

The influence of hypoxic physical activity on cfDNA as a new marker of vascular inflammation

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Abstract

The phenomenon of circulating cell-free DNA (cfDNA) is important for many biomedical disciplines including the field of exercise biochemistry and physiology. It is likely that cfDNA is released into the plasma by apoptosis of endothelial cells and circulating endothelial progenitor cells (EPCs), and/or by NETosis of immune cells induced by strenuous exercise. Increases of cfDNA are described to be a potential hallmark for the overtraining syndrome, and might be related to aseptic vascular inflammation in athletes. Yet, the relevance of systemic inflammation and cfDNA with endothelial dysfunction in athletes still remains unclear. In this review article, we provide a current overview of exercise-induced cfDNA release to the circulation with special emphasis on its relationship with apoptosis and NETosis and the effect of hypoxic physical activity on vascular inflammation in athletes.

Key words: apoptosis, NETosis, cytokines, overtraining.

Introduction

It has been shown that vascular endothelial dysfunction precedes the development of arteriosclerosis, and thereafter plays an important role in its progression. Therefore, preservation or recovery of endothelial function is important to inhibit the development of cardiovascular events [1]. Several biomarkers have been proposed to investigate endothelial dysfunction, including insulin resistance, homocysteinemia, inflammatory cytokines, oxidized lipoproteins LDL and HDL, reactive oxygen and nitrogen species, vasodilators and vasoconstrictors (Table I) [2–21]. Recent studies have also suggested that dysfunctional HDL might be very effective and predictive biomarker of endothelial impairment as well as cardiovascular risk [22–26].

The newest studies demonstrate that cell-free DNA (cfDNA) could serve as an auxiliary biomarker of vascular endothelial dysfunction and cardiometabolic risk assessment [6, 27, 28]. The mechanisms of the occurrence of cfDNA fragments in blood under normal and pathological conditions are not yet fully understood. The sequence analysis of circulat-

Table I. Changes in biomarkers of vascular endothelial dysfunction

Markers	Direction of change	References
Adiponectin	↓	[2–4]
Adhesion molecules: vascular cell adhesion molecule (VCAM), intercellular adhesion molecule (ICAM), P-selectin, E-selectin	↑	[5]
Circulating cell-free DNA (cfDNA)	↑	[6]
Circulating microRNA (miRNA)	↑	[7]
Endothelial progenitor cells (EPCs)	↓	[8, 9]
Endogenous inhibitors of nitric oxide synthase (NOS): asymmetric dimethyl-arginine (ADMA)	↑	[10]
Homocysteine (Hcy)	↑	[11]
Insulin resistance	↑	[12]
Inflammatory factors: C-reactive protein (CRP), interleukin 1 β (IL-1 β), IL-6, IL-12, IL-17, tumor necrosis factor- α (TNF- α), monocyte chemoattractant protein-1 (MCP-1)	↑	[6, 9, 13–15]
Lipoproteins LDL, oxidized LDL (oxLDL) and oxidized HDL (oxHDL)	↑	[6, 16, 17]
Peroxyntirite (ONOO $^-$)	↑	[9, 18, 19]
Thrombotic hemostatic factors: plasminogen activator inhibitor-1 (PAI-1), tissue plasminogen activator (TPA), von Willebrand factor, thrombomodulin	↑	[9, 20]
Vasoconstrictors: endothelin, thromboxane A $_2$, reactive oxygen and nitrogen species (RONS)	↑	[9, 19, 21]
Vasodilators: nitrite and nitrate, 6-keto PGF 1α	↑	[9]

ing DNA in normal human plasma demonstrated that cfDNA fragments are derived from apoptotic cells but not from necrotic cells. The mean values of cfDNA length were \sim 180 bp in the culture supernatant of apoptotic cells, while DNA fragments larger than \sim 10,000 bp are observed in the culture of cells undergoing necrosis. Their 5' and 3' ends were rich in cytosine and guanidine, respectively, and they presented in the 5' end characteristic (protruding) forms of double-stranded DNA. The cfDNA concentrations in normal plasma samples were 3.6–5.0 ng/ml [29]. The cfDNA fragments apparently circulate as nucleoprotein complexes, but the main part of cfDNA is found adsorbed to the surface of blood cells in healthy individuals [30].

A novel fascinating explanation of how DNA can actively be released under inflammatory conditions has recently become apparent by the discovery of an evolutionarily highly conserved first-line defense mechanism that allows neutrophils to expel their DNA in response to infectious or endogenous factors, thereby forming a meshwork of chromatin and proteins, termed neutrophil extracellular traps (NETs). Accumulating evidence indicates that formation of NETs plays a pivotal role in the immune response to both pathogens and physical exercise [27].

