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ORIGINAL ARTICLE

Effects of ultra-marathon on circulating DNA and mRNA expression of pro- and anti-apoptotic genes in mononuclear cells

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Abstract We investigated the effects of an ultra-marathon on cell-free plasma DNA as well as on mRNA expression of pro-apoptotic (Bax, Bad), anti-apoptotic (Bcl-2) and cell-protective (Hsp70, Hsp27 and Hsp32) genes in mononuclear blood cells (MNCs). Blood samples were drawn from 14 athletes before and immediately after 6-h run. In addition, blood samples were also collected and analyzed 2 and 24 h after the end of the run. Levels of plasma DNA were significantly increased immediately after the marathon ($P < 0.001$) and were still higher 2 h later ($P < 0.005$), but significantly lower than those immediately after the race ($P < 0.05$). Cell-free plasma DNA returned to pre-race levels 24 h after the run. mRNA expressions of Hsp70, Hsp32 and Bax significantly increased in MNCs after the race, whereas Hsp27 and Bad mRNA expression levels showed no significant changes. Bcl-2 expressions decreased immediately after the race ($P < 0.001$), but increased in the 24 h later ($P < 0.05$). We conclude that apoptotic ladders of cell-free DNA following exhaustive exercise originate from apoptotic cells and that not only skeletal muscle cells but also leukocytes contribute to this phenomenon.

Keywords Cell-free plasma DNA · Apoptotic genes · Anti-apoptotic genes · Ultra-marathon

Introduction

Exhaustive exercise may result in muscle damage and DNA degradation. In this process, hypoxia and increased radical production during exercise have been shown to be causative factors for cellular damage (Goodman et al. 1997). Moreover, excessive stress can induce DNA damage like oxidized nucleosides or strand breaks resulting in apoptosis or necrosis (Chevion et al. 2003).

For apoptosis, three phases can be described: induction phase, effector phase and degradation phase. The induction phase depends on cell death-inducing signals, like activation of ROS or Bcl-2 family proteins (Bax, Bad) stimulating pro-apoptotic signal transduction cascades. In the effector phase, cells become committed to die by various actions of mitochondria. The degradation phase involves both cytoplasmatic and nuclear events. Cytochrome c released from mitochondria forms a complex with Apaf-1 to catalyze the activation of caspase 9 in a dATP-dependent manner. Activated caspase 9 cleaves and activates caspase 3 leading to apoptosis (Nishimura et al. 2001). Finally, the cell is fragmented into apoptotic bodies (Pollack and Leeuwenburgh 2001).

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Cell death or survival depends on the ratio of pro- and anti-apoptotic gene expression. High levels of anti-apoptotic Bcl-2 in relation to pro-apoptotic Bax promote survival, whereas the reverse ratio promotes cell death (Oltvai et al. 1993). Thereby, the anti-apoptotic effect of Bcl-2 is achieved by preventing an opening of permeability transition pores (Susin et al. 1999).

Apart from cellular damage, exhaustive exercise can furthermore induce an acute response of the immune system (Pedersen and Hoffman-Goetz 2000). Stimulated by chemotactic factors like tumor necrosis factor-α, infiltrating neutrophils and macrophages were found in skeletal muscles after exercise (Stupka et al. 2000), and exercise-induced activation of immunocompetent cells was found to be followed by oxidative and cytokine stress resulting in production of heat shock proteins (HSP) (Niess et al. 1999). Increased cellular HSP can protect proteins that are essential in controlling the apoptotic cascade and may hereby provide cell protection (Mosser et al. 1997) in skeletal muscle (Febbraio and Koukoulas 2000), heart muscle (Plumier and Currie 1996) and in leukocytes (Fehrenbach et al. 2000).

In a previous study, we demonstrated elevated levels of cell-free DNA after a half-marathon (Atamaniuk et al. 2004). Although the origin and release mechanisms of circulating DNA still remain unclear (Lui and Dennis 2002), circulating DNA might serve as an unspecific but sensitive marker for cellular apoptosis or necrosis in the quantification of cellular damage (Atamaniuk et al. 2006). Hypothesizing that there is a correlation between cell-free plasma DNA and leukocyte apoptosis after strenuous exercise, we investigated cell-free DNA, mRNA expression levels of apoptotic and anti-apoptotic genes as well as leukocyte heat shock protein production in athletes undergoing ultra-marathon.

