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Stevioside Enhances Satellite Cell Activation by Inhibiting of NF-κB Signaling Pathway in Regenerating Muscle after Cardiotoxin-Induced Injury

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ABSTRACT: Stevioside, a noncaloric sweetener isolated from *Stevia rebaudiana*, exhibits anti-inflammatory and immunomodulatory effects through interference of nuclear factor (NF)-kappa B pathway. We investigated whether this anti-inflammatory property of stevioside could improve muscle regeneration following cardiotoxin-induced muscle injury. Adult male Wistar rats received stevioside orally at an accepted daily dosage of 10 mg kg^{-1} for 7 days before cardiotoxin injection at the tibialis anterior (TA) muscle of the right hindlimb (the left hindlimb served as control), and stevioside administration was continued for 3 and 7 days. TA muscle was examined at days 3 and 7 postinjury. Although stevioside treatment had no significant effect in enhancing muscle regeneration as indicated by the absence of decreased muscle inflammation or improved myofibrillar protein content compared with vehicle treated injured group at day 7 postinjury, the number of MyoD-positive nuclei were increased ($P < 0.05$), with a corresponding decrease in NF-κB nuclear translocation ($P < 0.05$). This is the first study to demonstrate that stevioside could enhance satellite cell activation by modulation of the NF-κB signaling pathway in regenerating muscle following injury. Thus, stevioside may be beneficial as a dietary supplementation for promoting muscle recovery from injury. However, its pharmacological effect on muscle function recovery warrants further investigation.

KEYWORDS: *stevioside, muscle regeneration, muscle injury, satellite cell, NF-κB pathway*

INTRODUCTION

Skeletal muscles play an essential role in locomotion and maintenance of daily activities, but they are frequently susceptible to injury due to bruising and contusion and by contraction, especially during eccentric exercises.[1] Following injury, skeletal muscles are able to repair themselves, with the degree of recovery depending on the duration and severity of the injury. Normally, after injury, muscle fibers undergo necrosis and are infiltrated by neutrophils, which is the first step of inflammation. Subsequently, macrophage invades into the site of injury to remove cellular debris and triggers satellite cell activation via the release of cytokines and growth factors.[2] The activation of myogenic satellite cells initiates the process of muscle regeneration.[3] Under normal conditions, satellite cells remain in a quiescent state located between the basal lamina and sarcolemma.[4] Following injury, activated satellite cells proliferate in order to renew the satellite cell pool and fuse with existing fibers in order to repair injured myofibers, so that muscle mass and muscle function can be restored. These processes are regulated by the expression of myogenic regulatory factors, MyoD, Myf5, myogenin, and MRF4.[2]

The NF-κB signaling pathway acts as a negative regulator of skeletal muscle myogenesis through the suppression of MyoD synthesis, activation of cyclin D1, and activation of peptide YY1, a transcription repressor of muscle formation.[5−7] The disruption of NF-κB signaling in mature muscle accelerates muscle recovery, which is characterized by an increase in the cross-sectional area of regenerating muscle, by a rise in the numbers of centrally positioned myonuclei (a hallmark of muscle regeneration), and by enhanced satellite cell proliferation in response to cardiotoxin-induced muscle injury.[8] In addition, systemic administration of NF-κB inhibitors, such as curcumin and pyrrolidine dithiocarbamate (PDTC), to freeze-induced muscle injury or to dystrophin-deficient mdx mice stimulates muscle regeneration.[9−11] Thus, disruption of NF-κB signaling pathway facilitates muscle regeneration and restores function of injured muscle.

Stevioside, a major component in *Stevia rebaudiana* leaves, has long been used as a noncaloric natural sweetener and in traditional medicine by native people in South America.[12] Due to increased health concerns of sugar consumption, stevioside has been used widely as a sugar substitute and as an ingredient in a number of products.[12] Stevioside has been approved as a food additive in Japan, Korea, and Brazil but only as a dietary supplement by the U.S. Food and Drug Administration (FDA).[13] In addition to its sweet property, stevioside has been reported to have several therapeutic properties, including antihyperglycemic,[14,15] antihypertensive,[16,17] antitumor progression,[18] and antidiarrheal effects.[19] In addition, its major metabolite, steviol, also is able to interact with renal organic anion and cation transporters, with the potential to be used to develop as modulator of the therapeutic efficacy of drugs.[20] Interestingly, stevioside was found to suppress TNF-α, IL-1, and NO release in LPS-stimulated human THP-1 monocyte cells via disruption of IKKβ and NF-κB (p50) complex,[21]

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whereas steviol attenuates TNF-α-induced IL-8 release by interfering with NF-κB signaling pathway in colonic epithelial cells including T84, Caco-2, and HT 29 cells.[22]

As stevioside is able to disrupt NF-κB function, it was of interest to determine whether oral consumption of stevioside at an acceptable daily intake (ADI) could enhance muscle regeneration after cardiotoxin-induced muscle injury in rat. The effects of stevioside on parameters related to inflammatory processes also were investigated. The present study reveals that daily consumption of stevioside as food supplement could promote satellite cell activity in regenerating muscle after cardiotoxin-induced injury. In addition, we found that NF-κB signaling pathway was attenuated in stevioside treated injured muscle.

