

Steroidal Nondepolarizing Muscle Relaxants Do Not Simulate the Effects of Glucocorticoids on Glucocorticoid Receptor–mediated Transcription in Cultured Skeletal Muscle Cells

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MUSCLE wasting and persistent muscle weakness occur in critically ill patients who are treated in the intensive care unit.[1,2] The incidence of this complication varies between 47 and 96%.[3] The etiology of this muscle weakness is multifactorial[4,5] and includes the disease itself, the associated immobilization,[6,7] and the concomitant use of muscle relaxants alone or in combination with corticosteroids.[8,9] An enquiry by the US Food and Drug Administration concluded that factors that increase susceptibility to myopathy and muscle weakness are the administration of high doses over several days primarily of steroidal relaxants (pancuronium and vecuronium) to patients who may also be receiving corticosteroids, aminoglycoside antibiotics, or both.[10] Other recent reports have concurred with this notion.[11,12] Although these reports of myopathy implicated steroidal relaxants as an important contributing factor,[9–12] other reports implicate benzoylisoquinolinium-type relaxants also to have myopathic effects when combined with corticosteroids.[13,14] It has been postulated that steroidal relaxants can behave like corticosteroids or, by some other interaction, potentiate the glucocorticosteroid actions of endogenous steroids. Conversely, steroids are known to inhibit nicotinic receptors[15] and can also allosterically potentiate neuromuscular blocking effects of vecuronium in functional acetylcholine receptor channels expressed *in vitro*.[16]

Denervation itself results in an up-regulation of glucocorticoid receptors in skeletal muscle that might make the muscle more susceptible to the effects of steroids.[17] Muscle relaxants, when used for prolonged periods, by binding to the acetylcholine receptors on the muscle membrane and preventing neurotransmission induce a chemical denervation confirmed by the up-regulation of acetylcholine receptors.[18,19] All muscle relaxants, therefore, have the potential to enhance the interaction of corticosteroids, steroidal relaxants, or both on the muscle steroidal receptors. Whether steroidal relaxants do in fact behave like or potentiate corticosteroids has not been tested because it has been difficult to differentiate the denervation effects induced by the steroidal muscle relaxants from those of steroids.

Corticosteroids activate transcription of target genes by binding to the glucocorticoid receptor,[20] which belongs to an important class of ligand-regulated transcription factor. Regulation of the transcription of genes plays a central role in a variety of cellular processes and is tightly controlled. The glucocorticoid receptor, when activated by steroids, translocates to the nucleus and recognizes and binds to the specific DNA motif in the regulatory elements of target genes, termed *glucocorticoid-responsive element* (GRE).[21,22] The sequence of the canonical GRE is 5'-GGTACA nnn TGTTCT-3'.[21,22] When a ligand (corticosteroid) is bound, glucocorticoid receptor complexes with GRE and alters transcription of target genes.

An *in vitro* system using muscle cell cultures is more amenable to elucidate the interaction of corticosteroids and steroidal relaxants on muscle steroidal receptors because the confounding effect of nerve denervation does not come into play; muscle cells in culture devoid of the nerve input behave like denervated muscle cells.[23] The current study, using C2C12 cultured skeletal myocytes and luciferase assay, compares the intrinsic steroidal effects of prototypical steroid relaxants, pancuronium, and vecuronium to d-tubocurarine and dantrolene, representing nonsteroidal compounds, which also have actions on muscle. The interaction of these compounds with dexamethasone was also examined.

Materials and Methods

Dexamethasone, dantrolene, d-tubocurarine, and pancuronium were purchased from Sigma (St. Louis, MO). Vecuronium was provided by Akzo Nobel (Chicago, IL). Mouse C2C12 myoblasts (American Tissue Culture Col-

