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European Journal of Pharmacology 420 (2001) 175–179

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Short communication

Helicobacter pylori lipopolysaccharide-provoked injury to rat gastroduodenal microvasculature involves inducible nitric oxide synthase

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Received 18 April 2001; accepted 24 April 2001

Abstract

The actions of a purified *Helicobacter pylori* lipopolysaccharide (3 mg kg^{-1}, i.v.) on rat gastric antral and duodenal microvascular integrity (determined as radiolabelled albumin leakage) and the expression of the inducible nitric oxide (NO) synthase (iNOS; assessed by the citrulline assay) were investigated 4 h after challenge. Significant increases of albumin leakage and expression of iNOS in both antral and duodenal tissues were observed following challenge. Concurrent administration of the selective iNOS inhibitor, 1400W (*N*-(8-(aminomethyl)benzyl)-acetamidine; 0.2–1 mg kg^{-1}, s.c.), with lipopolysaccharide, caused a dose-dependent attenuation of the gastric and duodenal albumin leakage. Thus, *H. pylori* lipopolysaccharide can initiate the expression of iNOS in the stomach and duodenum following systemic challenge, which can provoke gastroduodenal microvascular dysfunction.

Keywords: *Helicobacter pylori*; Lipopolysaccharide; Gastritis; Duodenum; Inflammation; Nitric oxide (NO) synthase inhibitor; Nitric oxide (NO) synthase, inducible

1. Introduction

The acid-resistant Gram-negative bacterium, *Helicobacter pylori*, grows predominantly in the antral region of the stomach and at sites of gastric metaplasia in the duodenum (Blaser, 1990). The pathological involvement of *H. pylori* is recognized in gastric mucosal inflammation and in gastroduodenal peptic ulceration (NIH, 1994). To understand the mechanisms that are involved in such gastroduodenal tissue inflammation and injury, it is important to evaluate the effects of the soluble mediators produced by *H. pylori* and their actions in promoting cytotoxicity.

The purification of lipopolysaccharide from *H. pylori* has provided an important tool for exploring the cytotoxic processes related to the bacterium and its products (Moran, 1999). One such potential mechanism involves the inappropriate production of nitric oxide (NO), since the expression of the inducible NO synthase enzyme (iNOS) can be triggered by the lipopolysaccharide component of bacterial endotoxins, as well as the cytokines produced by lipopolysaccharide challenge (Moncada and Higgs, 1995; Perez-Perez et al., 1995). This overproduction of NO has cytotoxic potential and provokes widespread microvascular inflammatory reactions with albumin leakage into the interstitium (László et al., 1995). Recent studies have shown that the purified lipopolysaccharide from *H. pylori* can cause cytotoxicity in rat duodenal epithelial cells following in vivo challenge (Lamarque et al., 2000). Pharmacological studies indicate that this cytotoxic action involves the expression of iNOS in the epithelial cells and the production of superoxide (Lamarque et al., 2000), thus implicating a role of the cytotoxic species, peroxynitrite (Beckman et al., 1990).

In our present study, we have investigated whether administration of a purified *H. pylori* lipopolysaccharide

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0014-2999/01/$ - see front matter
PII: S0014-2999(01)01008-1

leads to the expression of iNOS, determined by the citrulline assay, and the production of microvascular dysfunction, determined as the leakage of radiolabelled albumin, in both the gastric antrum and duodenum of the rat. The association of such microvascular injury with iNOS expression was explored by the use of the bisisothiourea derivative, 1400W (Garvey et al., 1997; László and Whittle, 1997), a potent and selective inhibitor of the iNOS isoform.

2. Materials and methods

2.1. Experimental procedures

Male Wistar rats (230–250 g) were fasted overnight but were allowed free access to water. Under transient ether anaesthesia, purified *H. pylori* lipopolysaccharide (3 mg kg^{-1}) was administered into the tail vein, and the gastric antrum and the duodenum were removed 4 h later.

2.2. Albumin leakage

For the measurement of vascular permeability, [^{125}I]human serum albumin was administered (2 μCi kg^{-1}, i.v.) and its leakage into the tissue determined in segments of the gastric antrum and duodenum, 4 h after lipopolysaccharide administration. Blood was collected from the abdominal aorta into syringes containing trisodium citrate (final concentration 0.318%) and centrifuged (10,000 × *g*, 10 min, 4°C). The [^{125}I]human serum albumin in the plasma and segments of tissues was detected using a gamma-spectrometer (Nuclear Enterprises NE 1600). Subsequently, the tissues were dried at 80°C over 48 h and the albumin content on a weight basis was calculated.

