Pharmacological Reports
2012, 64, 369–376
ISSN 1734-1140

Single bout of endurance exercise increases NNMT activity in the liver and MNA concentration in plasma; the role of IL-6

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Abstract:
Background: Methylnicotinamide (MNA) displays vasoprotective activity, however, the regulation of the activity of nicotinamide-N-methyltransferase (NNMT), is largely unknown. We analyze a possible involvement of IL-6 in the activation of NNMT-MNA pathway during an endurance exercise.
Methods: FVB, C57Bl/6J IL6$^{+/+}$ and C57Bl/6J IL-6$^{-/-}$ mice were subjected to the single bout of endurance exercise consisting of 90 min of swimming. Thereafter, exercise-induced changes in NNMT activity in the liver as well as concomitant changes in the concentration of MNA and its further metabolites in plasma were analyzed.
Results: In two strains of mice (FVB and C57Bl/6J IL6$^{+/+}$) 90 min of swimming resulted in approximately 2–3 folds increase in NNMT activity (from 0.14 ± 0.03 to 0.421 ± 0.02 pmol/min/mg, $p < 0.05$ and from 0.2 ± 0.06 to 0.35 ± 0.07 pmol/min/mg, $p < 0.01$, respectively) and concomitant increase in the plasma concentration of MNA (from 157 ± 15.06 to 230 ± 16.2 ng/ml, $p < 0.01$, and from 77.05 ± 14.6 ng/ml to 152.55 ± 58.4 ng/ml; $p < 0.01$, respectively). However, in C57Bl/6J IL-6$^{-/-}$ mice 90 min of swimming did not change liver NNMT activity (from 0.25 ± 0.07 to 0.23 ± 0.06 pmol/min/mg), while MNA concentration in plasma rose approximately two-fold (from 65.3 ± 30.9 ng/ml to 124.8 ± 35.8 ng/ml; $p < 0.05$).
Conclusions: We demonstrated for the first time that NNMT – MNA pathway is activated by a single bout of endurance exercise. Interestingly, exercise-induced activation of NNMT in the liver involves IL-6, while the rise in MNA concentration in plasma was partially IL-6-independent. Taking into the consideration the pharmacological activity of MNA, IL-6-dependent and IL-6-independent activation of NNMT, may contribute to the exercise capacity. The physiological role of NNMT in the exercise warrant further studies.

Key words:
endurance exercise, interleukin-6, nicotinamide N-methyltransferase, 1-methylnicotinamide

Introduction

N-methyltransferase (NNMT) is an enzyme responsible for N-metylation of nicotinamide based on the transferring a methyl group of S-adenosylmethionine (SAM) to nicotinamide (NA) and the synthesis of 1-methylnicotinamide (MNA) and S-adenosylhomocysteine (SAH) [1, 18]. Although NNMT is found mainly in the liver, its expression has been reported in other tissues e.g., in the kidney, lung, skeletal muscle, heart and brain [1, 27]. In number of pathologies for instance, cancer [27], atherosclerosis [18] or liver diseases [25] the up-regulation of NNMT was reported, however, the pathways of regulation of the activity and expression of this enzyme remain largely unknown.

It was demonstrated that in skeletal myoblast *in vitro* NMMT expression was enhanced by cytokines such as IL-6, TNFα and TGFβ [15]. Previous work also showed that NNMT gene expression in some type of cancer cells (thyroid and liver) is stimulated by HNF-1 β (hepatic nuclear factor-1), which works as a transcription activator and binds to NNMT basal promoter region identified as HNF-1 binding site [33]. HNF-1 binding sites were also detected in metabolic genes including glucose-6-phosphatase (G6P), phosphoenolpyruvate-carboxykinase (PEPCK) as well as acute phase proteins. Interestingly, HNF-1 dependent activation of genes responsible for metabolic homeostasis and acute phase response may be dependent on IL-6 [5, 17, 29, 31].

It is well known that IL-6 plays a central role in variety of inflammatory conditions including cardiovascular diseases [10, 14]. Interestingly, it has been recently appreciated that IL-6 production is stimulated by prolonged exercise and IL-6-dependent response may play an adaptive role in human body during exercise [20]. For example, IL-6-dependent pathways regulate fatty acid oxidation, liver and muscle glucose uptake and gluconeogenesis [2, 3, 7, 9, 13]. Furthermore, IL-6 increases AMPK (AMP-activated protein kinase) activity [7] and mediates anti-inflammatory effects by stimulating the production of anti-inflammatory cytokines and by suppressing the production of TNFα [19, 23, 24]. Accordingly, it might well be that muscle-derived IL-6 released during demanding exercise may influence exercise performance [12, 22]. In this context, we aimed to test whether in the physiological setting of endurance exercise the activity of NNMT-MNA pathway is also regulated by IL-6. Potentially, increased production of MNA may activate prostacyclin release [8] and thus contribute to the adaptive vascular response during exercise.

