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A. MOGIELNICKI, K. KRAMKOWSKI, L. PIETRZAK, W. BUCZKO

N-METHYLNICOTINAMIDE INHIBITS ARTERIAL THROMBOSIS IN HYPERTENSIVE RATS

Department of Pharmacodynamics, Medical University in Bialystok, Poland

There are few findings indicating that nicotinamide may potentially influence intravascular thrombosis. Interestingly, N-methylnicotinamide, one of the metabolites of nicotinamide - could be more potent than its parent compound. In the present study we have investigated the influence of N-methylnicotinamide on arterial thrombosis in normotensive and renovascular hypertensive rats. The contribution of platelets, coagulation and fibrinolytic systems in the mode of N-methylnicotinamide action was also determined. Furthermore, we examined the role of nitric oxide/prostacyclin in the mechanisms of N-methylnicotinamide action. N-methylnicotinamide, but not nicotinamide, administered intravenously into renovascular hypertensive rats developing electrically induced arterial thrombosis caused dose-dependent decrease of thrombus weight, collagen-induced platelet aggregation and plasma antigen/activity of plasminogen activator inhibitor - 1, without changing of occlusion time, routine coagulation parameters and plasma activity of tissue plasminogen activator. Indomethacin - an inhibitor of prostacyclin synthesis, completely abolished the antithrombotic and antiplatelet effect of N-methylnicotinamide, and the plasma level of 6-keto-$PGF_{1\alpha}$, prostacyclin metabolite, increased simultaneously with the inhibition of thrombus formation. Our study shows that N-methylnicotinamide *via* production/release of prostacyclin inhibits arterial thrombosis development. The antithrombotic effect of N-methylnicotinamide is accompanied by platelet inhibition and enhanced fibrinolysis, due to the decrease production of plasminogen activator inhibitor - 1.

Key words: *nicotinamide metabolite, arterial thrombosis, platelets, fibrinolysis, prostacyclin*

List of abbreviations:
6-keto-$PGF_{1\alpha}$ - 6-keto prostaglandin $F_{1\alpha}$; NA - nicotinamide; TNFα - tumor necrosis factor α; PGI_2 - prostacyclin; NMN^+ - N-methylnicotinamide; NO - nitric oxide; 2K-1C - two-kidney one-clip; SO - sham-operated; L-NAME - N^G-nitro-L-arginine methyl ester; PAI-1 - plasminogen activator inhibitor; t-PA - tissue plasminogen activator; PT - prothrombin time; APTT - activated partial thromboplastin time; Fg - fibrinogen; SBP - systolic blood pressure; MBP - mean blood pressure; CBF - carotid blood

flow; HR - heart rate; FG - fibrin generation; tFG - time to fibrin generation; AUC - area under the curve; INDO - indomethacin; Ang - angiotensin; ACE-Is - angiotensin converting enzyme inhibitors;

INTRODUCTION

Nicotinamide (NA) has been shown to possess a variety of anti-inflammatory properties. Indeed, in whole blood, NA dose-dependently decreases endotoxin-induced production of four proinflammatory cytokines, interleukin-1,6,8 and TNFα (1). Recently, it has been demonstrated that NA may improve neurological outcome and reduce infarct volume after transient focal cerebral ischemia (2). The results of animal studies indicate that NA may prevent onset of type 1 diabetes (3). Furthermore, only a few studies show that NA may also influence intravascular thrombosis. It is able to inhibit endotoxin-induced monocyte tissue factor expression in concentration dependent manner (4). Moreover, 1,2-bis(nicotinamido)propane, two NA particles connected with hydrocarbon chain, prevented mice, rats and rabbits from death induced by acute cerebral or pulmonary thromboembolism following the injection of arachidonate or collagen. Antithrombotic action of NA was accompanied by increased prostacyclin (PGI_2)/thromboxane A_2 ratio (5). NA, similarly to PGI_2, enhanced thrombolysis (6) at *in vivo* conditions. However, NA exerts its vascular effects only when administered in huge, parenteral doses, like 500 mg/kg. Although, it had looked promising in animal tests, an extensive trial which evaluated some of the therapeutic effects of NA, *i.e.* the prevention of the onset of type 1 diabetes, ended in disappointment (7). Interestingly, there is an evidence in the literature that one of the metabolites of NA could be more potent than its parent compound (7). N-methylnicotinamide (NMN^+) is one of the two major primary metabolites of NA, along with nicotinamide-N-oxide, endogenously present in the human plasma. NMN^+ has been shown at *in vitro* study to be a potent anti-inflammatory agent (8 - 10). It is more protective in the prevention of the onset of alloxan-induced diabetes in mice (11). Thus, we can not exclude that *in vivo* some of the biological effects of NA are mediated by NMN^+. According to our knowledge there is no data concerning the influence of NMN^+ on the hemostasis.

