

ORIGINAL ARTICLE

The administration of high-dose methylprednisolone for 24 h reduced muscle size and increased atrophy-related gene expression in spinal cord-injured rats

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Objective: Administration after spinal cord injury (SCI) of methylprednisolone (MP) for 24–48 h has been suggested to improve functional outcome. The safety of this approach has been questioned because of the known adverse effects of glucocorticoids on skeletal muscle and the immune system. The purpose of this study was to explicitly test adverse effects of regimen of MP administration on skeletal muscle.

Study design: Male rats underwent spinal cord transection at T9-T10, followed by an intravenous injection of MP and subsequent infusion of MP for 24 h.

Results: MP significantly reduced the weight of the triceps, soleus, plantaris and gastrocnemius muscles, with the greatest effect being a 63% decrease in triceps weight (for example, muscle above the level of lesion) at 7 days; below the level of lesion, gastrocnemius weight was reduced by 33% by SCI alone, and by 45% by SCI and MP. Centralized nuclei were found in myofibers of the gastrocnemius and triceps from the MP-SCI group, but not other groups. MP increased expression in the triceps, soleus and plantaris of FOXO1, MAFbx, MuRF1 and REDD1 at 1 day, and, in plantaris, at 7 days.

Conclusions: Thus, 1 day of MP at a dose comparable to those routinely employed in clinical practice immediately after SCI resulted in marked atrophy of functionally intact muscle above the level of lesion, and worsened atrophy of paralyzed muscle below the level of lesion, associated with elevations in expression of four genes involved in pathways associated with muscle atrophy.

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Introduction

When begun within 8 h after spinal cord injury (SCI), administration for 24–48 h of gram doses of the glucocorticoid methylprednisolone (MP) has been suggested to improve functional neurological outcomes.¹ Adverse effects of this treatment may include immunosuppression and muscle atrophy, as well as glucocorticoid-induced myopathy. Because the duration of administration of MP for this purpose is short, and the drug is rapidly eliminated,² one might argue that the brief period of treatment minimizes adverse effects. Even so, a case series has found histological evidence of glucocorticoid myopathy and electrophysiological evidence of impairments in neuromuscular function in

persons with SCI treated with MP at the time of injury.³ Direct evidence that short-term administration of very high-dose MP causes muscle atrophy, or alterations in molecular markers consistent with atrophy, has not been reported.

Muscle atrophy arises largely through the accelerated breakdown of proteins by the ubiquitin–proteasome pathway, in part through the action of two muscle-specific E3 ubiquitin ligases, muscle atrophy F-box (MAFbx, also called atrogen-1) and muscle ring finger 1 (MuRF1).^{4,5} Expression of these genes is upregulated in all forms of muscle loss,^{4,6} and loss of either slows denervation atrophy.⁷ Loss of MuRF1 also diminishes loss of myosin heavy chain in glucocorticoid-induced atrophy.⁸ Upregulation and activation of the transcription factor FOXO1 is an important molecular event leading to increased expression of both MAFbx⁹ and MuRF1.¹⁰ In glucocorticoid-induced atrophy, upregulation of REDD1, an inhibitor of protein synthesis, has been suggested to explain the reduced protein synthetic rates found in this form of muscle atrophy.¹¹ Whether the brief

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period of MP administration used at the time of SCI to improve function results in significant or lasting changes in the expression of muscle atrophy genes, (for example, FOXO1, MAFbx, MuRF1 or REDD1) is not known.

In the present study, we characterized the effects over time of MP administered in the doses and temporal sequence used to attempt to improve neurological function after SCI on muscle mass and histology, as well as the expression of genes strongly linked to muscle atrophy. A rat model of complete spinal cord transection at the mid-thoracic level was used for these studies. Because the majority of persons with SCI are male, male rats were selected for this work.

Materials and methods

Animal studies

We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during the course of this research. Animal studies were approved by the Institutional Animal Care and Use Committee at the James J Peters VA Medical Center. Male Wistar rats weighing 250 g (Taconic Farms) were fed food and water ad libitum and housed in a temperature and humidity-controlled facility with a 12:12 h day/night cycle.

