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Effect of nitric oxide on apoptotic activity in the rat gastrointestinal tract

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Abstract

The effect of nitric oxide (NO) on apoptosis in the gastrointestinal mucosa was investigated. Experiments involved long-term exposure of rat gastric mucosal cells in vitro to exogenous NO delivered from the NO, donor *S*-nitroso-*N*-acetyl-penicillamine, and the effect of intravenous administration of lipopolysaccharide in vivo, in the presence and absence of the selective inhibitor of inducible NO synthase *N*-(3-(aminomethyl)benzyl) acetamidine (1400 W). *S*-nitroso-*N*-acetyl-penicillamine produced a dose-related inhibition of caspase 3-like activity and DNA fragmentation in isolated gastric mucosal cells. Caspase 3-like activity and DNA fragmentation in gastric, ileal and colonic mucosa were increased both 5 and 24 h after injection of lipopolysaccharide (3 mg/kg, i.v.) to rats in vivo. Administration of 1400 W (5 mg/kg, i.v.) immediately after lipopolysaccharide enhanced caspase 3-like activity and DNA fragmentation above that found with lipopolysaccharide alone. In conclusion, data obtained both in vitro and in vivo suggest that NO exerts an anti-apoptotic effect on rat gastrointestinal mucosal cells. © 2001 Published by Elsevier Science B.V.

Keywords: Nitric oxide (NO); Apoptosis; Gastrointestinal tract; Lipopolysaccharide

1. Introduction

The Ca^{2+}-independent inducible isoform of nitric oxide (NO) synthase (iNOS) is capable of sustained production of NO (Knowles and Moncada, 1994). iNOS can be induced in the gastrointestinal tract of experimental animals by both intravenous and intragastric lipopolysaccharide, including that from *Helicobacter pylori* (Boughton-Smith et al., 1993b; Lamarque et al., 1998; Slomiany et al., 1999). The presence of iNOS in the human colon is associated with inflammatory bowel disease (Boughton-Smith et al., 1993a; Singer et al., 1996), and in the human stomach with infection with *H. pylori* (Mannick et al., 1996). iNOS is found under such circumstances not only in inflammatory cells of the lamina propria (Mannick et al., 1996), but also in epithelial cells (Tepperman et al., 1993; Brown et al., 1994; Lamarque et al., 1998) and in neurons of the muscle layer (Yugai et al., 1997). iNOS increases vascular permeability (Boughton-Smith et al., 1993b) and mucosal barrier dysfunction (Unno et al., 1997) in response to lipopolysaccharide, and mucosal cells isolated from animals treated with lipopolysaccharide exhibit increased permeability to trypan blue, which may be a consequence of the induction of iNOS (Tepperman et al., 1993; Lamarque et al., 1998). Inhibitors of nitric oxide (NO) synthase have been used in the above and other studies to establish such links, but except for highly selective iNOS inhibitors such as *N*-(3-(aminomethyl)benzyl) acetamidine (1400 W) (Garvey et al., 1997; Laszlo and Whittle, 1997), interpretation may be complicated by inhibition of other isoforms of NO synthase (Laszlo and Whittle, 1997; Kubes, 2000).

Apoptosis is a cell death programme, which is characterised by controlled fragmentation of nuclear material and by the specific cleavage of DNA between nucleosomes (Song and Steller, 1999). Proteolytic enzymes called caspases are essential components of the apoptotic pathway (Song and Steller, 1999). NO can potentially induce a stress response (Byrne and Hanson, 1998; Bersimbaev et al., 1999; Yugai et al., 2000), trigger or inhibit apoptosis (Brune et al., 1998), or initiate a proliferative response (Brune et al., 1998), depending on the tissue. The apoptotic response of rat gastrointestinal cells to NO has not been established. In the present work, we have modelled the effect of sustained exposure of gastric mucosal cells to

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exogenous NO by using the NO donor *S*-nitroso-*N*-acetyl-penicillamine, and we have investigated the involvement of NO in apoptosis caused by administration of lipopolysaccharide in vivo by using the selective iNOS inhibitor 1400 W. Apoptotic activity was assessed by the measurement of activation of the late stage effector caspase, caspase 3, and from fragmentation of DNA.

