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Skeletal muscle: a dual system to measure glucocorticoid-dependent transactivation and transrepression of gene regulation

Ester Carballo-Jane[a], Shilpa Pandit[b,c], Joseph C. Santoro[b,c], Concetta Freund[b], Silvi Luell[a], Georgianna Harris[b], Michael J. Forrest[a], Ayesha Sitlani[b,c,*]

[a] *Merck Research Laboratories, Department of Pharmacology, P.O. Box 2000, Rahway, NJ 07065, USA*
[b] *Merck Research Laboratories, Department of Metabolic Disorders, P.O. Box 2000, Mail Code: R80Y-300, Rahway, NJ 07065, USA*
[c] *Merck Research Laboratories, Department of Atherosclerosis and Endocrinology, P.O. Box 2000, Mail Code: R80Y-300, Rahway, NJ 07065, USA*

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Abstract

The use of chronic glucocorticoid (GC) therapy for the treatment of inflammatory diseases is limited by associated metabolic side effects, including muscle atrophy. Therefore, selective glucocorticoid receptor-(GR)-binding ligands that maintain anti-inflammatory activity and demonstrate diminished side-effect profiles would have great therapeutic utility. In this work, we use Taqman PCR and ELISA methods to show that GCs can inhibit basal, and lipopolysaccharide (LPS)-stimulated levels of cytokines IL-6 and TNFα, and also the chemokine MCP-1 in a non-inflammatory system such as primary human skeletal muscle cells. In the murine C2C12 skeletal muscle cell line we observe a similar effect of GCs on IL-6 and MCP-1; however, in contrast to previous reports, we observe a time-dependent repression of TNFα. Furthermore, in skeletal muscle cells, concomitant with cytokine repression, GCs transcriptionally induce glutamine synthetase (GS), a marker for muscle wasting, in an LPS independent manner. Similarly, administration of dexamethasone to mice, previously administered LPS, results in an increase in GS and an inhibition of TNFα and MCP-1 in skeletal muscle tissue. Thus, skeletal muscle cells and tissues present a novel system for the identification of selective GR-binding ligands, which simultaneously inhibit cytokine expression in the absence of GS induction.

Keywords: Glucocorticoid receptor; Dexamethasone; TNFα; IL-6; MCP-1; Glutamine synthetase; Skeletal muscle

1. Introduction

Glucocorticoids (GCs) are extensively used for the suppression of inflammation in chronic inflammatory diseases such as asthma, rheumatoid arthritis, and several autoimmune diseases, all of which are associated with increased expression of inflammatory genes [1–4]. GCs bind to the glucocorticoid receptor (GR), a transcription factor protein that inhibits expression of multiple inflammatory genes including cytokines, enzymes, receptors, and adhesion molecules [2,5]. Anti-inflammatory effects of glucocorticoids are believed to derive from their combined effects at multiple cell types including cells of the immune system (e.g. macrophages, mononuclear cells and T-lymphocytes), epithelial and endothelial cells [3,4,6]. The mechanism of GC-induced downregulation of cytokines, whose promoters often lack GR binding elements (GREs), is believed not to occur predominantly through a direct GR–GRE interaction. Instead, several lines of evidence indicate that the inhibitory activity of GR arises from a direct interaction between activated GRs and other transcription factors such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), both of which regulate inflammatory gene expression [2,3]. In addition to inducing a beneficial anti-inflammatory effect, GC-bound GR directly interacts with promoter elements and regulates genes to promote undesirable side effects in metabolic tissues including liver, adipose tissue and muscle [7–11].

Chronic glucocorticoid treatment often leads to severe muscle catabolism and impairment of muscle glucose utilization, phenotypes that characterize a diabetic state. The mechanism of GC-induced skeletal muscle atrophy is not well understood; however, recent work has focused on GC-dependent upregulation of two genes: myostatin (MyoS), a member of the TGFβ growth factor family, and the enzyme glutamine synthetase (GS), both implicated in muscle degradation. A lack or suppression of functional MyoS expression has been associated with an increase in

* Corresponding author. Tel.: +1-732-594-0788; fax: +1-732-594-1169.
E-mail address: ayesha_sitlani@merck.com (A. Sitlani).

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muscle mass [12–14] and an increase in the expression levels of MyoS by glucocorticoids is associated with muscle degeneration and weight loss [15]. The precise link between GS, an enzyme that converts glutamic acid to glutamine, and muscle atrophy is not clear; however, it has been shown that concentrations of cellular glutamine are regulated in response to catabolic states. In rat skeletal muscle cells and tissues, glucocorticoids transcriptionally upregulate GS through a direct GR–GRE interaction in the promoter of the GS gene [16,17]. In addition, glutamine has a negative feedback effect on GS expression and loss of body weight. Specifically, infusion of the dipeptide alanyl-glutamine in rats treated with GCs significantly suppressed GC-induced GS mRNA, total body weight and muscle loss [18–20].

To overcome the side effects of GCs, there is a significant pharmaceutical interest to develop selective GCs, with improved therapeutic profiles [1,21,22]. For example, one type of selective GC would be a ligand that induces potent anti-inflammatory activity with reduced or no associated muscle wasting effects. It is likely that such a ligand would promote cytokine downregulation (transrepression activity) with diminished levels of GS upregulation (transactivation activity). Given the complex and cell-specific transcriptional profiles induced by GCs [23], we directed our efforts at developing a simple system in which both the endogenous transrepression and transactivation activities of genes regulated by GCs could be simultaneously and readily measured in skeletal muscle cells. Recent reports have shown that primary human myoblasts express high basal levels of cytokines and chemokines and these can be regulated further by pro-inflammatory cytokines [24–27]. In addition, it was observed that lipopolysaccharide (LPS), in a concentration-dependent manner, can induce TNFα and IL-6 mRNA in mouse myoblasts and skeletal muscle [28]. In the present study, we have investigated the broader effects of GCs on cytokines and chemokines in human and murine skeletal muscle systems, both in the absence and presence of LPS stimulation. In addition, we have extended our previous work on GS upregulation in human skeletal cells [29] to characterize the GC-dependent induction of GS, in the presence and absence of LPS, in murine skeletal muscle cells and tissue.