The resting value for albumin leakage was taken as the mean of the data of untreated control animals. In each experiment and for each procedure, this basal control mean value was subtracted from the values of treatment groups. The data were expressed as changes in albumin accumulation (Δ plasma leakage, μl plasma g^{-1} dry tissue) as described previously (László et al., 1994, 1995).

2.3. Nitric oxide synthase enzyme activity

NO synthase (NOS) activity was determined as the conversion of L-[^{14}C]arginine monohydrochloride to L-[^{14}C]citrulline (László et al., 1995). After lipopolysaccharide administration (4 h), gastric antral and duodenal tissues were homogenized (15 s) in buffer (250 mg ml^{-1}, 4°C) containing HEPES (10 mM), sucrose (32 mM), dithiothreitol (1 mM), EDTA (0.1 mM), soybean trypsin inhibitor (10 μg ml^{-1}), leupeptin (10 μg ml^{-1}) and aprotinin (2 μg ml^{-1}). This tissue-containing buffer, adjusted to pH 7.4, was centrifuged at 10,000 × *g* for 20 min at 4°C. A 40-μl sample of supernatant was incubated for 10 min at 37°C in 110 μl of reaction buffer comprising (final concentrations): 50 mM KH_2PO_4, 1 mM $MgCl_2$, 0.2 mM $CaCI_2$, 50 mM valine, 1 mM dithiothreitol, 15 nM L-arginine, 1 mM L-citrulline, 0.3 mM NADPH, 3 μM FAD, 3 μM FMN and 157 pM [^{14}C]L-arginine. The reaction was arrested by the addition (0.5 ml) of a 1:1 suspension of Dowex (AG 50W-8) in water. The mixture was dispersed and diluted with 0.85 ml of distilled water, and allowed to settle for 30 min. The supernatant was removed for estimation of the radiolabelled products by scintillation counting (2 ml Pico-Fluor). Sample protein content was estimated via spectrophotometric assay (Bio-Rad Protein Assay), and NOS activity was expressed as pmol min^{-1} mg^{-1} protein.

Total NOS activity was defined as citrulline formation that was abolished by incubation in vitro with N^G-monomethyl-L-arginine (L-NMMA, 700 μM). The L-NMMA-sensitive NOS activity that was not inhibited by EGTA (1 mM) incubation was taken as iNOS activity (László et al., 1995).

2.4. Effect of selective inhibition of inducible nitric oxide synthase on gastric and duodenal albumin leakage

In a separate experiment, the selective iNOS inhibitor, *N*-(8-(aminomethyl)benzyl)-acetamidine (1400W; 0.2–1 mg kg^{-1}, s.c.) was administered concurrently with *H. pylori* lipopolysaccharide. The dose, timing and route of administration of 1400W have been established in previous studies (Garvey et al., 1997; Lamarque et al., 2000). The gastric antrum and the duodenum were removed 4 h following treatment.

2.5. Chemicals

Purified *H. pylori* lipopolysaccharide was prepared as previously described (Nielsen et al., 1994; Moran, 1999). 1400W (*N*-(8-(aminomethyl)benzyl)-acetamidine) was obtained from GlaxoWellcome (Stevenage, UK). L-[U-^{14}C]arginine monohydrochloride was obtained from Amersham International (UK). [^{125}I]Human serum albumin was purchased from IZINTA (Budapest, Hungary). All other compounds were from Sigma.

2.6. Statistics

The data are expressed as mean ± S.E.M. from *n* rats per experimental group. For statistical comparisons, the Mann–Whitney non-parallel *U*-test and the One-way Analysis of Variance followed by the Tukey–Kramer Multiple Comparisons Test were utilised, where appropriate. $P < 0.05$ was taken as a significant difference.

3. Results

3.1. Induction of nitric oxide synthase and albumin leakage

Administration of purified *H. pylori* lipopolysaccharide (3 mg kg^{-1}, i.v.) led to the expression of iNOS activity in the whole tissue of the gastric antrum (Fig. 1) and duodenum (Fig. 2) when determined 4 h later. The activity of iNOS following challenge was significantly higher in the duodenum than in the antrum (144 ± 58 and 9 ± 5 pmol min^{-1} mg^{-1} protein, respectively; $n = 4$, $P < 0.05$).