2. Materials and methods

2.1. Animals

Male rats (200–250 g body weight) were obtained from the experimental clinic of the Kazakh State National University, Almaty, Kazakhstan.

2.2. Materials

Phenylmethylsulphonyl fluoride, pepstatin A, leupeptin, soybean trypsin inhibitor, aprotinin, Asp-Glu-Val-Asp-*p*-nitroanilide (DEVD-pNA), Tris, *S*-nitroso-*N*-acetyl-penicillamine, lipopolysaccharide (from *Escherichia coli* serotype 0111:B4), Eagle's minimum essential medium, penicillin, streptomycin and EDTA were obtained from Sigma. Pronase E (95,000 PU units per g) was purchased from Merck, Germany. *N*-(3-(aminomethyl)benzyl) acetamidine (1400 W) was obtained from Alexis Corporation, Laufelfingen, Switzerland. Other reagents were from Sigma.

2.3. Isolation and culture of rat gastric mucosal cells

Dispersed gastric cells were prepared as described previously (Bersimbaev et al., 1985). The stomachs were removed, washed with ice-cold saline, everted into sacs and filled with pronase solution (500 I.U. /ml of medium A). Medium A consisted of: 50 mM Tris–HCl, 0.5 mM NaH_2PO_4, 1 mM Na_2HPO_4, 20 mM $NaHCO_3$, 70 mM NaCl, 80 mM glucose, 2 mM EDTA and 2% bovine serum albumin (bovine serum albumin), pH 7.4. Sacs were incubated in 10 ml of medium A, equilibrated with 95% O_2–5% CO_2, for three 30-min periods at 37 °C with gentle stirring. This medium was replaced with fresh medium A at 30-min intervals. Dispersed cells were filtered through nylon mesh to remove coarse fragments, centrifuged at $100 \times g$ for 10 min, and the pellet suspended in medium B. Medium B had the same composition as medium A, except that it was free of EDTA and bovine serum albumin and contained 1 mM $CaCl_2$ and 1.5 mM $MgCl_2$.

The pooled cells were washed twice in medium B and were finally resuspended under sterile conditions in Eagle's minimal essential medium containing 10% foetal calf serum supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin at 10^6 cells per ml of suspension. Cultures were incubated in a six-well culture plates for 24, 36, 48 and 60 h in an incubator at 37 °C in the presence of 5% CO_2 with *S*-nitroso-*N*-acetyl-penicillamine added daily to appropriate wells to give a concentration of newly added material equal to that shown in the figures (see Kim et al., 1997 for a similar procedure using hepatocytes).

2.4. Experiments in vivo

Animals were anaesthetised with sodium pentobarbitone (60 mg/kg i.p.). Lipopolysaccharide (3 mg per kg of body weight) was injected intravenously. Control animals received sterile saline. The selective inhibitor of iNOS, *N*-(3-(aminomethyl)benzyl) acetamidine (1400 W), (5 mg/kg, i.v.) was injected immediately after administration of lipopolysaccharide.

Stomach, ileum (defined as the lower half of the small intestine) and colon were removed 5 or 24 h later, washed thoroughly with isotonic saline, and the mucosal layer was separated from the muscle by scraping gently with a scalpel.

2.5. Measurement of caspase 3-like activity

Gastric cells or tissue scrapes were immediately frozen in liquid nitrogen. Samples were then freeze-thawed three times in homogenization buffer consisting of: 100 mM HEPES, 140 mM NaCl, 1 mM EDTA, pH 7.4, and containing phenylmethylsulphonyl fluoride 0.43 mM, aprotinin 5 μg/ml, leupeptin 10 μg/ml, and pepstatin 5 μg/ml. After homogenization, the samples were centrifuged at $12,000 \times g$ for 20 min. Aliquots of the resulting cytosolic fraction were incubated with assay buffer containing 100 mM HEPES, pH 7.4, 20% (v/v) glycerol, and phenylmethylsulphonyl fluoride 0.43 mM, aprotinin 5 μg/ml, leupeptin 10 μg/ml, pepstatin 5 μg/ml, and 50 μM DEVD-pNA for 1 h at 37 °C. The caspase 3-like activity was calculated from the change in absorbance at 405 nm and the activity was expressed as pmol of *p*-nitroanilide formed per mg of protein in 1 h.

