Ph.D. THESIS

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AGGRESSIVE AND PROTECTIVE MEDIATORS IN THE REGULATION OF INTESTINAL MUCOSAL INTEGRITY

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CONTENTS

Own articles, the thesis based on[1]:

I. **Szepes Z.,** Kiss J., Molnár T., Lamarque D., Jancsó G., László F. Capsaicin-sensitive mechanisms in the modulation of rat colonic vascular permeability under physiological and pathological conditions. J. Physiol. (Paris) 1997;91:123-126.

II. **Szepes Z.,** Morschl É., Kiss J., Pávó I., Whittle B.J.R., Varga Cs., László F.A., László F. Detrimental effects of oestradiol on cysteamine-induced gastroduodenal ulceration in the female rat. J. Physiol. (Paris) 1999;93(6):491-494.

III. Lamarque D., Moran A.P., **Szepes Z.,** Delchier J.C., Whittle B.J.R. Cytotoxicity associated with induction of nitric oxide synthase in rat duodenal epithelial cells in vivo by lipopolysaccharide of *Helicobacter pylori:* inhibition by superoxide dismutase. Br. J. Pharmacol. 2000;130:1531-1538.

IV. **Szepes Z.,** Kiss J., Lamarque D., Moran A.P., Nemcsik J., Morschl É., László F, Whittle BJR. Attenuation of *Helicobacter pylori* endotoxin-provoked rat intestinal inflammation by selective inhibition of the inducible nitric oxide synthase. J. Physiol. (Paris) 2001;95:453-455.

Own articles, the thesis related to[2]:

1. Varga Cs., Pavo I., Lamarque D., **Szepes Z.,** Kiss J., Karacsony G., Laszlo F.A., Laszlo F. Endogenous vasopressin increases acute endotoxin shock-provoked gastrointestinal mucosal injury in the rat. Eur. J. Pharmacol. 1998;352:257-261.

2. Laszlo F , Pavo I , **Szepes Z.,** Varga Cs., Laszlo F.A. Deleterious action of vasopressin in gastroduodenal ulceration: experimental and clinical observations. Scand J Gastroenterol (Suppl., review) 1998;228:62-67.

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[1]Articles of which results are fully demonstrated and discussed in the thesis (see as attachment)
[2]Articles of which results are related to the thesis and cited in the text where appropriate (see attached)

3. Pavo I, Morschl E, **Szepes Z.,** Kiss J, Boda K, Vetro G, Varga C, Laszlo FA, Laszlo F. Vasopressin deficiency decreases the frequency of gastroduodenal ulceration in humans. J. Physiol. (Paris) 2000;94:63-66.

4. Blanchard H.S., Dernis-Labous E., Lamarque D., Nhieu J.T., **Szepes Z.,** Flejou J.F., Wollman E., Whittle B.J.R., Breban M. Inducible nitric oxide synthase attenuates chronic colitis in human histocompatibility antigen HLA-B27/human beta2 microglobulin transgenic rats. Eur. Cytokine Netw. 2001;12:111-118.

5. Lamarque D., Nhieu J.T., Breban M., Bernardeu C., Martin-Garcia N., **Szepes Z.,** Whittle B.J.R., Claudepierre P. Lymphocytic infiltration and expression of inducible nitric oxide synthase in human duodenal and colonic mucosa is a characteristic feature of ankylosing spondylitis. J Rheumatol. 2003;30:2428-2436.

6. Molnar T., **Szepes Z.,** Nagy F., Lonovics J. Successful treatment of steroid resistant ulcerative colitis associated with severe autoimmune hemolytic anemia with oral microemulsion cyclosporin--a brief case report. Am J Gastroenterol. 2003;98:1207-1208.

7. Molnar T., **Szepes Z.,** Nagy F., Szenohradszki P., Lonovics J. Open questions concerning cyclosporine therapy in ulcerative colitis. Gastroenterology 2004;126:1495-1296.

List of abbreviations

CGRP	calcitonine gene-related peptide
CSN	capsaicine-sensitive sensory nerves
cNOS	constitutive nitric oxide synthase
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
GMP	guanidine monophosphate
H. pylori	*Helicobacter pylori*
IBD	inflammatory bowel diseases
iNOS	inducible nitric oxide synthase
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
NOS	nitric oxide synthase
L-NAME	N^G-nitro-L-arginine methyl ester
L-NNA	N^G-nitro-L-arginine
LPS	lipopolysaccharide
SOD-PEG	long-lasting conjugate of superoxide dismutase
SP	substance P
THB_4	tetrahydrobiopterin
TNBS	trinitro-benzene sulphonic acid

1. INTRODUCTION

1. Mediators affecting mucosal defense

Numerous endogenous substances affect the defense mechanisms of the small and large intestine. The balance between the generation of aggressive and protective mediators is the rate-limiting step in the maintenance of intestinal mucosal integrity. In the present work, the involvement of endogenous estrogens, nitric oxide (NO) and capsaicine-sensitive innervation are described in this complex system.

1.1. Estrogens and duodenal ulceration

On the basis of experimental and clinical observations, it is known that ulceration of the duodenal mucosa shows sexual dimorphism. For example, in the fertile age, peptic ulcer disease occurs more frequently among men than in women (Aston *et al.*, 1991; Michaletz-Onody, 1992). In experimental models of mucosal damage, this sexual difference of mucosal injury has also been found. Oral administration of ethanol generated more severe gastric mucosal erosions in male rats than in females (László *et al.*, 1992, 1993). In this model of mucosal damage, gonadectomy protected the stomach against ethanol-induced injury only in male rats, but not in females (László *et al.*, 1992). Moreover, administration of the testosterone-synthase inhibitor, cyproterone acetate attenuated gastric haemorrhagic erosions in intact male rats following ethanol challenge (László *et al.*, 1992). By contrast, in a different model of gastroduodenal ulceration (induced by restraint and activity stress), the stomach of male rats was revealed to be less sensitive to mucosal damage compared to that in females (Robert *et al.*, 1966; Robert & Nezamis, 1975). In conjunction with these findings, it can be suspected that sex hormones might possibly play a role in the development of duodenal mucosal ulceration.

1.2. Helicobacter pylori infection and nitric oxide generation

Infection with *Helicobacter pylori* (*H. pylori)* is a dominant pathogenic factor in peptic ulcer disease (Baldwin *et al.*, 1991). This bacterium colonises in the gastric antrum and in the sites of gastric metaplasia in the duodenum, and induces local inflammation (Carrick *et al.*, 1989; Blaser, 1990). *H. pylori* infection may provoke damage in the stomach and duodenum by releasing soluble cytotoxic factors that activate inflammatory cells such as neutrophils, that are implicated in tissue damage

including superoxide (Mooney *et al.*, 1991) and nitric oxide (NO) (McCall *et al.*, 1989). High concentration of NO are known to be cytotoxic, and in combination with the superoxide radical, leads to the subsequent formation of the moities, peroxynitrite and the hydroxyl radicals, which are highly injurious to cells (Beckman *et al.*, 1990; Ischiropoulos *et al.*, 1995). Indeed, it has been previously shown that administration of a long-lasting conjugate of superoxide dismutase (SOD-PEG), which scavenges superoxide, reduced the injury provoked by NO donors in the rat gastric mucosa (Lamarque & Whittle,1995).

The inducible isoform of NO synthase (iNOS) is capable of sustaining high levels of NO, and can be expressed in gastrointestinal epithelial cells and is associated with cytotoxicity (Tepperman *et al.*, 1993, 1994; Brown *et al.*, 1994). Since *H. pylori* can synthesize an endotoxin (Moran *et al.*, 1992), expression of iNOS in the duodenal epithelial cells could also play a role in the pathogenesis of mucosal lesions related to infection by this organism.

Recent studies have shown that challenge with a water extract of *H. pylori* can express iNOS and lead to epithelial injury in the rat duodenum. These effects were inhibited by polymixin B, suggesting the involvement of a lipopolysaccharide (LPS) (Lamarque *et al.*, 1998). However, although *in vitro* studies have shown that *H. pylori* LPS can lead to the expression of iNOS in murine and human macrophage cell lines in culture, it was only weakly active (Perez-Perez *et al.*, 1995).

All over the world, the incidence of *H. pylori* infection is high, e.g. it approaches 50-80% in European adults. Although the clinical impact of a better understanding how *H. pylori* affects gastrointestinal mucosa can not be a concept of debate, only few investigations have been carried out by using its purified endotoxin for exploring its systemic effect, e.g. in the gut or in other organs. For example, in chronic inflammatory diseases of the gut (e.g. in inflammatory bowel diseases [IBD]), the overproduction of NO by the expression of the iNOS is cytotoxic, and has pathological impact (Whittle *et al.*, 1995). It is known that the excess of NO by iNOS increases small and large intestinal vascular permeability, adhesion of neutrophils to the vascular endothelium and leads to vasocongestion (Moncada & Higgs, 1993).

1.3. Capsaicine-sensitive innervation and intestinal inflammation

IBD is the collective term used to describe the two gastrointestinal disorders, ulcerative colitis and Crohn's disease. Although these diseases have distinctive

pathophysiological characteristics, they are frequently considered together due to several clinical and therapeutic similarities (Riis, 1990; Molnar *et al.*, 2003). Implication of a causal factor remains elusive, and there is no known cure (Riis, 1990; Hay & Hay, 1992).

Capsaicin-sensitive sensory nerves (CSN) play an important role in the maintenance of the structural and functional integrity of the gastrointestinal mucosa (Holzer, 1988). There is ample evidence that this effect is mediated by neuropeptides released from afferent nerve endings. Capsaicin selectively stimulates these nerves and causes the release of vasoactive peptides, such as calcitonine gene-related peptide (CGRP) and substance P (SP). Hence, mucosal application of capsaicin at low concentrations may affect mucosal protection by releasing peptides (e.g. CGRP), which increase mucosal blood flow (Holzer & Sametz, 1986; Holzer, 1988, 1991). In contrast, systemic administration or local application of capsaicin at higher concentrations results in a selective destruction of CSN (for a review see Jancsó, 1992). Thus, pre-treatment with capsaicin may lead to aggravation of tissue damage. Available experimental evidence indicates that sensory nerves may be involved in the development of colonic IBD (Evangelista & Meli, 1989; Reinshagen *et al.*, 1994).

2. OBJECTIVES

1. Estrogen effect on duodenal ulceration

The aim of our studies was to evaluate the actions of the female sex steroid, estradiol on the generation of duodenal ulceration and vascular endothelial damage provoked by cysteamine in rats. Therefore, in female rats, we examined the effects of ovariectomy and exogenous oestradiol on the severity of macroscopically detectable lesions as well as the changes in microvascular permeability in the duodenal tissue.

2. Helicobacter pylori endotoxin-provoked induction of nitric oxide synthase, and its actions on the intestinal vasculature and on epthelial cell viability

The first objective of our investigations was to examine the ability of a purified *H. pylori* LPS to induce iNOS activity in duodenal epithelial cells following its administration *in vivo,* and determine its association with cell damage in the rat. The effects of a novel highly selective inhibitor of iNOS, 1400 W (Garvey *et al.*, 1997; László & Whittle, 1997) on the cytotoxic actions were evaluated. In addition, the involvement of the superoxide, and hence peroxynitrite, on epithelial cell injury provoked by the *H. pylori* LPS was investigated using a long-lasting conjugate of superoxide dismutase (SOD-PEG).

