British Journal of Pharmacology (2007) 152, 230–239
 0007–1188/07 $30.00
www.brjpharmacol.org

RESEARCH PAPER

# 1-Methylnicotinamide (MNA), a primary metabolite of nicotinamide, exerts anti-thrombotic activity mediated by a cyclooxygenase-2/prostacyclin pathway

S Chlopicki[1], J Swies[1], A Mogielnicki[2], W Buczko[2], M Bartus[1], M Lomnicka[1], J Adamus[3] and J Gebicki[3]

[1]*Department of Experimental Pharmacology, Chair of Pharmacology, Jagiellonian University Medical College, Krakow, Poland;* [2]*Department of Pharmacodynamics, Medical University of Bialystok, Bialystok, Poland and* [3]*Institute of Applied Radiation Chemistry, Technical University of Lodz, Lodz, Poland*

**Background and purpose:** 1-methylnicotinamide (MNA) has been considered to be an inactive metabolite of nicotinamide. Here we assessed the anti-thrombotic activity of MNA *in vivo*.
**Experimental approach:** Antithrombotic action of MNA was studied in normotensive rats with extracorporeal thrombus formation (thrombolysis), in renovascular hypertensive rats with intraarterial thrombus formation (arterial thrombosis) and in a venous thrombosis model in rats (venous thrombosis).
**Key results:** MNA (3-100 mg $kg^{-1}$) induced a dose-dependent and sustained thrombolytic response, associated with a rise in 6-keto-$PGF_{1\alpha}$ in blood. Various compounds structurally related to MNA were either inactive or weaker thrombolytics. Rofecoxib (0.01-1 mg $kg^{-1}$), dose-dependently inhibited the thrombolytic response of MNA, indomethacin (5 mg $kg^{-1}$) abolished it, while L-NAME (5 mg $kg^{-1}$) were without effect. MNA (3–30 mg $kg^{-1}$) also reduced arterial thrombosis and this effect was abrogated by indomethacin (2.5 mg $kg^{-1}$) as well as by rofecoxib (1 mg $kg^{-1}$). MNA, however, did not affect venous thrombosis. *In vitro* MNA did not modify platelet aggregation nor induce vasodilation.
**Conclusions and implications:** MNA displayed a profile of anti-thrombotic activity *in vivo* that surpasses that of closely related compounds. MNA inhibited platelet-dependent thrombosis by a mechanism involving cyclooxygenase-2 and prostacyclin. Our findings suggest that endogenous MNA, produced in the liver by nicotinamide N-methyltransferase, could be an endogenous activator of prostacyclin production and thus may regulate thrombotic as well as inflammatory processes in the cardiovascular system.
*British Journal of Pharmacology* (2007) **152**, 230–239; doi:10.1038/sj.bjp.0707383; published online 16 July 2007

**Keywords:** 1-methylnicotinamide; thrombolysis; thrombosis; $PGI_2$; COX-2; platelets

**Abbreviations:** 6-ANA, 6-aminonicotinamide; ASA, acetylsalicylic acid; COX, cyclooxygenase; (EtOH)NA, 1-(2-hydroxyethyl)-nicotinamide; L-NAME, L-$N^G$-nitroarginine methyl ester; MNA, 1-methylnicotinamide; MNA-$Et_2$, 1-methyl-N′,N′-diethylnicotinamide; MNA-Me, 1,N′-dimethylnicotinamide; MAP, 1-methyl-3-acetylpyridine; NNMT, nicotinamide *N*-methyltransferase; N-Ox, nicotinamide-*N*-oxide; $PGI_2$, prostacyclin; $PGE_2$, prostaglandin $E_2$; PNA, 1-propylnicotinamide; 2-PYR, 1-methyl-2-pyridone-5-carboxamide; 4-PYR, 1-methyl-4-pyridone-5-carboxamide; RibNA, 1-ribosylnicotinamide; SNAP, *S*-nitroso-*N*-acetyl-penicillamine; $TXB_2$, thromboxane $B_2$

## Introduction

Nicotinamide is an essential nutrient, a precursor of nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate, known to reverse the symptoms of pellagra (Pellagra Preventive vitamin—vitamin PP, also known as vitamin $B_3$). More recently a number of other biological activities of nicotinamide have been described, including its cytoprotective effects on neural and vascular tissues (Chong *et al.*, 2002; Maiese and Chong,

Correspondence: Professor S Chlopicki, Department of Experimental Pharmacology, Chair of Pharmacology, Jagiellonian University Medical College, Grzegorzecka 16, Krakow, 31-531 Poland.
E-mail: s.chlopicki@cyfronet.krakow.pl
or Professor J Gebicki, Institute of Applied Radiation Chemistry, Technical University of Lodz, Zeromskiego 116, Lodz, 90-924 Poland.
E-mail: jgebicki@p.lodz.pl
Received 5 March 2007; revised 25 April 2007; accepted 8 June 2007; published online 16 July 2007

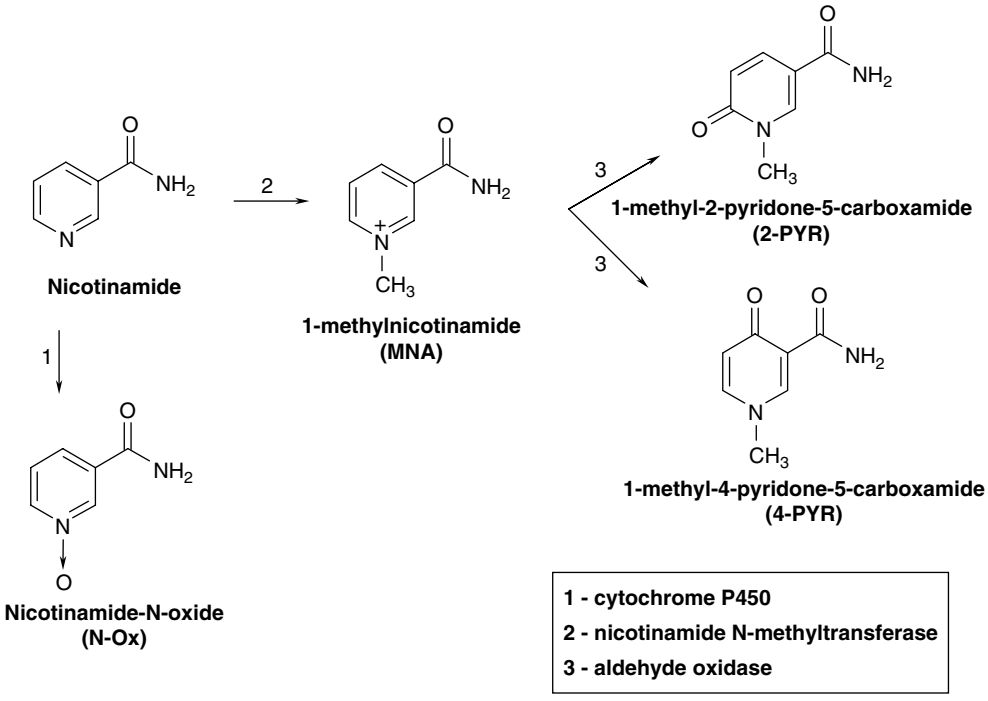



**Figure 1** Major pathways of nicotinamide metabolism (see text for details).

2003) as well as its anti-inflammatory activity (Ungerstedt *et al*., 2003).

Nicotinamide is metabolized in the liver by cytochrome P450 to nicotinamide-*N*-oxide (N-Ox) but mainly by nicotinamide *N*-methyltransferase (NNMT) to 1-methylnicotinamide (MNA) that is further metabolized to 1-methyl-2-pyridone-5-carboxamide (2-PYR) or 1-methyl-4-pyridone-5-carboxamide by aldehyde oxidase (Aoyama *et al*., 2000) (Figure 1). Despite the early discovery of nicotinamide and its metabolites, NNMT, a major liver enzyme in nicotinamide metabolism, has only recently been genetically characterized in mouse and human (Yan *et al*., 1999).