MATERIALS AND METHODS

Chemicals. Stevioside (approximately 90% pure) used in the study was prepared and analyzed for its purity with standard compound as described below. Cardiotoxin was purchased from Calbiochem (San Diego, CA, USA). Murine anti-MyoD monoclonal and rabbit anti-pp65 (Ser 536) polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), and secondary goat anti-rabbit/mouse IgG conjugated to Alexa Fluor 488 and TOPRO-3 from Molecular Probes, Invitrogen (Carlsbad,CA). Krebs−Henseleit solution and protease inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MO).

Preparation of Stevioside. A commercial stevioside was purchased from Thai's company, Thailand. It was further purified as previously described by Adduci et al.[23] The purity of stevioside (approximately 90%) was validated using the HPLC method following the conditions previously developed by Hutapea et al.[24] Its purity was calculated from area under the peak of the chromatogram by dividing the peak area of interest by the total peak areas obtained in the chromatogram derived from HPLC.[25] The purity of the compound was expressed as percent purity. In order to identify the peak of stevioside used in this study (which was obtained from the purification process following the method of Adduci et al.[23]), the standard stevioside (a kind gift from Dr. Patoomratana Tuchinda, Department of Chemistry, Faculty of Science, Mahidol University, Thailand) was used to compare the peak height and retention time with those of our purified stevioside.

Animals. Adult male Wistar rats (250−350 g) were obtained from the National Laboratory Animal Centre of Thailand and housed at the Faculty of Science, Mahidol University, Bangkok, Thailand. Rats were fed with standard rat chow and water ad libitum and were housed under a 12:12 h dark:light cycle at 22−25 °C. Animals were acclimatized for a week before experimentation. All procedures were approved by the Animal Care and Use Committee of Faculty of Science, Mahidol University, and performed in accordance with the guidelines of the National Laboratory Animal Center of Thailand.

Experimental Design. Rats were randomly divided into 4 groups (n = 6 per group) as follows: group 1, given vehicle (control); group 2, given stevioside; group 3, given vehicle + injury to muscle; and group 4, given stevioside + injury to muscle.

Stevioside was freshly prepared in distilled water (10 mg/mL) and given to rats at a dose of 10 mg/kg body weight (BW) by oral gavage for 7 days before induction of muscle injury, and stevioside administration was continued until the end of the experimental period. In vehicle treated groups, distilled water was given at the same volume and time as that of stevioside treated groups. This dosage was based on the Joint FAO/WHO Expert Committee on Food Additives, which established an acceptable daily intake (ADI) for stevioside.[26]

Skeletal Muscle Injury Induction. The right tibialis anterior (TA) muscle was injected with 0.3 mL of 10 μM cardiotoxin in saline at the proximal, middle, and distal muscle belly (approximately 0.1 mL for each part).[27] The left TA muscle of the same rat served as uninjured control leg and was injected with saline in the same volume as cardiotoxin solution. All procedures were conducted under anesthesia using pentobarbital sodium intraperitoneal injection (60 mg/kg BW ip). TA muscles from both legs were dissected on days 3 and 7 following cardiotoxin-induced injury for assessment of muscle function, muscle degeneration, and regeneration.

In Situ Measurement of Muscle Function. Rats were anesthetized as described above, and supplementary doses were applied to maintain deep anesthesia. The TA muscle and common peroneal nerve were exposed taking care to avoid blood vessel and nerve damage. During this period, the tissue was kept moist and maintained at 37 °C using Krebs−Henseleit solution. The knee and ankle joints were tightly secured on a stable platform, and the distal part of the TA tendon was tied with a 3-0 silk suture and connected to a force transducer (FT-03, Grass, Rhode Island, USA) and muscle tension was recorded using PowerLab 400 recorder equipped with Chart 4 software (Castle Hill, NSW, Australia). The common peroneal nerve was attached to a bipolar silver electrode and stimulated with supramaximal square wave pulses (5 V, 0.2 ms duration). In order to obtain a force frequency relationship, the TA muscle was stimulated at frequencies of 10, 20, 40, 60 80, 100, 120, and 150 Hz with a 2 min pause period so as to avoid muscle fatigue.

Maximal tetanic tension (P_o) was determined from the plateau phase of the force frequency relationship. Optimal muscle length (L_0) was measured from the muscle length that produced the maximum twitch tension (P_t). Optimal fiber length (L_f) was calculated from the L_f/L_0 ratio of 0.6.[28] Muscle cross-sectional area was determined by dividing TA muscle mass by L_f and the density of mammalian skeletal muscle (1.06 g/m^3).[29] Tetanic muscle force was expressed as specific tetanic force (sP$_o$, N/cm^2) by calculating maximal tetanic force (P_o) per muscle cross-sectional area. To assess muscle fatigability and recovery of force, the TA muscle was continuously stimulated at 60 Hz for 3 min, the muscle was rested for 5 min, and then P_o was determined every min for 3 min.

