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Honokiol protects rats against eccentric exercise-induced skeletal muscle damage by inhibiting NF-κB induced oxidative stress and inflammation

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ABSTRACT

Honokiol, a bioactive component isolated from the Chinese herb *Magnolia officinalis*, is known for its potent antioxidative and anti-inflammatory effects. To study whether honokiol can protect skeletal muscle from sports injuries, we set up an eccentric exercise bout protocol for rats consisting of downhill running on a treadmill and examined the effect of oral administration of honokiol at 1 h before eccentric exercise at a dose of 5 mg/kg on day 1 (HK5×1) or 1 mg/kg/day for 5 consecutive days (HK1×5). Eccentric exercise was implemented for 3–5 consecutive days, and induced remarkable tissue damage. This damage was associated with an increase in serum creatine levels, increase in protein nitrotyrosylation, poly-ADP-ribose-polymerase (PARP) upregulation, lipid peroxidation, and leukocyte infiltration. The degree of muscle damage also paralleled dramatic gene expression for cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and inflammation-associated cytokines (interleukin (IL)-1β, IL-6, tumor necrosis factor-α, and monocyte chemoattractant protein-1), possibly through activation of nuclear factor kappa-B (NF-κB), a crucial proinflammatory transcription factor. Both honokiol treatments (HK5×1 and HK1×5) significantly ameliorated eccentric exercise-induced muscle damage as revealed by suppression of cell fragmentation, protein nitrotyrosylation and PARP upregulation, as well as reductions in lipid peroxidation and leukocyte infiltration, possibly through downregulating gene expression for COX-2, iNOS, and the proinflammatory cytokines by modulation of NF-κB activation. In conclusion, the present study demonstrates for the first time that honokiol exhibits protective effects against eccentric exercise-induced skeletal muscle damage in rats, probably by modulating inflammation-mediated damage to muscle cells.

1. Introduction

Honokiol, an active component isolated from the herb 'houpo' (*Magnolia officinalis* Rehd. et Wils.), is an effective antioxidant (Taira et al., 1993) that protects animal tissues against lipid peroxidation (Lo et al., 1994; Chiu et al., 1997). It also serves as an antiplatelet agent (Teng et al., 1988) and displays an anti-inflammatory effect in activated macrophages (Son et al., 2000; Matsuda et al., 2001). We previously reported that honokiol reduces myocardial infarct size and exhibits an antiarrhythmic effect in rats subjected to coronary artery occlusion (Tsai et al., 1996, 1999). Recently, we found that honokiol can ameliorate focal cerebral ischemic reperfusion-induced brain infarction (Liou et al., 2003a), possibly through its anti-inflammatory effects against leukocyte activation (Liou et al., 2003b; Chiang et al., 2006; Munroe et al., 2007).

Proper physical activity is known to attenuate chronic diseases such as cardiovascular diseases, hypertension, diabetes, and obesity (AlJaroudi and Petersen, 2006; Volker, 2006; Giada et al., 2008). Acute exercise, particularly eccentric exercise, induces skeletal muscle damage that causes infiltration of neutrophils to the site of injury within several hours (Beaton et al., 2002). The increased infiltration of neutrophils may last for

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up to 24 or even 48 h after exercise (MacIntyre et al., 2000; Mahoney et al., 2008). Macrophages are also present in muscle from 1 to 14 d after exercise (Round et al., 1987; Malm et al., 2000; Mahoney et al., 2008). These reports reveal that infiltration of neutrophils and macrophages for such long time may be one of the causes of delayed on-set muscle soreness by eccentric exercise. After being recruited to skeletal muscles, neutrophils and macrophages release reactive oxygen species (e.g., O_2^-, OH^-, and H_2O_2), nitrogen species (e.g., NO and $ONOO^-$) (Nguyen and Tidball, 2003a,b), and proinflammatory cytokines, which damage muscle tissue by oxidative stress and inflammatory responses (Cannon and St Pierre, 1998). It has been reported that the proinflammatory cytokines interleukin (IL)-1β and tumor necrosis factor (TNF)-α are detected in skeletal muscle up to 5 d after exercise (Cannon et al., 1989; Fielding et al., 1993; Hamada et al., 2005) and play a role in initiating the breakdown of damaged muscle tissue (Cannon and St Pierre, 1998). Other study indicated that after a marathon, plasma concentrations of TNF-α (2.3-fold), IL-1β (2.1-fold), IL-6 (128-fold), and IL-10 (27-fold) are all elevated immediately after running, while soluble TNF-α receptors (2-fold) and IL-1ra (39-fold) peak 1–1.5 h later (Ostrowski et al., 1999). Therefore, the local immune responses within skeletal muscle after eccentric exercise is predominantly proinflammatory, although other cytokines such as IL-6 and transforming growth factor (TGF)-β1 are also expressed in skeletal muscle following eccentric exercise (Malm et al., 2004; Hamada et al., 2005). The latter cytokines may play important roles to mediate restriction of inflammation (Petersen and Pedersen, 2005). In addition to cytokines, Mahoney et al. also identify many novel genes that respond to skeletal muscle damage are likely involved in recovery from and/or adaptation to damaging exercise in healthy men (Mahoney et al., 2008).

Nuclear factor-κB (NF-κB) is one of the most important signaling pathways that are activated during eccentric exercise (Jimenez-Jimenez et al., 2008). As a nuclear transcription factor, NF-κB directly alters the expression of a large number of genes, including those encoding cytokines, immune and antigen-presenting receptors, and regulators of the redox status, the acute-phase response, apoptosis, cachexia, atrophy, and host defense (Pahl, 1999). Most genes activated by NF-κB have been shown to be proinflammatory and to be involved in the inflammatory process (Chung et al., 2005). The expression of these genes, including those encoding the proinflammatory cytokines, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), is induced in inflammatory cells, and their products work in concert to cause tissue inflammation by diverse mechanisms including activation of protein nitrotyrosylation, lipid peroxidation, DNA breaking, and the production of proinflammatory prostanoids such as prostaglandin E_2 (Ischiropoulos, 1998; Cipollone et al., 2001). It has recently been reported that an acute bout of eccentric exercise induces muscular injury and a change in NF-κB activation in healthy adults (Garcia-Lopez et al., 2007). Therefore, it is conceivable that NF-κB activation could be a consequence of stimuli arising from the damaged skeletal muscle to mediate eccentric exercise-induced oxidative and inflammatory cell injury.

For these reasons, we hypothesized that honokiol, a potent antioxidant with powerful anti-inflammatory properties, will be beneficial for protecting the skeletal muscle from eccentric exercise-induced injury. Therefore, the present study examined the effects of honokiol on the oxidative and inflammatory tissue damage and the expression of inflammation-associated genes, as well as changes in NF-κB activation induced by an acute bout of eccentric exercise in muscles from downhill-running rats.