Therefore, the aim of the present study was to investigate the effect of NMN^+ on the experimental arterial thrombosis electrically induced in normotensive and renal hypertensive rats. The influence of NMN^+ on the collagen-induced platelet aggregation and the markers of coagulation and fibrinolysis were examined. We also elucidated the involvement of nitric oxide (NO) and PGI_2 in the mechanism of NMN^+ observed effects.

MATERIALS AND METHODS

Animals and the induction of renovascular hypertension

Male Wistar rats (180-200 g) were used in the experiments. They were housed in a room with a 12 h light/dark cycle, in group cages as appropriate, were given tap water and fed a standard rat

chow. Rats were anesthetized with pentobarbital (40 mg/kg, *i.p.*). Two-kidney one-clip (2K-1C) renovascular hypertension was induced by a partial, standardised clipping of the left renal artery (12). After 6 weeks most of the animals developed hypertension which was confirmed by the blood pressure measurement. Sham-operated rats (SO) served as a control to 2K-1C hypertensive rats. They received the same surgical intervention without the clipping of the renal artery.

Ethics

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 (13).

Chemicals and drugs

N-methylnicotinamide (NMN^+) as chloride salt was kindly provided by Accos; Pharmena Ltd, (Lodz, Poland). Nicotinamide, NO-synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) and indomethacin were all bought in Sigma-Aldrich Chemicals (USA). 6-keto-$PGF_{1\alpha}$ ELISA kit was bought in Cayman Chemicals (USA), rat plasminogen activator inhibitor - 1 (PAI-1) ag/activity Zymutest Kits in HYPHEN BioMed (France), whereas rat tissue plasminogen activator (t-PA) activity kit in Innovative Research Inc (USA). Reagents to prothrombin time (PT), activated partial thromboplastin time (APTT) and fibrinogen (Fg) level measurements were bought in HemosIL, Instrumentation Laboratory (USA). Moreover, collagen (Chrono-log, USA), heparin (Heparinum, Polfa, Poland), Tris buffer [Tris(hydroxymethyl)-aminomethane hydrochloride (Sigma Chemical Co., USA) and Tris(hydroxymethyl)-aminomethane (Merck, Germany)], pentobarbital (Vetbutal, Biovet, Poland), trisodium citrate (Polish Chemicals, Poland) were used in the study.

Blood Pressure, Heart Rate and Carotid Blood Flow measurement

To verify hypertension development we measured the systolic blood pressure (SBP) using the "tail cuff" method (Student Oscillograph, Harvard Rat Tail Blood Pressure Monitor, UK) in conscious rats (14). Each value was the average of three consecutive readings. Hypertensive rats (SPB higher than 145 mmHg) were used in the further experiments.

The mean blood pressure (MBP) was measured directly through a cannula filled with heparin solution (150 IU/ml), placed in the left common carotid artery and connected to the pressure transducer (Plugsys, Transonics System, USA) in anaesthetized rats, receiving intravenously NMN^+ in dose of 30 mg/kg or 0.9% NaCl (VEH), as described previously (15). The carotid blood flow (CBF) was measured by placing flow probe on the right carotid artery connected to the blood flow module of the same Plugsys. Heart rate (HR) was measured from ECG and was also automatically calculated from the values of MBP and CBF. In these animals, arterial thrombosis was not induced.

Drugs administration

NMN^+ (3, 10, 30 mg/kg), NA (30 mg/kg) or VEH were administered into right femoral vein of 5, 7, 7, 8 and 14 rats, respectively, in a volume not exceeding 1ml/kg 10 min before the induction of arterial thrombosis. In other experiments animals received the examined drugs dissolved in drink water in dose of 100 mg/kg per day for 10 days before induction of thrombosis. The 5 rats treated with NA and 7 rats treated with NMN^+ had the same food and water intake and the same bodyweight after 10 days as the 9 control rats. In experiments concerning the mechanism of antithrombotic action of NMN^+, NO-synthase inhibitor (L-NAME; 30mg/kg; *s.c.*) or PGI_2 synthesis inhibitor indomethacin (INDO; 2,5 mg/kg; *i.v.*) were administered alone (n = 4 and n = 5,

respectively) or additionally to NMN^+ (n = 7), 20 or 10 min before arterial thrombosis induction, respectively, as described previously (16).

