



## Original article

## Increased TNF- $\alpha$ and TGF- $\beta$ concentrations in rat liver after intense exercise

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## ABSTRACT

**Introduction:** Intense exercise can cause cellular damage, resulting in activation of inflammatory and fibrotic reactions.

**Aim:** The aim of the present study was to investigate how one session of intense exercise affected liver gene expression and concentrations of tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and profibrotic transforming growth factor  $\beta$  (TGF- $\beta$ ) in untrained and trained rats.

**Material and methods:** Here we investigated the impact of intense exercise on liver gene expression and concentrations of TNF- $\alpha$  and TGF- $\beta$  in untrained and trained healthy rats ( $n = 30$  each). The trained rats underwent 6 weeks of endurance training with increasing load. From each group, liver samples were collected before, immediately after, and 3 h after one session of intense exercise. Gene expression was evaluated with quantitative real-time polymerase chain reaction. Protein content was measured by enzyme immunoassay.

**Results and discussion:** One session of intense exercise did not influence gene expression at any time point. In trained rats TNF- $\alpha$  and TGF- $\beta$  was increased immediately after exercise ( $P = 0.011$  and  $P = 0.009$ , respectively). The increase in TGF- $\beta$  persisted 3 h post exercise ( $P = 0.045$ ). In untrained rats the concentration of TNF- $\alpha$  did not change in any time point, while TGF- $\beta$  was decreased both immediately and 3 h after intense exercise ( $P = 0.01$  and  $P = 0.03$ , respectively).

**Conclusions:** Intense exercise led to increased proinflammatory and profibrotic activity in the liver of trained rats.

## 1. INTRODUCTION

Physical activity induces the production and release of wide range of cytokines. They exert their effects in autocrine, paracrine, and endocrine manners and play an important role in driving adaptive changes to physical exercise.<sup>1</sup> Such changes occur mainly in muscles,<sup>2</sup> but physical exercise also influences many other organs and tissues in the organism, including liver, which serves as a very important energy source during exercise. While moderate activity provides health benefits, intense exercise can negatively affect metabolic processes, cause cellular damage,<sup>3</sup> and provoke transient inflammatory responses.<sup>4</sup> It has been shown in human studies that strenuous exercise is a strong stressor and could result in splanchnic hypoperfusion with subsequent disturbances in liver homeostasis and energy.<sup>5,6</sup> Rats that run on a treadmill to exhaustion exhibit liver damage marked by increased serum levels of aspartate transaminase (AST) and alanine transaminase (ALT). These animals also show increased serum levels of the proinflammatory cytokine tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), and histological evaluation reveals periportal leukocyte infiltration and hepatocyte vacuolization.<sup>7,8</sup>

## 2. AIM

The aim of the present study was to investigate how one session of intense exercise affected liver gene expression and concentrations of TNF- $\alpha$  and profibrotic transforming growth factor- $\beta$  (TGF- $\beta$ ) in untrained and trained rats.

## 3. MATERIAL AND METHODS

### 3.1. Rats and exercise protocol

The experimental protocol was approved by the Ethics Committee of the Medical University in Białystok, and was performed according to EU regulations governing laboratory animal treatment.

This study included 60 male Wistar rats that were randomly assigned to the untrained (UT,  $n = 30$ ) or trained (T,  $n = 30$ ) group. The exercise protocol was previously described.<sup>9</sup> Trained rats were subjected to endurance training (treadmill running, 5 days per week, 6 weeks). During week 1, the speed was 1200 m/h. The initial running time was 10 min/day and was increased by 10 minutes each day. Over the remaining 5 weeks, the exercise time was 60 min/day. The speed was 1500 m/h for week 2, and 1680 m/h for weeks 3–6. The untrained rats stayed sedentary during the training period.

At 24 h after training cessation, each group was randomly divided into 3 subgroups (each  $n = 10$ ). Two subgroups (UT-pre and T-pre) were sacrificed before exercise. The remaining subgroups performed 60 minutes of treadmill running at 1680 m/h. Two subgroups (UT0h and T0h) were sacrificed immediately after this exercise session. The remaining sub-

groups (UT3h and T3h) were sacrificed 3 h after exercise. Liver tissue samples were collected under anesthesia with intraperitoneal chloral hydrate (1 mL/100 mg body mass).