2. Materials and methods

2.1. Cell culture

Murine skeletal muscle cells C2C12 were obtained from American Type Culture Collection (Rockville, MD) and grown in medium A (Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin) at 37 °C in 5% CO_2. FBS was from Gemini Bioproducts (Woodland, CA). Primary human skeletal muscle cells (HSMC) were obtained from Clonetics (San Diego, CA) and cultured and maintained in medium A.

2.2. Glutamine synthetase activity assay

In this work, we use a whole cell culture colorimetric assay to measure the γ-glutamyl transferase (GT) activity of GS. This assay uses absorbance ($\lambda = 540$ nM) to measure GS catalyzed conversion of L-glutamine to glutamyl-γ-hydroxamate, a salt which forms a purplish brown complex in the presence of trivalent iron [30,31]. This method has been used to measure corticosteroid-induced GS-catalyzed GT activity in rat and human skeletal muscle cells and in extracts of rat skeletal muscle tissue [29,32]. To measure time-dependent dexamethasone induction of GS in C2C12, a 96-well plate containing 40,000 cells per well (in glutamine free DMEM media containing 10% charcoal stripped FBS, 100 units/ml penicillin, and 100 μg/ml) was treated with dexamethasone (100 nM) at different time points. Similarly, dose-dependent titrations of dexamethasone and prednisolone were carried out by treating the cells for 16–24 h with varying amounts of compound. At the end of the incubation period, cells were lysed and GS activity measured as described above [29]. The specific activity of GS is expressed as nmol/(min g) of total protein. Absorbance values of the product glutamyl-γ-hydroxamate (γ-GH) formed in the enzyme assay are converted to nanomoles of product by a calibration concentration curve obtained using commercially available γ-GH. The total protein in cell lysates prepared for the enzyme activity assay is measured using the Pierce Coomassie Plus Assay reagent kit based on the Bradford method of protein determination [33]. Measured protein concentration values were adjusted for cell number and volumes of dilution.

2.3. Cell treatment for Taqman experiments

Skeletal muscle cells (C2C12, or HSMC) were plated in 96-well plates at a density of 4×10^4 cells per well in medium A. The following day, the media was switched to medium B (DMEM containing 10% charcoal stripped FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin). Varying concentrations of dexamethasone, dissolved in DMSO (both from Sigma, St. Louis, MO), was added to the wells at a final DMSO concentration of 0.5% in the media. The cells were incubated with GCs for 30 min, followed by the addition of LPS from *E coli* serotype 0111:B4 (Sigma), which was added to selected cultures, to a final concentration of 100 ng/ml. After 4–24 h of treatment, the experiment was stopped by cell lysis with the addition of Trizol reagent (Life Technologies, Rockville, MD) as described below. For time course experiments, a single dose of dexamethasone or prednisolone was added to the cultures, and RNA extracted at different times.

2.4. RNA preparation

Total RNA was extracted from skeletal muscle cells or tissues with Trizol reagent according to the protocol

provided by the manufacturer. The concentration of extracted RNA was determined spectrophotometrically by measuring the absorbance of the sample at 260 nm on a SPECTRAmax Plus microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). RNA (10 μg) was then DNase treated, to remove contaminating genomic DNA, using a DNA-free™ kit (Ambion, Austin, TX). This procedure accomplishes DNase removal by a procedure that does not require phenol/chloroform extraction, alcohol precipitation, heating or the addition of EDTA. The concentration of RNA obtained after DNase treatment was then quantitated spectrophotometrically as described above.

2.5. Taqman real-time reverse transcriptase (RT)-PCR assay

PCR primers and Taqman probes for mouse and human GS were designed based on published cDNA sequences (Genbank accession numbers: U09114 for mouse GS and NM_002065 for human) for these genes using the software program Primer Express™ 1.0 (PE Applied Biosystems, Foster City, CA). The primer and probe sequences for glutamine synthetase are as follows: murine glutamine synthetase, forward primer 5′-GGACATCGTGGAGGCTCACT-3′ (624–644 bp); reverse primer 5′-GCATTTGTCCCCGTAATCTTG-3′ (689–668 bp); probe (sense) 5′-CCGGGCCTGCTTGTATGCTGGAG-3′ (645–668 bp); human glutamine synthetase, forward primer 5′-GGAGGCCATTGAGAAACTAAGC-3′ (812–832 bp); reverse primer 5′-GGAGGTTTCATGGAATCCAGTTA-3′ (1040–1018 bp); probe (sense) 5′-ACCACATCCGTGCCTATGATCCCAAG-3′ (967–992 bp). Primers and probes for human and mouse TNFα, IL-6, and MCP-1 were obtained as pre-developed assay reagents from PE Applied Biosystems. The Taqman probes were labeled with a reporter fluorescent dye, FAM (6-carboxyfluorescein) at the 5′-end, and a fluorescent dye quencher, TAMRA (6-carboxy-tetramethyl-rhodamine) at the 3′-end.

Expression levels of specific mRNAs were quantitated using quantitative fluorescent real-time polymerase chain reaction (RT-PCR). RNA is first reverse transcribed using random hexamers in a protocol provided by the manufacturer (PE Applied Biosystems). The RT reaction contains 500 ng of total RNA in a total volume of 50 μl containing Taqman RT buffer, 5.5 mM $MgCl_2$, 500 μM of each dNTP, 2.5 μM random hexamers, 0.4 U/ml RNase inhibitor and 1.25 U/l of multiscribe reverse transcriptase. The RT reaction was carried out at 25 °C for 10 min, 48 °C for 30 min, and 95 °C for 5 min in a PTC-200 peltier thermal cycler (MJ Research, Waltham, MA).

The cDNA obtained from the RT reaction was used for TaqMan PCR amplification. TaqMan PCR was carried out in duplicate for each sample in a 96-well plate using TaqMan Universal PCR master mix (PE Applied Biosystems) with corresponding primers and TaqMan probes. PCR reactions were done at 50 °C for 2 min, and 95 °C for 10 min for 1 cycle, then 95 °C for 15 s and 60 °C for 1 min for 40 cycles on an ABI PRISM 7700 sequence detection system (PE Applied Biosystems).

