-Glucuronidase (huGUS), hypoxanthine ribosyl transferase (huHPRT), phosphoglycerokinase (huPGK), TATA binding protein (huTBP) and transferrin receptor (huTfR)) because it proved to be the most stable across all differentiation stages and treatments (data not shown).

Western Blot analysis

Cells were washed 2 times with ice-cold phosphate buffered saline and harvested in 200 µl lysis buffer (20 mM Tris, 1 mM EDTA, 10% (w/v) sucrose, 0,1% Triton X-100, and 1% (v/v) Protease Inhibitor Cocktail (all obtained from Sigma), pH=7.4). Insoluble components were removed by centrifugation (12.000 x g, 10 minutes, at 4^{o}C). Protein content was determined by the Lowry protein assay (Sigma). Protein samples, prepared in Laemmli buffer, were separated in 10% NuPage Novex Bis-Tris Gel (Invitrogen) by using XCell *SureLock* electrophoresis system (Invitrogen) and transferred to PDVF membrane (Millipore, Billerica, MA). Membranes were blocked in blocking buffer (0.2% (w/v) I-Block (Applied Biosystems), 0.3% (v/v) Tween 20 (Sigma) prepared in phosphate buffered saline). After an

overnight incubation at 4°C with appropriate primary antibodies membranes were incubated with alkaline phosphatase-conjugated secondary antibodies (Sigma). Blots were developed in 2% (v/v) NBT/BCIP (Roche, Mannheim, Germany) solution prepared in developing buffer (0.1 M Tris, 0.05 M $MgCl_2$, 0.1 M NaCl, pH 9.5). Quantifications were performed with Chemi Genius BioImaging System (Syngen, Cambridge, UK) and actin was used as the loading control. We used the following primary antibodies: GRα (rabbit polyclonal antibody PA1-516, ABR-Affinity BioReagents Inc, Rockford, IL; diluted 1:200)**,** GRβ (rabbit polyclonal antibody PA3-514, ABR-Affinity BioReagents Inc; diluted 1:200) and actin (rabbit polyclonal antibody SC-1616-R, Santa Cruz Biotechnology Inc., Santa Cruz, CA; diluted 1:1000). A representative Western Blot is shown below (Figure 1).

Statistical Analysis

Two-way ANOVA, followed by Bonferroni's t-test, or Student t-test (as appropriate) was used to test for differences amongst groups. Results are expressed as means ± SEM (standard error of the mean).

Results

Expression pattern of GRα and GRβ mRNA in myoblasts and myotubes

GRα and GRβ mRNA (Fig. 2) were expressed already at the level of non-differentiated mononuclear myoblast and differentiation into myotubes did not trigger any profound changes in their expression. There was a tendency towards an increase in GRα mRNA and a decrease in GRβ mRNA in myotubes compared to myoblasts, but these changes were not statistically significant ($p=0.63$ and $p= 0.19$, respectively). GRβ mRNA level was 3 orders of magnitude lower than GRα mRNA ($p<0.0001$) and was close to the limit of reliable

detection (35-40th cycle). Two-way ANOVA did not show interaction between differentiation stage and GR isoform expression (p=0.67).

Effect of dexamethasone on GRα expression in human myoblasts and myotubes

After establishing that both GR isoforms are expressed in myoblasts and myotubes, we evaluated GR response to DEX treatment. Myoblasts and myotubes treated for 24 hours with 0.2 μM or 1.0 μM DEX down-regulated GRα mRNA by 30-50% (Fig. 3). Response to 24-hour DEX treatment was not affected by the differentiation stage. Changes in GRα protein followed changes in GRα mRNA and GRα protein was reduced by 15-20% compared to Basal. GRα protein level tended to be lower in cells treated with 1.0 μM DEX compared to 0.2 μM DEX, but it did not reach the level of statistical significance.

Effect of dexamethasone on GRβ expression in human myoblasts and myotubes

GRβ mRNA was expressed at very low level in non-stimulated condition (Basal) and was not significantly affected neither by differentiation nor DEX treatment (Fig. 4). Interestingly there was a tendency towards a decrease in GRβ level in 0.2 μM DEX treated myoblasts and especially myotubes (although it was not statistically significant). GRβ protein could not be reliably detected neither in myoblasts nor myotubes. Western Blot is therefore not shown.

