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REDD1 (Regulated in DNA Damage and Development 1) Expression in Skeletal Muscle as a Surrogate Biomarker of the Efficiency of Glucocorticoid Receptor Blockade

Rashmi Kumari¹, Lisa Willing¹, Leonard S. Jefferson², Ian A. Simpson¹, and Scot R. Kimball²

¹Dept of Neural & Behavioral Sciences, The Pennsylvania State University College of Medicine, Hershey, PA 17033

²Dept of Cellular & Molecular Physiology, The Pennsylvania State University College of Medicine, Hershey, PA 17033

Abstract

Glucocorticoids are potent regulators of cell metabolism, and in part act through a receptor-based mechanism to alter the transcription of target genes. A plethora of studies have utilized the glucocorticoid receptor antagonist, RU-486, both in vivo and in vitro, to reverse or prevent hormone-induced alterations in gene transcription. However, although RU486 potentially blocks many of the functions of the receptor, it does not lower plasma concentrations of the hormone, and a biomarker for the effectiveness of RU-486 in blocking receptor activation is lacking. In the present study, we demonstrate glucocorticoid-induced changes in expression of a protein referred to as regulated in development and DNA damage (REDD1) in a variety of mouse models of hypercortisolemia including stroke, type 2 diabetes, and stress induced by confinement. Notably REDD1 expression in skeletal muscle positively correlated with changes in corticosterone concentrations in all conditions. RU-486 had no effect on corticosterone concentrations, but strongly attenuated the stroke-, diabetes-, and stress-induced changes in REDD1 expression. Overall, the results of the present study suggest that changes in REDD1 expression in skeletal muscle represent an excellent surrogate biomarker for the efficacy of RU-486 treatment in repressing glucocorticoid action.

Keywords

Corticosterone; glucocorticoid; stroke; diabetes; REDD1; RU-486

Introduction

Glucocorticoids (e.g. cortisol in humans, corticosterone in rodents) are potent regulators of cell metabolism that act in a tissue-specific manner to control various metabolic processes [1,2]. One of the first steps in glucocorticoid action involves the binding of the hormones to

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Corresponding Author: Scot R. Kimball, Ph.D. Dept of Cellular & Molecular Physiology, The Pennsylvania State University College of Medicine, 500 University Drive, Hershey, PA 17033, Phone no. – (717) 531-8970, Fax No. – (717) 531-7667, skimball@psu.edu.

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a specific intracellular receptor, referred to as the glucocorticoid receptor. In the ligand-free state, the receptor resides in the cytosol, but in response to hormone binding, it translocates to the nucleus where it acts as a transcriptional regulator of specific target genes. In numerous studies, the glucocorticoid receptor antagonist RU-486 has been used to block the transcriptional changes induced by glucocorticoids. However, although RU-486 blocks many of the actions of glucocorticoids, it does not attenuate the elevation of glucocorticoid concentrations associated with catabolic conditions such as stress and diabetes [3]. Consequently, changes in serum glucocorticoid concentrations cannot be used to assess the effectiveness of RU-486 treatment in attenuating glucocorticoid signaling.

In the present study we assess the possible role of glucocorticoid-induced elevation of the mRNA encoding a protein referred to as regulated in development and DNA damage response 1 (REDD1) in skeletal muscle, as a biomarker for RU-486-induced modulation of glucocorticoid signaling. In a previous study, we showed that in both rats *in vivo* and L6 myoblasts in culture, treatment with the synthetic glucocorticoid dexamethasone results in rapid upregulation of REDD1 mRNA and protein expression [4]. More recently, we showed that REDD1 expression is elevated in the gastrocnemius muscle of rats fasted overnight, and that treatment with the glucocorticoid receptor antagonist RU-486 prior to an overnight fast prevents the fasting-induced increase in REDD1 expression [5]. We also showed that both glucocorticoid levels and REDD1 expression in muscle are increased in a rat model of type 1 diabetes, and that RU-486 treatment reverses the increase in REDD1 expression, but not the increase in serum glucocorticoid concentrations [5]. Combined, the results of these and other studies suggest that REDD1 expression might represent a biomarker for glucocorticoid action and the effectiveness of RU-486 in preventing such action.