Animals were weighed and then anesthetized by inhalation of isoflurane. A laminectomy was performed, after which animals underwent complete transection of the spinal cord at the interspace between the 9th and 10th thoracic vertebrae. Skin and muscle were closed with suture. Animals were administered an intravenous injection of freshly prepared MP (30 mg kg⁻¹) or vehicle (propylene glycol) by tail vein, followed by implantation into a subcutaneous pocket of an Alzet model 2001 pump (Durect Co, Cupertino, CA, USA) which provided a 24-h infusion of MP at 5.4 mg kg⁻¹ hr⁻¹, or vehicle (propylene glycol). This dosing of MP corresponds on a mg per kg basis to that prescribed by the Bracken protocol.¹ Urine was expressed from bladders of transected animals at least four times daily, and these animals were administered Baytril 2.5 mg kg⁻¹ subcutaneously twice daily for 3–5 days, then as indicated based on the presence of discolored urine or urethral plugs. Sham-transected animals underwent laminectomy and implantation of the same Alzet pumps, which infused vehicle (propylene glycol) without additives. At 1, 7 or 14 days, animals were euthanized by inhalation of carbon dioxide. Muscles were removed by careful dissection, weighed and flash frozen in liquid nitrogen; specimens were then stored at –80 °C.

Quantitative real-time PCR

Extraction of total RNA from skeletal muscle and quantitative real-time PCR was performed as described.^{12,13} Real-time PCR determinations of mRNA levels were performed in triplicate. To minimize RNA degradation and permit analysis of the large number of samples included in our study, the following additional procedures were included. Samples were processed batch-wise by muscle and time point. For example, all triceps muscles collected at 7 days were

processed together. To minimize RNA degradation, total RNA was extracted over several days from 6 to 12 samples at a time. For each piece of skeletal muscle, immediately after being cut from the frozen block of muscle, it was plunged into ice-cold homogenization buffer and immediately homogenized. Real-time PCR reactions were performed in consecutive 96-well plates. In preliminary studies, when real-time PCR reactions are repeated even months later, the crossing points obtained by real-time PCR vary by ± 0.5 or less when aliquots from a specific complementary DNA library are used, suggesting that interassay variability was small, particularly when compared with the magnitude of changes in gene expression in our study. Changes in expression levels of mRNA transcripts were expressed as fold-change as compared with the 7-day Sham-SCI group treated with vehicle using the $2^{-\Delta\Delta C_t}$ method. This method was selected rather than normalizing mRNA levels for each SCI group to those for the Sham-SCI from the same time point, because mRNA levels for the genes of interest were significantly elevated at 1 day in the Sham-SCI group, perhaps reflecting the effects of the stress associated with laminectomy, and because these levels appeared to have returned to baseline by 7 days. By 7 days these levels appeared to have returned to baseline. The internal control for these analyses was 18S RNA.

Histology

Pieces of gastrocnemius muscle were frozen in isopentane pre-cooled on dry ice. Tissues were kept at –80 °C. Sections (16 μ m) were cut on a cryotome and mounted directly onto cooled glass slides then stained with hematoxylin-eosin. Sections were viewed at $\times 200$ using a Nikon Eclipse E600 microscope (Nikon Instruments Inc., Melville, NY, USA) fitted with a Nikon DXM1200 CCD camera for image capture.

Statistics

Data are expressed as means \pm s.e.m. The significance of differences for means among groups over time was determined by two-way analysis of variance with a Bonferonni test *post-hoc* to establish the significance of differences at specific times. Differences were considered significant at $P < 0.05$.

Results

Body weights were significantly lower in the SCI-vehicle group compared with the Sham-SCI group at 7 and 14 days (Figure 1a). Body weights were significantly lower in the SCI-MP group at 1 and 7 days, compared with the SCI-vehicle group, with the greatest effect at 7 days (Figure 1a).

As might have been expected, when normalized for body weight, muscle weights for the SCI-sham group for triceps, soleus, plantaris and gastrocnemius muscles were unchanged between days 1 and 14 (Figures 1b–e). In the SCI-vehicle group, weights of all muscles caudal to the level of lesion were significantly reduced at 7 and 14 days, with decreases of 32–35% at 7 days and 40–47% at 14 days. For triceps, which remained subject to voluntary motor control, MP