2.6. Quantification of DNA fragmentation.

Gastric cells or tissue scrapes were resuspended in 10 mM Tris, 1 mM EDTA, pH 8.0 (TE-buffer), and an equal volume of lysis-buffer (5 mM Tris, 20 mM EDTA, pH 8.0, 0.5% Triton X-100) was added. After incubation at 4 °C for 30 min, the intact chromatin (pellet) was separated from DNA fragments (supernatant) by centrifugation for 15 min at $13,000 \times g$. Pellets were resuspended in 500 μl of TE-buffer and all samples were precipitated by adding 500 μl of 10% (w/v) trichloroacetic acid at 4 °C. Samples were subjected to centrifugation at $3000 \times g$ for 10 min, 300 μl of 5% trichloroacetic acid was added to the pellets

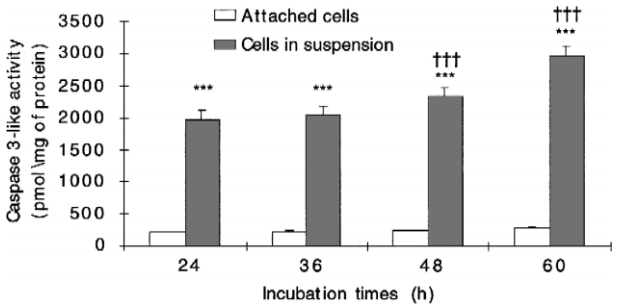

Fig. 1. Effect of attachment to the culture plate and of incubation time on caspase 3-like activity in rat gastric mucosal cells. Results are means ± S.E. from five separate batches of cells and have been analysed by analysis of variance followed by a Newman–Keul's multiple comparison test. $^{***}P < 0.001$ for comparison between attached and detached cells; †††$P < 0.001$ for comparison with appropriate 24-h result.

and samples were boiled for 15 min. DNA contents were quantified using the diphenylamine reagent (Burton, 1956). Fragmentation of DNA was given as the DNA in the supernatant expressed as a percentage of that in the pellet and supernatant.

2.7. Detection of apoptosis using fragment-end labelling of DNA (TUNEL assay)

Stomach, ileum and colon were removed 24 h after administration of agents in vivo and fixed, overnight at room temperature, with freshly prepared and buffered formaldehyde 9% (w/v). Tissues were dehydrated, embedded in paraffin and sections of 5-μm thickness were mounted on poly-L-lysine-treated slides. Sections were de-paraffinised and rehydrated, permeabilised with proteinase K (20 μg/ml) for 20 min, rinsed in Tris-buffered saline (TBS) and then exposed to 3% (v/v) hydrogen peroxide in methanol for 5 min. After preincubation with buffer (200 mM sodium cacodylate, 30 mM Tris, 0.3 mg/ml bovine serum albumin and 0.75 mM CoCl2, pH 6.6) for 20 min, a reaction mixture containing biotinylated deoxynucleoside triphosphates and 15 units of 'Klenow' DNA polymerase was incubated with the slides at 37°C for 1.5 h. The reaction was stopped with 0.5 M EDTA, pH 8.0, the slides were rinsed with TBS, blocked with 4% bovine serum albumin in PBS for 10 min and the presence of biotin

