Biochemical and Biophysical Research Communications **261,** 15–20 (1999)
Article ID bbrc.1999.1003, available online at http://www.idealibrary.com on IDEAL®

Gastric Mucosal Inflammatory Responses to *Helicobacter pylori* Lipopolysaccharide: Down-regulation of Nitric Oxide Synthase-2 and Caspase-3 by Sulglycotide

Bronislaw L. Slomiany, Jerzy Piotrowski, and Amalia Slomiany
Research Center, University of Medicine and Dentistry of New Jersey, Newark, New Jersey 07103-2400

Received June 16, 1999

We applied the animal model of *H. pylori* lipopolysaccharide-induced gastritis to assess the effect of antiulcer agent, sulglycotide, on the mucosal inflammatory responses by analyzing the interplay between the activity of a key apoptotic caspase, caspase-3, epithelial cell apoptosis, and the expression of constitutive (cNOS) and inducible (NOS-2) nitric oxide synthase. *H. pylori* lipopolysaccharide applied intragastrically elicited within 4 days a pattern of mucosal responses resembling that of acute gastritis. This was accompanied by an 11.2-fold increase in epithelial cell apoptosis, a 6.5-fold induction in mucosal expression of NOS-2 and a 2.2-fold decline in cNOS, and a 5.4-fold increase in caspase-3 activity. Treatment with sulglycotide led to a 56.7% reduction in the extent of mucosal inflammatory changes elicited by *H. pylori* lipopolysaccharide and an 88.3% decrease in the epithelial cells apoptosis. Furthermore, this effect of sulglycotide was associated with a 51% decrease in mucosal expression of caspase-3 activity, a 73.7% decline in NOS-2, and a 64.1% increase in cNOS. The findings suggest that sulglycotide suppresses the *H. pylori*-induced mucosal inflammatory responses by up-regulating cNOS and interfering with the events propagated by NOS-2 and caspase-3. © 1999 Academic Press

***Key Words: Helicobacter pylori;* lipopolysaccharide; acute gastritis; cNOS; NOS-2; caspase-3; sulglycotide.**

Infection with *Helicobacter pylori* is a primary factor in the etiology of gastric disease, and the relationship between the bacterium and gastric mucosal inflammatory changes that characterize gastritis and duodenal ulcers is well established (1–4). The product of particular significance to the virulent action of *H. pylori* is its cell wall lipopolysaccharide (5–8). The pathogenic effects of *H. pylori* lipopolysaccharide are manifested by a marked up-regulation in gastric mucosal proinflammatory cytokine expression, excessive nitric oxide generation, repression of regulatory cytokine production, and abrogation of the processes associated with cell cycle progression and cellular proliferation (5, 8–10).

Other mucosal responses elicited by the lipopolysaccharide involve stimulation transcriptional factor NFκB nuclear translocation, and the disturbances in nitric oxide synthase (NOS) activity responsible for NO generation (11–15). Indeed, the sustained activation of NOS-2 has been identified as a culprit of transcriptional disturbances leading to apoptosis (14–17, 19), while the cNOS appears to play an active role in the inhibition of apoptogenic signals through S-nitrosylation of cysteine residue on the catalytic site of the major executioner caspase, caspase-3.

Induction of apoptosis by lipopolysaccharide of Gram-negative bacteria is mediated by cytokines of the TNF family and involves the transmembrane type 1 receptor, TNFR1 (12, 13). The ligand binding induces trimerization of the receptor molecules, eliciting recruitment to its cytosolic face of several death domain-containing adaptor proteins that interact with caspase zymogens causing their autocatalytic activation (17, 18). The activation of caspases is recognized as an irreversible commitment to the execution phase of apoptosis characterized by cytoplasmic shrinkage, cleavage of cytoskeletal and nuclear proteins, and DNA fragmentation (17, 18). An extensive apoptotic DNA fragmentation and up-regulation of proinflammatory cytokine expression are also characteristic feature of gastric mucosa exposed to *H. pylori* lipopolysaccharide and in patients with *H. pylori*-associated gastritis (6, 8, 20).