2. Materials and methods

2.1. Animals and exercise protocol

All animal procedures and protocols were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH, 1996) and were reviewed and approved by the Animal Research Committee of National Research Institute of Chinese Medicine. Male adult Wistar rats weighing 250–300 g (6–7 weeks old; National Laboratory Animal Breeding and Research Center, Taipei, Taiwan) were used. Rats were treated with honokiol (HK) or the vehicle solution (0.1% propylene glycol in saline) by gastric gavage (1 ml/kg). Rats were assigned to one of four groups ($n = 5$–10 for each group). The first group received the vehicle solution with no exercise whereas the second group received the vehicle solution but was subjected to 5 or more consecutive days of eccentric exercise as indicated. The third and fourth groups were subjected to 3–5 consecutive days of eccentric exercise as indicated after receiving HK at the dose of 5 mg/kg for 1 d on day 1 (HK5 × 1) or HK for 5 consecutive days at the dose of 1 mg/kg (HK1 × 5) from day 1. The eccentric exercise was performed on a rodent treadmill using the following protocol as described by Cabral de Oliveira et al. (2001) with some modifications. In brief, rats were subjected to a protocol of intermittent downhill (at a −16° incline) at 16 m/min for a total of 90 min/day that included eighteen 5-min bouts separated by 2-min rest period for 3 to 5 consecutive days as indicated. At the time of sacrifice the animals were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and the muscles rapidly excised, trimmed of extraneous fat and connective tissue, immersed in isopentane, dropped into liquid nitrogen, and stored at −70 °C until further analysis. The muscles studied were the soleus, rectus, extensor digitorum longus, tibialis anterior, and vastus intermedius. Additional animals were taken from the groups described above for real-time PCR analyses.

2.2. Biochemical analysis

Blood was sampled from the tail vein and used to determine serum creatine kinase levels immediately after eccentric exercise. Serum creatine kinase levels were analyzed using a commercially available kit of Fuji dry-chem slides (Fujifilm, Tokyo, Japan).

2.3. Histological analysis

The muscles were removed from all rats and fixed in 4% (v/v) formalin, embedded in paraffin, cut longitudinally into 5 μm sections and stained with hematoxylin–eosin (H&E). The density of leukocyte infiltration and the extent of muscle fiber damage (fragmentation) were observed under microscope (10× or 40×) and estimated using 10 randomly selected fields per section.

2.4. Western immunoblot analysis

Frozen muscle tissue was homogenized by a homogenizer (Bullet Blender™, Next Advance Inc., NY, USA) containing stainless steel beads (100 mg) for 3 times (3 min each) in lysis buffer (50 mM Tris-HCl (pH 7.4), 50 mM 2-mercaptoethanol, 1 mM phenylmethyl sulfonyl fluoride, 1 mg/ml leupeptin, 1 mM NaF, 1 mM Na_3VO_4, 2 mM EDTA, and 5 mM EGTA). After centrifugation at 18,000 ×*g* for 30 min at 4 °C, the supernatant was collected and the protein concentration was determined using a BioRad protein assay kit (BioRad Laboratory, Hercules, CA, USA). Equal amounts of protein (around 40–80 μg) were subjected to 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) and electrotransferred to a hydrophobic polyvinylidene difluoride (PVDF) membrane. After blocking with 5% nonfat milk in PBS containing 0.05% Tween 20 (PBST) for 1 h at 4 °C, the membrane was washed 3 times with PBST and incubated overnight at 4 °C with proper dilution of the first antibody against nitrotyrosine (Upstate, New York, NY, USA), myeloperoxidase (MPO, Abcam, Cambridge, UK), 4-hydroxy-2-nonenal (4-HNE) (JalCA, Shizuoka, Japan) for malondialdehyde (MDA) formation, phosphorylated p65 (pP65) (Cell Signaling Technology, Hitchin, UK), COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), poly-ADP-ribose-polymerase (PARP), iNOS, IκB-α, or p65 (all from BD Biosciences, San Diego, CA, USA), or β-actin (Sigma-Aldrich, St. Louis, MO, USA) as the loading control. After additional washes with PBST, the membrane was