Our second proposal was to explore 1./ whether systemic administration of a purified *H. pylori* endotoxin (LPS) leads to the expression of iNOS in other parts of the small and large intestine, and 2./ whether *H. pylori* LPS is capable to provoke jejunal or/and colonic inflammation, and how they relate to each other.

3. Capsaicine-sensitive innervation in colonic mucosal defense under physiological and pathological circumstances

The present experiments were initiated in an attempt to reveal a possible participation of local capsaicin-sensitive sensory innervation in changes of colonic vascular permeability under physiological and pathological conditions, i.e. in experimental colitis provoked by trinitrobenzene-sulphonic acid (TNBS) in the rat.

MATERIALS AND METHODS

3.1. Animals

Wistar rats (10-12 week-old) were used throughout our studies. Rats were housed 5 per cage in a room under constant conditions of illumination (12 hours light-dark cycle), temperature (22±2 °C) and humidity (20-25%). Standard diet and tap water were available *ad libitum*. After a week of habituation in the facilities, animals were admitted to the experimental sessions. All experiments were carried out according to the directives of the local Ethical Committee of the University and also of the European Communities Council Directive 86/609/EEC. Efforts were made to minimize animal suffering and to reduce the number of animals used.

3.2. Ovariectomy

Bilateral ovariectomy and sham-operation have been performed under ketamine and medetomidine (75 mg/kg and 0,5 mg/kg, respectively; i.p.) narcosis. Animals were allowed to recover over two weeks.

3.3. Provocation of duodenal, jejunal and colonic damage

3.3.1. Duodenal mucosal ulceration and vascular leakage

Female Wistar rats (200-220 g) were injected with cysteamine (400 mg/kg, s.c.). Twenty-four hours after cysteamine administration, the animals were killed by ether overdose, and their duodenum was removed. During this 24-h period, rats were deprived of food, but received water ad libitum. In some groups of rats, ovariectomy was performed. For additional groups of rats, a depot injection of oestradiol (1-5 mg/kg, i.m.) was administered before cysteamine.

The area of damage and the total mucosal surface of the duodenum were measured by a digital planimeter (Sokkia KP-82-N, Japan), and the injured area was expressed as a percentage of the total mucosal surface. We determined vascular leakage simultaneusly.

3.3.2. Jejunal inflammation induced by purified Helicobacter pylori endotoxin (LPS)

3.3.2.1. Preparation of H. pylori LPS

Biomass of H. pylori (NCTC 11637 strain) was grown in brain-heart infusion containing 2% foetal calf serum to ensure expression of high molecular weight LPS.

Extraction of LPS was performed using a phenol-water procedure. Subsequently, extracted LPS was purified by treatment with RNase A, DNase II and proteinase K, and by ultracentrifugation at 100.000xg at 4°C for 18 h (Moran *et al.*, 1992). For suspension, purified LPS was dispersed in endotoxin-free water sonication.

3.3.2.2. Provocation of jejunal vascular leakage

We used male Wistar rats (230-250 g). They were fasted overnight, but received water *ad libitum*. Under transient ether anaesthesia, purified *H. pylori* LPS (3 mg/kg) was administered intravenously. In a separate experiment, we administered a bisisothiourea derivative selective iNOS inhibitor, N-(8-(aminomethyl)-benzyl)-acetamidine (1400W; 0.2-1 mg/kg, s.c.; [Garvey *et al.*, 1997]) concurrently with endotoxin. For the measurement of vascular permeability or iNOS enzyme activity, segments of the jejunum and colon were removed from standard sites 4 h following treatments.

3.3.3. Colonic mucosal injury

3.3.3.1. Defunctionalization of colonic capsaicin-sensitive neurons (CSN-depletion)

Rats were anaesthetised with urethane (1 g/kg, i.p.). A small piece of gelfoam was moistened with capsaicin (1%, 100 µl) and placed onto the serosal surface of the lower part of the colon (1-4 cm from the anus) for 30 min. Sham-operated rats were treated with the solvent of capsaicin. For the determination of vascular permeability changes, tissues samples of the lower and upper colon were removed 2 months later. This examination period was chosen, since among capsaicin-treated (CSN-depleted) rats moderate diarrhoea occurred during the postoperative survival time. The sham-operated group did not show this symptom of inflammation.

3.3.3.2. Induction of colitis in CSN-depleted and CSN-intact rats

Colonic inflammation was induced with TNBS according to the method of Morris *et al.* (1989). TNBS (30 mg/rat) or/and ethanol (50%) were administered through the anus via a plastic tube advanced 8 cm into the colon of CSN-depleted or control (CSN-intact) rats. Plasma leakage, in the upper and lower colon, was determined in TNBS-alone and TNBS+ethanol groups one week later.

3.3.3.3. Colonic inflammation induced by purified H. pylori LPS

We used male Wistar rats (230-250 g). They were fasted overnight, but received water *ad libitum*. Under transient ether anaesthesia, purified *H. pylori* LPS (from Prof. Moran's laboratory, Ireland; 3 mg/kg) was administered intravenously. In a separate experiment, we administered a bisisothiourea derivative selective iNOS inhibitor, 1400W (0.2-1 mg/kg, s.c.) concurrently with endotoxin. For the measurement of vascular permeability or iNOS enzyme activity, segments of the jejunum and colon were removed from standard sites, 4 h following treatments.

3.4. Vascular leakage

As an index of vascular endothelial damage, leakage of [^{125}I]human serum albumin was determined in the duodenum, jejunum (4 cm-long segment, 10 cm-far from the pylorus) and in the upper and lower part of the colon (4-4 cm-long segments from the coecum and anus, respectively). Under light ether anesthesia [^{125}I]human serum albumin (2 μCi/kg) was administered into the tail vein 2 hours before autopsy. Immediately before autopsy (also under ether anesthesia) blood was collected from the abdominal aorta into syringes containing trisodium citrate (final concentration 0,318%) and centrifuged (10 000 g, 10 minutes, 4°C). The [^{125}I]human serum albumin content of the stomach and plasma was determined in a gamma spectrometer (Nuclear Enterprises NE1600). Control values (from rats that had received saline) were subtracted from the values of treated animals and data were expressed as changes in albumin accumulation (μl plasma/g tissue) as described previously (Boughton-Smith *et al.*, 1993).

3.5. Investigation of H. pylori LPS-provoked duodenal epithelial cell damage

3.5.1. Animal preparation

Male Wistar rats, weighing 200-250 g, were fasted overnight but allowed free access to water. The animals received 0.75-3 mg/kg of *H. pylori* purified LPS dissolved in saline and administered via the tail vein under transient anaesthesia induced by ether. In control experiments, rats were pre-treated with saline.

3.5.2. Duodenal epithelial cell isolation

Duodenal epithelial cells were isolated as described previously (Roth *et al.*, 1985). A 5 cm segment of duodenum was slowly flushed with 50 ml of a solution containing 0.15 M NaCl and 0.1 mM dithiothreitol (DTT). The segment was then filled with 5 ml of a solution containing (in mM) 1.5 KCl, 96 NaCl, 27 sodium citrate, 8 KH_2PO_4, and 5.6 Na_2HPO_4 (pH 7.3), and the proximal and the distal ends were ligated. The segment was then immersed in phosphate-buffered saline (PBS) kept at 37°C, which was bubbled with 95% O_2-5% CO_2. After 15 min, the instilled solution was removed and another solution containing 1.5 mM EDTA and 0.5 mM DTT was instilled over 5 min, as described previously (Tepperman *et al.*, 1993). The epithelial cells were collected in suspension in this solution. The cells were washed twice with PBS (pH 7.4) and centrifuged for 5 min at 800 g. The cells were suspended in a buffer containing HEPES (10 mM), sucrose (320 mM), DTT (1 mM), soybean trypsin inhibitor (10 µg/ml), leupeptin (10 µg/ml), aprotonin (2 µg/ml).

To assess the purity in epithelial cells in the aliquots isolated from duodenum, in some experiments the cells were fixed with formaldehyde, stained by hematoxylin-eosin-safran (HES) and counted under light microscopy and expressed as the percentage of epithelial cells by fields. The percentage of epithelial cells in the cell suspension isolated from the duodenum by dispersion was 98±1 % (n=8), as determined by microscopy, the other cells identified by morphological analysis being mastocytes.

3.5.3. Duodenal epithelial cell viability

The cell viability was determined in duodenal epithelial cells collected from rats that has been challenged, 5 h previously, with purified *H. pylori* LPS or saline. The viability of cells was determined by trypan blue dye exclusion (0.5%, trypan blue in PBS) as described previously (Tepperman *et al.*, 1991). The number of non-viable cells was determined by light microscopy (x40 magnification) by counting those cells that failed to exclude the dye. Cells were counted in a randomised manner using a hemocytometer.

Duodenal epithelial cells viability was also determined by the conversion of the tetrazolium salt (3-[4.5-dimethylthiazol-2yl]-2.5-diphenyl tetrazolium bromide, MTT) in the formazan salt by mitochondrial dehydrogenases. Briefly, 300 µl of MTT

was added to 50 µl suspension of the homogenised cells prepared as previously described and suspended in a buffer containing HEPES (10 mM), sucrose (320 mM), DTT (1 mM), soybean trypsin inhibitor (10 µg/ml), leupeptin (10 µg/ml), aprotonin (2 µg/ml). After 4 h of incubation at 37°C, the suspension was centrifuged for 2 min at 10,000 g, the pellet was solubilized in DMSO (1 ml) and centrifuged again for 2 min at 10,000 g. The spectrophotometric absorbance of the formazan salt was measured in the supernatant at 540 nm. Protein content was measured by the Bradford's method in the initial suspension of homogenised cells. Results were expressed as OD/mg of protein.

3.5.4. Effects of H. pylori LPS on NOS and viability in duodenal epithelial cells

At 5 hours after administration of *H. pylori* purified LPS (0.75-3 mg/kg), the animals were killed by cervical dislocation, duodenum removed and duodenal epithelial cells isolated as described above. In control experiments, rats were pre-treated with saline. The NO synthase activity was determined in epithelial cells. Cells viability was estimated by MTT conversion and trypan blue exclusion.

3.5.5. Effects of N-(3-(aminomethyl)benzyl)acetamidine (1400W) on iNOS activity and viability of duodenal epithelial cells

In further experiments, rats were treated with the selective iNOS inhibitor, 1400 W (0.2-1 mg/kg i.v.) or saline, concurrently administered with *H. pylori* LPS (3 mg/kg). The NO synthase activity was determined in the duodenal epithelial cells isolated 5 h after challenge with *H. pylori* LPS. The doses and timing of administration of 1400W was taken from previous studies on rat gastrointestinal tissue (László & Whittle, 1997).

