Neutrophils release extracellular DNA traps in response to exercise

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Neutrophils release extracellular DNA traps in response to exercise. J Appl Physiol 117: 325–333, 2014. First published May 15, 2014; doi:10.1152/japplphysiol.00173.2014.—In- tense exercise evokes a rapid and transient increase in circulating cell-free DNA (cf-DNA), a phenomenon that is commonly observed in a variety of acute and chronic inflammatory conditions. In this study, we aimed to shed new light on the release and clearance mechanisms of cf-DNA in response to exercise. We hypothesized that activated neutrophils may primarily contribute to exercise-evoked cf-DNA levels by releasing neutrophil extracellular traps (NETs). Analysis of plasma and/or serum samples from male athletes at rest and in response to exhaustive treadmill exercise revealed an immediate and transient increase in cf-DNA that was concomitantly counterbalanced by an increase in serum DNase activity. Consistently, rapid release and clearance kinetics for cf-DNA could also be observed in response to intensive cycling exercise, with no significant differences between endurance-trained (VO2max > 57 ml·min−1·kg−1) and healthy (VO2max < 49 ml·min−1·kg−1) sedentary individuals. In postexercise blood smear samples, we detected seemingly intact neutrophils displaying morphological signs of NET release, as indicated by abnormal swollen nuclei and emanating DNA fibers. In support, we observed a striking correlation of postexercise cf-DNA concentrations with plasma levels of the granule-derived enzyme myeloperoxidase. Our study indicates that intense exercise induces liberation of NETs, which is sufficiently counterbalanced in healthy individuals by a concomitant increase in serum DNase activity. As aberrant release of NETs has been linked to diverse disease states, monitoring of cf-DNA/DNase levels or activities in response to standardized exercise testing could provide a valuable tool to identify people who are at increased risk for cardiac ischemia, thrombosis, autoimmunity, or chronic fatigue.

AT THE ADVENT OF THE 21st century, the prevalence of chronic diseases in developed and developing countries is increasing at an alarming rate (37). Chronic conditions like obesity, diabetes, and cardiovascular disease contribute largely to morbidity and mortality all over the globe and place increasing pressure on health care systems (4). Because chronic disorders are primarily attributable to lifestyle-related factors, a sizable fraction of this disease burden could substantially be minimized by nonpharmacological interventions.

Adopting a regular exercise routine is widely recognized as offering protection against obesity, insulin resistance, and atherosclerosis (28, 57). Moreover, there is accumulating knowledge that exercise intervention provides a true treatment mo-
diverse autoinflammatory conditions, vascular inflammation, and atherogenesis (19, 41, 48, 53).

By monitoring the time course of cf-DNA plasma concentrations in healthy individuals during and after incremental treadmill running, we recently reported rapid release and clearance characteristics of cf-DNA in response to exhaustive exercise (5). Based on this observation, we hypothesized that the immediate exercise-induced increase in cf-DNA might be indicative of NETs that could be formed within the vasculature, as has recently been observed in septic blood (12, 48, 59). We further speculated that release of cf-DNA in response to acute exercise might be efficiently counterbalanced in healthy individuals by an increase in serum DNase 1 activity. To test our hypothesis, we measured cf-DNA concentrations as well as DNase 1 activity in the blood of healthy individuals at rest and in response to intensive endurance exercise. As regular physical activity is generally considered to exert beneficial effects on immune functions, we included sedentary as well as well-trained individuals in our study. In addition, we quantified plasma markers of inflammation, muscle injury, and neutrophil activation. We further attempted to visualize exercise-induced NET formation in the bloodstream by immunocytochemical staining of blood smear samples. Our data indicate that intravascular NETs are released during intensive exercise.

**MATERIALS AND METHODS**

**Study design.** In the first part of the study, we attempted to provide initial evidence for the proposed release and clearance mechanisms of cf-DNA. We chose an exhaustive treadmill protocol that has recently been shown as suitable for provoking immediate and pronounced increases in cf-DNA (5). The second part of the study aimed to confirm these results in a different exercise setting that allowed assessment of whether the outcome variables would be influenced by the training status. All experimental procedures were approved by the Institutional Review Board of the University of Tuebingen according to the Declaration of Helsinki.