Strenuous exercise, which consists of high-force eccentric exercises interspersed with long-duration endurance exercises at mild- to moderate-intensity work or regular high-intensity workouts, induces a slow and constant release of cfDNA.

During incremental treadmill running the capillary cfDNA concentrations increased nearly parallel to the lactate values. The values correlated best with heart rate and energy expenditure, followed by oxygen consumption and lactate levels [24]. After chronic excessive resistance exercise, cfDNA level increased in proportion to muscle damage (CK increase) and systemic inflammation (hsCRP increase), suggesting that plasma DNA may be a sensitive marker for overtraining-induced inflammation [30–33].

It is likely that cfDNA is released into the plasma by apoptosis of endothelial cells and circulating endothelial progenitor cells, and/or by NETosis of immune cells induced by strenuous exercise [34]. According to Angeli *et al.* [35] the generation of pro-inflammatory and pro-apoptotic mediators disturbs vascular endothelial activity. However, the relevance of cfDNA with inflammation and endothelium function in athletes still remains unclear. It is well known that marathon, rowing, soccer, tennis, handball and basketball cause activation of immune cells, which synthesize large quantities of pro-inflammatory and pro-apoptotic cytokine tumor necrosis factor- α (TNF- α) that can be responsible for vascular endothelial dysfunction [1, 36–43].

In this review article, we provide a current overview of exercise-induced cfDNA release to the circulation with special emphasis on its relationship with apoptosis and NETosis and the effect of hypoxic training on vascular inflammation in athletes.

Physical activity and vascular inflammation

Physical activity has significant beneficial effects on overall health, and especially on cardiovascular morbidity and mortality. Regular physical activity significantly attenuates the atherosclerotic process by reducing atherosclerotic risk factors, retarding arterial wall aging, delaying development of endothelial dysfunction and preserving vascular function. Furthermore, regular physical activity reduces vascular oxidative stress, increases NO production via endothelial NOS (eNOS), modifies the lipid profile [44–46], inhibits the production of pro-inflammatory and pro-apoptotic cytokine TNF- α [47], and also decreases both oxidative stress and the circulating concentrations of ADMA [48]. It is stressed that observations were performed on healthy non-active subjects or patients with coronary artery disease [49, 50]. It is still not known how pro-inflammatory and pro-apoptotic molecules affect endothelial cells' activity in highly trained subjects.

There were a few observations concerning changes in markers of endothelial activity in elite athletes following intense training periods or after finishing their sport career. It is known that the detraining period induces a rapid increase in total cholesterol (TC) and lipoprotein LDL, which elevates the risk of cardiovascular disease [51]. In our recent project, a high level of total cholesterol in 43% of elite Greco-Roman wrestlers was observed. The average concentrations of serum total cholesterol, lipoprotein HDL and LDL as well as triglycerides were 193, 51, 120 and 108 mg/dl, respectively (unpublished data). We do not know whether the observed changes in lipid profile are temporary, whether they are related to endothelial dysfunction, and what the health implications for the future are. Maron *et al.* [52] estimated that 18.5% of incidents of sudden death in young athletes (< 35 years old) were related to vascular endothelial dysfunction and atherosclerotic coronary artery disease. According to Suarez-Mier *et al.* [53], the sports most frequently associated with sudden death were cycling (29%), soccer (25.5%), running (8.9%) and gymnastics (6.5%). Approximately 70% cases were not related to some personal pathological antecedents or familial sudden deaths. De Van and Seals [54] observed that masters endurance athletes demonstrate a more favorable arterial phenotype and lower risk of CVD compared with untrained middle-aged and older adults. In contrast, masters athletes for whom training and competitive sport require primarily or exclusively intensive resistance muscle activities exhibit a less favorable arterial function-structure profile. The differences in arterial properties between masters athletes participating in endurance sports vs. resistance training-requir-

ing sports are likely explained by differences in the intravascular mechanical forces generated during these activities. Agrotou *et al.* [55] demonstrated that the type of anaerobic exercise, e.g. weightlifting, is an important determinant of subclinical atherosclerosis such as intima-media thickness and flow-mediated dilation.

Apoptosis and vascular inflammation

Vascular inflammation is an early marker of endothelial dysfunction prior to the development of structural changes and clinical symptoms, contributes to the progression of atherosclerosis, and increases the risk of coronary events. Studies over the past year have demonstrated the significance of inflammation in endothelial apoptosis [56–58].