Methods

Subjects

Fourteen experienced ultra-marathon runners (nine males, five females) from local running clubs all over Austria volunteered to participate in this study. The endurance-trained subjects, nine males (mean ± SD age 43.3 ± 6.0 years; mass 76.8 ± 6.7 kg; height 180.8 ± 5.7 cm) and five females (mean ± SD age 51.6 ± 7.6 years; mass 60.8 ± 7.1 kg; 159.8 ± 6.3 cm), completed medical screening questionnaires and a training diary over the 4-week training period prior to the race (number and duration of workouts, distance covered, past running experience). All subjects had participated in regular training for ultra-endurance running events for more than 3 years (10.5 ± 6.5 years, range 3–25) and in at least four sessions per week of running exercise. These subjects ran 30–130 km weekly (71.7 ± 30.1 km) during the last 4 weeks in preparation for this ultra-endurance event. All subjects had participated in at least three competitive ultra-marathon events at the national level. The rate of exertion was measured in all participants after the race using the 20-point Borg scale. All participants gave written informed consent prior to their inclusion in the study and the study was performed in accordance with the ethical standards laid down Declaration of Helsinki.

Blood collection and cell separation

Venous blood was drawn before and after a 6-h race, as well as 2 and 24 h later and were collected in Vacutainers (Becton Dickinson, Austria) containing EDTA and immediately placed on ice. Phosphate-buffered saline, without calcium and magnesium, kept at 4°C, was used to dilute samples before layering blood onto Histopaque-1077 (Sigma-Aldrich, Vienna, Austria). The cell separation procedure was carried out according to instructions provided by the manufacturer of Histopaque-1077. With the exception of Histopaque-1077 (kept at 18°C), and the 30-min centrifugation period at 18°C, care was taken to keep samples and solutions at 4°C throughout the isolation procedure. After the last washing step, cells were resuspended in TRIOZOL solution (Sigma-Aldrich, Austria) and kept at −80°C until further use. Aliquots of the cell suspensions were used to analyze cell viability and purity. Such analysis revealed a viability of >95% (Trypan blue exclusion assay) and less than 3% PMN content in the mononuclear cell preparations.

All samples were processed within 1 h of collection. In addition, agitation of blood and cell-containing solutions during transport and isolation procedure was avoided to prevent activation and/or cell damage.

RNA isolation and RT-PCR

RNA was isolated from MNCs and used to determine the fold-change of steady-state mRNA levels relative to baseline using real-time reverse transcription-polymerase chain reaction. RNA was isolated using a standard protocol (Stuhlmeier and Pollaschek 2004).

Reverse transcription was performed using Strata Script First Strand Synthesis System (Stratagen, Netherlands). Five hundred nanograms of RNA was transcribed using hexamer primers. SYBR Green RT-PCR amplification was performed on a Mx3000P real-time PCR system (Stratagen, Netherlands), using Brillant SYBR Green QPCR Master Mix (Stratagen, Netherlands). PCR was started with an initial denaturation for 10 min at 95°C, followed by denaturation

for 10 s at 57°C and extension for 15 s at 72°C. mRNA for hypoxanthine-guanine phosphoribosyl transferase 1 (HPRT) was used as control for amplification. All primers were purchased from MWG Biotech AG (Ebersberg, Germany) and were dissolved at a concentration of 100 pmol/μL in Tris–EDTA.

Calibrators and samples were assayed in a 25 μL reaction mixture: 2× Brillant SYBR Green QPCR Master Mix, 30 nM Reference dye ROX (Stratagen, Netherlands), 1.5 μL forward primer, 1.5 μL reverse primer and 2.5 μL cDNA and water.

Primer sequences were as follows: Bad sense primer 5′-TGA CAC TGG CAA AAC AAT GCA-3′, Bad anti-sense primer 5′-GGT CCT TTT CAC CAG CAA GCT-3′; Hsp27 sense primer 5′-ACG AGC ATG GCT ACA TCT CC-3′, Hsp27 anti-sense primer 5′-GAC TGG GAT GGT GAT CTC GT-3′; Hsp70 sense primer 5′- CTG TTT GAG GGC ATC GAC TT-3′, Hsp70 anti-sense primer 5′-AGG ACC AGG TCG TGA ATC TG-3′; Bax sense primer 5′-TTC CGA GTG GCA GCT GAC AT-3′, Bax anti-sense primer 5′-TTC CAG ATG GTG AGT GAG GC-3′; Bcl-2 sense primer 5′-GGT GAA CTG GGG GAG GAT TGT-3′, Bcl-2 anti-sense primer 5′-CTT CAG AGA CAG CCA GGA GAA-3′; Hsp32 sense primer 5′-CAG GCA GAG AAT GCT GAG TTC-3′, Hsp32 anti-sense primer 5′-GCT TCA CAT AGC GCT GCA-3′.

