Interleukin-6 and hepcidin expression changes in cardiac tissue of long-term trained and untrained rats after exhaustive exercise*

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Background/aim: Exercise benefits the cardiovascular system, but strenuous exercise can cause cardiac damage and induce cytokine production, particularly that of interleukin-6 (IL-6). Hepcidin, which is primarily regulated by IL-6, increases after exercise. Hepcidin is a possible protective factor against the adverse effects of strenuous exercise such as oxidative stress. The aim of the study is to reveal that training increases hepcidin and attenuates increased levels of IL-6 in the hearts of exhaustively exercised rats by comparing the IL-6 and hepcidin mRNA expression levels in trained and untrained groups.

Materials and methods: Thirty male Wistar albino rats were assigned to the following groups: sedentary controls (Con); untrained animals that acutely completed exhaustive exercise and were sacrificed immediately after exhaustion (UT-i) or 1 day after exhaustion (UT-1); and long-term trained animals that completed exhaustive exercise and were sacrificed immediately after exhaustion (T-i) or 1 day after exhaustion (T-1). mRNA levels were examined by reverse transcription PCR.

Results: IL-6 levels significantly increased in the UT-i, T-i, and T-1 groups compared to the Con group (P = 0.000, P = 0.024, P = 0.001), with maximal IL-6 expression found in the UT-i group. Hepcidin levels significantly increased in the T-1 group (P = 0.000) compared to the control.

Conclusion: Increased IL-6 levels in rats show that exhaustive exercise can cause cardiac inflammation. However, long-term training attenuated the severity of the inflammation. The possible protective effect of increased hepcidin in the trained groups can be explained by the antiinflammatory effects of IL-6 and long-term training.

Key words: Hepcidin, interleukin-6, heart, exhaustive exercise

1. Introduction

Exercise in daily life is conducted for elite sports performance, maintenance of health, and management of disease and disability, among other reasons (1). As a physical stress, exercise challenges homeostasis, and the body generates an acute and subclinical inflammatory response to it (2). However, many studies have demonstrated that regular exercise is associated with a reduction in the long-term risk of myocardial events (3).

Interleukin-6 (IL-6) is a cytokine that is produced during strenuous exercise more than any other proinflammatory or antiinflammatory cytokines and may be involved in mediating exercise-related metabolic changes (4). An increase in cytokines such as IL-6, systemic inflammation (5), and oxidative stress induced by strenuous exercise has been suggested to lead to cardiomyocyte dysfunction and cardiac injury (3).

Severe exercise yields an increase in cytokine production, especially that of IL-6, which is the predominate mediator of the iron regulatory hormone hepcidin (6). Hepcidin activity may be elevated at the conclusion of physical activity as a result of exercise-induced inflammation (7). Additionally, plasma IL-6 could be involved in the exercise-induced increase in hepcidin gene expression (8). Hepcidin is secreted in response to iron overload and/or inflammation and hypoxia. Its preventive role in cardiac injury has been suggested in several studies (9). It is hypothesized that training attenuates the IL-6 increase due

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* Long-term trained groups' results in this study were presented as a poster at the 40th National Meeting of Turkish Physiological Sciences; 2–6 September 2014; Kayseri, Turkey.

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to inflammation and oxidative stress caused by exhaustive exercise and increases hepcidin expression. It is not known whether cardiac hepcidin expression changes in response to exercise. Therefore, the differences between IL-6 levels, which may represent the cardiac inflammation caused by exhaustive exercise, and hepcidin levels, which are known as cardiac protective factors against the proinflammatory cytokine IL-6, were investigated in long-term trained and untrained rats forced to complete exhaustive exercises.

2. Materials and methods

2.1. Animals and study design
Three-month-old male Wistar albino rats (n = 30) were housed under standard conditions (21 ± 2 °C, 12-h light/12-h dark cycle) with free access to tap water and food pellets. The rats were randomly assigned to 5 groups: sedentary controls (Con; n = 6) that never ran in the experiment; untrained animals that were acutely forced to complete exhaustive exercise and sacrificed immediately after exhaustion (UT-i; n = 6) or 1 day after exhaustion (UT-1; n = 6); and trained animals that received long-term exercise training, were forced to complete exhaustive exercise, and were sacrificed immediately after exhaustion (T-i; n = 6) or 1 day after exhaustion (T-1; n = 6).