In athletes, endothelial apoptosis can be triggered through several pathways. One of them can be induced by the binding of TNF- α to one or more of the extracellular receptors of the tumor necrosis factor receptor (TNFRs) superfamily located on the surface of macrophages, T lymphocytes and endothelial cells. The binding of this ligand to its receptors ultimately leads to the activation of a specific set of proteases crucial for the execution of apoptosis. These proteases are collectively referred to as caspases, and they are activated by proteolytic cleavage. Caspases have the ability to cleave and activate other caspases in a “cascade-like” fashion. This serves as an efficient and potent mechanism for amplifying the cell death signal. Ultimately, caspase activation is responsible for the biochemical breakdown of cytosolic and nuclear targets leading to the distinct morphological features of apoptosis. Recruitment of procaspase 8 to the plasma membrane results in feedforward recruitment of more procaspase-8 proteins. This caspase-8 accumulation forms a death-inducing signaling complex at the plasma membrane, resulting in caspase-8 activation. Activated caspase-8 can then directly cleave and activate caspase-3. Caspase-3 exerts its apoptotic effect by cleaving key structural proteins of the plasma membrane and nuclear envelope, leading to the structural breakdown of the cell. However, this enzyme mediates its primary effects within the nucleus of the cell. Activated caspase-3 translocates from the cytosol to the nucleus, where it cleaves and deactivates an inhibitor of caspase-activated DNase. This releases caspase-activated DNase, and endonuclease, enabling the cleavage of genomic DNA. This DNA fragmentation is one of the hallmark morphological features of apoptosis [34, 59].

In athletes, a high concentration of TNF- α can also induce endothelial apoptosis through disturbance of the equilibrium between eNOS and inducible NOS (iNOS) activities, resulting in pro-apoptotic NO activity, and finally disturbanc-

es in vascular endothelial activity which precedes the development of arteriosclerosis [19, 58]. The excess NO reacts with the superoxide anion to produce peroxynitrite (ONOO⁻) by 1 000 000-fold. ONOO⁻ in turn can “uncouple” eNOS to become a dysfunctional superoxide-generating enzyme that contributes to vascular oxidative stress. Oxidative stress and endothelial dysfunction can promote atherogenesis. Without superoxide, the formation of ONOO⁻ by the reaction of NO with oxygen is minimal. Nitric oxide and superoxide do not even have to be produced within the same cell to form peroxynitrite, because NO can so readily move through membranes and between cells [19].

Athletes have demonstrated significantly higher levels of reactive oxygen and nitrogen species [60] and TNF- α [35–41], which can serve as predictors of overtraining-induced inflammation [37, 43]. Survey research involving endurance athletes who completed monocycle training indicated a rate of overtraining syndrome of approximately 10% (range: 7–21%) [61].

Netosis and vascular inflammation

Recently, a novel mechanism of cfDNA release at inflammation sites was described. Upon activation, neutrophils release NETs composed of DNA fibers decorated with granular proteins. Neutrophil extracellular traps are produced by neutrophils and other immune cells, in contact with pathogens, a variety of host factors such as activated platelets, inflammatory mediators or reactive oxygen and nitrogen species (Table II) [62–76].

Neutrophil extracellular traps are the results of a unique form of cell death that morphologically is characterized by the loss of intracellular

membranes before the integrity of the plasma membrane is compromised. To release NETs, activated neutrophils undergo dramatic morphological changes. Minutes after activation, they flatten and firmly attach to the substratum. During the next hour, the nucleus loses its lobules, the chromatin decondenses, and the inner and outer nuclear membranes progressively detach from each other. Concomitantly, the granules disintegrate. After 1 h, the nuclear envelope disaggregates into vesicles and the nucleoplasm and cytoplasm form a homogeneous mass. Finally, the cells round up and seem to contract until the cell membrane ruptures and the interior of the cell is ejected into the extracellular space, forming NETs. Notably, despite the intermixing of cellular compartments, during the last phase of NETosis, < 30 proteins are present in NETs. Most of them originate from granules, a few are from the nucleus, and cytoplasmic NET components are rare. NETosis is morphologically quite different from apoptosis and necrosis (Figure 1) [62, 70, 77, 78].

Beiter *et al.* [27] were the first to demonstrate evidence of exhaustive exercise-induced release of NETs. Their observation opened a new avenue to understand exercise immunology. The authors stressed that future work is necessary to explain whether this phenomenon is a remnant of the ancient human fight-or-flight response or whether NETs represent a fundamental layer of the immune response to exercise.