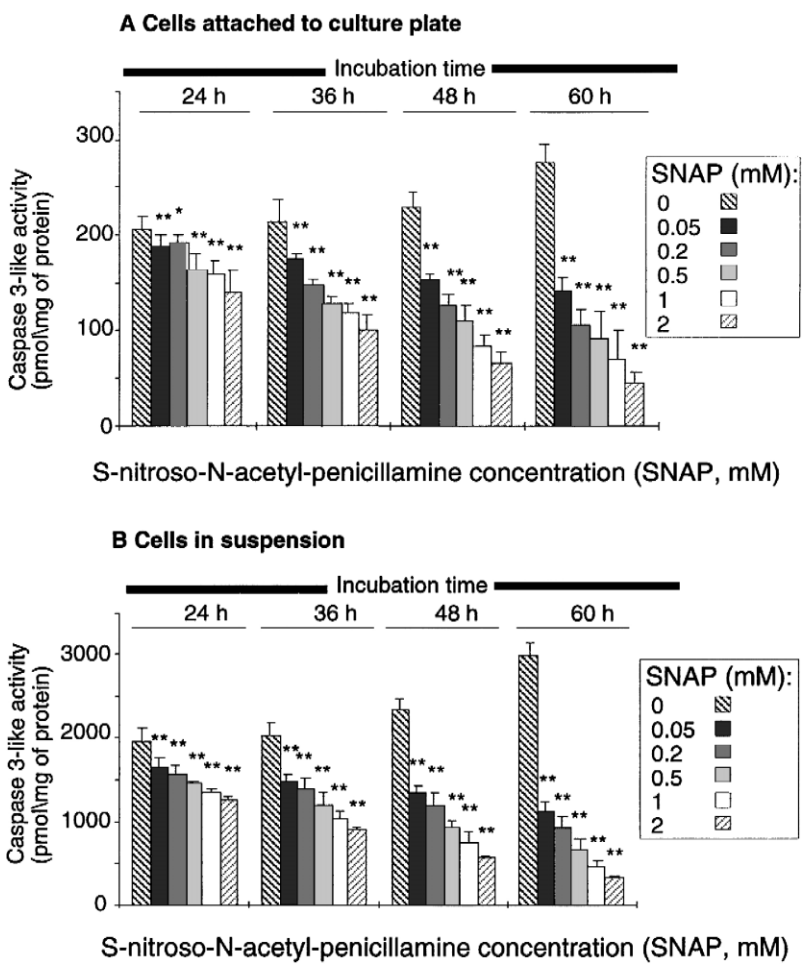

Fig. 2. Effect of concentration of *S*-nitroso-*N*-acetyl-penicillamine on caspase 3-like activity in rat gastric mucosal cells (A), attached to the culture plate and (B), in suspension. Results are means ± S.E. from five separate batches of cells, and for each incubation time have been analysed by analysis of variance followed by Dunnett's test. $^{*}P < 0.05$; $^{**}P < 0.01$ for comparison with the result in the absence of *S*-nitroso-*N*-acetyl-penicillamine.

detected by using a streptavidin–peroxidase conjugate and diaminobenzidine as substrate.

2.8. Statistical analysis

Results are expressed as means ± S.E., with the number of animals or cell batches in parentheses, or given in the figure legends. Statistical comparisons were made by analysis of variance (ANOVAR), followed by a Dunnett's or Newman–Keul's multiple comparison test.

3. Results

3.1. Effect of S-nitroso-N-acetyl-penicillamine on apoptosis in isolated rat gastric mucosal cells

The effect of exogenous NO on apoptosis in primary cultures of rat gastric mucosal cells was investigated by incubating cells for 24, 36, 48 and 60 h at 37 °C with different concentrations (0.05, 0.2, 0.5, 1.0 and 2.0 mM) of *S*-nitroso-*N*-acetyl-penicillamine. Caspase 3-like activity was employed as an indicator of apoptosis. Caspase 3-like activity in attached cells was much lower than in cells, which had not attached to the culture plate (Fig. 1). Caspase activity in detached cells was higher at both 48 and 60 h than at 24 h (Fig. 1) but no significant changes with incubation time were found with cells attached to the culture plate (Fig. 1).