Additionally, no-template-controls were included in all experiments. Delta values were calculated as the difference between CT (threshold cycle) values of HPRT1 and selected genes ($\Delta CT = CT_{Hprt1} - CT_{selected\ genes}$).

Hematological and chemical analyses

White blood cells and differential analysis of lymphocytes, monocytes and neutrophils were determined using an automated haematology analyzer (XE 2100; Sysmex Corporation, Japan). For myoglobin measurements, a commercially available enzyme immunoassay was used (Dimension RXL; Siemens Health Care Diagnostic Inc., Deerfield, USA) in which the analytical imprecision (CV) was 2.9%. Creatine kinase was determined by standardized methods (Creatine Kinase liquid, Modular P800; Roche, Germany) with a CV of 2.7%.

Isolation of cell-free DNA

DNA was isolated from 800 μL of plasma and quantified by use of a DNA reagent set (Roche, Germany), according to the manufactures′ recommendations. Isolated DNA was diluted with elution buffer to a final volume of 50 μL. An aliquot of 5 μL was stained with Vistra Green for DNA quantification. The amount of cell-free plasma DNA was measured on a Light Cycler real-time PCR system (Roche, Germany). Human placental DNA calibrators (Sigma, USA) were dissolved in Tris–EDTA buffer (1 mmol/L Tris–HCL, 1 mmol/L EDTA, pH 8.0; Sigma, USA) to create a calibration curve ranging from 100 to 5,000 pg/μL. Calibrator or sample (5 μL) was pipetted into pre-cooled capillary tubes. Vistra Green (stock solution in dimethyl sulphoxide; Amersham Biosciences, United Kingdom) was diluted 1:1,000 in Tris–EDTA buffer, and 5 μL was added to each sample or calibrator. Subsequently, samples were centrifuged at 800×*g* for 30 s. The emitted fluorescent signals from samples and calibrators were measured in the LightCycler at 530 nm. The cell-free plasma DNA concentrations were reported in picograms per microliter (pg/μL). Repeated measurements of 300 pg/μL DNA calibrators yielded an analytical imprecision (CV) of 3.31%.

Statistics

Data were analyzed with STATISTICA for Windows, version 6.0. Descriptive data are reported as mean, median and standard deviation (SD). Statistical significance was determined by Wilcoxon-matched pairs test for nonparametric variables. P values <0.05 were considered statistically significant.

Results

In MNCs, mRNA expression of Hsp70 was significantly increased immediately after the race ($P < 0.005$), remained significantly increased 2 h post-exercise ($P < 0.005$), but returned to baseline levels 24 h after the race. In contrast, Hsp32 expression was significantly increased immediately after the ultra-marathon ($P < 0.05$), but rose further 2 h after the running competition ($P < 0.005$).

While mRNA expression levels of Hsp27 and Bad did not show significant changes, MNC's Bcl2 expression decreased immediately after the marathon ($P < 0.001$) and returned to baseline levels within 2 h. However, 24 h after the race, mRNA levels of Bcl2 were significantly higher than those measured before the marathon ($P < 0.05$).

In MNCs, mRNA expression of Bax was increased in 8 of 14 subjects immediately after the race. It stayed elevated 2 h ($P < 0.005$) as well as 24 h after the race ($P < 0.05$) (Table 1).

Cell-free plasma DNA increased to highest levels immediately after the race (162.5 pg/μL ± 201.4; $P < 0.001$). Two hours later, plasma DNA levels were still increased when compared with pre-race levels (30.9 pg/μL ± 171.4; $P < 0.005$). Cell-free plasma DNA levels returned to baseline after 24 h of rest (9.0 pg/μL ± 5.6) (Fig. 1). Agarose gels showed ladders typical for apoptosis immediately after and 2 h after the run (Fig. 2).

