SPORTS MEDICINE

Short-Term Highly Intense Physiological Stress Causes an Increase in the Expression of Heat Shock Protein in Human Leukocytes

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Extracellular heat shock protein with molecular weight of 70 kDa is a signal molecule of the immune system. It is secreted by the peripheral blood, liver and muscle cells in response to physiological, thermal, and mental stresses. The main goal of our study was to compare the levels of expression of heat shock protein (70 kDa) matrix ribonucleic acid in leukocytes and serum concentrations of the protein before and after physiological stress. In order to solve this problem, we developed enzyme immunoassay of serum heat shock (70 kDa) protein concentration and a method for evaluating the expression of matrix ribonucleic acid of this protein in leukocytes by the real time PCR. The concentration of 70 kDa heat shock protein in the serum increased 1.7 times as a result of even a short-term highly intense physiological stress, while the expression of its matrix ribonucleic acid in leukocytes increased 1.5 times. The individual features determine the response to physiological stress. Probable sources of 70 kDa heat shock protein are discussed.

Key Words: *70 kDa heat shock protein; enzyme immunoassay; expression of matrix ribonucleic acid in leukocytes; physiological stress*

Heat shock proteins (HSP) are highly conservative proteins found in all prokaryotes and eukaryotes and located mainly in the cell. They are involved in the processes controlling the activities of regulatory proteins, folding of synthetic polypeptides, prevention of protein refolding and aggregation [3,11], and hence, in the defense from stress, cytotoxic, and pathogenic factors (heat or cold shock, oxidative stress, highly acid environment, food deficit, infections, *etc.*) [8,9,11].

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These proteins were detected on cell surface [9] and in the blood stream [3,9,14]. Serum HSP with molecular weight of 70 kDa (HSP-70) serves as the signal molecule of the immune system and activates macrophages, neutrophils, mast and dendritic cells, and stimulate the production of cytokines [3], while in complex with Tag7 the protein acts as a cytotoxic agent [12].

The level of HSP-70 expression is an indicator of adaptive processes in the cell, caused by various stress types, and a marker of a previous stress exposure [5]. The expression of HSP-70 has been detected in many body tissues, including muscles, leukocytes, and liver, and increases in response to exercise of different intensity [9,13]. Human peripheral

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blood mononuclears [4,7,8], hepatic and splenic cells release HSP into the blood as a result of exocytosis. The mechanism of secretion has been described in detail [9,10].

We developed test systems for measuring serum concentrations of HSP-70 (stress marker) and level of expression of its matrix ribonucleic acid (mRNA) in leukocytes by the real time PCR method. These test systems are intended for evaluation of short-term highly intense physiological stress in athletes during competitions.

MATERIALS AND METHODS

Four trained skiers (M1-M4; age 19.3±0.7 years, body length 175±7 cm, body weight 68.0±4.6 kg; maximum oxygen consumption (MOC) 59.8±1.5 ml/kg/min). After answering the questionnaire on their health status, the participants gave voluntary written consent to participation in experiment. The protocol of experiment was approved by the Ethical Committee of Institute of Physical Culture. The volunteers were subjected to treadmill test as described previously [1] with slight modifications (initial treadmill velocity 3.0 m/sec, slope angle 1°, velocity increment 0.5 m/sec). Venous blood was collected at rest before and directly after the exercise. Serum was separated and frozen at -20°C. Venous blood for isolation of mRNA was placed into PAXgene Blood RNA Tubes (PreAnalytiX GmbH). The saliva was collected using a Salivette device (Sarstedt) consisting of a neutral cotton tampon and a tube. The saliva was separated from the tampon by centrifugation.

Recombinant HSP-70 was expressed in *E. coli* cells transformed by pQE30 plasmid with integrated genetic sequence, coding for HSP-70 with a hexahistidine Tag at the N terminal (kind gift from Prof. L. P. Sashchenko, Laboratory of Molecular Immunogenetics of Cancer, Institute of Gene Biology). The resultant protein was purified by metal chelate chromatography.

Polyclonal antibodies were obtained by immunization of rabbits by purified recombinant HSP-70. Polyclonal antibodies were affinely isolated on an adsorbent containing 0.25 mg/ml HSP-70. The activity and purity of affinely purified antibodies were tested in EIA and PAAG electrophoresis.