Over the years, nicotinamide metabolites have been frequently measured in rodents and humans. The level of MNA in plasma and urine was analysed initially to diagnose niacin deficiency (Vivian *et al*., 1958) and then to monitor renal tubular excretion (Maiza *et al*., 1992) or peroxisome proliferation in the liver (Delaney *et al*., 2005). An increased concentration of urine MNA was found for example in patients with Parkinson's disease (Aoyama *et al*., 2000) or liver cirrhosis (Pumpo *et al*., 2001). In all these studies, MNA was considered as an inactive biomarker. Other nicotinamide metabolites were also considered as inactive.

In light of the above, it was quite surprising that MNA applied topically proved to be effective in alleviating the inflammatory responses of various skin diseases (Gebicki *et al*., 2003; Wozniacka *et al*., 2005). These pilot studies provided the first evidence for the biological activity of MNA *in vivo*, although the mechanism of MNA activity was not defined. The present study was undertaken to characterize the profile of cardiovascular action of MNA and its *in vivo* anti-thrombotic activity, relative to those of structurally related compounds. We have demonstrated that MNA is a unique anti-thrombotic agent as it limits platelet-dependent experimental thrombosis by a mechanism dependent on prostacyclin ($PGI_2$) synthesized by the inducible isoform of cyclooxygenase (COX-2).

## Methods

*Animals*

All animal procedures conform 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.

*Assay of the thrombolytic effect of MNA and related compounds* in vivo *in rats*

For assessment of thrombolysis a model developed originally by Gryglewski *et al*. (2001) was used. Briefly, in anaesthetized heparinized Wistar rats (body weight of 300–350 g) with extracorporeal circulation, arterial blood from the carotid artery superfused a collagen strip and was returned to the jugular vein. The mass of the collagen strip was continuously registered. After 20–30 min of superfusion, the mass of platelet-rich thrombus adhering to the collagen strip reached approximately 100 mg, and this weight was maintained for 3–4 h as long as the blood superfusion lasted. MNA, nicotinamide itself, other metabolites of nicotinamide (nicotinamide-*N*-oxide, 1-methyl-2-pyridone-5-carboxamide) or various compounds structurally related to MNA (nicotinic acid, trigonelline, 1,*N*′-dimethylnicotinamide,

1-methyl-*N*′,*N*′-diethylnicotinamide, 1-ribosylnicotinamide, 1-propylnicotinamide, 1-(2-hydroxyethyl)nicotinamide, 1-methyl-3-acetylpyridine, 6-aminonicotinamide), were injected intravenously into the femoral vein (30 mg kg$^{-1}$or 3–300 mg kg$^{-1}$ for MNA, nicotinamide, nicotinic acid and 1-methyl-3-acetylpyridine). The thrombolytic response of the platelet-rich thrombus that was bathed in arterial blood was recorded and expressed as a percentage of initial thrombus weight. The details of the method were described previously (Gryglewski *et al*., 2001). To differentiate between a transient and sustained thrombolytic response, the magnitude of thrombolytic response in the present work was characterized at two time points. After reaching the maximum thrombolysis (usually within 30 min after drug injection) and 1 h after drug injection (sustained thrombolysis).

The mechanism of the thrombolytic response induced by MNA was assayed using a non-selective COX inhibitor (indomethacin, 5 mg kg$^{-1}$), a selective COX-2 inhibitor (rofecoxib, 0.01–1 mg kg$^{-1}$), an NO synthase inhibitor (L-$N^G$-nitroarginine methyl ester, L-NAME, 5 mg kg$^{-1}$) or an antiplatelet dose of acetylsalicylic acid (ASA, 1 mg kg$^{-1}$). All drugs were injected intravenously 15 min before MNA. To exclude the involvement of *de novo* induction of COX-2 in the thrombolytic response to MNA, dexamethasone was injected intravenously before initiating extracorporeal circulation, approximately 3 h before injecting MNA. Experimental groups consisted of $n=3$–11 rats (altogether $n=125$ rats).

*Assay of the anti-thrombotic effect of MNA in a model of arterial thrombosis in hypertensive rats*

Two kidney-one clip (1K-1C) renovascular hypertension was induced as described previously (Mogielnicki *et al*., 2005). Rats were used for experiments 6 weeks after the induction of hypertension. Male Wistar rats with confirmed renovascular hypertension (145 mm Hg) were anaesthetized by intraperitoneal injection of pentobarbital (40 mg kg$^{-1}$) and then fixed in the supine position on a heated operating table. The left femoral vein was cannulated to administer drugs. Arterial thrombosis was induced by electrical stimulation of the carotid artery according to the method described by Schumacher *et al*. (1993). Briefly, a segment of the left common carotid artery, about 15 mm long, was exposed and cautiously dissected free of surrounding tissue. A piece of parafilm (5 mm × 20 mm) was placed under the exposed vessel to provide electrical isolation. Two electrodes were used. The anode, a stainless steel L-shaped wire, was inserted under the artery and connected to a circuit with a constant current generator. The cathode was a subcutaneous metal needle attached to the hindlimb. The electrical stimulation (1 mA) lasted 5 min. 45 min after stimulation, the segment of the carotid artery with the formed thrombus was dissected, opened lengthwise. Then the thrombus was completely removed, air-dried at 37°C and weighed 24 h after the end of the experiment.

MNA in doses of 3, 10 and 30 mg kg$^{-1}$ or 0.9% NaCl were administered intravenously (i.v.) 5 min before the induction of arterial thrombosis. To investigate the mechanism of the anti-thrombotic action of MNA in the arterial thrombosis model, a non-selective COX inhibitor (indomethacin, 2.5 mg kg$^{-1}$, i.v.) or a selective COX-2 inhibitor (rofecoxib, 1 mg kg$^{-1}$, i.v.), was administered into the left femoral vein 10 min before MNA administration. Experimental groups consisted of $n=4$–13 rats (altogether $n=67$ rats).

*Assay of the anti-thrombotic effect of MNA in a model of venous thrombosis*

For the assessment of the anti-thrombotic effects of MNA in venous thrombosis an experimental model in rats was used, in which venous thrombosis was induced by the ligation of the vena cava as described previously (Pawlak *et al*., 1996). Briefly, rats (350–500 g) were anaesthetized with pentobarbital (40 mg kg$^{-1}$, i.p.). 10 min after MNA or a vehicle administration, the abdomen was opened and the vena cava was carefully separated from the surrounding tissues and then ligated tightly with a cotton thread, just below the left renal vein. Subsequently, the abdomen was closed with a double layer of sutures (peritoneum with muscles and the skin separately). After 2 h the abdomen was reopened, the vena cava was carefully dissected and inspected for the presence of a thrombus. The thrombus was air-dried at 37°C, and after 24 h, its weight was measured. Experimental groups consisted of $n=5$ rats (altogether $n=10$ rats).

*Platelet aggregation assay*

Rat blood was withdrawn from anaesthetized Wistar rats and sodium citrate (3.2%, 1:9 v/v) was added as anticoagulant. Venous blood was also obtained from human volunteers at the University Hospital Blood Bank Centre. Blood was collected into vials containing sodium citrate (3.2%, 1:9 v/v) as the anti-coagulant agent. Volunteer donors had not taken any medicines for the preceding 2 weeks.

To obtain platelet-rich plasma (PRP), blood was centrifuged at 250 × *g* for 20 min. The PPP fraction was obtained by centrifugation of the remaining blood for 5 min at 2000 × *g*.

Aggregation of blood platelets was assessed in PRP using a dual channel Chrono-log aggregometer. The baseline value of the aggregometer was set using PRP whereas PPP was used to set the full transmittance. PRP (500 $\mu$l) was equilibrated for 3 min at 37°C with continuous stirring at 1100 r.p.m. and then stimulated with collagen to cause aggregation. At the beginning of each experiment, concentrations of collagen were determined that induced sub-maximum aggregation response inhibited by >90% with acetylsalicylic acid (ASA, 300 $\mu$M). Usually it was in the range of 1.2–2 $\mu$g ml$^{-1}$ of collagen.