Immediately after functional assessment, TA muscles from injured and uninjured legs were dissected out, trimmed free of tendon, weighed, and cut at the midbelly of the TA muscle. One section of the muscle was embedded with optimal cutting temperature Tissue-Tek compound (OCT) and snap-frozen in isopentane prechilled in liquid nitrogen and stored at −80 °C until used for morphological and immunofluorescence analysis. The other muscle section was frozen in liquid nitrogen and stored at −80 °C until used for myofibrillar protein content determination.

Morphological Analysis. Frozen TA muscle sections were cut into 6 μm thick slices starting from the midbelly at −20 °C in a cryostat and placed on microscope glass slides. Transverse sections were stained with hematoxylin and eosin (H&E). Microscope images were viewed under an inverse electron microscope (Nikon TE 2000-S, Nikon Corporation Tokyo, Japan). Degenerative area was analyzed over the entire muscle section at 10× magnification. This area was identified by presence of infiltration of inflammatory cells and muscle fiber necrosis and then measured using Image J software and expressed as percent total cross-sectional area. In addition, muscle fiber with central nuclei, a characteristic of recent muscle regeneration, was analyzed from a minimum of 500 muscle fibers per animal.

Immunofluorescence. Six μm frozen muscle sections were incubated with primary mouse anti-MyoD monoclonal or rabbit anti-pp65 (Ser 536) polyclonal antibodies (both at 1:50 dilution) for 24 h at 4 °C. The sections were washed with 0.1 M phosphate-buffered saline (PBS) containing 0.1% Triton X-100 and then incubated with Alexa Fluor 488-labeled goat anti-mouse/rabbit secondary antibody (1:1000 dilution) for 1 h at 25 °C. For myonucleus determination, sections were stained with TOPRO-3 (1:500 dilution). The numbers of MyoD- and p-NFκB-positive nuclei were determined under a confocal microscope (FV-1000; Olympus) at 40× magnification and expressed as percent MyoD- or p-NFκB-positive nuclei to total nuclei.

Myofibrillar Protein Content. TA muscle (∼100 mg) was homogenized in ice-cold homogenization buffer (50 mM Tris, 250 mM NaCl, 5 mM EDTA, and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO)) using a Polytron tissue homogenizer. All procedures were conducted on ice. The homogenate was centrifuged

Table 1. Muscle Mass and Contractile Properties of Vehicle and Stevioside Treated Groups at 3 and 7 Days Postinjury[#]

	vehicle		stevioside	
	uninjured leg	injured leg	uninjured leg	injured leg
		3 Days Postinjury		
n	6	6	6	6
TA mass, mg	608.1 ± 25.3	↓451.9 ± 22.0[b]	607.6 ± 17.8	↓385.0 ± 42.1[c]
TA mass/body mass, mg/g	1.9 ± 0.04	↓1.4 ± 0.03[b]	1.9 ± 0.05	↓1.2 ± 0.14[c]
sP_o, N/cm^2	1757.5 ± 62.6	↓917.9 ± 87.6[c]	1774.5 ± 25.3	↓872.5 ± 95.9[c]
recovery sP_o, %	100.0	↓52.3 ± 5.7[c]	100.0	↓49.4 ± 5.6[c]
		7 Days Postinjury		
n	6	6	5	5
TA mass, mg	585.4 ± 12.5	↓430.1 ± 17.7[c]	↑663.5 ± 14.4[a]	↓453.1 ± 17.4[c]
TA mass/body mass, mg/g	1.8 ± 0.14	↓1.3 ± 0.06[c]	↑2.0 ± 0.05[a]	↓1.4 ± 0.14[c]
sP_o, N/cm^2	1819.0 ± 77.3	↓1410.3 ± 68.7[b]	1667.2 ± 37.7	↓1404.7 ± 56.9[b]
recovery sP_o, %	100.0	↓78.2 ± 4.7[c]	100.0	↓84.6 ± 4.6[b]

[#]Values are means ± SE; *n*, number of animals. sP_o, specific or normalized muscle force. Superscript "a" indicates $P < 0.05$ vs same-day vehicle uninjured. Superscript "b" indicates $P < 0.01$ and "c" indicates $P < 0.001$ vs same-day vehicle uninjured and stevioside uninjured. ↓ and ↑ indicate significant difference vs same-day vehicle uninjured.

Table 2. Fatigability and Recovery Muscle Force Following 3 min Continuous Stimulation in TA Muscle from Vehicle and Stevioside Treated Groups at 3 and 7 Days Postinjury[#]