In the present work, firstly, we analyzed whether in untrained, healthy mice (FVB, C57Bl/6J IL-$6^{+/+}$) a single bout of endurance exercise display en effect on NNMT activity in the liver as well as on the plasma concentration of MNA. Secondly, we tested whether IL-6 plays a role in the activation of liver NNMT-derived MNA using IL-6 knockout mice (C57Bl/6J IL-$6^{-/-}$).

Materials and Methods

All animal procedures conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996), and the experimental procedures used in the present study were approved by the local Jagiellonian University Ethical Committee on Animal Experiments.

Animals and exercise protocol

FVB, C57Bl/6J IL-$6^{+/+}$ C57Bl/6J IL-$6^{-/-}$ mice were used for the experiments. FVB mice were bred in the Animal Laboratory of the Polish Academy of Science Medical Research Centre in Warszawa, while C57Bl/6J IL-$6^{+/+}$, C57Bl/6J IL-$6^{-/-}$ mice in Centre for Experimental Medicine of Medical University in Białystok. Mice were housed in pathogen free conditions, fed a standard laboratory diet and given water *ad libitum*.

Animals were divided in the six experimental groups: FVB mice – basal (not subjected to exercise), (n = 20), FVB mice subjected to exercise (n = 19), C57Bl/6J IL-$6^{+/+}$ and C57Bl/6J IL-$6^{-/-}$ mice – basal (not subjected to exercise) and subjected to exercise (n = 8 in each group). Before the main bout of swimming exercise, mice were familiarized to the swimming condition by placing them in a glass (24 cm high, 25 cm diameter) filled with warm water (30–32°C) twice in the two following days, each time for 15 min. After one day of rest, the main exercise bout was performed which consisted of 90 min swimming session. Immediately after exercise animals were anesthetized with thiopental (Sandoz

Biochemie GmbH, Kundl, Austria; 10 mg/kg, *ip*) and blood samples as well as liver samples were collected. Blood samples were taken from the left ventricle of the heart into heparinized tubes and were centrifuged for 8 min (13,000 × g) to obtain plasma. Then, samples were stored frozen at –80°C until analyzing the concentration of MNA and its metabolites. A liver sample (approximately 300 mg) was taken and frozen in liquid nitrogen until assayed for hepatic NNMT activity.

Measurements of NNMT activity

To evaluate the activity of NNMT, liver homogenates were assayed for the enzymatic conversion of 4-methylnicotinamide (4-MNA) to 1,4-dimethylnicotinamide (1,4-MNA) by the modified fluorometric method of Sano et al. [26]. The method consists of a determination of the product, 1,4-MNA, where 4-MNA is the methyl acceptor substrate in the presence of cofactor Sadenosyl-L-methionine (AdoMet).

Liver samples were collected and washed with cold 0.9% (w/v) sodium chloride, immediately frozen in liquid nitrogen and kept at –80°C. The tissue was homogenized 1:9 (w/v) in cold PBS buffer with a teflon-glass homogenizer and centrifuged at 12,000 × g for 20 min at 4°C. The supernatant was used immediately to assay NNMT activity. Protein concentration was determined by the method of Bradford [6] using BSA as the standard. The reaction mixture consisted of 12.5 µl of 2 mM dithiothreitol, 12.5 µl of 0.8 M Tris-HCl buffer (pH = 8.6), 25 µl of 16 mM 4-MNA, 50 µl of 0.4 mM AdoMet in 0.1 mM sulfuric acid and 100 µl of the enzyme preparation. The mixture was incubated at 37°C for 80 min. The enzyme reaction was terminated by heating in a boiling water bath for 2 min. To prepare the blank, the same procedure was used but the reaction was stopped immediately without incubation. Reaction mixtures were centrifuged at 13,000 × g for 5 min at room temperature. A 100-µl aliquot of the supernatant was poured into 1.5-ml stopper test tubes containing 1 ml of 0.02 M 4-methoxybenzaldehyde in 35% (v/v) aqueous ethanol and 100 µl of 0.5 M aqueous sodium hydroxide. The tube was heated in a boiling water bath for 15 min. After cooling, the mixture was centrifuged again at 13,000 × g for 10 min at room temperature. The fluorescence intensity was measured with excitation at 418 nm and emission at 490 nm. To quantify the reaction product, 1,4-MNA was calculated using a calibrating curve for 1,4-DMN at the concentration range 0.2–2.0 nM, where the curve was linear.