An EIA test system was developed for evaluation of the serum HSP-70. Affinely purified polyclonal rabbit antibodies to HSP-70 were adsorbed on an EIA plate. After washing and blocking of nonspecific binding sites, test sera and reference samples were applied to the plate. The plates were then incubated (1 h) with biotinilated polyclonal rabbit antibodies to HSP-70 and horseradish streptavidin peroxidase. Detection was carried out using orthophenylene diamine/H_2O_2 solution.

Serum growth hormone (GH) was measured as described previously [1] using affinely purified polyclonal rabbit antibodies to recombinant human GH. Hydrocortisone was measured by EIA using commercial EIA-1887 kit (DRG Instruments). Activities of creatine phosphokinase (CPK), AsAT, and AlAT were measured by spectrophotometry with ALT R2 and AST R2 commercial kits (Randox).

Total mRNA was isolated according to the instruction using PAXgene Blood RNA Kit (PreAnalitiX GmbH). The quality of the resultant mRNA — RIN (RNA Integrity Number) was evaluated on an Agilent 2100 Bioanalyzer. The RIN was above 8 for all RNA specimens.

Reverse transcription was carried out using a reverse transcription kit (DNA-Technology Firm)

TABLE 1. Primers for HSPA1A, B2M, and HPRT

Primer	Nucleotide sequence
HSPA1A-s	GTGGGGAGGACTTTGACAACA
HSPA1A-as	CCCTGGTGATGGACGTGTAG
HSPA1A-TM	(BHQ1)-5'-ACAAGAAGGACA(FDT)CAGCCAGAACAAGC-3'P
B2M-s	AGCGTACTCCAAAGATTCAGGTT
B2M-as	ATGATGCTGCTTACATGTCTCGAT
B2M-TM	(BHQ1)-5'-TCCATCCGACATTGAAGTTGACTTACTG-3'P
HPRT-s	CTCAACTTTAACTGGAAAGAATGTC
HPRT-as	TCCTTTTCACCAGCAAGCT
HPRT-TM	(BHQ1)-5'-TTGCTTTCCTTGGTCAGGCAGTATAATC-3'P

Note. HSPA1A (GeneID 3303) is an HSP with molecular weight of 70 kDa 1A, B2M (GeneID 567) is a β_2-microglobulin, HPRT (GeneID 3251) is hypoxanthine phosphoribosyltransferase 1.

TABLE 2. Values after Physiological Stress

Volunteer	Time, min	Time after TAM, min	MOC/kg	Respiration coefficient	Heart rate, str/min	Lactate, mmol/liter	TAM
M1	15	6	59.6	1.26	210	12.59	50.9
M2	15.5	6	61.3	1.24	201	11.22	51.8
M3	14	3.5	58.2	1.17	200	9.9	53.1
M4	14	4	59.9	1.21	216	9.35	54.9

according to the instruction. The resultant cDNA was diluted 10-fold and used in the real time PCR.

Specific detection of amplification products was carried out using complementary oligonucleotides labeled by fluorophores and fluorescence quenchers (Table 1). Amplification was carried out on a DT-96 device (DNA Technology Firm) in the real time mode.

RESULTS

The volunteers were tested on a treadmill at step-by-step increasing power. The duration of exercise was 15.0±0.5 min. Oxygen consumption, carbon dioxide content in exhaled air, respiration rate, pulse rate, and lactate concentration in capillary blood were measured during exercise. The individual threshold anaerobic metabolism (TAM) of each athlete was calculated from oxygen consumption and lactate concentration values and the duration of work after TAM attaining was estimated. Maximum oxygen consumption, peak heart rate, lactate concentration, respiration coefficient (VO_2/VCO_2) are presented (Table 2). The TAM was calculated from lactate concentration (4 mmol/liter) and the duration of work after anaerobic threshold was calculated.