Table 1 mRNA expression in MNCs relative to pre-race

	Pre-race	Post-race (mean ± SD)	*P*	2 h post-race (mean ± SD)	*P*	24 h post-race (mean ± SD)	*P*
Pro-apoptotic genes							
Bax	1	1.2 ± 0.5	NS	2.0 ± 0.8	<0.005	1.9 ± 2.3	<0.05
Bad	1	1.2 ± 0.3	NS	1 ± 0.3	NS	1 ± 0.3	NS
Anti-apoptotic gene							
Bcl-2	1	0.5 ± 0.2	<0.001	0.6 ± 0.6	NS	1.5 ± 0.2	<0.05
Cell-protective genes							
Hsp70	1	2.6 ± 1.7	<0.005	2.9 ± 2.1	<0.005	1 ± 0.4	NS
Hsp32	1	1.3 ± 1	<0.05	1.8 ± 0.7	<0.005	1 ± 0.7	NS
Hsp27	1	1.1 ± 0.4	NS	1 ± 0.6	NS	1.2 ± 0.5	NS

mRNA expression in MNCs of genes, given in fold expressions relative to baseline levels (mean ± SD). Pre-race mRNA expressions of all genes were defined as 1. *P* values at all time points (*post-race* immediately after the race, *2 h post-race* 2 h after the race, *24 h post-race* 24 h after the race) were compared with pre-race data. $N = 14$

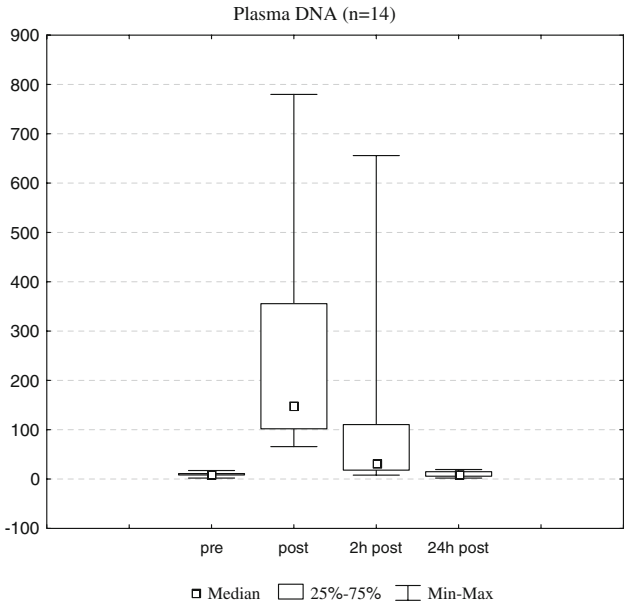

Fig. 1 Cell-free plasma DNA is significantly elevated at the end as well as 2 h after a run that lasted for 6 h. Shown is a comparison of cell-free plasma DNA concentrations in 14 marathon runners measured at rest (*pre*), immediately after a 6-h race (*post*), 2 h after the race (*2 h post*) and 24 h after the race *(24 h post*). *Boxes* indicate the 25th–75th percentiles; *error bars* indicate the maximum and minimum concentrations; *open squares* (within the *boxes* that indicate 25th–75th percentiles) indicate the median

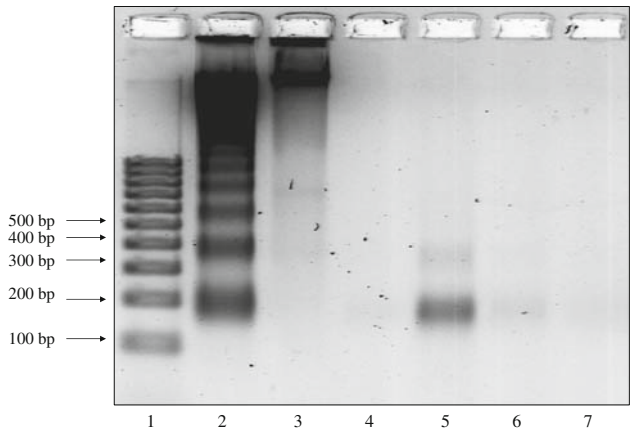

Fig. 2 Cell-free apoptotic DNA fragments are detectable in plasma samples at the end as well as 2 h after a 6-h marathon shown here is an agarose gel separation of plasma DNA samples collected before and after an ultra-marathon. *Lanes 1–3* represent controls [size marker (100–1,000 bp) (*lane 1*), apoptotic DNA (*lane 2*) and apoptosis negative control DNA (*lane 3*)]. Loaded on *lane 4* is a sample collected from a runner before the race. Samples that were collected immediately after the race (*lane 5*) show apoptotic DNA pattern. Samples collected 2 h after the race still show increased concentrations of apoptotic DNA (*lane 6*). However, as compared to the pre-run sample (*lane 4*), increased apoptotic plasma DNA can no longer be seen in samples that were collected 24 h after the race (*lane 7*)

Established parameters for muscle damage were significantly increased at all times after the race ($P < 0.005$) (plasma myoglobin before the race 51.0 ± 12.8, immediately after the race 1123.6 mg/dL ± 640.8, 2 h after the race 1,050.1 mg/dL ± 565.3, after 24 h 134.6 mg/dL ± 71.2; creatine kinase before the race 127 U/L ± 43.3, immediately after the race 647.8 U/L ± 310.1, 2 h after the race 808.4 U/L ± 451.3, after 24 h 1,057.0 U/L ± 617.1) (Table 2).

