Nitric oxide-releasing aspirin protects gastric mucosa against ethanol damage in rats with functional ablation of sensory nerves

P. C. Konturek¹, T. Brzozowski², J. Kania¹, S. J. Konturek² and E. G. Hahn¹

¹ First Department of Medicine, University Erlangen-Nuernberg, D-91064 Erlangen, Germany
² Institute of Physiology, University School of Medicine, Jagiellonian University, Krakow, Poland

Received 22 October 2002; returned for revision 28 March 2003; returned for final revision 10 April 2003; accepted by M. Parnham 25 April 2003

Abstract. Objective and Design: The aim of the present study was to investigate, whether sensory nerves are involved in the gastroprotection induced by NO releasing non-steroidal anti-inflammatory drugs (NO-NSAID).

Material: Studies were performed in Wistar rats with intact or inactivated sensory nerves by pretreatment with large dose of capsaicin (125 mg/kg sc).

Treatments: Acute gastric lesions were induced by 100% ethanol (100% EtOH). 1 h before exposure to 100% EtOH, rats received vehicle, aspirin (ASA) or NO-releasing aspirin (NO-ASA) in the same dose (50 mg/kg). The animals were killed 1 h after exposure to 100% EtOH.

Methods: Determinations were made of gastric mucosal injury, mucosal gastric blood flow, mucosal mRNA expression of glutathione peroxidase (GPx), zinc copper superoxide dismutase (SOD) and heat shock protein (HSP70) by RT-PCR and protein expression for HSP70 by Western blotting.

Results: Pretreatment with ASA aggravated the acute gastric injury induced by 100% EtOH, whereas pretreatment with NO-ASA led to a significant reduction in this injury. Administration of 100% EtOH was accompanied by a pronounced upregulation of HSP70, which was reduced by ASA, but enhanced by NO-ASA application. Sensory deactivation with capsaicin enhanced acute ethanol lesions and led to a significant attenuation in HSP70 expression. In contrast to ASA, NO-ASA attenuated gastric mucosal lesions and significantly upregulated HSP70 expression despite blockade of sensory nerves. NO-ASA, but not ASA, caused an upregulation of SOD and GPx mRNA in gastric mucosa with or without sensory denervation.

Conclusions: NO-ASA protects gastric mucosa even after blockade of sensory nerves due to the upregulation of HSP70 expression and attenuation of the oxidative injury resulting from strong upregulation of genes for antioxidant enzymes.

Key words: NO-releasing aspirin – Heat shock proteins – Sensory neurons

Introduction

Nitric oxide (NO) releasing non-steroidal anti-inflammatory drugs (NO-NSAID) do not cause gastric mucosal damage, despite inhibition of the cyclooxygenase activity to a similar extent as conventional NSAID that induce such damage [1, 2, 3]. The mechanisms underlying the much lower gastrototoxicity of NO-NSAID as compared with native NSAID are multifactorial and still not fully explained. Previous studies showed that NO released from these derivatives enhances the gastric mucosal defense by the stimulation of gastric microcirculation, the inhibition of leucocyte adhesion, the attenuation of lipid peroxidation and the inhibition of apoptosis and proinflammatory cytokine release [3, 4, 5].

Recently, we demonstrated a possible link between NO-NSAID and inducible heat shock proteins (HSP70) showing that this new class of drugs stimulate the expression of HSP70 in the gastric mucosa. However, the link between NO, HSP70 and sensory neurons is still poorly understood [6].

Concerning the gastric mucosal defense, peptidergic sensory fibers seem to play a pivotal role in the protection of gastric mucosal integrity against acute and chronic injury. There is an evidence that the stimulation of sensory nerve fibers in the gastroduodenal mucosa leads to a local release of neuropeptides such as CGRP and neurokinin A, which directly or via formation of NO, promote mucosal protection and repair. In addition, sensitive neurons also fascilate repair of the ulcerated mucosa [7, 8]. The implication of sensory nerves in gastric mucosal defense was discovered with the help of capsaicin, which specifically targets on afferent neurons via vanilloid receptors of type 1 (VR1) [9]. Capsaicin at a low dose stimulates sensory nerves leading to the enhancement of gastric mucosal blood flow and the inhibition of gastric motor activity and gastric emptying [7]. In contrast, high dose of capsaicin shows neurotoxic effect and induces an irreversible long-standing inactivation of the capsaicin-sensitive nerve endings with a loss of their sensory-afferent functions and their ability to release of sensory neuropeptides [10].