	vehicle		stevioside	
	uninjured leg	injured leg	uninjured leg	injured leg
		3 Days Postinjury		
n	6	6	6	6
fatigability (mN)				
0 min	6231.9 ± 160.1	2385.3 ± 380.8[a]	6379.5 ± 153.3	2047.2 ± 305.4[a]
1 min	803.7 ± 38.1	223.3 ± 84.2[a]	801.1 ± 58.4	192.8 ± 36.7[a]
2 min	470.2 ± 41.5	107.5 ± 56.1[a]	476.9 ± 32.5	107.9 ± 21.0[a]
3 min	292.9 ± 50.3	76.3 ± 48.9[b,c]	361.7 ± 16.4	75.8 ± 14.5[b,c]
recovery of force following 3 min fatigue protocol (mN)				
1 min	510.0 ± 22.3	120.2 ± 5.6[a]	525.7 ± 32.4	131.5 ± 20.6[a]
2 min	512.2 ± 29.7	165.0 ± 31.2[a]	507.6 ± 45.4	141.6 ± 24.3[a]
3 min	475.5 ± 23.0	161.9 ± 32.3[a]	473.9 ± 50.1	142.3 ± 25.8[a]
		7 Days Postinjury		
n	6	6	5	5
fatigability (mN)				
0 min	6569.7 ± 139.8	3218.3 ± 295.1[a]	6738.5 ± 80.4	↑3755.5 ± 190.3[a]
1 min	950.5 ± 43.9	373.2 ± 69.3[a]	970.9 ± 91.9	↑484.9 ± 22.5[a]
2 min	640.1 ± 55.6	112.7 ± 56.9[a]	641.9 ± 53.7	↑219.3 ± 16.4[a]
3 min	480.8 ± 54.2	50.4 ± 49.9[a]	490.1 ± 74.4	↑138.2 ± 24.6[a]
recovery of force following 3 min fatigue protocol (mN)				
1 min	543.4 ± 19.0	237.1 ± 23.5[a]	537.3 ± 22.6	↑281.0 ± 12.9[a]
2 min	498.2 ± 19.8	251.4 ± 22.7[a]	501.8 ± 33.3	↑301.1 ± 11.9[a]
3 min	461.7 ± 21.1	248.7 ± 21.4[a]	469.9 ± 36.4	↑296.5 ± 11.3[b]

[#]Values are means ± SE; *n*, number of animals. Superscript "a" indicates $P < 0.001$ vs same-day vehicle and stevioside uninjured. Superscript "b" indicates $P < 0.01$ vs same-day vehicle uninjured. Superscript "c" indicates $P < 0.001$ vs same-day stevioside uninjured, ↑ indicates tended to be increased vs same-day injured legs.

at 4 °C for 15 min at 2500*g* and the supernatant containing sarcoplasmic protein was discarded. The myofibrillar pellet was resuspended and centrifuged three times in the homogenization buffer containing 0.5% Triton X-100. The myofibrillar pellet then was solubilized in 0.3 N NaOH and protein content was determined using the Lowry method.

Statistical Analysis. Results are expressed as mean ± SE. For multiple comparisons, statistical analysis was performed using multivariate ANOVA (MANOVA) followed by Tukey post hoc test to determine the effects of treatment. For individual comparison, statistical analysis was performed using Student's *t* test. The data were calculated as percentage for myofibrillar protein, number of MyoD, NFκB-positive nuclei, and central located nuclei; the statistical analysis was also performed using Student's *t* test. All data were analyzed using SPSS software and significant difference was accepted at *P* value <0.05.

RESULTS

Muscle Mass and Contractile Properties. In order to determine whether stevioside treatment had any effects on muscle mass and its contractile function after cardiotoxin-induced injury, body mass, TA muscle mass, tetanic muscle force (P_o), fatigability, and recovery of muscle force were measured. For both vehicle and stevioside treated groups, the left leg of each rat served as its own control (uninjured leg). There was no significant difference in body mass of vehicle