Arterial thrombosis

Male renovascular hypertensive Wistar rats were anesthetized by intraperitoneal injection of pentobarbital (40 mg/kg) and then fixed in the supine position on a heated operation table. The left femoral vein was cannulated to administer drug. We induced arterial thrombosis by electrical stimulation of the common carotid artery according to the method described by Schumacher *et al.* (17) and Guarini (18). Briefly, the thrombosis was induced by providing electrical stimulation (1mA) and additionally clipping of the carotid artery for 5 min. A Doppler flow probe was placed in contact with the carotid artery downstream from the site of electrical stimulation and connected to the flowmeter (Hugo Sachs Elektronik, Germany). CBF was continuously monitored before, during and after electrical stimulation. The time from the end of the electrical stimulation to the point at which the CBF decreased to zero was defined as the occlusion time. 45 min after stimulation the segment of the common carotid artery with the formed thrombus was clipped at both sides, dissected opened lengthwise and the thrombus was completely removed, air-dried in 37°C and weighed 24 hours after the end of experiment.

After removal of formed thrombus blood samples were taken from the heart. Platelet aggregation was assessed in whole blood directly after experiment. The rest blood samples were drawn into 3.13% trisodium citrate in a volume ratio of 10:1, then centrifuged at 2000 x g in 4°C for 20 min and plasma was deep-frozen (- 70°C) in aliquots of 1 ml until further assays could be preformed.

Coagulation parameters

PT and APTT were automatically determined by optical method (Coag-Chrom 3003; Bio-ksel, Poland) adding routine laboratory reagents to collected rat plasma. Fg was evaluated according to Clauss method (19). Fibrin Generation (FG) and Time to Fibrin Generation (tFG) was measured according to previously described methods (20,21). FG curve was created by adding $CaCl_2$ (36 mM) to the Tris buffer (66 mM Tris and 130 mM NaCl, pH = 7.4) and mixed with rat plasma sample. Optical density was measured *via* microplate reader (Dynex Tech., USA) in 1 min intervals for 15 min. The FG was expressed as area under the curve (AUC). Time to Fibrin Generation was measured from the start of optical density registration to the noticeable increase.

Fibrinolysis parameters

The rat PAI-1 antigen and activity and t-PA activity were measured by ELISA techniques in a microtiter plate at 25°C using a microplate reader (Dynex Tech., USA) to monitor the changes in absorbance at 450 nm according to the manufacturer's directions.

Platelet aggregation

Whole blood platelet aggregation was monitored by measuring electrical impedance in Chronolog aggregometer (Chrono-log Corp., USA) according to the method described by Cardinal *et al.* (22). Blood samples were drawn into 3.13% trisodium citrate in a volume ratio 10:1. Collagen (10 mg/ml) was added after 15 min of incubation at 37°C with 0.9% NaCl in a volume ratio 1:2. Then changes in impedance were registered for 6 min. The maximal extension of the aggregation curve at 6^{th} minute was expressed in Ohms (Ω). In addition at *in vitro* study blood samples were mixed with NMN^+ (10^{-7}M) or VEH solution before incubation.

Plasma level of 6-keto-PGF$_{1\alpha}$ - stable metabolite of PGI$_2$

We also measured plasma level of 6-keto-PGF$_{1\alpha}$ (Cayman Chemicals, USA) by ELISA technique in a microtiter plate at 25°C using a microplate reader (Dynex Tech., USA) to monitor the changes in absorbance at 405 nm according to the manufacturer's directions.

Statistical analysis

The data are shown as mean ± SEM. In calculating the thrombus weight, the lack of the thrombus was regarded as 0 mg. To test whether the mean of a variable differs between two groups two-tail, Mann-Whitney test was used consistently throughout the study. Multiple group comparisons were preformed by Kruskal-Wallis nonparametric ANOVA, followed by Dunn's multiple comparisons test. Correlations were analyzed using a Pearson test. The p values less than 0.05 were considered significant.

Table 1. Presents thrombus weight, coagulation and fibrinolytic parameters, platelet aggregation and 6-keto-PGF$_{1\alpha}$ concentration measured in hypertensive rats treated intravenously with 0.9% VEH or NMN$^+$ (3, 10 and 30 mg/kg) and *per os* with VEH or NMN$^+$ (100 mg/kg/day for 10 days).