Discussion

We demonstrated that GRα and GRβ mRNA were expressed in human myoblasts and myotubes. However, the GRβ mRNA level was at least 1000-times lower than the GRα

mRNA and GRβ protein could not be detected. These results suggest that the predominant splicing pathway is the one leading to GRα mRNA, as has already been pointed out by Oakley et al. (1996). The alternative splicing event leading to GRβ mRNA is probably only a minor pathway during early skeletal muscle regeneration. Although GRβ was detected in skeletal muscle biopsies, our results are consistent with earlier studies, which showed either very low or no expression of GRβ in human skeletal muscle cultures (Whorwood *et al*. 2001, Pujols *et al*. 2002, De Bleecker *et al*. 2007). GRβ expression in skeletal muscle biopsies could in fact be mainly due to other cell types (i.e. not skeletal muscle fibers) (De Bleecker *et al*. 2007). However, methodological differences (Northern Blot vs. real-time PCR) could also explain failure to detect GRβ mRNA in some earlier papers. Like in our case, Pujols *et al*. (2002) were unable to detect GRβ protein although cultured skeletal muscle cells expressed GRβ mRNA.

GRα and GRβ level was relatively constant in myoblasts and myotubes and induction of differentiation did not have a major impact on expression of either isoform. This observation does not support the notion of differential GC sensitivity in myoblasts and myotubes. Our result that GR level was independent of differentiation stage was similar to the result in paper by Sun *et al*. (2008) who did not see major changes in GR expression during C2C12 myogenesis. In contrast, Aubry and Odermatt (2009) reported that C2C12 cells displayed a pronounced GR up-regulation during differentiation process.

24-hour DEX treatment down-regulated GRα mRNA with corresponding changes protein level. Although mRNA response was larger in relative terms, protein changes were entirely consistent. Moreover, kinetics of GR protein depends not only on the rate of transcription and translation but also on stability of the GR protein. These results are consistent with earlier reports from studies on human myoblasts (Whorwood *et al*. 2001) and L6 and C2C12 cells (Dekelbab *et al*. 2007, Menconi *et al*. 2008). Although changes in GRβ

mRNA expression were rather challenging to quantify due to its very low level, we could not see any effect of differentiation or DEX treatment, which was consistent with our earlier observation that GRβ mRNA in human myoblasts had not responded to DEX and/or hypoxia (Pirkmajer *et al*. 2010). These results contrast with earlier reports that GC treatment could induce GRβ in human skeletal muscle cell culture (Whorwood *et al*. 2001).

Results presented in this paper and our previous results (Pirkmajer *et al*. 2010) have established that early precursors of human muscle regeneration already possess an elaborate molecular machinery to respond to GCs. Since no differences in GRα expression pattern or DEX-mediated response could be observed in myoblasts and myotubes, this suggests that response to GCs at the receptor level is established very early and is not affected by myogenic differentiation program that takes place before the innervation of myotubes. Further studies are needed to evaluate the effect of innervation on GRα expression and function in skeletal muscle. Functional role of GRβ in early stages of human skeletal muscle regeneration remains questionable due to its very low expression level and apparent lack of GRβ protein.

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Legends to figures

Fig. 1. GRα protein expression in human myoblasts and myotubes (Western Blot).

A representative Western Blot from myoblasts (left column) and myotubes (middle column) grown in basal conditions is shown. Molecular weight marker (Marker) is shown on the right.

Fig. 2. GRα and GRβ mRNA expression in human myoblasts and myotubes (real-time PCR). GRα and GRβ mRNA levels are reported in relative units, where GRα mRNA level in myoblasts was arbitrarily chosen as 1. None of the differences in GR expression between myoblasts and myotubes was statistically significant. GRβ mRNA level was significantly lower in both differentiation stages (see text). 18S rRNA was used as the endogenous control. Columns are ± SEM (N=3-4).

Fig. 3. GRα mRNA and protein expression in DEX-treated human myoblasts and myotubes. Top panels represent GRα mRNA level (real-time PCR) in myoblasts (left) and myotubes (right) after 24 hours of DEX treatment. Corresponding changes in GRα protein expression (Western Blot) are shown in the bottom panels. 18S rRNA was used as the real-time PCR endogenous control. Columns are means ± SEM (N=3-5), *$p<0.05$ vs. Basal (t-test).