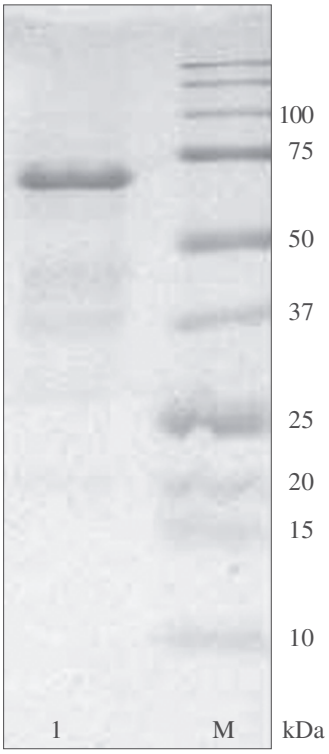

Fig. 1. Recombinant HSP-70. M: molecular weight marker.

The mean duration of work after TAM was 4.9±1.3 min, peak lactate concentration 10.8±1.5 mmol/liter, heart rate 206±7 bpm, and respiration coefficient 1.22±0.40.

Hence, the athletes experienced a short-term, but strong physiological stress, which was seen from lactate concentration, pulse rate, duration of work after TAM, and respiration coefficient.

Two athletes (M3 and M4) attained TAM later and the duration of work after it was shorter (3.3 and 4.0 min, respectively). Their peak lactate values were consequently lower (9.90 and 9.35 mmol/liter, respectively) than in M1 and M2 athletes (12.59 and 11.22 mmol/liter, respectively).

In order to evaluate serum concentration of HSP-70 (stress marker), we obtained highly purified recombinant HSP-70. According to PAAG electrophoresis, the protein purity was at least 90% (Fig. 1). Recombinant HSP-70 was used for immunization of rabbits and obtaining polyclonal antibodies and their affine purification.

Polyclonal affinely purified antibodies to HSP-70 were used for the development of a method for measurement of serum concentration of HSP-70 by EIA. The range of detectable concentrations is 0.5-100 ng/ml. Before measurements the serum samples were diluted 2- or 4-fold in PBS—Twin—BSA in order to reduce the matrix effects. All sera were analyzed repeatedly. The data of the reference curve were approximated using 4 parametrical functions. Calibration curve for the HSP-70 concentrations of 0.5-35 ng/ml is presented (Fig. 2).

Serum concentrations of HSP-70, GH, hydrocortisone, CPK, AsAT, and AlAT were measured in all athletes before and after exercise. Hydrocortisone was also measured in the saliva (Table 3).

The enzymes (CPK, AsAT, AlAT) are detected in the serum if muscle fibers are damaged, but we

detected no signs of cardiomyocyte and skeletal muscle injury in the athletes before or after testing. The mean levels of the enzymes before and after physiological stress were: 151.2±50.0 and 146.0± 16.0 U/liter for CPK, 12.0±3.8 and 14.5±2.4 U/liter for AlAT, and 21.5±9.7 and 26.5±14.9 U/liter for AsAT, respectively. The increase in the enzymes activities indicates probable negligible injuries in the cells. However the enzyme levels were within the reference range of values: up to 190 U/liter for CPK and up to 41 U/liter for AlAT and AsAT. No appreciable differences were detected in the athletes by these parameters.

Hydrocortisone plays the key role in the defense reactions to stress. It possesses a catabolic effect, and its serum and salivary levels can serve as stress markers. Blood and salivary hydrocortisone concentrations in the four athletes increased significantly after exercise (367±87 and 433±33 nmol/liter in the blood and 15.9±3.0 and 19.7±4.3 nmol/liter in the saliva), but remained within the population range of values (150-700 and 13.8-48.9 nmol/liter).

The concentration of GH increased in response to stress in all athletes. The final GH concentration in athletes with a lower level of lactate (M3 and M4) was significantly higher.

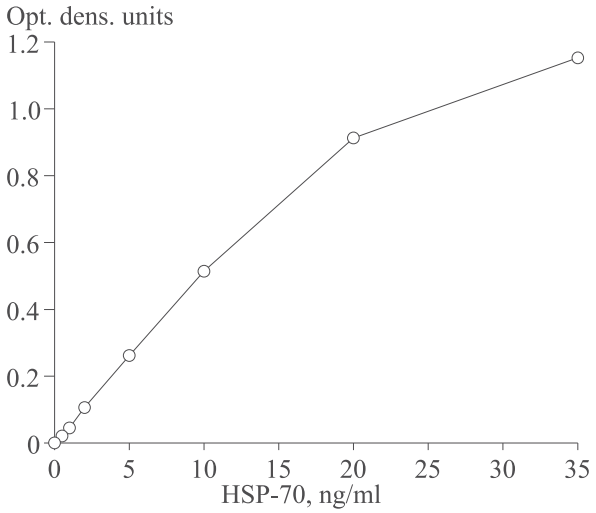

Fig. 2. Calibration curve for measurements of serum HSP-70 concentrations.