	VEH	NMN$^+$ 3	NMN$^+$ 10	NMN$^+$ 30	VEH	NMN$^+$100
Treatment	Intravenous				*Per os*	
n	14	5	7	7	8	7
			Arterial thrombosis			
Thrombus weight (mg)	0.88±0.10	0.90±0.10	0.48±0.20	0.44±0.07**	0.96±0.16	0.67±0.21
			Coagulation parameters			
Fg [ng/ml]	2.57±0.09	3.00±0.14	2.69±0.14	2.45±0.11	2.51±0.24	3.03±0.27
PT [sec]	14.92±0.32	15.50±0.29	15.56±0.34	14.21±0.18	15.36±0.44	15.30±0.29
APTT [sec]	32.38±0.89	28.47±0.86	31.72±2.22	29.80±1.67	28.07±2.05	27.68±2.03
FG [AUC]	267±20	326±34	257±20	261±24	293±27	341±39
tFG [min]	7.00±0.58	6.80±0.80	7.86±0.77	6.43±0.53	6.90±1.1	6.8±1.47
			Fibrinolysis parameters			
PAI-1 antigen [ng/ml]	1.73±0.22	1.46±0.32	1.23±0.31	0.99±0.20*	1.22±0.29	1.19±0.13
PAI-1 activity [ng/ml]	2.58±0.45	2.30±0.78	1.24±0.45	1.01±0.15*	0.97±0.15	1.15±0.23
t-PA activity [ng/ml]	3.03±0.31	3.58±1.43	2.96±0.50	3.15±0.50	2.54±0.67	2.61±0.62
			Platelet aggregation			
Impedance (Ω)	3.19±0.19	3.59±0.61	2.82±0.24	2.3±0,29*	2.74±0.30	2.58±0.28
			PGI$_2$ relaese/production			
6-keto-PGF$_{1\alpha}$ concentration (pg/ml)	313.5±59.6	322.5±41.7	366.8±92.2	345.3±51.2	97.6±20.2	207.2±48.9*

NMN$^+$ - N-methylnicotinamide; n - number of animals; PT - prothrombin time; APTT - activated partial thromboplastin time; Fg - fibrinogen; FG - fibrin generation; tFG - time to fibrin generation; PAI-1 - plasminogen activator inhibitor; t-PA - tissue plasminogen activator; 6-keto-PGF$_{1\alpha}$ - 6-keto prostaglandin F$_{1\alpha}$; * p<0.05, ** p<0.01, *** p<0.001 *vs* VEH. Data are expressed as mean ± SEM.

RESULTS

The effect of NA and its metabolite - NMN^+ on arterial thrombosis

Intravenous NMN^+ treatment of 2K-1C rats resulted in a dose-dependent decrease of arterial thrombus weight (0.90 ± 0.10, 0.48 ± 0.20 and 0.44 ± 0.07 mg, for doses of 3, 10 and 30 mg/kg, respectively *vs* 0.88 ± 0.10 mg in VEH treated group; ns, ns, $p<0.01$, *Table 1*). Since we did not observe the antithrombotic effect of the highest dose of NMN+ in SO rats (1.28 ± 0.14 *vs* 1.28 ± 0.24 mg in VEH treated group, ns), the effects of NMN+ in normotensive animals were not further elucidated. Interestingly, NA administered in the same as potent dose of NMN^+ (30 mg/kg) failed to influence arterial thrombosis (0.94 ± 0.11 *vs* 0.88 ± 0.10 mg in VEH treated group, ns). Although there was a trend to decrease thrombus weight, both NA and NMN^+ administered *per os* (100 mg/kg/day) did not significantly influence arterial thrombus weight (0.67 ± 0.21 and 0.72 ± 0.20, respectively *vs* 0.96 ± 0.16 mg in VEH treated group; ns, *Table 1*) in renal hypertensive rats. There was no significant change in occlusion time after administration of examined compounds when compared to control (data not shown).

The effect of NMN^+ on mean blood pressure, heart rate and carotid blood flow

The initial MBP measured directly in carotid artery did not significantly differ between groups. We observed rise in MBP passing 3 min after NMN^+ (30 mg/kg)