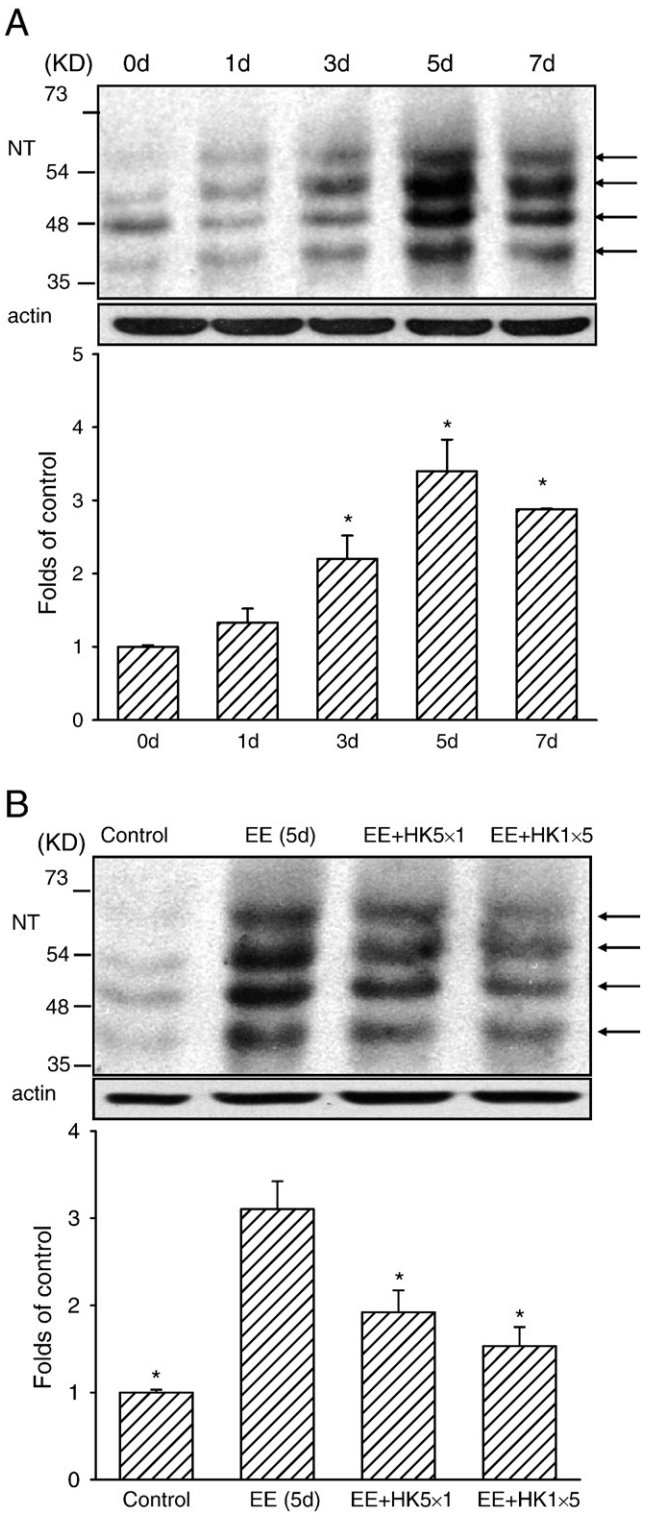


Fig. 1. Honokiol reduces eccentric exercise-induced protein nitrosylation in skeletal muscles of rats subjected to eccentric exercise. (A) Upper panel, representative immunoblots for time-dependent (0–7 d) protein nitrosylation (NT) formation after eccentric exercise. Lower panel, four major nitrosylation bands (arrow) included for statistical analysis. (B) Upper panel, honokiol treatments on NT from rats received the vehicle solution with no exercise (control) or subjected to eccentric exercise for 5 consecutive days (EE (5d)). Honokiol (HK) treated-rats received HK 5 mg/kg for only 1 d on day 1 (HK5 × 1) or HK 1 mg/kg for 5 consecutive days from day 1 (HK1 × 5) were subjected to eccentric exercise for 5 successive days (EE + HK5 × 1 and EE + HK1 × 5). Lower panel, statistical analysis. Data were calculated as multiples (folds) of the control after normalization to β-actin, and are expressed as the mean ± S.E.M. ($n = 5–10$ for each data point). $^{*}P<0.05$, compared to (A) 0 d or (B) EE (5d) by one-way ANOVA followed by *post-hoc* S–N–K test.

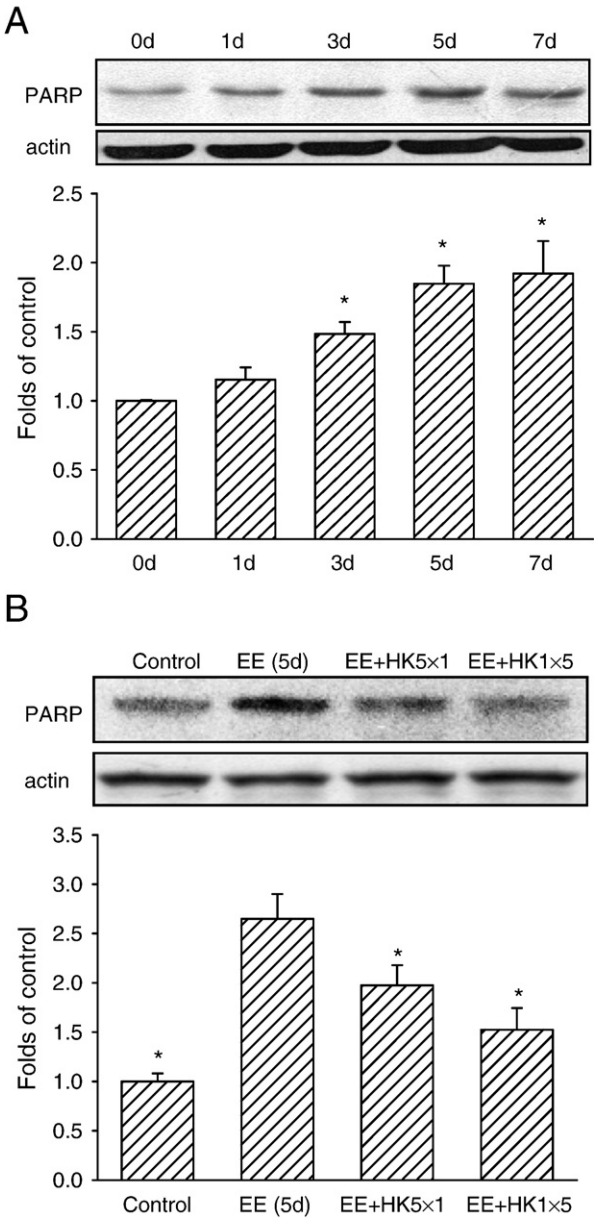
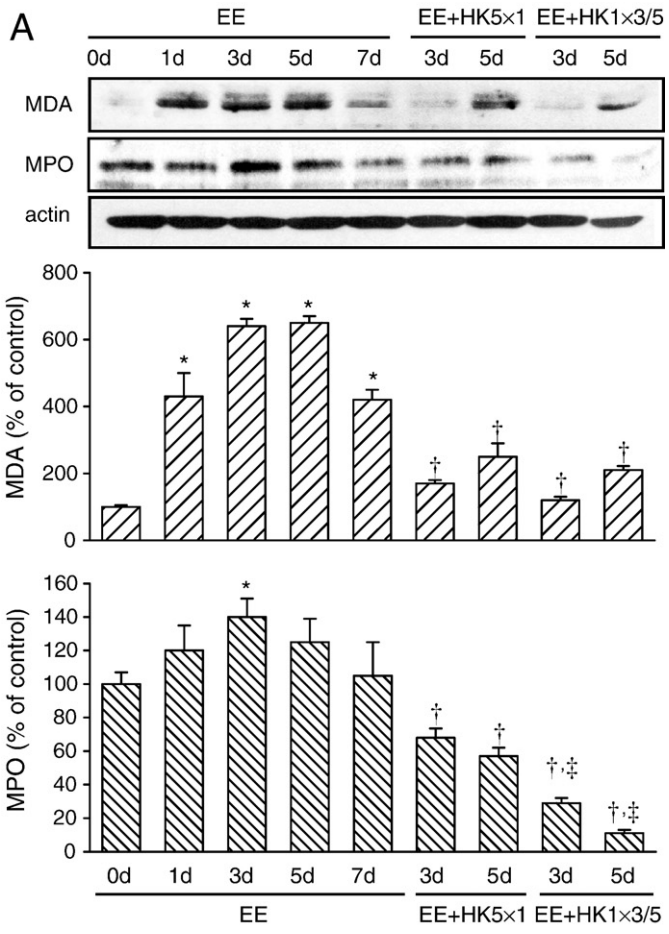
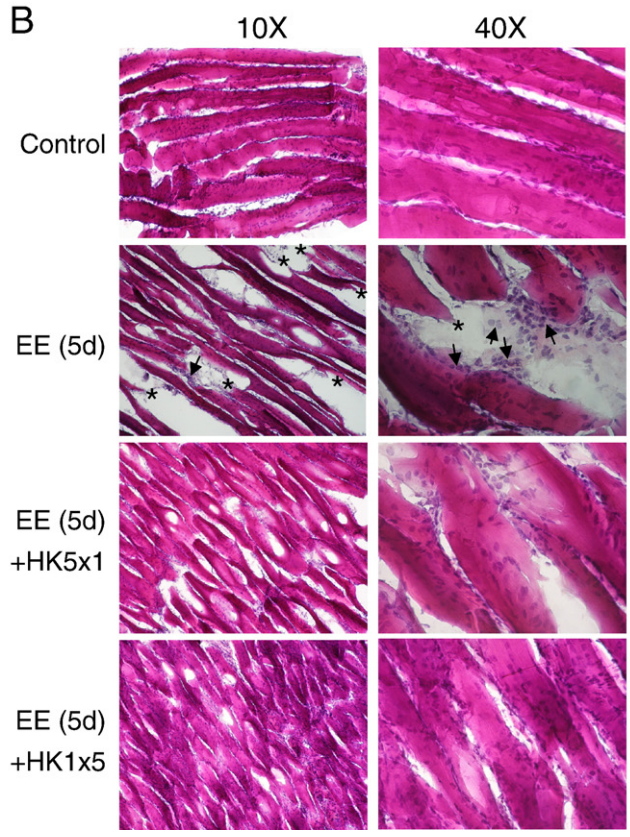