**Study participants and exercise setups.** All subjects were fully informed of the experimental protocols and gave written informed consent before the study commenced. Vital signs, anthropometric measurements, and physical examinations were conducted to evaluate health status. Subjects were excluded if they presented with any acute or chronic disease, major or minor injuries, or use of prescribed medication. In the initial study part, six well-trained male athletes (median age, 24 yr; range, 20–33 yr) who were reenrolled from our previous study (5) repeated a graded exercise test until volitional exhaustion on a motorized treadmill (Sartum, HP COSMOS, Traunstein, Germany) at a constant incline of 1% with an increment of 2 km/h every 3 min. Serum and EDTA blood samples were taken before (pre), immediately after (post), and 30 min (+30 min) after the run.

To determine effects of training status, healthy male subjects were recruited who were either engaged in regular endurance exercise of more than 5 h/wk or habitually spent less than 2 h/wk with physical activity. All subjects underwent preliminary baseline screening including lactate diagnostic and maximal bicycle spiroergometry to determine aerobic capacity; ET, endurance trained; UT, untrained. *P < 0.01, significantly different compared with UT group.

**Quantification of cf-DNA.** DNA was extracted from 400 μl of plasma according to the blood and body fluid protocol of the QiAamp Blood Mini Kit (Qiagen, Hilden, Germany) with a final elution volume of 100 μl. cf-DNA equivalents were quantified by SYBR green real-time PCR that targeted an 88-bp fragment of the chromosomal myostatin (MSTN) gene locus, as described previously (5). Briefly, samples were run in triplicates on an iCycler single-color detection system (BioRad, Munich, Germany) using the QuantIFast SYBR Green PCR Kit (Qiagen). DNA levels were calculated from a standard curve generated from serial dilutions of a genomic calibrator by plotting the threshold cycle value against the log of calibrator copy numbers. Copies of the target sequence per plasma sample volume were then calculated as described by Chiu et al. (11), and total quantities of cf-DNA are given as genome equivalents (GE) per milliliter of plasma, with one GE being defined as the amount of the target sequence contained in a single haploid cell. The lower quantification and detection limits of the method were reproducibly identified at 500 GE/ml and 250 GE/ml plasma, respectively.

**Quantification of serum DNase 1 activity.** DNase activity in serum samples was determined by using Org 590 DNase activity ELISA (OriGene, Mainz, Germany) according to the manufacturer’s instructions. This assay measures the intrinsic DNA degradation capacity of a serum sample by the turnover of an immobilized DNase substrate coated on the chamber surfaces of a microwell plate. Remaining substrate is quantified photometrically at 450 nm, and the amount of color reaction is inversely proportional to the DNase activity in the serum sample (activity reduction). To make data more amenable for quantitative comparison, we included a standard curve [5.31 up to 277.53 milli-Kunitz units (mKuU)/ml] with defined activities of a recombinant human DNase 1 standard provided by the manufacturer.

**Staining of blood smears.** Blood droplets (35 μl) from EDTA whole blood samples were placed on glass slides and gently spread using the edge of a second glass slide. For histological staining, blood smears were air-dried, fixed in methanol, and stained by standard Giemsa stain (Carl Roth, Karlsruhe, Germany) procedure.

**For immunohistochemical staining of neutrophils, blood smears were fixed in 4% paraformaldehyde in PBS for 2 h at room temperature, rinsed with PBS, permeabilized with 0.5% Triton X-100 PBS for 1 min, and blocked overnight in 2% BSA PBS at 4°C.** Incubation