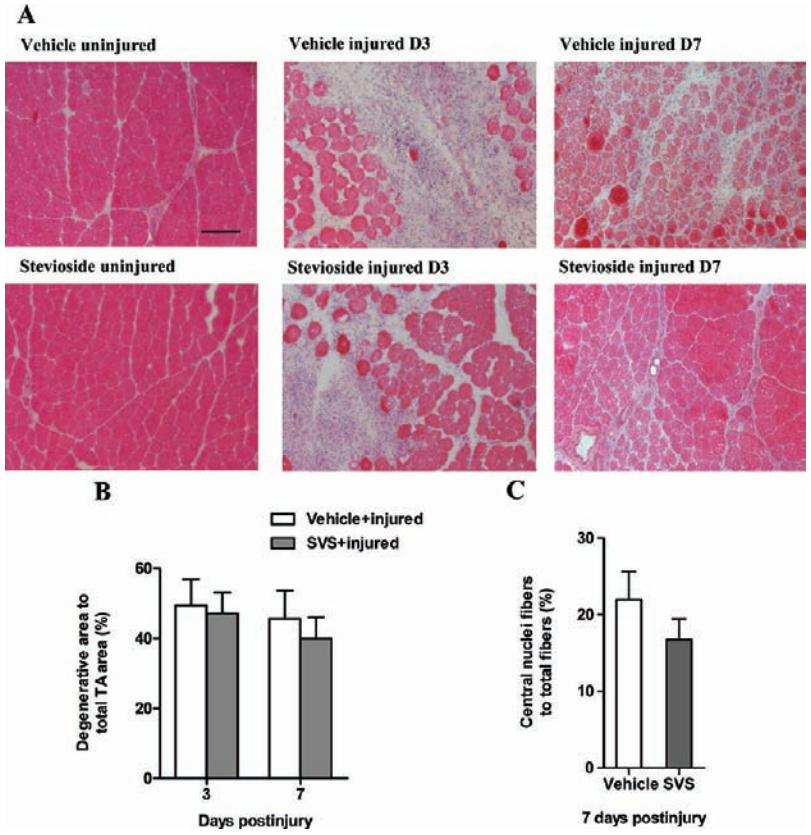

Figure 1. Morphology of uninjured and injured TA muscle from vehicle and stevioside treated rats. (A) Representative hematoxylin and eosin stained sections at days 3 and 7 post-cardiotoxin-induced muscle injury. Scale bar = 100 μm. (B) the proportion of degenerative area in cardiotoxin-induced injured TA muscle from vehicle and stevioside treated rats at day 7 postinjury. (C) number of central nucleus fibers in cardiotoxin-induced injured TA muscle from vehicle and stevioside treated rats at day 7 postinjury. There were no significant difference effects of stevioside.

treated rats compared with that of stevioside treated rats at days 3 (312.8 vs 316.4 g) and 7 (326.4 vs 331.0 g) after cardiotoxin injection. In addition, both the absolute and relative TA muscle mass of vehicle treated uninjured legs were similar to those of stevioside treated uninjured legs at day 3 after injury. However, at day 7, the absolute muscle mass of uninjured leg in stevioside treated group was about 13% larger than that of uninjured muscle in vehicle treated group ($P < 0.05$). As for the injured leg, muscle mass in both vehicle and stevioside treated groups was less than that of uninjured leg at both time points ($P < 0.001$). The muscle mass of injured leg in both vehicle and stevioside treated groups was not statistically different at both time points (Table 1).

Tetanic muscle force was measured in order to examine whether stevioside treatment could improve muscle function after injury. At days 3 and 7 after injury, specific muscle force (sP_o) of injured muscles from both stevioside and vehicle treated groups were similar (~50% and 21% lower than that of uninjured muscle at days 3 ($P < 0.001$) and 7 ($P < 0.01$) after injury respectively. However, at day 7, the percent recovery of sP_o in stevioside treated group tended to be greater, although not statistically significantly, than in vehicle treated group (Table 1).

Fatigability of injured muscles in both groups was not different at day 3 postinjury. However, injured muscle from stevioside treated group tended to be less fatigued than that of vehicle treated injured muscle during the 3 min continuous stimulation at day 7 after injury. In addition, the recovery of muscle force in injured muscle from stevioside treated group following the 3 min fatigue protocol was slightly greater than that of vehicle treated group at day 7 after injury (Table 2). These results indicated that the degree of muscle fatigue in stevioside treatment after cardiotoxin-induced injury at day 7 is slightly less than that of vehicle treated group.

Muscle Histological Analysis. Muscle histological analysis was performed in order to quantify muscle degenerative phase following injury. Muscle degeneration is defined as increased necrotic myofibers and inflammatory cell infiltration in the injured area. Degenerative area (50%) of injured TA muscle in vehicle treated group was not significantly different from that (47%) of stevioside treated group at day 3 after injury. However, at day 7, degenerative area of injured TA muscle in vehicle treated group was decreased to 45% whereas in stevioside treated group the decrease was 39% (Figure 1**B**).

Central Nucleus Fiber Status. The presence of centrally located nuclei in myofiber is a marker for the recent muscle regeneration. The mean number of central nucleus fibers was 23% in injured muscle of vehicle treated group at day 7 after cardiotoxin-induced injury. On the other hand, muscle fibers in stevioside treated group had 19% central (Figure 1C). At day 3 after injury central nucleus fibers could not be quantified due to increased inflammatory response and presence of necrotic myofibers. Interestingly, injured muscle fibers from vehicle treated group had a tendency to have a larger number of central nucleus fibers compared to those of stevioside treated group. These results implied that stevioside treated group had a tendency to reduced muscle fiber damage after cardiotoxin-

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induced injury as indicated by the lower percent of central nuclei.

Myofibrillar Protein Content. Myofibrillar protein content represents the functional contractile machinery in muscle fibers. At day 3 after injury, muscle of vehicle injured group contained only 52% of myofibrillar protein compared with vehicle uninjured group ($P < 0.01$), whereas more myofibrillar protein content of injured muscle in stevioside treated group was observed, 68%, compared with vehicle uninjured group ($P < 0.05$). At day 7 after injury, myofibrillar protein content of injured muscles in vehicle treated group was still decreased (~56%) compared to that of vehicle uninjured group ($P < 0.01$). Interestingly, myofibrillar protein content of injured muscle in stevioside treated group at day 7 after injury slightly increased (Figure 2). These results indicated that stevioside may exert some stabilizing effect against loss of myofibrillar protein content following cardiotoxin-induced injury.