### 3.2. Tissue homogenization

About 25 mg of tissue was homogenized with a steel ball (diameter 5 mm) in the TissueLyser (Qiagen, Germany). Homogenization was performed at room temperature at a frequency of 25 Hz for 5 minutes. The tissues intended for real-time polymerase chain reaction (RT-PCR) were homogenized in 750  $\mu$ L of QIAzol Lysis Reagent (Qiagen, Germany). The tissues used to determine tissue protein levels were homogenized in 1 mL of 1  $\times$  PBS. Samples used to determine protein concentrations were subjected to two cycles of freezing at  $-20^{\circ}\text{C}$  and thawing at room temperature, then centrifuging at  $5000 \times g$  for 5 minutes at  $4^{\circ}\text{C}$ .

### 3.3. Isolation of total RNA

We isolated total RNA from rat liver tissue with a BioRobot EZ1 (Qiagen, Germany) and an EZ1 RNA Universal Tissue Kit (Qiagen, Germany), according to the manufacturer's instructions. RNA quality and quantity were evaluated with a camera ND-1000 (NanoDrop Technologies, Wilmington, Delaware, USA). Then, total RNA samples were stored at  $-80^{\circ}\text{C}$ .

### 3.4. Quantitative, real-time reverse-transcription polymerase chain reaction

RT-PCR reactions were performed with an ABI 7500 (Applied Biosystems, Waltham, Massachusetts, USA) and MicroAmp Optical 96-Well Reaction Plates with Barcodes (Applied Biosystems, Waltham, Massachusetts, USA). To each well, we added the reaction mixture, which included the TaqMan RNA-to-CT 1-Step Kit (Applied Biosystems, Waltham, Massachusetts, USA) and primers appropriately selected for the TaqMan Gene Expression Assay (Applied Biosystems, Waltham, Massachusetts, USA). Primers specifically targeted sequences of the following genes: *TNF- $\alpha$* , *TGF- $\beta$ 1*, and the control gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). Next, we added total RNA to the reaction mixture in all wells, except the no template control (NTC), which received water instead of RNA (Sigma-Aldrich, Saint Louis, Missouri, USA). The test samples and control were assayed in duplicate, and the NTC was assayed in triplicate.

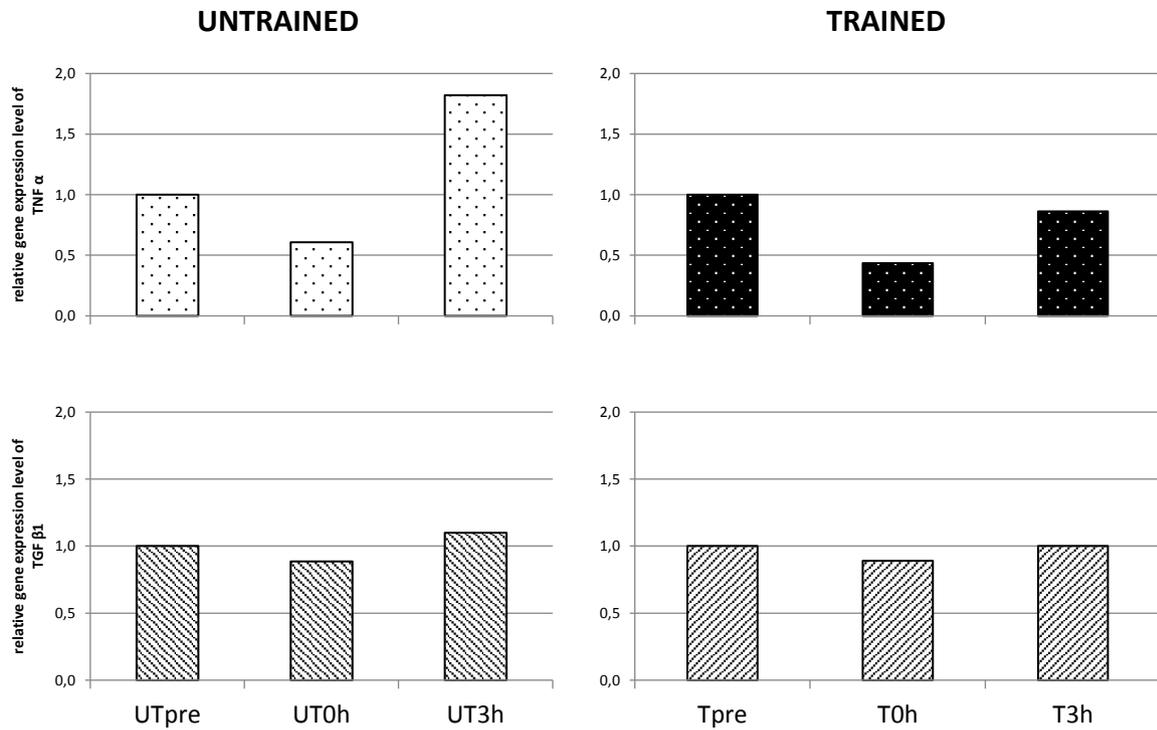
Gene expression levels were calculated with the comparative cycle threshold ( $C_T$ ) method. The  $C_T$  of each sample was normalized to the  $C_T$  of GAPDH as follows:

$$\Delta C_T = C_{T,\text{research gene}} - C_{T,\text{GAPDH}}$$

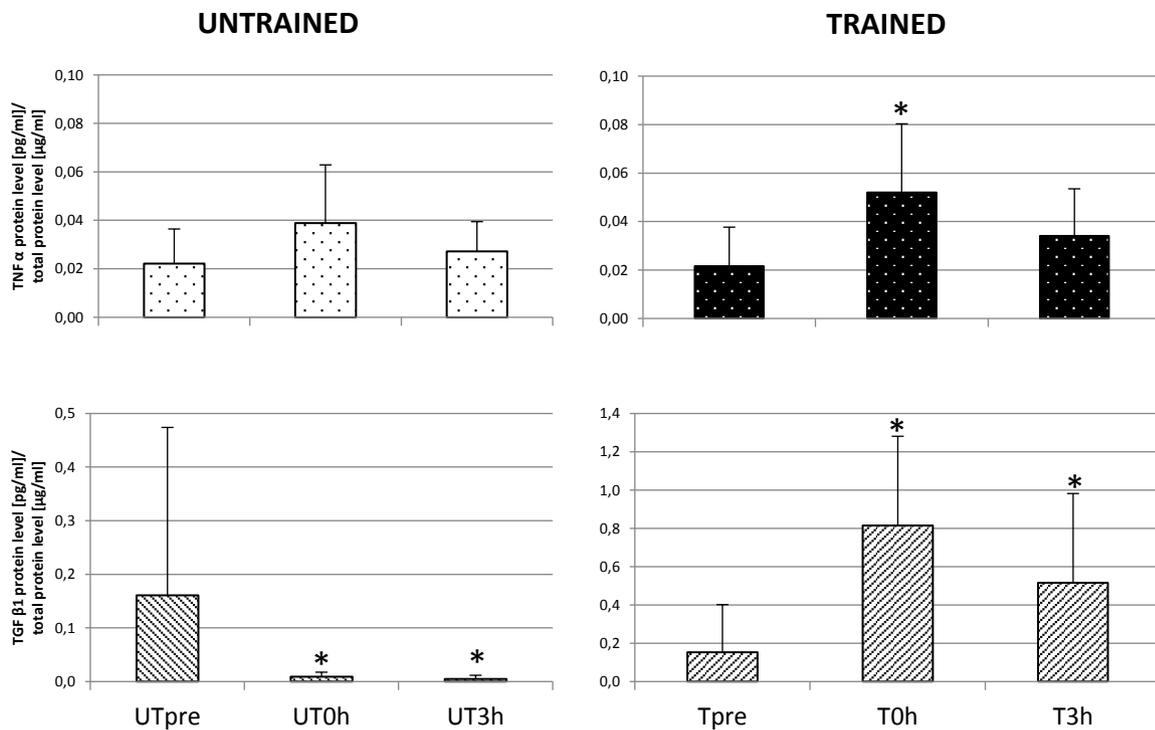
The relative gene expression levels ( $\Delta\Delta\text{CT}$ ) were calculated by subtracting normalized  $C_T$  values for the trained group from those of the untrained group, as follows:

$$\Delta\Delta\text{CT} = \Delta\text{CT}_{\text{trained}} - \Delta\text{CT}_{\text{untrained}}$$

Finally, the fold change in the mRNA level after treatment was calculated as  $2^{-\Delta\Delta\text{CT}}$ .



**Figure 1.** Relative changes in liver mRNA expression with exercise. Relative mRNA levels (dCt) of *TNF- $\alpha$*  and *TGF- $\beta$*  were measured in livers of UT and T rats prior to exercise (UTpre,  $n = 10$ ; Tpre,  $n = 10$ ) and immediately after (UT0h,  $n = 10$ ; T0h,  $n = 10$ ) and 3 h after (UT3h,  $n = 10$ ; T3h,  $n = 10$ ) one session of exercise. There were no statistical differences between investigated groups.



**Figure 2.** Levels of *TNF- $\alpha$*  and *TGF- $\beta$*  proteins, relative to total proteins, were measured in UT and T rats prior to exercise (UTpre,  $n = 10$ ; Tpre,  $n = 10$ ), immediately after exercise (UT0h,  $n = 10$ ; T0h,  $n = 10$ ) and 3 h after exercise (UT3h,  $n = 10$ ; T3h,  $n = 10$ ). \* $P < 0.05$ .

