The Effects of Taurine Administration Against Inflammation in Heavily Exercised Skeletal Muscle of Rats

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Abbreviations

CD68	Cluster of differentiation 68
EDL	Extensor digitorum longus
IHC	Immunohistochemistry
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
SOL	Soleus
Tau	Taurine
TNF-α	Tumor necrosis factor-α

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1 Introduction

Taurine (2-aminoethanesulfonic acid) is the most abundant amino acid in the body. It is found in high concentrations in most tissues, including skeletal muscle, heart, nerve, brain, and liver in vertebrates (Jacobsen and Smith 1968). Its cytoprotective activity against tissue damage involves several essential biological, physiological, and pharmacological mechanisms, including membrane stabilization (Pasantes-Morales et al. 1985), antioxidation (Gordon and Heller 1992; Sugiura et al. 2013) and osmoregulation (Thurston et al. 1980). Because of the absence of taurine biosynthesis, the large intracellular taurine pool of skeletal muscle is maintained by a specific transporter (Ramamoorthy et al. 1994). The uptake of taurine differs with muscle type (Kim et al. 1986), as the steady state level of taurine concentration of the slow-twitch fiber is higher than that of the fast-twitch fiber (Iwata et al. 1986). Heavy exercise increases oxidative stress and damages skeletal muscle (Sugiura et al. 2013). The damage is characterized by enhanced muscle protein catabolism and acute inflammation, followed by muscle tissue regeneration (Meeson et al. 2004; Ciciliot and Schiaffino 2010). The progression of muscle repair is modulated by essential and semi-essential amino acids and involves the modulation of inflammation and protein anabolism (Dort et al. 2013; Miyazaki et al. 2004). Taurine protects skeletal muscle against intense exercise-induced nitrosative inflammation and ensuing DNA damage by preventing iNOS expression and the increase in nitrosative stress mediated by heavy exercise (Sugiura et al. 2013). Therefore, the regulation of inflammation is likely to differ with muscle type. To evaluate differences related to muscle type, we observed the effect of taurine administration on inflammation in heavily exercised skeletal muscle of rats.

2 Methods

2.1 Ethics Statement

All experimental protocols were approved by the Animal Ethics Committee of Suzuka University of Medical Science, Japan.

2.2 Animal and Experimental Design

Eight-weeks-old male Wistar rats were obtained from SLC (Hamamatsu, Japan) and weighed 250–260 g on arrival. They were housed in cages (Max, six per cage) and maintained on water and food *ad libitum*. All animals were maintained at 24.0 °C (45–55 % of humidity) with a 12 h light and 12 h dark cycle. The 18 rats were randomly divided into three groups; Exercise plus saline group (n=6), Exercise plus taurine group (n=6), and Control group (n=6).

2.3 Exercise Protocol and Taurine Supplementation

The exercise protocol of the present study used a widely recognized animal model to study post exercise inflammatory responses. We designed a heavy treadmill exercise protocol on a motor-driven tread-mill (model MK-680; Muromachi Kikai, Tokyo, Japan) in which rats began exercising at 8 m/min for 10 min during the week preceding the actual exercise experiments. The rats became accustomed to locomotion in preparation of the final exercise experiments without stimulating development of skeletal muscle as an adaptation to training. Each group ran on the treadmill at 20 m/min, 25 % grade, for 20 min or until exhaustion. Exhaustion was determined as the point when an animal was unable to right itself when placed on its side. The workload was ~75 % of the rat's maximal aerobic capacity. Rats subjected to the exercise protocol were either treated with saline or taurine (intra-abdominally) 1 h prior to the onset of the exercise protocol.

2.4 Surgical Method and Muscle Collection

In the control group, extensor digitorum longus and soleus muscles of both legs were removed in the morning of the 10th day. Then, muscle tissue was formalin-fixed and the samples paraffin-embedded.

In the exercise plus saline group and the exercise plus taurine group, the animals were euthanized by cardiac exsanguination under anesthesia 48-h after the 10th day of exercise, and the muscles were immediately excised. The protocol was designed to restore basal conditions prior to sacrifice.