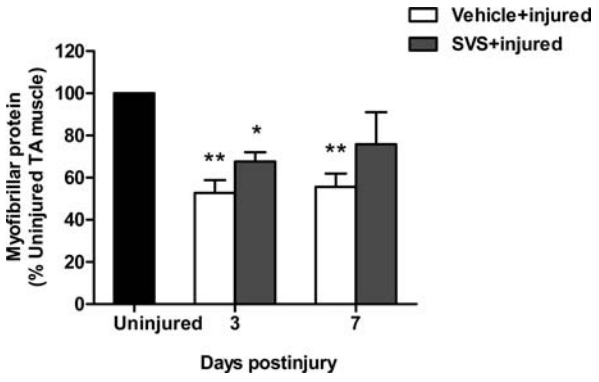

Figure 2. Myofibrillar protein content in uninjured and cardiotoxin-induced injured TA muscle from vehicle and stevioside treated rats at days 3 and 7 postinjury. Values are mean ± SE. *$P < 0.05$ and **$P < 0.01$ vs uninjured muscle.

Satellite Cell Activation. MyoD is a myogenic regulatory factor and is expressed during early regenerative phase in differentiated satellite cells.[30] At day 3 after injury, the numbers of MyoD-positive nuclei in muscle of both vehicle and stevioside treated groups is not statistically different. However, at day 7 after injury, numbers of MyoD-positive cells in muscle of stevioside treated group was 1.6-fold higher than that of vehicle treated group ($P < 0.05$) (Figure 3B), indicating that stevioside treatment enhances myogenic differentiation.

NF-κB Expression. NF-κB translocation from cytosol to nucleus can activate expression of genes involved in inflammation.[31] Therefore, the presence of phosphorylated NF-κB (p65) in nuclei was determined in order to test if the enhancement of MyoD level in stevioside treated injured muscle was due to the suppression of NF-κB translocation. NF-κB has been shown to be activated in injured muscle caused by cardiotoxin.[8,32] In uninjured muscle, there was no significant difference in percent p-NF-κB-positive nuclei in both vehicle and stevioside treated groups. At days 3 and 7 after injury, muscles from vehicle and stevioside treated groups had higher numbers of p-NF-κB-positive nuclei than that of vehicle treated uninjured muscles ($P < 0.001$). However, in injured muscle of stevioside treated group, the number of p-NF-κB-positive nuclei was significantly lower than that of vehicle treated group at day 7 after injury ($P < 0.05$) (Figure 4B), supporting the notion that stevioside treatment exerts an anti-inflammatory effect via inhibition of NF-κB activation (nuclear translocation).

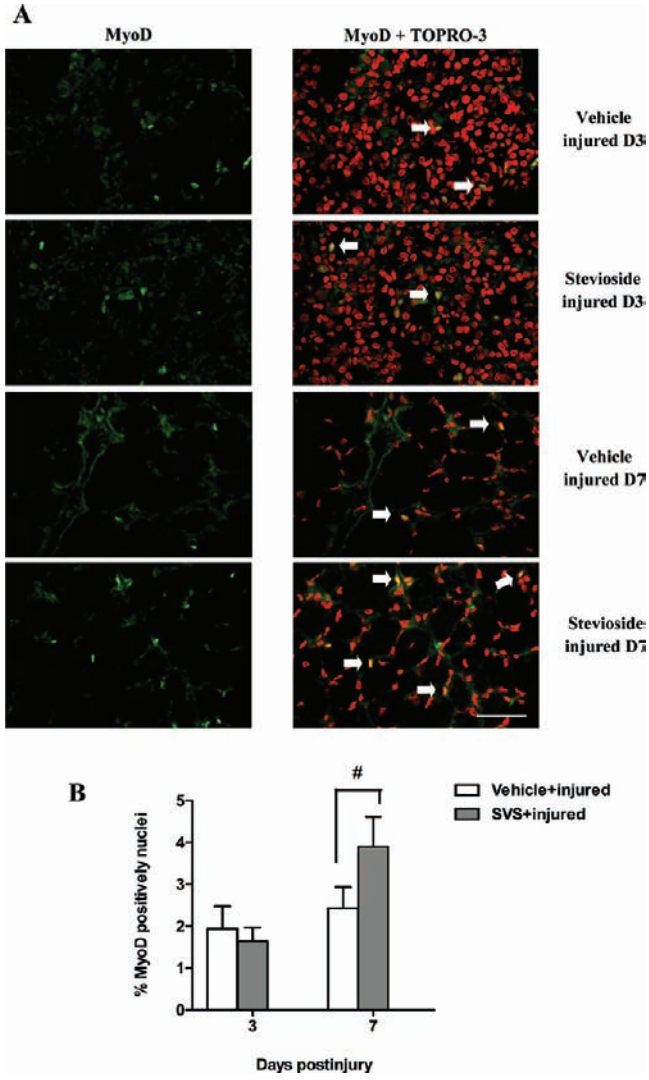

Figure 3. MyoD expression in cardiotoxin-induced injured TA muscle from vehicle and stevioside treated rats at days 3 and 7 postinjury. (A) Representative immunofluorescent staining of MyoD (green) and TOPRO-3-labeled nuclei (red). Left panels, MyoD staining; right panels, merge of MyoD and TOPRO-3 stainings. Arrowhead indicates presence of MyoD (yellow) in nucleus. Scale bar = 50 μm. (B) the number of MyoD-positive nuclei in cardiotoxin-induced injured TA muscle from vehicle and stevioside treated rats at day 3 and 7 postinjury. #$P < 0.05$ vs injured muscle.