To check the viability of platelets the response to carbaprostacyclin (cPGI$_2$, 100 nM), or *S*-nitroso-*N*-acetylpenicillamine (SNAP, 3 $\mu$M) was tested. Then the effect of MNA and nicotinamide was assayed. All compounds were added 2 min before stimulation of platelets with collagen. Transmittance was read 5 min after simulation of platelets with an agonist.

*Vasodilation assay*

Rats were anaesthetized with thiopental-sodium (120–150 mg kg$^{-1}$ body weight) (Biochemie GmbH, Kundl-Rakusko, Austria) and the aorta or the mesenteric vascular bed was

quickly removed and placed in cold Krebs–Heinseleit buffer of the following composition (mM): NaCl 118, $CaCl_2$ 2.52,. $MgSO_4$ 1.64, $NaHCO_3$ 24.88, $K_2PO_4$ 1.18, KCl 4.7, glucose 10.0.

Aortic rings were mounted in organ chambers filled with 5 ml of Krebs–Heinseleit solution maintained at 37°C, pH 7.4, and gassed with carbogen. After mounting of the rings, the resting tension was increased stepwise to reach a final 4 g and then the rings were incubated to equilibrium for 30 min. After testing the viability of the tissue by contractile response to potassium chloride (KCl $3 \times 10^{-2}$ to $9 \times 10^{-2}$ M), the aortic rings were precontracted with phenylephrine (Phe, $10^{-8}$–$3 \times 10^{-7}$ M) to obtain submaximal contraction (60–80% of KCl-induced maximum response). Relaxation to cumulative concentrations of acetylcholine ($10^{-9}$–$10^{-5}$ M) or SNAP ($10^{-9}$–$10^{-5}$ M) were induced to check the viability of the preparation, and then the effects of MNA and nicotinamide, were assayed. The relaxation response was expressed as a percentage of the precontraction induced by phenylephrine.

Mesenteric arteries, were mounted in the pressure myograph (JP Trading, Aarhus, Denmark). Cannulated vessels were filled with the Krebs–Heinseleit buffer containing 1% of albumin. The buffer in the chamber was bubbled with a gas mixture containing 21% $O_2$, 5% $CO_2$ and 74% $N_2$. The outer diameter of the vessel was continuously monitored by a video camera attached to the inverted microscope. After 30 min of stabilization, the vasoconstrictor response to KCl (60 mM) as well as the cumulative concentration-dependent response to acetylcholine, SNAP and 2-(N,N-diethylamino)-diazenolate 2-oxide (DEA-NO) (all in a concentration from $10^{-7}$ to $10^{-5}$ M) on phenylephrine-preconstricted vessels were tested to check the viability of the tissue. Thereafter the response to MNA and nicotinamide was analysed. The relaxation response was expressed as a percentage of the precontraction induced by phenylephrine.

*Measurements of $PGI_2$ release by MNA* in vivo

For determination of prostanoid (6-keto-$PGF_{1\alpha}$, $TXB_2$, $PGE_2$) levels in blood, arterial blood samples (500 $\mu$l) were drawn before injection of MNA, as well as 15, 30 and 60 min after MNA injection. Samples were collected in Ependorff tubes with indomethacin 10 $\mu$M and EDTA 1 mM (final concentrations), and immediately centrifuged for 5 min at $2000 \times g$ to obtain plasma. Samples of plasma were stored at −70°C. The concentrations of 6-keto-$PGF_{1\alpha}$, $TXB_2$ and $PGE_2$ in plasma were assayed using commercially available enzyme immunoassay kits (Cayman Chemical, Ann Arbor, MI, USA) and expressed in pg ml$^{-1}$. The limit of detection for 6-keto-$PGF_{1\alpha}$, $PGE_2$ and $TXB_2$ in blood was 20, 15 and 13 pg ml$^{-1}$, respectively.

*Data analysis*

Results were expressed as means ± s.e.means. Differences between means were evaluated by Kruskal–Wallis test followed by Dunn's multiple comparison test, by Friedman test or by Mann–Whitney test. A *P*-value of less than 0.05 was considered statistically significant.

*Reagents and drugs*

The pyridinium compounds (chloride salts): MNA, 1-propylnicotinamide (PNA), 1,N′-dimethylnicotinamide (MNA-Me), 1-methyl-N′,N′-diethylnicotinamide (MNA-$Et_2$), 1-(2-hydroxyethyl)nicotinamide [(EtOH)NA] and 1-methyl-3-acetylpyridine (MAP) were synthesized by alkylation of the corresponding 3-substituted pyridine derivatives with the appropriate reagent (methyl iodide, n-propyl iodide or 2-iodoethanol) in methanol solution, as described by Shaw (1961). The resulting iodide salts were converted to chlorides in aqueous solutions using freshly precipitated silver chloride and then purified by repeated crystallization from acetone–methanol. 1-ribosylnicotinamide (RibNA) was prepared by ribosylation of nicotinamide according to a published procedure (Tanimori *et al*., 2002). 1-methyl-2-pyridone-5-carboxamide (2-PYR) was synthesized from nicotinic acid as described previously (Grudzinski, 1962). Trigonelline, nicotinamide (purity >99%), nicotinic acid (purity >98%), 6-aminonicotinamide (6-ANA, purity 99%) and other 3-substituted pyridines were purchased from Sigma-Aldrich and used as received.

The chemical structures of pyridinium derivatives and nicotinamide analogues that were used in the study are shown in Tables 1a and b.

Collagen was obtained from Chrono-log (Havertown, PA, USA), acetylsalicylic acid (ASA) from Bayer (Dormagen, Germany), SNAP and indomethacin were from Sigma-Aldrich Chemicals, whereas carbaprostacyclin ($cPGI_2$) was purchased from Biomol Research Lab Inc. (USA). Rofecoxib was a gift from MSD Poland.

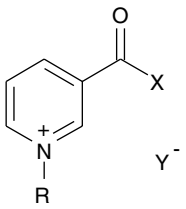
## Results

*Thrombolytic effects of MNA and structurally similar compounds* in vivo *in rats*

In Wistar rats with extracorporeal circulation, MNA administered intravenously produced a dose-dependent and sustained thrombolytic response (Figures 2a and 3a and b). The response was seen at a dose of 3 mg kg$^{-1}$ reaching a maximum value at doses of 30–100 mg kg$^{-1}$. The thrombolysis induced by MNA (10 mg kg$^{-1}$ or more) was noticeable within 2 min after MNA administration. It reached its maximum within 20–25 min following MNA administration and stayed at approximately the same level for at least 2–3 h (Figures 2a and 3a and b). MNA-induced thrombolytic response was not accompanied by a change in arterial blood pressure (data not shown).

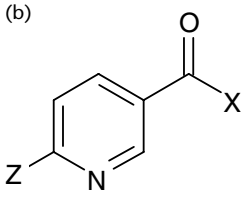
In contrast to MNA, nicotinamide (at a dose of 30 mg kg$^{-1}$) induced only a weak and transient thrombolysis. Even at a dose of 300 mg kg$^{-1}$, the effect of nicotinamide was still much weaker than that of MNA (Figures 2b and 3a and b). Nicotinamide metabolites other than MNA, such as 2-PYR or N-Ox, failed to induce significant thrombolysis (data not shown). Nicotinic acid (30–300 mg kg$^{-1}$) produced a transient thrombolytic response that was even weaker than that induced by nicotinamide, while 1-methyl nicotinic acid (trigonelline) was inactive even at a dose of 300 mg kg$^{-1}$ (Figures 2c and d and 3a and b).