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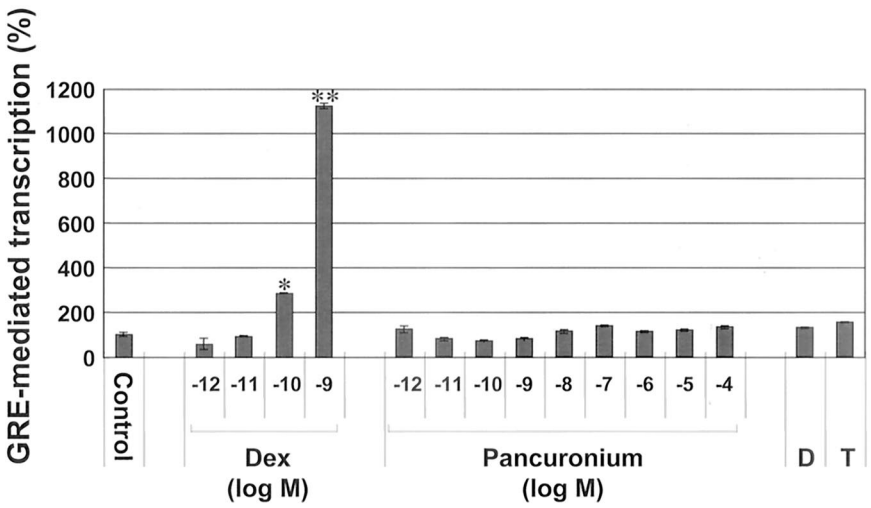

Fig. 1. The effects of pancuronium on glucocorticoid-responsive element (GRE)–mediated transcription. C2C12 myoblasts were transfected with glucocorticoid receptor expression vector, GRE-linked firefly luciferase, and control sea pansy luciferase. GRE-mediated transcription was evaluated by GRE-driven firefly luciferase activity that was normalized to sea pansy luciferase activity and was expressed as percent control (Cont). The figure shows the representative results of three independent experiments, each of which were performed in triplicate. * $P < 0.005$ *versus* control; ** $P < 0.002$ *versus* control. Dexamethasone (Dex) induced GRE-mediated transcription, whereas pancuronium did not alter GRE-mediated transcription. Dantrolene (D) and d-tubocurarine (T) showed no effects on GRE-mediated transcription, either.

lection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Sigma). To avoid the influence of glucocorticoids contained in fetal bovine serum, the medium was replaced with serum-free medium, Opti-MEM (Invitrogen, Carlsbad, CA), and then C2C12 myoblasts were seeded onto a 12-well plate. At 24 h after plating, the cells were transfected with pMMTV-Luc (0.8 μg), p6RGR (0.1 μg), and pRL-TK (0.05 μg) (Promega, Madison, MI) using Lipofectamine 2000 (Invitrogen). p6RGR is a mammalian expression vector containing complementary DNA (cDNA) for full-length glucocorticoid receptor driven by viral promotor, RSVLTR. The pRL-TK (Promega) contains cDNA for luciferase of sea pansy (*Renilla reniformis*) that is linked to the constitutive eukaryotic promoter.

To assess the effects of muscle relaxants on glucocorticoid receptor and GRE-mediated transcription, the mouse mammary tumor virus (MMTV) promoter that contains multiple GREs was fused upstream of the firefly luciferase. The expression of firefly luciferase in this plasmid, pMMTV-Luc, is strongly activated in the presence of glucocorticoid ligand and glucocorticoid receptor. Luciferase assay is one of reporter gene assays to study gene transcription and is currently in more extensive use for promoter analysis because of better sensitivity than chloramphenicol acetyltransferase or other commonly used reporter gene assays. Luciferase enzyme of the firefly (*Photinus pyralis*)[24] catalyzes the reaction for the luciferase assay; adenosine triphosphate + luciferin + oxygen, when catalyzed by luciferase, → adenosine monophosphate + oxyluciferin + pyrophosphate + light (560 nm). Because posttranslational modification is not required for its activity, the enzyme activity, as measured by the intensity of light output, is a sensitive surrogate marker of transcription. Luciferases of the firefly and sea pansy use different substrates and possess distinct biochemical properties.[25,26] The activity of luciferase of sea pansy derived from pRL-TK construct, which reflects transfection efficiency, was used as an interassay standardization control. GRE-mediated transcription was determined by the activity of firefly luciferase normalized to that of sea pansy luciferase.

At 24 h after the transfection, the various concentrations of muscle relaxants (pancuronium: 10^{-12}∼10^{-4} M; vecuronium: 10^{-12}∼10^{-4} M; dantrolene: 10^{-6} M; d-tubocurarine: 10^{-6} M) and dexamethasone (10^{-12}∼10^{-9} M) were added to the culture media, and the cells were incubated for an additional 24 h. Then, the cells were harvested for luciferase assay. Luciferase activities were measured using the Dual-Luciferase Reporter System (Promega) and a luminometer (Turner Designs Instrument, Sunnyvale, CA). The treatment with these agents for 24 h did not affect the viability of the cells, as shown by trypan blue exclusion test.