In the present study, we extend the results of previous studies and show that upregulated expression of REDD1 in skeletal muscle correlates with increased corticosterone levels in mice subjected to immobilization-induced stress, in a mouse model of type 2 diabetes (i.e. the *db/db* mouse), and in a mouse stroke model using *db/db* mice, and that treatment with RU-486 strongly attenuates the increase. In addition, we present the novel finding that corticosterone-induced upregulation of REDD1 expression occurs in both fast- and slow-twitch skeletal muscle. Combined, the results of the present and previous studies suggest that changes in REDD1 expression represent a surrogate biomarker for both glucocorticoid signaling in skeletal muscle and the effectiveness of RU-486 in repressing glucocorticoid action in intact animals.

Material and Methods

Animal procedures

Animal facilities and experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of The Pennsylvania State University College of Medicine. Stroke was induced in 8-week old male *db/db* and *db/+* mice (Jackson laboratories Bar Harbor, Maine, USA) as described earlier [6,7]. Briefly, mice were anesthetized with isoflurane (3.5% in 70% nitrous oxide/30% oxygen) and a small midline neck incision was made. The right carotid artery was exposed and ligated twice using 3-0 surgical silk. The incision was then sutured and the animals returned to their cages for 3 hours of recovery during which time they had free access to food and water. Following recovery, the animals were placed in individual jars maintained at 35.5°C and exposed to a humidified gas mixture of 8% O₂ balanced with N₂ for 20 minutes. Blood samples were collected two days before surgery to establish a baseline, and after 24 h of recovery from hypoxia/ischemia for corticosterone measurement. Animals were injected subcutaneously with RU-486 (40 mg/kg in polyethylene glycol 400; Sigma Aldrich) 24 h and 2 h prior to and 8 h post surgery. Mice were sacrificed after 24 h of recovery, and the

soleus and gastrocnemius muscles were removed and stored at -80°C for mRNA and protein measurement.

Psychological stress was induced by confining C57Bl/6 mice in a well-ventilated 50 ml conical tube for 90 min, as described previously [8]. Approximately 24 h prior to confinement, mice were randomly separated into three groups. Mice in the first group received two injections of RU-486 as described above, whereas mice in the second group received two injections of solvent. Mice in the third group were not injected or restrained, and served as controls.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Soleus and gastrocnemius muscles were homogenized in Trizol (Invitrogen, CA, USA) and RNA was isolated using the Trizol method according to the manufacturer's instructions. An Omniscript kit (Qiagen Inc, CA, USA) was used to generate cDNA from the RNA samples. REDD1 mRNA expression was measured by qRT-PCR using 0.4 μM forward and reverse primers (Integrated DNA technology Inc. CA, USA) as described earlier [4], except that the samples were analyzed in 384 well plates using an ABI Prism 7700 Sequence Detection System (Applied Biosystems Branchburg, NJ, USA). REDD1 mRNA expression was normalized to expression of GAPDH mRNA.

Western blot analysis

The gastrocnemius muscle was homogenized in ice-cold lysis buffer, centrifuged at 1000g for 3 min at 4°C , and the supernatant was collected and boiled in SDS sample buffer for 5 min. Proteins were then resolved by electrophoresis in 7.5% polyacrylamide gels and immunoblotted for ribosomal protein S6 kinase 1 (S6K1) using antibodies from Bethyl Laboratory as described previously [9]. Blots were developed using chemiluminescence detection reagents (Pierce) and visualized using a GeneGnome HR bioimaging system (Syngene).

Radio immunoassay (RIA) for corticosterone measurement

Blood samples were allowed to clot for 30 min, centrifuged at 10,000 rpm for 10 min, and serum was stored at -80°C until analyzed. RIA was performed using ImmuChemTM Double Antibody Corticosterone ^{125}I RIA Kit (MP Biomedicals, LLC Diagnostic division, NY, USA) as described previously [10]. Samples were mixed with corticosterone labeled ^{125}I tracer and anti-corticosterone antibody and then incubated for 2 hr. The solution was precipitated with precipitant solution, centrifuged at 1000 $\times$ g for 15 min, and the supernatant was decanted. The pellet was counted in a gamma counter (Micromedic 4/200 Plus Automatic gamma counter). A standard curve was plotted from 25 ng/ml to 1000 ng/ml for each experiment, and sample values were interpolated from the standard curve.

Statistics

Data were analyzed using Graph Pad Prism 5.0 (Graph Pad Software Inc., San Diego, CA, USA) and are presented as mean $\pm$ SEM. If a difference among groups was detected by one-way analysis of variance, data were further analyzed by unpaired t-tests; $p < 0.05$ was considered statistically significant.

