Transcriptional and translational regulation of heat shock proteins in leukocytes of endurance runners

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Fehrenbach, Elvira, Andreas Michael Niess, Elke Schlotz, Frank Passek, Hans-Herrmann Dickhuth, and Hinna Nordhoff. Transcriptional and translational regulation of heat shock proteins in leukocytes of endurance runners. J Appl Physiol 89: 704–710, 2000.—Heat shock proteins (HSP) represent cell-protective and antioxidant systems that may be induced by reactive oxygen species, cytokines, and hyperthermia. In the present study, we evaluated the influence of heavy endurance exercise and training on HSP27 and HSP70 in peripheral leukocytes of 12 athletes (before and at 0, 3, and 24 h after a half-marathon) and 12 untrained controls on protein and mRNA levels by flow cytometry and RT/PCR, respectively. HSP transcripts increased significantly immediately after acute exercise accompanied by elevated levels of corresponding proteins. HSP protein expression remained high until 24 h postexercise. Significant increases of plasma interleukin-8, myeloperoxidase, and creatine kinase occurred after exercise. Basal HSP expression was usually lower in trained compared with untrained subjects. Applying in vitro heat shock to resting blood samples of all subjects significantly stimulated HSP mRNA, showing higher increases in trained individuals. The exercise-induced alterations indicate that immunocompetent cells became activated. In addition to heat stress, other exercise-associated stress agents (oxidants, cytokines) may have also participated in stimulation of HSP expression in leukocytes. The expression pattern of HSP due to training status may be attributed to adaptive mechanisms.

MATERIALS AND METHODS
Twelve well-trained male athletes (53.3 ± 18.4 km/wk running; 32.3 ± 9.3 yr; 175.0 ± 3.3 cm; 64.4 ± 3.7 kg) performed an official half-marathon under competition conditions (21.1 km), which started at 10:00 AM on a cool and humid December day (1°C). Venous blood samples were taken with the subjects in a sitting position with EDTA used as anticoagulant. The samples were collected at rest 24 h before (9:00 AM), directly after (11:30 AM-12:00 noon), and 3 h (2:30–3:00 PM) and 24 h (9:00 AM) after competition. The trained individuals were engaged in specific endurance training. During the last 3 days before the race, the athletes performed only moderate endurance runs lasting up to 40 min with a running intensity below the lactate threshold. Athlete 8 did not finish the half-marathon due to a knee injury; his postexercise values were excluded from the statistics. Additionally, blood samples obtained at rest from 12 healthy and normally conditioned male adults, who did not participate in the half-marathon, were collected (9:00 AM) as control samples.

STRENUOUS PHYSICAL EXERCISE has been shown to induce an acute response of the immune system, including the activation of inflammatory cells (17, 22). Metabolic activation correlates with an augmented generation of reactive oxygen species (ROS) and reactive nitrogen intermediates by leukocytes, a mechanism that is partly mediated by cytokines such as plasma interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) (2, 31, 32). Protection and/or tolerance against exercise-induced oxidative, heat, cytokine, and inflammatory stress in leukocytes may be in part provided by heat shock proteins (HSPs) (7). The metabolic changes caused by exercise are similar to those known to induce stress protein synthesis (8, 12). HSPs play a role in protein translocation, stabilization, assembly, and degradation processes, functions that could be important in leukocytes activated by heavy exercise (10, 11, 27).