The aim of the present study was to investigate, whether the sensory nerves are involved in the protection induced by NO-NSAID such as NO-aspirin (NO-ASA).
Materials and methods

Male Wistar rats (n = 50) weighing 180–220 g were used in all studies. Rats were fasted 18 h before experiments, but they had free access to the drinking water. Studies were approved by Ethical Committee for Animal Research of Jagiellonian University College of Medicine.

Production of gastric lesions and measurement of gastric blood flow (GBF)

Gastric lesions were produced by an intragastric application of 1.5 ml of 100% ethanol by means of metal orogastric tube as described in detail elsewhere [3]. Native ASA (50 mg/kg) and equimolar doses of NO-ASA were introduced ig 30 min prior to exposure of the gastric mucosa to 100% ethanol. ASA was obtained from Sigma Chemical Co. (St. Louis, USA) while NO-ASA (NCX 4016; 2-(Acetyloxy)benzoic acid 3-(nitrooxymethyl)phenyl ester was kindly provided by Mrs. Nathalie Baudry from NicOx S.A., Sophia Antipolis, France.

One h after application of ethanol, the animals were anesthetized with ether, their stomach were exposed and the gastric blood flow was measured by means of local H₂-gas clearance technique as described elsewhere [3]. Briefly, double needle electrodes were inserted into the mucosa through the serosa with the tips located in the mucosa, one electrode being used for local generation of H₂-gas and the other for the measurement of tissue level of H₂. With this method the H₂ generated by water hydrolysis is carried away by the blood and the polarographic current detector shows the decreasing tissue H₂ concentration as a clearance curve which is used to calculate absolute flow rate (ml/min/100 g). The GBF was measured in three areas of the oxyntic portion of the stomach macroscopically not involving visible mucosal lesions, and the mean value of three recordings was calculated and expressed as a percentage of the flow rate recorded in the intact mucosa. Using this technique, the results of the repeated blood flow measurements gave highly reproducible results and inter- and intraassay variability of these measurement were 3.5% and 5.8%, respectively. The area of gastric lesions reproducible results and inter- and intraassay variability of these measurements gave highly reproducible results and inter- and intraassay variability of these measurement were 3.5% and 5.8%, respectively. The area of gastric lesions reproducible results and inter- and intraassay variability of these measurement were 3.5% and 5.8%, respectively.

NO-ASA, ASA and implication of sensory afferent nerves in acute gastric injuries

In order to test the implication of sensory nerves, rats with intact nerves (control) and capsaicin-induced deactivation of these nerves were used. For this purpose, the animals were pretreated with higher doses of capsaicin (Sigma Co, St. Louis, USA) injected for 3 consecutive days at a dose of 25, 50 and 50 mg/kg (sc) about two weeks before the start of acute experiments as described elsewhere [11]. All injections of capsaicin were performed under ether anesthesia to counter pain reactions and respiratory impairment associated with injection of such high doses of this agent. To check the effectiveness of the capsaicin denervation, a drop of 0.1 mg/ml solution of capsaicin was instilled into the eye of each rat and the protective movements were counted as described previously [11]. All animals pretreated with capsaicin showed negative wiping movement test, thus confirming functional denervation of the capsaicin sensitive nerves.

Reverse-transcriptase polymerase chain reaction for detection of mRNA for HSP-70, SOD and GPx