2.2. Exercise training and adaptation on the rat treadmill
Exercise-trained rats (T) ran on the treadmill for 30 min, 5 days a week for 12 weeks (10). Each session included warm-up and cool-down periods (running 7 m/min, 0° incline). Between these periods, they were forced to run 15 m/min on a 15° incline for 20 min (10). Untrained rats (UT) were adapted to the treadmill by running at a speed of 7 m/min with no incline for 10 min over 5 days (11). One day after these exercise periods ended, both groups were forced to do exhaustive exercise: animals were forced to run at a rate of 20 m/min on a 5° incline until they refused to run any further, lay on their backs, and failed to respond to an electrical shock of about 1.5 mA (12).

Rats were sacrificed using ketamine hydrochloride (45 mg/kg) and xylazine hydrochloride (5 mg/kg) anesthesia by intracardiac puncture to remove blood. Heart tissues were removed from the body and kept at –80 °C until analysis.

2.3. Total RNA isolation
Total RNA samples of heart tissues were isolated using a commercial isolation kit for fibrous tissues (Fibrous Tissue Mini Kit K74704, QIAGEN). Briefly, mechanochemical tissue homogenization was performed in liquid nitrogen using a tissue homogenizer system (Glas-Col, 099C-K5424). Following proteinase incubation of the homogenate, total RNA was eluted in spin columns with several centrifuging and washing steps. Subsequently, the concentration and quality of total RNA samples were

determined by measuring the absorbance at 230, 260, and 280 nm (NanoDrop, ND-1000). The 260/280 and 260/230 ratios were used to assess the purity and quality of the RNA, and extractions were repeated until the ratio values were 1.8–2. All total RNA samples were run on 1% agarose gels to check their integrity. Observance of intact 28S and 18S RNA bands was required to continue further experiments with that sample.

2.4. Reverse transcription polymerase chain reaction (RT-PCR)
Briefly, 2 µg of total RNA per sample was converted to cDNA by reverse transcriptase using a commercially available reverse transcription (RT) kit (RevertAid First Strand cDNA Synthesis Kit, Fermentas, Life Sciences). To obtain specific mRNAs, total cDNAs were amplified by PCR using primers specific for rat IL-6, hepcidin, and a housekeeping gene (*GAPDH*). The gene regions corresponding to these primers were double-checked using the NCBI Nucleotide Database (http://www.ncbi.nlm.nih.gov/nucleotide) and the Ensemble Genome Browser Database (http://useast. ensembl.org). Base-pair counts of targeted PCR products were calculated and optimal PCR conditions were adjusted according to the base sequences (Table).

2.5. Agarose gel electrophoresis and mRNA analysis
PCR products (20 µL) were run on a 2% agarose gel with ethidium bromide at 100 V for 1 h. The ethidium bromide-stained mRNA bands in the gel were visualized under UV light with a digital camera (Cleaver Scientific, DI-HD), and the pictures were transferred to a computer. The band density was measured using a software program (Image J 1.38X, National Institutes of Health). The relative levels of IL-6 and hepcidin mRNAs were normalized to *GAPDH* mRNA for each sample (Figures 1a and 1b). All measurements were performed in triplicate.

2.6. Statistical analysis
Statistical analyses were performed using SPSS 20.0 (IBM Corp., Armonk, NY, USA). The results from five experimental groups were compared using parametric one-way ANOVA tests for the two different genes separately. Statistical significance was defined as $P \leq 0.05$. When $P \leq 0.05$, the relationship between each of the two groups was further evaluated using Tukey's post hoc test.

3. Results
The IL-6 mRNA expression level was significantly higher in the UT-i group (P = 0.000), T-i group (P = 0.024), and T-1 group (P = 0.001) than in the Con group, but no significant difference was observed in the UT-1 group (P = 0.923). Maximum IL-6 expression was found in the UT-i group (Figure 2).

The hepcidin mRNA expression level was significantly higher in the T-1 group than in all other groups (T-1 group

Table. PCR conditions and base sequences of the primers.

