Therapeutic Potential of 1-Methylnicotinamide against Acute Gastric Lesions Induced by Stress: Role of Endogenous Prostacyclin and Sensory Nerves

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Received January 16, 2008; accepted April 1, 2008

ABSTRACT

1-Methylnicotinamide (MNA) is one of the major derivatives of nicotinamide, which was recently shown to exhibit antithrombotic and antiinflammatory actions. However, it is not yet known whether MNA affects gastric mucosal defense. The effects of exogenous MNA were studied on gastric secretion and gastric lesions induced in rats by 3.5 h of water immersion and water restraint stress (WRS) or in rats administered 75% ethanol. MNA [6.25–100 mg/kg intragastrically (i.g.)] led to a dose-dependent rise in the plasma MNA level, inhibited gastric acid secretion, and attenuated these gastric lesions induced by WRS or ethanol. The gastroprotective effect of MNA was accompanied by an increase in the gastric mucosal blood flow and plasma calcitonin gene-related peptide (CGRP) levels, the preservation of prostacyclin (PGI2) generation (measured as 6-keto-PGF1α), and an overexpression of mRNAs for cyclooxygenase (COX)-2 and CGRP in the gastric mucosa. R-3-(4-Fluoro-phenyl)-2-[5-(4-fluoro-phenyl)-benzofuran-2-ylmethoxy-carbonylamino]-propionic acid (RO 324479), which is the selective antagonist of IP/PGI2 receptors, reversed the effects of MNA on gastric lesions and GBF. MNA-induced gastroprotection was attenuated by suppression of COX-1 [5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole; SC-560] and COX-2 [4-(4-methylsulfonylphenyl)-3-phenyl-5H-furan-2-one; rofecoxib] activity, capsacin denervation, and by the pretreatment with CGRP8-37 or capsazepine. Addition of exogenous PGI2 or CGRP restored the MNA-induced gastroprotection in rats treated with COX-1 and COX-2 inhibitors or in those with capsacin denervation. WRS enhanced MDA content while decreasing superoxide dismutase (SOD) activity in the gastric mucosa, but pretreatment with MNA reversed these changes. MNA exerted potent gastroprotection against WRS damage via mechanisms involving cooperative action of PGI2 and CGRP in preservation of microvascular flow, antioxidizing enzyme SOD activity, and reduction in lipid peroxidation.

Nicotinamide (NA) is a precursor for the coenzyme β-nicotinamide adenine dinucleotide (NAD+), which serves as an essential nutrient for cellular growth, and it participates in the regulation of multiple cellular functions, including apoptosis and response to injury (Mukherjee et al., 1997; Maiese and Chong, 2003). Indeed, NA has been reported to exert several physiological and pharmacological effects, including the prevention of ATP depletion, inhibition of poly(ADP-ribose) polymerase, prevention of apoptosis, and suppression of lipid peroxidation (Klaidman et al., 1996, 2003). NA also possesses anti-inflammatory properties (Ogata et al., 2002; Ungerstedt et al., 2003) and exhibits angiogenic activity by promoting the growth of capillaries (Morris et al., 1989).

This work was supported by Grant PBZ-KBN-101/T09/2003 from the Polish Ministry of Science and Higher Education.


Article, publication date, and citation information can be found at http://jpet.aspetjournals.org. doi:10.1124/jpet.108.136457.

ABBREVIATIONS: NA, nicotinamide; MNA, 1-methylnicotinamide; M2PY, 1-methyl-2-pyridone-5-carboxamide; M4PY, 1-methyl-1-4-pyridone-5-carboximide; PGI2, prostacyclin; PGE2, prostaglandin E2; COX, cyclooxygenase; RO 3244794, R-3-(4-fluoro-phenyl)-2-[5-(4-fluoro-phenyl)-benzofuran-2-ylmethoxy-carbonylamino]-propionic acid; CGRP, calcitonin gene-related peptide; WRS, water immersion and restraint stress; GBF, gastric blood flow; SOD, superoxide dismutase; MDA, malonyldialdehyde; GF, gastric fistula; SC-560, 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole; i.g., intragastrically; TRPV1, transient receptor potential vanilloid type 1; RT-PCR, reverse transcriptase-polymerase chain reaction; 4-HNE, PG, prostaglandin; 4-HNE, trans-4-hydroxy-2-nonenal.
1-Methylnicotinamide (MNA) is the major primary metabolite of NA, which is formed in the liver by the enzyme nicotinamide N-methyltransferase, and it is further metabolized to 1-methyl-2-pyridone-5-carboxamide (M2PY) or 1-methyl-4-pyridone-5-carboxamide (M4PY) by aldehyde oxidase (Aoyama et al., 2000). For many years, MNA has been considered inactive. Recent studies, however, have indicated that MNA applied topically exhibits remarkable anti-inflammatory properties against skin diseases such as acne vulgaris, contact dermatitis (Gebicki et al., 2003), and rosacea (Wozniacka et al., 2005). Recently, it has been reported that MNA displays a potent anti-arthritogenic activity in vivo that was mediated by the prostacyclin (PGI2) pathway (Chlopicki et al., 2007). By using the mouse model of contact hypersensitivity, it was demonstrated that MNA affords anti-inflammatory action that also seems to be mediated by PGI2 (Bryniaski et al., 2008). Little is known about the action of MNA in the gut; however, the gastric uptake of nicotinic acid via mechanism involving bilitranslocase was recently demonstrated, suggesting that significant amounts of niacin-related compounds could be taken up at the gastric level from food during digestion (Passamonti et al., 2000). Conversely, the gastroprotective effect of PGI2 and PGE2 is well established (Konturek et al., 1981). Accordingly, the recently discovered vasoprotective action of MNA mediated by PGI2 implies that there is a possibility for the involvement of MNA in gastric pathophysiology, particularly in the mechanism of gastric secretory functions, mucosal defense, and gastroprotection against acute gastric lesions.