Results

Previous studies have shown that circulating glucocorticoid levels are elevated in various animal models of type 1 diabetes, e.g. treatment with alloxan or streptozotocin, and type 2 diabetes, e.g. *ob/ob* and *db/db* mice [5,10,11,12], as well as in models designed to induce stress [8]. In confirmation of those reports, in the present study we found that corticosterone

levels were increased approximately 2.5-fold in control C57Bl/6 mice confined for 90 min in a conical tube (Fig. 1A). Moreover, corticosterone concentrations were more than 3-fold higher in serum from *db/db* diabetic compared to *db/+* non-diabetic mice (Fig. 1B). Concentrations of the hormone were also increased in both control and diabetic mice subjected to unilateral brain ischemia/reperfusion. Notably, treatment with RU-486 did not prevent the stress-, diabetes-, or hypoxia/ischemia-induced increase in serum corticosterone levels, and in fact, in some cases, concentrations of the hormone were significantly greater in RU-486-treated compared to vehicle-treated animals (Figs. 1A and 1B). RU-486-mediated increases in serum corticosterone have been observed in previous studies [e.g. 13,14], an effect that is likely a consequence of attenuated feedback of the hormone on CRH release from the hypothalamus and/or ACTH release by the anterior pituitary.

Because RU-486 did not prevent the increase in serum corticosterone levels, a different index of its effectiveness in blocking corticosterone action was required. In a previous study [5], we showed that RU-486 had no effect on serum corticosterone levels, but blocked the induction of REDD1 expression in the gastrocnemius in a rat model of type 1 diabetes. As shown in Fig. 2A, REDD1 mRNA expression was increased approximately four-fold in both the soleus and gastrocnemius muscles of C57Bl/6 mice subjected to 90 min of confinement. REDD1 mRNA expression was also significantly greater in the soleus muscle of *db/db* diabetic compared to *db/+* control mice, and was further increased in response to unilateral brain ischemia/reperfusion in both control and diabetic animals (Fig. 2B). Interestingly, the response was greater in *db/db* compared to *db/+* mice, a finding consistent with the greater increase in serum corticosterone concentrations observed in diabetic compared to non-diabetic mice (Fig. 1B). Treatment with RU-486 attenuated the increase in REDD1 expression in all conditions.

To further confirm the effectiveness of RU-486 in blocking corticosterone action in muscle, mTORC1 signaling was assessed as changes in phosphorylation of the mTORC1 substrate, S6K1. Consistent with the increase in REDD1 expression, phosphorylation of S6K1 was reduced in the gastrocnemius of *db/db* diabetic compared to *db/+* control mice (Fig. 3). Moreover, S6K1 phosphorylation was decreased in response to hypoxia/ischemia in both control and diabetic animals. RU-486 had little effect on S6K1 phosphorylation in control mice, but in *db/db* diabetic mice it restored S6K1 phosphorylation to values not significantly different than in control *db/db* mice.

Discussion

Previous studies have shown that in rats treated with dexamethasone, REDD1 expression is upregulated in skeletal muscle within a few hours [e.g. 4,15,16]. Similarly, in L6 myoblasts in culture, addition of dexamethasone to the culture medium leads to upregulated REDD1 expression, an effect that can be mimicked using a luciferase reporter construct driven by a promoter bearing the glucocorticoid-response element from the REDD1 gene [15,17]. Thus, dexamethasone upregulates REDD1 expression through a glucocorticoid receptor-dependent increase in the transcription of the REDD1 gene. Further support for this conclusion is the recent identification of a functional glucocorticoid response element in the promoter region of the REDD1 gene [15].

In a previous study [5], we provided evidence that physiological changes in circulating corticosterone concentrations are sufficient to alter REDD1 expression in gastrocnemius. Thus, treatment with RU-486 prevented the fasting-induced increase in REDD1 expression, in the absence of changes in corticosterone concentrations. In the present study, the increases in serum corticosterone concentrations observed in C57Bl/6 mice following 90 min of confinement and in *db/db* diabetic mice following a period of unilateral brain