3.5.6. Effects of SOD-PEG on epithelial cell viability and NO synthase activity

In groups of rats, a systemically acting conjugate of polyethylene glycol and superoxide dismutase (SOD-PEG; 125-500 IU/kg) or isotonic saline was administered by an intravenous bolus injection, 15 min prior *H. pylori* LPS administration (3 mg/kg). The doses of SOD-PEG were taken from previous studies on its inhibitory action on the inflammatory response in the rat skin following systemic administration

(Boughton-Smith *et al*, 1993). The epithelial cells viability and NO synthase activity were determined in duodenal cells, 5 h after *H. pylori* LPS administration.

3.6. Measurement of nitric oxide synthase (NOS) enzyme activity

The NOS enzyme activity was determined as the conversion of L-[^{14}C]-arginine monohydrochloride to L-[^{14}C]-citrulline based on the method described previously [Salter *et al.*, 1991; Boughton-Smith *et al.*, 1993; Kiss *et al.*, 2001, Blanchard *et al.*, 2001; Lamarque *et al.*, 2003]. We sacrificed the animals by decapitation, and immediately after autopsy, we prepared the fresh tissues for measurements. The tissues were homogenized (15 s, Ultra-Turrax homogenizer, 5 mm blade) in buffer (250 mg/ml, 4°C) containing 10 mM Hepes, 32 mM sucrose, 1 mM dithiothreitol, 0.1 mM EDTA, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, and 2 μg/ml aprotinin at pH 7.4. Homogenates were centrifuged for 20 min (10000 g, 4 °C). Supernatants were mixed with Dowex (AG 50W-8; 200-400, 8% cross-linked, Na^+ form) resin and centrifugated for a further 10 min (10000 g, 4 °C). Sample supernatant (40 μl) was incubated for 10 min at 37°C in reaction buffer comprising final concentrations of 50 mM KH_2PO_4, 10 μg/ml calmodulin, 2.5 mM $CaCI_2$, 50 mM valine, 1 mM dithiothreitol, 15.5 nM L-arginine, 1 mM L-citrulline, 0.3 mM NADPH, 3 μM FAD, 3 μM FMN, 3 μM THB_4 and 0.17 μM of [^{14}C]L-arginine. The reaction was arrested by the addition (0.5 ml) of a 1:1 v/v suspension of Dowex:water. After addition of 0.85 ml distilled water and settling for 30 min, the supernatant was removed for scintillation counting. Protein content was estimated via spectrophotometric assay (Bio-Rad Protein Assay), and NOS activity was expressed as pM/min/mg protein. Total NOS activity was defined as citrulline formation that was abolished by incubation *in vitro* with N^G-nitro-L-arginine (L-NNA, 1 mM). Basal L-NNA-sensitive activity that was abolished by EGTA, was taken as calcium-dependent cNOS activity. Calcium-independent NOS activity (iNOS) was determined as the difference between samples containing 1 mM EGTA and samples containing 1 mM L-NNA.

3.7. Chemicals

L-[^{14}C]arginine monohydrochloride was purchased from Amersham International (U.K.). [^{125}I]human serum albumin was obtained from Izinta, Budapest,

Hungary. The selective iNOS inhibitor, 1400W was a gift from Wellcome Research Laboratories. Ketamine and medetomidine (Domitor) were purchased from a commercial pharmacy. All other compounds were from the Sigma-Aldrich Chemical Company.

3.8. Statistics

The data are expressed as the mean ± S.E.M. of (n) rats or tissue samples per experimental group. Data were analyzed with the Mann-Whitney non-parallel U-test, one way ANOVA followed by the Tukey-Kramer or by the Bonferroni multiple comparisons test, where appropriate. $P<0.05$ was taken as significant.

4. RESULTS

7.1. Actions of estrogen on duodenal ulceration

Administration of cysteamine (400 mg/kg, s.c.) provoked macroscopic injury of the duodenal mucosa, and increased vascular permeability after 24 h. Ovariectomy decreased, while exogenous oestradiol dose-dependently increased cysteamine-induced macroscopic lesion formation and vascular leakage, as demonstrated in Figure 1.

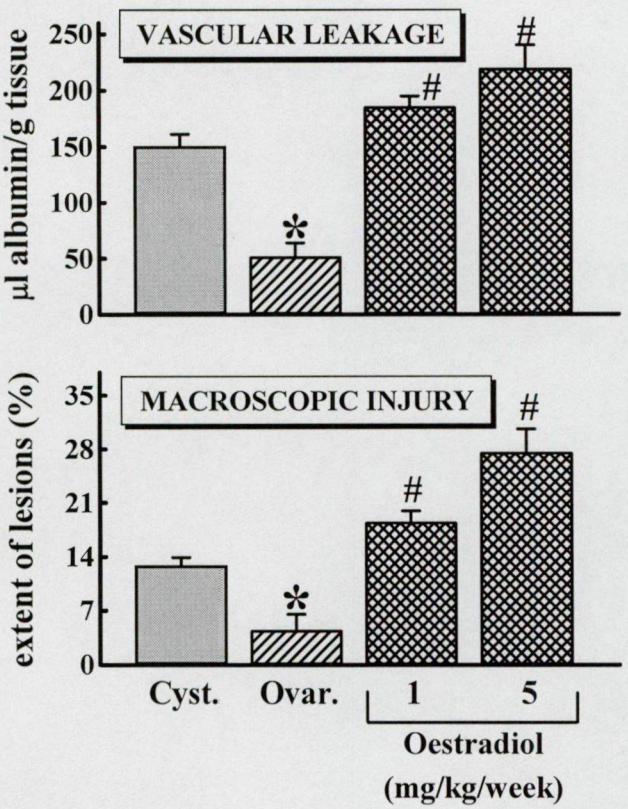

Figure 1.

Induction of vascular leakage of plasma (determined by using radiolabeled albumin) and macroscopic injury by cysteamine (Cyst., 400 mg/kg, s.c.) after 24 h in the rat duodenum. The inhibition and potentiation of cysteamine-provoked microvascular and macroscopic lesion formation by ovariectomy (Ovar., two weeks before cysteamine challenge) and by oestradiol pretreatment (1-5 mg/kg/week, i.m.), respectively. The columns show the extent of lesions (in %, lower panel) and the leakage of plasma (Δ µl/g tissue, upper panel). Data are given as the mean±S.E.M. of 4-7 rats per group; statistical significance is shown as *$P<0.05$: inhibition of cysteamine induced macroscopic injury and vascular leakage; $^{\#}P<0.05$: potentiation of cysteamine induced macroscopic injury and vascular leakage.

4.2. Actions of nitric oxide

4.2.1. Induction of iNOS in duodenal epithelial cells after H. pylori LPS

NO synthase activity, which was inhibited by L-NMMA but not by *in vitro* incubation with EGTA (1mM), was determined in the supernatants of duodenal epithelial cells. A significant dose-dependent increase in NO synthase activity was detected 5 h after administration of LPS (0.75-3 mg/kg), as shown in Figure 2. This Ca^{2+}-independent activity remained at a similar level when determined 7 h after challenge.

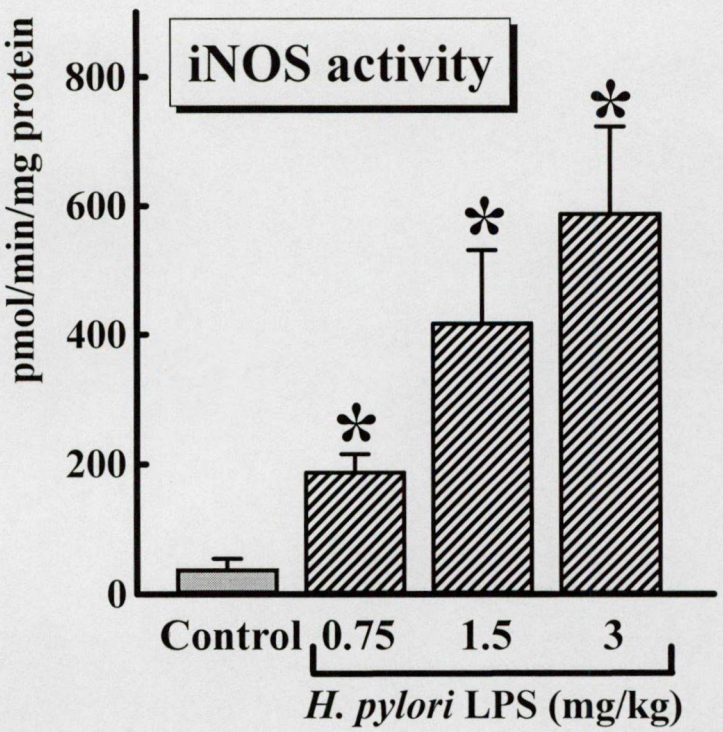

Figure 2.

Dose-dependent increase in inducible nitric oxide (iNOS) activity in isolated duodenal epithelial cells, harvested 5 h following challenge with *H. pylori* lipopolysaccharide administration (LPS, 0.75-3 mg/kg, i.v.) in the rat. Data, shown as the Ca^{2+}-independent iNOS activity (pmol/min/mg protein), are means± S.E.M. of 5-10 experiments, where * denotes significant difference from the control group ($P<0.005$).

4.2.2. Effect of H. pylori LPS on duodenal epithelial cell viability

The proportion of epithelial cells in the cell suspension isolated from the duodenum by dispersion was 98±2 % (n=4), as determined by microscopy, the other cells identified by morphological analysis being mastocytes. The proportion of non-viable intestinal cells isolated from control rats, assessed by Trypan blue staining, was 13.1±1 % (n=12). Administration of *H. pylori* LPS (0.75-3 mg/kg, i.v.) provoked a significant dose-dependent increase of the number of non-viable cells when assessed by the dye exclusion 5 h later (Figure 3). Likewise, 5 h following *H. pylori* LPS (0.75-3 mg/kg, i.v.) administration, the percentage of non viable cells, estimated by MTT conversion, dose-dependently increased, as shown in Figure 3.

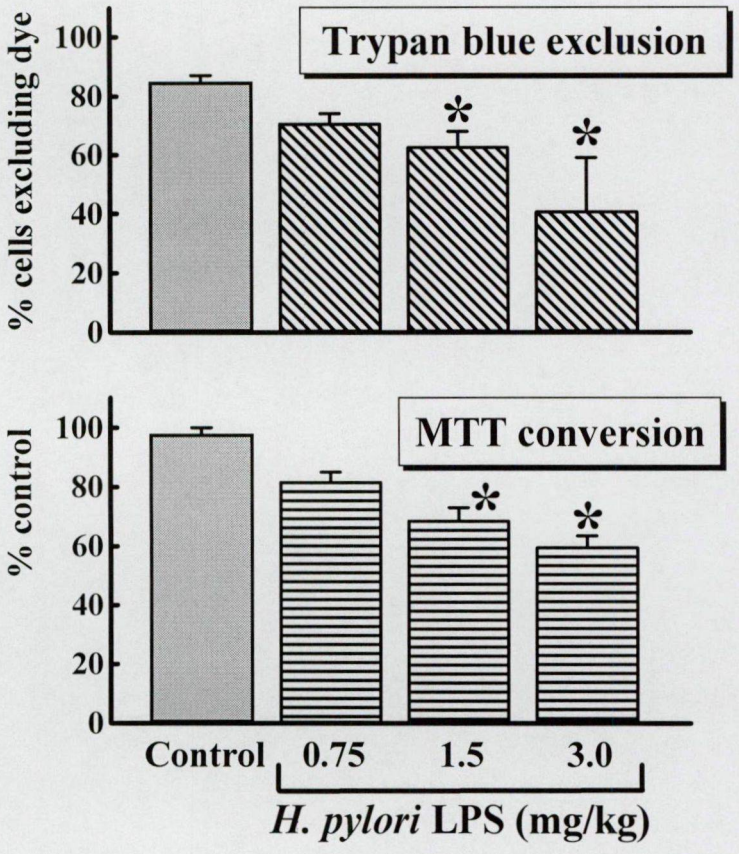

Figure 3.