The influence of MNA to the mechanism of gastric mucosal defense against the damage induced by noxious agents has not been studied so far. This prompted us to examine whether exogenous MNA affects the formation of stress-induced gastric damage, which is a serious clinical entity in humans ultimately resulting in gastric mucosal bleeding and erosions. Furthermore, the gastroprotective effect of MNA was compared with that exhibited by potent gastric acid inhibitors such as proton pump inhibitor omeprazole and histamine H2-receptor antagonist ranitidine against the formation of acute gastric lesions induced by stress. In a separate group of rats, the protective effects of MNA against the formation of ethanol lesions were determined to check whether MNA provided protection against acid-independent necrotizing type of gastric injury. An attempt was made to assess the mechanism of gastroprotective action of MNA by focusing on the involvement of PGI2 and sensory nerves. We measured the gastric mucosal generation of PGI2 (6-ketoprostaglandin F1α) and by assessment of mRNA expression for COX-1 and COX-2 as well as by pharmacological tools such as specific IP/PGI2 receptor antagonist RO 324479 (Bley et al., 2006), the nonselective (indomethacin) and selective inhibitors of COX-1 (SC-560) and COX-2 [4-(4-methylsulfonylphenyl)-3-phenyl-5H-furan-2-one rofecoxib] activity. In addition, an attempt was made to determine the involvement of sensory nerves in MNA-induced gastroprotection, considering that reciprocal interactions between activities of PGI2 and sensory nerve-derived CGRP have been reported previously (Holzer et al., 1991; Arai et al., 2003). The mechanism of gastroprotection induced by MNA in water restraint stress (WRS) model of gastric damage was determined by studying the influence of MNA on the gastric blood flow (GBF), plasma levels of MNA assessed by liquid chromatography-mass spectrometry, antioxidizing enzyme superoxide dismutase (SOD), and malonyldialdehyde (MDA) content in the gastric mucosa, as an index of lipid peroxidation.

**Materials and Methods**

Male Wistar rats, weighing 180 to 220 g and fasted for 24 h, were used in studies on gastric secretion and gastroprotection. This study was approved by the Institutional Animal Care and Use Committee of Jagiellonian University Medical College in Cracow and run in accordance to the statements of Helsinki Declaration regarding handling of experimental animals.

**Gastric Secretory Studies.** The effects of MNA (synthesized in the Institute of Applied Radiation Chemistry Technical University of Lodz, Lodz, Poland) on gastric acid secretion were examined in 40 conscious rats equated approximately 1 month earlier with a Thomas-type gastric fistula (GF) as described previously (Brzozowski et al., 2000). The animals were fasted overnight, but they had free access to water 24 h before the experiment. They were placed in individual Bollman-type cages to maintain the minimum restraint necessary. The gastric fistulas were opened and the stomachs were gently rinsed with approximately 5 ml of tap water at 37°C. The basal gastric secretions were then collected for 60 min, and MNA and NA were administered intragastrically (i.g.) in one of drug solution ranging from 6.25 to 100 μg/kg, with each dose being dissolved in 1 ml of saline and administered on a separate test day. In control tests, vehicle (1 ml of saline (i.g.) was given in the same dose as in tests with MNA or NA, with the collection of gastric juice being continued for the final 60 min. Vehicle (1 ml of saline) was applied i.g. in control animals parallel to animals administered with MNA and NA. The volume and acid concentration of each collected sample of gastric juice were measured, and acid outputs (expressed in term of micro moles of acid per 30 min) and pepin output (expressed in term of milligrams per 30 min) were determined as described previously (Brzozowski et al., 2000).

**Gastroprotection Studies and Measurement of GBF.** Acute gastric lesions were induced by exposing animals to 3.5 h of water immersion and WRS according to the procedure described by our group previously (Brzozowski et al., 2004). In addition, the efficacy of MNA applied i.g. to prevent gastric injury induced by corrosive agent such as ethanol was examined in an acute model of gastric damage induced by i.g. application of 75% ethanol in a volume of 1 ml using a metal catheter (Brzozowski et al., 2006). At 1 h upon the ethanol application, or 3.5 h upon the termination of WRS, the animals were lightly anesthetized with phenobarbital (60 mg/kg i.p.), their abdomens were opened by a midline incision, and the stomachs were exposed for the purpose of measuring GBF by means of H2 gas clearance technique as described previously (Brzozowski et al., 1999). For this purpose, double electrodes of electrolytic regional blood flowmeter (model RBF-2; Biotechnical Science, Osaka, Japan) were inserted into the gastric mucosa. The measurements were made in three areas of the oxyntic mucosa, and the mean values of the measurements were calculated and expressed as percentage of changes of those recorded in the vehicle (saline)-treated animals. After the GBF measurement, the venous blood samples were withdrawn from the vena cava for the determination of plasma levels of MNA as described below, and the stomach was removed, rinsed with saline, and pinned open for macroscopic examination. Rats were sacrificed 1 h after i.g. ethanol instillation and 3.5 h after the end of WRS. Their stomachs were excised and opened along the greater curvature, and the number of gastric bleeding erosions in case of WRS lesions and the area of ethanol-induced gastric lesions expressed in square millimeters were determined by computerized planimetry (Morphomat; Carl Zeiss, Jena, Germany) (Brzozowski et al., 1996) by an individual who was unaware of the rat groupings.

In some experiments with i.g. administration of MNA with or without 75% ethanol application or exposure to 3.5 h of WRS, the
standardized specimens from the corpus of the stomachs were fixed in 10% buffered formalin, and the paraffin sections were stained with hematoxylin-eosin for histology evaluation (Brzozowski et al., 2006). A Nikon microscope equipped with Microplan II digital image system was used for the quantitative histology examination (morpheometry) of the sections. Coded specimens of mucosa stained with hematoxylin and eosin were evaluated quantitatively at 500× magnification under blinded conditions.

**Involvement of COX-PGI2 and Sensory Nerve Systems in Gastroprotection Induced by MNA.** In subsequent studies four major series (A, B, C, and D) of experiments were carried out. Series A was used to test the effect of exogenous MNA given i.g. (6.25–100 mg/kg). For comparison purposes, NA was administered i.g. in single doses of 50 or 200 mg/kg against the mucosal lesions induced by WRS or ethanol, respectively. In series B and C, the involvement of COX-PGI2 and the efficacy of antisecretory agents, namely, omeprazole, a proton pump inhibitor, and ranitidine, a histamine H2 receptor antagonist, in protection afforded by MNA against WRS-induced mucosal damage was determined. To establish the role of PGI2 in the MNA-induced gastroprotection, we used a specific IP receptor antagonist, RO 324479, that has been described recently (Bley et al., 2006). RO 324479 was shown to be devoid of a significant effect on prostaglandin receptors and many other receptors, and its in vivo efficacy to blunt PGI2-mediated responses has been demonstrated previously (Bley et al., 2006; Bryniarski et al., 2008).

