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# Vitamin $D_3$ supplementation modulates inflammatory responses from the muscle damage induced by high-intensity exercise in SD rats

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ABSTRACT

Vitamin D is an important factor for calcium and phosphorus homeostasis. A negative relationship has been observed between vitamin D status and diseases such as cancer, arthritis, diabetes, and muscle fiber atrophy. However, the relationship between vitamin D and prevention of skeletal muscle damage has not been clearly elucidated. The purpose of this study was to investigate the effects of vitamin D on exercise-induced muscle changes. Rats were divided into 3 groups: (1) sedentary control (C: $n = 10$), (2) high-intensity exercise (HE: $n = 10$), and (3) high-intensity exercise with vitamin D supplementation (HED: $n = 10$; i.p. 1000 IU/kg body weight). Rats were trained for 30 min/day on treadmills (5 days/week for 8 weeks) with the running speed gradually increased up to 30 m/min at a 3° incline. At the end of the training period, the running speed was 38 m/min at a 5° incline. The high-intensity exercise significantly increased plasma creatine kinase (CK) and lactate dehydrogenase (LDH) activity. In addition, IL-6 and TNF-α levels as well as phosphorylation of AMPK, p38, ERK1/2, IKK, and IκB were significantly increased. Vitamin D-treated rats showed a significant decrease in plasma CK level, phosphorylation of AMPK, p38, ERK1/2, IKK, and IκB, and gene expression of IL-6 and TNF-α. Furthermore, the protein expression of vitamin D receptor (VDR) was highly increased in the muscles of HED-treated rats, respectively. Therefore, we concluded that vitamin D may play a pivotal role in exercise-induced muscle damage and inflammation through the modulation of MAPK and NF-κB involved with VDR.



## 1. Introduction

Muscle tissues may be damaged following high-intensity exercise. High-intensity exercise results in structural damage of muscle cells, as evidenced by an increase in the plasma activity of cytosolic enzymes such as creatine kinase (CK) and lactate dehydrogenase (LDH) [1–3]. Furthermore, inflammation during exercise is linked to muscle damage. When exercising at high intensity, leukocytes stimulate the release of pro-inflammatory cytokines such as TNF-α, IL-8, and IL-6.

The physiological conditions such as influx of intracellular calcium, ROS accumulation, and mitogen-activated protein kinases (MAPKs) activation have all been shown to activate NF-κB, leading to the hypothesis that exercise may also activate NF-κB [4,5] NF-κB is one of the signaling molecules activated during exercise and most genes activated by NF-κB have been shown to be pro-inflammatory and to be involved in the inflammatory process [6]. High-intensity exercise also activates MAPK, stress-activated proteins, such as p38 and ERK1/2 [7] p38 MAPK shares common kinase substrates with ERK1/2 is rapidly activated in rat and mouse models of exercise [8–10], as well as in both trained and untrained human skeletal muscles following acute submaximal cycling [11,12] and marathon running exercise [13]. Exercise-induced changes are not limited to the activation of the ERK signaling cascade, as activation of p38 MAPK has also been observed [9,10,13]. It is reported that MAPK may fulfill an important function as a cellular intermediary coupling perceived alterations in stress with adaptive changes in oxidative stress, metabolic actions, and gene regulation [7].

Vitamin $D_3$ (cholecalciferol) is synthesized by ultraviolet irradiation (UV) in the skin from its precursor 7-dehydrocholesterol or ingested with food. The active form of vitamin D, $1,25(OH)_2D_3$, is an important factor for calcium and bone homeostasis that acts by binding to the vitamin D receptor (VDR), which belongs to the nuclear receptor superfamily. From epidemiologic and clinical studies, a negative relationship has been observed between vitamin D status and diseases such as cancer [14], arthritis, diabetes [15], muscle fiber atrophy, and cardiovascular disease [16]. A strong correlation has been described between low serum 25(OH)D levels and higher rates and longer duration of generalized

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muscle aches and pains [17]. In addition, vitamin D may improve muscle strength through a highly specific nuclear receptor in muscle tissues [18]. Recently, vitamin D was shown to potentially improve athletic performance and protect muscle damage in vitamin D-deficient athletes [18]. Other study in healthy endurance-trained runners has shown the inverse association between 25(OH)D and TNF-α concentrations [19]. However, one study reported a not significant changes in TNF-α and IL-6 level in high dose vitamin D supplementation-healthy overweight and obese subjects following a 12-week progressive resistance exercise training program [20]. The physiologic relevance of such vitamin D effects in vivo has not been proved yet, nor has the role of the VDR.

Therefore, we hypothesized that vitamin $D_3$ would be beneficial for skeletal muscles damages from high-intensity exercise and we suggested that one of reasons would be the modulation of inflammation via the MAPK-NF-κB signaling involved the VDR.