The principle of real time detection was based on the fluorogenic 5′-nuclease assay [34]. During the PCR reaction, the AmpliTaq Gold DNA polymerase cleaves the Taqman probe at the 5′-end and separates the reporter dye from the quencher dye only if the probe hybridizes to the target. This fluorescent signal generated by the cleaved reporter dye is directly monitored by the ABI PRISM 7700 detection system. The increase in the fluorescence signal is proportional to the amount of the specific PCR product. Amplification of the gene for rodent GAPDH (for mouse) or human 18S ribosomal protein was carried out for each sample to control for variations in absolute RNA levels. The absence of contaminating genomic DNA in the samples is demonstrated by the lack of a fluorescent signal observed in PCR reactions carried out in the absence of reverse transcriptase. After normalizing for levels of the control gene, gene-specific mRNAs are presented as fold increases for stimulated cells (or treated animals) compared with unstimulated cells (or vehicle animals). Unless otherwise specified all the data are represented as means of three experiments with the error bars representing standard deviations. The data are considered significant for *P*-values less than 0.05.

2.6. Cytokine determination

Skeletal muscle cells (HSMC or C2C12) were grown to sub-confluence in medium A. Cultures were trypsinized and cells plated in fresh medium in 48-well plates (100,000 cells per well) for 24 h to allow attachment. After 24 h, culture medium was replaced by fresh medium containing DMSO, GCs or RU486 (Sigma), at the concentrations indicated in the figures. The cells were incubated with GCs for 30 min, followed by the addition of LPS, which was added to selected cultures, to a final concentration of 100 ng/ml and the incubation carried out for 24 h. Supernatants were harvested and either immediately analyzed by ELISA, or frozen at −80 °C until used. TNFα and IL-6 were measured by standard ELISA methodology, using the DuoSet ELISA development kits from R&D Systems (Minneapolis, MN), according to the manufacturer's specifications.

2.7. Mouse LPS challenge

Female Balb/c mice (12–16 weeks old) from Taconic (Germantown, NY) were used in these studies. Mice were housed in a controlled environment, on a 12-h light/dark cycle, for at least 4 weeks prior to the studies, and were fed water and standard chow ad libitum. In the morning of the study, mice were dosed orally with dexamethasone (Sigma), prepared in 10% Tween 80 (Fisher Scientific, Fair Lawn, NJ) in water. Mice were challenged 2.5 h later by intraperitoneal injection of LPS (10 μg/mouse) and D-galactosamine (800 mg/kg, Sigma). Mice were euthanized 90 min later by

CO_2 inhalation and heparinized blood was collected by cardiac puncture. Skeletal muscle (gastrocnemius) was harvested and flash-frozen in liquid nitrogen. Muscle samples were kept frozen at −80 °C until processed for RNA. Plasma samples were assayed for TNFα and IL-6 by an ELISA method as described above. The Institutional Animal Care and Use Committee at Merck Research Laboratories approved all experiments.

3. Results

3.1. Glucocorticoids repress basal and stimulated IL-6 in primary human skeletal muscle cells

Primary HSMC expressed high levels of basal IL-6 mRNA, as measured by Taqman PCR, and dexamethasone concentration-dependently downregulated IL-6 mRNA expression (Fig. 1A). Stimulation of HSMC cells with LPS (100 ng/ml for 4 h) increased the expression of IL-6 mRNA (Fig. 1B) and treatment with dexamethasone (Fig. 1B) concentration dependently prevented the accumulation of IL-6 mRNA. The clinically used glucocorticoid prednisolone had similar inhibitory effects on basal and stimulated IL-6 mRNA in these cells (data not shown).

The effects of glucocorticoids on IL-6 mRNA translated to a repression of IL-6 protein secretion by HSMC cells under both basal and LPS-stimulated conditions (Fig. 1C and D). Interestingly, different batches of HSMC cells (derived from different donors) produced significantly different levels of IL-6 protein under non-stimulated conditions (compare DMSO column in Fig. 1C with control column in Fig. 1D). In addition, HSMC cells cannot tolerate many passages in culture medium, and the accumulation of IL-6 decreases as cells reach senescence (data not shown). Regardless of the different levels of IL-6 expression in different donors, treatment of the cells with dexamethasone (Fig. 1C) or prednisolone (data not shown) showed significant concentration-dependent reductions in the accumulation of IL-6 in all donors. Moreover, in the LPS-treated cells GCs