Several groups of rats, each consisting of six to eight animals, were pretreated 30 min before the WRS either with 1) vehicle (saline); 2) MNA (standard dose 50 mg/kg i.g.); 3) RO 324479 (10 mg/kg i.p.); 4) SC-560 (5 mg/kg i.g.), a selective COX-1 inhibitor (Brzozowski et al., 1999); 5) rofecoxib (10 mg/kg i.g.), a highly selective COX-2 inhibitor (Takeuchi et al., 2004); or 6) indomethacin (5 mg/kg i.p.), a nonselective COX inhibitor (Brzozowski et al., 1999). At the dose used in the present study, indomethacin has been shown in previous studies to inhibit gastric PGE2 generation by ~90% without itself causing any mucosal damage (Konturek et al., 1981; Brzozowski et al., 1999). The doses of SC-560 and rofecoxib were selected on the basis of previous studies showing that these agents almost completely suppressed PGE2 generation in exudates of air-pouch inflammation and inhibited gastric PGE2 production in mucosa with pre-existing gastric ulcer (Brzozowski et al., 2001; Takeuchi et al., 2004). SC-560 (Cayman Chemical, Ann Arbor, MI) or rofecoxib (Merck Sharp and Dhome, Warsaw, Poland) was first dissolved in absolute ethanol or methanol to obtain stock solutions of 50 mg/ml or 75 mg/ml, and then it was diluted to the desired concentrations with isotonic saline.

Control rats received the corresponding vehicle. Our preliminary studies (data not included) showed that none of the COX inhibitors used in this study alone produced any gastric lesions at the doses tested. In addition, in separate groups of rats the effect of antisecretory agents omeprazole and ranitidine applied in a comparable dose of 20 mg/kg i.g. was compared with that of MNA applied in a standard dose of 50 mg/kg i.g. The dose of these antisecretory agents was selected on the basis of our previous study (Brzozowski et al., 2000) showing that omeprazole and ranitidine were highly effective in the attenuation of acute gastric mucosal lesions induced by ischemia-reperfusion.

Samples of the oxyntic gland area were taken by biopsy (approximately 100 mg) immediately after the animals were euthanized to determine the mucosal generation of 6-keto-PGF1α, the stable metabolite of PGI2. After 30 min of incubation of the biopsies of gastric mucosa in Eppendorf tubes (37°C), samples of effluent were taken for analysis as described previously (Whittle et al., 1990). Samples were stored at ~70°C until assayed by commercially available enzyme immunoassay kits (R&D Systems, Minneapolis, MN) (Chlopicki et al., 2007), and the concentration of 6-keto-PGF1α was expressed in picograms per milliliter per milligram of wet tissue weight.

The role of sensory afferent nerves (series D) and neuropeptides such as CGRP released from sensitive afferent nerve endings in gastroprotection by MNA was tested in rats with capsaicin-induced deactivation of these nerves (Konturek et al., 1995; Brzozowski et al., 1996) or in those pretreated either with capsaazpine, the antagonist of transient receptor potential vanilloid type 1 (TRPV1) (Caterina et al., 1997), or the antagonist of CGRP receptors, CGRPα1-3 (series C) (Konturek et al., 2000). Chemical ablation of sensory afferent nerves was achieved with capsaicin (Sigma-Aldrich, St. Louis, MO) injected s.c. for three consecutive days at a respective dose of 25, 50, and 50 mg/kg (total dose of 125 mg/kg) approximately 2 weeks before the experiment (Holzer et al., 1991; Konturek et al., 1995). Capsazepine was dissolved in 10% Tween 20 and 10% ethanol with normal saline. Both capsaazpine and CGRP antagonist CGRPα1-3 were applied 30 min before i.g. administration of MNA or vehicle followed 30 min later by WRS. The experimental protocol included the following study groups, each consisting of six to eight animals: 1) vehicle (saline 1 ml i.g.) followed 30 min later by WRS in rats with intact afferent nerves; 2) MNA (standard dose 50 mg/kg i.g.) followed 30 min later by WRS in rats with intact sensory nerves; 3) vehicle (saline 1 ml i.g.) followed 30 min later by WRS in rats with capsaazpine deactivated afferent nerves or those pretreated with capsaazpine (5 mg/kg i.g.) or CGRPα1-3 (100 µg/kg i.g.); and 4) MNA (50 mg/kg i.g.) followed 30 min later by WRS in rats with capsaazpine-deactivated afferent nerves or those pretreated with capsaazpine (5 mg/kg i.g.) or CGRPα1-3 (100 µg/kg i.g.).

**Determination of Plasma MNA Levels in Rats Exposed to WRS with or without MNA Pretreatment.** As mentioned, immediately after GFB measurement, a venous blood sample was withdrawn from the vena cava into EDTA-containing vials, and the concentration of endogenous MNA and its metabolites in plasma samples was measured using liquid chromatography mass spectrometry as described previously (Slominska et al., 2006). Chromographic separation was performed using 3-μm Hypersil C18-BDS 150 × 2.0-mm column. Buffer A was 10 mM nonafluoropentanoic acid in water, and buffer B was 100% acetonitrile. The mobile phase was run at 0.2 ml/min in a gradient from 0 to 60% B in 12 min. The mass detector (LCQ Advantage; Thermo Electron Corporation, Waltham, MA), with an electrospray ion source, was operated in a positive single-ion monitoring mode for detection of [M + H]+ species of NA, MNA, M2PY, and M4PY, with the collision energy setting at 25%. Internal standard (2-chloroadenosine) signal was extracted from full MS mode. Electrospray cone voltage was set at 4.5 kV, and heated capillary temperature was 275°C. Sheath gas flow was set at 35 arbitrary units. The ion optics was optimized using standard instrument procedures during infusion of NA. Rat plasma was deproteinized using 10% trichloroacetic acid followed by ether extraction. Recovery of M2PY, M4PY, and NA added to the samples with known concentration was 75 to 95%. The coefficient of variation was below 10% for repeated injections on the same day. However, a much larger (>20%) variation was observed for repeated injections between days (Slominska et al., 2006).