Fig. 2. Honokiol reduces eccentric exercise-induced poly-ADP-ribose-polymerase upregulation in skeletal muscles of rats subjected to eccentric exercise. (A) Upper panel, representative immunoblots for time-dependent (0–7 d) analysis of poly-ADP-ribose-polymerase (PARP) upregulation after eccentric exercise. Lower panel, statistical analysis. (B) Honokiol treatments on PARP upregulation. PARP was measured from rats received the vehicle solution with no exercise (control) or subjected to eccentric exercise for 5 consecutive days (EE (5d)). Honokiol (HK) treated-rats received HK 5 mg/kg for only 1 day on day 1 (HK5 × 1) or HK 1 mg/kg for 5 consecutive days from day 1 (HK1 × 5) were subjected to eccentric exercise for 5 consecutive days (EE + HK5 × 1 and EE + HK1 × 5). Data were calculated as multiples (folds) of the control after normalization to β-actin, and are expressed as the mean ± S.E.M. ($n = 5–10$ for each data point). $^{*}P<0.05$, compared to (A) 0 d or (B) EE (5d) by one-way ANOVA followed by *post-hoc* S–N–K test.

incubated with a second antibody at a proper dilution (1:5000–10,000) (anti-rabbit, anti-goat, or anti-mouse, respectively, conjugated with horseradish peroxidase, Jackson Laboratories, Bar Harbor, Maine, USA) for 1 h at room temperature. The immunoblot on the membrane was visible after development with an enhanced chemiluminescence (ECL) system (Perkin-Elmer, Foster City, CA, USA). Quantification of the Western blot analysis was performed using a Multi Gauge program (Fujifilm).





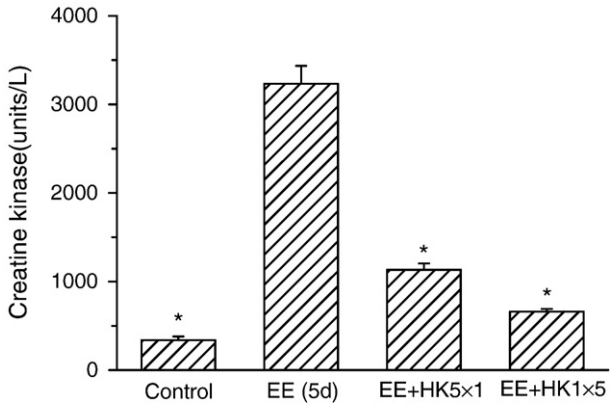


Fig. 4. Honokiol reduces eccentric exercise-induced changes of serum creatine kinase levels in rats subjected to eccentric exercise. Serum creatine kinase levels were measured from rats received the vehicle solution with no exercise (control) or subjected to eccentric exercise for 5 consecutive days (EE (5d)). Honokiol (HK) treated-rats received HK 5 mg/kg for only 1 day on day 1 (HK5×1) or HK 1 mg/kg for 5 consecutive days from day 1 (HK1×5) were subjected to eccentric exercise for 5 consecutive days (EE + HK5×1 and EE + HK1×5). Data are expressed as the mean ± S.E.M. ($n = 5$ for each data point). $^{*}P<0.05$, compared to EE (5d) by one-way ANOVA followed by *post-hoc* S–N–K test.

2.5. Measurement of expression levels of inflammatory genes

For gene expression study, the real-time PCR was performed according to the manufactory protocol by using TaqMan Gene Expression Assays (Applied Biosystems, USA). Briefly, the TaqMan Universal PCR Master Mix (10 μl) containing cDNA (1 μl, 100 ng) prepared from frozen muscle, and 20×TaqMan Gene Expression Assay Mix (1 μl) for iNOS, COX-2, IL-1β, IL-6, monocyte chemoattractant protein-1 (MCP-1), TNF-α and GAPDH were performed in 96-well plates in an ABI Prism 7700 real-time PCR instrument (Applied Biosystems). The thermal cycle conditions were as follows: 2 min at 50 °C and 10–15 min at 95 °C, followed by 35–40 cycles at 95 °C for 15 s and 60 °C for 60 s. Fluorescence signals measured during amplification were considered positive if the fluorescence intensity was more than 20-fold greater than the standard deviation of the baseline fluorescence. The $\Delta\Delta CT$ method of relative quantification was used to determine the multiples of change in expression. This was done by first normalizing the resulting threshold cycle (CT) values of the target gene (targ) to the CT values of the internal control, a housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH), in the same samples ($\Delta CT = CT\text{targ} - CT\text{GAPDH}$). The expression of target gene in the eccentric exercise (EE) or honokiol-treated (HK) groups was further normalized to the solvent control groups (control)

Fig. 3. Honokiol reduces eccentric exercise-induced lipid peroxidation and leukocyte infiltration in skeletal muscles of rats subjected to eccentric exercise. (A) Upper panel, representative immunoblots for time-dependent (0–7 d) analysis of lipid peroxidation (MDA formation) and leukocyte infiltration (MPO immunoblotting) after eccentric exercise and the effects of honokiol treatment. Rats receiving 7 successive days of oral solvent control (EE) for 7 consecutive days (0–7 d) of eccentric exercise. Honokiol (HK) treated-rats received HK 5 mg/kg for only 1 day on day 1 (HK5×1) or HK 1 mg/kg for 3 or 5 consecutive days (HK1×3/5) from day 1 were subjected to eccentric exercise for 3 or 5 consecutive days (EE + HK5×1 and EE + HK1×3/5). Lower panel, data were calculated as multiples (folds) of the control (0 d) after normalization to β-actin, and are expressed as the mean ± S.E.M. ($n = 5$–10 for each data point). $^{*,\dagger}P<0.05$, compared to the control (0 d) or respective EE-only group by one-way ANOVA followed by *post-hoc* S–N–K test. $^{\ddagger}P<0.05$, compared to respective EE + HK5×1 group by two-way ANOVA followed by *post-hoc* S–N–K test. (B) The H&E staining (10× and 40× objective) of skeletal muscle after eccentric exercise. Leukocyte inflammation and muscle fragmentation from rats received the vehicle solution with no exercise (control) or subjected to eccentric exercise for 5 consecutive days (EE (5d)). Honokiol (HK) treated-rats received HK 5 mg/kg on day 1 (HK5×1) or HK (1 mg/kg) for 5 consecutive days (HK1×5) from day 1 were subjected to eccentric exercise for 5 consecutive days (EE + HK5×1 and EE + HK1×5). Arrows point indicates the extensive leukocyte infiltration. *indicates muscle cell fragmentation.