The question arose whether strenuous exercise such as a half-marathon influences HSP expression in blood at the cellular, protein, and/or transcriptional level. Semiquantitative RT/PCR and flow cytometry were used for analysis of HSP27 and HSP70 alterations in leukocytes due to strenuous endurance exercise. We used a competitive half-marathon as an in vivo stress model. Furthermore, we intended to verify whether the regulation of basal HSP expression in immunocompetent cells exhibits adaptation due to regular endurance training. A control group of untrained men at rest was compared with half-marathon runners at rest to examine the influence of regular endurance training on the baseline expression of HSPs in leukocytes. In vitro stimulation of leukocytes with heat shock was used to evaluate the individual heat shock response to a defined stimulus in differently trained subjects.
sedentary control (45.4 ± 11.4 yr; 176.7 ± 2.8 cm; 75.9 ± 3.2 kg). These untrained individuals did not perform any kind of sports conditioning and did not have any stress situation during the 3 days before the collection of blood samples. The preexercise samples of the trained persons represent the corresponding resting (baseline) value. None of the subjects used drugs or mineral or vitamin supplements. Each gave written informed consent before participation in the study. The experimental protocol was approved by the institute’s human ethics committee according to the principles set forth in the Declaration of Helsinki of the World Medical Association.

Analytic methods. The lactate concentrations of the hemolyzed capillary blood samples were measured electrochemically by use of a lactate analyzer (EBIO, Eppendorf, Hamburg, Germany). Plasma creatine kinase (CK) activity and uric acid concentrations were determined by enzymatic analyses (Hitachi 717, Boehringer, Mannheim, Germany). Hematocrit, hemoglobin concentration, and total and differential counts of white blood cells were determined by using an automated hematometry analyzer (Coulter Junior JS, Coulter Electronics). Differential analyses of lymphocytes, monocytes, and neutrophils were conducted automatically. Hematocrit and hemoglobin were used to correct plasma concentration of CK, myeloperoxidase (MPO), IL-8, and TNF-α with regard to changes in plasma volume after exercise (6).

Preparation of leukocytes from peripheral blood for flow cytometry. Five milliliters of EDTA blood were carefully layered over 5 ml of Lymphoflot (Biotest, Dreieich, Germany), a solution containing diatrizoate (9.6% wt/vol) and Ficoll (5.6% wt/vol), and allowed to settle by gravity without centrifugation for 60 min. The erythrocytes were aggregated at the interface and sedimented to the bottom of the tube. The majority of the leukocytes remained in the plasma layer and were removed. The overlay was washed two times with PBS, and the cell concentration was adjusted with PBS to 1 × 10^7 cells/ml; 100 μl of the suspension was used for analysis by flow cytometry.

Flow cytometry. The cells were analyzed by intracellular, indirect immunofluorescence by using HSP-specific monoclonal antibodies (StressGen, Biotechnologies, Victoria, BC, Canada; Biomol, Hamburg, Germany): SPA-800 (HSP27; IgG1, clone G3.1) and SPA-810 (specific for the inducible form of human HSP70; IgG1, clone C92F3A-5). Cells (1 × 10^6) were first fixed at room temperature in a solution containing formaldehyde (reagent A) according to the manufacturer’s instructions (Fix & Perm kit, An der Grub, Vienna, Austria) and washed twice. Then the cells were permeabilized with reagent B and at the same time incubated with the primary HSP-specific monoclonal antibody to show the maximum of positive cells or isotype-matched monoclonal antibodies at the same concentration (1 μg/test) and incubated for 15 min at room temperature. After washing the cells twice and incubating in the presence of the secondary FITC-conjugated goat anti-mouse F(ab')2 IgG (Dianova, Hamburg, Germany), we analyzed the cells using the flow cytometer EPICS-XL-MLC (Coulter, Krefeld, Germany). Dead cells were excluded by electronic gating, and fluorescence histograms were area corrected to 10,000 cells. The lymphocyte, monocyte, and granulocyte populations were differentiated according to granularity and size in the forward vs. side scattergram and were gated. For each of the three special gates, data were presented as percent positive cells and mean fluorescent channel (MFC), corrected for background fluorescence by the negative controls.