DISCUSSION

Inhibition of NF-κB signaling pathway accelerates muscle regeneration in several degenerative−regenerative models, including dystrophin-deficient mdx mice, freezing, and myotoxin-induced muscle injury.[9−11] Stevioside has been found to disrupt the NF-κB pathway involved in the inflammatory responses of immune and colonic cell lines.[21,22] Thus, the anti-inflammatory and myogenic roles of stevioside in an experimental model of muscle injury, which mimics physiological response in a living system, were investigated.

The pharmacokinetics study has shown that stevioside is absorbed into the blood circulation in the form of its aglycon steviol. Our previous studies have also demonstrated that steviol is able to interact with renal transporters.[20,33] In addition, steviol absorption in the intestinal epithelial Caco-2 cells line has been shown to be mediated by monocarboxylic

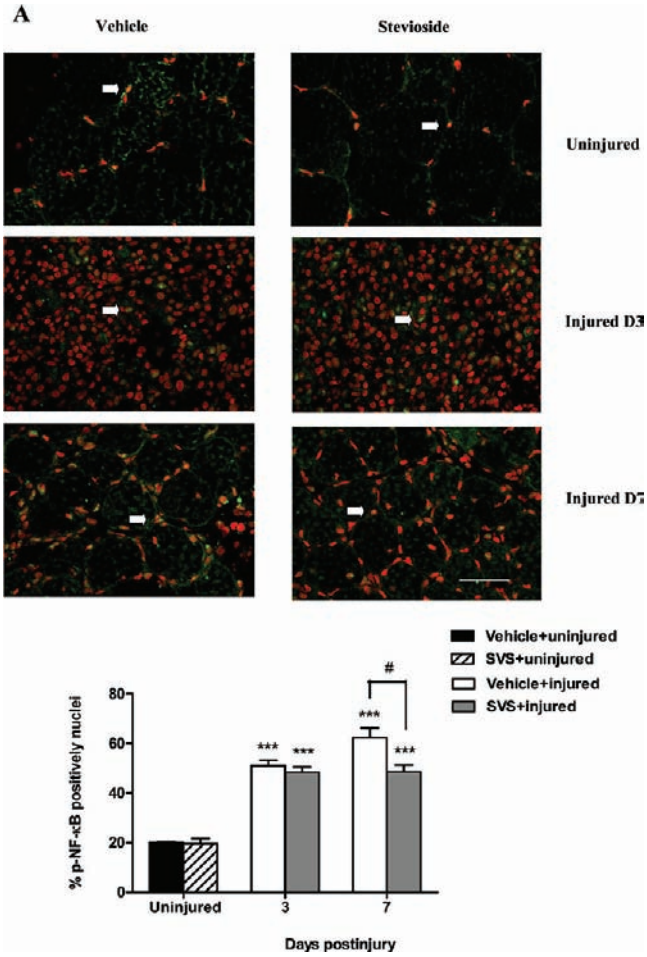

Figure 4. NF-κB localization in uninjured and cardiotoxin-induced injured TA muscle from vehicle and stevioside treated rats. (A) Representative merged immunofluorescent staining of p-NF-κB (green) and TOPRO-3-labeled nuclei (red). Arrowhead indicates presence of NF-κB (yellow) in nucleus. Scale bar = 50 μm. (B) The number of p-NF-κB-positive nuclei in cardiotoxin-induced uninjured and cardiotoxin-induced injured TA muscle from vehicle and stevioside treated rats at days 3 and 7 postinjury. ***$P < 0.001$ vs uninjured muscle. $^{\#}P < 0.05$ vs injured muscle.

transporters.[34] Collectively, this evidence suggested that steviol may enter the muscle cell via transporter-mediated processes. The regenerative capacity of stevioside ingestion observed in our study may be due to the direct action of steviol on muscle cells.

The model of cardiotoxin injection into tibialis anterior muscle used in this study is a well reproducible approach and has been widely employed in several studies to induce the degeneration−regeneration process.[2] Cardiotoxin injection damages all skeletal muscle fibers but does not affect microvasculature and myogenic satellite cells. Histological analysis of injured muscle has shown that following cardiotoxin injection inflammatory cell infiltration significantly increases in the injured muscle fibers at day 3 after cardiotoxin treatment.[35] At day 7 after injury, myofibers exhibit small diameter with central nuclei indicating new formation of muscle fibers, but infiltration of inflammatory cells still is present in this period.[27,35] Thus, we selected day 3 post-cardiotoxin-induced injury for the examination of the anti-inflammatory effect of stevioside. The early regenerative capacity of muscle was investigated at day 7 postinjury in order to observe the role of stevioside on muscle regeneration.

In cardiotoxin treated rats, stevioside treatment (10 mg/kg BW) had no effect on body mass and had no detrimental effect on the (self-control) uninjured skeletal muscle as characterized by the normal morphology and muscle contractile properties, which were similar to those of vehicle treated cardiotoxin injured group. JECFA reported that the ADI dose of stevioside (up to 11 mg/kg BW) has no detectable adverse effects on human health.[26,36] Indeed, TA mass of uninjured muscle from stevioside treated group was higher than that of vehicle treated group at day 7. Also, the total protein content of stevioside treated uninjured muscle was higher than that of vehicle treated uninjured muscle at day 7 after injury (data not shown). Thus, the increase in mass of the uninjured muscle in the stevioside treated group may be partially mediated through enhanced protein synthesis, but the exact mechanism underlying this phenomenon requires further investigation.

