

Plasma ADMA Decrease Following a Single Bout of Physical Exercise is Related to Reduced Homocysteine Lowering

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ARTICLE INFO

Article history:

Received: 05 August 2017

Accepted: 11 September 2017

Published: 18 September 2017

Keywords:

ADMA; DDAH;
Physical exercise;
Homocysteine;
Nitric oxide

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J Pharm Sci Biomed Anal

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Citation this article: Zinellu A, Sotgia S, Usai MF, Tangianu F, Caria M. Plasma ADMA Decrease Following a Single Bout of Physical Exercise is Related to Reduced Homocysteine Lowering. J Pharm Sci Biomed Anal. 2017; 1(1):112.

ABSTRACT

This study describes as a single bout of physical exercise reduce plasma Asymmetric Dimethylarginine (ADMA) levels. ADMA is a naturally occurring aminoacid that aroused interest because inhibits Nitric Oxide Synthases (NOS) enhancing atherogenesis and producing sustained hypertensive damage to end organs. We reported experimental evidences that ADMA decrease is due to the lowering of reduced homocysteine during the physical efforts. A regular physical activity may have a positive effect on healthy status since it reduces two important risk factor of vascular disease as homocysteine and ADMA. Therefore, a regular physical activity may exert a positive effect on healthy status even through the reduction two important risk factors of vascular disease such as homocysteine and ADMA.

Introduction

The role of physical exercise in disease prevention and quality life improvement is amply reported in literature. Evidence indicates that physical inactivity leads to increased incidences of cardiovascular disease (heart disease, hypertension, stroke, intermittent claudication, platelet adhesion and aggregation) [1-3], metabolic diseases (type 2 diabetes, obesity and dyslipidemia) [4,5], cancer (breast, colon, prostate, pancreatic and melanoma) [6,7], immune dysfunction [8] and neurological disorders (cognitive impairment, Alzheimer's disease, and dementia) [9]. Evidence also indicates that physical inactivity worsens asthma and accelerates bone loss [10]. A great variety of mechanisms are involved in health improvement induced by physical exercises. Daily physical activity can help prevent heart disease and stroke by strengthening heart muscle, lowering blood pressure, reducing body fat, raising High-Density Lipoprotein (HDL) and lowering Low-Density Lipoprotein (LDL) levels, improving blood flow, and increasing heart's working capacity [1,2]. We recently reported as a single bout exercise decreases homocysteine plasma levels which is a well known risk factor for vascular disease [11,12]. Several studies suggest that homocysteine plasma levels are related to Asymmetric Dimethylarginine (ADMA) concentrations [13-15]. ADMA is a post-translationally modified form of arginine that is generated in all cells during

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protein turnover. ADMA is released from hydrolysis of proteins containing methylated L-arginine residues. L-arginine and free L-methylarginines are detectable in cell cytosol, plasma, tissues, and urine. L-arginine is metabolized by nitric oxide synthase to Nitric Oxide (NO), ADMA acts as an endogenous competitive inhibitor of this reaction. The inhibition by ADMA of NO synthesis is well known to assume a pathophysiological significance. Defects in NO generation have been associated with the pathogenesis of diseases such as arteriosclerosis, hypertension, and endothelial dysfunctions [16-18]. In the cardiovascular system NO is known for being the most potent vasodilator, and decreased NO biosynthesis has the potential to increase blood pressure, enhance platelet aggregation and leucocyte adhesion, increase vascular smooth muscle growth, alter mitochondrial oxygen consumption, and accelerate the development of arteriosclerotic lesions [16,18,19]. Recently has been reported as a regular physical activity reduce the ADMA plasma levels in subjects with type 1 diabetes mellitus [20]. Aim of this study is to evaluate whether a single bout of physical exercise affects ADMA plasma levels in healthy young subjects as well as to investigate whether ADMA variations may be related to the decreased levels of plasma homocysteine that we have already observed in the same subjects after a single bout of exercise [11,12].