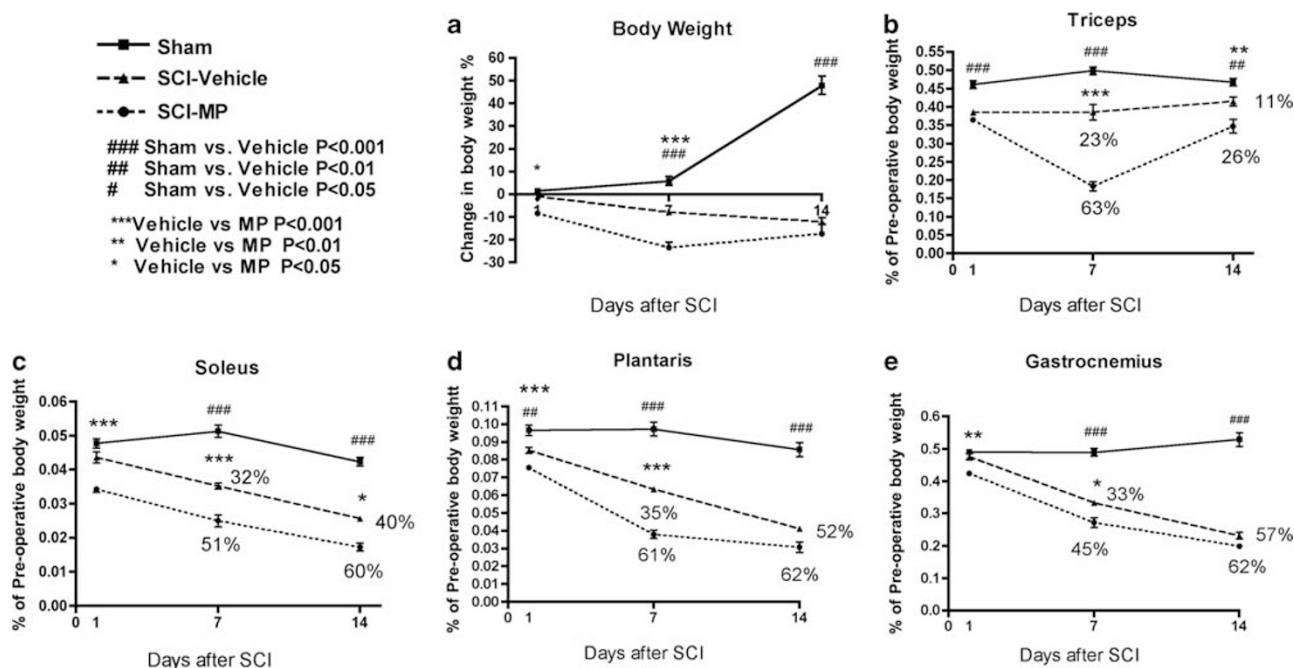


Figure 1 Body and muscle weights over time. (a) The change in body weight over time is expressed as a percentage of body weight on the day of surgery. (b–e) Weights of the triceps, soleus, plantaris and gastrocnemius muscles over time, where weights are normalized relative to body weight on the day of surgery. $N = 6–10$ animals per group per time point, except for 7-day SCI-vehicle for which $n = 12$ animals. There was a significant main effect for time and SCI/MP by two-way analysis of variance for all panels. The symbols for significance of differences between groups (#, *) indicate the results of a *post-hoc* Bonferonni test.

significantly reduced muscle mass at 7 and 14 days compared with the SCI-vehicle group (Figure 1b), although the difference in muscle mass between SCI-MP and SCI-vehicle groups was smaller at 14 versus 7 days. At 7 days, triceps weight was reduced by nearly two-thirds in the SCI-MP group, but by only about one-fifth in the SCI-vehicle group. Muscle weights were reduced in the SCI-MP group at 1 and 7 days for the paralyzed gastrocnemius, soleus and plantaris muscles when compared with the same muscle from the SCI-vehicle group (Figures 1c–e); MP increased muscle loss at these time points by 10–25 %, depending on the time post-injury and the specific muscle. For the soleus of the SCI-MP group, a significant decrease was also found at 14 days, while for the plantaris and gastrocnemius, weights were not significantly different from those for the SCI-vehicle group at this time point.

Examination of hematoxylin-eosin-stained sections of triceps and gastrocnemius muscle from SCI-Sham, SCI-vehicle and SCI-MP animals was performed to evaluate effects of MP on muscle structure. Occasional fibers with centralized nuclei were found in both the triceps and gastrocnemius from SCI-MP animals, but not SCI-vehicle animals or sham-transected animals. These were rare, and occurred in only a few muscle fibers in each section. No necrotic fibers or fibers invaded by inflammatory cells were observed (Figure 2) in any of the experimental groups.