Regenerating muscles of stevioside treated group tended to be less susceptible to fatigue than those of vehicle treated group. It is well-known that skeletal muscle composition can shift from one fiber type to another muscle fiber type under conditions of adaptation to training or myotoxic injury.[37,38] The alteration of twitch-to-tetanus ratio indicates the shift of skeletal muscle between fast and slow phenotype.[39] However, regenerating muscle from stevioside treated group did not show any change in this ratio at days 3 and 7 after injury (data not shown). Another cause of muscle fatigue is energy depletion in skeletal muscle.[40] Stevioside has been shown to improve glucose transport into the skeletal muscles of insulin resistance rat,[41] thereby this process may account for the reduced susceptibility of regenerating muscle in stevioside treated group to fatigue.

MyoD is myogenic regulatory factor expressed in activated and differentiated satellite cells.[30] In this study, stevioside treatment significantly increases MyoD expression in injured TA muscle at day 7 after injury. Megeney et al.[42] showed that mice lacking MyoD exhibit impaired muscle regeneration. This provides another explanation for the effectiveness of stevioside in accelerating the differentiation of regenerating fibers.

In damaged muscle fibers, satellite cells are responsible for repair and the nuclei of newly fused satellite cells nuclei are present in the center of the damaged fibers, and such cells subsequently migrate to the periphery as myonuclei.[2] The continued existence of centrally located nuclei in regenerating muscle fibers indicates the existence of an abnormal process impairing the regenerative capacity.[43,44] In injured muscle, stevioside treatment tended to reduce ($P = 0.14$) the numbers of central nuclei, suggesting that stevioside supplementation partially accelerates satellite cell fusion in repairing muscle fibers. However, there was no significant difference in the number of central nuclei in stevioside treated injured muscle, which could be explained by the fact that stevioside acts at the early step of muscle regeneration (satellite cell activation and differentiation) to promote muscle regeneration.

Indexes of acute inflammation (percentage of degenerative area) and NF-κB translocation were quantified in order to determine how stevioside supplementation modulated inflammation. There was no difference in the percentage of degenerative area between stevioside treated and control injured legs. Although stevioside has been shown to have anti-inflammatory properties in cell culture[21,22] and animal models,[14] it was not found to reduce inflammatory cell

infiltration in injured muscle at days 3 and 7 after cardiotoxin-induced injury. This could be speculated that mediators or factors from inflammatory cells impacting inflammatory response after cardiotoxin-induced injury may not be affected by stevioside treatment. The recovery should thus likely be the effect of steviol/stevioside on regenerative capability of satellite cells. Concomitantly, we observed an increase in satellite cell activation in regenerating muscle of stevioside treated group.

NF-κB is a transcriptional factor that modulates many gene expressions involved in inflammation and immune responses.[31,45] Here, NF-κB activation (as determined by nuclear localization of the phosphorylated form) in injured muscle from stevioside treated group was lower than that of vehicle treated group at day 7 after injury, indicating that stevioside impaired the NF-κB signaling pathway. These results are consistent with those of Geeraert et al.[14] showing that stevioside treatment decreased the expression of nuclear factor-B subunit 1 (NFκb1) gene in vascular wall of obese insulin resistant mice. Recently, NF-κB has been shown to inhibit myogenesis via suppression of MyoD expression.[6] Furthermore, Mourkioti et al.[8] found that in IKK-2 (upstream catalytic subunit of NF-κB pathway) depleted mice there is an acceleration of muscle recovery after cardiotoxin induced injury and regenerating muscle of IKK-2 depleted mice showed reduced inflammatory response and enhanced satellite cell activation.

Unexpectedly, we did not find any significant difference in TA mass, myofibrillar protein, central located nuclei, and contractile function between stevioside treated and vehicle treated injured legs. Skeletal muscle regenerative process can be divided into 2 phases: inflammation and regeneration phases.[3] Inflammatory phase is defined as the event by which muscle fibers undergo necrosis with accompanying inflammatory cell infiltration.[3] Regenerative processes are initiated by activation of satellite cell (early regeneration).[3] Subsequently, satellite cell fuses to injured fibers and restores normal muscle function.[3] Although there was no change in TA mass, myofibrillar protein, central located nuclei, and contractile function in stevioside treated injured muscle, the stevioside treatment could enhance muscle recovery at the early regenerative stage. These findings suggested that increased satellite cell activation and decreased NF-κB activity in skeletal muscle after injury are not the main factors in the improvement of muscle function.

In summary, the present study demonstrated that stevioside supplementation (at accepted daily intake) could enhance muscle recovery from cardiotoxin induced injury. Enhanced satellite cell activity and reduced NF-κB activation were observed in regenerating muscle. However, the molecular mechanisms by which stevioside is able to exert these effects and therapeutic potential of stevioside in muscle injury require further investigation.

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