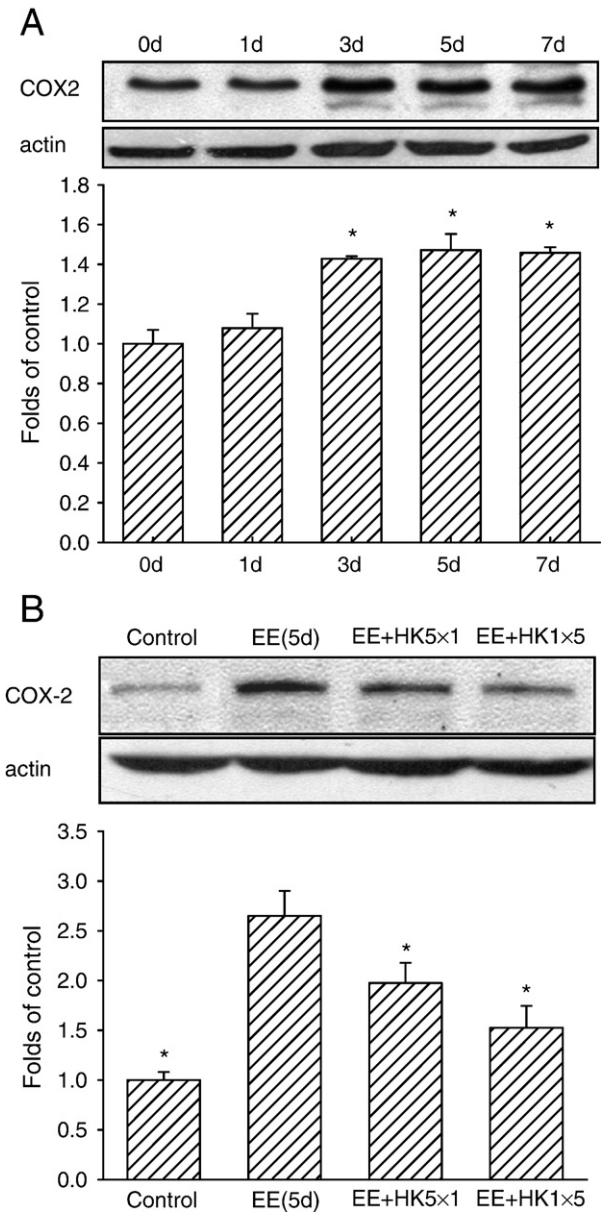


Fig. 5. Honokiol reduces eccentric exercise-induced cyclooxygenase (COX)-2 protein expression in skeletal muscles of rats subjected to eccentric exercise. COX-2 protein levels was determined by immunoblotting in skeletal muscles removed from rats received (A) the vehicle solution and subjected to eccentric exercise (EE) for 0 to 7 successive days (0 d to 7 d) or (B) the vehicle solution with no exercise (control) or subjected to eccentric exercise for 5 consecutive days (EE (5d)). Honokiol (HK) treated-rats received HK 5 mg/kg for only 1 day on day 1 (HK5×1) or HK 1 mg/kg for 5 consecutive days (HK1×5) from day 1 were subjected to eccentric exercise for 5 consecutive days (EE + HK5×1 and EE + HK1×5). The lower panel of each figure shows the statistical results (mean ± S.E.M., $n = 5$–10) of the densitometric measurements after normalization to β-actin. $^{*}P<0.05$, compared to the (A) 0 d or (B) EE (5d) by one-way ANOVA followed by *post-hoc* S–N–K test.

($\Delta\Delta CT = \Delta CT$EE − ΔCTcontrol or $\Delta\Delta CT = \Delta CT$HK − ΔCTcontrol). The multiples of change in expression were then obtained ($2^{-\Delta\Delta CT}$).

2.6. Determination of NF-κB activation

Frozen muscle was homogenized and extracted by a nuclear extraction reagent (NE-PER; Pierce, Rockford, IL, USA). An NF-κB p65 Transcription Factor Assay ELISA Kit (Pierce) consisting of a 96-well plate onto which the oligonucleotide duplex containing the NF-κB consensus site (5'-GGGACTTTCC-3') pre-coated was used to monitor NF-κB activation. The active form of NF-κB in the nuclear extract specifically binds to this consensus site and is recognized by the primary antibody. A horseradish peroxidase-conjugated secondary antibody provides for chemiluminescent quantification. Results were expressed as relative light units (RLU). TNF-α-activated HeLa whole-cell extract provided by the manufacturer was used as a positive control for NF-κB activity. Wild-type (5'-CACAGTTGAGGGGACTTTCCCAGGC-3') and mutant (5'-CACAGTTGAGGCCACTTTCCCAGGC-3') portions of the NF-κB competitor duplex were included to contrast with the specific binding of NF-κB.

2.7. Honokiol and other chemicals

Honokiol was purchased from Nacalai (Kyoto, Japan). It was first dissolved in propylene glycol as stock solutions and then serially diluted in normal saline immediately prior to the experiments. The stock solution was used within 1 week after preparation. Normal saline containing 0.1% (v/v) of propylene glycol was used as the vehicle control. Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), except where otherwise indicated.

2.8. Statistical analysis

All values in the text and figures are presented as the mean ± S.E.M. Data were analyzed by one- or two-way analysis of variance (ANOVA) depending on the number of parameters for comparison, followed by *post-hoc* Student–Newman–Keuls (S–N–K) test for multiple comparisons. Values of $P<0.05$ were considered significant.