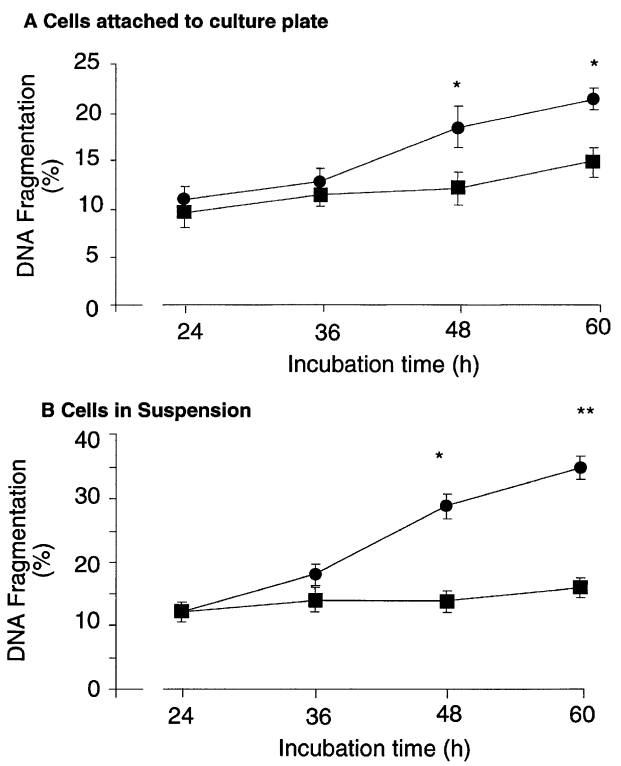

Fig. 3. Effect of *S*-nitroso-*N*-acetyl-penicillamine (2 mM) on DNA fragmentation in rat gastric mucosal cells (A), attached to the culture plate and (B), in suspension. Results are means ± S.E. from five separate batches of cells. For each incubation time, data in the presence (square symbols) and absence (circles) of *S*-nitroso-*N*-acetyl-penicillamine have been compared by a *t*-test: $^{*}P < 0.05$; $^{**}P < 0.01$.

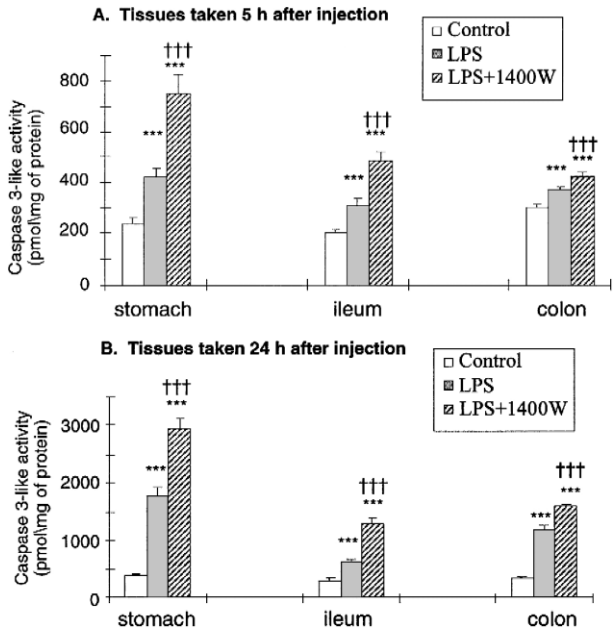

Fig. 4. Effect of intravenous administration of lipopolysaccharide (3 mg/kg), in the presence and absence of the inhibitor of inducible nitric oxide synthase 1400 W (5 mg/kg), on caspase-3 like activity in mucosa from stomach, ileum and colon taken (A) 5 h and (B) 24 h after injection. Results are means ± S.E. from five animals in each case and have been analysed by analysis of variance followed by a Newman–Keul's multiple comparison test. $^{***}P < 0.001$ for comparisons with control; †††$P <$ 0.001 for comparison between lipopolysaccharide and lipopolysaccharide plus 1400 W.

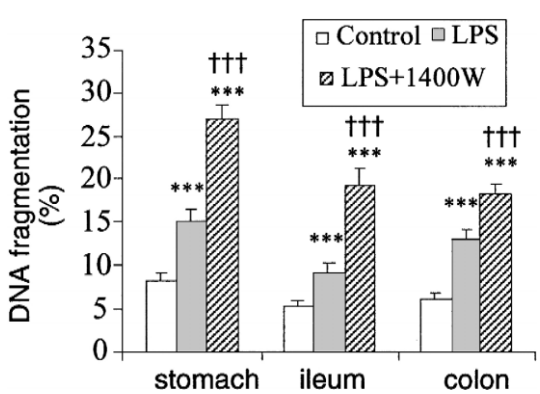

Fig. 5. Effect of intravenous administration of lipopolysaccharide (3 mg/kg), in the presence and absence of the inhibitor of inducible nitric oxide synthase 1400 W (5 mg/kg), on DNA fragmentation in mucosa from stomach, ileum and colon taken 24 h after injection. Results are means ± S.E. from five animals in each case and have been analysed by analysis of variance followed by a Newman–Keul's multiple comparison test. $^{***}P < 0.001$ for comparisons with control; †††$P < 0.001$ for comparison between lipopolysaccharide and lipopolysaccharide plus 1400 W.