Furthermore, white blood cell counts were significantly increased at all time points after the race (before the race 5.6 G/L ± 1.5, immediately after the race 13.9 G/L ± 2.6, 2 h after the race 13.2 G/L ± 2.4, 24 h after the race 6.9 G/L ± 3). In contrast, the total circulating lymphocyte concentration was significantly decreased immediately after the race (before the race 1.71 G/L ± 0.5, immediately after the race 1.29 G/L ± 0.7, 2 h after the race 1.15 G/L ± 0.6), but significantly increased 24 h after the ultra-marathon when compared with pre-race concentrations (2.06 G/L ± 0.5, $P < 0.01$) (Table 2).

All runners rated their perceived exertion at the end of this 6-h race as hard to very hard on the 20-point Borg scale

Table 2 Established parameters for muscle damage and white blood cell counts

	Pre-race (mean ± SD)	Post-race (mean ± SD)	2 h post-race (mean ± SD)	24 h post-race (mean ± SD)
Leukocytes (G/L)	5.56 ± 1.5	13.95 ± 2.6**	13.16 ± 2.4**	6.85 ± 3.1*
Lymphocytes (G/L)	1.71 ± 0.5	1.29 ± 0.7*	1.15 ± 0.6**	2.06 ± 0.5**
Neutrophils (G/L)	3.12 ± 1.0	11.58 ± 2.0**	11.10 ± 1.9**	3.99 ± 2.7
Myoglobin (mg/dL)	51.0 ± 12.8	1123.6 ± 640**	1050.1 ± 565**	134.6 ± 71.2**
Creatine kinase (U/L)	127.7 ± 43.4	647.8 ± 310**	808.4 ± 451**	1057.0 ± 617**

$N = 14$

Circulating total leukocytes (G/L), neutrophils (G/L) and lymphocytes (G/L) in peripheral blood before (pre-race), immediately after (post-race), 2 h after and 24 h after the ultra marathon.

Plasma myoglobin (mg/dL) and plasma creatine kinase (U/L) before (pre-race), immediately after (post-race), 2 h after and 24 h after the ultra marathon

*$P < 0.05$ versus pre-race; **$P < 0.01$ versus pre-race

(18.4 ± 1.0, range 17–20), which is a clear indication that the exercise intensity was close to the possible maximum in all athletes.

Discussion

Hypoxia and increased radical production during exhaustive exercise are known causative factors for DNA degradation and apoptosis (Goodman et al. 1997) resulting in increased post-exercise levels of cell-free plasma DNA. While high concentrations of plasma myoglobin and creatinine kinase let us believe that muscle cell damage is responsible for a considerable fraction of apoptotic cell-free plasma DNA, it is still under discussion whether leukocytes might also contribute to this phenomenon. Exercise-induced activation of immunocompetent cells was found to be followed by oxidative and cytokine stress and by the production cell-protective heat-shock protein production (Niess et al. 1999). This has been shown not only in skeletal muscles (Febbraio and Koukoulas 2000) but also in leukocytes (Fehrenbach et al. 2000). MNC may contribute to the post-exercise plasma levels of cell-free DNA. We therefore investigated the MNC mRNA expression of pro- and anti-apoptotic genes as well as cell-protective genes in athletes undergoing ultra-marathon to elucidate whether apoptotic cell-free plasma DNA may also be derived from exercise-induced MNC apoptosis.

Similar to published data, we found that exhaustive exercise influences the plasma lymphocyte count. While Mooren et al. described an increased lymphocyte count during exercise, which was followed by a decrease to levels below pre-exercise values in the recovery period (Mooren et al. 2002), we could only observe the post-exercise lymphocyte decrease. There are different explanations for the lymphopenia during the recovery period. While Simpson et al. reflected an extravasation of specific lymphocyte populations (Simpson et al. 2006), Mars et al. concluded that apoptosis could be a cause for this phenomenon (Mars et al. 1998). On the other hand, in a study reported by Steensberg et al., lymphocyte apoptosis after 2.5 h of treadmill run did not contribute to post-exercise lymphopenia (Steensberg et al. 2002). Our observations of post-exercise lymphopenia, increased concentrations of cell-free DNA and ladder-patterns typical for apoptosis seem to point to cell apoptosis rather than extravasation of lymphocytes. However, data reported by Steensberg et al. may not be comparable to ours because of the athletes' different stress exposure resulting from a 2.5-h race versus a 6-h ultra-marathon.