Fig. 4. **GRβ mRNA expression in DEX-treated myoblasts and myotubes.** GRβ mRNA level (real-time PCR) in myoblasts (left) and myotubes (right) after 24 hours of DEX treatment. 18S rRNA was used as the endogenous control. Columns are means ± SEM (N= 4). All the observed changes in GRβ level were statistically non-significant.

Figures

Fig.1

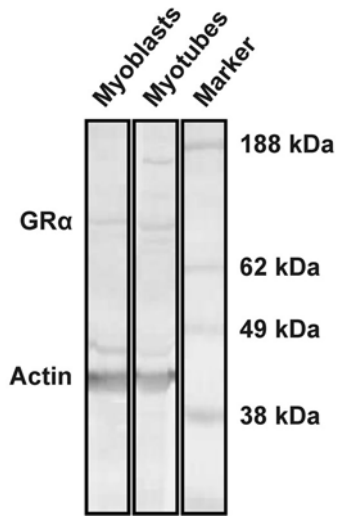


Fig. 2

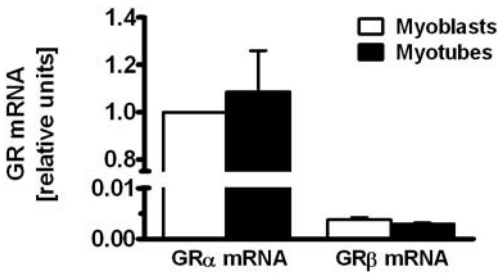

GR mRNA
[relative units]
1.4
1.2
1.0
0.8
0.01
0.00
Myoblasts
Myotubes
GRα mRNA
GRβ mRNA

Fig. 3

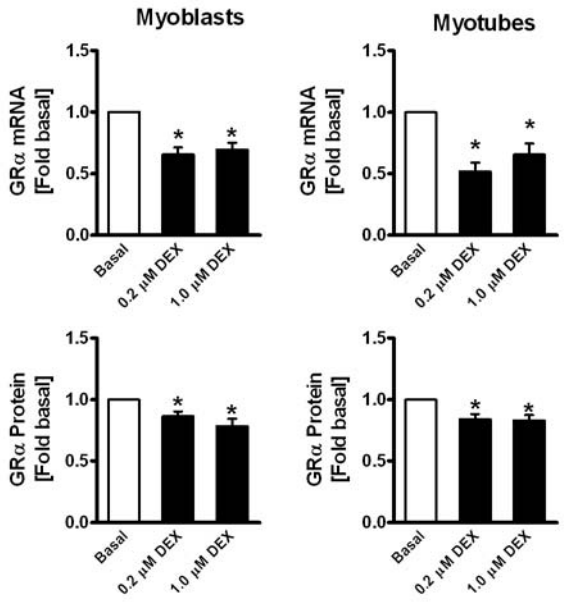
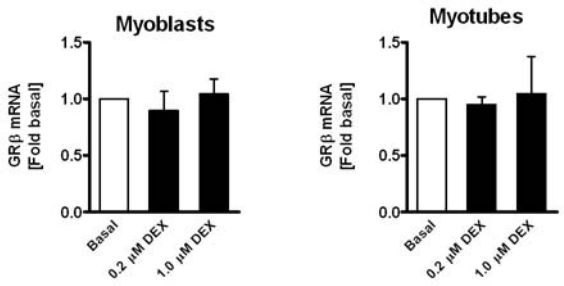

Myoblasts
Myotubes
GRα mRNA [Fold basal]
GRα Protein [Fold basal]
1.5
1.0
0.5
0.0
*
Basal
0.2 μM DEX
1.0 μM DEX

Fig. 4


Myoblasts
GRβ mRNA [Fold basal]
1.5
1.0
0.5
0.0
Basal
0.2 μM DEX
1.0 μM DEX
Myotubes
GRβ mRNA [Fold basal]
1.5
1.0
0.5
0.0
Basal
0.2 μM DEX
1.0 μM DEX