Percentage of viable isolated duodenal epithelial cells damaged, as assessed by Trypan blue exclusion (per cent cells excluding dye; upper graph) or MTT conversion (per cent control, lower graph), 5 h following challenge with *H. pylori* lipopolysaccharide (LPS; 0.75-3 mg/kg, i.v.) in rats. Data are means ± S.E.M. of 5-12 experiments where * significant difference from the same control group ($P<0.01$).

4.2.3. Effects of 1400W on H. pylori LPS-provoked iNOS activity in rat duodenal epithelial cells

The increase in iNOS activity, determined in the rat duodenal epithelial cells 5 h after intravenous challenge with *H. pylori* LPS, was inhibited dose-dependently by concomitant administration of 1400W (0.2-1 mg/kg, i.v.), as shown in Figure 4. The cNOS activity measured 5 h after LPS challenge was not significantly modified by the treatment with 1400W (5 mg/kg, i.v.), being 643±234 and 684±176 pmol/min/mg protein, respectively (n=8).

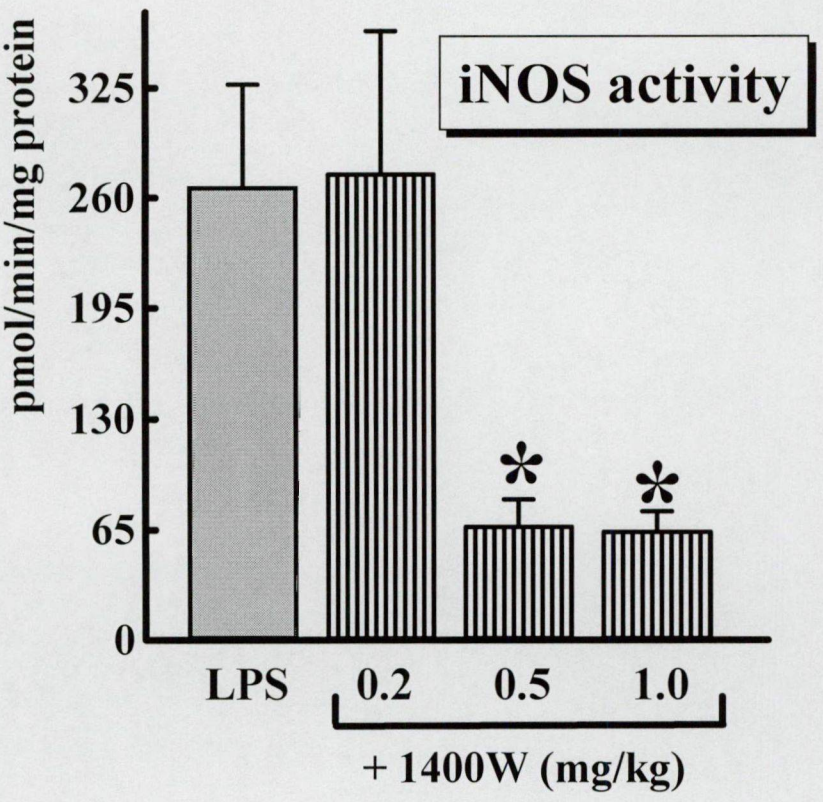

Figure 4.

Inducible nitric oxide (iNOS) activity in duodenal epithelial cells following challenge with *H. pylori* lipopolysaccharide (LPS; 3 mg/kg, i.v.) in the rat, and the actions of concurrent administration of N-(3-(aminomethyl)benzyl) acetamidine (1400W; 0.2-1 mg/kg, i.v.). Data, shown as iNOS activity (pmol/min/mg protein), are means ± S.E.M. of 5-9 experiments, where * denotes significant difference from LPS ($P<0.01$).

4.2.4. Effects of 1400W on the viability of rat duodenal epithelial cells exposed to H. pylori LPS

Five hours after the *H. pylori* LPS injection (3 mg/kg, i.v.), the increase in non-viable cells, estimated by Trypan blue dye exclusion or MTT conversion, was reversed by concomitant treatment of the rats with 1400W (0.2-1 mg/kg, i.v.), as demonstrated in Figure 5.

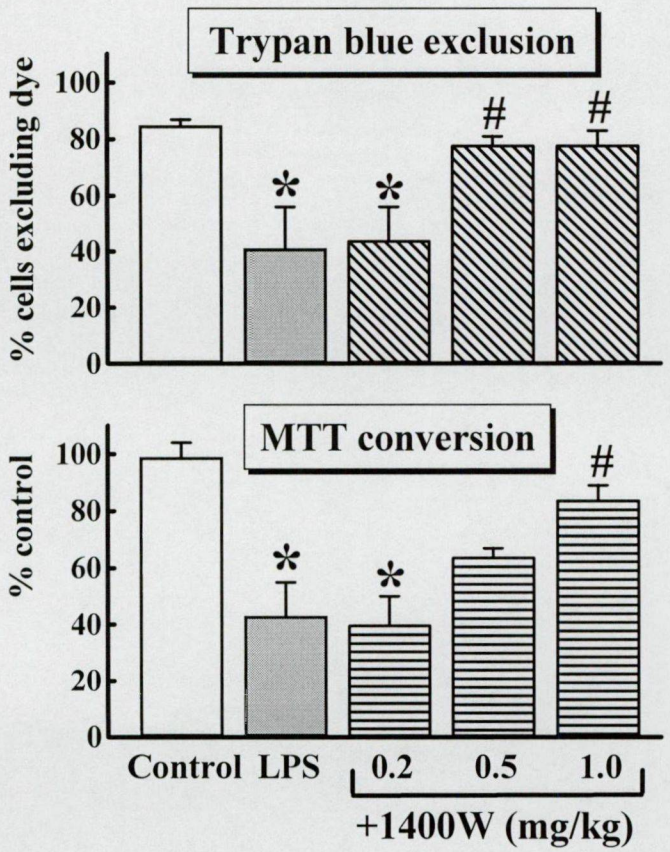

Figure 5.

Percentage of viable isolated duodenal epithelial cells, as assessed by Trypan blue exclusion (per cent cells excluding dye; upper graph), or MTT conversion (per cent control; lower graph), 5 h following challenge with *H. pylori* lipopolysaccharide (LPS; 3 mg/kg, i.v.) in rat treated concurrently with saline (control) or with N-(3-(aminomethyl)benzyl)-acetamidine (1400 W; 0.2-1 mg/kg, i.v.). Data are means ± S.E.M. of 5-9 experiments, where * denotes a significant difference from the control ($P<0.01$), and # shows a significant difference from the LPS alone group ($P<0.05$).

4.2.5. Effects of SOD-PEG on H. pylori LPS-provoked iNOS activity in duodenal epithelial cells

Administration of SOD-PEG (500 IU/kg, i.v.), 15 min prior to challenge with *H. pylori* LPS, did not significantly affect the increase in iNOS activity in duodenal epithelial cells, determined 5 h after challenge (Figure 6). The cNOS activity measured 5 h after *H. pylori* LPS challenge was likewise not affected following SOD-PEG administration (data are not shown; n=10).

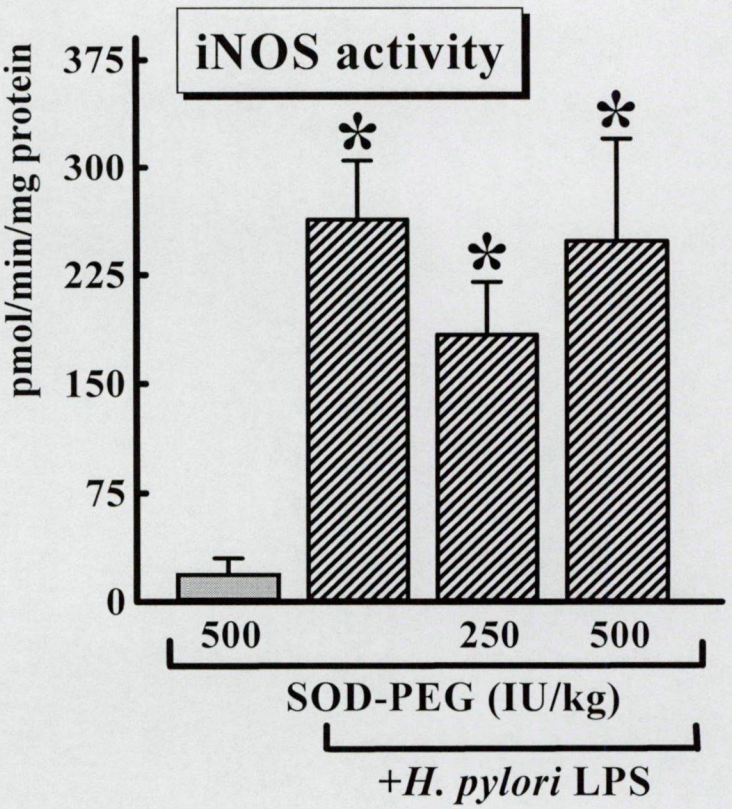

Figure 6.

Inducible nitric oxide (iNOS) activity in duodenal epithelial cells, 5 h following challenge with *H. pylori* lipopolysaccharide (LPS; 3 mg/kg, i.v.) in rat treated concurrently with saline or with superoxide dismutase conjugated with polyethylene glycol (SOD-PEG; 250-500 IU/kg, i.v.). Data shown as iNOS activity (pmol/min/mg protein) are means ± S.E.M. of 5-10 experiments, where * denotes a significant difference from the control ($P<0.01$). In control experiments, a group of rats received SOD-PEG (500 IU/kg, i.v.). There was no significant difference ($P>0.05$) between values for LPS alone and LPS with either dose of SOD-PEG.

4.2.6. Effects of H. pylori LPS and SOD-PEG on viability of epithelial cells

The proportion of non-viable intestinal cells, assessed by Trypan blue staining, that was isolated from rats treated 5 h previously with SOD-PEG (500 IU/kg, i.v.) alone, was not different from those of cells taken from control rats (12±1%, n=6 compared with 12.4±2%, n=5). However, the increase in non-viable cells 5 h after the *H. pylori* LPS administration was prevented by pretreatment (15 min) of the rats with SOD-PEG (250-500 IU/kg, i.v.). Likewise, the increase in non-viable cells assessed by MTT conversion after *H. pylori* LPS challenge was prevented by pretreatment with these doses of SOD-PEG, as demonstrated in Figure 7.