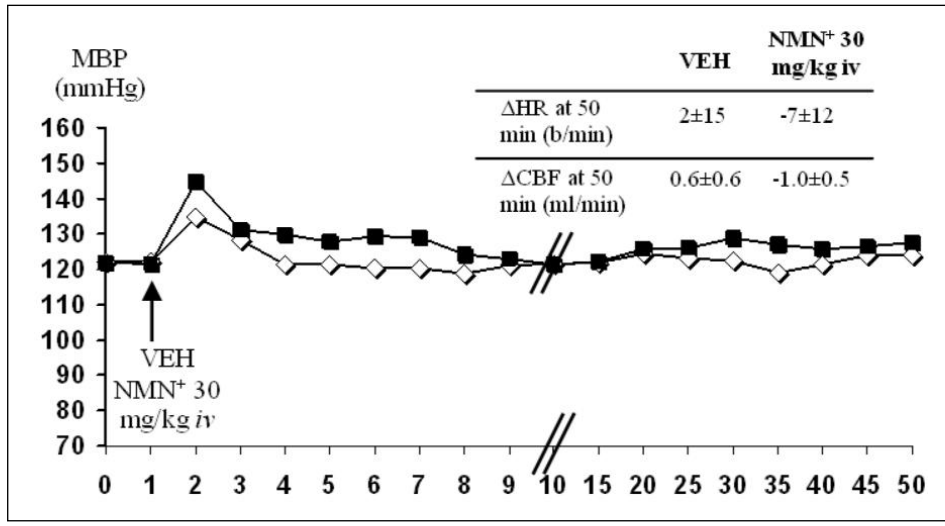

Fig. 1. The lines represent course of MBP registered for 50 min after *iv* administration of VEH (white squares) or NMN^+ (30 mg/kg) (black squares) (n=6); ΔHR, ΔCBF - the difference between initial HR, CBF value and at 50 min after VEH or NMN^+ administration. Positive values represent increases and negative values decreases in comparison to initial values. Data are expressed as mean ± SEM.

or VEH intravenous bolus, related to the administration of fluid volume. After 50 min of monitoring, NMN^+ failed to influence MBP, HR and CBF (*Fig. 1*).

The effect of NMN^+ on coagulation and fibrinolysis

NMN^+ failed to influence FG, TFG, ACLT, PT and Fg (*Table 1*).

Interestingly, NMN^+ decreased PAI-1 antigen and activity in plasma in dose-dependent manner, whereas it did not change t-PA activity (*Table 1*).

The role of platelets in the antithrombotic action of NMN^+ in 2K-1C rats

NMN^+ administered intravenously, but not *per os*, decreased collagen induced platelet aggregation in dose-dependent manner (3.59 ± 0.61; 2.82 ± 0.24 and 2.3 ± 0,29 Ω for doses 3, 10 and 30 mg/kg respectively *vs* 3.19 ± 0.19 Ω in VEH treated group; ns, ns, $p<0.05$, (*Table 1*). NMN^+ (10^{-7}M) did not change platelet aggregation (3.11 ± 0.09 *vs* 3.12 ± 0.13 Ω in VEH treated group; ns) at *in vitro* experiments.

The involvement of NO and PGI_2 in the antithrombotic action of NMN^+

Concurrently, 30 mg/kg intravenously of NMN^+ and either indomethacin (INDO), (PGI_2 synthesis inhibitor) and L-NAME (NO synthase inhibitor) was

