Short-Term Treadmill Running as a Model for Studying Cell-Free DNA Kinetics In Vivo

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BACKGROUND: Increased plasma concentrations of cell-free DNA (cf-DNA) are considered a hallmark of various clinical conditions. Despite intensive research in this field, limited data are available concerning the time course of release and clearance of cf-DNA in vivo.

METHODS: We extracted cf-DNA from plasma samples taken before and immediately after a 10-km cross-country run, and from samples taken before, immediately after, and 30 min after exhaustive short-term treadmill exercise. The contribution of nuclear (nDNA) and mitochondrial DNA (mtDNA) was measured by quantitative real-time PCR. The incremental treadmill exercise setup was exploited to delineate the precise sequencing and timing of cf-nDNA, lactate, and high-mobility group box 1 protein (HMGB1) release during the exercise and recovery phases.

RESULTS: Postexercise plasma cf-nDNA concentrations in cross-country and treadmill runners were significantly increased, by 7.6-fold and 9.9-fold, respectively (P < 0.001). cf-nDNA concentrations were not correlated with age, sex, or body mass index. Plasma concentrations of cf-nDNA and HMGB1 in postexercise samples of treadmill runners were significantly correlated (r = 0.84; P = 0.004). cf-mtDNA concentrations were not affected by treadmill exercise. Time-course analyses demonstrated that cf-nDNA is released within minutes after the onset of exercise and is rapidly cleared from the circulation after the cessation of exercise. Nearly congruent kinetics for cf-nDNA, lactate, and HMGB1 were observed during the exercise phase.

CONCLUSIONS: A single bout of exhaustive short-term treadmill exercise constitutes a versatile model system suitable for addressing basic questions about cf-DNA biology.

Immune-response patterns observed after acute bouts of physical activity show similarities to those induced by acute infection and trauma (1). Intense exercise has recently been reported to provoke increases in the plasma and serum concentrations of circulating cell-free DNA (cf-DNA)2 (2, 3), a phenomenon considered a hallmark of various pathologic conditions, including cancer, autoimmune diseases, trauma, sepsis, stroke, and myocardial infarction (4). Despite intensive research, the sources, triggers, and mechanisms that cause increases in cf-DNA remain enigmatic. Descriptive in vivo studies that try to address these questions with regard to clinical conditions are limited by one major drawback: They do not allow monitoring of cf-DNA release at the onset of disease. Rather, they provide snapshots of the cf-DNA status after clinical symptoms have already manifested. This limitation is further aggravated by the considerable biological interindividual variation in cf-DNA concentrations, necessitating a large number of individuals for study cases and control groups to draw statistically significant conclusions. In this respect, physical exercise provides a unique opportunity to deliberately induce an increase in cf-DNA in the human in vivo setting, thereby allowing intraindividual monitoring of cf-DNA concentrations in serial blood samples taken at defined time points.

We demonstrate that a single bout of exhaustive short-term treadmill exercise constitutes a versatile model system suitable for addressing basic questions about cf-DNA biology.

Fifty-three recreational runners [34 men and 19 women; mean (SD) age, 34.8 (12.7) years; range, 17–60 years] of a public 10-km cross-country interval run volunteered to participate in this study. Nine well-trained male athletes performed incremental tests on a motorized treadmill (Saturn; H/P/COSMOS). The treadmill inclination was held constant at 1%. The running velocity was increased by 2 km/h every 3 min until exhaustion. A time-course kinetic analysis was performed with 3 participants. Each individual gave written informed consent before participating in the study. All experimental protocols were approved by the Institute’s Human Ethics Committee according to the principles set forth in the Declaration of Helsinki of the World Medical Association.

Venous blood samples (EDTA-anticoagulated blood) were collected before and immediately after exercise. In the treadmill experiment, an additional sam-

‐Nonstandard abbreviations: cf-DNA, cell-free DNA; V̇O2max, velocity corresponding to the individual anaerobic threshold; nDNA, nuclear DNA; mtDNA, mitochondrial DNA; GE, genome equivalent; HMGB1, high-mobility group box 1 protein; NET, neutrophil extracellular trap.
ple was taken 30 min after the run. For time-course kinetic analysis, blood samples were collected after each stage of the treadmill protocol and at indicated time points after exercise.

Lactate measurement and assessment of the running speed at the individual anaerobic threshold (VIAT) were performed as described previously (5).