Target DNA	Primer sequences (5'–3')	PCR program (/30 cycle/)	Target base #
IL-6	forward 5'-TTC ACA AGT CCG GAG AGG AG -3' reverse 5'-GAG CAT TGG AAG TTG GGG TA -3'	94 °C (4′)/94 °C (30″) – 60 °C (40″) – 72 °C (1′)/72 °C (7′)	437 bp
HEP	forward 5'-CAA GAT GGC ACT AAG CAC TCG -3' reverse 5'-GCT GGG GTA GGA CAG GAA TAA -3'	94 °C (4′)/94 °C (30″) – 60 °C (40″) – 72 °C (1′)/72 °C (7′)	337 bp
GAPDH	forward 5'-TCC ACC ACC CTG TTG CTG TA-3' reverse 5'-ACC ACA GTC CAT GCC ATC AC-3'	94 °C (3′)/94 °C (30″) – 60 °C (30″) – 72 °C (1′)/72 °C (5′)	452 bp

vs. control, P = 0.000; UT-i, P = 0.000; UT-1, P = 0.000; and T-i, P = 0.000) (Figure 3). The other groups were not significantly different from each other.

4. Discussion

We found significant elevations in IL-6 and hepcidin mRNA expression levels in the cardiac tissues of rats that were forced to complete exhaustive exercise. Raised IL-6 levels during strenuous exercise can be associated with cardiac damage (13) and can cause hepcidin alterations in the heart (14).

Strenuous exercise enhances whole-body and tissue oxygen consumption up to 20-fold. This increases electron leakage from the mitochondrial transport system, and

Figure 1a. Samples of 2% agarose gel showing IL-6 mRNA expressions.

Figure 1b. Samples of 2% agarose gel showing hepcidin mRNA expressions.

intracellular prooxidant and antioxidant homeostasis is disturbed (15). Increased oxidative stress and acute inflammatory conditions are associated with increased production of cytokines, including IL-6, IL-1β, and tumor necrosis factor-α (TNF-α), in various tissues such as skeletal and cardiac muscle (16). McKay et al. showed that the oxidative effect of strenuous exercise is mostly seen in acutely exercising animals. On the contrary, there was evidence that long-term physical activity increased antioxidant defenses by upregulating antioxidant enzymes (17).

In our study, maximal IL-6 expression was seen in the UT-i group, in which animals were forced to run acutely and were sacrificed immediately after exhaustion. On the other hand, although there was a significant increase in IL-6 expression in the T-i group compared to the controls, this increase was less than that observed in the UT-i group (P < 0.05).

Parallel to our study, Kasapis and Thomson found that levels of inflammatory markers were lower in joggers and aerobic dancers than in young, healthy humans who rarely exercised (18). We suggest that although exhaustive exercise generates systemic or tissue inflammation, trained rats were resistant to proinflammatory activity. Therefore, our findings suggest that cardiac inflammation levels were different in both groups, probably due to different pathophysiologic mechanisms. According to Jia et al., low-concentration IL-6 preconditioning could rescue cell viability by activating cellular defense machinery to protect cells from oxidative damage (19). Based on this proposition, we think that the increased IL-6 expression in rats forced to run acutely might be a pathological process, but the increase in IL-6 expression in trained rats could be a metabolic process, which causes IL-6 to act as
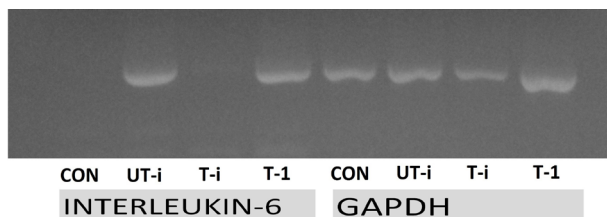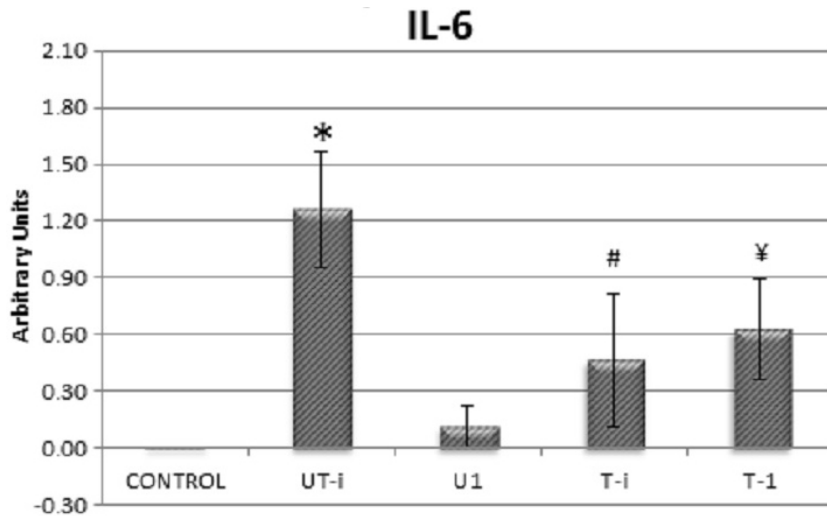
IL-6