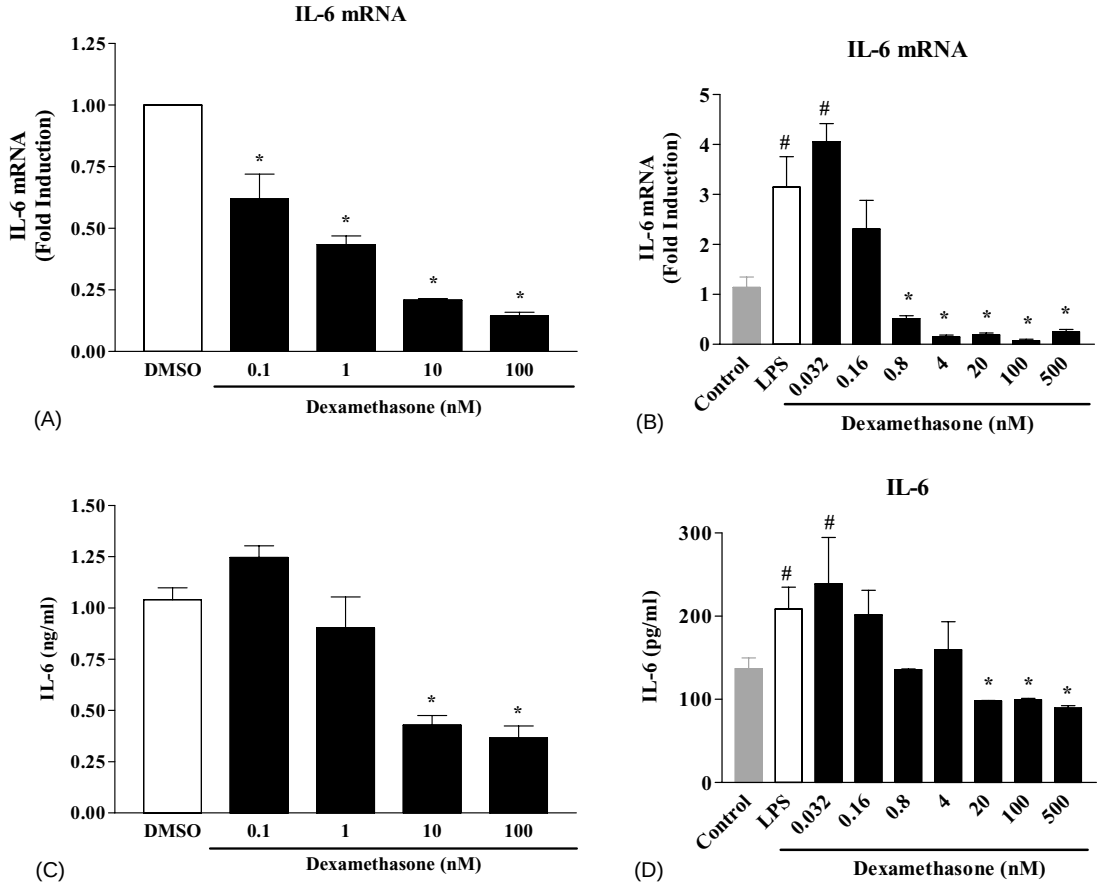

Fig. 1. Dexamethasone regulates the expression of IL-6 in HSMC cells. (A) Un-stimulated HSMC cells, cultured for 24 h, express basal IL-6 mRNA as measured by Taqman PCR. IL-6 mRNA levels are down regulated by dexamethasone, in a concentration-dependent manner ($^{*}p < 0.05$ vs. DMSO). (B) LPS (100 ng/ml) treatment of HSMC cells for 4 h significantly upregulates the expression of IL-6 mRNA (three-fold) in HSMC cells, and dexamethasone dose-dependently represses IL-6 mRNA to levels below the control ($^{\#}p < 0.05$ vs. CT, $^{*}p < 0.05$ vs. LPS). (C) Un-stimulated HSMC cells secrete IL-6 protein (as measured by ELISA) at basal conditions, and treatment dexamethasone for 24 h dose-dependently inhibits IL-6 protein secretion ($^{*}p < 0.05$ vs. DMSO). (D) LPS-stimulated (100 ng/ml) IL-6 protein secretion is repressed by dexamethasone to levels below the control ($^{\#}p < 0.05$ vs. CT, $^{*}P < 0.05$ vs. LPS). These experiments are representative of at least two independent studies.

repressed IL-6 mRNA and protein to levels below basal levels of expression (Fig. 1B and D).

3.2. Glucocorticoids repress basal and LPS-stimulated IL-6 in the murine skeletal muscle cell line C2C12

C2C12 cells, when cultured for 24 h in the absence of LPS, produced very modest amounts of basal IL-6 in the culture medium (<100 pg/ml), which made it difficult to evaluate inhibitory effects of glucocorticoids by an ELISA method (data not shown). However, treatment of C2C12 cells with 100 ng/ml of LPS (Fig. 2A) or 10 ng/ml of TNFα (data not shown) for 4 h resulted in a three-fold induction of IL-6 mRNA, which could be inhibited by pre-treatment with dexamethasone or prednisolone (Fig. 2A). Compared to the LPS response in HSMC cells, treatment of C2C12 cells with LPS (100 ng/ml) resulted in a relatively robust and reproducible increase in the amount of IL-6 protein secreted (Fig. 2B). Pre-treatment of the cells with dexamethasone (Fig. 2B and C) or prednisolone (Fig. 2B) resulted in significant concentration-dependent inhibition of IL-6 accumulation. Furthermore, the GR antagonist RU486 inhibited the effect of dexamethasone on IL-6 accumulation in C2C12 cells (Fig. 2C) and HSMC cells (data not shown) in a concentration-dependent manner.

3.3. Effects of glucocorticoids on TNFα and MCP-1 in skeletal muscle cells

In HSMC and C2C12 cells, the basal levels of TNFα mRNA were very low for detection by Taqman (data not shown). However, LPS induced a robust expression of TNFα mRNA, the levels of which were concentration-dependently repressed by dexamethasone or prednisolone (Fig. 3A and B). Interestingly, TNFα protein was not detectable by ELISAs both in basal and LPS-stimulated C2C12 cells (data not shown). Under the same conditions, in HSMC (Fig. 3C) and C2C12 cells (data not shown) LPS also induced mRNA for the chemotactic factor MCP-1, and this induction was significantly inhibited by treatment with dexamethasone

