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Short communication

N-methylnicotinamide failed to induce endothelial prostacyclin release in perfused rat hindquarters

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Abstract:
N-methylnicotinamide, a nicotinamide derivative, possesses anti-thrombotic activity, although the mechanism of its action is unclear. Using a rat model of isolated perfused hindlimb, we tested whether this metabolite of nicotinamide is able to inhibit the vasoconstrictive effects of epinephrine, norepinephrine, and angiotensin II, thereby releasing prostacyclin from the endothelium. We found that N-methylnicotinamide administration by infusion or bolus injection did not change the course of perfusion pressure and did not inhibit the vasoconstrictive action of epinephrine, norepinephrine, or angiotensin II. In contrast, prazosin was able to completely abolish the constriction induced by epinephrine. Moreover, we did not find any changes in the level of a stable prostacyclin analog measured in the collected perfusate samples. Thus, we did not observe any endothelial prostacyclin-releasing properties of N-methylnicotinamide in the perfused rat hindquarters model.

Key words:
N-methylnicotinamide, endothelium, prostacyclin, rat

Abbreviations: COX – cyclooxygenase, EPI – epinephrine, NA – nicotinamide, NMN^+ – N-methylnicotinamide, PGI_2 – prostacyclin

Introduction

It has been previously shown that 1,2-bis(nicotinamido)propane, two nicotinamide (NA) particles connected by a hydrocarbon chain, prevented mice, rats, and rabbits from experimentally induced cerebral or pulmonary thromboembolism. The anti-thrombotic action of NA was accompanied by an increased prostacyclin (PGI_2)/thromboxane A_2 ratio [10]. NA, similar to PGI_2, enhanced thrombolysis *in vivo* [5]. Interestingly, there is evidence in the literature to suggest that N-methylnicotinamide (NMN^+), one of the two major primary metabolites of NA, could be more potent anti-inflammatory agent than its parental compound [4].

Recently, we have demonstrated the anti-thrombotic activity of NMN^+ [11]. Furthermore, NMN^+ was only shown to inhibit platelet aggregation *in vivo*. Both of these effects of NMN^+ were blocked by indomethacin, a non-selective prostaglandin synthesis inhibitor, and by rofecoxib, a selective cyclooxygenase

2 (COX-2) inhibitor [1]. The source of PGI_2 formation was unknown and NMN^+ failed to decrease systemic blood pressure [11] or to relax the aorta of mesenteric vascular beds [1].

Hence, we aimed to test the vascular response to NMN^+ and to determine whether it is able to release PGI_2 from the vascular endothelium. We have chosen a model of isolated perfused hindlimb that is suitable for the measurement of vascular response without involvement of other hemostatic elements, such as platelets.

Materials and Methods

Animals

Male Wistar rats (180–200 g) were used in these experiments. They were housed in a room with a 12 h light/dark cycle, in group cages when appropriate, given tap water to drink, and fed a standard rat chow diet. Rats were anesthetized with pentobarbital (40 mg/kg, *ip*). Procedures involving the animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and Guidelines for the Use of Animals in Biomedical Research [3] and were approved by a local bioethics committee.

Chemicals and drugs

NMN^+ as chloride salt was kindly provided by Accos; Pharmena Ltd, (Łódź, Poland). 6-Keto-$PGF_{1\alpha}$ ELISA kits were purchased from Cayman Chemicals (USA). Epinephrine (Injec. Adrenalini, Polfa, Poland), norepinephrine (Levonor, Polfa, Poland), angiotensin II, prazosin hydrochloride, BSA, and Tyrode's solution (Sigma Chemical Co., USA), and pentobarbital (Vetbutal, Biovet, Poland) were also used in this study.

Rat hindlimb perfusion model

The method used in this study was described by Emeis [2] and previously used by us [7]. Briefly, the abdominal cavity of a rat anaesthetized with pentobarbital (40 mg/kg) was cut open and the aorta and inferior vena cava were carefully dissected out. Separate double ligatures were loosely applied around the aorta and the vena cava. A 22-gauge needle was inserted into the aorta up to the ileofemoral bifurcation and fixed by a ligature. Blood circulation was intact and perfusion was started immediately by peristaltic pump (Miniplus 2; Gilson, USA) at a constant flow of 10 ± 1 ml/min using Tyrode's solution, oxygenated with 95% O_2 and 5% CO_2, containing 0.1% BSA, pH 7.4 at 37°C. The vena cava was then closed at the level of the renal vein and was cut just below the ligature to allow the blood to flow out. After 20 min, when the hindlimb circulation was totally emptied of blood, a plastic cannula (inside diameter: 1 mm) was inserted to collect perfusate samples after 5 min. Perfusion pressure was measured just proximal to the inflow by a transducer connected to a monitoring unit. After a 5-min equilibration period with Tyrode's solution, the tested substances were administered as a bolus for a 5- or 10-min perfusion. Samples were collected after 5 min, cooled to 4°C, centrifuged (12,000 × g for 1 min), and the 6-keto-$PGF_{1\alpha}$ level was measured.