At all incubation times, *S*-nitroso-*N*-acetyl-penicillamine exerted a dose-dependent inhibition of caspase 3-like activity in both attached and detached cells (Fig. 2A,B). The inhibitory effect of 2 mM *S*-nitroso-*N*-acetyl-penicillamine increased with incubation time. Thus, inhibition of caspase activity by 2.0 mM of *S*-nitroso-*N*-acetyl-penicillamine in attached cells after 24 h was $32 \pm 4\%$, and after 60 h $83 \pm 2\%$ ($P < 0.001$, $n = 5$). In detached cells, inhibition by 2.0 mM of *S*-nitroso-*N*-acetyl-penicillamine after 24 h was $36 \pm 4\%$ and after 60 h $89 \pm 1\%$ ($P < 0.002$, $n = 5$).

To confirm that changes in caspase activity reflected apoptosis, DNA fragmentation, as evidenced by the presence of low molecular weight DNA in the cytosol, was determined. *S*-nitroso-*N*-acetyl-penicillamine (2 mM) reduced DNA fragmentation compared with control in both attached cells and in those in suspension after 48 and 60 h of incubation (Fig. 3A,B).

3.2. Effect administration of lipopolysaccharide in vivo on caspase activity in rat gastric, ileal and colonic mucosa

Lipopolysaccharide (3 mg per kg of body weight, i.v.) elevated caspase 3-like activity 5 h after administration in gastric, ileal and colonic mucosa by $82 \pm 5\%$ (5), $53 \pm 2\%$ (5) and $23 \pm 3\%$ (5), respectively (Fig. 4A). Twenty-four hours after lipopolysaccharide pretreatment, caspase 3-like activity was further elevated by $357 \pm 24\%$ (5) in stomach, by $109 \pm 10\%$ (5) in ileum and by $261 \pm 24\%$ (5) in colon (Fig. 4B).

3.3. Effect of the selective iNOS inhibitor, 1400 W, on the lipopolysaccharide-induced elevation of caspase activity in vivo

The selective iNOS inhibitor, 1400 W administered to rats at the same time as lipopolysaccharide (Laszlo and