Using animal model of *H. pylori* lipopolysaccharide-induced gastritis (6, 8), in this study, we assessed the effect of gastroprotective agent, sulglycotide, on gastric mucosal inflammatory responses to *H. pylori* lipopolysaccharide by analyzing the interplay between activity of a key executioner caspase, caspase-3, apoptotic DNA

0006-291X/99 $30.00

fragmentation, and the expression of cNOS and NOS-2.

MATERIALS AND METHODS

Animals. The study was conducted with Sprague-Dawley rats weighing 180 to 200 g, and cared for by the professional personnel of the Research Animal Facility of UMDNJ. The animals were maintained on a regular chow diet, and deprived of food 24 h before the experimentation. Water was withheld for 2 h before the procedure. All experiments were conducted with groups of eight animals per treatment. The animals received twice daily for 3 consecutive days the intragastric pretreatment with either sulglycotide at 200 mg/kg or the vehicle, and were then subjected to intragastric surface epithelial application of *H. pylori* lipopolysaccharide at 50 μg per animal, and maintained on the drug or vehicle regimen for an additional 4 days (10). The rats in each group were killed 16 h after the last treatment and their stomachs dissected.

Mucosal histology. The sections of gastric mucosa were cut into 4-μm strips, fixed in 10% buffered formalin, and stained with hematoxylin and eosin (6). The morphological pattern of gastritis was graded in accordance with the Sydney system (21), and the changes in mucosal histology were quantified on the basis of the scoring system of Rauws *et al.* (22) as detailed earlier (10).

Apoptosis assay. Measurements of apoptosis was carried out with epithelial cells prepared from gastric mucosal scrapings (10). The cells were incubated in the lysis buffer in accordance with the manufacturer's (Boehringer Mannheim) instruction, centrifuged, and the diluted supernatant containing the cytoplasmic histone-associated DNA fragments reacted in the microtitrator wells with immobilized anti-histone antibody. The retained complex was reacted with anti-DNA peroxidase, and probed with ABTS reagent for spectrophotometric quantification (10).

cNOS and NOS-2 activity assay. Nitric oxide synthase activities of gastric mucosa was measured using a NOS-detect kit (Stratagene). The individual specimens of gastric mucosa were homogenized in a sample buffer containing either 10 mM EDTA (NOS-2) or 6 mM $CaCl_2$ (cNOS), and centrifuged at 800 $\times$ g for 10 min (8). The aliquots of the resulting supernatants were incubated for 30 min at 25°C in the presence of 50 μCi/μl L-[2,3,4,5-^{3}H]arginine, 10 mM NADPH, 5 μM tetrahydrobiopterin, and 50 μM Tris-HCl buffer, pH 7.4, in a final volume of 250 μl. The reaction was terminated by adding to each sample a 0.4 ml of stop buffer followed by 0.1 ml of Dowex-50W (Na^+) resin. The mixtures were transferred to spin cups, centrifuged, and the formed L-[^{3}H]citrulline contained in the flow through was quantitated by scintillation counting (8).

Caspase-3 activity assay. Caspase-3 activity measurements were carried out with gastric epithelial cells using a Quanti Zyme assay system (Biomol Res. Lab., Inc.). The epithelial cells, prepared from gastric mucosal scrapings (15), were incubated at 4°C with the lysis buffer according to the manufacturer's instruction, and the lysates were centrifuged at 10,000 $\times$ g for 10 min. The aliquots of the resulting cytosolic fraction were incubated in the microtitrator wells with 50 μM of DEVD-pNA (Asp-Glu-Val-Asp-p-nitroanilide) substrate for 1 h at 37°C, and the caspase-3 activity measured spectrophotometrically (8).

H. pylori lipopolysaccharide. H. pylori ATCC No. 4350 clinical isolate was used for lipopolysaccharide preparation (6). The cells were washed with water, treated with ethanol and acetone, dried and homogenized with liquid phenol-chloroform-petroleum ether (23). The resulting suspension was centrifuged, and the lipopolysaccharide contained in the supernatant was precipitated with water, washed with 80% phenol solution, and dried with ether. The dry residue was dissolved in a small volume of water at 45°C, centrifuged at 10,000 $\times$ g for 4 h, and the resulting lipopolysaccharide sediment subjected to lyophilization (6).