Drug treatment

NMN^+ was dissolved in Tyrode's solution so that the final concentration was 1, 10, or 1000 μM and administered during the 5 min perfusion. In addition, we injected NMN^+ intra-arterially at a dose of 30 mg/kg. Epinephrine (EPI, 3 μM) was infused alone or together with NMN^+ (10 μM). Norepinephrine (NEPI, 100 nM) and angiotensin II (Ang II, 1 nM) were both injected intra-arterially in a total volume of 0.1 ml alone or together with NMN^+ (10 μM). Prazosin was infused for 10 min, starting 5 min before EPI at a concentration of 10 μM.

Level of 6-keto-$PGF_{1\alpha}$ – stable metabolite of PGI_2

We measured the level of 6-keto-$PGF_{1\alpha}$ (Cayman Chemicals, USA) by ELISA in a microtiter plate at 25°C using a microplate reader (Dynex Tech., USA) according to the manufacturer's directions and literature. This was done in order to monitor the changes in absorbance at 405 nm [6].

Statistical analysis

The data are shown as the mean ± SEM. A two-tailed Mann-Whitney test was used consistently throughout the study to test whether the mean of a variable differs

between two groups. Multiple group comparisons were performed by Kruskal-Wallis non-parametric ANOVA, followed by Dunn's multiple comparisons test; $p < 0.05$ were considered significant.

Results

Perfusion pressure

The basal level of perfusion pressure was 26.2 ± 0.6 mmHg (n = 69) (Fig. 1). EPI, NEPI, and Ang II increased perfusion pressure to 171.8 ± 19.7 mmHg (n = 8; $p < 0.001$), 53.0 ± 2.2 mmHg (n = 4; $p < 0.001$), and 45.5 ± 1.3 mmHg (n = 6; $p < 0.001$), respectively (Fig. 1, 2). Neither perfusion (10 μM) nor bolus injection (30 mg/kg) of NMN^+ changed the course of perfusion pressure (23.0 ± 3.0 mmHg; n = 5 and 25.5 ± 0.2 mmHg; n = 6, respectively) (Fig. 1). Moreover, NMN^+ administered with EPI and NEPI failed to change the increase in perfusion pressure (178.2 ± 10.8 mmHg; n = 8; and 54.3 ± 3.9 mmHg, n = 4; respectively; ns) (Fig. 1, 2A), whereas prazosin completely abolished the effect of EPI (25.5 ± 0.7 mmHg; n = 4) (Fig. 1). NMN^+ slightly enhanced the vasoconstrictive effect of Ang II (55.8 ± 1.9 mmHg; n = 6; $p < 0.01$) (Fig. 2B).

Level of 6-keto-$PGF_{1\alpha}$ – stable metabolite of PGI_2

The 6-keto-$PGF_{1\alpha}$ level in perfusate was collected from EPI-treated animals after 5 min and was significantly higher when compared to the control (Thyrode's perfused animals) (498 ± 88 pg/ml for EPI-treated animals; n = 5 *vs.* 156 ± 23 pg/ml for control animals; n = 10; $p < 0.01$). NMN^+ failed to release PGI_2 from the endothelium (165 ± 28, 167 ± 40, and 137 ± 27 pg/ml for NMN^+ at a concentration of 1 (n = 6), 10 (n = 4), or 1000 μM (n = 7); ns *vs.* control animals).

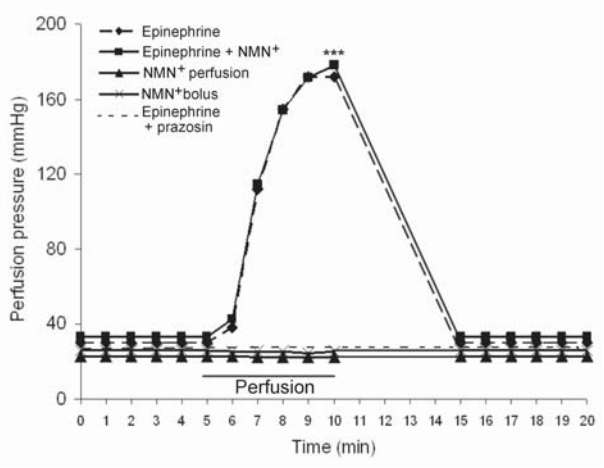

Fig. 1. Effect of epinephrine administered alone (3 μM), together with NMN^+ (10 μM), or prazosin (10 μM) during perfusion. NMN^+ administered as a perfusion (10 μM) or bolus injection of 30 mg/kg on the course the perfusion pressure. *** $p < 0.001$ maximal *vs.* basal value