The albumin accumulation of non-challenged control rats was 217 ± 11 and 383 ± 10 μl g^{-1} dry tissue in the gastric antrum and duodenum, respectively ($n = 6$). In a separate group of animals, a significant increase in gastric antral and duodenal vascular albumin leakage occurred 4 h after the injection of purified *H. pylori* lipopolysaccharide (3 mg kg^{-1}, i.v.) as shown in Figs. 1 and 2, respectively. The enhancement of albumin leakage after lipopolysaccharide challenge was significantly greater in the duodenum compared to the gastric antrum (Δ 322 ± 27 and Δ 201 ± 22 μl g^{-1} dry tissue, respectively; $n = 6$, $P < 0.01$).

Macroscopically visible gross damage did not develop either in the gastric antrum or duodenum, over the 4 h following lipopolysaccharide administration, nor was it observed in any other region of the gastrointestinal tract.

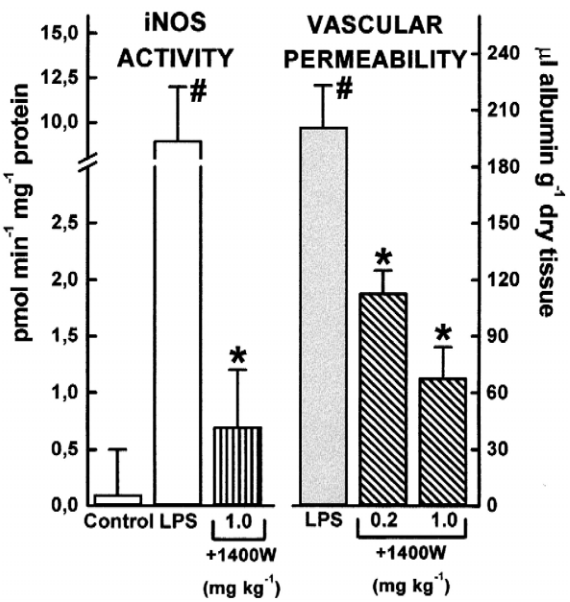

Fig. 1. Increase in inducible nitric oxide synthase enzyme activity (iNOS; pmol min^{-1} mg^{-1} protein; left panel) and in vascular leakage (μl albumin g^{-1} dry tissue; right panel) in the rat gastric antrum provoked by the intravenous administration of a purified endotoxin (LPS) of *H. pylori* (3 mg kg^{-1}), determined 4 h later. The effects of the concurrent administration of the selective iNOS inhibitor, 1400 W (1 mg kg^{-1} and 0.2–1 mg kg^{-1}, respectively; s.c.) 4 h later are also shown. Data are expressed as mean ± S.E.M., where $n = 4$–6 rats were in a group, where $^{\#}P < 0.05$ is a significant increase in iNOS activity and albumin leakage following lipopolysaccharide administration; $^{*}P < 0.05$ is a significant reduction of lipopolysaccharide-provoked iNOS expression and vascular permeability.