The relationship between HSP-70 concentration and other biochemical values could not be traced. The concentration of HSP-70 virtually did not change in M1 and increased 2.5 times in M2; in M3 and M4 it increased 1.5 times. The final concentration of HSP-70 was about 10 ng/ml in all athletes. This indicates that this protein can serve as a marker of physiological stress.

TABLE 3. Biochemical Values before and after Physiological Stress

Parameter; time of measurement		Volunteer			
		M1	M2	M3	M4
Serum HSP-70, ng/ml	before	10.8	4.5	6.1	3.8
	after	11.8	11.9	9.8	6.8
	increment	1.09	2.64	1.61	1.78
Expression of HSP-70 mRNA in leukocytes, arb. units	before	1.189	1.366	1.366	1.515
	after	1.866	1.569	2.000	2.878
	increment	1.57	1.15	1.46	1.90
Serum GH, ng/ml	before	0.1	0.1	0.2	?.1
	after	3.9	3.9	22.7	14.6
CPK, U/liter	before	87	138	176	204
	after	164	153	141	128
Serum hydrocortisone, nmol/liter	before	462	266	408	330
	after	406	479	435	411
Salivary hydrocortisone, nmol/liter	before	17.1	11.7	16.4	18.3
	after	14.8	25.3	18.5	20.0
AlAT, U/liter	before	7	15	11	15
	after	13	17	12	16
AsAT, U/liter	before	13	35	16	22
	after	18	48	15	25

The level of mRNA expression was evaluated using HSPA1A (HSP-70 1A), B2M (β_2-microglobulin), and HPRT (hypoxanthine phosphoribosyltransferase-I) kits for measuring the levels of these genes' mRNA by the real time semiquantitative PCR. The initial RNA was not fragmented (RIN>8). Specimens of cDNA before and after exercise were analyzed twice (three repeats) after reverse transcription. The real time PCR experiments were carried out using specific primers to the studied gene and two housekeeping genes (B2M, HPRT). The expression of B2M and HPRT is constant in various cell types and does not depend on external factors, but the number of their copies in the cell is different: B2M is a well presented gene, while the presentation of HPRT is low. Normalization by these two genes helps quantitatively evaluate the expression of HSP-70 in leukocytes.

A significant ($p<0.05$) increase of HSP-70 mRNA expression in leukocytes indicates triggering of the adaptive processes in response to physiological stress (Table 3). However it does not explain the observed increase in the protein concentration in the serum. Presumably, during the first minutes the protein is released into the serum not only from leukocytes, but from other producer cells as well.

The release of HSP-70 into the blood is not caused by cell necrosis, as serum concentrations of CPK, AlAT, and AsAT increase negligibly. Hence, the increase in HSP-70 concentration is a result of adaptation to stress. Serum concentration of HSP-70 did not increase in athlete M1 and its initial level was high, while the expression of mRNA increased 1.6 times. In the rest athletes the increase in HSP-70 concentration was paralleled by an increase of its expression in leukocytes.

A short-term highly intense physiological stress was detected in the group of well-trained athletes. The duration of exercise, leading to changes in the biochemical values and level of mRNA expression, was 15 min, which is much shorter than test exercises described previously (30-260 min) [6,14]. The duration of work after TAM was 3.3-6.0 min. Serum biochemical parameters (GH, HSP-70, hydrocortisone, CPK, AlAT, AsAT concentrations), which help evaluate the level of stress, were studied before and after a short-term exercise. Serum concentration of HSP-70 increased for a reason other than cell necrosis.

Hence, we developed an enzyme immunoassay of human serum HSP-70 (stress marker) and a method for evaluation of HSP-70 mRNA expression in leukocytes by the real time PCR. Short-term highly intense exercises led to physiological stress, which induced significant changes in HSP-70 mRNA expression and in the serum concentration of this protein. The response to stress was individual in the athletes.

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