Physical activity in hypoxia and endothelial repair

Endothelial cell injury initiates regenerative processes. The first step is the mobilization of

Table II. Exogenous and endogenous factors which stimulate the formation of NETs

Factors	References
Calcium ions Ca ²⁺	[63]
Glucose oxidase (GOx) catalyzes the oxidation of β -D-glucose to gluconic acid with simultaneous production of H ₂ O ₂	[64]
Granulocyte-macrophage colony-stimulating factor (GM-CSF) + C5a Granulocyte-macrophage colony-stimulating factor (GM-CSF) + LPS	[65, 66]
Inflammatory factors: interleukin 8 (IL-8), interleukin 1 β (IL-1 β), tumor necrosis factor- α (TNF- α), interferon γ (IFN- γ)	[63, 66, 67]
Lipopolysaccharide (LPS) Lipophosphoglycan (LPG)	[68–70]
M1-protein-fibrinogen complex	[71]
Panton-Valentine leukocidin (PVL)	[72]
Pathogens: bacteria, fungi, viruses, parasites	[68–70, 72]
Platelet activating factor (PAF)	[73]
Reactive oxygen and nitrogen species: hydrogen peroxide (H ₂ O ₂), nitric oxide (NO)	[74]
Statins: lovastatin, simvastatin, fluvastatin and mevastatin	[75]
Platelet via toll-like receptor 4 (TLR-4)	[76]

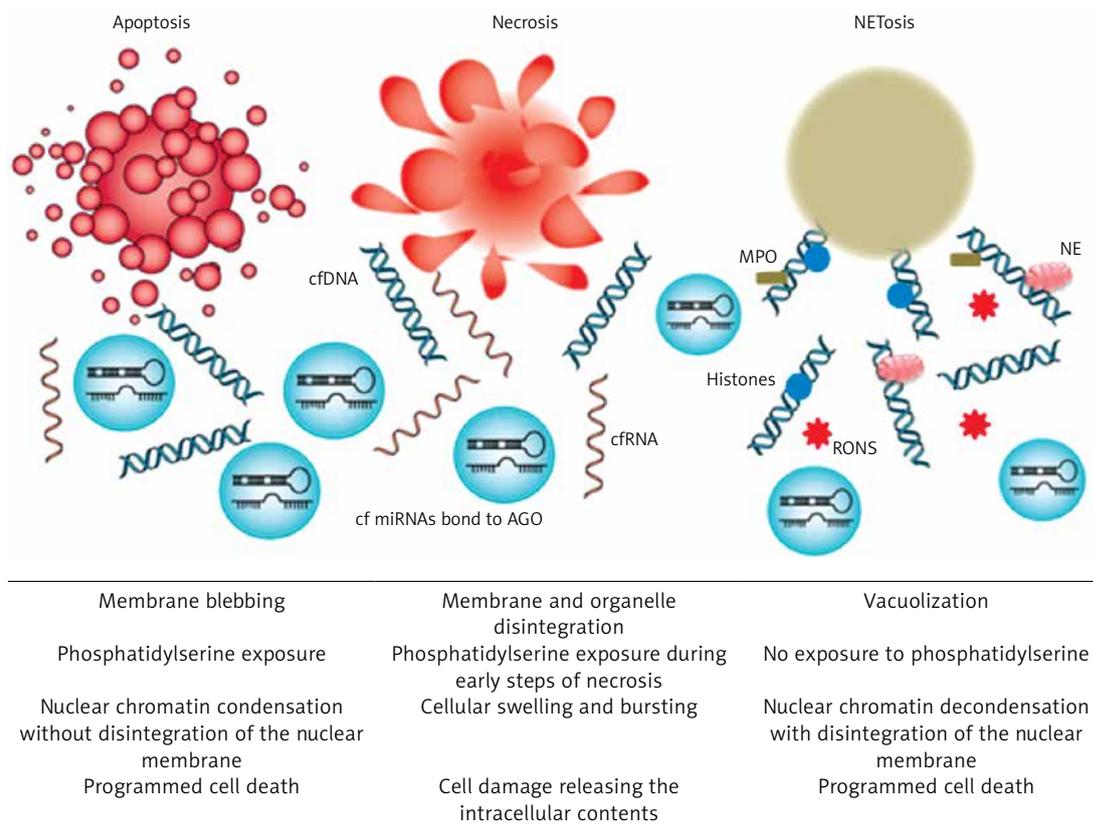


Figure 1. Schematic representation and differences between apoptosis, necrosis and NETosis