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<th>Table 1. <strong>Physical characteristics of endurance-trained and untrained participants</strong></th>
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<td><strong>Age, yr</strong></td>
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<td><strong>UT</strong></td>
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Values are median (range); BMI, body mass index; VO₂max, maximal aerobic capacity; ET, endurance trained; UT, untrained. *P < 0.01, significantly different compared with UT group.
with primary rabbit anti-human neutrophil elastase antibodies (Abcam, Cambridge, United Kingdom) or the corresponding negative control antibodies (Ab 46540, Abcam) was performed for 1 h at room temperature in 2% BSA PBS, followed by secondary staining with Cy3-conjugated donkey anti-rabbit IgG (711-166-152, 1:400, Dianova, Hamburg, Germany) for 1 h at room temperature. DNA was labeled with 4,6-diamidino-2-phenylindole (1.25 μg/ml; Invitrogen, Life Technologies, Darmstadt, Germany). Specimens were mounted with Vectashield (Vector Laboratories, Peterborough, United Kingdom), and neutrophils were visualized by immunofluorescence microscopy (Axiovert, Zeiss, Goettingen, Germany).

Quantification of plasma markers. Quantification of 89 inflammation-associated proteins in plasma samples was performed at Rules Based Medicine (Myriad RBM, Austin, TX) using their human MAP v1.6 panel of Luminex-based multiplex assays as described by the vendor. Analytes containing values below the lower detection limit were excluded from analysis.

Plasma volume changes. Study participants were instructed to drink water at two defined time points during the cycling exercise protocol. To confidently exclude biased measures due to plasma volume shift, changes in plasma concentrations were calculated from hemoglobin and hematocrit values and concentrations of postexercise variables were corrected according to Kraemer and Brown (35). For all analyzed postexercise samples, calculated losses in plasma volume were below 6% (range: 1.5% to 5.4%).

Statistical analysis. Statistical analysis was performed using JMP (version 10; SAS Institute, Cary, NC) and SPSS statistical software (version 15; SPSS, Chicago, IL). Differences within groups over time were analyzed by Friedman’s two-way nonparametric ANOVA, followed by Wilcoxon’s matched-pairs signed-rank test. The Mann-Whitney U-test was used to compare between group variables. Spearman rank correlation coefficients (CCs) were calculated with Bonferroni-adjusted significance levels to estimate the correlation between variables. A value of \( P < 0.05 \) was regarded as statistically significant. Data are presented as median (range).

RESULTS

Release of cf-DNA in response to acute exercise is counter-balanced by serum DNase I. An immediate, pronounced, and transient increase in cf-DNA levels could be observed in six balanced by serum DNase 1.

Postexercise blood smears reveal NET-like structures. Considering the rapid appearance of excess cf-DNA in the circulation of exercising subjects, we analyzed Giemsa-stained blood smears from pre- and postexercise blood samples to screen for suspicious signs of damaged or dying blood cells. In peripheral blood smear slides prepared immediately after the exhaustive treadmill run, we observed the scattered but striking appearance of seemingly disintegrated cells with a decondensed chromatin structure (Fig. 2A). Similar structures are apparent in blood smears from patients with malaria (3), and have recently been documented in blood smears from stored red blood cell units (24). Here, these cells were recognized as circulating neutrophils that are releasing extracellular DNA webs, termed NETs. To obtain proof of principle, we used immunocytochemistry to visualize intravascular neutrophils by neutrophil elastase staining and to clearly characterize NET-like structures in postexercise blood smears. As shown in Fig. 2B, suspicious NET-like structures emerged as seemingly intact neutrophils with abnormal swollen nuclei and dispersed nucleic acid staining patterns that were surrounded by a delicate network of emanating DNA (Fig. 2B). Control stainings of isolated neutrophils treated with phosphol myristate acetate (PMA; data not shown) exemplified that the observed phenotype is clearly distinct from the commonly described lytic mechanism of NET release that culminates in the rupture of the cellular membrane and the concomitant expulsion of filamentous chromatin structures (8), but could rather be attributed to the recently discovered rapid mechanism of vesicular-mediated NET release (48).