2.5 Immunofluorescence Study of TNF-α, CD68, and IL-6

Standard immunofluorescence methods were used to examine the distribution of TNF-α, CD68, and IL-6 in muscle tissue and normal controls. After deparaffinization and rehydration, antigen was retrieved in 5 % urea by microwave heating for 5 min and then incubated in 1 % H_2O_2 for 30 min to block endogenous peroxidase activity. Sections of 5 μm thickness were incubated overnight at room temperature with the following antibodies: TNF-α goat polyclonal antibody, CD68 mouse monoclonal antibody, and IL-6 rabbit polyclonal antibody (1:200, Santa Cruz Biotechnology Co., Ltd). For the goat antibodies (TNF-α), the sections were incubated with Donkey anti-goat IgG-Alexa 594 for 2 h. For mouse antibody (CD68), the sections were incubated with Donkey anti-mouse IgG-Alexa 488 for 2 h. For the rabbit antibodies (IL-6), the sections were incubated with Goat anti-Rabbit IgG-Alexa 594 for 2 h.

2.6 Immunohistochemical Grading

Immunohistochemical (IHC) grading, which is based on intensity and frequency of staining, was performed by two independent investigators. The staining intensity was scored as negative (−), weak (+), moderate (++), strong (+++), or very strong (++++). In addition, we measured the brightness of the image by using ImageJ (Wayne Rasband) (Schneider et al. 2012).

2.7 Statistical Analysis

Data are presented as means ± SEM. The two-tailed Student's *t*-test was performed. Differences were considered statistically significant at $P < 0.05$.

3 Results

3.1 Body Weight Progression and Consumption of Water and Food

Over the duration of the experiments, there was no significant difference in body weight progression between the Control and Taurine groups. All rats were in good health, with no pathological signs observed throughout the period of study.

3.2 Localization of TNF-α, IL-6 and CD68 in Normal and Exercised EDL and SOL Skeletal Muscle

To observe TNF-α, IL-6 and CD68 expression, immunohistochemistry was performed on normal and exercised skeletal muscle. TNF-α and IL-6 immunofluorescence staining shows intense immunoreactivity in the stromal cell of endomysium and blood vessels of EDL and SOL muscle (Figs. 1, 2, 3, and 4). In contrast, CD68 immunoreactivity was localized in some of the muscle fibers of EDL and SOL (Figs. 1, 2, 3, and 4).
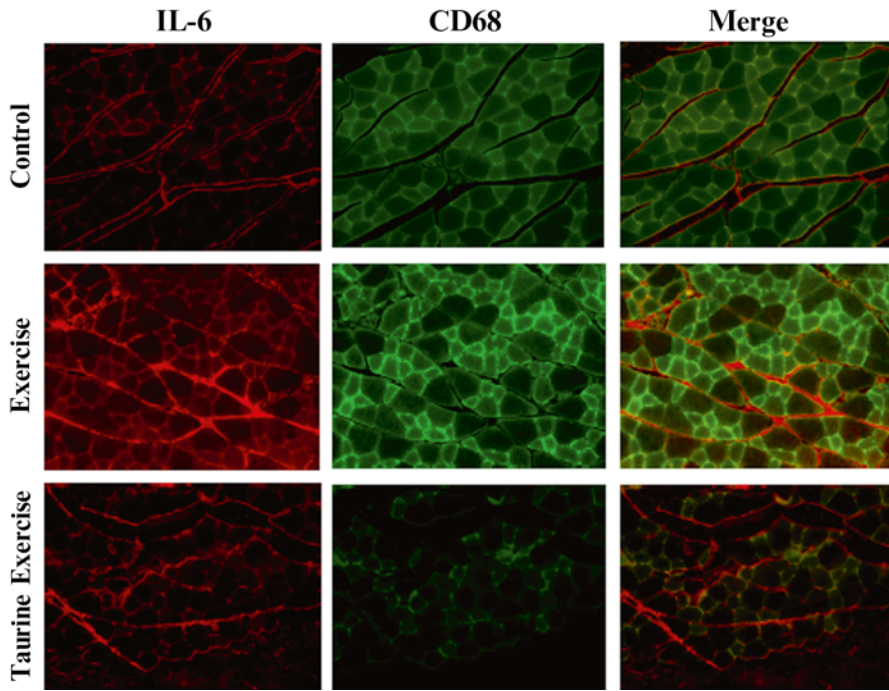
Fig. 1 Expression of IL-6 and CD68 in EDL of each experimental group (Control, Exercise plus saline, Exercise plus taurine). The expression of IL-6 and CD68 in EDL of the Exercise plus taurine administration group shows low immunoreactivity compared to that of the Exercise plus saline group