**Determination of Lipid Peroxidation and SOD Activity and in Gastric Mucosa Exposed to WRS with and without Pre-treatment with MNA.** Given that lipid peroxidation is a well-established mechanism of cellular injury induced by reactive oxygen metabolites, we measured the changes in the MDA as an indicator of the lipid peroxidation in gastric mucosa exposed to WRS with and without treatment with MNA. For the measurement of lipid peroxidation, each gastric mucosa sample was weighed, transferred to the ice-cooled test tube, and homogenized in 400 μl of 20 mM Tris buffer, pH 7.4, containing 5 mM butylated hydroxytoluene to prevent new lipid peroxidation, which can occur during homogenization. The homogenate was then centrifuged at 4°C for 10 min, and resultant supernatant (200 μl) was stored at ~80°C until an assay of lipid peroxidation. The content of lipid peroxidation was measured at 37°C by spectrophotometer at wavelength of 586 nm and compared with the absorbance of purified MDA as the standard (Kwiecien et al., 2004).

The activity of SOD was measured in the gastric mucosa of rats exposed to WRS with or without pretreatment with MNA using SOD...
The mixture was overlaid with 25 mM Tris-HCl, pH 8.3, and primers used at final concentration of 0.5 mM. 10 μl of 100 mM phosphate, deoxyguanosine triphosphate, and deoxycytidine triphosphate; 5 μl of a 100 mM mixture of deoxyadenosine triphosphate, deoxyribothymidine triphosphate, deoxyguanosine triphosphate, and deoxyctydine triphosphate; and 5 μl of 100 mM formaldehyde gel electrophoresis and ethidium bromide staining, the 5 μl of mineral oil to prevent evaporation. The reaction mixture that contained 50 U of Moloney murine leukemia virus reverse transcriptase and oligo(dT) primers from Stratagene (Heidelberg, Germany). Using 1% agarose gel electrophoresis and ethidium bromide staining, the total RNA concentration in each sample was determined. Aliquot RNA samples were stored at −80°C until analysis. Single-stranded cDNA was generated from 5 μg of total cellular RNA using StrataScript reverse transcriptase and oligo(dT) primers (Stratagene). In brief, 5 μg of total RNA was uncoiled by heating (65°C for 5 min), and then it was reversed by transcribing into cDNA in a 50-μl reaction mixture that contained 50 U of Moloney murine leukemia virus reverse transcriptase; 0.3 mg of oligo(dT) primer; 1 ml of RNase block ribonuclease inhibitor (40 U/μl); 2 ml of a 100 mM mixture of deoxyadenosine triphosphate, deoxyribothymidine triphosphate, deoxyguanosine triphosphate, and deoxyctydine triphosphate; and 5 ml of 10× RT buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 5 mM MgCl₂). The resultant cDNA (2 μl) was amplified in a 50-μl reaction containing 0.3 ml (2.5 U) of Taq polymerase, 200 mM (each) dNTP (GE Healthcare, Chalfont St. Giles, UK), 1.5 mM MgCl₂, 5 ml of 10× polymerase chain reaction buffer (50 mM KCl and 10 mM Tris-HCl, pH 8.3), and primers used at final concentration of 0.5 mM. The mixture was overlaid with 25 μl of mineral oil to prevent evaporation. The polymerase chain reaction mixture was amplified in a DNA thermal cycler (PerkinElmer Life and Analytical Sciences, Waltham, MA) in the area dedicated for performing PCR reaction. The polymerase chain reaction mixture was amplified in a DNA thermal cycler (PerkinElmer Life and Analytical Sciences), and the incubation and thermal cycling conditions were as follows: denaturation at 94°C for 1 min, annealing at 60°C for 45 s, and extension at 72°C for 2 min. The number of cycles was 30 for β-actin, 32 for COX-1, 33 for COX-2, and 31 for CGRP. The nucleotide sequences of the primers for COX-1 and COX-2 were selected on the basis of the published cDNA encoding COX-1, COX-2, and β-actin (Brzozowski et al., 2001, 2006), respectively, and they were synthesized by Invitrogen (Eggenstein, Germany). The nucleotide sequences of the primers for CGRP were identical to those published by Peng et al. (2002). Polymerase chain reaction products were detected by electrophoresis on a 1.5% agarose gel containing ethidium bromide. Location of predicted products was confirmed with the use of DNA 100-base pair ladder (Invitrogen) as a standard size marker. The intensity of bands was quantified using densitometry (LKB Ultrascan; GE Healthcare) as described in details in a previous study (Brzozowski et al., 2000). The signals for COX-1 and COX-2 mRNAs were standardized against the β-actin signal for each sample, and results are expressed as COX-1 and COX-2/β-actin mRNA ratios.

**Statistical Analysis.** Results are expressed as means ± S.E.M. Statistical analysis was done using analysis of variance and the two-way analysis of variance test with a Tukey’s post hoc test where appropriate. Differences of p < 0.05 were considered significant.

### Results

**Effects of Exogenous MNA Applied i.g. on Gastric Acid and Pepsin Secretions.** The effects of vehicle (saline) or MNA applied in the graded doses ranging from 6.25 to 100 mg/kg (i.g.) on gastric acid and pepsin secretions from the GF in conscious rats are shown in Table 1. In control vehicle-treated rats, basal acid output averaged 135 ± 5 μmol/30 min, whereas pepsin output reached 0.78 ± 0.03 mg/30 min. MNA, which was given i.g. in the graded doses ranging from 6.25 mg/kg up to 100 mg/kg, dose-dependently inhibited gastric acid and pepsin secretions, with a maximal inhibition of the gastric acid and pepsin output reached at the dose of 100 mg/kg (Table 1). NA administered i.g. in the dose of 100 mg/kg also significantly decreased the gastric acid and pepsin outputs, but this inhibition was significantly less pronounced compared with the respective values observed in MNA-treated rats.