Trained and untrained healthy individuals do not differ in their cf-DNA/DNase responses to exercise. Because aberrant release of cf-DNA/NETs has been implicated in diverse chronic inflammatory conditions, we wanted to address whether sedentary (untrained, UT) and habitually exercising (endurance-trained, ET) individuals display distinct cf-DNA/DNase responses to acute exercise. To achieve comparable workload and duration for both groups, we chose a 1-h high-intensity cycling protocol adjusted to the individual \( V_{\text{O2max}} \) level. Both groups did not differ in their baseline cf-DNA levels, with a median plasma concentration of 2,078 (638–

![Fig. 1. Values of cell-free DNA (cf-DNA) plasma concentrations (A) and serum DNase 1 activity (B) in the blood of well-trained individuals (n = 6), determined at rest (pre), in response to exhaustive treadmill exercise (post), and after 30 min of recovery (+30 min). Bar, median of the group. **P ≤ 0.01, significant difference between sampling points.](http://jap.physiology.org/; by 10.2203/24.246 on April 11, 2017)
exercise (Spearman’s CC = 0.699, P = 0.011; Fig. 3E). Of note, no significant association could be observed with the muscle damage marker myoglobin (Spearman’s CC = −0.028, P = 0.931; Fig. 3F). Pronounced liberation of myoglobin became apparent at 3 h after cessation of exercise (Fig. 3D), with a significant correlation to FABP plasma levels (Spearman’s CC = 0.937, P < 0.0001), another early cytoplasmic muscle damage marker. Because MPO is particularly abundant in the primary granules of neutrophils, correlation of cf-DNA and MPO levels in postexercise samples further supports the idea that netting neutrophils might substantially contribute to the immediate cf-DNA release in response to exercise.

DISCUSSION

Increased concentrations of cf-DNA are frequently observed in acute and chronic inflammatory conditions (54). While the prognostic and diagnostic value of this phenomenon has been extensively attributed in diverse malignancies as well as in infectious and autoinflammatory diseases (32), the biological significance of nonpathological release of cf-DNA in the course of physical exercise remains enigmatic. It has initially been assumed that leakage of nuclear content from traumatized muscle fibers may primarily account for exercise-induced cf-DNA levels, thus simply representing a bystander marker of muscle damage (2). This view has recently been challenged by thorough time-course analyses, showing clearly distinct release kinetics of cf-DNA and cytosolic markers of muscle damage during and following strenuous endurance exercise (5, 23). Here, we provide evidence that release of cf-DNA in response to exercise rather reflects an active effector mechanism of innate immunity, namely the formation of NETs by peripheral blood neutrophils. Our results show that 1) exercise-induced cf-DNA is efficiently counterbalanced in healthy individuals by a concomitant increase in serum DNase 1 activity; 2) the immediate appearance of cf-DNA in the circulation clearly precedes and is not correlated with the early muscle damage.

Fig. 2. Representative images of neutrophil extracellular trap (NET)-like structures in the postexercise blood of a well-trained subject. Blood smears were prepared immediately after exhaustive treadmill exercise and Giemsa stained for cytological examination (A). Intact neutrophils are clearly delineated by their lobular and segmented nuclei (A, overview image and top left). NET-like structures appear as seemingly disintegrated cells with a decondensed chromatin structure and fibrous protrusions of DNA (A, overview image and top right). Blood smears were further analyzed by immunocytochemistry for neutrophil elastase (NE; B). Bottom row panels show merged immunofluorescence images of representative neutrophils in postexercise blood smears. NE is shown in red (top row). DNA was labeled with 4,6-diamidino-2-phenylindole (DAPI) and is shown in blue (middle row). Intact neutrophils were identified as NE-positive cells with typical segmented nuclei (B, left). Netting neutrophils were characterized as NE-positive cells with seemingly intact membrane structure and disintegrated nuclei (B, middle and right columns), and NET-like structures were identified as delicate networks of extracellular DNA. Bar, 10 μm.
markers myoglobin and FABP; 3) postexercise concentrations of cf-DNA are significantly correlated with circulating levels of the neutrophil granule enzyme MPO; and 4) neutrophils with delobulated nuclei and emanating DNA fiber structures are apparent in postexercise blood smears, indicating that intravascular NETs are released in response to intense exercise.