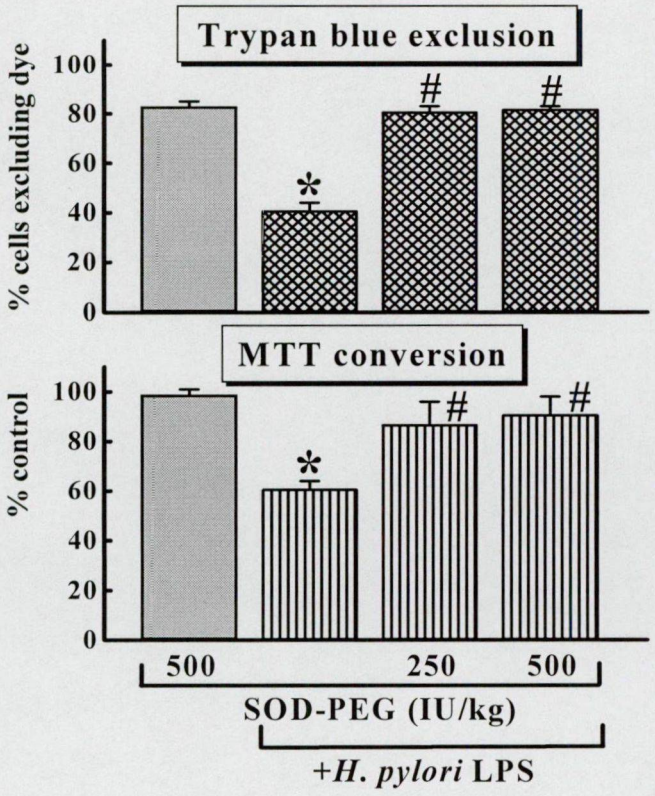

Figure 7.

Percentage of viable isolated duodenal epithelial cells, as assessed by Trypan blue exclusion (per cent cells excluding dye; upper graph) and by MTT conversion (per cent control, lower graph) 5 h following challenge with *H. pylori* lipopolysaccharide (LPS; 3 mg/kg, i.v.) in rat treated concurrently with saline or with superoxide dismutase conjugated with polyethylene glycol (SOD-PEG; (250-500 IU/kg, i.v.). In control experiments a group of rats received SOD-PEG alone (500 IU/kg, i.v.). Data are means ± S.E.M. of 5-10 experiments where * denotes a significant difference from the control ($P<0.05$) and # denotes a significant difference from the *H. pylori* LPS group alone ($P<0.05$).

4.2.7. Effects of H. pylori LPS on jejunal and colonic expression of iNOS, and its reversal by 1400W

Administration of *H. pylori* endotoxin led to the expression of iNOS in the jejunum and colon (n=4, P<0.001) 4 h later, an effect which was abolished by the administration of 1400W (p=4, P<0.001) in both intestinal tissues (Figure 8). Basal cNOS activities remained unchanged throughout the experiments (n=4, data are not shown).

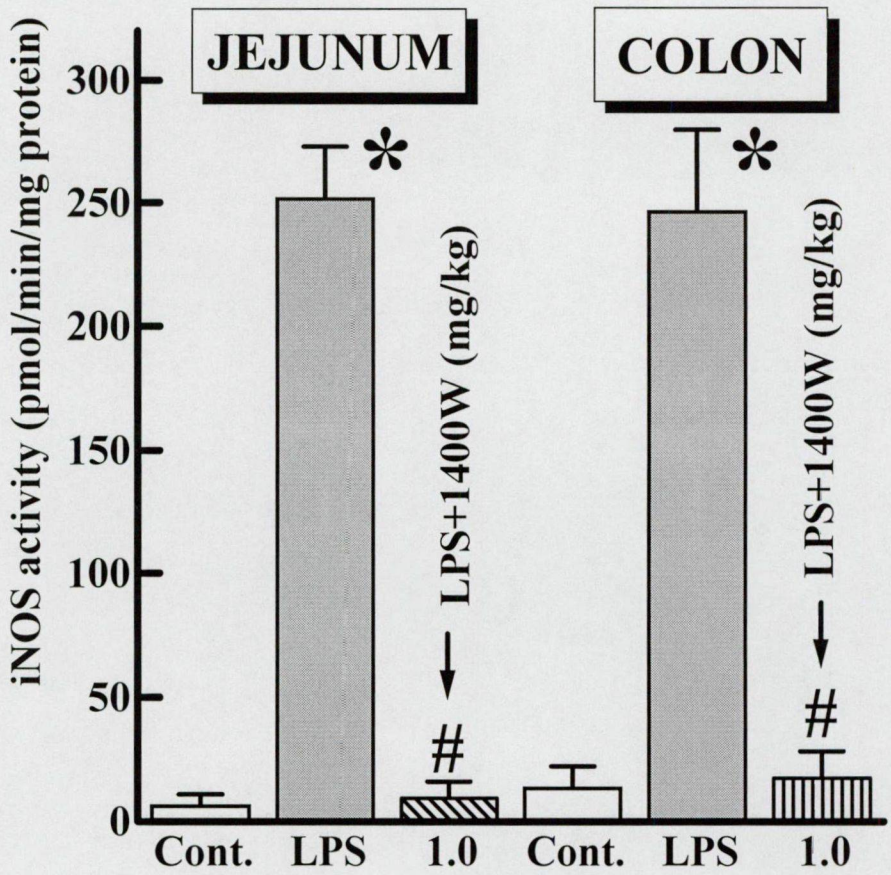

Figure 8.

Expression of iNOS (described in pmol/min/mg protein units) in the rat jejunal and colonic tissue 4 h after the administration of purified *Helicobacter pylori* endotoxin (LPS; 3 mg/kg, i.v.), and its reversal by the selective iNOS inhibitor 1400W (1 mg/kg, s.c., concurrently with LPS). Data are expressed as mean±S.E.M., where n=4 rats in a group. *P<0.05 means significant increase in iNOS activity compared to the untreated control (Cont.) group; #P<0.05 means significant decrease in iNOS activity compared to the LPS alone group.

4.2.8. Effects of H. pylori LPS on jejunal and colonic albumin leakage, and its reversal by 1400W

H. pylori endotoxin (3 mg/kg, i.v.) alone provoked significant small and large intestinal albumin leakage 4 h later (Figure 9). Concurrent administration of the selective inhibitor of iNOS, 1400W with *H. pylori* endotoxin dose-dependently attenuated jejunal and colonic albumin leakage after 4 h (83±5% and 94±3% maximal protection, respectively; n=4-6, P<0.01) as demonstrated in Figure 9.

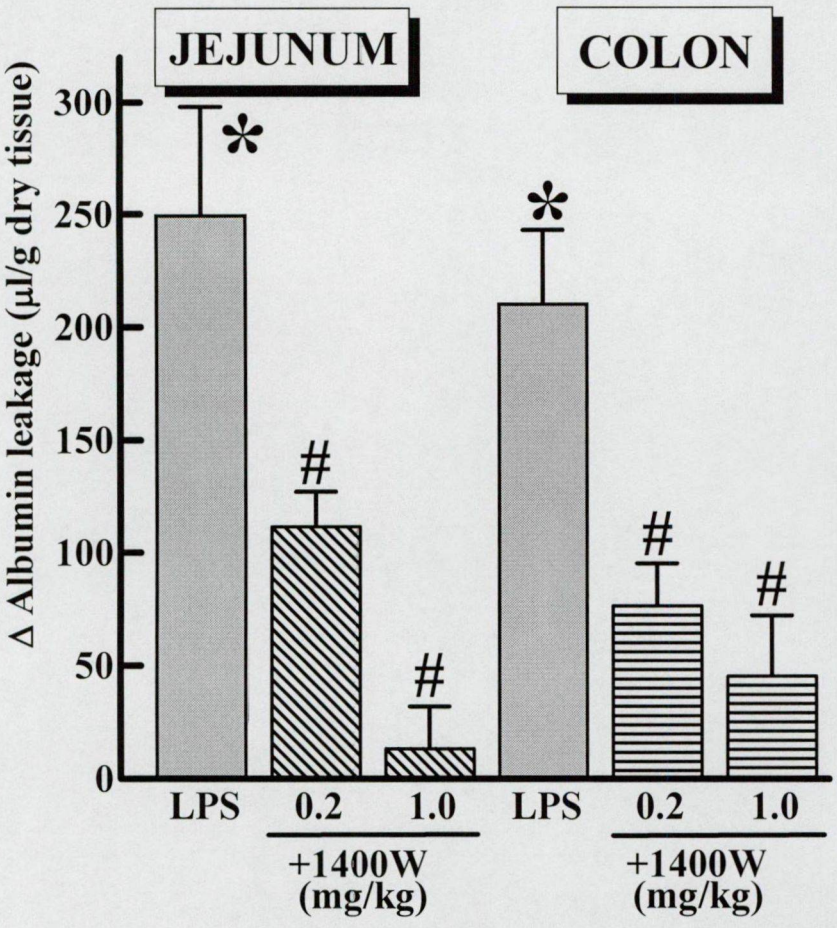

Figure 9.

Provocation of jejunal and colonic albumin leakage (expressed as µl albumin/g dry tissue) by the administration of a purified *Helicobacter pylori* endotoxin (LPS; 3 mg/kg, i.v.) 4 h later, and its dose-dependent attenuation by the concurrent administration of a selective iNOS inhibitor (1400W, 0.2-1 mg/kg, s.c.). Data are expressed as mean±S.E.M., where n=4-6 rats in a group. *P<0.05 means significant increase in albumin leakage; #P<0.05 means significant decrease in albumin leakage compared to the LPS alone group.

4.3. Role of local capsaicin-sensitive nerves (CSN) in colonic defence

4.3.1. Effect of CSN-depletion on colonic albumin leakage

CSN-depletion in the lower colon led to a significant plasma leakage ($P<0.005$, $n=8$) after two months. There was no difference in albumin extravasation in the lower colon of sham-operated animals as compared to the intact control group ($n=5$ and 8, respectively). In CSN-depleted and sham-operated animals, albumin extravasation in the upper colon was unaffected, as demonstrated in Figure 10.