Isolation of RNA and RT/PCR. Cytoplasmic RNA for RT/PCR analyses was isolated from whole blood with the RNeasy-blood kit (Qiagen, Hilden, Germany). Three hundred nanograms of RNA were reverse transcribed (10˚ 20°C; 15˚ 42°C; 5˚ 99°C; 5˚ 5°C) and amplified (3˚ 95°C; 1˚ 95°C; 1˚ 55°C; 1˚ 72°C) in the thermal cycler (MJ Research, PTC200) using specific primers for HSP27 and HSP70B [StressGen, Biotechnologies, (38)] and for β-actin (34). RT and subsequent amplification by the PCR were performed by using a GeneAmp RNA PCR kit (Perkin-Elmer). The RT master mix contained 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 5 mM MgCl₂; 1 mM each of deoxyribonucleoside triphosphates, 1 U/μl RNase inhibitor; 2.5 U/μl RT; and 2.5 μl of oligo(dT). The final RT reaction volume was 20 μl. The PCR master mix contained 50 mM KCl; 10 mM Tris-HCl, pH 8.3; 1.25 mM MgCl₂; 200 μM each of deoxyribonucleoside triphosphates; 2.5 U/100 μl AmpliTaq DNA polymerase, and 0.15 μM of each primer. A final 25 μl of PCR reaction solution contained 5 μl RT product (cDNA) and 20 μl PCR master mix. For each primer pair, control experiments were performed to determine the range of cycles in which a given amount of cDNA would be amplified in a linear fashion: HSP27, 25 cycles; HSP70, 30 cycles; and actin, 27 cycles. Furthermore, a dilution assay was performed to determine the proper input RNA concentration. The resulting amplified products for HSP27 (285 bp) and HSP70B (234 bp) were confirmed by sequence analysis (SEQLAB, Goettingen, Germany). Photographs of ethidium bromide-stained DNA gels (2%) were scanned by the Lumi-Imager System (Boehringer), which allowed semiquantitative analyses of the specific HSP27 and HSP70 expression. The data generated were normalized to transcript levels for the constitutively expressed β-actin gene.

In vitro stimulation with heat shock. EDTA blood of 12 healthy, normally conditioned (untrained) male adults at rest and of 12 well-trained athletes at rest was incubated for 2 h in a water bath heated to 42°C (heat shock). Experimental heat shock conditions were chosen to induce an intensive stress response in vitro without cell damage (8, 13, 27). The

| Table 1. White blood cell counts (×10^9 per liter), CK, MPO, TNF-α and IL-8 at rest and after the half-marathon |
|--------------------------------------------------------|-----------------------------|-----------------------------|-----------------------------|
| Rest | +0 h | +3 h | +24 h |
| Leukocytes | 6.9 ± 2.0 | 13.1 ± 4.9* | 14.7 ± 2.1* | 7.9 ± 1.8 |
| Lymphocytes | 2.4 ± 0.6 | 2.4 ± 1.2 | 1.0 ± 0.2* | 2.5 ± 0.6 |
| Monocytes | 0.7 ± 1.8 | 0.9 ± 0.5 | 1.2 ± 0.2* | 0.9 ± 0.2 |
| Granulocytes | 3.5 ± 1.2 | 9.7 ± 3.8* | 12.4 ± 3.8* | 4.7 ± 1.3* |
| CK, U/l | 53.4 ± 25.0 | 108.5 ± 50.7* | 179.9 ± 125.5* | 289.4 ± 221* |
| MPO, pg/ml | 9.4 ± 3.3 | 30.6 ± 15.2* | 15.6 ± 4.8 | 11.8 ± 3.8 |
| IL-8, pg/ml | 5.0 ± 6.5 | 30.7 ± 5.3* | 8.9 ± 11.3 | 3.9 ± 6.3 |
| TNF-α, pg/ml | 0.3 ± 0.2 | 1.2 ± 0.9 | 0.6 ± 0.6 | 0.3 ± 0.5 |

Values are means ± SD. CK, creatine kinase; MPO, myeloperoxidase; TNF-α, tumor necrosis factor-α; IL-8, interleukin-8; +0 h, +3 h, and +24 h, immediately, 3 h, and 24 h after half-marathon. *P < 0.05 post-vs. preexercise.
5.3 Leukocyte counts of untrained controls at rest were regarded as significant.