Blood samples were centrifuged at 1600 g for 10 min and then for 5 min at 16 000 g to obtain plasma samples. For cf-DNA quantification, we extracted DNA from 400 μL plasma according to the Blood and Body Fluid Protocol of the QIAamp Blood Mini Kit (Qiagen) into a final elution volume of 100 μL.

Nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) equivalents were analyzed by a quantitative real-time PCR that targeted an 88-bp fragment of the chromosomal myostatin (MSTN) gene locus and an 85-bp fragment of the mitochondrial genome (Mt: 15195–15279). A detailed description is given in the Supplemental Methods of the Data Supplement that accompanies the online version of this Brief Communication at http://www.clinchem.org/content/vol57/issue4. cf-nDNA results are expressed as genome equivalents (GEs) per liter of plasma; cf-mtDNA concentrations are given as copies of the target sequence per liter of plasma.

High-mobility group box 1 protein (HMGB1) in plasma was measured with a commercially available ELISA (Shino-Test Corporation, distributed by IBL Hamburg) according to the manufacturer’s directions.

The normalized and normally distributed data showed equal variances across groups; therefore, we used pairwise Student t-tests for statistical analysis. We present 95% CIs and P values, which were adjusted for multiple comparisons with the Bonferroni correction.

Immediately after the volunteers completed the 10-km interval run, we observed a significant 7.6-fold increase (95% CI, 5.7- to 10-fold; P < 0.001) in plasma cf-nDNA concentration (median, 2.3 × 10^6 GE/L vs 17.4 × 10^6 GE/L) and noted a high interindividual variation in postexercise concentration (minimum, 9.9 × 10^5 GE/L; maximum, 212 × 10^6 GE/L). We found no correlation between cf-nDNA concentration and age, sex, or body mass index.

To assess cf-DNA concentrations in a standardized exercise setting, we had 9 well-trained male study participants [mean (SD) age, 29.3 (8.5) years; VIAT, 13.8 (2.4) km/h] perform an incremental treadmill test until exhaustion. Blood samples were collected before exercise, immediately after exercise, and 30 min after the cessation of exercise. cf-nDNA concentrations were significantly increased immediately after the treadmill exercise (mean relative increase, 9.9-fold; 95% CI, 6.7- to 14-fold; P < 0.001) and declined to 4.1-fold higher than the baseline concentration by the end of the 30-min recovery period (Table 1). Plasma HMGB1 concentrations were significantly increased immediately after the run (mean increase, 3.5 μg/L; 95% CI, 2.2–4.7 μg/L; P < 0.001) and returned to baseline by 30 min after the exercise (Table 1). Bivariate regression analysis revealed a significant relationship between plasma cf-nDNA and HMGB1 concentrations in postexercise samples (r = 0.84; P = 0.004). In contrast to cf-nDNA, plasma cf-mtDNA concentrations were not affected by treadmill exercise (Table 1).

To monitor the time course of plasma cf-nDNA concentration kinetics in the initiation phase of cf-nDNA generation, we collected serial blood samples from 3 participants every 3 min during the course of the strenuous treadmill exercise protocol. Fig. 1 illustrates the kinetics of plasma cf-nDNA, lactate, and HMGB1 concentrations during and after exercise for data from a well-trained endurance athlete (26-year-old male; VIAT, 18.1 km/h). The profiles of a well-trained recreational runner (42-year-old male; VIAT, 14.4 km/h) and a moderately trained participant (25-year-old female; VIAT, 9.7 km/h) are presented in Fig. 1 in the online Data Supplement. Pronounced increases in the cf-nDNA plasma concentration started at about 70% of maximal exercise intensity and reached a peak concentration (range, 10- to 28-fold increase) immediately or shortly after the run (range, 0–10 min). The time frame between the start of exercise and the onset of

### Table 1. Plasma concentrations of cf-nDNA, cf-mtDNA, and HMGB1 measured sequentially before, immediately after, and 30 min after exhaustive short-term treadmill exercise (n = 9).