**Table 1** Chemical structures of various pyridinium derivatives (a) and nicotinamide analogues (b) assayed for thrombolytic activity *in vivo*



| *Chemical name* | *R* | *X* | *Y* |
|---|---|---|---|
| (a) | | | |
| 1-Methylnicotinamide (MNA) | $CH_3$ | $NH_2$ | Cl |
| 1,N′-Dimethylnicotinamide (MNA-Me) | $CH_3$ | $N(H)CH_3$ | Cl |
| 1-Methyl-N′,N′-diethylnicotinamide (MNA-$Et_2$) | $CH_3$ | $N(CH_2CH_3)_2$ | Cl |
| 1-Propylnicotinamide (PNA) | $CH_2CH_2CH_3$ | $NH_2$ | Cl |
| 1-(2-Hydroxyethyl)nicotinamide ((EtOH)NA) | $CH_2CH_2OH$ | $NH_2$ | Cl |
| 1-Ribosylnicotinamide (RibNA) | $\beta$-D-ribofuranosyl | $NH_2$ | $CF_3SO_3$ |
| 1-Methyl-3-acetylpyridinium (MAP) | $CH_3$ | $CH_3$ | Cl |
| Trigonelline (TRIG) | $CH_3$ | OH | Cl |

(b)



| *Chemical name* | *X* | *Z* |
|---|---|---|
| Nicotinamide | $NH_2$ | H |
| *N,N*-Diethylnicotinamide | $N(CH_2CH_3)_2$ | H |
| 6-Amino nicotinamide | $NH_2$ | $NH_2$ |
| Nicotinic acid | OH | H |

Furthermore, closely related MNA analogues (Table 1) such as PNA and (EtOH)NA induced significantly weaker responses (Figure 3c). Moreover, the thrombolytic responses induced by PNA or (EtOH)NA were not immediate as that induced by MNA, but were delayed (data not shown). RibNA, 6-amino nicotinamide and analogues of MNA with methyl (MNA-Me) or ethyl (MNA-Et2) substituted amide groups were inactive (each at a dose of 30 mg kg$^{-1}$) (Figure 3c).

On the other hand, the MNA analogue with an acetyl group in the 3-position of the pyridine ring (MAP) induced dose-dependent and sustained thrombolysis (3–30 mg kg$^{-1}$). This response was, however, still less pronounced than that for MNA (Figure 3).

*The mechanism of thrombolytic effect of MNA*

The COX-2 inhibitor, rofecoxib (0.01–1 mg kg$^{-1}$), dose-dependently inhibited the thrombolytic response to MNA (Figure 4a) and a low dose of acetylsalicylic acid (ASA, 1 mg kg$^{-1}$) slightly amplified this response, while L-NAME (5 mg kg$^{-1}$) and dexamethasone (1 mg kg$^{-1}$) were without effect (Figure 4b). The thrombolytic response induced by MNA was also abolished by indomethacin (5 mg kg$^{-1}$) (Figure 4b).

*Release of $PGI_2$ by MNA* in vivo

The thrombolytic response to MNA was associated with a significant increase in concentrations of 6-keto-$PGF_{1\alpha}$ in blood, while the levels of $TXB_2$ displayed a modest time-dependent rise (Figure 5a). On the other hand, $PGE_2$ concentrations did not change in response to MNA (145.7±3.7, 145.5±4.5, 142.5±4.5 and 144.5±5.1 pg ml$^{-1}$before MNA, as well as 15, 30 and 60 min after MNA, respectively).

Pretreatment with rofecoxib (1 mg kg$^{-1}$) or indomethacin (5 mg kg$^{-1}$) decreased blood levels of 6-keto-$PGF_{1\alpha}$ to values close to the detection limit of the assay (20 pg ml$^{-1}$) and these levels did not change in response to MNA. The thrombolytic response to MAP was also associated with a concomitant rise in 6-keto-$PGF_{1\alpha}$ in blood, while the levels of $TXB_2$ did not change significantly (Figure 5b).

*Anti-thrombotic effects of MNA in the arterial thrombosis model in rats with renovascular hypertension*

In the model of arterial thrombosis in rats with renovascular hypertension, whereby the thrombus was induced by electrical stimulation, MNA reduced the thrombus formation in a dose-dependent manner over a range of MNA doses (3–30 mg kg$^{-1}$) comparable to those used in the thrombolysis model (Figure 6a). The anti-thrombotic effect of MNA in this model was abolished by pretreatment with indomethacin (2.5 mg kg$^{-1}$) or with rofecoxib (1 mg kg$^{-1}$) (Figure 6b). As in the thrombolysis model, the MNA-induced antithrombotic response in the arterial thrombosis model in renovascular hypertensive rats was not accompanied by a change in mean arterial blood pressure (117±4 vs 118±4 mm Hg before and after injection of MNA, 30 mg kg$^{-1}$).

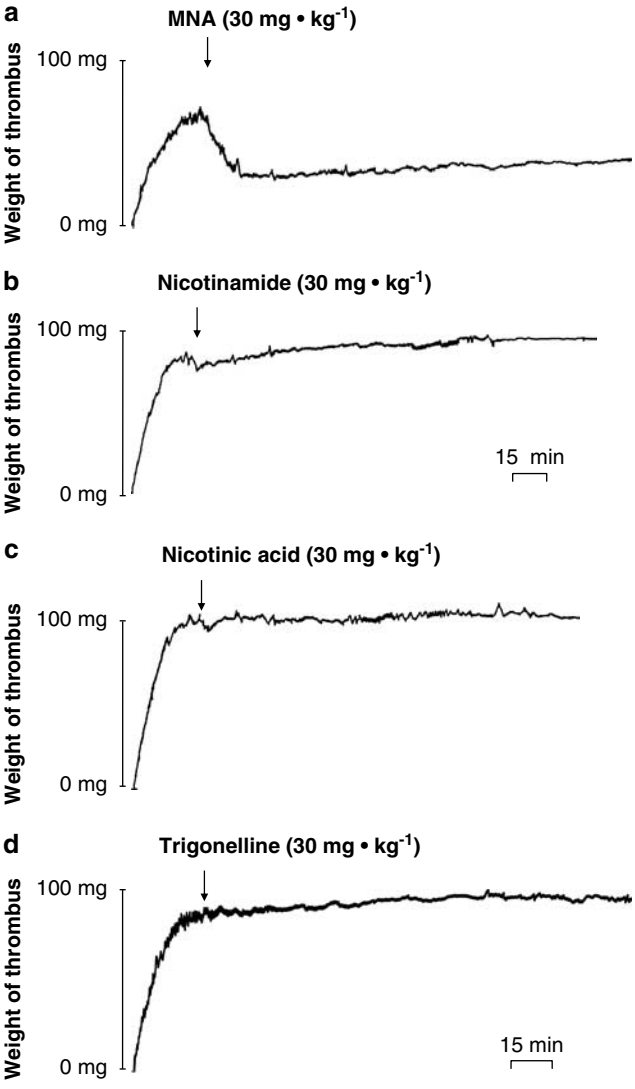



**Figure 2** Representative tracing showing pronounced and sustained thrombolytic response induced by 1-methylnicotinamide (30 mg kg$^{-1}$) *in vivo* in normotensive Wistar rats with extracorporeal circulation (**a**). For comparison, the lack of a significant thrombolytic response to nicotinamide (30 mg kg$^{-1}$) (**b**), nicotinic acid (30 mg kg$^{-1}$) (**c**) and 1-methylnicotinic acid (trigoneline, 30 mg kg$^{-1}$) (**d**) is shown.

*Anti-thrombotic effects of MNA in the venous thrombosis model in rats*

In contrast to its pronounced effectiveness in thrombolysis and arterial thrombosis models, MNA administered at a high dose (30 mg kg$^{-1}$) failed to modify thrombus formation in the rat model of venous thrombosis induced by ligation of the vena cava ($4.75 \pm 0.32$ mg in MNA treated group vs $4.08 \pm 1.11$ mg in vehicle treated group, $n = 5$, not significant).

*Antiplatelet effects of MNA* in vitro

*In vitro*, MNA did not affect collagen-induced human platelet aggregation over a micromolar range of concentrations and only minor effects of MNA on platelets were seen in the millimolar range of concentrations. Thus at 1 and 10 mM MNA, inhibition of platelet aggregation amounted to $4.7 \pm 2.3$ and $3.5 \pm 2.0$%, respectively. In contrast, nicotinamide (10 mM) inhibited collagen-induced platelet aggregation by $93.0 \pm 1.8$%. MNA also did not exhibit significant antiplatelet effects on rat platelets.