At 1 day, MP significantly increased mRNA levels in the triceps for FOXO1, MAFbx, MuRF1 and REDD1 (Figure 3). At 7 days, elevations in mRNA levels for these genes were no longer significant, except for FOXO1; mRNA levels had

returned to baseline levels by 14 days (Figure 3). When comparing SCI-sham and SCI-vehicle groups, a significant elevation of mRNA levels for these genes was also observed in the SCI-vehicle group at 1 day, but was no longer evident at 7 days (Figure 3).

A similar effect of MP on gene expression was observed for the soleus, with significant increases found in the SCI-MP group as compared with SCI-vehicle group for FOXO1, MAFbx, MuRF1 and REDD1 at 1 day. This increase was not significant at 7 days, and was absent by 14 days (Figure 4). Comparison of SCI-vehicle and SCI-sham groups revealed a significant increase in REDD1 at 1 day, whereas non-significant changes were observed for the other genes evaluated (Figure 4).

The pattern of gene expression changes in plantaris was different, with peak effects of MP occurring at 7 days for FOXO1, MAFbx and MuRF1 (Figure 5). Compared with the SCI-vehicle group, mRNA levels in the SCI-MP group were significantly elevated at 1 and 7 days for FOXO1, MAFbx, MuRF1 and REDD1; these elevations had resolved by 14 days (Figure 5). When comparing SCI-vehicle and SCI-sham groups, significant elevations were observed for all four transcripts at 1 day, and for FOXO1, MAFbx and MuRF1 at 7 days (Figure 5).

A comparison of the magnitude of gene expression among these three muscles revealed that the greatest effect of MP was observed for the triceps. Upregulation of REDD1 at day 1 approached 50-fold for the triceps, but was more than threefold less for the plantaris, and nearly 10-fold lower for soleus.

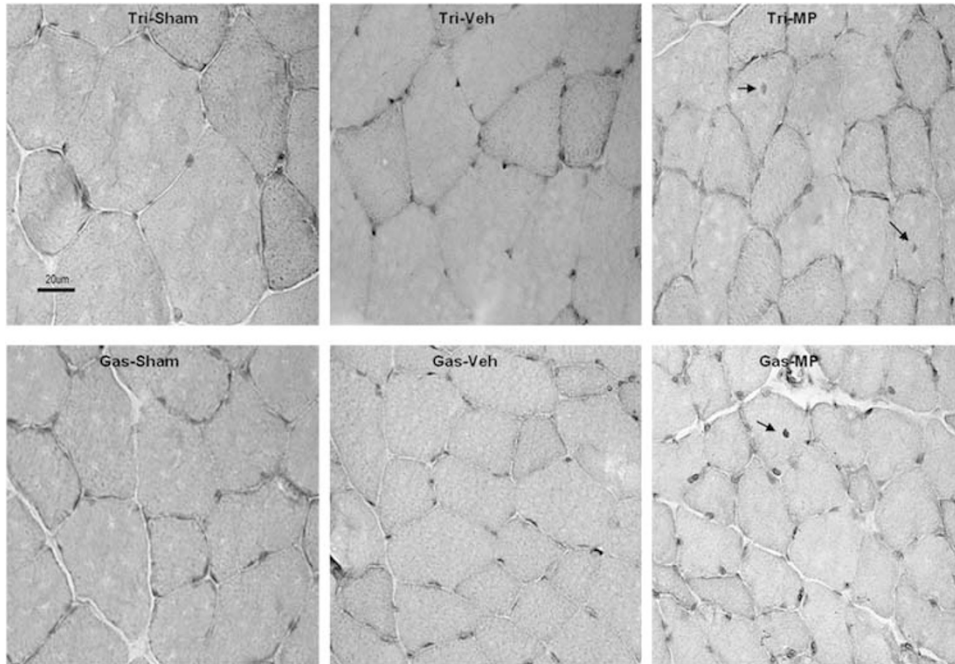


Figure 2 Histology of the triceps and gastrocnemius muscles at 7 days. Hematoxylin-eosin-stained sections of the gastrocnemius (gas, lower row) and triceps (Tri, upper row) viewed at a magnification of $\times 200$ are shown. The arrows indicate centralized nuclei.