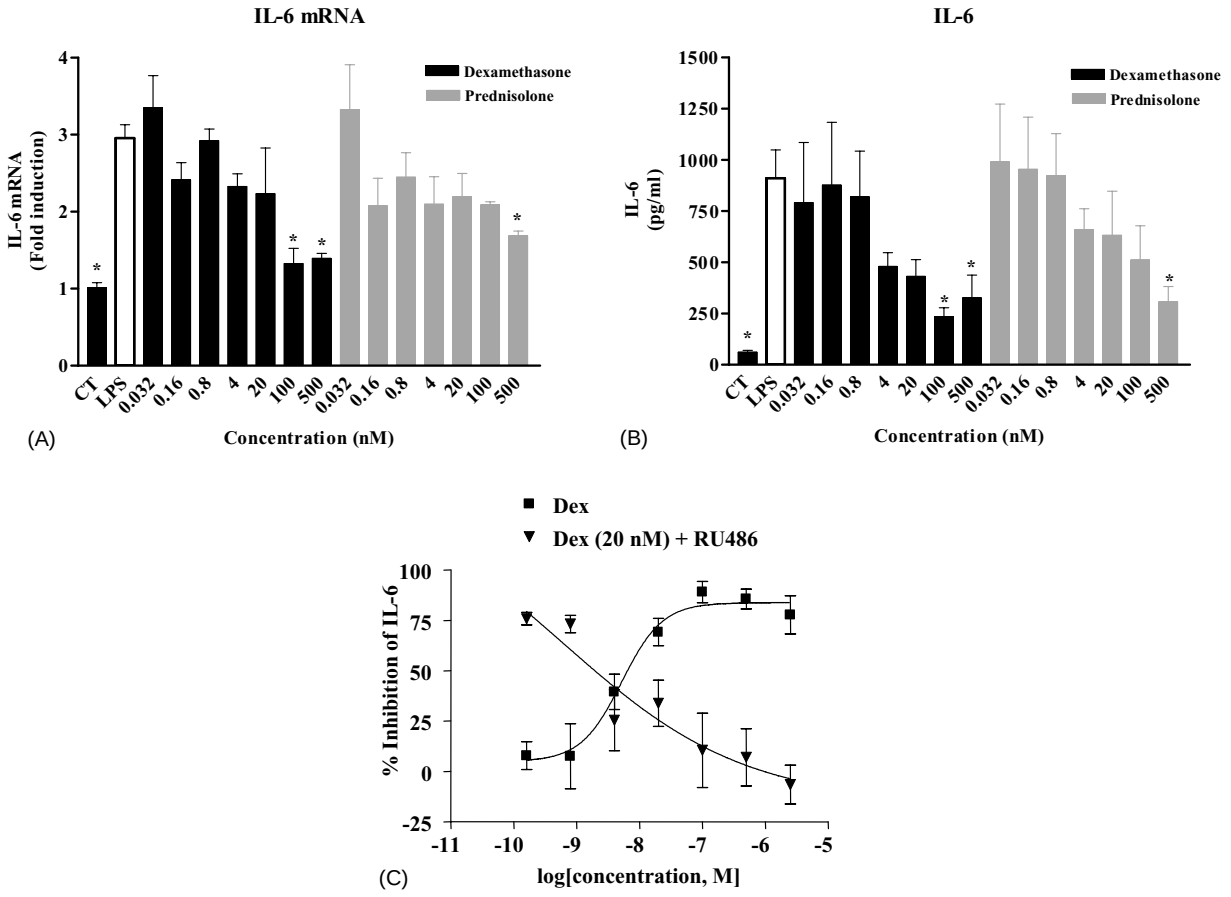

Fig. 2. Glucocorticoids regulate the expression of IL-6 in stimulated C2C12 cells. (A) LPS (100 ng/ml) treatment of C2C12 cells for 4 h upregulates the expression of IL-6 mRNA three-fold. Pre-treatment of the cells with dexamethasone or prednisolone (30 min prior to LPS addition) dose-dependently represses the LPS-dependent expression of IL-6 mRNA to control levels. (B) LPS-stimulated (100 ng/ml) IL-6 protein secretion (measured by ELISA) is repressed by dexamethasone or prednisolone to basal levels ($^{*}p < 0.05$ vs. LPS). (C) Dexamethasone inhibits the accumulation of IL-6 protein in C2C12 cells in a concentration-dependent manner. RU486 concentration-dependently prevents the downregulation of IL-6 production by dexamethasone (20 nM). These experiments are representative of three independent studies.

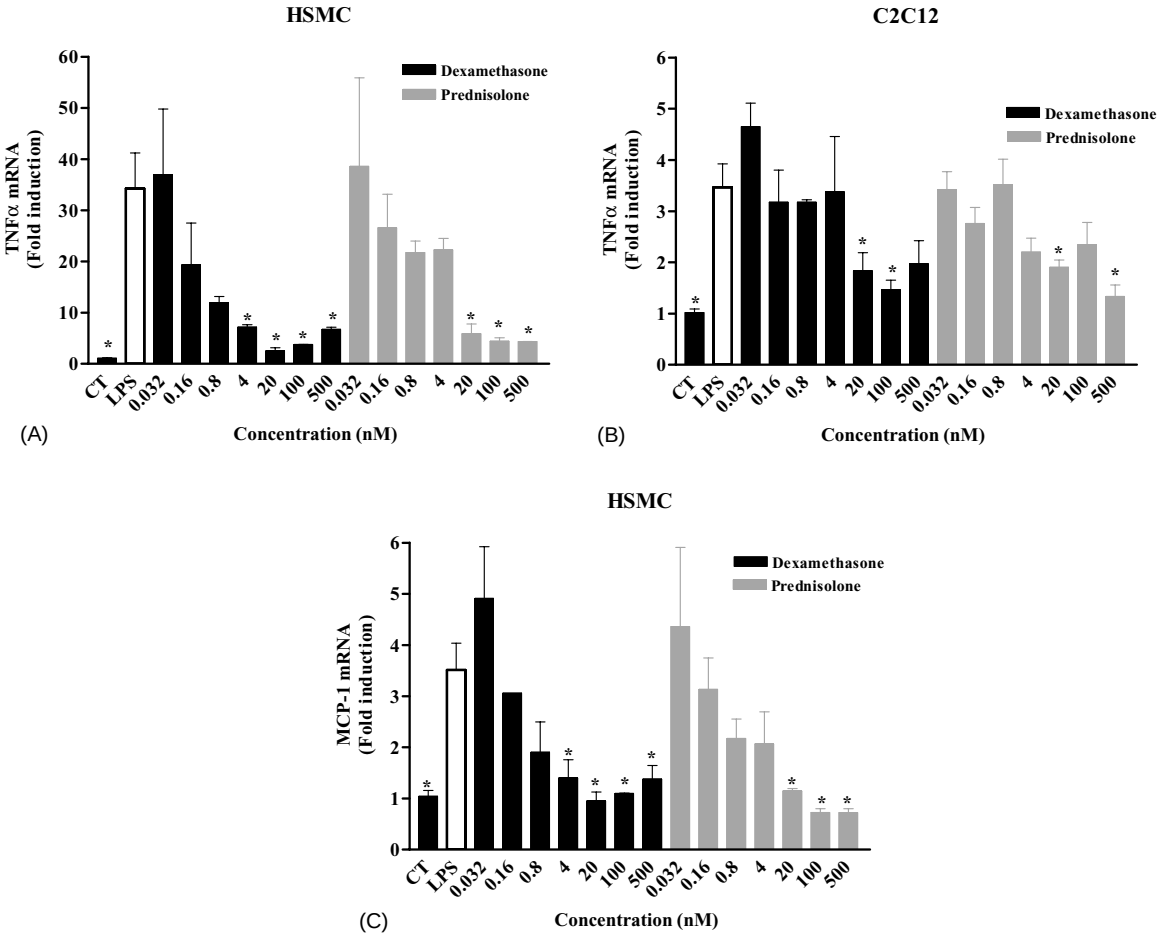

Fig. 3. Glucocorticoids regulate TNFα and MCP-1 expression in skeletal muscle cells. LPS (100 ng/ml) treatment of HSMC cells (A) and C2C12 cells (B) for 4 h significantly upregulates the expression of TNFα mRNA, and pre-treatment of the cells with dexamethasone or prednisolone promotes a dose-dependent repression of TNFα mRNA to un-stimulated levels (* $P < 0.05$ vs. LPS). (C) In HSMC cells, LPS induces the expression of MCP-1 mRNA ~3.5-fold and dexamethasone or prednisolone inhibits the effect of LPS in a concentration-dependent manner. * $P < 0.05$ vs. LPS. These experiments are representative of three independent studies.

or prednisolone. Moreover, high basal (1500 pg/ml) and LPS-stimulated (~10,000 pg/ml) levels of MCP-1 protein were detected in C2C12 cells, and dexamethasone concentration-dependently repressed MCP-1 protein levels (data not shown).