Figure 2. Relative mRNA expression levels of IL-6 in five experimental groups. IL-6/GAPDH levels are presented as mean ± standard deviation. * Control vs. untrained immediate (P < 0.001), # control vs. trained immediate (P < 0.05), ¥ control vs. trained 1 (P = 0.001).
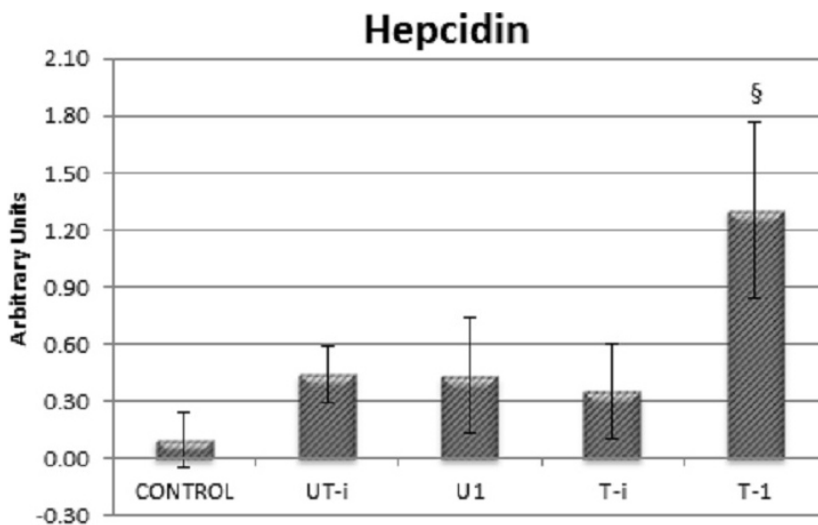
Hepcidin

Figure 3. Relative mRNA expression levels of hepcidin in five experimental groups. Hepcidin/GAPDH levels are presented as mean ± standard deviation. § Trained 1 (T-1) vs. control, untrained immediate, untrained 1, trained immediate (P = 0.001).

an antioxidant. Most studies suggest that the increase in cytokines in tissues due to strenuous exercises may not indicate tissue necrosis, but may be indicative of increased oxidative stress, which can return to basal levels after a few days (20). In this study, the moderate increase in IL-6 expression was thought to have an antiinflammatory effect on the hearts of the trained rats and was not an indicator of cardiomyocyte injury. It is known that IL-6 signaling in monocytes and macrophages due to injury creates a proinflammatory response, whereas IL-6 activation and

signaling in muscle due to increased metabolic activity and oxidative stress might be antiinflammatory (20). Our hypothesis is supported by the findings of Noël et al., who claimed that prolonged and repeated ischemic training sessions could be well tolerated without evidence of myocardial injury, significant arrhythmias, or left ventricular dysfunction (21).