### Effects of Pretreatment with MNA on WRS- and Ethanol-Induced Lesions and the Alterations in the GBF and Plasma MNA.

The results of i.g. administration of MNA on the mean number of WRS-induced gastric lesions as well as accompanying changes in the GBF and plasma MNA levels are presented in Fig. 1. Such pretreatment with MNA applied i.g. dose-dependently reduced the number of gastric lesions, which were evoked by 3.5 h of WRS, with the threshold reduction occurring at a dose of 12.5 mg/kg, and with the ID₅₀ value averaging about 46 mg/kg MNA. The reduction of the lesion number of WRS damage by MNA was accompanied by a significant and dose-dependent rise in the GBF and plasma MNA levels (Fig. 1). Pretreatment with NA (50 mg/kg i.g.) also significantly reduced the number of WRS lesions, although this reduction in the number of WRS lesions was significantly less pronounced in comparison with that of MNA applied

### TABLE 1

<table>
<thead>
<tr>
<th>Type of Test</th>
<th>Acid Output</th>
<th>Pepsin Output</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>μmol/30 min</td>
<td>mg/30 min</td>
</tr>
<tr>
<td>Vehicle (control)</td>
<td>135 ± 5</td>
<td>0.78 ± 0.03</td>
</tr>
<tr>
<td>MNA (mg/kg i.g.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.25</td>
<td>129 ± 4</td>
<td>0.74 ± 0.05</td>
</tr>
<tr>
<td>12.5</td>
<td>119 ± 3*</td>
<td>0.65 ± 0.04**</td>
</tr>
<tr>
<td>25</td>
<td>104 ± 5*</td>
<td>0.58 ± 0.07**</td>
</tr>
<tr>
<td>50</td>
<td>94 ± 6**</td>
<td>0.52 ± 0.06**</td>
</tr>
<tr>
<td>100</td>
<td>60 ± 6**</td>
<td>0.45 ± 0.04**</td>
</tr>
<tr>
<td>NA (mg/kg i.g.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.25</td>
<td>131 ± 4</td>
<td>0.76 ± 0.03</td>
</tr>
<tr>
<td>12.5</td>
<td>126 ± 3</td>
<td>0.75 ± 0.06</td>
</tr>
<tr>
<td>25</td>
<td>130 ± 5</td>
<td>0.72 ± 0.05</td>
</tr>
<tr>
<td>50</td>
<td>126 ± 6**</td>
<td>0.63 ± 0.08**</td>
</tr>
<tr>
<td>100</td>
<td>111 ± 6**</td>
<td>0.62 ± 0.04**</td>
</tr>
</tbody>
</table>

*a Significant change compared with the value recorded in the vehicle-treated control rats.
** Significant change compared with the value obtained in test with MNA applied i.g. in a dose of 12.5 mg/kg.
* Significant change compared with the value obtained in test with MNA at a dose of 50 or 100 mg/kg.

Data are mean ± S.E.M. of six to eight rats.
The representative macroscopic example of the stomach pretreated with vehicle (saline) or MNA (50 mg/kg i.g.) is presented in Fig. 2. A dramatic reduction in the number of WRS-induced gastric lesions is observed in MNA-pretreated gastric mucosa compared with that pretreated with vehicle. As shown in Fig. 3, i.g. application of 75% ethanol resulted in the formation of gastric lesions followed by the significant reduction in GBF by approximately 30% compared with the intact gastric mucosa. With graded doses of MNA administered i.g. (12.5–200 mg/kg) before ethanol, the area of ethanol-induced gastric lesions was significantly attenuated, and a significant increase in the GBF starting with 25 mg/kg MNA was recorded (Fig. 3). As shown in Table 2, histologically, the exposure of gastric mucosa to WRS and ethanol in rats pretreated with vehicle resulted in a widespread denudation of mucosal surface and deep necrosis. However, rats pretreated with MNA applied i.g. in a standard dose of 50 mg/kg showed a significant reduction in the area of denuded surface and deep necrosis compared with those pretreated with vehicle. The administration of omeprazole significantly attenuated WRS-induced gastric lesions while raising the GBF; these effects were significantly more pronounced than those caused by MNA applied in a standard dose of 50 mg/kg (Fig. 4). In this dose used, MNA was almost equally effective in attenuation of WRS lesions, and it caused a similar increase in the GBF compared with those evoked by histamine H2-receptor antagonist ranitidine (Fig. 4).

Effect of Selective IP Receptor Inhibition and Non-selective and Selective COX-1 and COX-2 Inhibitors on MNA-Induced Gastroprotection against WRS-Induced Gastric Damage and Alteration in GBF. As shown in Fig. 5, exposure to WRS resulted in a significant decrease in the mucosal generation of 6-keto-PGF1α, compared with that measured in the intact gastric mucosa. This effect was partially reversed in rats pretreated with MNA (50 mg/kg i.g.). The increase in the mucosal generation of 6-keto-PGF1α was observed in MNA-treated gastric mucosa, which was completely suppressed in animals treated with indomethacin (5 mg/kg i.p.). The reduction in WRS-induced gastric damage and accompanying increase in the GBF evoked by MNA were abolished by the pretreatment with RO 3244794, the selective antagonist of IP receptors (Fig. 6). As shown in Fig. 7, MNA resulted in a similar attenuation in the number of...
gastroduodenal PGI2 (10^5 M) pretreatment of MNA with or without the pretreatment with vehicle (saline) or rofecoxib (10 mg/kg i.g.), the selective COX-1 and COX-2 inhibitors, respectively. The concurrent treatment with exogenous PGI2 (10 mg/kg i.g.) restored the gastroprotective activity of MNA applied in a standard dose of 50 mg/kg (i.g.).

Effect of Capsaicin Denervation and Treatment with Capsazepine and CGRP8-37 on MNA Afforded Gastroprotection and Hyperemia against WRS-Induced Gastric Damage. Deafferentation with parenteral pretreatment with neurotoxic dose of capsaicin (approximately 2 weeks before the experiment) significantly increased the number of WRS-induced lesions, and it considerably reduced the GBF compared with the vehicle-treated rats with intact sensory nerves (Fig. 8). In rats with capsaicin deafferentation, the protective activity of MNA applied in a standard dose of 50 mg/kg i.g. and accompanying rise in the GBF and plasma levels of MNA were significantly reduced compared with those in rats with intact sensory nerves. Administration of CGRP alone in a dose of 10 μg/kg s.c. resulted in a small but significant reduction in the number of WRS-induced gastric lesions in rats with intact sensory nerves without capsaicin treatment (22 ± 2.6 in vehicle pretreated versus 16 ± 1.8 in CGRP pretreated). The concurrent administration of CGRP (10 μg/kg s.c.) with MNA in rats with capsaicin denervation restored the protection and accompanying rise in the GBF and plasma MNA levels to the extent similarly observed in MNA-treated rats with intact sensory nerves.

As shown in Fig. 9, pretreatment with CGRP8-37 (100 μg/kg i.p.), a CGRP antagonist, or with capsazepine (5 mg/kg i.g.), an antagonist of TRPV1, which alone failed to influence significantly the number of gastric lesions and the GBF in gastroduodenal mucosa exposed to WRS, attenuated significantly the protection and hyperemia attained with standard dose of MNA (50 mg/kg i.g.).