3.4. Dexamethasone transcriptionally induces glutamine synthetase in skeletal muscle cells in an LPS-independent manner

The treatment of C2C12 cells with dexamethasone at 100 nM time–dependently induced GS mRNA with a peak induction observed at ~10 h (Fig. 4A). The induction of GS activity by dexamethasone (100 nM) was time-dependent, with a peak induction observed at ~24 h (Fig. 4B). Following 16 h of cell treatment, GCs concentration-dependently upregulated GS mRNA (Fig. 4C) and GS activity (Fig. 4D). Importantly, LPS does not induce GS mRNA expression (Fig. 5A) and the GC-dependent induction of GS mRNA (Fig. 5B) and protein (data not shown) is not affected by LPS treatment in C2C12 cells. The EC_{50} for dexamethasone induction of GS mRNA in C2C12 cells cultured in the absence of LPS was 6 nM and in the presence of LPS, the EC_{50} was 1.6 nM; this difference in the EC_{50} values was not statistically significant. Similar results were observed in HSMC cells (data not shown).

3.5. In vivo effects of dexamethasone on inflammatory cytokines and GS

The LPS/D-galactosamine challenge model in mice [35–37] is a widely used model of acute inflammation, and has been shown to be sensitive to the effect of GCs [38]. Fig. 6 shows the plasma levels of TNFα and IL-6 in the LPS/D-galactosamine challenged mice. Basal levels of both cytokines were undetectable in plasma (data not shown). Dexamethasone, dosed 2.5 h before the LPS/D-Gal challenge, reduced the accumulation of TNFα in plasma, and, to a lesser extent, that of IL-6 (Fig. 6). Total RNA was prepared from skeletal muscle isolated from the animals used

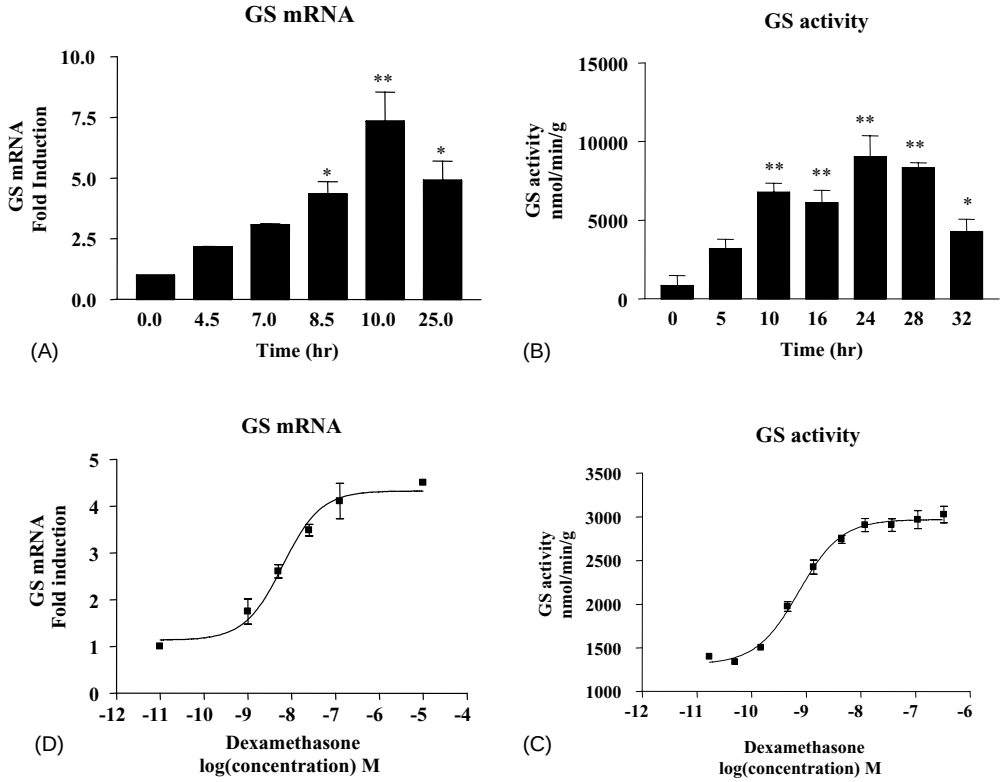

Fig. 4. Dexamethasone regulates glutamine synthetase in C2C12 cells. Treatment of C2C12 cells with dexamethasone (100 nM) promotes a time-dependent induction of GS mRNA (A), and GS activity (B) ($^{*}P < 0.01$ vs. $t = 0$ h, $^{**}P < 0.001$ vs. $t = 0$ h). Treatment of C2C12 cells with dexamethasone for 16 h promotes a dose-dependent increase of GS mRNA (C) and GS activity (D).

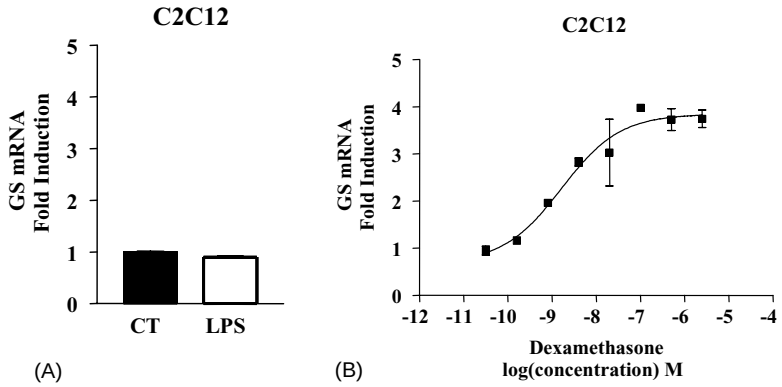

Fig. 5. LPS-independent induction of GS mRNA by dexamethasone in C2C12 cells. (A) LPS (100 ng/ml) alone has no effect on basal levels of GS mRNA in C2C12 cells (compare CT and LPS marked lane). (B) Treatment of these cells with increasing concentrations of dexamethasone in the presence of LPS induced a dose-dependent increase of GS mRNA ∼ four-fold. A similar fold induction is observed when these cells are treated with dexamethasone in the absence of LPS (Fig. 4C). These experiments are representative of three independent studies.