Data analysis. All experiments were carried out in duplicate, and the results are expressed as the means $\pm$ SD. The significance level was set at $p < 0.05$. The tests were performed using Soft Stat, STATISTICA, software. The protein content of samples was measured with BCA protein assay kit (Pierce), and the antiulcer agent, sulglycotide, was kindly donated by Crinos Industria, Italy.

RESULTS

The effect of antiulcer agent, sulglycotide, on the course of events associated with gastric mucosal inflammatory reaction to *H. pylori* infection was assessed in the animal model, using rats subjected to intragastric surface epithelial application of *H. pylori* lipopolysaccharide (6). The results of histologic examination revealed that the lipopolysaccharide at 50 μg per animal produced within 4 days a pattern of inflammatory responses characterized by the infiltration of lamina propria with lymphocytes and plasma cells, edema, hyperemia, and epithelial hemorrhage extending from the lamina propria to the surface of the mucosa. Treatment with gastroprotective agent, sulglycotide, led to a marked reduction in the severity pattern of mucosal inflammatory changes caused by *H. pylori* lipopolysaccharide. In the absence of the treatment, the mean grade of the mucosal pathologic condition caused by *H. pylori* lipoplysaccharide was 5.3, while that of the animals treated with sulglycotide showed a 56.7% (mean score 2.3) reduction in the severity of changes (Fig. 1).

The results of apoptotic DNA fragmentation assays conducted with the epithelial cells isolated from gastric mucosa during *H. pylori* lipopolysaccharide-induced inflammatory reaction are summarized in Fig. 1. The data obtained revealed that comparing to the controls, the animals subjected to *H. pylori* lipopolysaccharide application produced an 11.2-fold increase in gastric epithelial cells DNA fragmentation. On the other hand, the group treated with sulglycotide showed an 88.3% reduction in the extent of epithelial cell apoptosis

The data on the expression of gastric mucosal activity of cNOS and NOS-2 during *H. pylori* lipopolysaccharide-induced mucosal inflammatory reaction are shown in Fig. 2. In the absence of antiulcer agents, the lipopolysaccharide evoked a 6.5-fold increase in gastric epithelial expression of NOS-2 activity over that of the controls, while the activity of cNOS showed a 54.2% decrease. Treatment with sulglycotide reduced the lipopolysaccharide-induced increase in NOS-2 activity level by a 73.7%, wile the activity of cNOS increased by a 64.1%.

The expression of gastric mucosal caspase-3 activity in response to surface epithelial application of *H. pylori* lipopolysaccharide in the absence and the presence of treatment with sulglycotide is presented in Fig. 3. The assays established the mean value for caspase-3 activ-

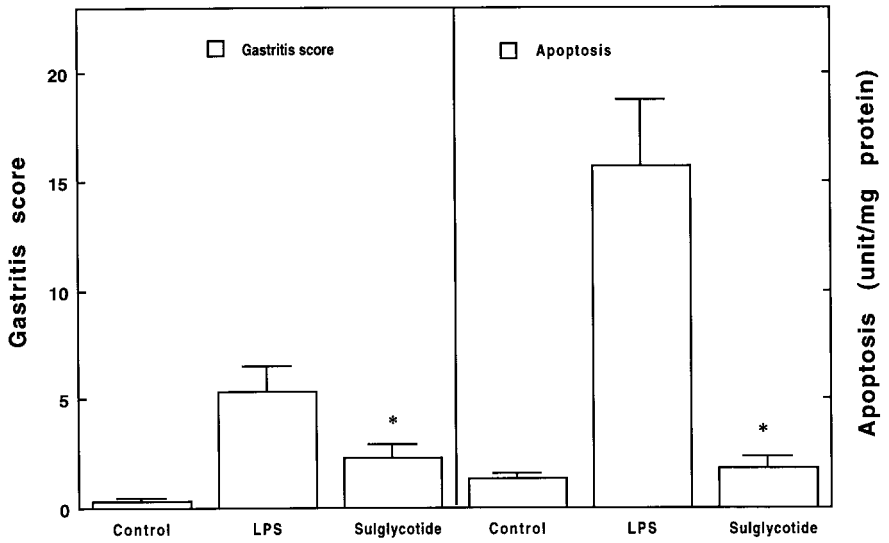

FIG. 1. Effect of treatment with sulglycotide on scores of acute gastritis and gastric epithelial cell apoptosis in rats subjected to intragastric surface epithelial application of *H. pylori* lipopolysaccharide (LPS). Values represent the means ± SD of duplicate analyses performed on 8 animals in each group.

ity in the controls at 3.4 pmol/mg protein, while that in gastric epithelial cells following lipopolysaccharide application reached the mean value of 18.3 pmol/mg protein. Treatment with sulglycotide caused a 51% decline in gastric epithelial level of caspase-3 activity.