hypoxia/ischemia were associated with increased REDD1 mRNA expression in skeletal muscle, providing further support for the conclusion that physiological changes in glucocorticoid levels are sufficient to modulate REDD1 expression. Interestingly, we found that increased serum corticosterone concentrations were associated with upregulated REDD1 expression in both gastrocnemius and soleus muscles. A similar observation has been reported in dexamethasone-treated rats [15]. This finding is unexpected because it suggests that glucocorticoids should repress mTORC1 signaling, and subsequently promote muscle wasting, in both fast-twitch type II glycolytic and slow-twitch type I oxidative muscle fibers. However, previous studies suggest that glucocorticoid-induced muscle atrophy occurs predominantly in muscle composed primarily of type I fibers [20]. However, in both the present study and the previous one [15], the glucocorticoid-induced upregulation of REDD1 expression was greater in gastrocnemius, composed primarily of type II fibers, than in soleus, composed primarily of type I fibers. Although unexplored, the differential response may be due to glucocorticoid receptor expression being lower in soleus compared to gastrocnemius [15], resulting in a more robust response in gastrocnemius compared to soleus muscle.

In the current study, changes in REDD1 expression and mTORC1 signaling occurred in a model-independent manner, correlating with increased serum glucocorticoid concentrations. Combined with the results of McGhee et al., the results of this study support a model in which glucocorticoids promote upregulated REDD1 expression, subsequently leading to a reduction in mTORC1 signaling [e.g. 5]. Furthermore, although not explored herein, the decrease in mTORC1 signaling would result in a fall in global rates of protein synthesis as well as an increase in autophagy, which combined would promote muscle wasting [9]. Finally, the robust, reproducible, and model-independent induction of REDD1 expression in response to increases in serum glucocorticoid concentrations suggest that changes in its expression may represent an excellent surrogate biomarker for the effectiveness of RU-486 in repressing the action of glucocorticoids in muscle.

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Highlights

- Glucocorticoids upregulate REDD1 mRNA expression in skeletal muscle
- The upregulation is robust, reproducible, and model independent
- The upregulation is blocked by the glucocorticoid receptor antagonist RU-486
- Upregulated REDD1 expression correlates with decreased mTORC1 signaling
- Changes in REDD1 expression represent a surrogate biomarker for RU-486 effectiveness

Fig. 1. RU-486 does not block the stress-induced increase in serum corticosterone concentrations
 Serum corticosterone concentrations were measured by RIA. (A) C57Bl/6 mice were randomly divided into two groups; mice in the first (control) group were not confined whereas mice in the second group were subjected to 90 min of confinement as described under “Materials and Methods”. Mice in the confinement group were further subdivided into two groups that were treated with either vehicle or RU-486. The results represent the mean $\pm$ SEM of 8 mice/group. (B) Non-diabetic (*db/+*) and diabetic (*db/db*) mice were randomly divided into two groups. Mice in the first group served as controls. Mice in the second group were subjected to surgery to induce unilateral brain hypoxia/ischemia as described under “Materials and Methods”, and further subdivided into two groups that were treated with either vehicle or RU-486, respectively. The results represent the mean $\pm$ SEM of 4–13 mice/

group. Statistically significance differences ($p < 0.05$) are noted by the absence of a shared letter above the bars, shared letters indicate an absence of statistical differences.

Fig. 2. REDD1 mRNA expression in gastrocnemius and soleus muscle

(A) The gastrocnemius and soleus muscles were removed from the hindlimb of mice used in the study described in the legend to Fig. 1A. RNA was subsequently isolated from the muscles, and qRT-PCR was performed for REDD1 and GAPDH mRNA content as described under “Materials and Methods”. The results represent the mean $\pm$ SEM of 4–8 mice/group. (B) RNA was isolated from the soleus muscle of the mice used in the study described in the legend to Fig. 1B, and REDD1 and GAPDH mRNA content was assessed by qRT-PCR. The results represent the mean $\pm$ SEM of 8–15 mice/group. Statistically significance differences ($p < 0.05$) are noted by the absence of a shared letter above the bars, shared letters indicate an absence of statistical differences.

Fig. 3. mTORC1 signaling in skeletal muscle

A portion of the gastrocnemius muscle from the mice used in the study described in the legend to Fig. 1B was subjected to Western blot analysis for S6K1 phosphorylation as described under "Materials and Methods". Representative blots are shown above the graph. All samples in the blot on the right were analyzed on the same blot, but not in adjacent lanes. Noncontiguous lanes are separated by a white line. The results represent the mean $\pm$ SEM of 5–10 mice/group. Statistically significant differences ($p < 0.05$) are noted by the absence of a shared letter above the bars, shared letters indicate an absence of statistical differences.