Mucosal Expression of COX-1, COX-2, and CGRP mRNAs in the Gastric Mucosa Subjected to WRS with or without MNA Pretreatment. Figures 10 and 11 (left

**Table 2**

Table 3 indicates the changes in plasma levels of CGRP and in the mucosal content of MDA plus 4-HNE, measured as an index of lipid peroxidation, and in the mucosal activity of SOD in the gastric mucosa of WRS animals with or without pretreatment with vehicle or MNA. In nonstressed rats treated with MNA, the significant increase in the plasma CGRP levels compared with that in intact animals without any significant alteration in gastric mucosal MDA plus 4-HNE content was observed (Table 3). The exposure of rats to 3.5 h of WRS resulted in a significant decrease in the plasma levels of CGRP, a significant rise in the mucosal MDA plus 4-HNE content, and a significant fall in the mucosal SOD concentration compared with respective values measured in the intact gastric mucosa. In contrast, pretreatment with MNA applied i.g. in a standard dose of 50 mg/kg significantly enhanced plasma CGRP concentration, significantly attenuated the mucosal MDA plus 4-HNE content, and in part, restored the SOD activity compared with those values attained in vehicle-control animals exposed to WRS alone (Table 3).

**Fig. 3.** Mean area of 75% ethanol-induced gastric lesions and the alterations in GBF in rats pretreated with vehicle (saline) or with various doses of MNA (6.25–200 mg/kg i.g.) and for comparison, with NA applied in a single dose of 200 mg/kg (i.g.). Mean ± S.E.M. of six to eight rats.* a significant change compared with the value obtained in vehicle-pretreated gastric mucosa.

**TABLE 2**

Quantitative histology in the gastric mucosa of rats exposed to WRS or 75% ethanol with or without the pretreatment with vehicle (saline) or MNA applied in standard dose of 50 mg/kg (i.g.). Results are expressed as percentage of the mucosal strip length. Data are mean ± S.E.M. of six to eight rats.

<table>
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<th>Type of Test</th>
<th>Denuded Surface</th>
<th>Deep Necrosis</th>
</tr>
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<tbody>
<tr>
<td>Vehicle + WRS</td>
<td>32 ± 4</td>
<td>14.2 ± 2.5</td>
</tr>
<tr>
<td>MNA + WRS</td>
<td>12 ± 3*</td>
<td>6.8 ± 0.3*</td>
</tr>
<tr>
<td>Vehicle + ethanol</td>
<td>49 ± 6</td>
<td>22.4 ± 2.5</td>
</tr>
<tr>
<td>MNA + ethanol</td>
<td>21 ± 3*</td>
<td>7.2 ± 0.3*</td>
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* Significant change compared with the value obtained in vehicle-pretreated gastric mucosa.

As shown in Fig. 1, Indomethacin (5 mg/kg i.p.), which by itself significantly aggravated gastric lesions, induced WRS, and produced a significant fall in GBF, in comparison with vehicle-pretreated animals, abolished the reduction in the number of the lesions and the accompanying rise in GBF and mucosal PGI2 generation evoked by MNA (Figs. 5 and 7). The decrease in the number of WRS lesions and accompanying increase in GBF caused by MNA were also significantly attenuated by pretreatment with SC-560 (5 mg/kg i.g.), and rofecoxib (10 mg/kg i.g.), the selective COX-1 and COX-2 inhibitors, respectively. The concurrent treatment with exogenous PGI2 (10 μg/kg i.g.) restored the gastroprotective and hyperemic activity of MNA in stressed rats treated with COX-1 and COX-2 inhibitors that were exposed to WRS (Fig. 7).
which were pretreated with vehicle. The ratio of COX-2 exposed 30 min later to 3.5 h of WRS compared with those mucosa of rats pretreated with MNA (50 mg/kg i.g.) and mucosa, but it was up-regulated in the WRS-exposed mucosa.

(right), the COX-2 mRNA was absent in the intact gastric tric mucosa (Fig. 10, top right). As shown in Fig. 10 (bottom right) show the RT-PCR expression of COX-1, COX-2, and CGRP mRNAs in gastric mucosa of intact rats and those exposed to WRS administered with vehicle (saline) and MNA (20 mg/kg i.g.) with or without the pretreatment with indomethacin (5 mg/kg i.p.). Data are mean ± S.E.M. of 8 to 10 rats. *, a significant change compared with the respective value obtained in animals pretreated with vehicle or MNA.

and right) show the RT-PCR expression of COX-1, COX-2, and CGRP mRNAs in gastric mucosa and the ratio of mRNA expression for COX-1, COX-2, and CGRP over β-actin mRNA expression of intact rats and those given vehicle or MNA (50 mg/kg i.g.) and exposed to WRS. The signals for COX-1 mRNA were detected in the intact gastric mucosa, and this remained unchanged in gastric mucosa exposed to WRS (Fig. 10). The ratio of COX-1 mRNA over β-actin mRNA confirmed that COX-2 mRNA was significantly increased over that observed in the gastric mucosa of WRS-exposed gastric mucosa without pretreatment with MNA (Fig. 10, bottom right). Figure 11 (left and right) shows the effect of vehicle (control) and MNA on the expression of CGRP mRNA in the gastric mucosa. In rats exposed to 3.5 h of WRS the expression of CGRP mRNA was significantly diminished (Fig. 11, left), and this effect was confirmed by the significant fall in the ratio of CGRP over β-actin determined by densitometry. In contrast, strong signal of mRNA for CGRP was detected in gastric mucosa of rats pretreated with MNA applied before the WRS exposure. The ratio of CGRP mRNA over β-actin mRNA confirmed that CGRP mRNA was significantly increased over that observed in the gastric mucosa of WRS-exposed gastric mucosa without pretreatment with MNA (Fig. 11, right).