in these studies, and mRNA levels of TNFα, IL-6 and GS mRNAs were quantitated in these samples by Taqman-PCR methodology. As shown in Fig. 7A, LPS treatment did not affect the level of expression of GS in skeletal muscle (consistent with our in vitro results), whereas dexamethasone significantly increased GS mRNA expression. In contrast, LPS treatment resulted in a 50–75-fold induction of TNFα, MCP-1 and IL-6 mRNAs in skeletal muscle (Fig. 7B–D). Pre-treatment with dexamethasone resulted in a partial but significant prevention in the LPS-induced increase in TNFα and MCP-1 mRNAs and a modest (not significant) effect on IL-6 mRNA; however, in contrast to the in vitro results in C2C12 cells, complete inhibition of LPS-induced increase of these genes by dexamethasone was not observed.

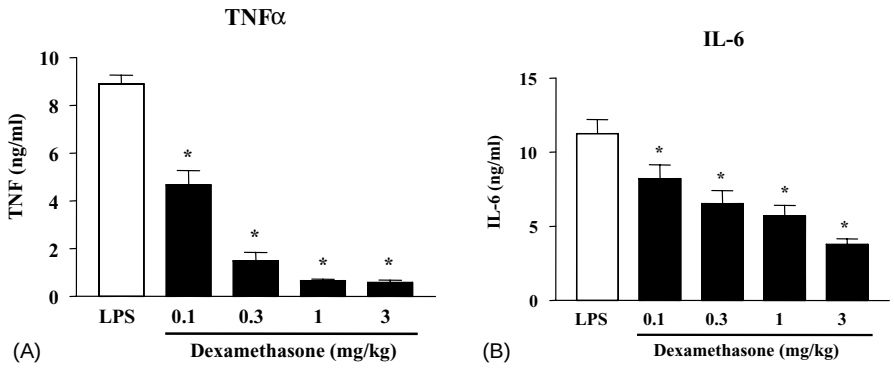

Fig. 6. Dexamethasone regulates LPS-induced cytokine accumulation in mouse plasma. Mice intra-peritoneally (IP) dosed with 10 μg of LPS plus 800 mg/kg of D-Galactosamine (D-Gal) for 90 min accumulate TNFα (A) and IL-6 (B) in their plasma. Pre-dosing mice with dexamethasone for 2.5 h prior to LPS treatment promotes a dose-dependent and significant ($^{*}p < 0.05$) repression of plasma protein levels of TNFα (A) and IL-6 (B). Neither cytokine is detectable in the plasma of animals not challenged with LPS (data not shown). This experiment is representative of two independent studies with $n = 8$ animals per group.

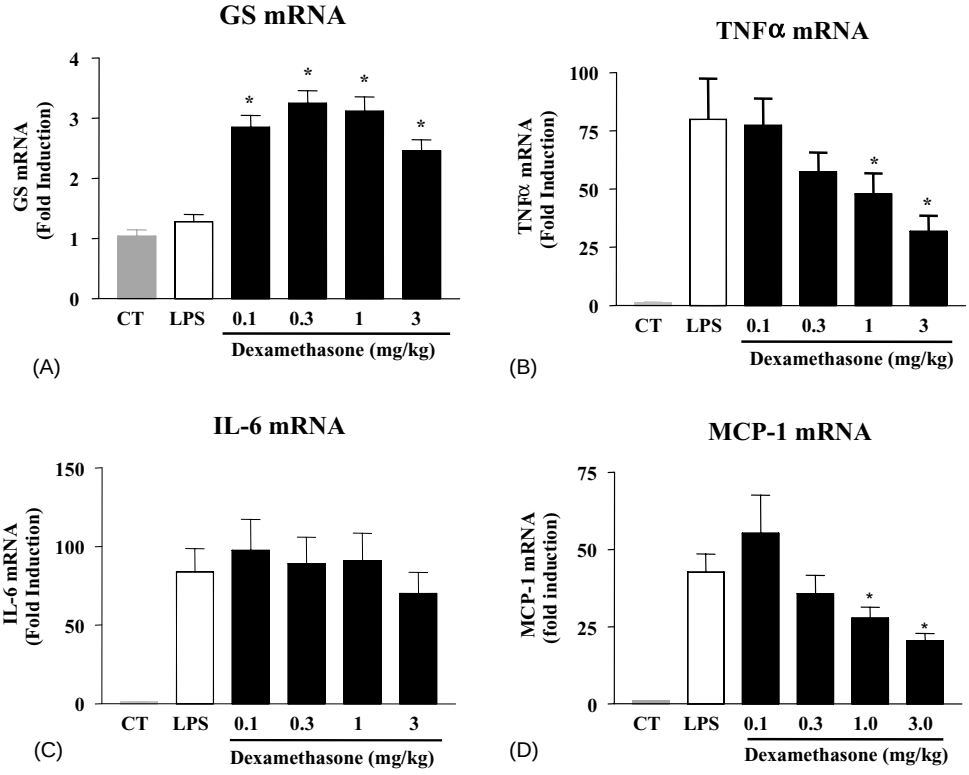

Fig. 7. Mouse skeletal muscle as a dual system to measure GC-dependent transactivation and transrepression. Skeletal muscle tissues harvested from LPS-challenged and dexamethasone treated mice were processed for GS mRNA (A), TNF-a mRNA (B), IL-6 mRNA (C), and MCP-1 mRNA (D). (A) LPS does not alter the basal expression of GS mRNA in mouse skeletal muscle (compare CT and LPS columns) and dexamethasone induces the expression of GS mRNA ($^{*}p < 0.05$ vs. CT). (B) LPS induces the expression TNFα mRNA in skeletal muscle ∼75-fold, and pre-treatment with dexamethasone (2.5 h) significantly inhibits the expression of TNFα mRNA. (C) LPS induces the expression of IL-6 mRNA in skeletal muscle ∼75-fold; however, pre-treatment with dexamethasone does not inhibit the induction to significant levels. (D) LPS induces the expression of MCP-1 mRNA in skeletal muscle ∼40-fold. Pre-treatment with dexamethasone dose-dependently inhibits this effect ($^{*}p < 0.05$ vs. LPS). This experiment is representative of two independent studies with $n = 8$ animals per group.