Statistical methods. All statistical analyses and descriptive methods were computed by the statistical software package JMP (JMP3.1 software, SAS Institute, Cary, NC) for personal computer. Data in Tables 1-3 are expressed as means ± SD. Comparisons of repeated measurements in the trained athletes were tested for significance by the Wilcoxon signed-ranks test. The nonparametric test of Mann-Whitney was used for evaluation of significant differences between the resting values and the heat shock-induced increases of the trained and untrained group. A P value of P < 0.05 was regarded as significant.

RESULTS

Leukocyte, monocyte, and granulocyte counts were significantly increased after exercise and declined 24 h later to baseline levels (P < 0.05). By contrast, lymphocyte counts decreased 3 h after the run (P < 0.05, Table 1). Leukocyte counts of untrained controls at rest were 5.3 ± 1.4 × 10⁹/l.

Table 2. Expression of the proteins HSP27 and HSP70 in the cytoplasm of mono- and granulocytes of 12 trained athletes analyzed by flow cytometry at rest and after a half-marathon

<table>
<thead>
<tr>
<th></th>
<th>Rest</th>
<th>+0 h</th>
<th>+3 h</th>
<th>+24 h</th>
</tr>
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<tbody>
<tr>
<td>HSP27</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M, %</td>
<td>20.6±5.8</td>
<td>60.9±26.8</td>
<td>73±15.7</td>
<td>67±19.6</td>
</tr>
<tr>
<td>G, %</td>
<td>9.3±4.4</td>
<td>19±12.1</td>
<td>20.2±4.4</td>
<td>26.3±16.5</td>
</tr>
<tr>
<td>M, MFC</td>
<td>2.2±0.3</td>
<td>3.3±1.6</td>
<td>2.9±0.7</td>
<td>2.0±0.7</td>
</tr>
<tr>
<td>G, MFC</td>
<td>2.2±0.3</td>
<td>1.8±0.2</td>
<td>1.7±0.3</td>
<td>1.5±0.1</td>
</tr>
</tbody>
</table>

Values are means ± SD. M, monocytes; G, granulocytes; HSP, heat shock proteins. Data are presented as percent positive cells (%) and fluorescence intensity (MFC). *P < 0.05 post- vs. preexercise.

Heat-shocked blood samples were prepared for further analyses as described above. Control blood samples of the same persons were treated identically except for the heat shock exposure.

ELISA. Testing for IL-8 (Genzyme, Duoset, Cambridge, MA, 1.0 pg/ml), TNF-α (R&D Systems, Minneapolis, MN, 1.0 pg/ml), and MPO (Calbiochem-Novabiochem, Bad Soden, Germany, 1.5 pg/ml) in the plasma samples of the athletes was done by ELISA according to the instructions of the manufacturer.

Table 3. Comparison of the basal HSP-positive cell counts of trained vs. untrained individuals at rest determined by flow cytometry

<table>
<thead>
<tr>
<th></th>
<th>Trained</th>
<th>Untrained</th>
</tr>
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<tbody>
<tr>
<td>HSP27-positive monocytes</td>
<td>20.6±5.8</td>
<td>41.5±22.7</td>
</tr>
<tr>
<td>HSP27-positive granulocytes</td>
<td>9.3±4.4</td>
<td>17.3±12.4</td>
</tr>
<tr>
<td>HSP70-positive monocytes</td>
<td>19.1±15.6</td>
<td>38.4±13.7</td>
</tr>
<tr>
<td>HSP70-positive granulocytes</td>
<td>39.5±16.9</td>
<td>76.3±15.3</td>
</tr>
</tbody>
</table>

Values are means ± SD. *P < 0.05, trained vs. untrained.

CK was also significantly increased and had its maximum 24 h postexercise (P < 0.05, Table 1). Lactate values after exercise were 5.1 ± 2.2 mmol/l.