Discussion

The present study shows for the first time that the intra-gastric administration of MNA, one of the major metabolite of NA, which was originally implicated in the synthesis of β-nicotinamide adenine dinucleotide and NADPH cofactors and in the regulation of lipid metabolism, exerts a potent gastroprotective effect against stress-induced gastric lesions, and this action is accompanied by an increase in the gastric blood flow. The mechanism of MNA-induced gastroprotection involves hyperemia mediated by COX-2/PGI2 system and capsaicin-sensitive afferent nerves releasing vasodilatory neuropeptides such as CGRP. We found that pretreatment with MNA also exhibited a dose-dependent protection against gastric lesions caused by noxious topically administered irritants such as ethanol, and this was also accompanied by gastric mucosal hyperemia. These data suggest that MNA-induced protection is not limited to stress-induced gastric lesions, but it could also be extended to the prevention of the damage induced by the direct contact of the gastric mucosa with a strong corrosive agent such as ethanol.

It is interesting that MNA exhibited dose-dependent reduction in WRS- and ethanol-induced gastric lesions, and this gastroprotective activity was much more pronounced
rats and in those exposed to 3.5 h of WRS
and the gastric mucosal MDA and SOD contents in intact nonstressed
Effect of vehicle or MNA (50 mg/kg i.g.) on the plasma CGRP levels
TABLE 3
those pretreated with vehicle and exposed to WRS.
This is supported by our preliminary observation that a sin-
then that exerted by NA, a biological precursor of MNA. With
respect to NA, this activity, in keeping with the previous observation that NA has been originally recognized as an
important cofactor for the formation of dinucleotide, exhibits anti-inflammatory and radical scavenging activity (Ogata et al., 2002) and affords neuroprotection against hypoxic brain injury and thermal and spinal cord damage (Smith et al., 1989; Feng et al., 2006). Moreover, the activation of adenylyl cyclase/protein kinase A system facilitates a neural release of NA adenine dinucleotide in the mesenteric artery system, suggesting the role for derivatives of NA, in the control of the vascular circulation in the upper gastrointestinal tract (Bobalova and Mutafova-Yambolieva, 2006). It is of interest that the protective effects of MNA against WRS-induced gastric damage were accompanied by a significant and dose-dependent rise in the plasma MNA increments, and marked attenuation of the fall in the gastric blood flow provoked by WRS and ethanol, suggesting that MNA-evoked increase in the gastric microcirculation could be an important component of the protective action of this amide in the stomach. This is supported by our preliminary observation that a sin-

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<th>Type of Test</th>
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<th>MDA + 4-HNE Content</th>
<th>SOD Activity</th>
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<tr>
<td></td>
<td>pg/ml</td>
<td>nmol/g</td>
<td>U/mg</td>
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<tr>
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<tr>
<td>MNA + WRS</td>
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* Significant change compared with the value obtained in intact gastric mucosa.
* Significant change compared with the value obtained in vehicle-pretreated animals without pretreatment with MNA.
** Significant change compared with the value obtained in intact animals and those pretreated with vehicle and exposed to WRS.

than that exerted by NA, a biological precursor of MNA. With respect to NA, this activity, in keeping with the previous observation that NA has been originally recognized as an...
gle application of MNA results in profound time-dependent increase in plasma MNA concentrations, reaching the peak at 3 h with only small increments in plasma concentrations of other MNA metabolites, such as Met4PY and Met2PY (data not shown). The activity of antioxidizing enzyme SOD, which was diminished in gastric mucosa of stressed animals, was preserved by treatment with MNA. Furthermore, the MDA content, which was significantly raised in the gastric mucosa of rats subjected to WRS, was significantly reduced in rats pretreated with MNA, suggesting that amelioration of WRS-induced gastric lesions by MNA depends upon the antioxidizing activity and attenuation of the lipid peroxidation process in gastric mucosa of stressed animals.

The results of secretory studies revealed that MNA applied in the doses that were gastroprotective against WRS injury dose-dependently inhibited gastric acid and pepsin secretion in well adapted conscious rats provided with GF, suggesting that its acid inhibitory effect could contribute to the gastroprotective effect of this amide. This is consistent with the previous report that NA, the precursor of MNA, inhibits gastric secretion after oral administration in humans (Stratford et al., 1996). In our study, omeprazole, the proton pump inhibitor, was superior to both MNA and the histamine H2-receptor antagonist ranitidine, but MNA exhibited comparable activity with ranitidine in attenuation of WRS-induced lesions. This antisecretory action could contribute to gastroprotection by MNA against acid-dependent WRS damage, but it cannot serve as satisfactory explanation for the efficacy of MNA to attenuate ethanol-induced gastric lesions, in which gastric acid plays a minor role. Further studies are necessary to explain the mechanism of gastroprotective action of this amide against necrotizing type of gastric lesions.

Fig. 8. Mean number of WRS-induced gastric lesions and changes in GBF in gastric mucosa and plasma MNA levels of rats with intact sensory nerves or those with capsaicin-deactivated sensory nerves with or without pretreatment with vehicle (control), MNA (50 mg/kg i.g.), and the combination of exogenous CGRP (10 µg/kg s.c.) added to MNA. Data are mean ± S.E.M. of six to eight rats. *, a significant change compared with the value obtained in rats with intact sensory nerves pretreated with vehicle. †, indicates a significant change compared with the value obtained in rats without capsaicin denervation. * and †, a significant change compared with the value obtained in MNA-pretreated animals with capsaicin-deactivated sensory nerves.

Fig. 9. Mean number of WRS-induced gastric lesions and changes in GBF in gastric mucosa in rats pretreated with vehicle (control) and MNA (50 mg/kg i.g.) with or without capsazepine (5 mg/kg i.g.), an inhibitor of TRPV1 receptors, or CGRP8-37 (100 µg/kg i.p.), an antagonist of CGRP receptors. Data are mean ± S.E.M. of six to eight rats. *, a significant change compared with the value obtained in vehicle-pretreated animals. †, a significant change compared with the value obtained in MNA-treated rats without capsazepine or CGRP8-37.
Gastrin, because in our study, the MNA-induced protection and hyperemia were attenuated by CGRP8-37, a CGRP receptor antagonist that was shown previously to inhibit protective effect of exogenous gastrin, leptin, and ghrelin as well as those released endogenously by peptone (Brzozowski et al., 1996). Given that NA and its metabolites reverse the symptoms of pellagra (pellagra preventive vitamin PP also known as vitamin B3) and they were reported to exert cytoprotective effects in the neural, vascular, and dermal tissues, we attempted to test the hypothesis that MNA protection involves the activation of afferent sensory nerves leading to the release of CGRP from sensory afferent nerves (Holzer et al., 1991; Kato et al., 1996). Activation of sensory nerves influences the secretory functions in the stomach and exhibits gastroprotection by increasing gastric mucosal blood flow via the release of CGRP (Holzer et al., 1991; Brzozowski et al., 1996). The binding places for the capsaicin, a selective stimulator of these afferent neurons, has been identified and 