3.3 Comparative Quantification of TNF-α, IL-6 and CD68 Immunoreactivities in EDL and SOL Skeletal Muscle

To compare the expression of TNF-α, IL-6 and CD68 in different skeletal muscle of each group, immunoreactive signal intensity was quantified by grading. TNF-α and IL-6 signal intensities exhibited a definitive trend in EDL that was less apparent in SOL. There was no difference in CD68 expression between EDL and SOL (Tables 1 and 2). In the heavy exercise group, TNF-α, IL-6 and CD68 signal intensities significantly increased compared to that of the control group and exercise plus taurine group (p < 0.05) (Figs. 1, 2, 5 and 6).
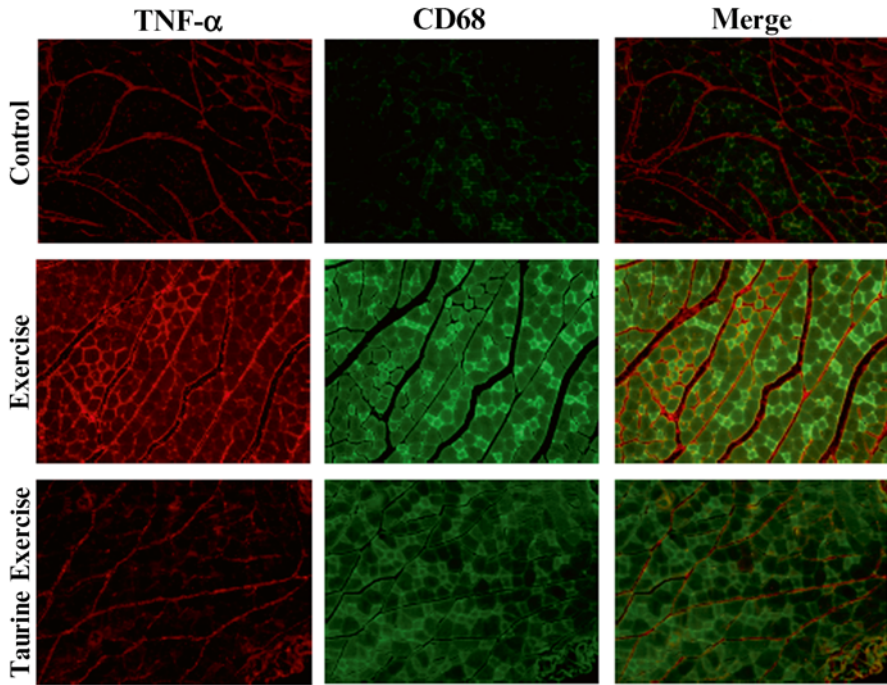
Fig. 2 The expression of TNF-α and CD68 in EDL of each group. The expression of TNF-α and CD68 in EDL of the Exercise plus taurine administration group shows low immunoreactivity compared to that of the Exercise plus saline group