Measurement of plasma concentrations of NA, MNA and further metabolites (Met-2PY and Met-4PY)

To measure plasma concentrations of NA, MNA, 1-methyl-2-pyridone-5-carboxamide (Met-2PY) and 1-methyl-4-pyridone-5-carboxamide (Met-4PY) liquid chromatography tandem mass spectrometry (LC-ESI/MS/MS) was used. Chloronicotinamide was used as an internal standard. Purification of the samples was performed by protein precipitation procedure. After centrifugation, plasma supernatant was transferred into conical vial and evaporated to dryness under the stream of nitrogen gas in a water bath at 37°C. Dry residue was reconstituted in the ACN/water mixture and 20 µl was injected into an HPLC system (Agilent 1100, Agilent Technologies, Waldbronn, Germany). Chromatography was performed on Waters Spherisorb® 5 µm CNRP 4.6 × 150 mm analytical column with gradient elution using a mobile phase containing acetonitrile and water. The full separation of all compounds was achieved within 15 min of analysis. Detection was performed by an Applied Biosystems MDS Sciex API 2000 triple quadrupole mass spectrometer set at unit resolution. The mass spectrometer was operated in the selected reactions monitoring mode (SRM), monitoring the transition of the protonated molecular ions *m/z* 153 to 110 for Met-2PY, 153 to 136 for Met-4PY, 123 to 80 for NA and 137 to 94 for MNA. The mass spectrometric conditions were optimized for each compound by continuously infusing the standard solution at the rate of 5 µl/min using a Harvard infusion pump. Electrospray ionization (ESI) was used for ion production. The validated quantitation ranges for this method were from 10 to 2,000 ng/ml for all analyzed compounds with precision 1.3–13.3% and accuracy 94.43–110.88%. No significant matrix effect was observed. Stability of compounds was established in a battery of stability studies, i.e., bench-top, autosampler and long-term storage stability as well as freeze/thaw cycles. The complete method and validation parameters were described previously [30].

Statistical analysis

All results are expressed as the mean ± SEM. Significance of differences between groups was established by Kruskal-Wallis test followed by Dunn's multiple comparison test. Statistical analysis was performed using Prism 5 (GraphPad Software Inc.).

Results

Exercise performance

All FVB, C57 IL-6$^{+/+}$ mice finished successfully 90 min of swimming session. After exercise session, mice were agile and moved normally along the cage taking care about their wet fur. In contrast, only 75% of the C57 IL-6$^{-/-}$ mice finished exercise protocol. The average time of swimming was 85 ± 5.5 min). After physical activity in all C57 IL-6$^{-/-}$ mice symptoms of exhaustion were observed (mice were less agile in comparison to FVB, C57 IL-6$^{+/+}$ and did not start to take care about their fur after the exercise).

Effect of single bout of endurance exercise on activity of NMMT in the liver

As shown in Figure 1, single bout of endurance swimming exercise induced the increase in hepatic NNMT activity by approximately three folds in FVB mice (0.421 ± 0.02 *vs.* 0.14 ± 0.03 pmol/min/mg; $p < 0.05$) and by approximately two-fold in C57Bl/6J IL-6$^{+/+}$ mice (0.35 ± 0.07 *vs.* 0.2 ± 0.06 pmol/min/mg, $p < 0.01$). In contrast, in C57 IL6$^{-/-}$ mice the same exercise protocol did not result in the increase in the activity of NMMT in the liver.

Effect of single bout of endurance exercise on plasma concentration of MNA and its further metabolites

In all three groups of mice (FVB, C57Bl/6J IL-6$^{+/+}$ and C57Bl/6J IL-6$^{-/-}$) single bout of endurance exercise induced a consistent rise in the plasma concentration of MNA (Fig. 2A). The plasma concentration of MNA increased from 157 ± 15.06 ng/ml to 230 ± 16.20 ng/ml ($p < 0.01$) and from 77.05 ± 14.6 ng/ml to 152.55 ± 58.449 ng/ml ($p < 0.01$) in FVB and C57IL-6$^{+/+}$, respectively. Surprisingly, in C57IL6$^{-/-}$ mice the plasma concentration of MNA also increased subsequent to exercise (from 65.3 ± 30.9 ng/ml to 124.8 ± 35.8 ng/ml ($p < 0.05$).