In line with our results, degranulation of neutrophils, as predicted by an immediate increase of MPO in the circulation, has commonly been reported in response to intense physical exercise (46, 47). Remarkably, the hemoprotein MPO is contained within the neutrophil primary granules, which are destined to fuse with the forming or formed phagosome after microbial engulfment, but are barely liberated by exocytosis (43, 52). As indicated by the observed association between plasma levels of cf-DNA and MPO, it has become evident that active release of MPO by neutrophils is primarily accomplished via colocalization with the chromatin backbone of NETs (55). Consistently, levels of MPO have been shown to strongly correlate with cf-DNA in the blood of patients with thrombotic microangiopathies (26), and in patients with deep vein thrombosis (17). Moreover, by use of capture ELISA protocols, true MPO-DNA complexes have recently been detected in the circulation of patients with small vessel vasculitis (SVV) (34) and in individuals with suspected coronary artery disease (6).

NETs were originally described as an innate defense mechanism to extracellularly capture and potentially kill invading pathogens. Nevertheless, recent findings suggest that NETs may also play a role in the regulation of inflammation and thrombosis. In this study, we have observed that the release of MPO from neutrophils is significantly correlated with cf-DNA levels in postexercise samples, indicating that NETs are released in response to intense exercise. This finding is consistent with previous reports demonstrating that NETs are released in response to intense physical activity (46, 47).

**Fig. 3. Effect of acute exercise and training status on plasma levels of cf-DNA (A), serum DNase activity (B), plasma concentrations of myeloperoxidase (MPO; C), and myoglobin (D).** Values were retrieved from blood samples of endurance-trained (ET; n = 6; closed circles; VO2max > 57 ml·min⁻¹·kg body wt⁻¹) and sedentary (untrained, UT; open circles; n = 6; VO2max < 49 ml·min⁻¹·kg body wt⁻¹) individuals at rest (pre), after 60 min of intense cycling exercise (post), and after 3 h of recovery (+3 h). Bar, median of the group. A significant positive association between plasma cf-DNA levels and MPO concentrations could be detected in postexercise samples (E), while no significant correlation could be seen between cf-DNA levels and the muscle damage marker myoglobin (F). mKU, milli-Kunitz units; GE, genome equivalents. *P < 0.05, significant difference within group. **P < 0.01, significant difference within group.
Table 2. Plasma concentrations of inflammation-associated blood markers at rest, immediately after, and 3 h after 60 min of intense cycling exercise in endurance-trained and untrained individuals

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<td>Pre</td>
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<td>4.1</td>
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<td>(1.4–2.4)</td>
<td>(2.8–7.3)</td>
<td>(1.8–3.4)</td>
<td>(3.7–6.9)</td>
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<td>18.8</td>
<td>31.0</td>
<td>0.5</td>
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<td>Pre</td>
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<td>Post</td>
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<td>(1–5.8)</td>
<td>(3.6–11.0)</td>
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<td>359</td>
<td>291</td>
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Values are median (range); n = 6. Pre, at rest; Post, immediately after; +3h, after 60 min of intense cycling exercise; ET, endurance-trained; UT, untrained; FABP, fatty acid-binding protein in heart; GH, growth hormone; IL, interleukin; MMP, matrix metalloproteinase; MPO, myeloperoxidase. *P < 0.05, significant difference compared to UT group at indicated time point. †P < 0.05, significant difference within group compared to preexercise level. ‡P < 0.01, significant difference within group compared to preexercise level.