<table>
<thead>
<tr>
<th></th>
<th>Before</th>
<th>Immediately after</th>
<th>30 min after</th>
</tr>
</thead>
<tbody>
<tr>
<td>cf-nDNA, GE/L × 10^6</td>
<td>1.8 (1.3–2.6)^a,c</td>
<td>25.3 (18.0–36.0)^c,d</td>
<td>7.6 (5.3–11.0)^b,d</td>
</tr>
<tr>
<td>cf-mtDNA, copies/L × 10^6</td>
<td>229 (110–500)</td>
<td>239 (110–520)</td>
<td>181 (83.0–400)</td>
</tr>
<tr>
<td>HMGB1, μg/L</td>
<td>1.5 (0.1–2.9)^b</td>
<td>5.0 (3.6–6.4)^c,d</td>
<td>1.6 (0.2–3.0)^b</td>
</tr>
</tbody>
</table>

* Data are given as the geometric mean, and 95% CIs are given in parentheses.
* P < 0.001, vs concentration immediately after exercise.
* P < 0.001, vs concentration 30 min after exercise cessation.
* P < 0.001, vs preexercise concentration.
cf-nDNA increase was within 10 min. Peak concentrations were reached within 20 min after the onset of cf-nDNA increases. Plasma cf-nDNA concentrations declined consistently to about 5-fold higher than the baseline concentration by 30 min after exercise, indicating a rapid clearance of excess cf-nDNA (mean half-life, approximately 16 min; range, 9–23 min). Remarkably, the kinetics for plasma lactate, cf-nDNA, and HMGB1 concentrations were nearly congruent during the exercise phase.

In agreement with our data, Fatouros et al. recently demonstrated that gradual increases in blood inflammatory markers in response to exhaustive treadmill exercise are preceded by an immediate and pronounced cf-nDNA peak (3). Rapid increases in cf-nDNA have also been demonstrated in patients after trauma (6). In contrast to trauma (7), however, the cf-nDNA response in our exercise setup was not accompanied by an increase in cf-mtDNA. Similarly, both down- and upregulation of cf-mtDNA have been reported in cancer patients (8, 9). Whether these discrepancies can be attributed to different sample-processing protocols (10) or are indicative of alternative/additive mechanisms of cf-DNA release requires further investigation.

In healthy individuals, excess cf-nDNA is rapidly cleared from the circulation after the cessation of exercise. Delayed or impaired clearance of cf-nDNA is especially implicated in the disease pathogenesis of autoimmune disorders, such as systemic lupus erythematosus (11), and, remarkably, athletes who experience chronic fatigue syndrome display increased seroprevalence of antinuclear antibodies (12). Recent studies have identified the endogenous nuclear protein HMGB1 as a master regulator of innate immunity and as a key player in the pathogenesis of systemic lupus erythematosus (13). HMGB1 can be passively released from necrotic or secondary necrotic cells, or actively secreted by activated leukocytes and oxidatively stressed nonimmune cells, thereby exhibiting particularly pleiotropic biological effects, including inflammation, stem cell recruitment/activation, and tissue repair (13).

Consistent with our results, a correlation between cf-nDNA concentrations and plasma lactate values has been described in patients with severe sepsis (14). Lactate accumulation has been shown to be directly related to an increased production of reactive oxygen species (15), and oxidative stress via sustained inflammation, cellular proliferation, or tissue damage constitutes a common denominator for conditions associated with increased cf-nDNA concentrations. Of note is that exogenous reactive oxygen species can trigger neutrophils to cast out their nDNA by a unique caspase-independent pathway, forming an antimicrobial meshwork of nDNA, histones, and antibacterial proteins that has been termed “neutrophil extracellular traps” (NETs) (16). Although extracellular DNA traps contribute to innate host defense, NETs have also been observed in noninfectious autoimmune diseases (17, 18). Recent data indicate that NETs can be released within minutes of activation (19, 20), a result that represents a feasible explanatory model for the selective and rapid-release kinetics of cf-nDNA that we observed in response to our exercise setup. Moreover, intravascular platelet–neutrophil interactions have recently been shown to trigger rapid NET extrusion and subsequent thrombus formation in vivo (21, 22). Acute strenuous exercise is known to transiently increase the risk of acute coronary syndromes (23), and thrombotic
complications have consistently been associated with disease progression in all conditions associated with increased cf-nDNA concentrations.

Assessment of cf-nDNA concentrations holds promise as a minimally invasive and sensitive diagnostic tool for monitoring cancer therapy and as a diagnostic and prognostic marker for both autoimmune disorders and acute pathologies (4). To fully exploit the diagnostic potential of cf-nDNA requires an improved insight into the cellular and molecular mechanisms underlying the release and clearance of cf-nDNA. We have shown that strenuous exercise can serve as a suitable surrogate model to study cf-nDNA biology in vivo.

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