4 Discussion

In the present study, we compared the effects of taurine administration on inflammation of various muscle types in rats subjected to a heavy exercise protocol. Our study revealed three findings. First, most of TNF-α and IL-6 preferentially accumulate in stromal cells of skeletal muscle. In contrast, CD68 accumulates within the muscle fibers. Second, signal intensity for TNF-α and IL-6 was observed in some muscle fibers after heavy exercise. Finally, taurine treatment reduced measures of inflammation in rat skeletal muscle after exercise injury. Skeletal muscle damage leads to changes in tissue morphology and function that may last for several weeks (Lapointe et al. 2002; Smith et al. 2008). Heavy exercise is characterized by muscle protein catabolism and acute inflammation, followed by muscle regeneration (Meeson et al. 2004). The progression of muscle repair is modulated by essential and semi-essential amino acids, which alter inflammation and protein anabolism (Miyazaki et al. 2004; Dort et al. 2013). After muscle injury, myocytes and other cells release a number of cytokines, including IL-1β, IL-6, IL-8 and TNF-α, which cause neutrophils to produce a host of cytotoxic substances, including reactive
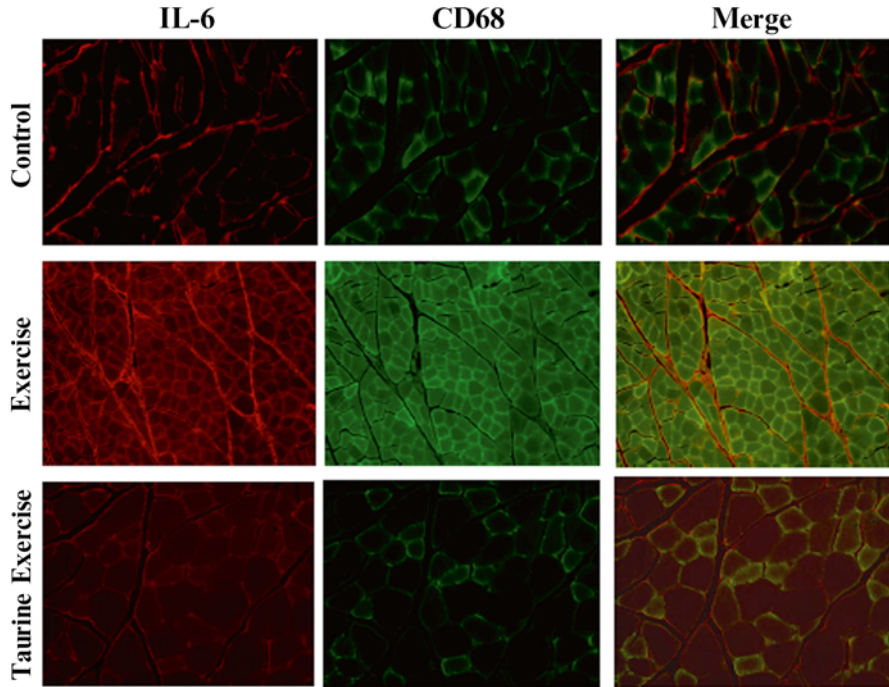
Fig. 3 The expression of IL-6 and CD68 in SOL of each group. The expression of IL-6 and CD68 in SOL of the Exercise plus taurine administration group shows low immunoreactivity compared to that of the Exercise plus saline group

oxygen species, such as superoxide anions, hypochlorite, and hydrogen peroxide (Best et al. 1999; Brickson et al. 2001). The cytokines IL-1, IL-6, and TNF-α stimulate pathways that contribute to activation of the enzyme NADPH-oxidase, which in turn generates reactive oxygen species. Previous research reported that taurine may exert various biological actions that contribute to muscle repair, such as improved outcome of an inflammatory insult, owing to its ability to decrease the production of major pro-inflammatory cytokines (TNF-α, IL-6) (Pilon et al. 2011; Rudkowska et al. 1995). Although the mechanism by which taurine reduces inflammation in skeletal muscle is not fully understood, taurine plays an important role in cytoprotection against ischemia and hypoxia in smooth muscle of stomach (Ma et al. 2003). Taurine efflux may also contribute to the regulatory volume decrease mediated by ion channels and triggered as a response to cell swelling. Released taurine can in turn react with $HOCl^-$ produced by activated leukocytes to form taurine chloramine, a strong reactant that inhibits the production of TNF-α and other proinflammatory mediators (Barua et al. 2001).
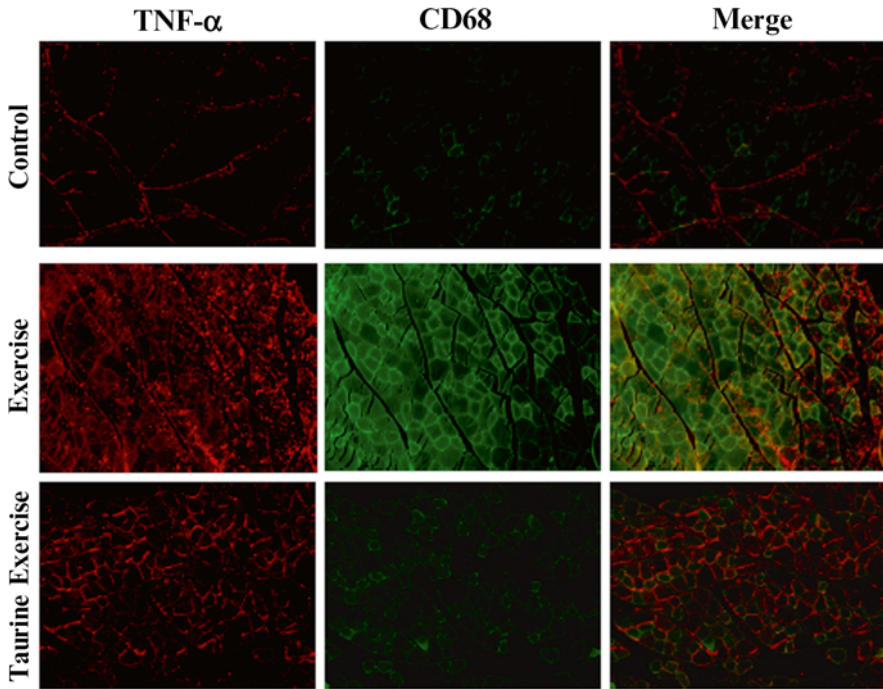
Fig. 4 The expression of TNF-α and CD68 in SOL of each group. The expression of TNF-α and CD68 in the Exercise plus taurine administration group show low immunoreactivity compared to that of the Exercise plus saline group