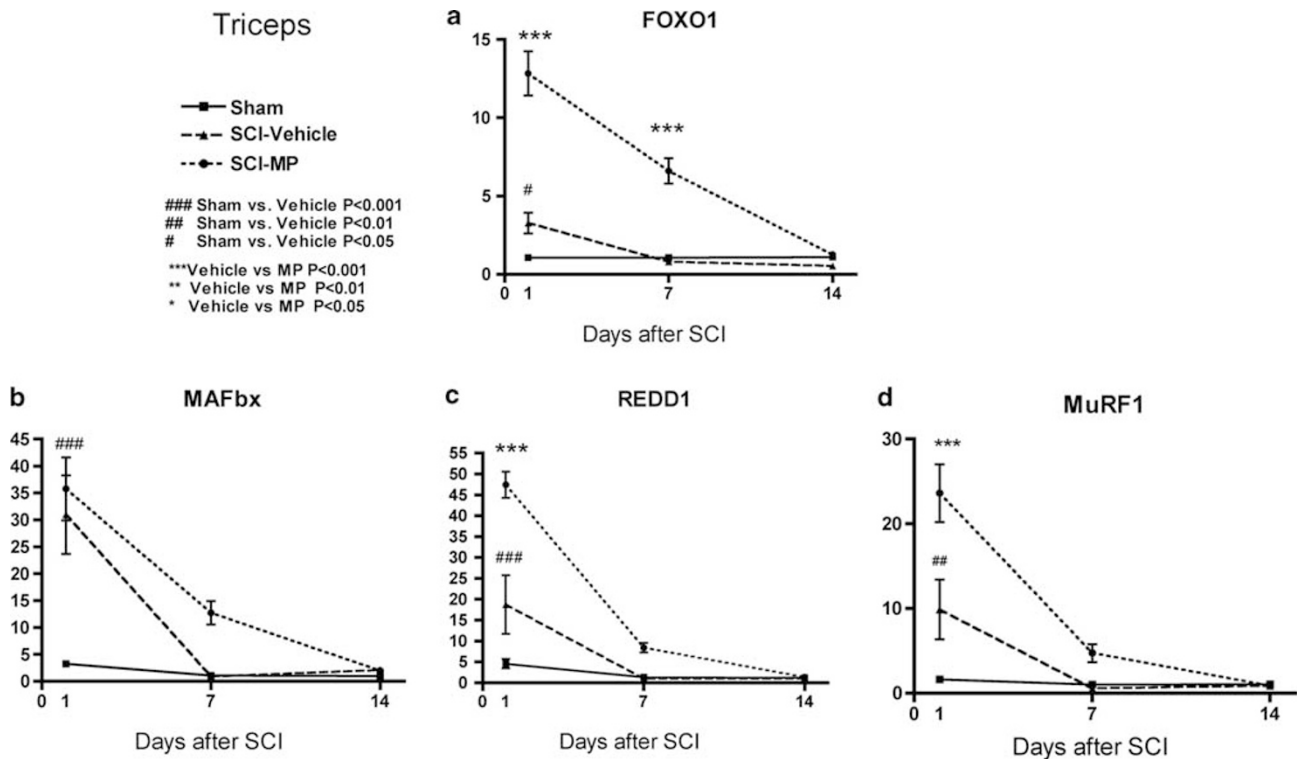


Figure 3 Changes in gene expression over time for the triceps muscle. (a–d). Gene expression for MAFbx, MuRF1, REDD1 and FOXO1 are shown. The data are normalized relative to the expression levels for SCI-sham animals at 7 days. The number of animals per time point is the same as previously described in Figure 1.