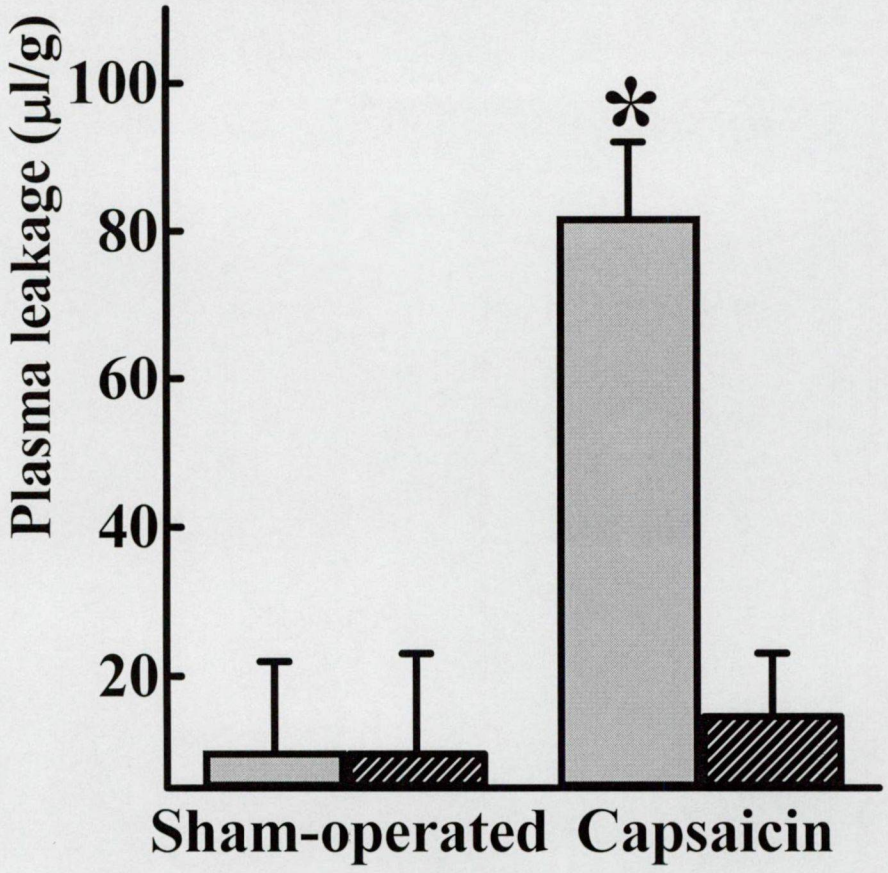

Figure 10.

Plasma leakage in the lower part (1-4 cm from the anus; grey column) of the rat colon induced by local capsaicin-sensitive nerve-depletion (CSN-depleted). The plasma leakage values in the upper part of the colon are demonstrated on hatched columns. Plasma leakage was expressed as Δ µl plasma/g dry tissue. Data are shown as means±S.E.M. of at least 5 experiments for each group, where statistical significant increase is shown as *$P<0.05$.

4.3.2. Effect of CSN-depletion on albumin leakage during colitis

In CSN-intact control rats administration of TNBS alone induced significant albumin extravasation in the upper and lower colon after one week ($P<0.001$, $n=5$). In contrast, in CSN-depleted rats TNBS alone produced a more marked increase in vascular permeability in the lower colon ($P<0.001$, $n=5$), while in the upper colon there was no difference in albumin extravasation between CSN-depleted and CSN-intact rats following the administration of TNBS-alone ($n=5$ and 8, respectively).

In CSN-intact rats administration of TNBS and ethanol caused marked plasma leakage in the upper and lower colon ($P<0.001$, $n=5$). In CSN-intact animals there was no significant difference in the enhancement of vascular permeability between the upper and lower parts of the colon. In the lower colon of CSN-intact rats TNBS+ethanol brought about similar increase in plasma leakage as TNBS alone in CSN-depleted animals (Figure 11).

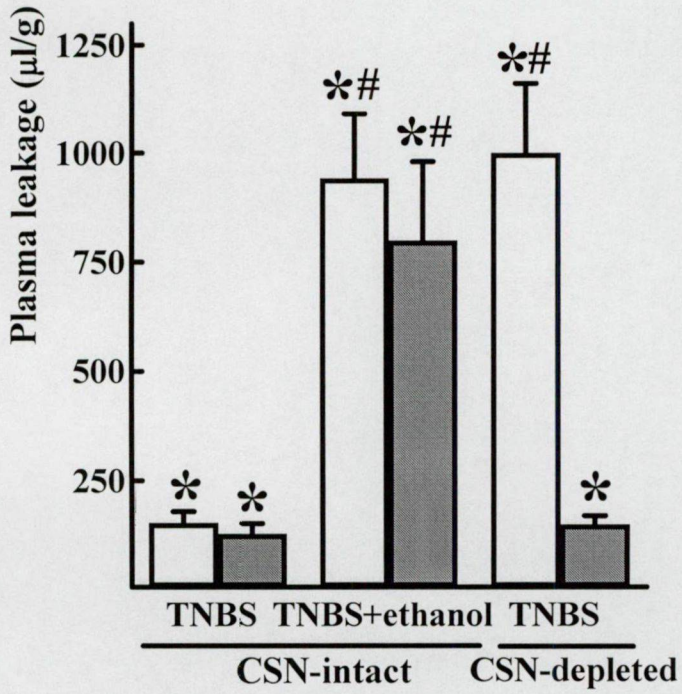

Figure 11.

Plasma leakage one week after intrarectal (8 cm from the anus) administration of trinitrobenzenesulphonic acid (TNBS, 30 mg/rat) or/and ethanol (50%) in lower (1-4 cm from the anus; white column) and upper (5-8 cm from the anus; grey column) parts of the colon following capsaicin-sensitive nerve-depletion (CSN-depleted) or in control (CSN-intact) rats. Plasma leakage was expressed as Δ µl plasma/g dry tissue. Data are shown as means±S.E.M. of at least 5 experiments for each group, where statistical significant increase is shown as $*P<0.05$. $^{\#}P<0.05$ represents statistical difference vs. the appropriate parts of the colon of TNBS in CSN-intact rats.

5. DISCUSSION

5.1. Aggressive actions of high doses of estrogens

This study confirms the previous observation (László *et al.*, 1992) that following ovariectomy the gastroduodenal mucosa is less sensitive to ulcerogenic stimuli. The reduction by ovariectomy of cysteamine-induced ulcers of the duodenum might relate to the decrease of endogenous plasma estradiol levels. Additionally, mucosal damage of the duodenum was increased by exogenous administration of oestradiol into intact female rats. Thus, our present findings indicate the involvement of estrogens among the female sexual steroids in the pathogenesis of duodenal mucosal ulceration.

For a better understanding of our results in relation to the pro-ulcerogenic actions of high doses of exogenous oestradiol, it should be mentioned here that administration of estrogens led to the inactivation of endogenous prostaglandins (Elegbe, 1978). Prostglandins have key importance in the defence mechanism of the duodenal mucosa (Robert, 1979; Robert & Kauffman, 1989). Furthermore, high doses of estrogens sensitize the vasculature towards the actions of vasopressin (László *et al.*, 1991), a pituitary hormone which plays an aggressive role in the development of experimental and human gastric and duodenal damage (Varga *et al.*, 1998; László *et al.*, 1998; Pávó *et al.*, 2000).

In the present study, we have shown that cysteamine provoked microvascular plasma leakage, as a measure of vascular endothelial damage. Our finding is in agreement with the previous suggestion that aggressive vascular factors are to be involved in the generation of cysteamine-induced gastric and duodenal ulcers (Bernardini *et al.*, 1989). On the other hand, it is well established that microcirculatory injury and the consequent hypoxia are among those important factors, which are considered to lead to mucosal ulceration (Robert & Kauffman, 1989). In the present work, it was also demonstrated that the reduction in endogenous estradiol level by ovariectomy caused the attenuation of cysteamine-induced microvascular leakage, which contrasts the harmful actions of exogenously administered estradiol following cysteamine challenge. It should be known that there are numerous biochemical and epidemiological evidences cumulated on the effects of estrogens on factors involved in the coagulation system (Williams & Stancel, 1996). A clear sense of the net effect on physiological or, more likely, pharmacological doses

of estrogens on coagulation has not yet been emerged (Beller, 1994). However, a focus should be given here that the vascular endothelium express oestrogen receptors and a number of special oestrogen derivatives can be used in certain cases as hemostatic agents (Williams & Stencel, 1996).

5.2. Attenuation of the intestinal barrier by H. pylori endotoxin-provoked induction of nitric oxide synthase

5.2.1. Actions of H. pylori LPS on duodenal epithelial cells

A dose-dependent elevation of Ca^{2+}-independent NOS activity was observed in rat duodenal epithelial cells 5h after challenge *in vivo* with a purified LPS of *H. pylori,* considered to reflect the expression of iNOS. Furthermore the appearance of this Ca^{2+}-independent activity was prevented by the novel highly selective iNOS inhibitor 1400W (Garvey *et al.*, 1997; László & Whittle, 1997). In previous studies, it has been suggested that circulating neutrophils are not the lonely source of the iNOS activity detected in duodenal tissue after *H. pylori* water extract challenge. Administration of anti-neutrophil serum in doses that reduced the circulating leukocytes count by 90% did not modify the level of the iNOS activity in duodenum (Lamarque *et al.*, 1998).

Induction of iNOS in colonic and small intestinal epithelial cells, that follows *E. coli* endotoxin challenge, is associated with a reduction in epithelial cells viability (Tepperman *et al.*, 1993, 1994). Likewise, in the present study, a reduction in duodenal epithelial cell viability, determined *ex vivo,* was observed four hours after *H. pylori* LPS administration. This reduction in cell viability, which accompanied with the expression of iNOS was inhibited by its highly selective inhibitor 1400W.

In the present study, intravenous administration of the systemically active superoxide scavenger, SOD-PEG inhibited the cell damage induced by *H. pylori* LPS. Such findings therefore, support the involvement of the superoxide anion in the tissue damage induced by *H. pylori* endotoxin. Sonicates of *H. pylori* have been shown to induce an oxidative burst in human polymorphonuclear and monocytes (Nielsen & Andersen, 1992b). Moreover, an increased luminol chemiluminesence, which reflects the generation of reactive oxygen species, has been found in the antrum of patients infected by *H. pylori* (Nielsen & Andersen, 1992a; Davies *et al.*, 1994). The local release of these cytotoxic moieties has been suggested to play a role in the duodenal

mucosal lesions observed in peptic ulcer disease associated with *H. pylori*. By interacting with NO, such oxygen species may form further damaging products such as peroxynitrite (Beckman *et al.*, 1990). Such reactive species may provokes epithelial cell injury by activating poly(ADP-ribose) synthase that depletes intracellular energy store (Kennedy *et al.*, 1998). Thus, by this mechanism, *H. pylori* could play a role in the apoptosis of gastric epithelial cells described following application of LPS on rat gastric mucosa (Piotrowski *et al.*, 1997).

The present study thus shows that a purified LPS from *H. pylori* can provoke the expression of iNOS *in vivo*. The expression of iNOS in duodenal epithelial cells could reflect a host-defence mechanism against the colonisation of *H pylori*, since NO can exert bacteriocidal actions (Granger *et al.*, 1988; Evans *et al.*, 1996). However, the release of *H. pylori* LPS and its passage into mucosa through damaged epithelium, could also play a role in a number of disease states. Indeed, expression of iNOS in gastric epithelial cells may stimulate apoptosis and contribute to induce gastric cancer (Anti *et al.*, 1998).

The current findings support the concept that the release of LPS stimulates the local production of high concentrations of NO from the duodenal epithelium and other cells through the expression of iNOS, which may play a role in the pathogenesis of duodenal ulceration associated with *H. pylori* infection.