Methods

1. Subjects recruitment

Sixteen young males, aged between 21 and 37 (mean 28.8 ± 4.8 years), were selected for the study on the analytes levels variation after a single bout exercise (incremental cycle ergometer stress test). In particular, 6 were sedentary individuals while 10 have practiced for 2 years a Thai Boxe training of 90 min, 3 days a week. Exclusion criteria included: hyperlipidemia, hypertension, use of alimentary integrators or vitamin therapy; renal disease (eGFR under $60 \text{ mL/min/1.73m}^2$); diabetes mellitus (fasting blood glucose $>125 \text{ mg/dL}$). Furthermore, all subjects participating at this study had normal values of TSH (normal ranges between 0.5-5.0

mU/L), that were measured at the beginning and at the end of the protocol. The body mass index of all individuals was between 19 and 27% ($24.4 \pm 2.2\%$). As confirmed by medical interviews, participants were not receiving dietary supplements of vitamin B6, B12, folate, or drug therapy. All the volunteers selected were non-smokers.

2. Single bout exercise - incremental cycle ergometer stress test till exhaustion

The test was performed in the morning (between 8:00 and 10:00) and was performed in fasted state. During the performance not drink or food was consumed. The test included a pre-test phase that lasted three minutes and was characterized by a standard work load of 25 W for all the subjects. This phase was followed by an incremental work load in steps of 50 W, each lasting 2 minutes until exhaustion (stress test phase). On subjects' demand or when the preset cycling frequency (60 rpm) was no longer maintained, the work load was brought down to 25 W and the subjects were asked to keep pedalling for three more minutes (active recovery phase). At the end of this phase the subjects stopped pedalling remaining seated on the cycle ergometer for three more minutes (passive recovery phase). Immediately after the end of the test a blood sample was taken for the evaluation of homocysteine and ADMA concentrations.

3. ADMA and homocysteine measurement

Blood was collected by venipuncture into evacuated tubes containing EDTA, and immediately centrifuged at $3000g \times 5\text{min}$ at 4°C for 2 minutes. Plasma was aliquoted and then stored at -80°C ; analysis of reduced homocysteine was performed by capillary electrophoresis with laser induced detection, as already reported [21]. In brief, 200 μL of plasma sample were deproteinized by adding 50 μL of 15% 5-sulfosalicylic acid and centrifuged at $2000g$ for 5 min. To 150 mL of supernatant was added 30 mL of 1 mmol/L NaOH. Then 50 μL sample was mixed with 100 μL of 100 mmol/L sodium phosphate buffer, pH 12.5, and 15 μL of 0.8 mmol/L 5-IAF. After vortex-mixing, samples were incubated for 15 min at room temperature. Derivatized

samples were diluted 100-fold in water and analyzed by a P/ACE 5510 capillary electrophoresis system equipped with laser-induced fluorescence was used. The dimension of the uncoated fused-silica capillary was 75 μm ID and 57 cm length (50 cm to the detection window). Analysis was performed applying 14 nL of sample under nitrogen pressure (0.5 psi) for 2s. A run buffer composed by 18 mmol/L sodium phosphate, 14.5 mmol/L boric acid as electrolyte solution with 75 mmol/L N-methyl-Dglucamine, pH 11.4, was employed. The separating conditions (22 kV, 150 μA at normal polarity) were reached in 20 s and held at a constant voltage for 9 min. All separations were carried out at 40°C.

Free ADMA was measured by capillary electrophoresis with UV detection as previously reported [22]. Briefly, 400 μL of obtained plasma were mixed with 50 μL I.S. homoarginine (50 $\mu\text{mol/L}$ final concentration). 900 μL of acetonitrile/ammonia (90/10) were then added to precipitate proteins. After centrifugation at 3000 g for 5 min the clear supernatant was evaporated in vacuum and the residue was redissolved with 500 μL of water filtered in Vivaspinn 500 microconcentrators by centrifugation at 3000xg for 20 min to further remove residual proteins. Filtered samples were finally evaporated in vacuum and the residue was re-dissolved with 50 μL of water and injected in CE. Analysis was performed in an uncoated fused-silica capillary, 75 μm I.D. and 60.2 cm length (50 cm to the detection window), injecting 1s water plug (0.5 psi) followed by 10s of sample (0.5 psi). Separation was carried out in a 50 mmol/L Tris buffer titrated with 1 mol/L phosphoric acid to the pH 2.30, 15°C at normal polarity 15 kV. Inter-assay CV of employed method was 3.3%.