Discussion

Administration for 1 day of MP at doses used to increase function after SCI led to marked loss of skeletal muscle mass

above the level of SCI that was greatest at 7 days and had largely resolved by 14 days. Loss of muscle in paralyzed regions was also increased by MP over the first week after SCI, although the magnitude of this effect was smaller,

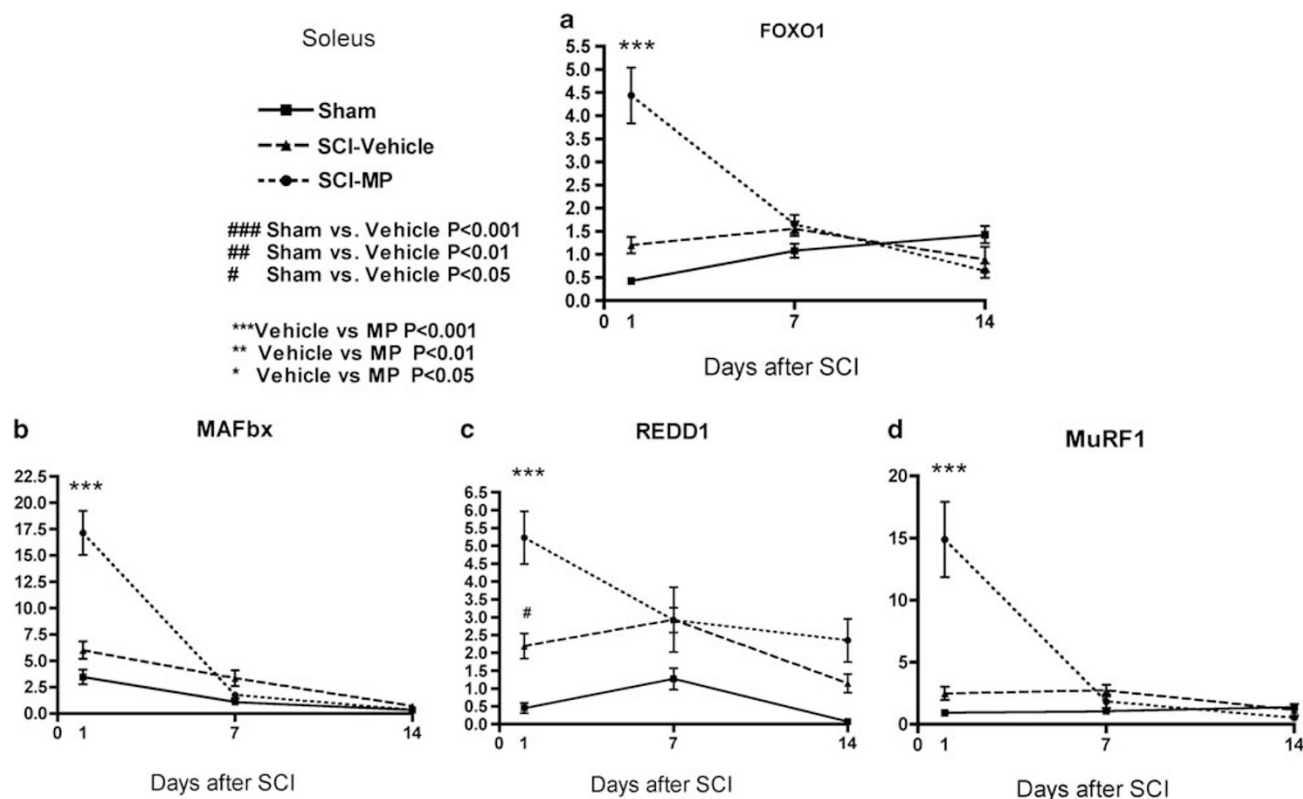


Figure 4 Changes in gene expression over time for the soleus muscle. (a–d) Gene expression for MAFbx, MuRF1, REDD1 and FOXO1 are shown. Data are normalized relative to the expression levels for SCI-sham animals at 7 days. The number of animals per time point is the same as previously described in Figure 1.

perhaps because of the extensive atrophy of such muscles resulting from SCI-related immobilization. Additionally, one might ask whether there is a maximum rate of muscle atrophy, beyond which even the combined actions of potent stimuli to muscle loss can not further increase muscle catabolism. MP administration was associated with early increases in expression of three genes linked to muscle catabolism, FOXO1, MAFbx and MuRF1, and of the protein synthesis inhibitor REDD1.