## 2. Materials and methods

### 2.1. Animals and exercise protocol

Thirty male Sprague–Dawley rats weighting 120–130 g (4 weeks old) were purchased from Nara Biotech (Seoul, Korea). Rats were fed a standard chow diet (Purina Mills Inc., Korea) and tap water ad libitum and housed in a room maintained at 23 ± 2 °C with a 12-h light–dark cycle for 8 weeks. The treatment of the rats and the study protocol were approved by the ethical committee of Sungshin University and carried out in an ethical manner by following the guidelines provided. Rats were randomly divided into 3 groups: a sedentary control group (C, $n = 10$); high-intensity exercise group (HE, $n = 10$); and high-intensity exercise plus vitamin $D_3$ supplementation group (HED, $n = 9$). Body weight did not differ between the groups at the beginning of the experiment. Rats in the vitamin $D_3$ treatment group were injected intraperitoneally with 25 μg cholecalciferol (Sigma, St. Louis, Mo) per kg of body weight, is equivalent to 1000 IU vitamin $D_3$ once a day for 8 weeks. This concentration of vitamin $D_3$ was decided by rat's requirement when Ca and P are fed in adequate amounts [21]. Vitamin $D_3$ was diluted in 100% propylene glycol (Junsei Chemical, Tokyo, Japan). Rats were trained for 30 min/day (5 days/week for 8 weeks) using a rodent treadmill (Dual treadmill; SK-DTR8L, Seoul, Korea). Max running speed was increased up to 32, 34, 36, and 38m/min at the beginning of 2nd, 4th, 6th, and 8th week of training at a 3°incline. So, at the end of the training period, running speed was 38m/min at a 5°incline.

### 2.2. Sample collection

After a rest period of 18 h after the last workout session, the rats were weighed and anesthetized with diethyl ether. Blood was rapidly collected from the abdominal aorta and then centrifuged (2000 rpm, 15 min, 4 °C) to isolate the plasma, which was stored at −80 °C until further analyses. The soleus and gastrocnemius muscles were removed, immediately soaked in liquid nitrogen, and stored at −80 °C.

### 2.3. Creatine kinase activity assay

CK activity was measured using a CK kit (Bio Assay, CA, USA) according to the manufacturer's instructions. The CK assay kit is based on enzyme-coupled reactions in which creatine phosphate and ADP are converted to creatine and ATP by CK; the generated ATP is used to phosphorylate glucose by hexokinase to generate glucose-6-phosphate, which is then oxidized by NADP in the presence of glucose-6-phosphate dehydrogenase. The produced NADPH, measured at 340 nm, is proportionate to the CK activity in the sample. Data are reported as U/L plasma. One unit of CK will transfer 1 μmol of phosphate from phosphocreatine to ADP per min at pH 6.0.

### 2.4. Lactate dehydrogenase activity assay

LDH activity was measured using a LDH kit (Bio Assay, CA, USA) according to the manufacturer's instructions. The LDH assay is based on the reduction of the tetrazolium salt MTT in a NADH-coupled enzymatic reaction to a reduced form of MTT that exhibits an absorption maximum at 565 nm. The intensity of the purple color formed is directly proportional to the enzyme activity. Data are reported as IU/L plasma. One unit (IU) of LDH will catalyze the conversion of 1 μmol of lactate to pyruvate per min at pH 8.2.

### 2.5. Cytokine assay

Skeletal muscle tissue was homogenized in 10 volumes of an ice-cold buffer (10 mM HEPES, 1.5 mM magnesium chloride, 10 mM potassium chloride, 0.5 mM dithiothreitol, and 0.05% Nonidet P-40) containing a protease inhibitor cocktail (Pierce, Rockford, IL). Homogenates were centrifuged at 14,000 rpm for 10 min at 4 °C; the supernatants were removed. The assay was performed according to the manufacturer's instructions (BioLegend, San Diego, CA). The change in color was measured at a wavelength of 450 nm using a microplate reader (Multiskan Spectrum; Thermo Electron Co., Vantaa, Finland). Measurements were performed in duplicate. Cytokine levels are expressed as pg/mg total protein.

### 2.6. Protein expression analysis by western immunoblot

Soleus and medial gastrocnemius muscles were collected and rinsed in PBS. Cytoplasmic and nuclear extracts of tissues were isolated using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL) and the Halt™ protease and phosphate inhibitor single-use cocktail (Pierce). Samples were boiled at 95 °C for 5 min in 2× Laemmli sample buffer (0.125 M Tris–HCl [pH 6.8], 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.004% bromophenol blue). Boiled samples were run on 8–10% SDS–polyacrylamide gels (20–40 μg/lane). The gels were blotted on nitrocellulose membranes (Bio-Rad, Hercules, CA) and stained with Ponceau red (Sigma Chemical, St. Louis, MO) to confirm equal loading and transferring of proteins to the membrane in each lane. The membranes were blocked in 5% skim milk or BSA in Tris-buffered saline with 0.1% Tween 20 and probed with corresponding antibodies against NFκB, phosphorylated IκB, IκB, phosphorylated IKK-α/β, IKKβ, phosphorylated AMPK, AMPK, phosphorylated p38 MAPK, p38 MAPK, phosphorylated ERK1/2 (all from Cell Signaling, Danvers, MA), ERK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA), and VDR (Abcam, Cambridge, MA) for 3 h. Additionally, as a second means of confirming equal loading, the membranes were also probed for α-tubulin (Cell Signaling) and lamin B1 (Abcam, Cambridge, MA). Secondary antibodies were conjugated to horseradish peroxidase (Enzo Life Sciences, Farmingdale, NY), and the signals were developed by chemiluminescence (BioFX, Owings Mills, MD). The signals were visualized by exposing the membranes to X-ray films (Agfa, Mortsel, Belgium) and quantified using an Image J Analyzer (NIH, Bethesda, USA).

### 2.7. Gene expression analysis by real-time PCR

For gene expression analysis, total RNA was isolated from medial gastrocnemius muscles using TRIzol Reagent (Favorgen Biotech Corp., Taiwan); 2 μg of total RNA was treated with RNase-free DNase I (Qiagen, Valencia, CA) to avoid genomic DNA