The effects of exercise on plasma concentration of NA, Met-2PY and Met-4PY are shown on Figure 2B-D. In all three groups of mice (FVB, C57Bl/6J IL-6$^{+/+}$ and C57Bl/6J IL-6$^{-/-}$) exercise induced a rise in the plasma concentration of NA, Met-2PY, and Met-4PY though in some cases the increase was not significant. Interestingly, in C57Bl/6J IL-6$^{-/-}$ mice exercise-induced rise in Met-2PY and Met-4PY was less pronounced as compared to C57Bl/6J IL-6$^{+/+}$, while the increase in NA was similar in C57Bl/6J IL-6$^{+/+}$ and C57Bl/6J IL-6$^{-/-}$ mice.

Discussion

In the present work we demonstrated that a single bout of the endurance exercise increased the activity of NMMT in the liver as well as increased the plasma concentration of MNA in untrained, healthy mice. We used FVB as well as C57Bl/6J mice to show that NNMT response to exercise is not limited to one given strain of mice but may represent a more general phenomenon. Most importantly, our results demonstrated that exercise-induced activation of NNMT in the liver was absent in IL-6 knockout mice, while exercise-induced rise in MNA concentration in plasma was only partially attenuated in IL-6 knockout mice. Altogether, we demonstrate for the first time that NNMT-MNA pathway is activated by exercise and displays IL-6-dependent component targeted to the NNMT in the liver and IL-6-independent compo-

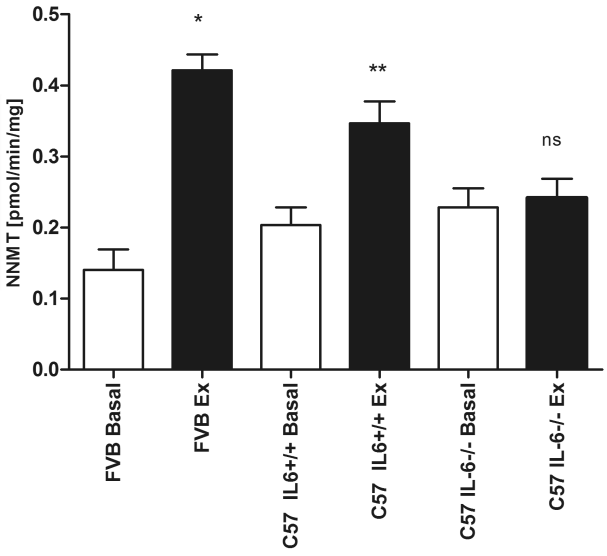

Fig. 1. Effects of a single bout of endurance exercise (90 min) on the activity of nicotinamide N-methyltransferase (NNMT) in the liver in FVB, C57Bl/6J IL-6$^{+/+}$ and C57Bl/6J IL-6$^{-/-}$ mice. Results are presented as the mean ± SEM (* denotes $p < 0.05$; ** denotes $p < 0.01$; ns denotes non-significant; for exercise *vs.* basal)