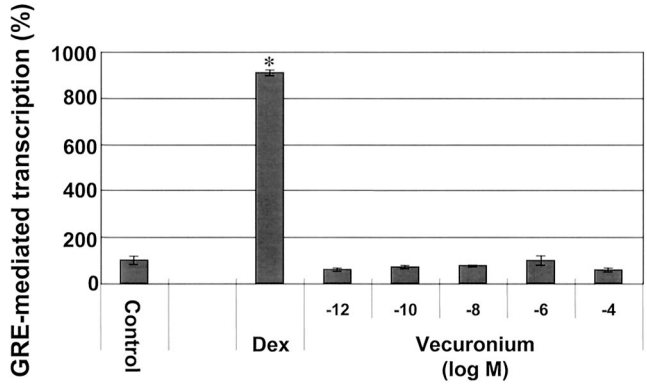

Fig. 2. The effects of vecuronium on glucocorticoid-responsive element (GRE)–mediated transcription. GRE-mediated transcription was evaluated by GRE-driven firefly luciferase activity that was normalized to sea pansy luciferase activity and was expressed as percent control (Cont). The figure shows the representative results of three independent experiments, each of which were performed in triplicate. * $P < 0.02$ *versus* control. Vecuronium did not affect GRE-mediated transcription in C2C12 myoblasts, whereas dexamethasone (Dex; 10^{-9} M) caused a marked induction.