cfDNA – Circulating cell free DNA, *cfRNA* – circulating cell free RNA, *AGO* – Argonaute protein, *MPO* – myeloperoxidase, *NE* – neutrophil elastase, *RONS* – reactive oxygen and nitrogen species, *miRNAs* – short noncoding RNAs (19–25 nucleotides) that modulate gene expression at the posttranscriptional level and play important roles in a wide range of physiological and pathological processes [62, 77, 78]

endothelial progenitor cells (EPCs) from the bone marrow into peripheral blood, which is followed by the recruitment of EPCs to the site of vascular injury. Decreased numbers of circulating EPCs are seen in patients with coronary risk factors and reflect senescence, endothelial dysfunction, impaired vascular repair, and increased cardiovascular events. Enhancement of EPCs is considered one of the most promising therapeutic alternatives for cardiovascular disease. The process of EPC mobilization leads to accelerated re-endothelialization, successfully achieved by erythropoietin and other growth factors [1].

Endothelial progenitor cells count is related to age and health status, and increases in response to physical exercise [8, 79–83] and hypoxia [84, 85] that occurs at high altitude. Ciulla *et al.* [84] observed in the Himalayas that high-altitude hypoxia and exercise oxygen demands are strong stimuli for clonogenic endothelial cell activation and activity, as shown by the increase in the number of EPCs. However, Mancuso *et al.* [86] found that circulating hematopoietic stem cells, circulating endothelial cells and EPC counts significantly decreased in 15 mountain trekkers after 12 days spent at > 3000 m. Strenuous exercise with expo-

sure to hypobaric hypoxia can be dangerous for athletes with endothelial dysfunction [87].

Physical training in hypoxia (hypobaric hypoxia) has been used for decades by Olympic and professional athletes to increase endurance, strength and speed, avoid fatigue and improve recovery [88, 89]. Recently, intermittent hypoxic training (IHT, normobaric hypoxia) was introduced into sport practice. Intermittent hypoxic training is a method by which athletes receive exposure to short bouts of severe hypoxia (9–12% O₂), interspersed with periods of normal air. The studies reported substantial improvements in sea level endurance and anaerobic performance after IHT at rest or during exercise. These enhancements suggest that IHT may be suitable for improving performance in high intensity team sports. Beside the effects of the hypoxic stimuli on exercise performance, there is recent evidence that intermittent hypoxic training or therapy (IHT) might be beneficial for endothelial cell activity [89, 90].

It is still not known whether normobaric hypoxia affects the circulating EPC number and whether IHT can reduce vascular inflammation in athletes. We observed a significant decrease in total cholesterol (by 10–15%) and its classes following IHT (unpub-

lished data). According to the consensus statement of the European Society of Cardiology, a decrease in serum cholesterol by 10% results in a reduction of cardiac artery disease by 20% [91]. However, we simultaneously observed an approximately 2-fold increase in hsCRP concentration in Greco-Roman wrestlers after 6-day normobaric hypoxia exposure (before IHT 1.45 ±0.33 mg/dl, after IHT 2.89 ±0.54) (unpublished data). The newest study indicates a significant correlation between serum hsCRP and the severity of coronary artery involvement [92]. Hence, further studies are recommended to be conducted on this issue in athletes.

Conclusions and future directions

The analysis of plasma cfDNA in relation to physical activity is currently being intensively studied, and this experience may determine the monitoring of overtraining-induced inflammation in the future.

Strenuous exercise, which consists of high-force eccentric exercises interspersed with long-duration endurance exercises at mild- to moderate-intensity work or regular high-intensity workouts, induces a slow and constant release of cfDNA. The elevated cfDNA level in both non-athletes and athletes is associated with markers of vascular endothelial dysfunction such as cytokines IL-1 β , IL-6 and TNF- α , hsCRP, oxidized lipoproteins as well as reactive oxygen and nitrogen species, etc. The main source of cfDNA is apoptosis of endothelial cells and circulating endothelial progenitor cells as well as NETosis of immune cells which move toward the site of vascular injury.

Physical exercise in hypoxia (hypobaric or normobaric hypoxia) is considered as a therapeutic modality in delaying development of endothelial dysfunction and preserving vascular function. However, to develop appropriate hypoxia intervention guidelines, it is necessary to gain a deeper understanding of how, when, and why hypoxia interferes with basic immune and repair mechanisms including EPC mobilization.

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Conflict of interest

The authors declare no conflict of interest.

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