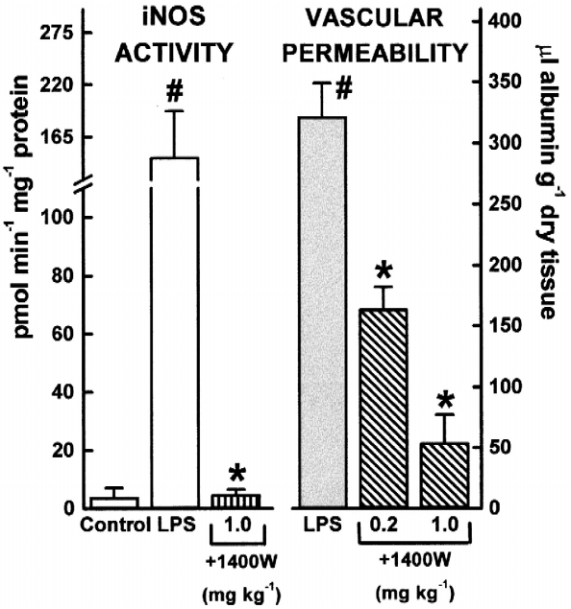

Fig. 2. Increase in inducible nitric oxide synthase enzyme activity (iNOS; pmol min^{-1} mg^{-1} protein; left panel) and in vascular leakage (μl albumin g^{-1} dry tissue; right panel) in the rat duodenum provoked by the intravenous administration of a purified endotoxin (LPS) of *H. pylori* (3 mg kg^{-1}), determined 4 h later. The effects of the concurrent administration of the selective iNOS inhibitor, 1400W (1 mg kg^{-1} and 0.2–1 mg kg^{-1}, respectively; s.c.) 4 h later are also shown. Data are expressed as mean ± S.E.M., where $n = 4$–6 rats were in a group, where $^{\#}P < 0.05$ is a significant increase in iNOS activity and albumin leakage following lipopolysaccharide administration; $^{*}P < 0.05$ is a significant reduction of lipopolysaccharide-provoked iNOS expression and vascular permeability.

3.2. Effect of selective inhibition of inducible nitric oxide synthase on gastric and duodenal albumin leakage

Administration of the selective iNOS inhibitor, 1400W (0.2–1 mg kg^{-1}, s.c.) concurrently with *H. pylori* lipopolysaccharide (3 mg kg^{-1}, i.v.) caused a significant and dose-dependent reduction of lipopolysaccharide-provoked vascular albumin leakage in the gastric antrum and duodenum determined after 4 h (Figs. 1 and 2). Thus, 1400W (1 mg kg^{-1}, s.c.) reduced albumin leakage in the duodenum and in the gastric antrum by $86 \pm 4\%$ and $68 \pm 6\%$, respectively ($P < 0.05$; $n = 4$).

Likewise, 1400W (1 mg kg^{-1}, s.c.) caused a $92 \pm 6\%$ ($n = 4$, $P < 0.05$) and $98 \pm 1\%$ ($n = 4$, $P < 0.05$) inhibition of iNOS activity, determined 4 h after challenge, in the gastric antrum and duodenum, respectively (Figs. 1 and 2).

4. Discussion

In the present in vivo study, an increase in albumin leakage, a sensitive index of vascular cell injury and

inflammation, in the rat gastric antrum and duodenum could be observed 4 h following the intravenous administration of *H. pylori* lipopolysaccharide. These findings of gastroduodenal microvascular dysfunction agree with previous observations, where increases in microvascular permeability to protein in the rat mesenteric circulation following local application of a water-extract of *H. pylori* have been observed (Kurose et al., 1994). In addition, administration of *H. pylori* extract or purified lipopolysaccharide has been shown to provoke injury of duodenal epithelial cells following intravenous challenge (Lamarque et al., 1998; Lamarque et al., 2000).

The mechanisms by which soluble mediators derived from *H. pylori* can injure the gastroduodenal microvasculature could involve a number of pathways. Early studies suggested that *H. pylori* extract-induced microcirculatory albumin leakage could be the consequence of interstitial and intravascular cell–cell interactions (Kurose et al., 1994). Thus, the lipopolysaccharide from *H. pylori* can activate polymorphonuclear cells (Nielsen et al., 1994). Administration of *H. pylori* extract can lead to the expression of a neutrophil-activating protein, which causes neutrophils to adhere to the vascular endothelium, an event that can initiate microcirculatory protein leakage (Evans et al., 1995; Takemura et al., 1996). Neutrophils are known to release a number of pro-inflammatory mediators, including platelet-activating factor and thromboxanes, which have an important role in promoting vascular albumin leakage following lipopolysaccharide challenge (László et al., 1994) as well as producing cytotoxic radicals such as superoxide and NO (Moncada and Higgs, 1995).

In the present study, the involvement of iNOS in the processes leading to microvascular injury following challenge with *H. pylori* lipopolysaccharide was evaluated by using a highly selective iNOS inhibitor, 1400W (Garvey et al., 1997; László and Whittle, 1997). Thus, in doses that reduced the iNOS enzyme activity following lipopolysaccharide challenge, 1400W substantially attenuated the albumin leakage in both gastric antrum and duodenum. These findings thus extend to the microvasculature, the previous observations that 1400W can prevent injury to duodenal epithelial cells provoked by *H. pylori* lipopolysaccharide (Lamarque et al., 2000). Such studies thus provide good evidence that iNOS is involved in both the epithelial cell cytoxicity and the microcirculatory injury brought about by systemic exposure to *H. pylori* lipopolysaccharide, a process that may involve the production of peroxynitrite through the combination of superoxide and NO (Lamarque et al., 2000). Despite substantially greater iNOS activity being detected in the duodenum compared to the gastric antrum, the relative expression of iNOS in these tissues appeared to contribute significantly to the microvascular injury, as 1400W prevented the leakage in both regions. The sites of iNOS expression that promote vascular leakage in the present model are not known but may involve the microvascular endothelium. In clinical studies on gastric mucosal biopsies from patients with gastritis associated with *H. pylori* infection, increased antral mRNA for iNOS, and iNOS protein in epithelium, endothelium and inflammatory cells have been reported (Fu et al., 1999).