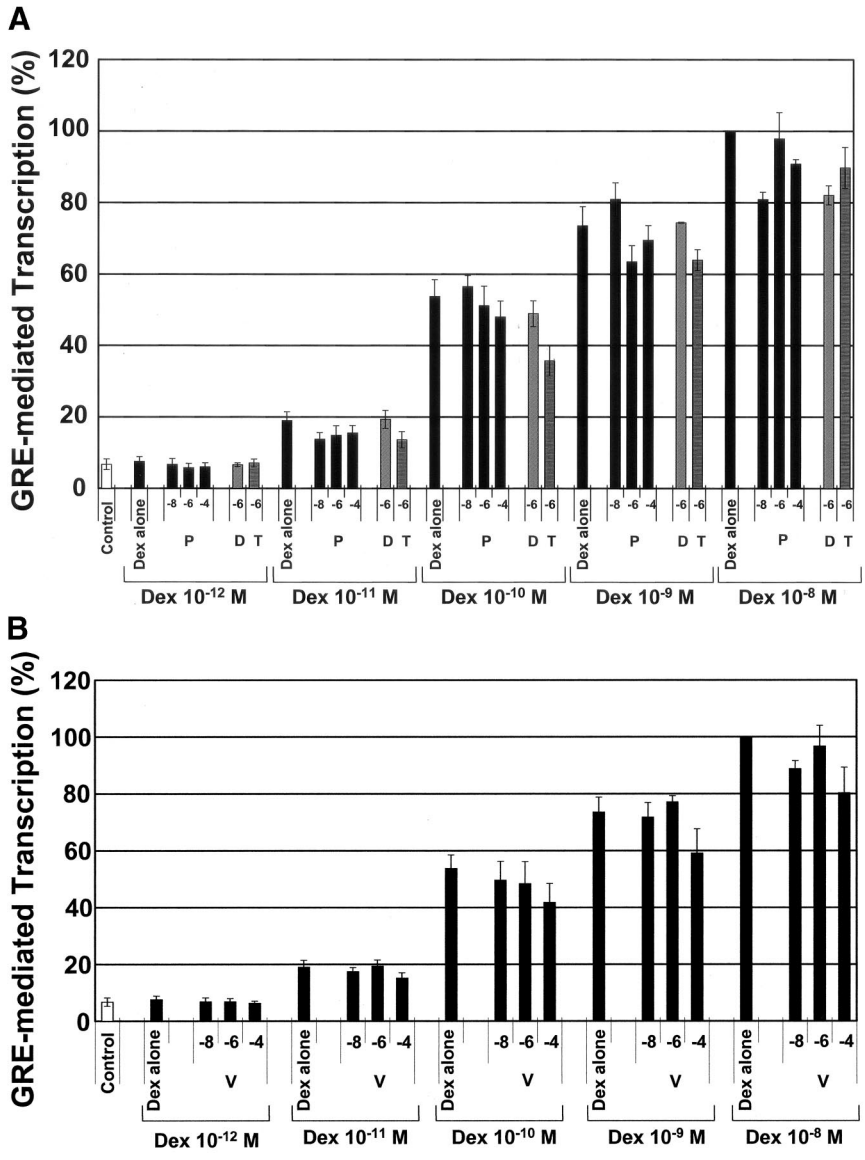

Fig. 3. The effects of muscle relaxants on dexamethasone-induced transcription. Glucocorticoid-responsive element (GRE-)–mediated transcription was expressed as percent of that induced by 10^{-8} M dexamethasone in the absence of muscle relaxants. The results are shown as the average of the data in three independent experiments performed in triplicate. Dexamethasone (Dex) up-regulated GRE-mediated transcription in a dose-dependent manner (*A* and *B*). Neither pancuronium (P; *A*), nor vecuronium (V; *B*) affected dexamethasone-induced transcription at any of the concentrations tested. Similarly, the negative controls, benzoylisoquinolinium relaxant, d-tubocurarine (T), and the ryanodine receptor antagonist dantrolene (D) did not alter dexamethasone-induced GRE transcription at any of the concentrations tested (*A*).

Glucocorticoid-responsive element–mediated transcription was compared using one-way analysis of variance followed by the Scheffé F test. The null hypothesis was rejected when P was less than 0.05. All values are reported as mean ± SEM.

Results

First, pancuronium effects on the transcription mediated by GRE were examined by luciferase assay in mouse C2C12 myoblasts transfected with the glucocorticoid receptor expression vector and the reporter genes. Dexamethasone was used as a positive control, and d-tubocurarine and dantrolene were used as negative controls. The treatment with dexamethasone resulted in a marked induction of luciferase activity in a dose-dependent manner. In contrast, pancuronium did not induce luciferase activity at the wide range of concentrations tested (fig. 1). Likewise, neither dantrolene nor d-tubocurarine affected luciferase activity. The other prototypical steroidal relaxant, vecuronium, also did not increase GRE-mediated transcription of luciferase in C2C12 myoblasts (fig. 2).

Next, to address whether pancuronium or vecuronium modulates dexamethasone-induced GRE-mediated transcription, we examined the effects of combined administration of varying concentrations of muscle relaxants and dexamethasone on the induction of luciferase. Dexamethasone caused luciferase induction in a dose-dependent manner, whereas the superimposition of neither pancuronium nor vecuronium altered dexamethasone-induced, GRE-mediated luciferase activities at all the concentrations of dexamethasone tested (10^{-12}∼10^{-8} M; figs. 3A and B). Dantrolene (10^{-6} M) and d-tubocurarine (10^{-6} M) also failed to show any effects on dexamethasone-induced GRE-mediated transcription (fig. 3A).

Discussion

Glucocorticoid receptor is a member of the nuclear receptor family of ligand-dependent transcription factors.[22] In the absence of its ligand, corticosteroid, the glucocorticoid receptor is inactive and forms a large heteromeric cytoplasmic complex with several other proteins, including heat shock proteins. The binding of corticosteroid to its receptor, however, leads to dissociation of heat shock proteins from the receptor, and homodimers of the ligand-glucocorticoid receptor are formed. Mobilization of the ligand-receptor complex to the nucleus, binding to GRE, and recruitment of coactivators, including histone acetyltransferases, to the receptors leads to activation of transcription; the whole process occurs in a matter of seconds to several minutes.

In our studies, we exposed the test drugs for 24 h, allowing sufficient time for translocation to nucleus and activation of GRE-mediated transcription. Our results demonstrate that neither pancuronium nor vecuronium modulated GRE-mediated transcription in the cultured skeletal muscle cells (figs. 1 and 2). In terms of GRE-mediated transcription, no difference was found between steroidal (pancuronium and vecuronium) and nonsteroidal (d-tubocurarine) muscle relaxant, as well as dantrolene, a ryanodine receptor antagonist in muscle. Based on our preliminary observation that pancuronium did not induce GRE-mediated transcription in simian kidney COS-7 cells and human Jurkat lymphoma cells (data not shown), the failure of pancuronium to induce GRE-mediated luciferase transcription should be considered not to be cell-type specific. It is important to note, however, that the main purpose of the study was to examine steroidal effects on muscle.