After the rats had been anesthetized and blood flow measured, the stomachs were removed and the gastric wall was rinsed with phosphate buffered saline, snap-frozen in liquid nitrogen, and then stored at −80 °C until the time of RNA extraction. Total RNA was extracted from mucosal samples by the method of Chomczynski and Sacchi [12] using extraction kit from Stratagene (Heidelberg, Germany). Following precipitation, RNA was resuspended in RNAse-free water and its concentration was estimated by absorbance at 260 nm wavelength. 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 (Stratagene, Heidelberg, Germany) and oligo-(dT)-primers (Stratagene, Heidelberg, Germany). Briefly, 5 μg of total RNA was co-uncouled by heating (65 °C for 5 min) and then reverse transcribed into complementary DNA (cDNA) in a 50 μl reaction mixture that contained 50 U Moloney murine leukemia virus reverse transcriptase (MMLV-RT), 0.3 μg oligo-(dT)-primer, 1 μl RNase Block Ribonuclease Inhibitor (40 U/μl), 2 μl of a 100 mM mixture of deoxyadenosine triphosphate (dATP), deoxythymidime triphosphate (dTTP), deoxyguanosine triphosphate (dGTP) and deoxyctytidine triphosphate (dCTP), 5 μl 10 × RT buffer (10 mM Tris-HCl, pH = 8.3, 50 mM MgCl₂, 5 mM MgCl₂). The resultant cDNA (2 μl) was amplified in a 50 μl reaction volume containing 2 U Taq polymerase, 200 μM (each) dNTPs (Pharmacia, Germany), 1.5 mM MgCl₂, 5 μl 10 × polymerase chain reaction buffer (50 mM KCl, 10 μM Tris-HCl, pH = 8.3) and specific primers used at final concentration of 0.5 μM. The polymerase chain reaction mixture was amplified in a DNA thermal cycler (Perkin-Elmer-Cetus, Norwalk, CT). The HSP 70 primer sequences were: sense, 5¢-GTG AAG ATC TGC TGC TGC TTG-3¢, antisense: 3¢-TTT GAC AAC AGG CTG GTG AAC C-5¢; the expected length of the product was 590 bp. The zinc/copper superoxide dismutase (SOD) primer sequences were: sense, 5¢-CAG CCT TGT GTA TTG TCT CC-3¢, antisense: 3¢-GCT TCT CTC GTC TCC TTG CF-5¢; the expected length of the product was 201 bp. The GPx primer sequences were: sense, 5¢-CAG GTG GTA TGG CTT-3¢, antisense: 3¢-GCC TCT CCA ACC TCC ACC-5¢; the expected length of the product was 232 bp. The β-actin primer sequences were: sense, 5¢-TTG TAA CCA ACT GGG ACG ATG TGG-3¢; antisense: 3¢-GAT CTT GAT CTG CAT GGT GCC AGG-5¢; the expected length of the product was 764 bp. The nucleotide sequence of the primers for HSP-70, β-actin, SOD and GPx were based on the sequences of the published cDNAs [13–16]. The primers were synthesized by GIBCO BRL/Life Technologies (Eggenstein, Germany). Polymerase chain reaction products were detected by electrophoresis on a 1.5% agarose gel containing ethidium bromide. Location of a predicted products was confirmed by using 100-bp ladder (Takara, Shiga, Japan) as a standard size marker. Expression of the product was quantified using video image system (Kodak, USA). The PCR product signal was standardised against the β-actin signal for each sample and result expressed as primer/β-actin ratio as described in detail elsewhere [17].

Western blot analysis

Shock frozen tissue from rat stomach was homogenized in lysis buffer (100 mM Tris-HCl, pH 7.4, 15% glycerol, 2 mM EDTA, 2% SDS, 100 mM DTT) by the addition of 1:20 dilution of aprotinin and 1:50 dilution of 100 mM PMSF. Insoluble material was removed by centrifugation at 12,000 g for 15 min. Approximately 50 μg of total protein extracts were loaded on SDS-polyacrylamide gels and run 40 μA, followed by transfer on nitrocellulose membrane (Protran, Schleicher & Schuell, Germany) by electroblotting. 3% BSA (Sigma Aldrich, Germany) in TBS/Tween-20 buffer (137 mmol NaCl, 20 mmol Tris-HCl, pH 7.4, 0.1% Tween-20) was used to block filters for at least 1 h at room temperature. Specific primary antibody against Hsp70 (mouse monoclonal, 1:200 dilution; StressGen Biotechnologies Corp., Canada) or β-actin (mouse monoclonal, dilution 1:5000; Sigma Aldrich, Germany) was added to the membrane, followed by an anti-mouse-alfG HRP-horseradish peroxidase conjugated secondary antibody (dilution 1:20000; Promega, WI USA) dissolved in 1% non-fat milk in TBS-Tween-20 buffer. Incubation of primary antibody was followed by 3 washes with TBS-Tween-20 buffer for 10 min. Incubation of the secondary antibody was followed by 5 washes for 10 min. Immunocomplexes were detected by the SuperSignal West Pico Chemiluminescent Kit ( Pierce, USA). Thereafter, the developed membrane was exposed to an X-ray film (Kodak, Wiesbaden, Germany). Comparison between dif-