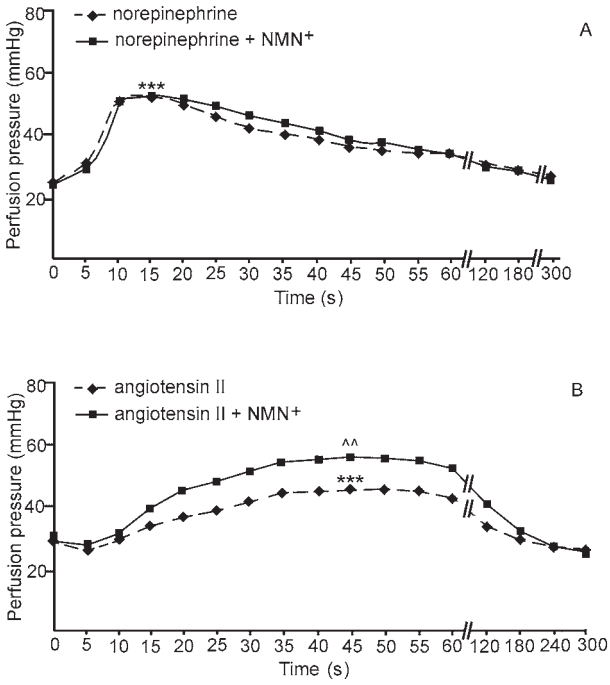

Fig. 2. Effect of norepinephrine **(A)** or angiotensin II **(B)** administered alone (100 nM and 1 nM, respectively) or together with NMN^+ (10 μM) on the course of the perfusion pressure. *** $p < 0.001$ maximal *vs.* basal value, ^^ $p < 0.01$ maximal effect of angiotensin II with the NMN^+ treated group *vs.* maximal effect of angiotensin II alone group

Discussion

In this study, we have tried to determine if the mechanism of various vascular effects of NMN^+, including

anti-thrombotic and anti-aggregatory action previously identified by us, depended on the involvement of vascular mechanisms. We have also recently shown increased plasma levels of a stable analog of PGI_2 – 6-keto-$PGF_{1\alpha}$ after administration of NMN^+ into normo- and hypertensive rats [1, 11]. Moreover, blocking anti-thrombotic action by indomethacin, a non-selective prostaglandin synthesis inhibitor, or rofecoxib, a selective COX-2 inhibitor, indicates that PGI_2-enhanced synthesis/release is involved in this effect. Nevertheless, the sources of PGI_2 production in the plasma remain unknown at this time. It seems that the source could be endothelium rather than platelets, since NMN^+ did not inhibit platelets *in vitro*. Therefore, we used a model of isolated hindlimb perfused by Thyrode's buffer, which is devoid of cellular elements and hemostatic factors and is suitable to selectively measure the endothelial response. This method was successfully used in rats to measure endothelial release of PGI_2 and tissue plasminogen activator in response to various factors, such as venous occlusion, EPI, or NEPI [8, 9].

We have found that NMN^+ administration by infusion or bolus injection did not change the course of perfusion pressure (Fig. 1). Moreover, NMN^+ did not change the vasoconstrictive activity of EPI and NEPI, and it only slightly enhanced the effect of Ang II. This was in agreement with our previous study, in which NMN^+ did not influence systemic blood pressure [11] and failed to relax the aorta of mesenteric vascular beds [1]. We next measured the plasma levels of a stable analog of PGI_2 – 6-keto-$PGF_{1\alpha}$ by ELISA in perfused samples in order to estimate the endothelial release of PGI_2. The time of collection was chosen according to published literature [8]. We did not find any changes in this parameter, using a wide range of NMN^+ concentrations. On the other hand, EPI strongly augmented the level of 6-keto-$PGF_{1\alpha}$ in the perfusate, which is in accordance with the published literature [7]. Thus, we have failed to show that NMN^+ has PGI_2 endothelial releasing properties in the perfused rat hindquarters model. It is possible that the NMN^+ anti-thrombotic and anti-aggregatory activities are similar to those of aspirin; thus, it could even inhibit endothelial PGI_2 release (a decreasing trend was observed at the highest concentration of NMN^+), like aspirin did in this model [8]. Therefore, to exert action of NMN^+, both platelets and endothelium must be present. Another explanation is that NMN^+ effects depend on the type and size of the examined vessels. For example, small resistance mesenteric vessels show 100% vasorelaxation to the cannabinoid anandamide, while the larger superior mesenteric artery has a maximal relaxation of around 40%. Moreover, in the human aorta, the maximum relaxation following administration of anandamide is approximately 20%, whereas rat and rabbit carotid arteries, respectively, do not relax in response to anadamide [13]. Indeed, we have observed that NA and NMN^+ can affect vascular permeability of small skin vessels by a mechanism that involves nitric oxide and prostaglandins [12]. It seems such differences between vessels could also be characteristic of NMN^+ and may be due to differences in the binding site populations or the prevailing mechanisms, which have yet to be identified.

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