5.2.2. Provocation of jejunal and colonic inflammation by H. pylori LPS

Our present results confirm previous observations, when a *H. pylori* extract was administered intravenously, and the expression of iNOS in the duodenal part of the small intestine and in its epithelial cells had been found (Lamarque *et al.*, 1998). The expression of iNOS correlated with epithelial cell death, and could be reversed by the administration of the non-selective iNOS inhibitor, dexamethasone, and by the treatment with N^G-nitro-L-arginine methyl ester, a non-specific NO synthase blocker at the time of the expression of iNOS (Lamarque *et al.*, 1998). Therefore, *H. pylori* extract could damage the intestinal mucosa by its direct toxic effect on epithelial cells, which is mediated by iNOS. The vascular injury is correlating with iNOS expression, and their reversal by the selective iNOS inhibitor, 1400W following the challenge with *H. pylori* purified endotoxin in our study suggests that, besides the damage of epithelial cells, microcirculatory dysfunction can also participate in *H. pylori*-related inflammation. Moreover, the induction of NO synthase and microvascular leakage in

the jejunum and colon might focus the impact of *H. pylori* endotoxin-provoked mucosal injury in other parts of the gastrointestinal tract.

5.3. Protection against colonic inflammation by local capsaicin-sensitive nerves

The present findings showed that local CSN-depletion led to a sustained increase in vascular permeability of the colon in control rats. Moreover, in CSN-depleted rats administration of TNBS produced a further increase in albumin leakage, which was similar in magnitude to that found after the application of TNBS+ethanol in the lower colon of CSN-intact animals. On the basis of these observations it appears that CSN are involved in the modulation of colonic vascular permeability under physiological and pathological circumstances.

Our results are in agreement with previous findings, where the defunctionalization of extrinsic CSN by systemic administration of capsaicin augmented 1./ immune-complex-induced colitis in rabbits (Reinshagen *et al.*, 1994), 2./ rat colonic inflammation provoked by acetic acid (Eliakim *et al.*, 1995) and 3./ TNBS+ethanol-induced mucosal injury in the rat colon (Evangelista & Meli, 1989). It has been also demonstrated that local activation of CSN by topical application of capsaicin protects against an acute colonic injury provoked by TNBS+ethanol in rats, an effect which is reversed by a competitive antagonist of capsaicin (Goso *et al.*, 1993). Taken together, these results suggest a protective role for CSN in experimental models of IBD.

It is known that CSN contain numerous neuropeptides (Holzer, 1991). The release of these neuropeptides may be responsible for the protective function of this neuronal system in IBD. During induction of colitis by acute immune complex exposure in rabbits, an early reduction of the two most important CSN neuropeptides, i.e. substance P (SP) and calcitonin gene-related peptide (CGRP) has been found (Eysselein *et al.*, 1991). These findings indicate that these peptides are released during inflammation. Because of its pharmacological actions (Holzer, 1991; Whittle, 1993) and of its predominant existence in the CSN of gastrointestinal tissues (Mulderry *et al.*, 1988; Reinshagen *et al.*, 1994), CGRP seems to be the most important peptide involved in the protective function of CSN in experimental models of IBD. Intravenous or intraarteriolar infusion of CGRP increases resting gastric mucosal blood flow (Dipette *et al.*, 1987; Holzer & Guth, 1991; Whittle *et al.*, 1992). In contrast, local infusion of the CSN neuropeptides, SP and neurokinin A did not elevate resting mucosal blood flow (Holzer

& Guth, 1991). Therefore, CGRP might promote mucosal integrity by increasing intestinal blood flow.

In conclusion, our results suggest that CSN may play a significant protective/anti-inflammatory role in the colon under physiological and pathological conditions. Finally, involvement of an impaired capsaicin-sensitive innervation of the intestine in the development of human IBD (especially Crohn's disease) can not be excluded. Indeed, it has been demonstrated that in patients with Crohn's disease the concentration of SP in rectal dialysates tends to be lower (Bernstein *et al.*, 1993) and in these patients the expression of SP receptors was shown to be increased in the intestinal tissue (Mantyh *et al.*, 1995).

6. SUMMARY

6.1. Actions of estrogens on duodenal ulceration

Our findings indicate that estradiol appears to exert a pro-ulcerogenic activity in the generation of macroscopic and microvascular injury in the duodenum. Thus, inappropriate high levels of estrogen might possibly have detrimental effects in the pathogenesis of duodenal ulceration.

6.2. Effects of H. pylori endotoxin on the intestinal mucosal barrier function

The expression of iNOS by *H. pylori* endotoxin results in duodenal epithelial cell injury by a process involving superoxide, suggesting the involvement of peroxynitrite. These events may contribute to the pathogenic effect of *H. pylori* in peptic ulcer disease.

On the basis of our present observations, *H. pylori* LPS seems to be capable to provoke damage not only in the duodenum, but its endotoxin is also able to induce jejunal and colonic microvascular albumin leakage, i.e. inflammation. This process is mediated by the overproduction of NO originating from the activation of the inducible NOS.

6.3. Role of capsaicin-sensitive innervation in colonic mucosal defence

Our results suggest that CSN may play a significant protective/anti-inflammatory role in the colon under physiological and pathological conditions, where the involvement of CGRP component of CSN is most likely to be involved. Since CGRP-depletion may

attenuate vasodilator tone and microvascular infarction(s) has significance in the pathways leading to Crohn's disease, the impact of an impaired CSN of the intestine in the development of human IBD (especially Crohn's disease) cannot be excluded.

7. PERSPECTIVES

Besides the injurious effect of *H. pylori* LPS in the duodenum, the induction of NO synthase and microvascular leakage in the jejunum and colon might focus the impact of *H. pylori* endotoxin-provoked mucosal injury in other parts of the gastrointestinal tract. We suspect that transmucosal invasion or/and surface translocation of *H. pylori* or its breakdown products might attenuate the mucosal barrier function of the jejunum and colon. This process is mediated by iNOS. Thus, the clinical implication of selective inhibitors of iNOS might have potential therapeutic benefit in the prevention and treatment of *H. pylori*-associated gastrointestinal mucosal inflammation. Finally, our results give further support of the importance of the eradication of *H. pylori*, even in asymptomatic cases.

REFERENCES

Anti M., Armuzzi A., Gasbarrini A., Gasbarrini G., Importance of changes in epithelial cell turnover during Helicobacter pylori infection in gastric carcinogenesis. *Gut* Suppl. 43 (1998) S27-32.

Aston N.O., Kalaichandran S., Carr J.V., Duodenal ulcer hemorrhage in the puerperum. *Can. J. Surg.* 34 (1991) 482-483.

Baldwin G., Alpert G., Caputo G.L., Baskin M., Parsonnet J., Gillis Z.A., Thompson C., Siber G.R., Fleisher G.R., Effect of polymyxin B on experimental shock from meningococcal and Escherichia coli endotoxins. *J. Inf. Dis.* 164 (1991) 542-549.

Beckman J.S., Beckman T.W., Chen J., Marshall P.A., Freeman B.A., Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. U.S.A.* 87 (1990) 1620-1624.

Beller F.K., Cardiovascular system: coagulation, thrombosis, and contraceptive steroids - is there a link? *In: Pharmacology of the Contraceptive steroids*. Ed.: Goldzieher J.W., Raven Press, New York, pp. 309-333 (1994).

Bernardini M.C., Blandizzi C., Morini G., Chiavarini M., Impicciatore, M., Del Tacca M., Pirenzepine prevents cysteamine-induced formation of gastroduodenal ulcers and reduction of mesenteric circulation. *Arch. Int. Pharmacodyn.* 302 (1989) 242-254.

Bernstein C.N., Robert M.E., Eysselein V.E., Rectal substance P concentrations are increased in ulcerative colitis but not in Crohn's disease. *Am. J. Gastroenterol.* 88 (1993) 908-913.

Blanchard H.S., Dernis-Labous E., Lamarque D., Nhieu J.T., Szepes Z., Flejou J.F., Wollman E., Whittle B.J.R., Breban M., Inducible nitric oxide synthase attenuates chronic colitis in human histocompatibility antigen HLA-B27/human beta2 microglobulin transgenic rats. *Eur. Cytokine Netw.* 12 (2001) 111-118.

Blaser M.J., Helicobacter pylori and the pathogenesis of gastroduodenal inflammation. *J. Infect. Dis.* 161 (1990) 626-633.

Boughton-Smith N.K., Evans S.M., László F., Whittle B.J.R., Moncada S., The induction of nitric oxide synthase and intestinal vascular permeability by endotoxin in the rat. *Br. J. Pharmacol.* 110 (1993) 1189-1195.

Brown J.F., Tepperman B.L., Hanson P.J., Whittle B.J.R., Lipopolysaccharide induces Ca^{2+}-independent nitric oxide synthase activity in rat gastric mucosal cells. *Eur. J. Pharmacol.* 292 (1994) 111-114.

Carrick W.J.A., Lee A., Hazell S., Ralston M., Daskalopoulos G., Campylobacter pylori, duodenal ulcer, and gastric metaplasia: possible role of functionnal heterotopic tissue in ulcerogenesis. *Gut* 30 (1989) 790-797.

Davies G.R., Simmonds N.J., Stevens T.R.J., Sheaff M.T., Banatvala N., Laurenson I.F., Blake D.R., Rampton D.S., Helicobacter pylori stimulates antral mucosal reactive oxygen metabolite production in vivo. *Gut* 35 (1994) 179-185.

Dipette D.J., Schwarzenberger K., Kerr N., Holland O.B., Systemic and regional hemodynamic effects of calcitonin gene-related peptide. *Hypertension* 9 Suppl. III. (1987) 142-146.

Elegbe R.A., Inactivation of prostaglandin E2 in the stomach of pregnant and oestrogen pretreated rats. *Biochem. Exp. Biol.* 14 (1978) 69-76.

Eliakim R., Karmeli F., Okon E., Rachmilewitz D., Ketotifen ameliorates capsaicin-augmented acetic acid-induced colitis. *Dig. Dis. Sci.* 40 (1993) 503-509.

Evangelista S., Meli A., Influence of capsaicin-sensitive fibres on experimentally-induced colitis in rats. *J. Pharm. Pharmacol.* 41 (1989) 574-575.

Evans T.J., Buttery L.D., Carpenter A., Springall D.R., Polak J.M., Cohen J., Cytokine-treated human neutrophils contain inducible nitric oxide synthase that

produces nitration of ingested bacteria. *Proc. Natl. Acad. Sci. U. S. A.* 93 (1996) 9553-9558.

Eysellein V.E., Reinshagen M., Cominelli F., Sternini C., Davis W., Patel A., Nast C.C., Bernstein D., Anderson K., Khan H., Snape W.J. Jr., Calcitonin gene-related peptide and substance P decrease in rabbit colon during colitis. *Gastroenterology* 101 (1991) 1211-1219.

Garvey E.P., Oplinger J.A., Furfine E.S., Kiff R.J., László F., Whittle B.J.R., Knowles R.G., 1400W is a slow, tight binding, and highly selective inhibitor of inducible nitric-oxide synthase in vitro and in vivo. *J. Biol. Chem.* 272 (1997) 4959-4963.

Goso C., Evangelista S., Tramontana M., Manzini S., Blumberg P.M., Szallasi A., Topical capsaicin administration protects against trinitrobenzenesulphonic acid-induced colitis in the rat. *Eur. J. Pharmacol.* 249 (1993) 185-190.