*Vasodilator effects of MNA* in vitro

MNA was also devoid of significant effects on the vascular tone in the aorta and mesenteric arteries. In aortic rings, MNA (10 mM) induced slight vasorelaxation ($6.7 \pm 1.0$%). In isolated mesenteric arteries, the relaxation induced by MNA (10 mM) was also weak ($6.02 \pm 6.22$%). Again, in these vascular preparations, nicotinamide in the millimolar range induced more marked vasorelaxation (in aortic rings $20.5 \pm 3.4$ and $101.5 \pm 7.4$% for 1 and 10 mM, respectively).

## Discussion

In the present work, we showed that MNA exhibited anti-thrombotic activity *in vivo* that was mediated by $PGI_2$ derived from vascular COX-2, while it was devoid of direct antiplatelet and vasodilator activity.

The anti-thrombotic effects of MNA were analysed in two complementary experimental models of platelet-dependent thrombosis. The first one involved extracorporeal thrombus formation, in which aggregates of platelets, formed on the collagen strip superfused with blood were dissipated by MNA. This response was considered as a thrombolytic response. The second one concerned intravascular thrombus formation in response to vascular injury, whereby MNA prevented the thrombus formation. Both models have been well-characterized in previous studies (Gryglewski *et al*., 2001; Wojewodzka-Zelezniakowicz *et al*., 2006). Importantly, in both models of arterial thrombosis platelets make a major contribution to the development of thrombosis, as the inhibition of platelet activation by ASA or stimulation of endothelial release of $PGI_2$ by angiotensin-converting enzyme inhibitors afforded pronounced anti-thrombotic effects (Schumacher *et al*., 1993; Gryglewski *et al*., 2001; Wojewodzka-Zelezniakowicz *et al*., 2006). In contrast, in venous thrombosis induced by the occlusion of the vena cava, platelets play a minor role and the stimulation of endothelial release of NO, rather then that of $PGI_2$, seems to provide the pronounced anti-thrombotic effect (Cylwik *et al*., 2004). Thus, our present findings that MNA with its $PGI_2$ releasing properties, while ineffective in venous thrombosis, displays the ability to limit platelet-dependent thrombosis is fully consistent with the nature of these experimental models and highlights the specificity of MNA and $PGI_2$-triggered mechanisms towards arterial, platelet-dependent, thrombosis. It would be still worthwhile to examine MNA activity in a different animal model of platelet-dependent thrombosis, sensitive to agents that modulate the COX pathways (Hennan *et al*., 2002) to confirm our conclusion.

We provide the following evidence for the involvement of the COX-2/$PGI_2$ pathway in the MNA-induced response. *In vitro,* MNA (up to high millimolar concentrations) did not affect platelet aggregation—hence mechanisms of the anti-

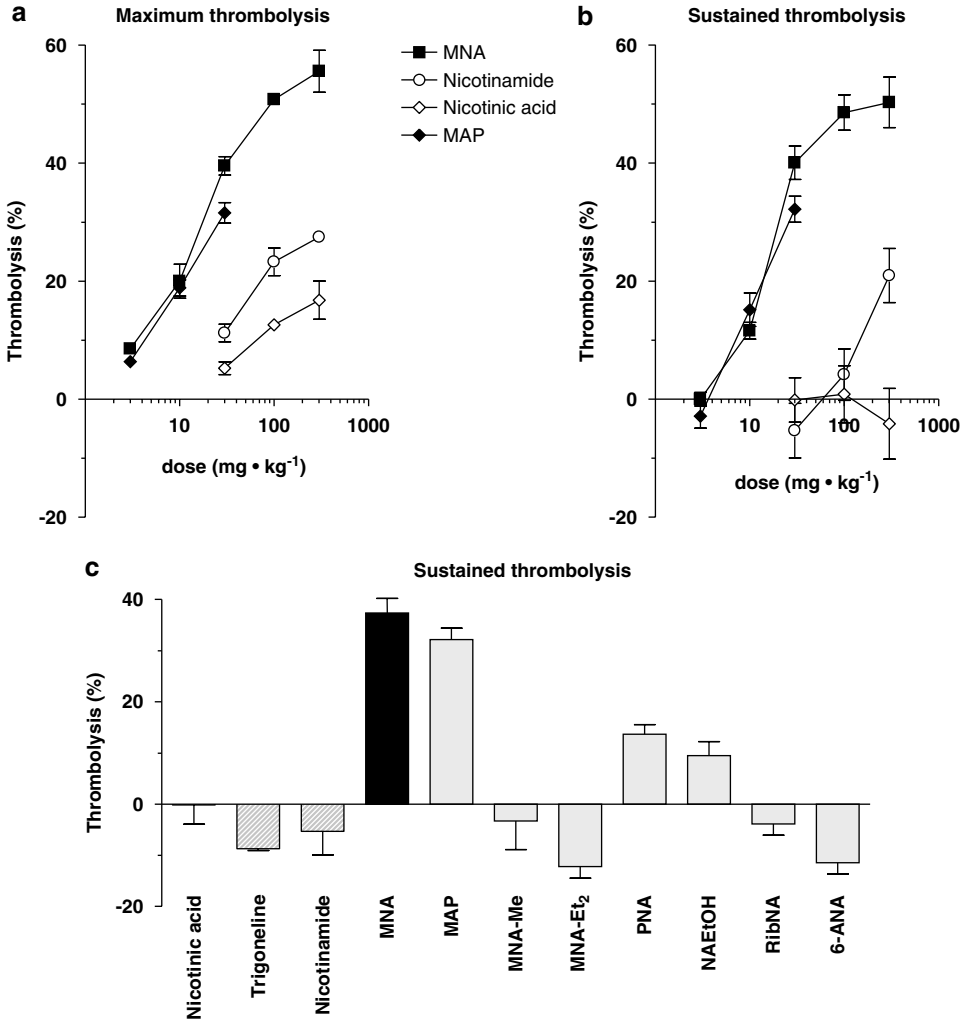



**Figure 3** Dose-dependent thrombolytic response induced by 1-methylnicotinamide (MNA) *in vivo* in normotensive Wistar rats with extracorporeal circulation as compared to the effect of nicotinamide, nicotinic acid and MAP. (**a**) Maximum thrombolytic response (attained within 30 min of drug injection) and (**b**) magnitude of sustained thrombolytic response (measured 1 h after drug injection) induced by MNA, nicotinamide, nicotinic acid and MAP are shown. Data represent the mean ± s.e.means from $n = 3$–11 experiments. (**c**) Comparison of the thrombolytic effect of MNA with that induced by structurally related compounds. The thrombolytic effect of each compound was assayed *in vivo* after intravenous injection (at a dose of 30 mg kg$^{-1}$) in normotensive Wistar rats with extracorporeal circulation. The sustained thrombolytic response (1 h after drug injection) of MNA is compared to the response induced by various pyridinium derivatives. Data represent mean ± s.e.means of 3–11 experiments. MAP, 1-methyl-3-acetylpyridine; MNA, Me - 1,N′-dimethylnicotinamide; MNA-$Et_2$, 1-methyl-N′,N′-diethylnicotinamide; PNA, 1-propylnicotinamide; (EtOH)NA, 1-(2-hydroxyethyl)nicotinamide; RibNA, 1-ribosylnicotinamide; 6-ANA, 6-amino nicotinamide (see also Table 1).

thrombotic action of MNA could not be explained by a direct antiplatelet action of MNA. On the other hand, the thrombolytic response to MNA was associated with a release of $PGI_2$ (assayed as 6-keto-$PGF_{1\alpha}$) into arterial plasma, while neither $TXB_2$ nor $PGE_2$ levels changed in response to MNA. Moreover, in the presence of a non-selective COX inhibitor (indomethacin) or a selective COX-2 inhibitor (rofecoxib) the thrombolytic response to MNA and concomitant 6-keto-$PGF_{1\alpha}$ release were abolished. Also in the arterial thrombosis model, the MNA-induced effect was markedly inhibited in the presence of indomethacin or rofecoxib, supporting our conclusion that the anti-thrombotic activity of MNA *in vivo* in both experimental systems was mediated by $PGI_2$ formed via COX-2.