The time course of these events, with maximal gene expression changes preceding declines in muscle mass, conforms to the current understanding of the role of these gene products in permitting or stimulating catabolism of muscle proteins, and reducing rates of protein synthesis. The time course of early gene expression changes after SCI with or without MP, and subsequent decreases in muscle mass by 7 days, agrees with that previously reported for a similar SCI model.¹⁴ In agreement with our study, in the SCI group, gene expression had returned to normal, or nearly normal values by 7 days, a change that was interpreted as reflecting the resolution of spinal shock and subsequent development of spasticity.¹⁴

The early effects of SCI on muscle above the level of the injury have not, to our knowledge, been reported in an animal model. The increases in expression of MAFbx, MuRF1 and REDD1 that occurred in triceps at 1 day may reflect stress responses to the surgery and/or recent SCI. Because REDD1 is quite sensitive to glucocorticoids, its upregulation in triceps

suggests that elevated levels of endogenous corticosteroids may be involved. An analysis of gene expression changes in skeletal muscle innervated by motor neurons originating above the level of injury, has also been conducted in human subjects;¹⁵ all subjects had received MP (M Urso, personal communication). Biceps muscle biopsies of these subjects performed at 2 days revealed elevated expression of MAFbx and MuRF1,¹⁵ as well as REDD1 (M Urso, personal communication).

The effects of MP on muscle mass and gene expression were associated with centralized nuclei in some muscle fibers in triceps and gastrocnemius in the MP-SCI group. Although this abnormality may reflect damage to myofibers, we did not observe the overt necrosis described in individuals with SCI after administration of MP.³ Additional studies will be needed to determine the functional significance of these abnormalities with respect to muscle contractile force, endurance and injury after exercise. The reason that MP did not cause myofiber necrosis in rats is unclear. This may reflect species differences, or the muscle-specific consequences of the greater severity and complexity of the medical problems of the typical patient with SCI, who may have concurrent trauma other than their SCI, as well as being administered additional medications, and experiencing stressors, such as starvation and intercurrent infection. With these considerations in mind, one must also ask whether the electromyographic abnormalities identified in individuals who received MP after SCI reflect neuropathy of critical