Table 1 The effect of exercise and taurine treatment on the immunoreactivities of IL-6, TNF-α and CD68 of EDL. Immunohistochemical (IHC) grading based on intensity and frequency of staining was performed by two independent investigators

EDL				
Biomarkers	Location	Control	Exercise plus saline	Exercise plus taurine
TNF-α	Stromal cell	+	+++	**+**
	Muscle fibers	−	−	−
IL-6	Stromal cell	+	++++	+
	Muscle fibers	−	−	−
CD68	Stromal cell	−	−	−
	Muscle fibers	+	+++	+

In a previous study by our laboratory, we found that taurine administration significantly reduces iNOS expression, indicating that heavy exercise-induced nitrosative and oxidative stress is modulated by taurine. Up-regulation of iNOS in skeletal muscle is responsible for nitrosative muscle damage induced by heavy exercise, a consequence of inflammation-mediated activation of NF-κB signaling (Sugiura

Table 2 The effect of exercise and taurine treatment on the immunoreactivities of IL-6, TNF-α and CD68 of SOL. Immunohistochemical (IHC) grading based on intensity and frequency of staining was performed by two independent investigators

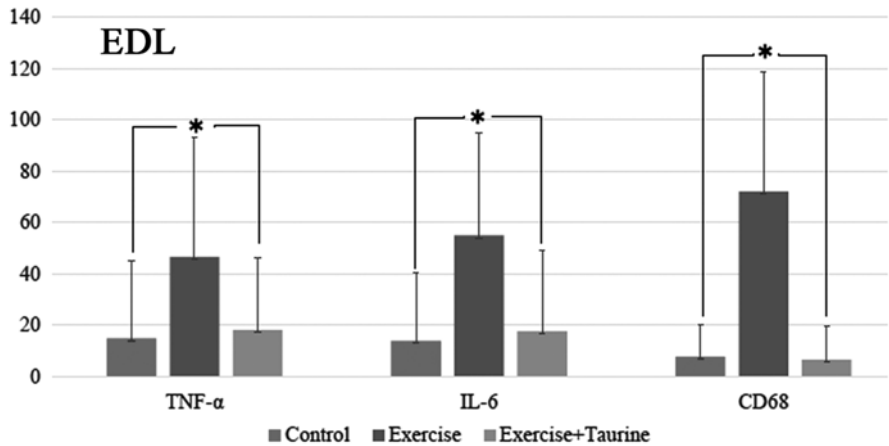| SOL				
Biomarkers	Location	Control	Exercise plus saline	Exercise plus taurine
TNF-α	Stromal cell	+	++	**+**
	Muscle fibers	−	−	−
IL-6	Stromal cell	+	+++	+
	Muscle fibers	−	−	−
CD68	Stromal cell	−	−	−
	Muscle fibers	+	+++	+