Granger D., Hibbs J.B., Perfect J.R., Durack D.T., Specific amino acid (L-arginine) requirement for the microbiostatic activity of murine macrophage. *J. Clin. Invest.* 81 (1988) 1129-1136.

Hay J.W., Hay A.R., Inflammatory bowel disease: medical costs and algorithms. *J. Clin. Gastroenterol.* 14 (1992) 309-317.

Holzer P., Sametz W., Gastric mucosal protection against ulcerogenic factors in the rat mediated by capsaicin-sensitive afferent neurons. *Gastroenterology* 91 (1986) 975-981.

Holzer P., Local effector functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, calcitonin gene-related peptide and other neuropeptides. *Neuroscience* 24 (1988) 739-768.

Holzer P., Capsaicin: cellular targets, mechanisms of action and selectivity for thin sensory neurons. *Pharmacol. Rev.* 43 (1991) 143-204.

Holzer P., Guth P.H., Neuropeptide control of rat gastric mucosal blood flow: increase by calcitonin gene-related peptide and vasoactive intestinal polypeptide, but not by substance P and neurokinin A. *Circ. Res.* 68 (1991) 100-105.

Ischioropoulos H., Al-Medhi, A.B., Fisher A.B., Reactive species in ischemic rat lung injury: contribution of peroxynitrite. *Am. J. Physiol.* 269 (1995) L158-L164.

Jancsó G., Pathobiological reactions of C-febre primary sensory neurones to peripheral nerve injury. *Exp. Physiol.* 77 (1992) 405-431.

Kennedy M., Denenberg A.G., Szabó Cs., Salzman A.L., Poly(ADP-ribose) synthetase activation mediates increased permeability induced by peroxynitrite in Caco-2BBe cells. *Gastroenterology* 114 (1998) 510-518.

Kiss J., Lamarque D., Moran A.P., Pozsár J., Morschl É., László F., Whittle B.J.R., *Helicobacter pylori* lipopolysaccharide-provoked injury to rat microvasculature involves inducible nitric oxide synthase. *Eur. J. Pharmacol.* 420 (2001) 175-179.

Lamarque D., Whittle B.J.R., Involvement of superoxide and xanthine oxidase in neutrophil-independent rat gastric damage induced by NO donors. *Br. J. Pharmacol.* 116 (1995) 1843-1848.

Lamarque D., Kiss J., Tankovic J., Flejou J.F., Delchier J.C., Whittle B.J.R., Induction of nitric oxide synthase in vivo and cell injury in rat duodenal epithelium by water soluble extract of Helicobacter pylori, *Br. J. Pharmacol.* 123 (1998) 1073-1078.

Lamarque D., Nhieu J.T., Breban M., Bernardeu C., Martin-Garcia N., **Szepes Z.**, Whittle B.J.R., Claudepierre P. Lymphocytic infiltration and expression of inducible nitric oxide synthase in human duodenal and colonic mucosa is a characteristic feature of ankylosing spondylitis. J Rheumatol. 2003;30:2428-2436.

László F.A., László F., de Wied D., Pharmacology and clinical perspectives of vasopressin antagonists. *Pharmacol. Rev.* 43 (1991) 73-108.

László F., Amani E., Varga Cs., László F.A., Influence of sex hormones on ethanol-induced gastric haemorrhagic erosions in rats. *Acta Physiol. Hung.* 80 (1992) 289-292.

László F., Amani E., Karácsony G., Szabó E., Rengei B., Varga Cs., László F.A., The modulatory role of endogenous vasopressin in the phenomenon that orally administered ethanol generates more severe gastric erosions in male than in female rats. *Ann. NY Acad. Sci.* 689 (1993) 623-626.

László F., Whittle B.J.R., Actions of isoform-selective and non-selective nitric oxide synthase inhibitors on endotoxin-induced vascular leakage in rat colon. *Eur. J. Pharmacol.* 34 (1997) 99-102.

László F., Pávó I., Szepes Z., Varga Cs., László F.A., Deleterious action of vasopressin in gastroduodenal ulceration: experimental and clinical observations. *Scand. J. Gastroenterol.* Suppl. 228 (1998) 62-67.

Mantyh C.R., Vigna S.R., Bollinger R.R., Mantyh P.W., Maggio J.E., Pappas T.N., Differential expression of substance P receptors in patients with Crohn's disease and ulcerative colitis. *Gastroenterology* 109 (1995) 850-860.

McCall T.B., Boughton-Smith N.G., Palmer R.M.J., Whittle B.J.R., Moncada S., Synthesis of nitric oxide from L-arginine by neutrophils. Release and interaction with superoxide anion. *Biochem. J.* 261 (1989) 293-296.

Michaletz-Onody P.A., Peptic ulcer disease in pregnancy. Gastroenterol. Clin. North Am. 21 (1992) 817-826.

Molnar T., Szepes Z., Nagy F., Lonovics J. Successful treatment of steroid resistant ulcerative colitis associated with severe autoimmune hemolytic anemia with oral microemulsion cyclosporin--a brief case report. Am J Gastroenterol. 98 (2003) 1207-1208.

Mooney C, Keenan J., Munster D., Wilson I., Allardyce R., Bagshaw P., Chapman B., Chadwick V., Neutrophil activation by Helicobacter pylori. *Gut* 32 (1991) 853-857.

Moncada S., Higgs E.A., Molecular mechanisms and therapeutic strategies related to nitric oxide. *FASEB J.* 9 (1995) 1319-1330.

Moran A.P., Helander I.M., Kosunen T.U., Compositional analysis of Helicobacter pylori rough-form lypopolysaccharides. *J. Bacteriol.* 174 (1992) 1370-1377.

Morris G.P., Beck P.L., Herridge M.S., Depew W.T., Szevczuk M.R., Wallace J.L., Hapten-induced model of chronic inflammation and ulceration in the rat colon. *Gastroenterology* 96 (1989) 795-803.

Mulderry P.K., Ghatei M.A., Spoke R.A., Jones P., Piersson A.M., Hamid Q.A., Kause S., Amara S.G., Burr J.M., Legon S., Polak J.M., Bloom S.R., Different expression of α-CGRP and β-CGRP by primary sensory neurons and enteric autonomic neurons of the rat. *Neuroscience* 25 (1988) 195-206.

Nielsen H., Andersen L.P., Activation of human phagocyte oxidative metabolism by Helicobacter pylori. *Gastroenterology* 103 (1992a) 1747-1753.

Nielsen H., Andersen L.P., Chemotactic activity of Helicobacter pylori sonicate for human polymorphonuclear leukocytes and monocytes. *Gut* 33 (1992b) 738-742.

Pávó I., Morschl É., Szepes Z., Kiss J., Boda K., Vetró G., Varga C, László FA, László F., Vasopressin deficiency decreases the frequency of gastroduodenal ulceration in humans. *J. Physiol. (Paris)* 94 (2000) 63-66.

Perez-Perez G.I., Sherpherd V.L., Morrow J.D., Blaser M.J., Activation of human THP-1 cells and rat bone marrow-derived macrophages by Helicobacter pylori lipopolysaccharide. *Inf. Immun.* 63 (1995) 1183-1187.

Piotrowski J., Piotrowski E., Skrodzka D., Slomiany A., Slomiany B.L., Induction of acute gastritis and epithelial apoptosis by Helicobacter pylori lipopolysaccharide. *Scand. J. Gastroenterol.* 32 (1997) 203-211.

Reinshagen M., Patel A., Sottili M., Nast C., Davis W., Mueller K., Eysselein E., Protective function of extrinsic sensory neurons in acute rabbit experimental colitis. *Gastroenterology* 106 (1994) 1208-1214.

Riis P., Differential diagnosis, ulcerative colitis, Crohn's disease and other disorders, including diverticular disease. *In: Inflammatory bowel disease*. Allan RN, Keighley MRB, Alexander-Williams J, Hawkins CF, eds. Edinburgh, Churchill Livingstone, pp. 191-198 (1990).

Robert A., Phillips J.P., Nezamis J.E., Production, by restraint, of gastric ulcers and of hydrothorax in the rat. *Gastroenterology* 51 (1966) 75-80.

Robert A., Nezamis J.P., Exertion ulcers. *In: Experimental ulcer*, ed. Gheorgiu T., Gerhard Witzstrock Publ., Baden-Baden-Brussels-Cologne, pp. 77-85 (1975).

Robert A., Cytoprotection by prostaglandins. *Gastroenterology* 77 (1979) 761-767.

Robert A., Kauffman G.L., Stress ulcers, erosions and gastric mucosal injury. *In: Gastrointestinal Disease, Pathophysiology, Diagnosis, Management.*, Eds.: Sleisenger M.H., Fordtran J.S., Saunders Co., Philadelphia, pp. 772-792 (1989).

Roth J., Lentze M.J., Berger E.G., 1985. Immunocytochemical demonstration of ecto-galactosyltransferase in absorptive intestinal cells. *J. Cell Biol.* 100, 118-125.

Salter M., Knowles R.G., Moncada S., Widespead tissue distribution, species distribution and changes in activity of Ca^{2+}-dependent and Ca^{2+}-independent nitric oxide synthases. *FEBS Lett.* 291 (1991) 145-149.

Tepperman B.L., Tan S.Y., Whittle B.J.R., Effects of calcium-modifying agents on integrity of rabbit isolated gastric mucosal cells. *Am. J. Physiol.* 261 (1991) G119-G127.

Tepperman B.L., Brown J.F., Whittle B.J.R., Nitric oxide synthase induction and intestinal epithelial cell viability in rats. *Am. J. Physiol.* 265 (1993) G214-G218.

Tepperman B.L., Brown J.F., Korolkiewicz R., Wittle B.J.R. (1994). Nitric oxide synthase activity, viability and cyclic GMP levels in rat colonic epithelial cells. Effects of endotoxin challenge. *J. Pharmacol. Exp. Ther.* 271 (1994) 1477-1482.

Varga Cs., Pávó I., Lamarque D., Szepes Z., Kiss J., Karácsony G., László F.A., László F., Endogenous vasopressin increases acute endotoxin shock-provoked gastrointestinal mucosal injury in the rat. *Eur. J. Pharmacol.* 352 (1998) 257-261.

Whittle B.J.R., Lopez-Belmonte J., Moncada S., Nitric oxide mediates rat mucosal vasodilatation induced by intragastric capsaicin. *Eur. J. Pharmacol.* 218 (1992) 339-341.

Whittle B.J.R., Neuronal and endothelium-derived mediators in the modulation of the gastric microcirculation: integrity in the balance. *Br. J. Pharmacol.* 110 (1993) 3-17.

Whittle B.J.R., Laszlo F., Evans S.M., Moncada S., Induction of nitric oxide synthase and microvascular injury in the rat jejunum provoked by indomethacin. *Br. J. Pharmacol.* 116 (1995) 2286-2290.

Williams C.L., Stancel G.M., Estrogens and progestins. *In: The Pharmacological Basis of Therapeutics.* Ed.: Goodman Gilman A., McGraw-Hill, New York, pp. 1411-1440 (1996).

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