Although *in vitro,* $PGI_2$ and NO seem to be released from the endothelium in a coupled manner (Gryglewski *et al.*, 1986), $PGI_2$-dependent thrombolysis induced by MNA *in vivo,* was not associated with NO-dependent hypotension. Indeed MNA did not change either mean blood pressure or carotid blood flow when applied intravenously. Moreover, the NOS inhibitor L-NAME did not modify the MNA-induced thrombolytic response. In line with this result, in aortic rings or mesenteric arteries *in vitro*, MNA was devoid of NO-dependent vasorelaxant activity. Accordingly, MNA appears to be a relatively selective releaser of vascular $PGI_2$ from COX-2 *in vivo*.

It was quite surprising that, among the various structurally modified MNA analogues, we have not found a compound with better thrombolytic activity than MNA itself. Only the

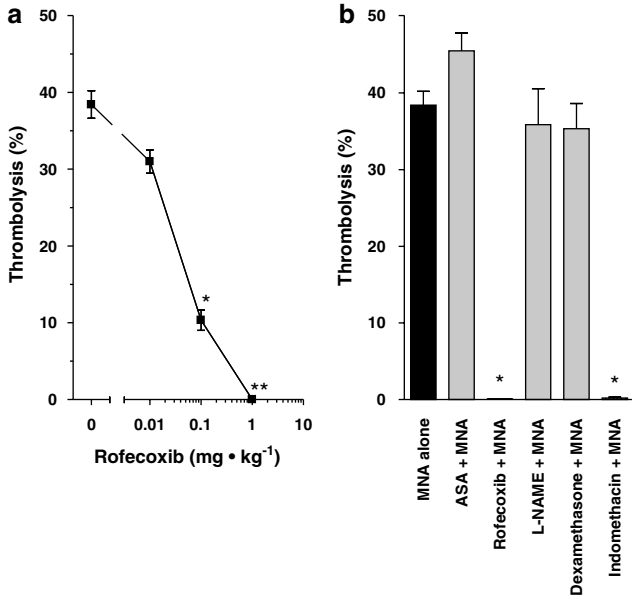



**Figure 4** Involvement of COX-2 in thrombolytic response induced by 1-methylnicotinamide (MNA) (30 mg kg$^{-1}$) *in vivo* in normotensive Wistar rats with extracorporeal circulation. (**a**) Dose-dependent inhibition of the thrombolytic response to MNA by the selective COX-2 inhibitor, rofecoxib. Data represent the mean±s.e.means from 3 to 10 experiments. * $P<0.05$ vs control, ** $P<0.01$ vs control as assessed by Kruskal–Wallis test followed by Dunn's multiple comparison test. (**b**) Effects of the non-selective COX inhibitor indomethacin (5 mg kg$^{-1}$), an antiplatelet dose of acetylsalicylic acid (ASA, 1 mg kg$^{-1}$), the non-selective NOS inhibitor (L-NAME, 5 mg kg$^{-1}$) and the inhibitor of COX-2 induction, dexamethasone (1 mg kg$^{-1}$) on the thrombolytic response to MNA (30 mg kg$^{-1}$). Inhibitors were administered 15 min before MNA injection, with the exception of dexamethasone, which was given 3 h before MNA. Data represent mean±s.e.means from 3 to 10 experiments. * indicates $P<0.05$, vs control response as assessed by Kruskal–Wallis test followed by Dunn's multiple comparison test.

replacement of the amide group at the three-position of the pyridine ring by an acetyl group, as in the case of MAP, resulted in the retention of sustained dose-dependent thrombolysis (3–30 mg kg$^{-1}$) with a concomitant rise in 6-keto-PGF$_{1\alpha}$ in blood. Other tested compounds were inactive or much weaker thrombolytic agents. For example, 6-amino nicotinamide—an inhibitor of pentose phosphate pathway (PPP) (Gupte *et al*., 2003) and 1-ribosylnicotinamide—a newly discovered precursor of NAD$^+$ (Bieganowski and Brenner, 2004)—were virtually inactive as thrombolytics. Thus the mechanism of MNA-induced thrombolysis would seem independent of PPP activity or of intracellular NAD$^+$. Nicotinic acid and trigonelline were also ineffective thrombolytically, even though nicotinic acid, at very high doses, has previously been shown to induce a remarkable thrombolytic response in cats that was attributed to the release of PGI$_2$ (Swies and Dabrowski, 1984). In turn, the nicotinamide-induced response was substantially weaker than that of MNA. The observed weak anti-thrombotic activity of nicotinamide may be explained by the fact that only a minor part of nicotinamide is transformed to MNA by liver nicotinamide-*N*-methyltransferase upon direct intravascular administration, while the different magnitude of the response to nicotinic acid in cats vs rats may underline species differences known to mark the selective biological response to nicotinic acid (Declercq *et al*., 2005).

It is of note that in patients with peripheral artery disease the drugs with the nicotinic acid moiety such as *β*-pyridylcarbinol (Ronicol) or xanthinol nicotinate (Sadamin) exert their antiplatelet actions through the release of endothelial PGI$_2$ (Dembinska-Kiec *et al*., 1983; Bieron *et al*., 1998), while nicotinic acid-induced flushing is mediated by a stimulation of the GPR109A receptor and the subsequent release of PGD$_2$ and PGE$_2$ from COX-1 (Benyo *et al*., 2005; Pike, 2005). It remains to be tested whether the MNA-induced release of PGI$_2$ from COX-2 involves this vascular nicotinic acid-like receptor or other mechanisms.

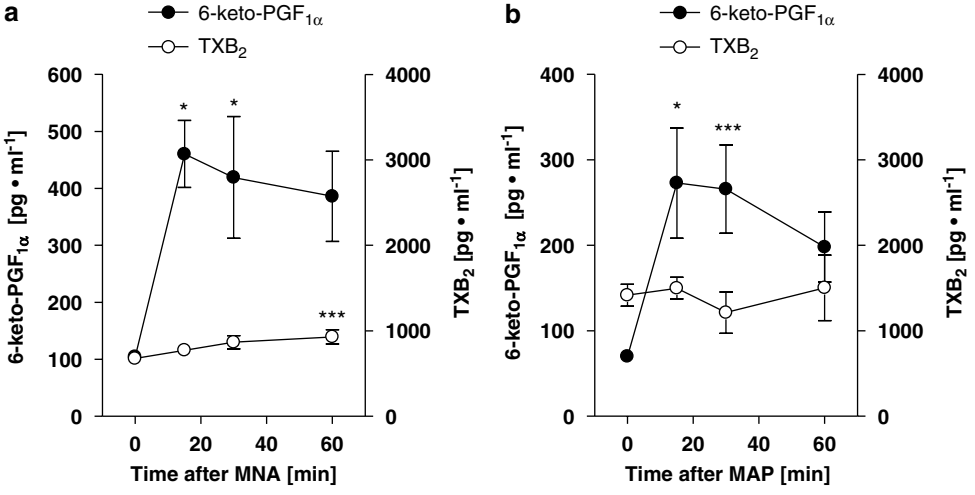



**Figure 5** Release of PGI$_2$ by 1-methylnicotinamide (MNA) (**a**) and MAP (**b**) *in vivo*. Levels of 6-keto-PGF$_{1\alpha}$ and of other prostanoids were measured in plasma following injection of MNA or MAP (30 mg kg$^{-1}$) in normotensive Wistar rats with extracorporeal circulation. Data represent the mean±s.e.means from $n=4$–7 experiments. * $P<0.05$ vs basal level, *** $P<0.001$ vs basal level as assessed by Friedman test followed by Dunn's multiple comparison test.