3. Results

3.1. Protective effect of honokiol against eccentric exercise-induced oxidative muscle damage

Repeated bouts of eccentric exercise for a period of 7 consecutive days induced extensive skeletal muscle damage in days 3–7 as revealed in the dramatic increase in protein nitrosylation (Fig. 1A, one-way ANOVA followed by *post-hoc* S–N–K test, $P<0.05$, $n = 5$–10 for each group) and upregulation of PARP (Fig. 2A, one-way ANOVA

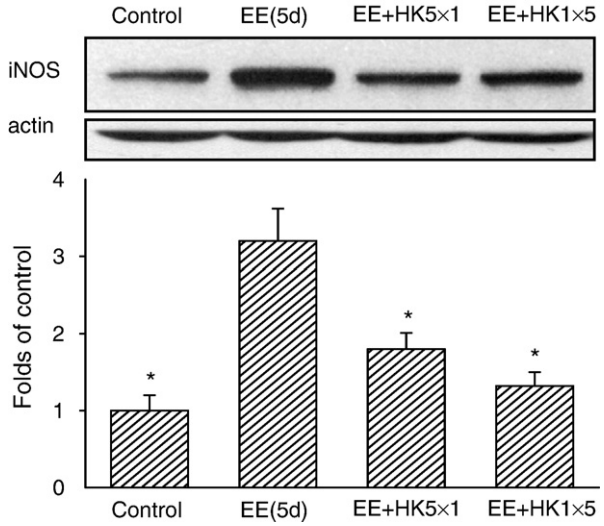


Fig. 6. Honokiol reduces eccentric exercise-induced inducible nitric oxide synthase (iNOS) protein expression in skeletal muscles of rats subjected to eccentric exercise. The iNOS protein was determined by immunoblotting. Skeletal muscles were removed from rats received the vehicle solution with no exercise (control) or subjected to eccentric exercise for 5 consecutive days (EE (5d)). Honokiol (HK) treated-rats received HK 5 mg/kg for only 1 day on day 1 (HK5×1) or HK 1 mg/kg for 5 consecutive days (HK1×5) from day 1 were subjected to eccentric exercise for 5 consecutive days (EE + HK5×1 and EE + HK1×5). The lower panel shows statistical results (mean ± S.E.M., $n = 5$–10) of the densitometric measurements after normalization to β-actin. $^{*}P<0.05$, compared to the EE (5d) by one-way ANOVA followed by *post-hoc* S–N–K test.

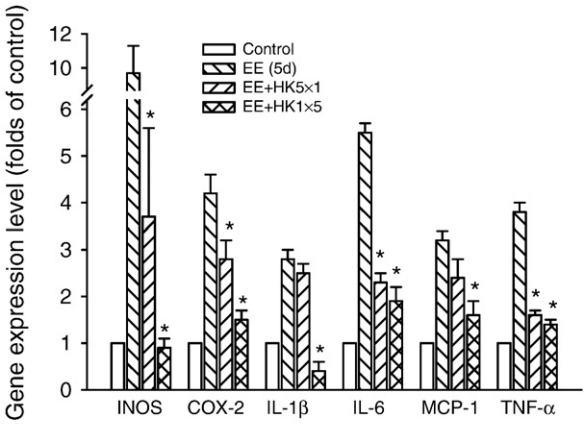


Fig. 7. Honokiol reduces eccentric exercise-induced gene expression levels of inflammatory genes in skeletal muscles of rats subjected to eccentric exercise. Gene expression levels (folds of control) were examined by real-time PCR using samples from skeletal muscle in rats received the vehicle solution with no exercise (control) or subjected to eccentric exercise for 5 consecutive days (EE (5d)). Honokiol (HK) treated-rats received HK 5 mg/kg for only 1 day on day 1 (HK5×1) or HK 1 mg/kg for 5 consecutive days (HK1×5) from day 1 were subjected to eccentric exercise for 5 consecutive days (EE + HK5×1 and EE + HK1×5). Data were expressed as the means ± S.E.M. (n = 3–5 for each group). $^{*}P<0.05$, as compared to EE (5d) by one-way ANOVA followed by *post-hoc* S–N–K test.

followed by *post-hoc* S–N–K test, $P<0.05$, n = 5–10 for each group), the biomarker for oxidative stress-induced protein denaturation and DNA damage, respectively. The damage also paralleled the significant enhancements of lipid peroxidation (MDA formation; Fig. 3A, one-way ANOVA followed by S–N–K test, $P<0.05$, n = 5–10 for each group), leukocyte accumulation (MPO level and leukocyte (H&E) staining) (Fig. 3A and B), and cell fragmentation and disintegration (Fig. 3B) in skeletal muscles, as well as the remarkable increase in serum creatine kinase levels on day 5 of eccentric exercise (Fig. 4, one-way ANOVA followed by *post-hoc* S–N–K test, $P<0.05$, n = 5 for each group). Both honokiol treatments (HK5×1 and HK1×5) significantly ameliorated the eccentric exercise-induced protein nitrosylation (Fig. 1B, one-way ANOVA followed by S–N–K test, $P<0.05$, n = 5–10 for each group) by 50%–76%, PARP upregulation by 37%–73% (Fig. 2B, one-way ANOVA followed by *post-hoc* S–N–K test, $P<0.05$, n = 5–10 for each group), lipid peroxidation (MDA, Fig. 3A, one-way ANOVA followed by S–N–K test, $P<0.05$, n = 5–10 for each group), leukocyte infiltration (MPO, Fig. 3A, one-way ANOVA followed by *post-hoc* S–N–K test, $P<0.05$, n = 5–10 for each group; Fig. 3B, leukocyte (H&E) staining), and muscle cell fragmentation (Fig. 3B), as well as the serum creatine kinase level (Fig. 4; one-way ANOVA followed by *post-hoc* S–N–K test, $P<0.05$, n = 5 for each group) measured at day 3 or 5 (as indicated in the figures) of eccentric exercise. There was no significant difference between HK treatments (HK5×1 and HK1×5) except in the reduction of MPO activity in which treatment of HK1×5 group was more potent than that of