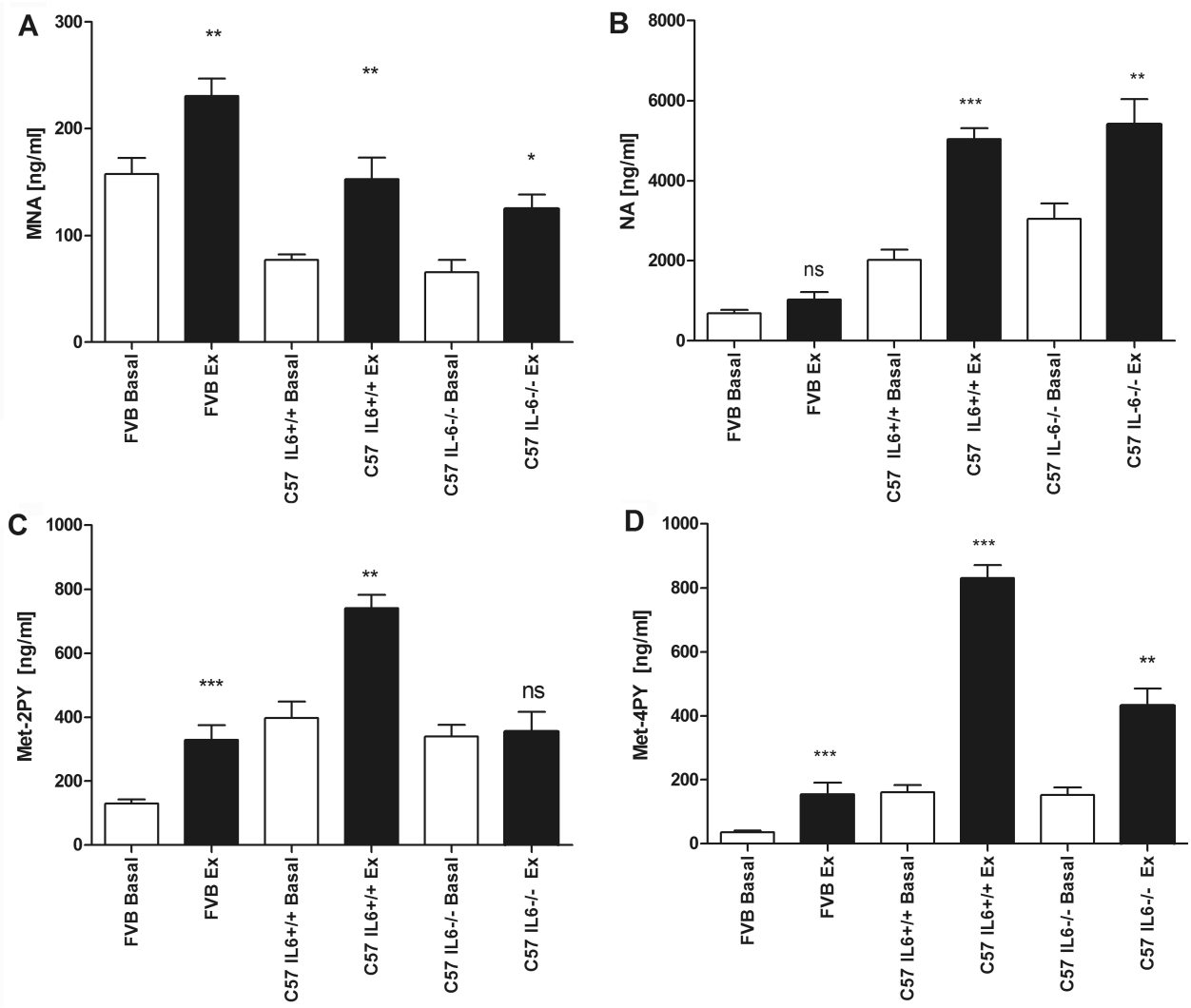

Fig. 2. Effects of single bout of endurance exercise (90 min) in FVB, C57Bl/6J IL-$6^{+/+}$ and C57Bl/6J IL-$6^{-/-}$ mice on the plasma concentration of 1-methylnicotinamide (MNA) (**A**), nicotinamide (NA) (**B**), 1-methyl-2-pyridone-5-carboxamide (Met-2PY) (**C**) and 1-methyl-4-pyridone-5-carboxamide (Met-4PY) (**D**). Results are presented as the mean ± SEM. * denotes $p < 0.05$; ** denotes $p < 0.01$;*** denotes $p < 0.001$; ns denotes non-significant; for exercise *vs.* basal)

nent most likely dependent on the activation of NNMT in other tissues.

There is overwhelming evidence that physical exercise has positive effects on cardiovascular health status [16, 21, 34]. A number of mechanisms have been postulated to mediate anti-inflammatory [23], anti-atherosclerotic [28], anti-platelet [32] and endothelial effects of physical exercise [28, 35]. Interestingly, increased activity of NNMT and subsequent increased formation of MNA may also contribute to the beneficial effects of exercise on vascular wall function. Indeed, MNA displays remarkable anti-thrombotic and anti-inflammatory activities, depending on PGI_2 derived from COX-2 [8]. MNA prevented also development of endothelial dysfunction in diabetes and hypertriglyceridemia [4] that could also be related to MNA-dependent stimulation of COX-2/PGI_2 pathway. In the light of the vasoprotective effects of exogenous MNA, it is tempting to speculate that an increase in activity of NNMT and subsequent elevation of plasma concentration of endogenous MNA provide an important pathway regulating thrombotic as well as inflammatory processes that might be activated by exercise [36]. In the present work, we did not measure PGI_2 release during

exercise nor we monitor pro-thrombotic, pro-inflammatory effects of exercise, we only examined the role of IL-6 in the activation of NNMT-MNA pathway. Accordingly, the hypothesis on the role of NNMT-MNA-PGI_2 pathway in the vascular adaptation to exercise needs to be addressed in more details in further experimental studies.