Protein incorporated ADMA was measured by capillary electrophoresis with UV detection as already reported [23]. In brief, blood proteins were treated with 1 mL of trichloroacetic acid (TCA) 5%. After centrifugation at 3000 g for 5 min pellet was washed two times with 1 mL TCA 3%. Purified proteins were then hydrolyzed by 400 μL of 6M HCl at 110°C for 16 hours. Then samples were dried and dissolved in 1 mL of water before capillary

electrophoresis analysis. A MDQ capillary electrophoresis system equipped with a diode array detector was used. Analysis was performed in an uncoated fused-silica capillary, 75 μm I.D. and 60.2 cm length (50 cm to the detection window), in a 50 mmol/L Tris buffer titrated with 1 mol/L phosphoric acid to the pH 2.15, 15°C at normal polarity 12 kV.

The Lowry's method was used to measure plasma protein concentration.

4. Statistics

Values were expressed as means \pm SD. The variables distribution within the study group was assessed by Kolmogorov-Smirnov test. Statistical differences among data were compared using the paired Student's t test. Correlation analysis between variables were performed by Pearson's correlation. A difference of $P < 0.05$ was considered to be statistically significant. Statistical was performed by Statgraphics plus 5.0.

Results and Discussion

Nitric oxide is a very active, but short living, molecule that is released in the circulation by endothelial cells. It is a potent vasodilator that regulates vascular resistance and tissue blood flow. In the last decade a body of evidence pointed out the role of reduced bioavailability of NO in the development of endothelial dysfunction, which is the first step in the process of atherosclerosis. NO inhibits, in fact, key processes of atherosclerosis such as monocyte endothelial adhesion, platelet aggregation, and vascular smooth muscle cell proliferation. It is synthesized by stereospecific oxidation of the terminal guanidino nitrogen of the amino acid, L-arginine, by the action of a family of nitric oxide synthases (NOS). Guanidino-substituted analogues of L-arginine such as asymmetric dimethylarginine can selectively inhibit NOS by competitive blockade of its active site [24]. ADMA is a post-translationally modified form of arginine that is generated in all cells during the process of protein turnover. Its biosynthesis is catalyzed by a family of enzymes named protein-arginine-N-methyltransferases [25]. These enzymes utilize the intermediate of methionine-homocysteine pathway S-adenosylmethionine as a methyl group donor [26]. After donating its methyl

group, SAM becomes S-adenosylhomocysteine, which then becomes homocysteine after losing its adenosine (a reversible reaction catalyzed by the enzyme SAH hydrolase). For this reason, it has been suggested that ADMA biosynthesis and homocysteine metabolism are linked to each other, and indeed elevated ADMA has been found in various models of hyperhomocysteinemia [27]. Moreover, recently, another link between ADMA and Hcy has been proposed. This involves the Dimethylarginine Dimethylaminohydrolase (DDAH) enzyme which is responsible for ADMA catabolism via the hydrolysis to dimethylamine and citrulline. It has been reported that in a cell-free system, homocysteine directly inhibits the activity of DDAH [28] leading to ADMA accumulation in presence of high Hcy levels.

Elevated ADMA concentrations have been described in several diseases that are associated with increased cardiovascular risk; for example, raised ADMA levels predict cardiovascular events in patients with renal failure and in men with coronary heart disease [16-18]. Recently has been reported as a regular aerobic exercise lowered ADMA plasma levels in subjects with type 1 diabetes mellitus [20]. To evaluate how asymmetric dimethylarginine concentration are affected from physical activity we measured plasma ADMA levels in 16 young volunteers before and after a single bout of physical exercise and found a significant decrease of this amino acid ($0.520 \pm 0.081 \mu\text{mol/L}$ vs $0.480 \pm 0.080 \mu\text{mol/L}$; $p < 0.001$) after the physical effort (Figure 1a). In the same subjects we have also found that reduced homocysteine decreased following the same exercise (Figure 1b) (rHcy $0.354 \pm 0.083 \mu\text{mol/L}$ vs $0.321 \pm 0.079 \mu\text{mol/L}$, $p < 0.001$), while total homocysteine levels were unaffected (tHcy $10.3 \pm 3.5 \mu\text{mol/L}$ vs $10.8 \pm 4.0 \mu\text{mol/L}$, $p = 0.35$). Circulating homocysteine should be found in small quantity (0.2-0.5%) in the reduced form, about 10-20% exists as low molecular weight oxidized disulfides (homocystine and homocysteine-cysteine mixed disulfide), and a major quantity (70-80%) is bound to protein cysteine residues via thiol disulfide bond [29,30]. The sum of the three forms is commonly defined as total homocysteine. The decrease of rHcy

concentrations rather than total form can be explained considering the physiological dynamism of rHcy concentrations in comparison to those of tHcy (that is principally linked with proteins or with other low molecular mass thiols), and taking into account that Hcy is synthesized in the reduced form. Therefore, acute perturbations of Hcy metabolism should influence primarily the reduced fraction.