In the current study, all athletes, independent of their exercise performance capacity, were exerted as shown by their rating to the Borg scale. A high to very high exertion corresponds to 51–55% of VO_2max (Dumke et al. 2006; Rauch et al. 1998).

Mooren et al. (2004) correlated the degree of apoptosis in lymphocytes with the athletes' training status. Since athletes participating in our study were experienced ultra-marathon runners, we attribute the occurrence of lymphocyte apoptosis once more to the enormous stress resulting from this race.

In addition, increased plasma catecholamine concentrations after endurance exercise were reported to cause lymphocyte apoptosis (Shephard 2003) and consequently post-exercise lymphopenia. However, Marra and Hoffman-Goetz showed in an animal model that catecholamines were not causative for post-exercise lymphopenia (Marra and Hoffman-Goetz 2004). For such reasons, the impact of catecholamines on exercise-induced lymphopenia still remains unclear.

With regards to cell-free plasma DNA during post-exercise recovery, we found elevated circulating DNA that showed apoptotic ladder-patterns in gel electrophoresis typical for apoptosis not only immediately after the race but also 2 h later. In a previous study (Atamaniuk et al. 2004),

increased cell-free DNA after a half-marathon could only be seen immediately after the race. Again, a possible explanation might lie in the different extent of stress resulting from a half-marathon versus an ultra-marathon. Such findings might also point to circulating DNA as being a sensitive marker for cellular apoptosis or necrosis and/or for the further quantification of cellular damage.

Regarding mRNA expression levels of the studied cell-protective genes, we found no change in Hsp27, but increased expressions of Hsp70 and Hsp32. Hsp32 is an ubiquitous heat-shock protein that is induced by several stress-related conditions (Maines 1997; Thompson et al. 2005; Willis et al. 1996). Hsp32 proteins play a role in controlling the apoptotic cascade; therefore, the post-exercise activation of expression of Hsp32 could be understood as an antagonism of pro-apoptotic mechanisms.

Increased expression of Hsp70 and Hsp32 returned to pre-race levels within 24 h of recovery, whereas mRNA expression of the pro-apoptotic Bax gene still stayed elevated 24 h after the ultra-marathon. Additionally, the mRNA expression of Bcl-2 was decreased until 2 h after the race, resulting in a lack of anti-apoptotic action of Bcl-2 in the early phase of recovery. Interestingly, Siu et al. reported increased Bcl-2 mRNA levels in muscle and ventricle of rats (Siu et al. 2004). However, elevated expression of Bax together with decreased expression of Bcl-2 in the early phase of recovery could be understood as a possible preponderance in MNC of the pro-apoptotic effect exerted by Bax resulting in MNC apoptosis and release of MNC-derived cell-free plasma DNA.

Shastry et al. described that, for a moderately trained subject, exercise within normal limits is not a sufficient stimulus for Hsp70 protein production in leukocytes (Shastry et al. 2002). It is therefore interesting that we found an increase of Hsp70 mRNA expression in MNCs in our study in which only well-trained volunteers participated. Hsp70 expression in well-trained individuals could be interpreted as an additional mechanism at work impending cell damage.

Enhanced cell apoptosis after physical exercise has also been reported for other tissues such as renal tubular cells (Podhorska-Okolow et al. 2004). In addition, apoptotic and necrotic polymorphnuclear neutrophils (PMNs) might also contribute to cell-free plasma DNA, since PMNs in the circulation are significantly increased in subjects exposed to physical stress. Therefore, high post-exercise levels of cell-free plasma DNA is likely due to apoptosis or necrosis of a number of cell types and not solely due to muscle cell damage.

Taken together, enhanced apotosis in skeletal muscles after exhaustive exercise (Arslan et al. 2002) accompanied by high concentrations of plasma myoglobin and creatinine kinase points at muscle cell damage as a major source of apoptotic cell-free plasma DNA. However, we conclude that after strenuous physical exercise, cell-free plasma DNA may additionally be derived from leukocytes. Such a conclusion is based on the observation that at least in MNCs, the balance between pro- and anti-apoptotic genes is shifted to a pro-apoptotic state.

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