Arachidonate metabolites were thought to act as the classic mediators of cytoprotection (Robert, 1979) cooperating with NO and sensory nerves in the mechanism of gastric mucosal defense (Whittle et al., 1990). Recent studies militate, however, against the role of NO in the action of MNA because the suppression of NO activity by N’-nitro-L-arginine methyl ester failed to modify the MNA-induced thrombolytic activity, but it involves PGL2 formation due to an activation of COX-2 activity (Chlopicki et al., 2007). This is why we tested whether MNA affects the generation of mucosal 6-keto-PGF1α, a stable metabolite of PGL2 in the gastric mucosa, and we used rats pretreated with a selective antagonist of IP receptors, RO 3244794. First, we established that MNA-induced protection and hyperemia are accompanied by the enhancement in the mucosal generation of 6-keto-PGF1α in the presence of WRS, an effect consistent with the disappearance of MNA-induced protection and mucosal hyperemia in RO 3244794-pretreated animals. Moreover, MNA-induced gastroprotection was accompanied by an overexpression of mRNA for COX-2, whereas the expression of COX-1 mRNA remained unchanged. It is interesting that WRS decreased gastric mucosal PGL2 while increasing the expression of COX-2 mRNA, and this effect was further enhanced by MNA, possibly resulting from a mucosal compensatory effect of PGL2 depletion observed in stressed animals. The suppression of COX-1 and COX-2 activity by nonselective COX inhibitor indomethacin or SC-560 and rofecoxib (Brzozowski et al., 2001; Takeuchi et al., 2004), respectively, greatly attenuated the protective and hyperemic effects of MNA, indicating that endogenous PG derived from the COX-1 and COX-2 pathway contribute to the beneficial effects of this amide in the stomach exposed to WRS.

The suppression of COX-1 and COX-2 activity by nonselective COX inhibitor indomethacin or SC-560 and rofecoxib (Brzozowski et al., 2001; Takeuchi et al., 2004), respectively, greatly attenuated the protective and hyperemic effects of MNA, indicating that endogenous PG derived from the COX-1 and COX-2 pathway contribute to the beneficial effects of this amide in the stomach exposed to WRS.

Capsaicin-sensitive afferent nerves releasing vasodilatory neuropeptides play a central role in the gastroprotection of the stomach (Stroff et al., 1995; Brzozowski et al., 1996). Activation of sensory nerves influences the secretory functions in the stomach and exhibits gastroprotection by increasing gastric mucosal blood flow via the release of CGRP from sensory afferent nerves (Holzer et al., 1991; Kato et al., 1996). Given that NA and its metabolites reverse the symptoms of pellagra (pellagra preventive vitamin PP also known as vitamin B3) and they were reported to exert cytoprotective effects in the neural, vascular, and dermal tissues, we attempted to test the hypothesis that MNA protection involves the activation of afferent sensory nerves leading to the release of CGRP. Indeed, one of such neurotransmitter could be CGRP, because in our study, the MNA-induced protection and accompanying hyperemia were attenuated by CGRP8-37, a CGRP receptor antagonist that was shown previously to inhibit protective effect of exogenous gastrin, leptin, and ghrelin as well as those released endogenously by peptone meal and cholecytokinin (Konturek et al., 1995; Brzozowski et al., 2004). The binding places for the capsaicin, a selective stimulator of these afferent neurons, has been identified and
named TRPV1 (Caterina et al., 1997). The capsaicin activation of TRPV1 could be abolished by capsaepine (Harada and Okajima, 2007). It was proposed that CGRP released from sensory nerve endings increases the production of PGs, especially PGL2, thus reducing stress-induced gastric damage (Shimozawa et al., 2006; Harada and Okajima, 2007). Since the mechanism of gastric mucosal defense includes cooperation between PG and sensory peptides (Whittle et al., 1990, Brzozowski et al., 1996), the MNA-induced gastrointestinal protection involving PG may originate not only from direct action but also from the activation of afferent sensory neurons by this agent. Indeed, MNA enhanced local expression of mRNA for CGRP, and it triggered release of this neuropeptide into the circulation as reflected by the enhancement in the plasma CGRP levels in MNA-pretreated animals. Furthermore, the capsaicin-deactivation of primary afferent nerves eliminated the protective activity of MNA, significantly attenuated the plasma increments of MNA, and abolished the rise in GBP induced by this amide, and these effects were restored by CGRP administered in rats with capsaepine denervation. These findings indicate that sensory nerves are essential for microcirculatory response and of significant importance for the gastrointestinal activity of MNA. The protective and hyperemic activity of MNA was mitigated by capsaepine, an antagonist of TRPV1, suggesting that MNA stimulates the afferent nerves through the activation of TRPV1 directly or indirectly, possibly via enhancement in PGL2 generation, resulting in the liberation of CGRP. MNA-induced gastrointestinal protection against damage induced by WRS might be due to the recruitment of endogenous endothelial and/or gastric mucosal PGL2, which cooperates with CGRP released from afferent sensory nerves. Our study indicates that MNA might be of clinical interest and deserves further clinical study trial because in addition to the recognized thrombolytic activity, this agent affords gastrointestinal protection against acute gastric lesions.

In summary, these results demonstrate that administration of exogenous MNA, which is accompanied by a significant plasma increment of this amide, exhibits dose-dependent gastrointestinal protection against the WRS-induced lesions. Evidence was provided that these protective and hyperemic effects of MNA against stress injury may involve cooperation between sensory nerves possibly sensitized by endogenous PGL2 to release CGRP acting via activation of TRPV1 receptors. Because gastroprotection by MNA was accompanied by the rise in the plasma levels of this amide, it is suggested that MNA may act locally to activate the above-mentioned protective mechanisms and to strengthen the gastric mucosal defense in animals exposed to adverse conditions such as stress.

Acknowledgments

We are grateful to Prof. Jerzy Gebicki for the encouragement to perform this study and to Dr. Jan Adamus for providing MNA. We also express our thanks Mary-Frances Jette (Roch, Palo Alto, CA) for a generous donation of RO 3244794 and to Nily Osman for the critical reading of this manuscript.

References


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