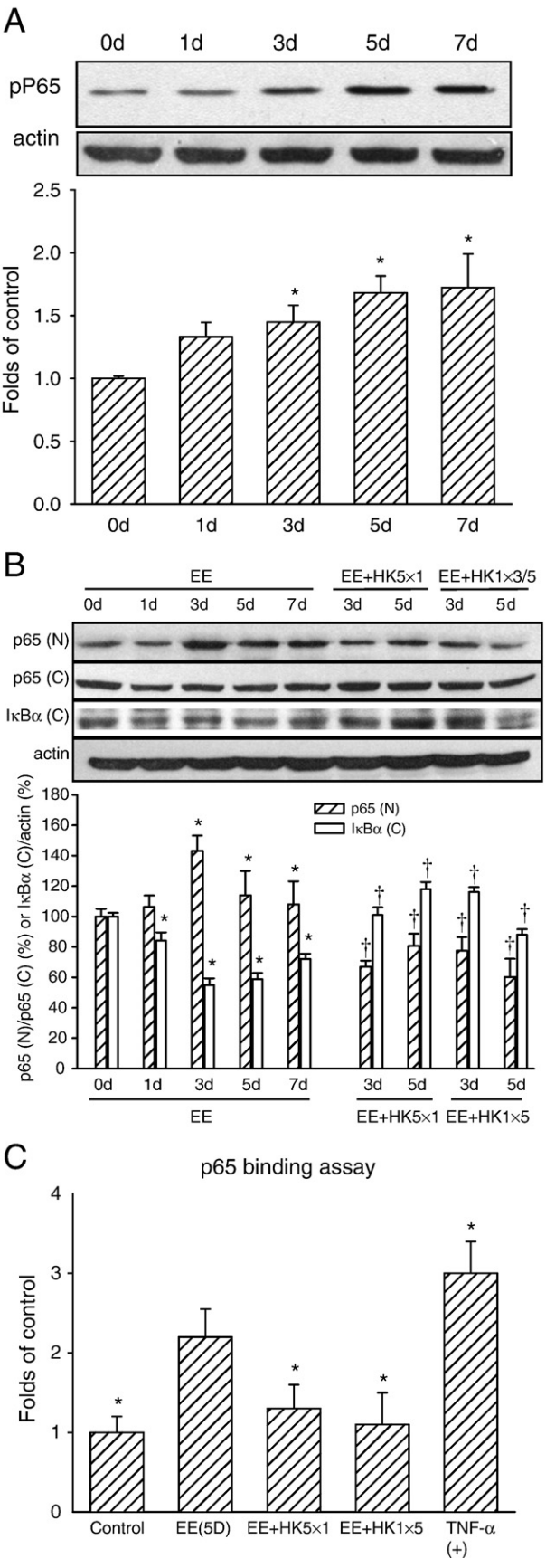


Fig. 8. Honokiol reduces eccentric exercise-induced nuclear factor-κB (NF-κB) activation in skeletal muscles of rats subjected to eccentric exercise. (A) The phospho-p65NF-κB (pP65) protein level, (B) degradation of IκBα and nuclear translocation of p65NF-κB, and (C) p65 binding activity were determined by immunoblotting ((A) and (B)) or ELISA (C). Skeletal muscles were removed from rats received the vehicle solution with no exercise (control) or subjected to eccentric exercise for 0–7 consecutive days (EE/0 d–7 d). Honokiol (HK) treated-rats received HK 5 mg/kg for 1 day on day 1 (HK5×1) or HK 1 mg/kg for 3 or 5 consecutive days (HK1×3/5) from day 1 were subjected to eccentric exercise for 3 or 5 consecutive days (EE + HK5×1 and EE + HK1×3/5). The lower panel of each figure shows the statistical results (means ± S.E.M., n = 5) of the densitometric measurements after normalization to β-actin. $^{*}P<0.05$, compared to 0 d (A, B) or EE(5 d) (C), and $^{\dagger}P<0.05$, compared to the respective time point control (EE3 d or EE5 d) by one-way ANOVA followed by *post-hoc* S–N–K test. TNF-α activated HeLa cells (TNF-α (+)) were included as positive control.

HK5×1-treated group (two-way ANOVA followed by *post-hoc* S–N–K test, $P<0.05$).

3.2. Effects of honokiol on eccentric exercise-induced COX-2 and iNOS expression

Many prooxidant enzymes, including COX-2 and iNOS, are responsible for mediating oxidative and nitrosative stress-induced tissue damage. In this study, significant upregulation of the protein levels of COX-2 and iNOS (Figs. 5A and 6; one-way ANOVA followed by *post-hoc* S–N–K test, $P<0.05$, $n=5$–10 for each group) was noted during the eccentric exercise period. Both honokiol treatments significantly reduced the immunoreactivity of COX-2 and iNOS (Figs. 5B and 6; one-way ANOVA followed by *post-hoc* S–N–K test, $P<0.05$, $n=5$–10 for each group) as well as relative gene expression levels of COX-2 and iNOS (Fig. 7) on day 5 of eccentric exercise, indicating that eccentric exercise induced upregulated expressions of prooxidant enzymes, especially COX-2 and iNOS, were limited by honokiol treatments. There was no significant difference between the honokiol treatments on their ability to reduce gene expression for COX-2 and iNOS (two-way ANOVA, $P>0.05$).

3.3. Effects of honokiol on eccentric exercise-induced gene expression levels of cytokines

Proinflammatory cytokines are important mediators of tissue injury. Eccentric exercise strongly enhanced the gene expression levels of inflammation-associated cytokines including IL-1β, IL-6, MCP-1 and TNF-α (Fig. 7). Treatment with HK1×5 significantly reduced eccentric exercise-induced upregulation of all these cytokines, whereas treatment with HK5×1 only significantly reduced upregulation of IL-6 and TNF-α (Fig. 7, one-way ANOVA followed by *post-hoc* S–N–K test, $P<0.05$, $n=3$–5 for each group).

3.4. Effect of honokiol on eccentric exercise-induced NF-κB activation

NF-κB has been reported to be a biomarker of eccentric exercise-induced tissue injury, because activation of NF-κB may function as an oxidative-stress responsive transcription factor for induction of many inflammation-related proteins (e.g., proinflammatory cytokines, COX-2, and iNOS). To examine whether honokiol treatments can affect the upregulaton of NF-κB signaling by eccentric exercise, the changes in the translocation of p65NF-κB, the degradation of cytosolic Iκ-Bα (an NF-κB inhibitor protein), and the binding activity of NF-κB in skeletal muscle tissues were examined. As shown in Fig. 8, eccentric exercise induced time-dependent phosphorylation/activation of p65NF-κB (pP65) (Fig. 8A, one-way ANOVA followed by *post-hoc* S–N–K test, $P<0.05$, $n=5$ for each data point) and enhanced the degradation of cytosolic Iκ-Bα and the nuclear translocation of p65NF-κB on days 3–7 of eccentric exercise as compared to those of the resting group (day 0) (Fig. 8B, one-way ANOVA followed by *post-hoc* S–N–K test, $P<0.05$, $n=5$ for each data point). Both honokiol treatments (HK5×1 and HK1×5) significantly reduced eccentric exercise-induced degradation of Iκ-Bα and nuclear translocation of p65NF-κB in skeletal muscles on days 3–5 (Fig. 8B, one-way ANOVA followed by *post-hoc* S–N–K test, $P<0.05$, $n=5$ for each data point), as well as the binding of nuclear p65NF-κB to its consensus binding sites (Fig. 8C), indicating that NF-κB activity was limited by honokiol treatments.