DISCUSSION

Sulglycotide is a potent gastroprotective antiulcer agent, derived from pig duodenal mucin by proteolysis and the chemical esterification of its carbohydrate chains with sulfate groups, recognized for its anti-*H. pylori* effects (24–26). The agent shows the ability to interfere with *H. pylori* mucosal attachment, exhibits a potent inhibitory effect on the protease, lipase and urease enzymes elaborated by the bacterium, and shows the ability to reverse the impairment caused by *H. pylori* lipopolysaccharide in feedback inhibition of gastrin release by somatostatin (7, 23, 25, 26). As the primary virulence factor of *H. pylori* that elicits muco-

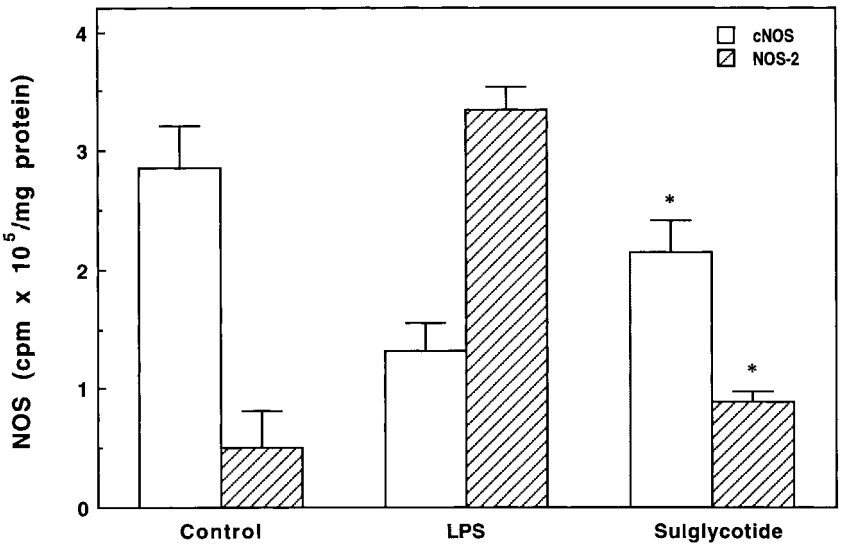

FIG. 2. Effect of treatment with sulglycotide on the expression of constitutive (cNOS) and inducible (NOS-2) nitric oxide synthase activities in gastric mucosa of the rats subjected to intragastric surface epithelial application of *H. pylori* lipopolysaccharide (LPS). Values represent the means ± SD of duplicate analyses performed on 8 animals in each group.