Fig. 5 The average immunoreactivities of IL-6, TNF-α and CD68 in EDL. TNF-α, IL-6 and CD68 immunoreactivities significantly increased in rats undergoing heavy exercise. The immunoreactivities significantly decreased in the taurine exercise group compared to the heavy exercise group. Result show Mean±SEM, for n=6 rats in each experimental group. *$P < 0.05$, exercise group versus exercise+taurine group or control group. The two-tailed Student's t-test

et al. 2013). ATP production and oxygen supply to mitochondria needs to be increased to activate skeletal muscle during exercise. An increase in oxygen consumption likely means an increase in the generation of superoxide radicals (O_2^-). Taurine appears to reduce the production of O_2^- via redox reaction, particularly in places with high production of O_2^-, such as mitochondria (Hansen et al. 2006). This involves the conjugation of taurine with tRNA[Leu(UUR)], which increases the biosynthesis of ND6, a subunit of complex I of the electron transport chain, and prevents the diversion of electrons to oxygen forming O_2^- (Jong et al. 2012). Type I muscle fibers contain a large number of mitochondria and perform aerobic energy metabolism by consuming a large number of oxygen molecules to produce ATP during
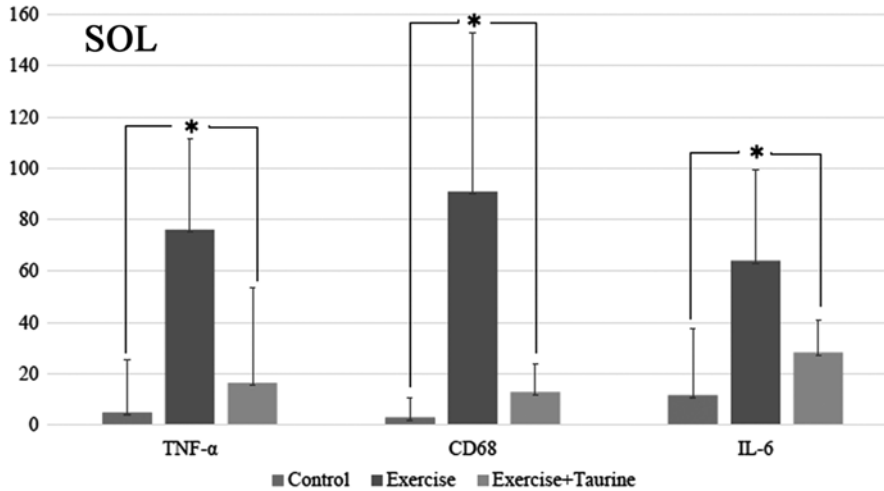
Fig. 6 The average immunoreactivity of IL-6, TNF-α and CD68 in SOL. TNF-α, IL-6 and CD68 immunoreactivities significantly increased in rats performing heavy exercise. Those immunoreactivities significantly decreased in the taurine exercise group compared to that of the heavy exercise group. Result show Mean ± SEM, for n = 6 rats in each experimental group. *P < 0.05, exercise group versus exercise + taurine group or control group. The two-tailed Student's *t*-test

exercise. Therefore, it is likely that type I muscle fibers generate a high level of free radicals from oxygen and are vulnerable to oxidative and nitrosative stress. Compared with type II muscle fibers, type I muscle fibers contain a higher level of polyunsaturated fatty acid and are reportedly vulnerable to lipid peroxidation (Nikolaidis and Mougios 2004; Nikolaidis et al. 2006) as well as to other types of oxidative damage. Because type I muscle fibers also contain a higher number of mitochondria (Moyes 2003) than type II muscle fibers, they may produce a higher level of free radical production during exercise and even at rest.

5 Conclusion

In conclusion, the present study demonstrates that taurine therapy protects against intense exercise-induced inflammation. Taurine may have various biological functions in muscle repair, such as an improved resolution of inflammation, owing to its ability to decrease the production of major pro-inflammatory cytokines.

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