One thing that may protect a trained rat's heart from damage associated with excessive increase in IL-6 expression during exhaustive running may be increased hepcidin

synthesis in parallel with the antiinflammatory effects of IL-6 during exercise (8,22–24). Isoda et al. suggested that the increase in cardiac inflammatory cytokines may induce hepcidin gene expression in cardiomyocytes and the liver (25). Hepcidin expression is increased by IL-6, but not by other inflammatory cytokines such as IL-1α or TNF-α (22,23). In cardiac tissue, cardiomyocytes, which contain myoglobin, are the primary cellular source of large amounts of iron. Therefore, destruction of cardiac tissue is thought to release large amounts of iron into the extracellular space (26). In animal studies, increased tissue iron content results in larger infarcts and more apoptosis when subjected to temporary coronary occlusion (27). Ischemia leads to iron accumulation in the myocardium, and increased myocardial iron content exacerbates ischemia/reperfusion injury (28). Hepcidin may inhibit the release of iron from macrophages or noncardiomyocytic noninflammatory cells with a high ferroportin expression in the heart (25). With regard to the idea that acute exhaustive running might cause coronary insufficiency in untrained rats, one can expect that these animals would be prone to changes in iron metabolism due to ischemia and ischemia/reperfusion injury (29). Because intracellular iron is sequestered, stored, and detoxified in ferritin, which is highly expressed in all cells, hepcidin may play an important cytoprotective role against extracellular free radical formation by inhibiting an increase in extracellular iron (30). In addition to its cytoprotective role, it has been suggested that hepcidin may be an antiinflammatory hormone because it reduces body iron stores (31).

Although there was no IL-6 expression in sedentary controls (Con), a likely physiological but minimal concentration of hepcidin was expressed in the cardiac tissues of the control rats. Hepcidin reached maximal levels in the trained group 1 day after exhaustion, instead of immediately afterwards. These results were in accordance with the study of Roecker et al., who claimed that after hepcidin synthesis begins, it reaches its peak level 24 h after running (32). Simonis et al. explained that hepcidin mRNA expression is temporally upregulated in ischemic cardiac tissue during the first 24 h after infarction, although its onset begins shortly after the beginning of ischemia (29). Simonis et al. also tested whether this regulatory process was specific for hepcidin, or whether other iron-regulatory proteins were also regulated, by assessing mRNAs specific for hemojuvelin and iron-regulated gene 1 (*IREG-1*). They found that iron-regulatory proteins were similarly regulated (29).

Interestingly, the tremendous increase in IL-6 expression in the untrained-immediate group (UT-i) did not cause the maximal increase of hepcidin expression among untrained animals. This result surprised us, because we expected that increased hepcidin would parallel the increase in IL-6, as in the study of Skarpanska-Stejnborn (33), and would reach its maximal level in acutely run rats. Skarpanska-Stejnborn et al. showed a significant increase in hepcidin and IL-6 in parallel with high-intensity, short-term exercise (33). However, some recent studies support our findings. Robson-Ansley et al. suggested that there was no dose-response relationship between IL-6 and hepcidin, and it is possible that prolonged exposure to IL-6, regardless of quantity, could increase hepcidin activity (24). Based on the results of previous studies, it was found that trained rats ran longer periods (34) and produced more hepcidin to protect the heart from injury, owing to preconditioning. Banzet et al. suggested that, in addition to IL-6, other biological signals possibly activate hepcidin transcription, primarily in response to intense and/or prolonged exercise (8). Diaz et al. claimed that iron-dependent signals as well as IL-6 signaling could be responsible for the increase of hepcidin (35). This knowledge and our findings suggest that hepcidin expression can be triggered by factors other than IL-6.

The iron content of the heart was not evaluated in our study, which is a limitation.

In conclusion, we have shown that exhaustive running produced different inflammatory effects in untrained and trained rat hearts. Although there were significant increases in both IL-6 and hepcidin mRNA expression levels in both groups, the excessive increase in IL-6 levels in untrained rats that were evaluated immediately after exhaustion did not cause a maximal increase in hepcidin expression. In fact, the largest increase in hepcidin was seen in the trained group 1 day after the completion of exhaustive exercise. Previous findings and our results suggest that the protective effect of hepcidin against oxidative stress and inflammation is stronger in long-term, exercise-trained rats and is not closely linked to the maximal increase of the proinflammatory cytokine IL-6, but is possibly related to the increase of antiinflammatory cytokine IL-6.

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