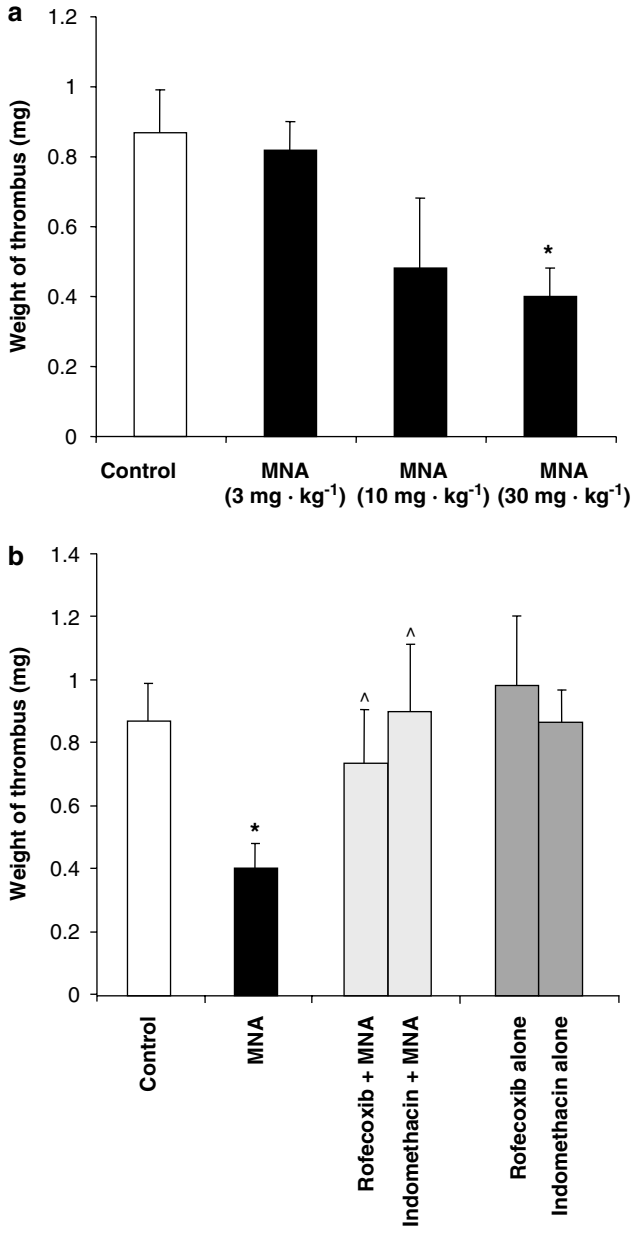



**Figure 6** Antithrombotic effects of 1-methylnicotinamide (MNA). (**a**) Dose-dependent antithrombotic effect of MNA in arterial thrombosis model in rats with renovascular hypertension. The columns represent the thrombus weight in hypertensive rats treated with 0.9% NaCl (Control), or MNA (3, 10 and 30 mg kg$^{-1}$ i.v.). (**b**) Inhibition of antithrombotic effect of MNA by the non-selective COX inhibitor indomethacin and the selective COX-2 inhibitor rofecoxib. The columns represent the thrombus weight in hypertensive rats treated with, 0.9% NaCl (Control), MNA (30 mg kg$^{-1}$ i.v.), indomethacin (2.5 mg kg$^{-1}$ i.v.) or rofecoxib (1 mg kg$^{-1}$ i.v.) followed by administration of MNA (30 mg kg$^{-1}$ i.v.) or the COX inhibitors alone. Data represent mean$\pm$s.e.means of $n=4$–13 experiments. * $P<0.05$ vs Control; ^ $P<0.05$ vs MNA 30 mg kg$^{-1}$ as assessed by two-tailed, Mann–Whitney test.

There is overwhelming evidence that COX-2 derived $PGI_2$ affords vasculoprotective, cardioprotective and anti-atherogenic activity (Gryglewski, 1980; Dowd *et al*., 2001; Grosser *et al*., 2006). The biological importance of the vascular COX-2/$PGI_2$ pathway has recently been emphasized by the increased risk of myocardial infarction and stroke reported in patients treated with selective COX-2 inhibitors (Grosser *et al*., 2006). It is clear today that the long-term use of drugs known to inhibit COX and subsequently to depress $PGI_2$ production proved to be harmful, while pharmacological stimulation of $PGI_2$ *in vivo* with the use of MNA might be beneficial in vascular diseases. Indeed MNA, being a stable and non-toxic molecule, seems to be a good candidate for a drug to boost the endogenous COX-2/$PGI_2$ pathway. So far, $PGI_2$ or its stable analogues have been widely used in the treatment of pulmonary hypertension (Wise and Jones, 1996) and have been proven effective in cases of peripheral arterial disease (Gryglewski, 1980) or liver injury (Ohta *et al*., 2005). It will be important to test the therapeutic effectiveness of MNA.

It is important to note that in our experiments, MNA afforded anti-thrombotic action, not only in normotensive rats, but also in rats with renovascular hypertension (2K-1C hypertension). Hypertension is one of the most important risk factors of arterial thrombosis and its clinical consequences such as acute coronary syndrome or ischaemic stroke. Therefore studying thrombosis in hypertensive rats more closely resembles a clinically relevant situation. In various cardiovascular pathologies, including hypertension, endothelial dysfunction develops that is characterized by an impaired production of NO, an impairment of basal $PGI_2$ production (Gryglewski, 1980; Frein *et al*., 2005) and a compensatory increase in $PGI_2$ formation by COX-2 (FitzGerald *et al*., 2000). Our results suggest that in the setting of impaired NO-dependent function, the COX-2/$PGI_2$ pathway is able to be stimulated pharmacologically with MNA. These findings have important therapeutic implications.

Finally, the demonstration of biological activity of MNA may bring a new understanding of the mechanism of the pharmacological activities of nicotinamide. Indeed, the anti-diabetic, neuroprotective (Satoh *et al*., 1999; Gosteli, 2005) as well as anti-inflammatory action of nicotinamide, at least in part, may be mediated by a MNA-COX-2/$PGI_2$ pathway, as outlined here.

Summing up, we demonstrate here—to our knowledge for the first time—the novel biological activity of MNA *in vivo* that greatly surpasses that of closely related compounds. MNA appears as an anti-thrombotic agent that limits platelet-dependent experimental thrombosis by a mechanism dependent on the COX-2/$PGI_2$ pathway. Although our study focused on exogenously applied MNA, our results could imply that endogenous MNA formed in the liver by nicotinamide N-methyltrasferase is an endogenous activator of the COX-2/$PGI_2$ pathway and may play an important regulatory role in limiting thrombosis, as well as inflammatory processes in the cardiovascular system. Our findings of novel *in vivo* biological activity of MNA may have potentially important physiological, biochemical as well as therapeutic implications and warrant further studies.

## Acknowledgements

This work was supported by the Polish Ministry of Science and Higher Education (grants no. PBZ-KBN-101/T09/2003).

Professor Stefan Chlopicki is the recipient of a Professorial grant from the Foundation for Polish Science (SP/04/04). We would like to thank Leonarda Stelmach for her excellent technical assistance.

## Conflict of interest

JG and SCH have filed a patent on the use of quaternary pyridinium salts as vasoprotective agents (International Patent Application: PCT/EP2005/050057). JG is involved in Pharmena Ltd to move MNA into clinical trials.

## References

Aoyama K, Matsubara K, Okada K, Fukushima S, Shimizu K, Yamaguchi S *et al*. (2000). N-methylation ability for azaheterocyclic amines is higher in Parkinson's disease: nicotinamide loading test. *J Neural Transm* **107**: 985–995.

Benyo Z, Gille A, Kero J, Csiky M, Suchankova MC, Nusing RM *et al*. (2005). GPR109A (PUMA-G/HM74A) mediates nicotinic acid-induced flushing. *J Clin Invest* **115**: 3634–3640.

Bieganowski P, Brenner C (2004). Discoveries of nicotinamide riboside as a nutrient and conserved NRK genes establish a Preiss-Handler independent route to NAD+ in fungi and humans. *Cell* **117**: 495–502.

Bieron K, Swies J, Kostka-Trabka E, Gryglewski RJ (1998). Thrombolytic and antiplatelet action of xanthinol nicotinate (Sadamin): possible mechanisms. *J Physiol Pharmacol* **49**: 241–249.

Chong ZZ, Lin SH, Maiese K (2002). Nicotinamide modulates mitochondrial membrane potential and cysteine protease activity during cerebral vascular endothelial cell injury. *J Vasc Res* **39**: 131–147.