### 3.5. Measuring TNF- $\alpha$ and TGF- $\beta$ 1 levels

TNF- $\alpha$  concentration was measured using the Rat TNF- $\alpha$  Quantikine ELISA Kit (R&D Systems, McKinley, Minnesota, USA). TGF- $\beta$ 1 concentrations were measured using the Mouse/Rat/Porcine/Canine TGF- $\beta$ 1 Quantikine ELISA Kit (R&D Systems, McKinley, Minnesota, USA).

### 3.6. Measuring of total protein content

Total protein contents of samples were measured using a colorimetric method (Pierce BCA Protein Assay Kit; Thermo Fisher Scientific, Waltham, Massachusetts, USA). All solutions were measured at 450 nm (total protein contents at 562 nm) using a BioTek Power Wave XS spectrophotometer (BioTek Instruments, Winooski, Vermont, USA).

### 3.7. Statistical analysis

Results are provided as the median with minimum and maximum values. In figures, the mean  $\pm$  standard deviations are presented. mRNA levels (for statistics,  $\Delta C_T$  was used) and protein levels in investigated groups were compared with the non-parametric Mann-Whitney  $U$  test.  $P$  less than 0.05 was considered statistically significant.

## 4. RESULTS

One session of exercise did not influence the gene expression of any of the investigated cytokines at any investigated time point (Figure 1).

The effects of one session of exercise on protein concentrations are presented in Figure 2.

In UT rats, TNF- $\alpha$  protein concentrations did not differ between UTpre and UT0h or UT3h, while TGF- $\beta$  concentrations significantly decreased from UTpre to UT0h ( $P = 0.01$ ) and UT3h ( $P = 0.03$ ). In trained rats, TNF- $\alpha$  concentrations significantly increased from Tpre to T0h ( $P = 0.011$ ), but not T3h, while TGF- $\beta$  concentrations significantly increased from Tpre to T0h ( $P = 0.009$ ) and T3h ( $P = 0.045$ ).

## 5. DISCUSSION

We investigated the influences of physical exercise on the generation of selected cytokines in the liver at the mRNA and protein levels. Our results suggested that one session of intense exercise could not influence gene expression of TNF- $\alpha$  and TGF- $\beta$ . However, one session of intense exercise influenced the liver protein content of these cytokines. The difference could be due to post-transcriptional modifications. It is estimated, that the correlation between mRNA and protein levels is lower than 0.5.<sup>10</sup> The fact that intense exercise suppressed toll-like receptor-4 production might, at least in part, explain the lack of change in pro-inflammatory cytokine gene expression.<sup>11</sup>

Intense exercise induces liver damage, resulting in aminotransferase release, oxidative stress, and ultrastructural abnormalities.<sup>7</sup> Here we showed that liver inflammation subsequent to such damage was driven by increased TNF- $\alpha$

concentration. TNF- $\alpha$ , a primary biomarker of tissue injury, activates T cells and macrophages and upregulates other proinflammatory cytokines. Intense exercise also led to increased liver concentrations of TGF- $\beta$ , which plays a crucial role in hepatic fibrosis. TGF- $\beta$  induces transdifferentiation of hepatic stellate cells into myofibroblasts, and promotes production and inhibits degradation of extracellular matrix. Hepatocytes affected by TGF- $\beta$  eventually become pre-neoplastic hepatocytes.<sup>12,13</sup>

Interestingly, TNF- $\alpha$  and TGF- $\beta$  levels only increased in trained animals. Gornicka et al. demonstrated that endurance training increased oxidative damage in rat livers and decreased concentrations of the antioxidant  $\alpha$ -tocopherol.<sup>14</sup> We hypothesize that untrained animals had sufficient antioxidant levels to protect from oxidative stress and subsequent damage after one session of intense exercise. However, antioxidants may have been partly consumed in the trained animals, potentially resulting in more pronounced damage and induction of inflammatory and fibrotic reactions. The results in trained animals could also be related to overtraining syndrome, defined as maladapted physiology following excessive exercise without adequate rest. The etiology of this syndrome is unknown, but overtraining is reportedly accompanied by immunologic abnormalities.<sup>15</sup>

## 6. CONCLUSIONS

Notably, our findings suggest that intense exercise increases liver concentration of TNF- $\alpha$  and TGF- $\beta$ , and these cytokines could drive liver damage after intense exercise. The intensity of one session of exercise and rest between sessions should be carefully planned to reduce the risk of potentially harmful and even cancerogenic reactions in the liver.

### Conflict of interest

The authors declare that there is no conflict of interest.

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