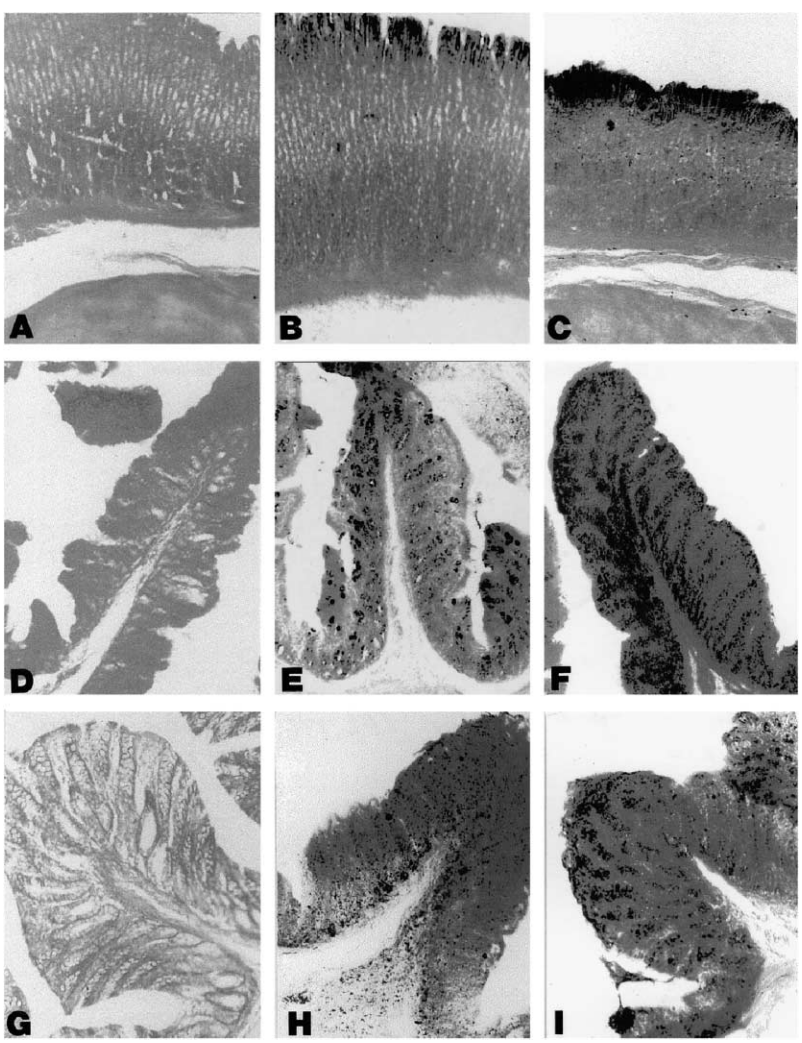

Fig. 6. Effect of intravenous administration of lipopolysaccharide (3 mg/kg) in the presence (C, F, I) and absence (B, E, H) of the inhibitor of inducible nitric oxide synthase 1400 W (5 mg/kg), on DNA strand breaks (TUNEL assay) in mucosa from stomach (A–C), ileum (D–F) and colon (G–I) taken 24 h after injection. (A, D, and G) are controls.

Whittle, 1997), raised caspase 3-like activity in gastric, ileal and colonic mucosa significantly above that found in animals that received lipopolysaccharide alone. Thus, 5 h after administration of 1400 W with lipopolysaccharide caspase like activity was 214 ± 4% (5), 140 ± 1% (5), and 40 ± 2% (5) above control in stomach, ileum and colon, respectively (Fig 4A). After 24 h, the corresponding increases were: 658 ± 30% (5), 314 ± 19% (5) and 382 ± 30% (5) (Fig. 4B).

3.4. Effect of lipopolysaccharide and 1400 W in vivo on DNA fragmentation

Results obtained from measurements of DNA fragmentation reflected the changes seen in caspase activity (Section 3.3), namely, the increases seen with lipopolysaccharide were even greater if lipopolysaccharide was administered with 1400 W (Fig. 5).

3.5. Effect of lipopolysaccharide and 1400 W in vivo on apoptosis detected by fragment-end labelling of DNA (TUNEL assay)

Lipopolysaccharide increased the number of cells exhibiting a positive result in the TUNEL assay in stomach, ileum and colon. In the stomach, the predominant site of labelling was the surface epithelial cells, also known as pit cells, while in the ileum and colon labelling was prominent in the lamina propria. Co-administration of 1400 W with lipopolysaccharide enhanced staining at the above sites in all three regions of the gastrointestinal tract with some epithelial cells becoming positive in ileum and colon (Fig. 6).

4. Discussion

The main finding of this work is that NO inhibited apoptosis in rat gastrointestinal cells both in vitro and in vivo. This result is based on three different measures of apoptotic activity. Proteases belonging to the caspase family are involved in coordinating the apoptotic process (Song and Steller, 1999). After apoptosis is triggered, caspases are activated by the cleavage of proenzymes into subunits that rearrange to form the active cysteine proteases. Initiator caspases transduce and augment the apoptotic signal by activation of other executor caspases. The substrate used in the present work measures primarily the activity of executor caspases like caspase 3. A key feature of apoptosis is chromatin condensation and the specific cleavage of double-stranded DNA, which involves the caspase-dependent removal of an inhibitory subunit from a DNAase and its subsequent transfer to the nucleus (Wilson, 1998). In the present work, DNA fragmentation was assessed by an established procedure (Messmer et al., 1998), which is based on the appearance of low molecular weight DNA in the cytosol, and by the labelling of the ends of fragmented DNA with biotinylated nucleotides (TUNEL assay).

There was reasonable agreement between the two methods which were used for cells incubated in vitro. Thus, in the absence of *S*-nitroso-*N*-acetyl-penicillamine, both caspase activity and DNA fragmentation (after 48 h) were higher in cells that were in suspension than in those attached to the culture plate. This effect of detachment on apoptosis in epithelial cells is a consequence of an absence of integrin signalling and is termed anoikis (Frisch and Francis, 1994). Furthermore, both methods demonstrated an inhibitory effect of *S*-nitroso-*N*-acetyl-penicillamine on attached cells and those in suspension. In experiments, in vivo changes in caspase activity paralleled changes in DNA fragmentation assessed either by assay for low molecular weight cytosolic DNA or by fragment end-labelling.