The present results thus suggest that the purified lipopolysaccharide from *H. pylori* is capable of inducing an iNOS-mediated microvascular inflammatory response in the gastric antrum and duodenum of the rat following its parenteral administration. Thus, the local liberation of *H. pylori* lipopolysaccharide into the mucosal tissue, or into the systemic circulation, may contribute to the pathogenic processes provoked by *H. pylori*, through the expression of iNOS.

Acknowledgements

This work was supported by the Hungarian Research Foundation (T-03 2143) and by the Hungarian Ministry of Health (ETT 281/2000). Dominique Lamarque was a recipient of a grant from Institut de Recherche Des Maladies de l'Appareil Digestif. Anthony P. Moran received a grant from the Irish Health Research Board. Ferenc László and Éva Morschl were sponsored by the Széchenyi Professor Fellowship of the Hungarian Ministry of Education and by the Soros Foundation, respectively.

References

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

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

Evans, D.J., Evans, D.G., Takemura, T., Nakano, H., Lampert, H.C., Graham, D.Y., Granger, D.N., Kvietys, P.R., 1995. Characterization of a *Helicobacter pylori* neutrophil-activating protein. Infect. Immun. 63, 2213–2220.

Fu, S., Ramanujam, K.S., Wong, A., Fantry, G.T., Drachenberg, C.B., James, S.P., Meltzer, S.J., Wilson, K.T., 1999. Increased expression and cellular localization of inducible nitric oxide synthase and cyclooxygenase 2 in *Helicobacter pylori* gastritis. Gastroenterology 116, 1319–1329.

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

Kurose, I., Granger, D.N., Evans, D.J., Evans, D.G., Graham, D.Y., Miyasaka, M., Anderson, D.C., Wolf, R.E., Cepinskas, G., Kvietys, P.R., 1994. *Helicobacter pylori*-induced microvascular protein leakage in rats: role of neutrophils, mast cells and platelets. Gastroenterology 107, 70–74.

Lamarque, D., Kiss, J., Tankovic, J., Flejou, J.F., Delchier, J.C., Whittle, B.J.R., 1998. 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, 1073–1078.

Lamarque, D., Moran, A.P., Szepes, Z., Delchier, J.C., Whittle, B.J.R.,

2000. 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. 130, 1531–1538.

László, F., Whittle, B.J.R., 1997. Actions of isoform-selective and non-selective nitric oxide synthase inhibitors on endotoxin-induced vascular leakage in rat colon. Eur. J. Pharmacol. 334, 99–102.

László, F., Whittle, B.J.R., Moncada, S., 1994. Interactions of constitutive nitric oxide with PAF and thromboxane on rat intestinal vascular integrity in acute endotoxaemia. Br. J. Pharmacol. 113, 1131–1136.

László, F., Evans, S.M., Whittle, B.J.R., 1995. Association of microvascular leakage with induction of nitric oxide synthase: effects of nitric oxide synthase inhibitors in various organs. Eur. J. Pharmacol. 283, 47–53.

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

Moran, A.P., 1999. *Helicobacter pylori* lipopolysaccharide-mediated gastric and extragastric pathology. J. Physiol. Pharmacol. 50, 787–805.

Nielsen, H., Birkholz, S., Andersen, L.P., Moran, A.P., 1994. Neutrophil activation by *Helicobacter pylori* lipopolysaccharides. J. Infect. Dis. 170, 135–139.

NIH, 1994. Consesus development panel on *Helicobacter pylori* in peptic ulcer disease. JAMA 272, 65–69.

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

Takemura, T., Granger, D.N., Evans, D.J., Evans, D.G., Graham, D.Y., Anderson, D.C., Wolf, R.E., Cepinskas, G., Kvietys, P.R., 1996. Extract of *Helicobacter pylori* induces neutrophils to injury endothelial cells and contains antielastase activity. Gastroenterology 110, 21–29.