pathsogens (8, 9). In their seminal study, Brinkmann et al. (8) reported that, on stimulation with PMA, neutrophils undergo a sequel of cytoplasmic and nuclear changes that culminate in the rupture of the cellular membrane to release web-like structures of DNA coated with nuclear and granule proteins (including MPO). This lytic process of NET release, termed NETosis, occurs slowly over 2–4 h, is dependent on formation of reactive oxygen species, and is clearly distinct from classical forms of cell death like apoptosis or necrosis. However, despite extensive research efforts in recent years, the molecular pathways and the biological significance of this phenomenon are just beginning to emerge. It appears that—dependent on stimulus, location, milieu, and activation state—several distinct but partially overlapping signaling pathways exist that culminate in the formation of NETs for varying purposes (30, 45). Recently, Paul Kubes and colleagues (49, 59) uncovered an alternative, nonlytic and very rapid process of NET formation that does not require cell death, thereby enabling intravascular neutrophils to expel their decondensed nuclear contents without losing their chemotactic and phagocytic abilities. The described phenotype intriguingly resembles that of the seemingly intact NET-forming neutrophils we observed in the blood smear specimens of postexercise samples.
SVV (51), rheumatoid arthritis (RA) (33), inflammatory skin disease (29), and type I diabetes (16). Moreover, aberrant release of NETs has been linked to cancer-associated thrombosis, tumor growth, and metastasis (13–15). At first sight, it might appear paradoxical that exercise should trigger an immune mechanism that seems crucially involved in many chronic diseases and conditions that regular exercise is meant to protect one from. However, one might speculate that, by allowing “safe” release of NETs in a noninflammatory or tolerogenic environment, exercise-induced formation of intravascular NETs might provide an essential and imminent strategy to establish self-tolerance to neutrophil-derived peptides and self-DNA, thus contributing significantly to the health benefits of physical activity.

Irrespective of its functional role(s), timely removal of exercise-induced NETs should be of indispensable importance to preserve tissue homeostasis, to avoid thrombotic complications, and to prevent aberrant presentation of self-antigens (10, 31, 38, 42). Here, we demonstrate that the rapid resolution of exercise-induced cf-DNA could be attributed to a concomitant and transient rise in serum DNase 1 activity. DNase 1 is the most important waste-management nuclease in serum with responsibility for degrading extracellular DNA. Consistently, DNase 1 has been depicted as the primary degrader of NETs, and impairment or deficiency in DNase 1 activity has been directly linked to the development of autoimmune diseases (31, 38). Remarkably, an increased seroprevalence of antinuclear antibodies, a hallmark of SLE and RA, has also been reported in athletes suffering from chronic fatigue (50). Thus, it seems reasonable to assume that aberrant or unresolved release of cf-DNA/NETs might profoundly contribute to unanticipated adverse outcomes of acute or prolonged exercise in susceptible individuals, evoked, for example, by excessive or unaccustomed training loads, high stress levels, or insufficient rest periods.

Considering the overall health-promoting effect of regular exercise, we wondered whether sedentary and physically active individuals in general display distinct cf-DNA/DNase responses to acute exercise. Our results indicate that the individual response to exercise is not directly interconnected to the training status, at least not in healthy young individuals—which of course does not rule out that exercise intervention might affect these responses in susceptible individuals. Likewise, it will be important to determine the responsiveness in individuals with known risk factors for lifestyle-related diseases. Remarkably, patients with severe coronary atherosclerosis or calcified coronary arteries have recently been shown to display increased plasma levels of cf-DNA, and quantification of circulating MPO-DNA complexes has been demonstrated of predictive value for future cardiovascular events (6). In this way, monitoring of cf-DNA/DNase levels or activities in response to a standardized exercise testing could provide a valuable tool to identify people who are at increased risk of developing cardiac ischemia, thrombosis, autoimmunity, airway hyperresponsiveness, or chronic fatigue.

In summary, we provide descriptive evidence of exercise-induced release of NETs. This observation might open a new avenue to improve our understanding of exercise immunology. Future work will show whether this phenomenon is rather a reminiscence of our ancient fight-or-flight response or whether it represents a fundamental layer of immune response to exercise.

GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: T.B., J.M.S., F.C.M., and A.M.N. conception and design of research; T.B., A.F., and J.H. performed experiments; T.B., A.F., and A.M.N. interpreted results of experiments; T.B. prepared figures; T.B. and A.M.N. edited and revised manuscript; T.B., A.F., J.H., M.S., J.M.S., F.C.M., and A.M.N. approved final version of manuscript.

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