In other experiments, incubation of rat gastric mucosal cells in vitro with NO donors increased cell damage (reduced exclusion of trypan blue, Tripp and Tepperman, 1996). However, Byrne and Hanson (1998) did not find damage associated with incubation of NO donors with guinea pig gastric mucous cells. The presence of serum used here and by Byrne and Hanson (1998), but not by Tripp and Tepperman (1996) may alter responsiveness of cells to NO. Alternatively, it is conceivable that NO might switch cells from an apoptotic to a necrotic death pathway.

The mechanism by which lipopolysaccharide induces apoptosis in vivo was not established in the present work, but release of tumour necrosis factor-α (TNF-α) from inflammatory cells could be a possible contributor. Certainly, TNF-α is implicated in the gastrointestinal apoptosis induced by non-steroidal antiinflammatory drugs (Fiorucci et al., 1999). Experiments with 1400 W in vivo suggest that it exhibits excellent selectivity for iNOS (Garvey et al., 1997; Laszlo and Whittle, 1997; Wray et al., 1998), and it therefore seems reasonable to conclude that the increased apoptosis seen in this work when 1400 W is administered with lipopolysaccharide stems from the removal of an inhibitory effect of NO derived from induction of iNOS. Effects of 1400 W on apoptotic activity in response to lipopolysaccharide were seen 24 h after administration. 1400 W is either an irreversible, or extremely slowly reversible, inhibitor of iNOS with a K_d value of ≤ 7 nM (Garvey et al., 1997). 1400 W may therefore exert a prolonged inhibition of iNOS activity even after its elimination from the blood. Alternatively, inhibition of iNOS might only be required in the early stages of the lipopolysaccharide response for exacerbation of subsequent apoptotic activity to occur.

In the liver, a selective inhibitor of iNOS, L-N^G-(1-iminoethyl) lysine) produced similar results by increasing apoptosis when administered with lipopolysaccharide (Ou et al., 1997). Moreover, pharmacological treatment with an NO donor can protect mice from lipopolysaccharide-

induced liver injury (Bohlinger et al., 1995). Finally, exogenous NO inhibits apoptosis in the HT 29 colon cell line (Madesh et al., 1999).

NO could inhibit apoptosis by inhibition of caspase activity as has been demonstrated with hepatocytes (Kim et al., 1997) gastric chief cells (Fiorucci et al., 1999) and gastric mucous cells (Potter and Hanson, 2000). Alternatively, effects of NO in vivo could be more indirect and involve modulation of cytokine production (Fiorucci et al., 2000). Lipopolysaccharide induces iNOS activity in gastric (Brown et al., 1994) and small intestinal (Tepperman et al., 1993) mucosal cells and increases nitrite production by mucosa throughout the gastrointestinal tract (Yugai, 2001). Consequently, the inhibitory effect of 1400 W in vivo could be exerted locally at the gastrointestinal cells. Furthermore, the finding that NO donors can directly inhibit apoptosis in gastric cells in vitro suggests that at least part of the effect of 1400 W in vivo may be to prevent a direct anti-apoptotic effect of NO on gastric cells. Finally, in the stomach, the predominant cell-type in which apoptosis was increased after administration of lipopolysaccharide, in vivo, and further enhanced by 1400 W, was clearly the surface mucous epithelial cell. Lipopolysaccharide increased iNOS in such cells (Brown et al., 1994), which again supports the contention that local inhibition of iNOS may be enhancing apoptosis.

In conclusion, while iNOS seems implicated in damage to the gastrointestinal tract induced by lipopolysaccharide (Boughton-Smith et al., 1993b; Unno et al., 1997; Lamarque et al., 1998), the present results suggest that NO from iNOS may serve to reduce cell loss through apoptosis under such circumstances, and support the contention that not all effects of iNOS are necessarily deleterious (Kubes, 2000).

Acknowledgements

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