Because some previous studies suggested that treatment of critically ill patients with the combination of corticosteroids and steroidal nondepolarizing muscle relaxants was associated with severe muscle atrophy and delayed functional recovery of muscle, a question was raised whether or not steroidal muscle relaxants may enhance the adverse effects of corticosteroids. The current study, however, clearly demonstrates that the combined administration of pancuronium or vecuronium together with corticosteroid does not influence the effects of dexamethasone on the glucocorticoid receptor and GRE-mediated transcription. Both dantrolene and d-tubocurarine also did not enhance steroidal effects of dexamethasone (fig. 3). Therefore, the neuromuscular dysfunction associated with steroidal relaxants and steroids in combination is unrelated to its glucocorticoid receptor–mediated effects on transcription. In retrospect, this misconception is probably related to the fact that pancuronium and vecuronium were overwhelmingly the most commonly administered relaxants at one time because of the lack of histamine-releasing properties. In the current intensive care unit setting, with availability of other relaxant drugs, myopathy of critical illness has now been observed even with nonsteroidal relaxants and steroids in combination (*e.g.*, atracurium).[13,14]

Steroids have a profound myopathic effect,[27–29] an effect seen even in the absence of denervation.[29] Similarly, muscle relaxants themselves induce a denervation-like state,[18,19] which can induce muscle wasting and weakness. Critical illness itself and the attendant immobilization, denervation, or both independent of each other lead to insulin resistance,[7,30,31] which can result in increased protein catabolism and decreased protein synthesis, partially accounting for the muscle wasting seen in critical illness. Finally, there is evidence that corticosteroids have additive effects on inhibition of neurotransmission block produced by muscle relaxants by allosteric actions on the nicotinic acetylcholine receptor itself.[15,16] All of these different effects, to a greater or lesser extent, may account for the myopathy when muscle relaxants are used in combination with steroids.

The current study documents that neither pancuronium nor vecuronium by itself exerts corticosteroid-like action on transcription, and neither of them affect genomic actions of corticosteroids. The myopathy and prolonged muscle weakness observed in critically ill patients is not compounded by an effect specific to steroidal relaxants mimicking a steroidal effect on the glucocorticoid receptor. The fact that prolonged paralysis was seen in patients receiving nonsteroidal relaxants, atracurium and cisatracurium,[13,14] confirms that neuromuscular relaxants may have direct neuromuscular toxicity and that the myopathy with relaxants is unrelated to the corticosteroid effect of the steroidal relaxants.

References

1. Fletcher SN, Kennedy DD, Ghosh IR, Misra VP, Kiff K, Coakley JH, Hinds CJ: Persistent neuromuscular and neurophysiologic abnormalities in long-term survivors of prolonged critical illness. Crit Care Med 2003; 31:1012–6

2. Herridge MS, Cheung AM, Tansey CM, Matte-Martyn A, Diaz-Granados N, Al-Saidi F, Cooper AB, Guest CB, Mazer CD, Mehta S, Stewart TE, Barr A, Cook D, Slutsky AS: One-year outcomes in survivors of the acute respiratory distress syndrome. N Engl J Med 2003; 348:683–93

3. De Jonghe B, Sharshar T, Lefaucheur J-P, Authier F-J, Durand-Zaleski I, Boussarsar M, Cerf C, Renaud E, Mesrate F, Carlet J, Raphaël J-C, Outin H, Bastuji-Garin S: Paresis acquired in the intensive care unit: A prospective multicenter study. JAMA 2002; 288:2859–67

4. Kane SL, Dasta JF: Clinical outcomes of critical illness polyneuropathy. Pharmacotherapy 2003; 22:373–9

5. Lacomis D, Campellone JV: Critical illness neuromyopathies. Adv Neurol 2002; 88:325–35

6. Ibebunjo C, Martyn JAJ: Fiber atrophy, but not changes in acetylcholine receptor expression, contributes to the muscle dysfunction after immobilization. Crit Care Med 1999; 27:275–85

7. Hirose M, Kaneki M, Yasuhara S, Sugita H, Martyn JAJ: Immobilization depresses insulin signaling in skeletal muscle. Am J Physiol (Endo Metab) 2000; 279:E1235–41

8. Giostra E, Magistris MR, Piczolato G, Cox J, Chevrolet JC: Neuromuscular disorder in intensive care unit patients treated with pancuronium bromide. Chest 1994; 106:210–20

9. de Letter MCJ, Schmitz PIM, Visser LH, Verheul FAM, Schellens RLLA, Op de Coul DAW, van der Meche FGM: Risk factors for the development of polyneuropathy and myopathy in critically ill patients. Crit Care Med 2001; 29:2281–6