4. Discussion

Skeletal muscle myoblasts have been recently reported to express several cytokines and chemokines that may play a role in the inflammation processes within muscle tissue [25]; however, a systematic study to evaluate the potential anti-inflammatory effects of GCs on muscle-specific cytokine/chemokine expression has not been documented. GCs

are known to induce several genes in skeletal muscle that contribute to some of the known GC-induced side effects such as insulin resistance and muscle atrophy. We investigated the possibility of using myoblast cells and skeletal muscle tissue as a dual system in which to study both the desirable anti-inflammatory and undesirable activities mediated by glucocorticoids.

We show that in vitro, GCs concentration-dependently inhibit basal and LPS-induced levels of IL-6, TNFα and MCP-1 mRNAs in HSMC. In contrast to previous reports, we also see inhibition of these cytokines in murine skeletal muscle C2C12 cells. Frost et al. [28] recently reported that dexamethasone (1 μM) selectively downregulates IL-6 mRNA but not TNFα mRNA in C2C12 cells. However, in their TNFα study, cells were incubated with GCs for 1 h, while in our study (Fig. 3) cells were incubated with GCs for 4 h. While levels of TNFα gene induction peak at 1–2 h following LPS treatment, significant levels of TNFα mRNA can be measured up to 4 h post LPS treatment [28]. Importantly, we found that treatment of the cells for 1–2 h with GCs was not sufficient to observe transrepression of these cytokines (data not shown) and that incubation of the cells with GCs for 4 h was necessary to observe a GC-dependent transcriptional effect on cytokines.

Protein levels of IL-6 (Fig. 2) and MCP-1 (data not shown) in HSMC [25] and C2C12 cells were readily measurable and inhibited by GCs, an effect that could be competed off with the GR antagonist RU486 (Fig. 2). The complete antagonism of dexamethasone by RU486 suggests that the effect of dexamethasone on IL-6 production by skeletal muscle cells is the result of a direct interaction with the glucocorticoid receptor. In comparison, neither HSMC nor C2C12 cells produced TNFα protein in amounts that were detectable by an ELISA, under basal or LPS-stimulated conditions, despite the induction of TNFα mRNA by LPS. Examples of high levels of expression of TNFα mRNA, with lack of associated protein synthesis can be found in the literature [28,39–41]. This lack of correlation between mRNA and protein expression could be explained by protein levels that are too low for detection by conventional ELISAs, or a lack of processing of the transmembrane form of TNFα. Moreover, TNFα is tightly regulated at the mRNA level, through several mechanisms involving its 3′ untranslated region (UTR) such as the p38 MAP kinase [42] and Tpl2/COT kinase [43] pathways. Any muscle-specific regulation of these pathways could explain the mRNA-protein dissociation observed in our experiments; thus, further studies need to be carried out to investigate this observation.

In vivo, dexamethasone robustly downregulates IL-6 and TNFα in mouse serum; however, in spite of significant muscle exposure to dexamethasone, as demonstrated by its profound effects on TNFα and MCP-1, dexamethasone has only a modest (did not reach statistical significance) inhibitory effect on IL-6 mRNA in mouse muscle. This is an unexpected finding, as GCs are highly effective in down-modulating the expression of IL-6 mRNA in both HSMC (this work) and C2C12 cells (this work and [28]) and in immune cells including macrophages and monocytes [3,4,6]. The discrepancy between the in vitro and in vivo results in skeletal muscle may simply reflect the fact that while undifferentiated HSMC and C2C12 cells are a homogenous population of myoblasts, muscle samples are composed of a mixture of mainly myotubes, satellite cells, and other cell types. An alternative and likely explanation is that there exist competing mechanisms of GC-dependent IL-6 regulation in skeletal muscle. Glucocorticoids have been shown to promote insulin resistance in skeletal muscle, a state that is correlated with higher levels of IL-6 [44]. Therefore, a potential feedback induction of IL-6 promoted by a GC-induced insulin resistant state may compensate for the anti-inflammatory effects of GCs on IL-6. Interestingly, a similar observation was recently reported in rats challenged with LPS and pre-treated with dexamethasone. Dexamethasone was shown to selectively inhibit LPS-induced TNFα but not IL-6 gene expression in rat gastrocnemius skeletal muscle tissue [45].

In comparison to the transcriptional downregulation of cytokines (transrepression activity) of GCs in skeletal muscle, the transcriptional upregulation of genes (transactivation activity) of GCs in skeletal muscle has been well documented. GCs have been shown to directly upregulate two well-characterized genes, GS and MyoS that each contribute to muscle atrophy, a key undesirable side-effect of GC therapy [15–17,29,46–48]. While the induction of MyoS by GCs is specific to myotubes, the upregulation of GS by GCs has been demonstrated in vitro in HSMC and L6 myoblasts and myotubes and in vivo in rat skeletal muscle [16,17,29,49,50]. We extend these observations to murine C2C12 cells and mouse tissue to show that GCs upregulate GS mRNA and GS enzymatic activity in these cells, in a concentration- and time-dependent manner. Importantly, we demonstrate in this work that LPS treatment does not affect the level of GS expression in HSMC, murine C2C12 myoblasts and mouse skeletal muscle. This result is in contrast to the robust induction of GS mRNA expression by LPS in rat skeletal muscle tissue [51]; however, this may reflect a species-specific phenomenon. Given the lack of an effect of LPS on GS induction in HSMC, C2C12 cells, and mice, we propose that C2C12 myoblasts and the LPS-challenged mouse are appropriate model systems for the simultaneous measurement of a transactivation marker (GS) and a transrepression (cytokine and chemokine) marker, and for the direct identification of selective GCs.

Selective or “dissociated” GCs have been recently identified by screening ligands for endogenous activities and/or using artificial promoter based luciferase systems in several different cell types [1,21,22]. However, the identification of mechanistically-based selective GCs by this approach is complicated by the fact that the concentration of GR and its coactivators vary significantly in different cell types, and that GR ligands demonstrate complex cell-specific effects [23]. The simplicity of the skeletal muscle/myoblast models presented in this work is that selective GCs can be directly

identified based on endogenous and physiologically relevant GC-dependent gene activities, measured simultaneously in a single cell-type. We propose that the development of these models will lead to the identification of novel selective GCs with improved therapeutic profiles.

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