It was recently demonstrated that IL-6 increases hepatic glucose production or lipolysis in adipose tissue during exercise [20]. Interestingly, in the present study we have observed that the physical performance during the 90 min swimming session of the C57 IL-$6^{-/-}$ mice was poorer than that of the FVB or C57 IL-$6^{+/+}$ mice. Namely, all mice from FVB or C57 IL-$6^{+/+}$ group successfully finished the 90 min swimming session without symptoms of exhaustion, whereas only 75% of the IL-6 knockout mice finished the exercise protocol and in all of them symptoms of exhaustion were observed. Therefore, the present study provide further evidence that IL-6 play an important role in endurance performance, most likely by improvement of energy homeostasis during exercise, as postulated previously [2, 3, 7, 9, 20].

In the present work, we demonstrated that in IL-6 knock-out mice the activation of NNMT activity in the liver by exercise was completely lost, while concomitant rise in MNA concentration in plasma was only slightly attenuated. These results suggest that exercise-induced activation of NNMT in the liver involves IL-6, while the rise in MNA concentration in plasma as well as its further metabolites such as Met-2PY and Met-4PY was partially IL-6-independent. Although the liver represents the major source of NMMT activity, it seems likely that the elevation of MNA in the plasma induced by exercise is not only due to the increased activity of NNMT in the liver, but also in other tissues such as lungs, kidneys or muscles, in all of which organs NNMT activity was shown [1, 27]. Accordingly, our results suggest that exercise- induced up-regulation of NNMT activity in the liver involved IL-6, while in other tissues activation of NNMT was IL-6-independent.

In previous work, it was suggested that IL-6 regulates NNMT activity hepatocytes *via* HNF 1 [17], but it is not known whether IL-6 is involved in the regulation of NNMT activity in skeletal muscle, lung or kidney. Interestingly, our unpublished results suggest that relatively high activity of NNMT is present in endothelium and in our hands IL-6 did not modulate NNMT activity in cultured endothelium. Thus, it may well be that IL-6-independent component of NNMT-derived MNA originated from vascular endothelium.

It is worth noting that regulation of NNMT activity may be also linked to the metabolic insult. NNMT methylation activity in the liver was shown to be inversely related to ATP content [25]. Ninety minutes of exhausting swimming session in mice represents a great metabolic challenge and could significantly reduce muscle and liver glucose level [11] and disturb muscle metabolic stability. We did not measure ATP or NA contents in the liver or skeletal muscle, but we demonstrated that 90 min of exercise in mice resulted in significant elevation of NA concentration in plasma that is most likely linked to the degradation of NAD^+ and oxidant and metabolic stress of the exercise. Thus, it could well be that exercise induced not only IL-6-dependent activation of NNMT in the liver but also, independently, the exercise-induced increase in the availability of NA, providing a substrate for increased NNMT activity.

It is worth noting that activation of NNMT may be also linked to alterations in concentration of SAH and homocysteine in the liver and in plasma as well as may lead to changes in the methylation status. Further studies are needed to address that issue.

In conclusion, in the present paper we demonstrated for the first time that NNMT-MNA pathway is activated by a single bout of endurance exercise in mice. IL-6-dependent activation of NNMT in the liver, and IL-6-independent activation of NNMT in other tissue(s), both contribute to that response. Taking into consideration the anti-thrombotic, anti-inflammatory and vasoprotective activity of MNA, it is tempting to speculate that NNMT-MNA pathway activation by exercise could contribute to the beneficial effects of exercise on vascular wall function. Further studies on the physiological role of NNMT-MNA pathway in physical performance are warranted.

Acknowledgment:
This work was supported by the European Union from the resources of the European Regional Development Fund under the Innovative Economy Programme (grant coordinated by JCET-UJ, No. POIG.01.01.02-00-069/09).

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Received: September 22, 2011; **in the revised form:** December 30, 2011; **accepted:** January 5, 2012.