4. Discussion

Skeletal muscle damage caused by eccentric exercise involves many factors and diverse mechanisms that begin from a greater extent of forced stretching of the sarcomeres, followed by intracellular calcium overloading activated by membrane damage, ultimately leading to the production of oxidative cellular responses generated from activation of inflammatory processes. Upregulation of inflammation-associated proteins, e.g., COX-2 and iNOS, mediates oxidative tissue damage by inducing protein nitrosylation and lipid peroxidation (Reid, 1998; Sorichter et al., 1999), both of which produce increased level of creatine kinase in the circulation as a response to myofibrilar disintegration, an optimum indicator of muscle damage in eccentric exercise (Armstrong et al., 1983). Consistent with these findings, the present study demonstrated that eccentric exercise induced remarkable muscle damage (cell fragmentation and disintegration) and increases in serum creatine kinase levels by inducing protein nitrotyrosylation, PARP upregulation, lipid peroxidation, and leukocyte infiltration. This damage also paralleled the dramatic induction of gene expressions of COX-2, iNOS, and proinflammatory cytokines (IL-1β, IL-6, TNF-α, and MCP-1), possibly through activation of NF-κB, a crucial proinflammatory transcription factor.

It is well known that honokiol is a potent antioxidant and anti-inflammatory agent against oxidative-related tissue damage (Lo et al., 1994; Chiu et al., 1997; Liou et al., 2003a,b). Herein, we demonstrate for the first time that honokiol also can reduce eccentric exercise-induced muscle injury. Both honokiol treatments significantly reduced the levels of creatine kinase in the circulation and displayed a protective effect by reducing eccentric exercise-induced oxidative and nitrosative tissue damage, possibly by blocking the upregulation of inflammation-related events and prooxidant proteins through modulation of NF-κB activation.

Activation and infiltration of leukocytes, predominantly neutrophil and monocyte/macrophage, may provide an important source of oxidative stress by producing large amounts of NO and reactive oxygen species during eccentric exercise-induced muscle injury (Nguyen and Tidball, 2003a,b). In the present study, we showed that eccentric exercise-induced extensive skeletal muscle damage can be uncovered by substantial staining of muscle fragmentation and disintegration, which are accompanied by significant leukocyte infiltration into skeletal muscles with paralleled lipid peroxidation (MDA formation) in skeletal muscles. Honokiol treatments prevented the eccentric exercise-induced muscle damage and leukocyte infiltration, and effectively decreased oxidative stress (MDA formation) revealing that both antioxidative and anti-inflammatory actions are responsible for honokiol's protective effect. Although the crucial physiological need of oxidative stress and inflammation are both important to induce molecular signals for muscle to recover from and/or adapt to damaging exercise (Hubal et al., 2008; Mahoney et al., 2008), in realms of eccentric exercise-mediated injuries, honokiol may be beneficial in attenuating the pathological levels of skeletal muscle damaging factors. Similar observations were reported in our previous studies demonstrating that honokiol can protect rat brain against focal cerebral ischemia–reperfusion injury by inhibiting neutrophil infiltration and reactive oxygen species production (Liou et al., 2003a), possibly through modulating enzyme systems related to reactive oxygen species production or metabolism, including NADPH oxidase, myeloperoxidase, COX, and GSH peroxidase in neutrophils (Liou et al., 2003b).

In addition to oxidative stress, nitrosative stress-mediated activation of inflammatory mediators is currently being emphasized as an important factor involved in inflammation-related disorders (Peake and Suzuki, 2004; Paula et al., 2005). Nitrosative stress arises mainly from the large accumulation of NO by the overexpression of iNOS in damaged tissue to form peroxynitrite ($ONOO^-$) when NO reacts with the superoxide anion ($O_2^{-\cdot}$) (Sugawara and Chan, 2003). Peroxynitrite initiates lipid peroxidation, oxidation of sulfhydryl groups, and nitrosation of tyrosine-containing molecules to damage tissue. In this study, we found that eccentric exercise induced time-dependent protein tyrosine nitrosylation. Upregulated expression of iNOS in skeletal muscles could be responsible for the nitrosative muscle damage induced by eccentric exercise. A similar result was also shown in other reports (Jungersten et al., 1997; Roberts et al., 1999; Paula

et al., 2005). Honokiol treatments significantly prevented protein tyrosine nitrosylation and iNOS expression, indicating that nitrosative stress raised by eccentric exercise could be modulated by honokiol.

Inflammatory cascades triggered by eccentric exercise injury can further amplify tissue damage (Peake et al., 2005a,b). For example, leukocytes and macrophages are recruited to damaged tissue where inflammatory mediators are generated by these immune cells (Peake and Suzuki, 2004; Mahoney et al., 2008). It is known that COX-2 plays a crucial role in damaging tissue induced by eccentric exercise (Jimenez-Jimenez et al., 2008). In particular, the administration of COX inhibitors was shown to reduce the normal increase in PGF2α in human skeletal muscle after eccentric resistance exercise through which to modulate skeletal muscle protein metabolism as well as inflammation and pain (Trappe et al., 2001). In this study, we observed that COX-2 expression was upregulated after eccentric exercise and honokiol significantly reduced COX-2 gene expression and protein levels. Whether COX-2 activity is also suppressed by honokiol requires further investigation.

In addition to prooxidant proteins (iNOS and COX-2), gene expression for some inflammation-associated cytokines (IL-1β, IL-6, MCP-1 and TNF-α) were also enhanced by eccentric exercise and were reduced by honokiol treatments, suggesting that inflammation-related transcription factors could be modulated by honokiol. Most of these inflammation-associated proteins are downstream gene products of the transcription factor NF-kB, which has been reported to be activated during oxidative and inflammatory stress. Accordingly, our data revealed that honokiol diminished NF-κB activation and NF-κB nuclear translocation by inhibiting Iκ-Bα degradation at sites of eccentric exercise-induced muscle injury.

In this study, there was no significant difference between HK treatments (HK5×1 and HK1×5) in reducing the pathological levels of skeletal muscle damage except in the reduction of MPO level and cytokines production where treatment of HK (1 mg/kg/day) for 3 to 5 consecutive days was relatively more potent than that of HK (5 mg/kg for 1 d)-treated group. Our data suggest that the antioxidant effect of honokiol is important early in the process of muscle damage, whereas its anti-inflammatory effect requires a longer treatment.

In conclusion, our study demonstrates that honokiol treatment effectively protects rat skeletal muscles against eccentric exercise-induced skeletal muscle injury, possibly through reducing lipid peroxidation (MDA formation) and protein nitrosylation by limiting leukocyte infiltration and the upregulated expression of inflammatory-related prooxidant enzymes (iNOS and COX-2) and cytokines in injured tissue *via*, at least in part, impeding the activity of NF-κB. As a potent antioxidative and anti-inflammatory agent in pharmacologically applicable doses, honokiol may be beneficial for the prevention and/or amelioration of eccentric exercise-induced skeletal muscle injury.

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