10. Anesthetic and Life Support Drugs Advisory Committee Report 1992. Rockville, Maryland, United Stated Food and Drug Administration Center for Drug Evaluation and Research, November 23, 1992

11. Elliot JM, Bion JF: The use of neuromuscular blocking drugs in intensive care practice. Acta Anaesthesiol Scand Suppl 1995; 106:70–82
12. Lewis KS, Rothenburg DM: Neuromuscular blockade in the intensive care unit. Am J Health Syst Pharm 1999; 56:72–75
13. Davis NA, Rodgers JE, Gonzalez ER, Fowler AA: Prolonged weakness after cisatracurium infusion: A case report. Crit Care Med 1998; 26:1290–2
14. Meyer KC, Prielipp RC, Grossman JE, Coursin DB: Prolonged weakness after infusion of atracurium in the intensive care unit. Anesth Analg 1994; 78:772–4
15. Paradiso K, Sabey Y, Evers AS, Zorumski CF, Covey DF, Steinbach JF: Steroid inhibition of rat neuronal nicotinic alpha4 beta2 receptor expressed in HFK 293 cells. Mol Pharmacol 2000; 58:341–51
16. Kindler, Verotta D, Gray AT, Gropper MA, Yost CS: Additive inhibition of nicotinic acetylcholine receptors by corticosteroids and neuromuscular blocking drug vecuronium. ANESTHESIOLOGY 2000; 92:821–32
17. DuBois DC, Almon RR: A possible role for glucocorticoids in denervation atrophy. Muscle Nerve 1981; 4:370–3
18. Yanez P, Martyn JAJ: Prolonged D-tubocurarine infusion and/or immobilization causes upregulation of acetylcholine receptors and hyperkalemia to succinylcholine. ANESTHESIOLOGY 1996; 84:384–91
19. Hogue CW, Ward JM, Itani MS, Martyn JAJ: Tolerance and upregulation of acetylcholine receptors follows chronic infusion of d-tubocurarine. J Appl Physiol 1992; 72:1326–31
20. Hollenberg SM, Weinberger C, Ong ES, Cerelli G, Oro A, Lebo R, Thompson EB, Rosenfeld MG, Evans RM: Primary structure and expression of a functional human glucocorticoid receptor cDNA. Nature 1986; 318:635–41
21. Beato M, Chalepakis G, Schauer M, Slater EP: DNA regulatory elements for steroid hormones. J Steroid Biochem 1989; 32:737–47
22. Schaaf MJ, Cidlowski JA: Molecular mechanisms of glucocorticoid action and resistance. J Steroid Biochem Mol Biol 2002; 83:37–48
23. Sanes JR, Lichtman JW: Development of the vertebrate neuromuscular junction. Annu Rev Neurosci 1999; 22:389–442
24. Matthews JC, Hori K, Cormier MJ: Substrate and substrate analogue binding properties of Renilla luciferase. Biochemistry 1977; 16:5217–20
25. de Wet JR, Wood KV, Helinski DR, DeLuca M: Cloning of firefly luciferase cDNA and the expression of active luciferase in Escherichia coli. Proc Natl Acad Sci U S A 1985; 8223:7870–3
26. Matthews JC, Hori K, Cormier MJ: Purification and properties of Renilla reniformis luciferase. Biochemistry 1977; 16:85–91
27. Marle WV, Woods KL: Acute hydrocortisone myopathy. Br Med J 1980; 281:871–3
28. Nava S, Grayan-Ramireza, Rollier H: Effects of acute steroid administration on ventilatory and peripheral muscles in rats. Am J Respir Crit Care Med 1996; 153:1888–96
29. Shin Y-S, Fink H, Khiroya R, Ibebunjo C, Martyn JAJ Prednisolone-induced muscle dysfunction is due more to atrophy than to altered acetylcholine receptor expression. Anesth Analg 2000; 91:322–8
30. Hirose M, Kaneki M, Sugita H, Yasuhara S, Ibebunjo C, Martyn JAJ: Long-term denervation impairs insulin receptor substrate (IRS)-1-mediated insulin signaling in skeletal muscle. Metabolism 2001; 50:216–22
31. Ikezu T, Okamoto T, Yonezawa K, Tompinks RG, Martyn JAJ: Analysis of thermal injury-induced insulin resistance in rodents: Implication of post-receptor mechanism. J Biol Chem 1997; 272:25289–95