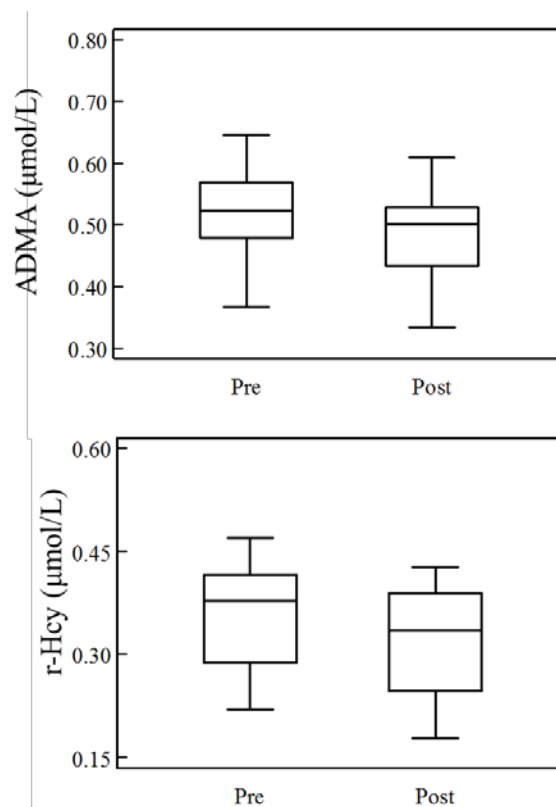


Figure 1: Box plots of ADMA and Hcy plasma concentrations. The central horizontal line on each box represents the median, the ends of the boxes are 25 and 75 percentiles and the error bars 5% and 95%.

We also evaluate the dehydration degree estimated from the difference between the post- and pre-exercise plasma proteins concentrations. We found that plasma proteins levels were significantly higher after exercise ($85.0 \pm 4.8 \text{ mg/mL}$ vs $78.9 \pm 5.2 \text{ mg/mL}$, $p < 0.001$) that correspond to a concentration factor of about 1.077. Thus the decrease of ADMA and rHcy concentrations should be greater than those observed since they are partially masked by haemoconcentration effects. Pearson's correlation between ADMA and reduced Hcy confirm the relationship, amply described in literature for total homocysteine, between these amino acids. We

found a positive significant correlation both before ($r=0.53$, $P=0.035$) and, even if slightly weaker, also after ($r=0.49$, $P=0.053$) physical exercise (Figure 2).

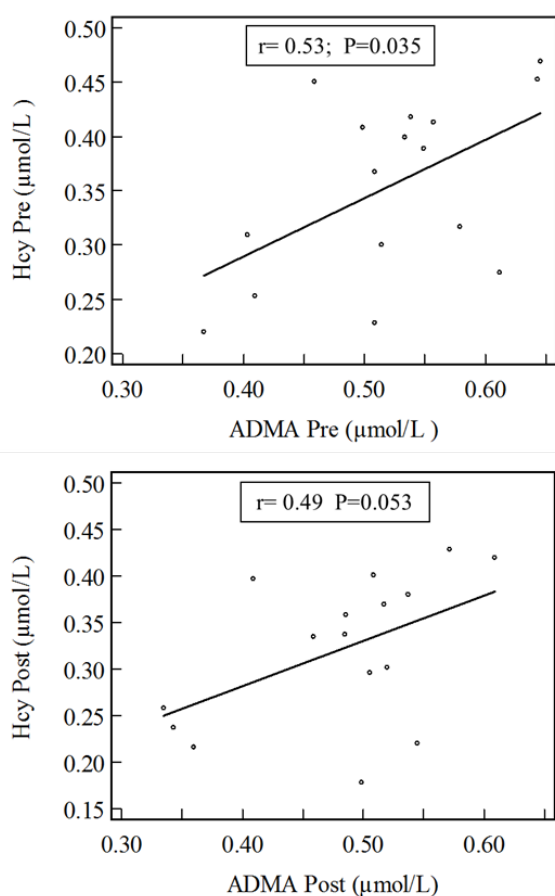


Figure 2: Pearson's correlation between reduced homocysteine and ADMA before and after the physical exercise.