MPO and IL-8 in plasma were significantly stimulated directly after exercise and were normalized thereafter (P < 0.05, Table 1). An elevation of TNF-α immediately and 3 h after the run could be detected, but only single samples attained values above the detection limit of 1.0 pg/ml (Table 1).

RT/PCR. Semiquantitative RT/PCR analyses revealed a significantly stimulated mRNA-expression of HSP27 (P < 0.05) and HSP70 in leukocytes of all athletes directly after the half-marathon (0 h) compared with preexercise levels (rest) (Figs. 1 and 2). In individual athletes, elevated levels of HSP70 transcript could be detected even 3 h after the competition. After 24 h, the values decreased to near preexercise levels.

HSP27, HSP70, and β-actin cDNAs were amplified under conditions to allow relative comparisons for a given mRNA species.
The comparison of trained vs. untrained persons revealed different HSP mRNA expression. Baseline HSP27 transcripts were significantly downregulated in leukocytes of athletes, whereas the HSP70 mRNA expression was significantly higher in trained compared with untrained men \( (P < 0.05, \text{Figs. 3 and 4}) \).

Applying heat shock in vitro \( (2 \text{ h}, 42^\circ \text{C}) \) to the blood samples of trained and of untrained individuals at rest revealed a significantly stimulated response concerning HSP27 and HSP70 mRNA expression \( (P < 0.05) \) compared with controls treated identically except for the 42°C exposure. The difference of the heat shock-induced HSP mRNA expression from the mRNA expression of the corresponding untreated controls was calculated and presented in Fig. 5. The increase of HSP27 and HSP70 mRNA expression due to heat shock in vitro was significantly more pronounced in leukocytes of trained athletes at rest compared with untrained subjects \( (P < 0.05, \text{Fig. 5}) \).

Flow cytometric analyses. After the race, there was a significantly greater percentage of leukocytes expressing cytoplasmic HSP27 and HSP70 \( (P < 0.05, \text{Table 2}) \).

The fluorescence intensity increased significantly in monocytes for HSP27 \( (0 \text{ h and } 3 \text{ h}, P < 0.05) \) and for HSP70 \( (0 \text{ h}, 3 \text{ h}, \text{ and } 24 \text{ h}, P < 0.05) \) \( (\text{Table 2}) \). In granulocytes, the HSP70 MFC was only stimulated 24 h postexercise \( (P < 0.05) \). The further increase 24 h after the run, which was detectable in all HSP70 values and only HSP27-positive granulocytes \( (\text{Table 2}) \), is remarkable. Lymphocytes were mainly negative for HSP.

Counts of HSP27- and HSP70-positive mono- and granulocytes of trained athletes were significantly lower compared with the corresponding cells of untrained persons \( (P < 0.05, \text{Table 3}) \).

DISCUSSION

The changes in cell counts and increases in MPO, IL-8, TNF-α, and CK in plasma reflect activation of immunocompetent cells as well as oxidative, cytokine, and muscular stress due to the half-marathon \( (1, 4, 25, 29, 35) \). This exercise-induced stress response may contribute to the induction of HSP in leukocytes of ath-
Influence of acute strenuous exercise on HSP expression. In our study, we described that strenuous endurance exercise such as a half-marathon stimulated HSP27 and HSP70 expression in leukocytes of human subjects at pretranslational and protein levels detected by RT/PCR and flow cytometry, respectively. HSP may play a protective role in leukocytes against exercise-induced stress, prepare them to survive new environmental challenges, and maintain cellular homeostasis. The increase in HSP expression is accompanied by the maintenance of the proliferative capacity and viability of the cells (13). HSP could further indicate selective mechanisms in protein conservation, regulation of the inflammatory response, and receptor function. HSPs are activated on cellular stress/injury and oxidative, heat, and cytokine stress and participate in the folding and intracellular transport of damaged proteins (7, 11).