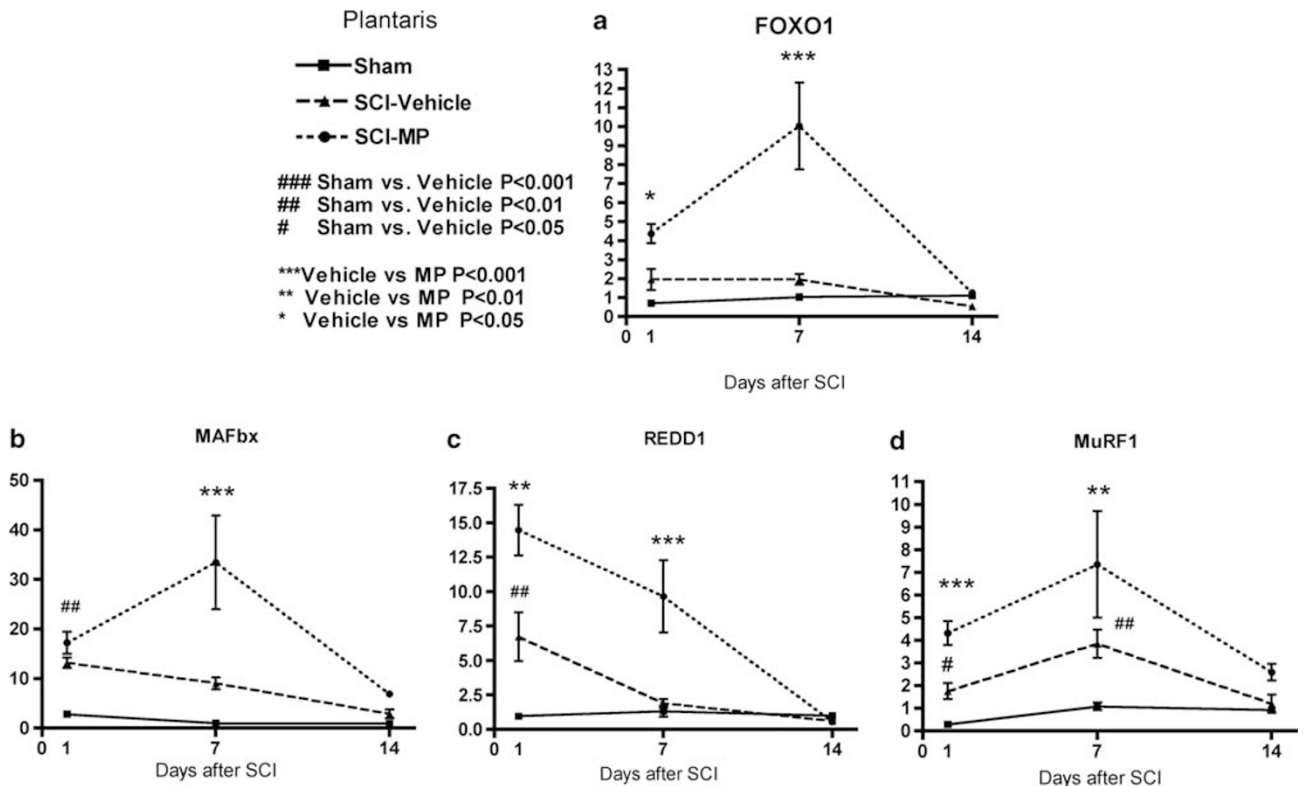


Figure 5 Changes in gene expression over time for the plantaris muscle. Gene expression for MAFbx, MuRF1, REDD1 and FOXO1 are shown. The data are normalized relative to the expression levels for the SCI-sham animals at 7 days. The number of animals per time point is the same as previously described in Figure 1.

illness rather than a specific effect of MP, or, possibly, the combined effects of multiple catabolic insults.

The loss of mass of neurologically intact triceps and paralyzed gastrocnemius caused by a 24-h infusion of MP and the associated increased expression of MAFbx, MuRF1, FOXO1 and REDD1 are consistent with changes, size and gene expression in neurologically intact rat gastrocnemius muscle during continuous infusion of dexamethasone.^{11,13,16} One difference between the findings in our study and prior work with dexamethasone is the more dramatic reduction in muscle size at 7 days in our study, which was approximately 63% for triceps from the SCI-MP group, compared with an approximately 20% decrease in weight of normally innervated gastrocnemius in prior reports. These differences may reflect muscle-specific differences in glucocorticoid sensitivity; the differences of muscle effects of MP on fold-change in expression of genes, such as REDD1, support this hypothesis. An additional consideration is the much greater glucocorticoid dose used in the current study of MP, and/or the additional effects of post-surgical stresses and inflammation resulting from SCI.

Whereas prior studies have evaluated gene expression changes during infusion of a glucocorticoid, in the current study gene expression was also determined 6 and 13 days after discontinuing MP. Although even in our study the time-course for resolution of the effect of MP on gene expression for the four key muscle atrophy genes cannot be completely determined from the data, it is clear that the effects of MP on

expression of these genes had resolved within 6 days, except for the plantaris. This delay in decline in expression in plantaris of the four muscle loss genes is striking, and suggests that MP can, in some situations, stimulate lasting changes in transcriptional programs that determine their expression. One explanation for the difference in time course of gene expression changes for soleus and plantaris is that the former is primarily composed of slow-twitch muscle fibers, whereas the latter is predominantly fast twitch. Much less likely explanations are slow elimination of the drug or slow turnover of the mRNA transcripts for these genes. The half-life of MAFbx mRNA is rapid,¹⁷ as is that for FOXO1 (W Qin, unpublished). Studies in rats have demonstrated that the half-life for MP after intravenous injection at doses similar to those used in our study is less than 1 h,² suggesting that the drug has been essentially eliminated from the circulation in less than 10 h (10 half-lives). Although absorption of MP from tissues has been reported to be delayed, this consideration does not appear to be significant.²

The use of MP during the period immediately after SCI with the goal of improving neurological function continues to be a topic of controversy. Evidence of benefit from clinical trials¹ led to use of this intervention in many newly injured individuals with SCI. A view that these benefits may not outweigh potential adverse actions of MP has been expressed.³ Our data do not address the question of the benefits of MP after SCI, but do provide experimental

support for the recent concerns regarding the adverse effects of MP when administered in the setting of acute SCI. The profound muscle atrophy above the level of SCI caused by a 24 h administration of MP may have significant implications for function of neurologically intact muscle. An area of vital concern is the potential implications for muscles necessary for respiration, particularly in individuals with higher cord lesions in whom function of diaphragm and accessory muscles of respiration becomes a limiting factor for independent ventilation. The potential of such atrophy to adversely impact the ability to transfer or use a wheel chair are other implications of these findings. It is appreciated, however, that there may be species-related differences between humans and rats that may limit extrapolation of our findings; the effects of MP on skeletal muscle in man may be milder, or more severe, than those we observed in rats.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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