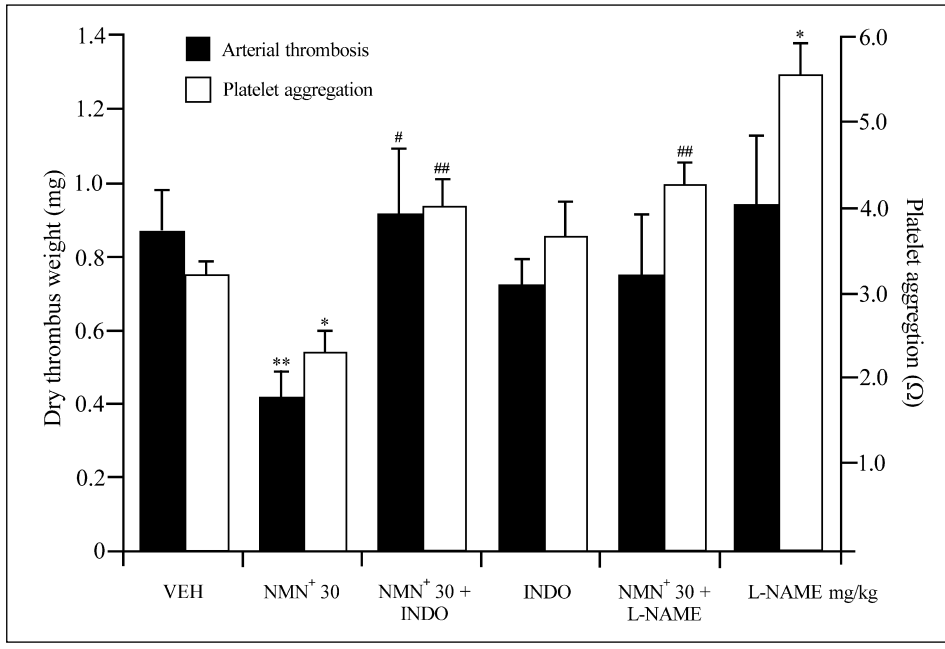

Fig. 2. The black columns represent dry thrombus weight and white columns platelet aggregation in hypertensive rats treated *i.v.* with VEH, NMN^+ (30 mg/kg), indomethacin - PGI_2 synthesis inhibitor (INDO; 2,5 mg/kg) or NO-synthase inhibitor (L-NAME; 30mg/kg; *s.c.*) followed by *iv* bolus of VEH or NMN^+ (30 mg/kg); * $p<0.05$, ** $p<0.01$ *vs* VEH; # $p<0.05$, ## $p<0.01$ *vs* NMN^+ (30 mg/kg). Groups consisted of 6 to 15 animals. Data are expressed as mean ± SEM.

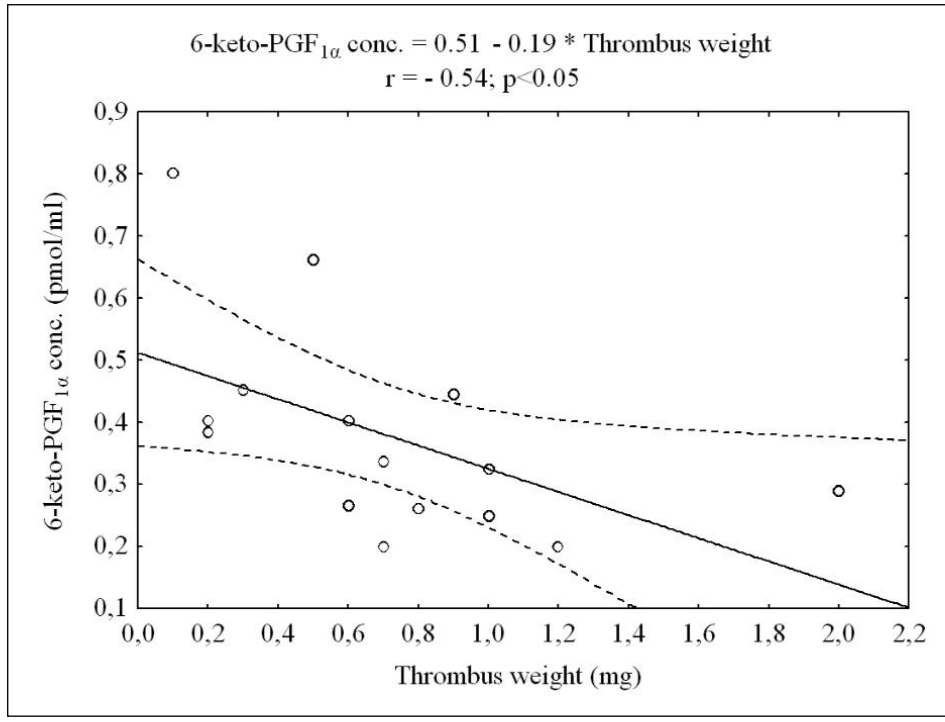

Fig. 3. Correlation between 6-keto-$PGF_{1\alpha}$ concentration and thrombus weight in hypertensive rats treated with NMN^+ (3, 10 and 30 mg/kg).

given. INDO, but not L-NAME, reversed the antithrombotic action of NMN^+ (0.44 ± 0.07 mg in NMN^+ 30 mg/kg treated group *vs* 0.93 ± 0.17 mg in INDO + NMN^+ 30 mg/kg treated group; $p<0.05$, *Fig. 2*). L-NAME or INDO failed to influence arterial thrombosis when administered to NMN^+-naive animals. INDO diminished antiplatelet effect of NMN^+ (2.30 ± 0.29 Ω in NMN^+ 30 mg/kg treated group *vs* 3.95 ± 0.28 Ω in INDO + NMN^+ 30 mg/kg treated group; $p<0.01$, *Fig. 2*). L-NAME also reversed the antiplatelet action of NMN^+ (2.30 ± 0.29 Ω in NMN 30 mg/kg treated group *vs* 4.16 ± 0.28 Ω in L-NAME + NMN^+ 30 mg/kg treated group; $p<0.01$, *Fig. 2*). However, L-NAME administered alone significantly increased (5.43 ± 0.53 Ω in L-NAME treated group *vs* 3.19 ± 0.19 Ω in VEH treated group; $p<0.05$, *Fig. 2*), whereas INDO did not influence platelet aggregation.