Cylwik D, Mogielnicki A, Kramkowski K, Stokowski J, Buczko W (2004). Antithrombotic effect of L-arginine in hypertensive rats. *J Physiol Pharmacol* **55**: 563–574.

Declercq V, Yeganeh B, Moshtaghi-Kashanian GR, Khademi H, Bahadori B, Moghadasian MH (2005). Paradoxical effects of fenofibrate and nicotinic acid in apo E-deficient mice. *J Cardiovasc Pharmacol* **46**: 18–24.

Delaney J, Hodson MP, Thakkar H, Connor SC, Sweatman BC, Kenny SP *et al*. (2005). Tryptophan-$NAD^+$ pathway metabolites as putative biomarkers and predictors of peroxisome proliferation. *Arch Toxicol* **79**: 208–223.

Dembinska-Kiec A, Korbut R, Bieron K, Kostka-Trabka E, Gryglewski RJ (1983). Beta-Pyridylcarbinol (Ronicol) releases a prostacyclin-like substance into arterial blood of patients with arteriosclerosis obliterans. *Pharmacol Res Commun* **15**: 377–385.

Dowd NP, Scully M, Adderley SR, Cunningham AJ, Fitzgerald DJ (2001). Inhibition of cyclooxygenase-2 aggravates doxorubicin-mediated cardiac injury *in vivo*. *J Clin Invest* **108**: 585–590.

FitzGerald GA, Austin S, Egan K, Cheng Y, Pratico D (2000). Cyclooxygenase products and atherothrombosis. *Ann Med* **32** (Suppl 1): 21–26.

Frein D, Schildknecht S, Bachschmid M, Ullrich V (2005). Redox regulation: a new challenge for pharmacology. *Biochem Pharmacol* **70**: 811–823.

Gebicki J, Sysa-Jedrzejowska A, Adamus J, Wozniacka A, Rybak M, Zielonka J (2003). 1-Methylnicotinamide: a potent anti-inflammatory agent of vitamin origin. *Pol J Pharmacol* **55**: 109–112.

Gosteli J (2005). Nicotinamide trials in diabetes intervention. Does a metabolite provide benefit? *Med Hypotheses* **64**: 1062–1063.

Grosser T, Fries S, FitzGerald GA (2006). Biological basis for the cardiovascular consequences of COX-2 inhibition: therapeutic challenges and opportunities. *J Clin Invest* **116**: 4–15.

Grudzinski S (1962). Preparation of N-methyl-2-pyridone-3-carboxamide. *Roczniki Chemii* **36**: 1705–1708.

Gryglewski RJ (1980). Prostaglandins, platelets, and atherosclerosis. *CRC Crit Rev Biochem* **7**: 291–338.

Gryglewski RJ, Moncada S, Palmer RM (1986). Bioassay of prostacyclin and endothelium-derived relaxing factor (EDRF) from porcine aortic endothelial cells. *Br J Pharmacol* **87**: 685–694.

Gryglewski RJ, Uracz W, Swies J, Chlopicki S, Marcinkiewicz E, Lomnicka M *et al*. (2001). Comparison of endothelial pleiotropic actions of angiotensin converting enzyme inhibitors and statins. *Ann N Y Acad Sci* **947**: 229–245.

Gupte SA, Arshad M, Viola S, Kaminski PM, Ungvari Z, Rabbani G *et al*. (2003). Pentose phosphate pathway coordinates multiple redox-controlled relaxing mechanisms in bovine coronary arteries. *Am J Physiol Heart Circ Physiol* **285**: H2316–H2326.

Hennan JK, Hong TT, Willens DE, Driscoll EM, Giboulot TA, Lucchesi BR (2002). Prevention of experimental carotid and coronary artery thrombosis by the glycoprotein IIb/IIIa receptor antagonist CRL42796. *Br J Pharmacol* **136**: 927–937.

Maiese K, Chong ZZ (2003). Nicotinamide: necessary nutrient emerges as a novel cytoprotectant for the brain. *Trends Pharmacol Sci* **24**: 228–232.

Maiza A, Waldek S, Ballardie FW, Daley-Yates PT (1992). Estimation of renal tubular secretion in man, in health and disease, using endogenous N-1-methylnicotinamide. *Nephron* **60**: 12–16.

Mogielnicki A, Chabielska E, Pawlak R, Szemraj J, Buczko W (2005). Angiotensin II enhances thrombosis development in renovascular hypertensive rats. *Thromb Haemost* **93**: 1069–1076.

Ohta S, Nakamuta M, Fukushima M, Kohjima M, Kotoh K, Enjoji M *et al*. (2005). Beraprost sodium, a prostacyclin (PGI) analogue, ameliorates concanavalin A-induced liver injury in mice. *Liver Int* **25**: 1061–1068.

Pawlak R, Chabielska E, Buczko W (1996). Antithrombotic effect of enalapril, an angiotensin-converting enzyme inhibitor, on venous thrombosis in rats. *Pol J Pharmacol* **48**: 117–120.

Pike NB (2005). Flushing out the role of GPR109A (HM74A) in the clinical efficacy of nicotinic acid. *J Clin Invest* **115**: 3400–3403.

Pumpo R, Sarnelli G, Spinella A, Budillon G, Cuomo R (2001). The metabolism of nicotinamide in human liver cirrhosis: a study on N-methylnicotinamide and 2-pyridone-5-carboxamide production. *Am J Gastroenterol* **96**: 1183–1187.

Satoh T, Ishikawa Y, Kataoka Y, Cui Y, Yanase H, Kato K *et al*. (1999). CNS-specific prostacyclin ligands as neuronal survival-promoting factors in the brain. *Eur J Neurosci* **11**: 3115–3124.

Schumacher WA, Steinbacher TE, Heran CL, Megill JR, Durham SK (1993). Effects of antithrombotic drugs in a rat model of aspirin-insensitive arterial thrombosis. *Thromb Haemost* **69**: 509–514.

Shaw EN (1961). Quaternary pyridinium compounds. In: E Klingsberg (ed). *Pyridine Its Derivatives*. Interscience Publishers: New York, pp 1–31.

Swies J, Dabrowski L (1984). Release of disaggregatory prostanoids into circulation by nicotinic alcohol, nicotinic acid and nicotinamide. *Pol J Pharmacol* **36**: 653–657.

Tanimori S, Ohta T, Kirihata M (2002). An efficient chemical synthesis of nicotinamide riboside (NAR) and analogues. *Bioorg Med Chem Lett* **12**: 1135–1137.

Ungerstedt JS, Heimersson K, Soderstrom T, Hansson M (2003). Nicotinamide inhibits endotoxin-induced monocyte tissue factor expression. *J Thromb Haemost* **1**: 2554–2560.

Vivian VM, Chaloupka MM, Reynolds MS (1958). Some aspects of tryptophan metabolism in human subjects. I. Nitrogen balances, blood pyridine nucleotides and urinary excretion of N1-methylnicotinamide and N1-methyl-2-pyridone-5-carboxamide on a low-niacin diet. *J Nutr* **66**: 587–598.

Wise H, Jones RL (1996). Focus on prostacyclin and its novel mimetics. *Trends Pharmacol Sci* **17**: 17–21.

Wojewodzka-Zelezniakowicz M, Chabielska E, Mogielnicki A, Kramkowski K, Karp A, Opadczuk A *et al*. (2006). Antithrombotic effect of tissue and plasma type angiotensin converting enzyme inhibitors in experimental thrombosis in rats. *J Physiol Pharmacol* **57**: 231–245.

Wozniacka A, Wieczorkowska M, Gebicki J, Sysa-Jedrzejowska A (2005). Topical application of 1-methylnicotinamide in the treatment of rosacea: a pilot study. *Clin Exp Dermatol* **30**: 632–635.

Yan L, Otterness DM, Weinshilboum RM (1999). Human nicotinamide N-methyltransferase pharmacogenetics: gene sequence analysis and promoter characterization. *Pharmacogenetics* **9**: 307–316.