The differential regulation of HSP mRNA expression, percent HSP-positive cells, and their MFC intensity started with an initial increase of HSP mRNA in the leukocytes immediately after the half-marathon. It was partially paralleled and followed by a prolonged high (0- to 3-h) protein expression level, mainly in monocytes. The deficiency of MFC increase in granulocytes in the first hours after exercise may be the result of less HSP-specific transcriptional and/or translational activation, potentially due to the short half-life of granulocytes. The stimulation of all HSP-positive cell populations lasted longer and even increased 24 h after exercise. The changes reflect activation of immunocompetent cells, particularly mono- and granulocytes, cells that are capable of producing relevant quantities of ROS as a result of their activation under physical stress (18). A systemic neutrophil activation and degranulation due to the half-marathon was also mirrored in a rise in plasma MPO (1). Together with the parallel, significant increase of IL-8, this is likely the basis for exercise-induced generation of ROS, which may partly be responsible for the HSP reaction. The delayed augmentation of HSP-positive cell counts may arise from an accumulation of activated cells, increased recruitment of the periphery, lower degrada-

Fig. 4. Comparison of the basal mRNA expression of HSP27, HSP70, and actin in leukocytes of UT with the resting values of TR. Presentation as ethidium bromide-stained RT-PCR agarose gel. HSP27, HSP70, and β-actin were amplified under conditions to allow relative comparisons for a given mRNA (n = 12).

Fig. 5. Comparison of the increase in basal HSP27 and HSP70 mRNA expression due to heat shock in vitro between leukocytes of UT (n = 12) and TR (n = 12) at rest. Results (means ± SD) are presented as differences between heat shock-induced expression vs. expression of the respective untreated controls. *Significant differences between the groups (P < 0.05).
exercise-induced stress. Nevertheless, it seems probable that HSPs in leukocytes were synthesized as a result of multiple mechanisms associated with exercise stress, including oxidative, cytokine, and muscular stress, and not only as a result of exercise-induced hyperthermia per se.

It is noteworthy that the heat shock-induced stimulation of HSP mRNA expression was significantly more pronounced in leukocytes of trained compared with untrained individuals. Regular endurance training seems to affect protective mechanisms in immunocompetent cells beneficially. The enhanced heat shock response in the athletes at rest may represent an activation of the protective resources in immune cells from denaturing heat. It may be interpreted as a training-induced mechanism of adaptation or as acquired thermotolerance (7, 15).

Training-related effects. The basal HSP expression of the trained athletes at rest displayed a unique pattern, different from that of nontrained individuals. HSP27- and HSP70-positive mono- and granulocytes were significantly diminished in trained compared with untrained persons. HSP27 transcripts were also decreased in the trained subjects, but HSP70 mRNA expression was significantly increased. The decrease in HSP expression may be explained by a lower pro-oxidant state due to intensive training, which is described by several authors (3, 9, 14, 24, 26). Nevertheless, the low HSP27 and the high HSP70 mRNA levels could both be significantly stimulated by a maximal stress such as a half-marathon or by heat shock in vitro, and the mRNAs were also translated, resulting in an enlargement of HSP-positive cells. The higher baseline expression of HSP70 mRNA in athletes as a result of intensive training may be attributed to a unique characteristic of HSP70 mRNA, which is unstable under normal physiological conditions and stabilizes under stress conditions (23). This may also be a mechanism of adaptation to regular, intensive endurance training. One can speculate that the training-stressed cells provide high HSP70 transcript levels for immediate translation whenever necessary. The resulting accumulation of HSP70 proteins themselves may regulate translation via a negative-feedback loop if they are not used for an immediate stress response (5, 16, 37).

The exact function of the differential regulation of HSP at the transcriptional and translational level in response to exercise and to experimental heat stress, as observed in our study, remains to be investigated further. Exercise-induced oxidative, cytokine, and heat stress may be involved in the stress response reported here. The results are potentially valid to monitor beneficial or unfavorable effects of intensive endurance exercise or extensive training and to estimate the relevance of individual stress gene responses in circulating leukocytes.

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REFERENCES