We found slight, but not significant, increase in the plasma level of 6-keto-$PGF_{1\alpha}$ (a stable metabolite of PGI_2) in rats treated intravenously with NMN^+. However, a significant inverse correlation between thrombus weight and the plasma level of 6-keto-$PGF_{1\alpha}$ could be observed in these animals ($r = - 0.54$; $p<0.05$, *Fig. 3*). Interestingly, the concentration of PGI_2 metabolite significantly increased in animals treated chronically (per os) (*Table 1*).

DISCUSSION

Few studies provided evidence that NA applied in high parenteral doses may inhibit intravascular thrombosis, by affecting some elements of hemostasis (4 - 6). Interestingly, one of the metabolites of NA - NMN^+ could be more potent than its parent compound (7). It exerts powerful anti-inflammatory effect (8, 9) and is even more protective than NA in the prevention of the onset of alloxan-induced diabetes in mice (11).

Following these signals we thought this compound should also affect thrombosis at *in vivo* conditions. Therefore, we checked if NMN^+, like NA, is able to influence arterial thrombus formation. Hypertension is one of the most important risk factors of arterial thrombosis and its clinical events such as acute coronary syndrome and ischemic stroke. Therefore we decided to examine the effect of NA and NMN^+ on arterial thrombosis induced in hypertensive animals. Since many evidences indicate that endothelial function impairment progressively occurs during renovascular hypertension development (23, 24), further experiments were performed on renovascular hypertensive rats. Using this model of hypertension, we have recently demonstrated that angiotensin (Ang) 1-7 is able to inhibit venous thrombosis (16), and in contrary, Ang II is a prothrombotic agent (20, 25).

To investigate the influence of examined compounds on arterial thrombus formation we used the model of experimental arterial thrombosis induced by electrical stimulation. Although thrombus formation was initiated by electrical stimulation producing arterial injury that is unrelated to clinical thrombosis, thrombus morphology suggests that growth of intravascular thrombotic material in response to electrolytic injury is physiologically relevant (18). Using this model, we have recently demonstrated the antithrombotic effect of angiotensin converting enzyme inhibitors (ACE-Is) (26) and prothrombotic action of Ang II (25).

Therefore, to study the influence of NA and NMN^+ on the thrombotic process, we applied these compounds to renovascular hypertensive rats before developing of the arterial thrombosis. When administered *i.v.* in a very low doses of 3 and 10 mg/kg, NMN^+ did not influence arterial thrombosis. However, the progress of electrically induced arterial thrombosis was significantly inhibited in the renovascular hypertensive rats treated with 30 mg/kg of NMN^+ (*Fig. 1*). Interestingly, we did not observed antithrombotic effect of NA administered in the same dose, confirming previous suggestion that NMN^+ may be much more potent than its parent compound (11). Our results are also in the line with others observation, who demonstrated cytoprotectant action of NA on vascular physiology only when administered *i.p*. and *i.v.* in higher than 100 mg/kg doses (2, 6). We were unable to show the antithrombotic effect of both: NA and its metabolite - NMN^+ when chronically administered *p.o.* Nevertheless, our results provide direct *in vivo* evidence pointing to NMN^+ as a potential moderate antithrombotic agent.

We next examined which element of hemostasis mediate the antithrombotic effect of NMN^+. Since there is no any information about the influence of NMN^+ on hemodynamic parameters, first we try to study the systemic response of vascular wall in the presence of NMN^+. To check this, we measured MBP, HR and CBF for 50 min after *i.v.* administration of VEH or NMN^+ into renal hypertensive rats, but found no significant difference between groups (*Fig. 1*). Thus, we concluded that the hemodynamic mechanisms are not involved in the antithrombotic action of NMN^+.

We next investigated the involvement of coagulation system in the mechanism of NMN^+ action. There is only one study demonstrating that NA may inhibit coagulation cascade. Ungerstedt *et al.* observed inhibition of endotoxin-induced monocyte TF expression in healthy volunteers by NA (4). Thus, we measured FG and tFG in the plasma of rats treated with VEH or NMN^+. It has been previously shown, that FG increases after adding exogenous TF factor and decreases when exogenous t-PA is added (27). Furthermore, we measured typical coagulation parameters, like APTT, PT or Fg level, using routine laboratory methods. We did not found any changes of examined parameters after treatment with NMN^+ (*Table 1*). Thus, we excluded the role of coagulation system in the antithrombotic effect of NMN^+.