To evaluate whether ADMA decrease was correlate to Hcy lowering, the differences between Hcy concentration pre- and post- exercise were plotted against the differences between ADMA levels after the physical activity. As shown in figure 3 there is a strong correlation between the differences of analytes before and after exercises ($r=0.75$, $P<0.001$) thus suggesting that the decrease concentration of the two analytes was effectively related. To verify whether this relationship was due to a decrease in protein methylation reaction (with a lower ADMA production) or to a reduced DDAH inhibition (due to Hcy lowering) we measured blood protein ADMA content in the 16 subjects. No significant differences were found in the blood protein methylation (4.335 ± 0.785 vs 4.321 ± 0.712 nmol/mg protein respectively for pre and post exercise, $p=0.96$)

suggesting that free ADMA decrease after exercise does not depend on a decrease in ADMA production, but it is probably due to ADMA elimination. Since ADMA was eliminated mainly by DDAH activity it is evident that after the single bout of exercise there is an increase activity of this enzyme almost certainly imputable to the decrease of its inhibitor homocysteine.

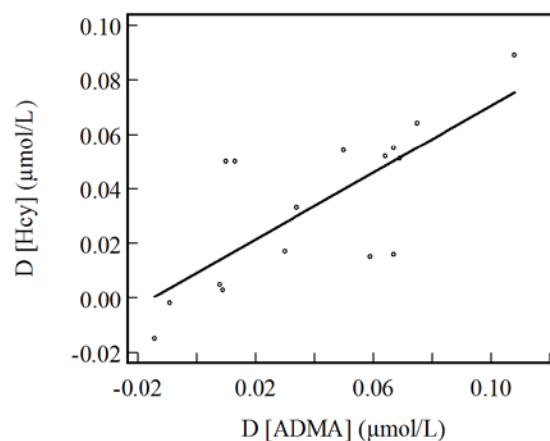


Figure 3: Pearson's correlation obtained plotting the differences between Hcy concentration before and after exercise with the differences between ADMA levels before and after the same exercise

Even though we did not evaluate DDAH levels in blood or tissues of studied subjects we assume that the levels of this enzyme is practically unchanged during exercise, considering that the blood samples (before and after exercise) were taken within 15-20 minutes. It is reasonable, in fact, that this time is not sufficient to stimulate and produce a neo-synthesis of enzyme DDAH. Therefore the decrease in Hcy concentration after physical exercise lead to an increase of DDAH activity (minor inhibition) which in turn reduces ADMA plasma levels. It seems important to emphasize that Hcy and ADMA lowering are already detectable after a single bout of exercise. On the basis of these results it can be hypothesized that more significant reductions of these analytes should be observed when the physical activity is performed constantly for a drawn out period.

6. Conclusion

The beneficial effects of physical exercise on health is well known. We recently reported as moderate physical activity may reduce plasma levels of homocysteine, an important risk factor for vascular disease. Moreover the

biochemical mechanism by which physical activity determine its lowering in plasma were also elucidated for the first time by our group [11,12]. The mechanisms responsible for endothelial dysfunction in hyperhomocyst(e)inemia remained unclear. In high concentrations, homocyst(e)ine is directly toxic to cultured endothelial cells, and it may decrease endothelial production of NO through oxidative mechanisms. Boger suggest that an increased generation of ADMA may be an alternative mechanism of endothelial dysfunction in hyperhomocyst(e)inemia [14]. In fact is amply reported as elevated concentration of Hcy is often accompanied with an increase in ADMA levels. This is probably due to the fact that Hcy and ADMA metabolism are strictly linked and that homocysteine is an important inhibitor of DDAH activity. In this study we described for the first time that plasma ADMA levels are lowered in healthy subjects after a single bout of physical exercise and we believe to have reported experimental evidences that its decrease is in turn determined by reduced homocysteine lowering. ADMA decrease may then have beneficial effects on health by increasing of NO bioavailability that in turn acts through inhibition of important processes of atherosclerosis such as monocyte endothelial adhesion, platelet aggregation, and vascular smooth muscle cell proliferation.

These findings may therefore shed some light on some of the biochemical mechanisms which, triggered by physical activity, may exert a positive effect in maintaining healthy status.

Acknowledgement

This study was supported by the "Fondazione Banco di Sardegna – Sassari – Italy" and by the "Ministero dell'Università e della Ricerca" Italy. The manuscript language revision by Mrs Maria Antonietta Meloni is greatly appreciated.

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