Next, we planned to estimate the involvement of fibrinolysis in the action of NMN^+. It has been shown that NA is able to disperse platelet thrombi in the extracorporeal circulation of anaesthetized cats (6). However, there is no any information about the mechanism of thrombolytic effect of NA and if its metabolites are involved. Therefore, we measured PAI-1 antigen/activity and t-PA activity using ELISA techniques and found significant dose-dependent PAI-1 antigen/activity decrease without changing that of t-PA in rats treated intravenously with NMN^+ (*Table 1*). Although these results show that plasmin production may be enhanced due to the decrease in antigen/activity of main fibrinolysis inhibitor - PAI-1 in the presence of NMN^+, they do not reveal a direct mechanism of the NMN^+ action. We thought, the activation of fibrinolysis by NMN^+ could be a consequence of the inhibition of platelets, which are known source of PAI-1 and other various active hemostatic factors (28).

To study this we induced platelet aggregation by collagen in whole blood of animals treated with VEH or NMN^+. Similarly to arterial thrombosis results, we observed dose-dependent inhibition of platelet aggregation by *i.v.* administered NMN^+ (*Table 1*).

However, we did not found a significant correlation between PAI-1 activity and the platelet aggregation ($r = 0.13$; ns). It is unlikely then that decrease of PAI-1 level is just the result of platelet inhibition. At this moment we thought there must be other mediator of antiplatelet and fibrinolytic activity of NMN^+. Interestingly, we observed antihrombotic effect of NMN^+ only in renal hypertensive rats but not in healthy animals. It is known that during development of renovascular hypertension, progressively occurs endothelial dysfunction (23).

Since NMN^+ failed to influence *in vitro* platelet aggregation, it seems that NMN^+ preferably acts on endothelium, than directly on platelets. Thus, we supposed NMN^+ could exert its action by improving endothelial function through release of endothelial mediators such as: NO or PGI_2, possessing both antiaggregatory and fibrinolytic properties (29).

We try to verify if NMN^+ acts in an NO/PGI_2 dependent manner. We found that indomethacin - an inhibitor of PGI_2 synthesis, completely abolished the antithrombotic effect of NMN^+, whereas L-NAME - NO synthase inhibitor did not alter the inhibition of thrombus formation caused by NMN^+ (*Fig. 2*). Similarly to our results, the antithrombotic effect of 1,2-bis(nicotinamido)propane was also accompanied by increased production of PGI_2 (5). Moreover, we have demonstrated that the antiplatelet action of NMN^+ was also abolished by the administration of INDO (*Fig. 2*). Interestingly, it has been shown by others that continuous infusion of PGI_2 decreases plasma levels of PAI-1 (30). Therefore, we thought the effects of NMN^+ observed in our study may be mediated by PGI_2. Indeed, we found that the antithrombotic effect of NMN^+ correlated with the increased plasma level of 6-keto-$PGF_{1\alpha}$, a metabolite of PGI_2. This slight local production of PGI_2 is not sufficient to lower systemic blood pressure, because we did not observed hipotensive action of intravenously administered NMN^+. Similarly, ACE-Is are still able to release of NO and PGI_2 from endothelium and inhibit thrombosis when administered in low non-hipotensive doses (15). Although, we found that L-NAME reversed inhibition of platelet aggregation by NMN^+, this could be due to the proaggregatory effect of L-NAME by itself, which not specifically mask antiplatelet action of NMN^+ (*Fig. 2*).

We have also shown, that NMN^+ inhibits extracorporal thrombus formation *via* cyclooxygenase-2/PGI_2 pathway (31). Our present study also documents a novel promising antithrombotic agent - NMN^+, a metabolite of nicotinamide, endogenously present in the human plasma. Although not complete at this moment, we provide essential information about the mechanism of the antithrombotic action of NMN^+, which is mediated by PGI_2 and may involve the inhibition of PAI-1 synthesis and platelet aggregation. We hope our finding will initiate series of studies, which help in better understanding of all NMN^+'s potential therapeutic activities and luckily move the compound under further investigation.

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Author's address: Prof. Wlodzimierz Buczko, MD, PhD, Department of Pharmacodynamics, Medical University, Mickiewicza Str. 2C, 15-089 Bialystok, Poland; tel/fax (48-85) 7485601; e-mail: pharmdyn@amb.edu.pl