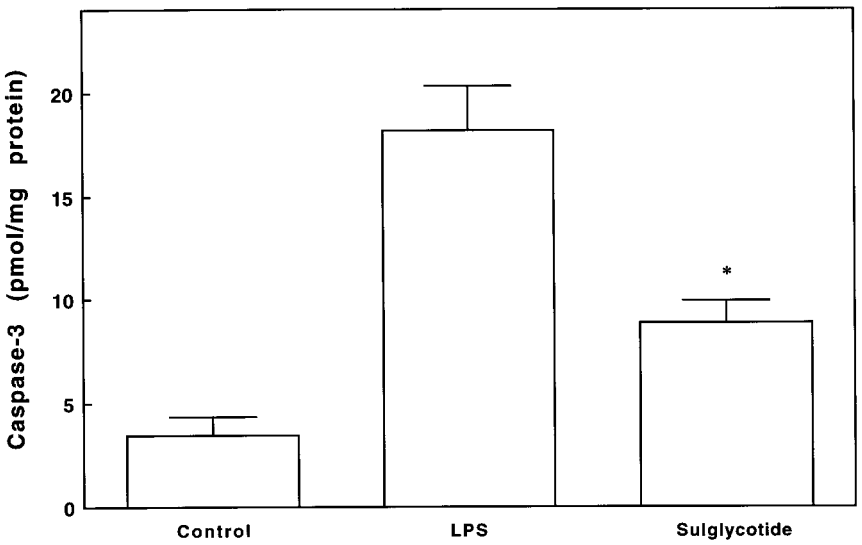

FIG. 3. Effect of treatment with sulglycotide on the expression of caspase-3 activity in gastric epithelial cells of the rats subjected to intragastric surface epithelial application of *H. pylori* lipopolysaccharide (LPS). Values represent the means ± SD of duplicate analyses performed on 8 animals in each group.

sal inflammatory responses that characterize gastritis is its cell wall lipopolysaccharide (6, 10), in this study, we investigated the effect of sulglycotide on the interplay between the extent of mucosal pathology, epithelial cell apoptosis, activity of a key executioner caspase, caspase-3, and the mucosal expression of cNOS and NOS-2.

The results revealed that gastric mucosal inflammatory responses to *H. pylori* lipopolysaccharide, in the absence of sulglycotide treatment, show a pattern resembling that of acute gastritis (6), and are accompanied by an 11.2-fold enhancement in epithelial cell apoptosis, 5.4-fold increase in caspase-3 activity, a 54.2% reduction in cNOS, and a 6.5-fold induction in mucosal expression of NOS-2. Treatment with sulglycotide elicited a 56.7% reduction in the extent of mucosal inflammatory changes caused by *H. pylori* lipopolysaccharide, and this effect of sulglycotide was reflected in an 88.3% decrease in epithelial cell apoptosis, a 64.1% increased in mucosal expression of cNOS activity, a 73.7% decline in NOS-2, and a 51% reduction in caspase-3 activity. These findings, together with the earlier results indicating that *H. pylori* as well as its cell wall lipopolysaccharide cause up-regulation of TNF-α and other proinflammatory cytokine expression (10, 27, 28), point toward the *H. pylori* lipopolysaccharide as a trigger initiating gastric mucosal inflammatory events that lead to caspase cascade activation and apoptotic death.

The caspase family of cysteine proteases consists of 12 members, of which caspases 8, 9 and 3 are situated at pivotal junctions in apoptosis pathways (17, 29). The implementation of the apoptotic program requires the participation of two classes of caspases, the initiator caspases with long N-terminal prodomains (caspases-8, -9, and -10) which activate the executioner caspases with short N-terminal prodomains (caspases-3, -6, and -7), that in turn cleave the targeted intracellular substrates. The activation of caspase-8, positioned to respond to extracellular apoptosis-inducing ligands, requires association with FADD (Fas-associated death domain) through the death effector domain, and this adaptor-mediated oligomerization brings procaspases molecules into close proximity to allow autocatalytic activation (17, 18, 30). On the other hand, caspase-9 is positioned at the apex of in the apoptotic signaling cascade activated by the release of cytochrome *c*. The activation of procaspase-9 occurs in response to the alteration in mitochondrial membrane permeability and the escape of cytochrome c from mitochondria, which, along with dATP, binds to APAF-1 (apoptotic protease activating factor-1), inducing it oligomerization and association through the CARD (caspase recruitment domain) with procaspase-9 (29–31).

Thus activated initiator caspases 8 and 9 then activate the pivotal executioner caspase, caspase-3, which lacks the long N-terminal prodomain required for the recruitment of APAF-1 complex (18, 29–31). The importance of caspase-3 to the apoptotic process is underscored by a recent finding that this caspase liberates a DNase termed CAD (caspase-activating DNase) from an inhibitor of CAD (ICAD) by cleaving the ICAD protein, thus leading to DNA degradation (29). The fact that gastric mucosal responses to *H. pylori* lipopolysaccharide were reflected by a marked increase (5.4-fold) in caspse-3 activity supports the notion that the apoptotic events elicited in mucosa by the lipopolysaccha-

ride display a pattern of apoptogenic signal propagation consistent with that of TNFR1 engagement.

Since the executioner caspases, including caspase-3, remain under the regulatory control of nitric oxide (11–14), it was of interest to examine the effect of *H. pylori* lipopolysaccharide on the interplay between the gastric mucosal caspase-3 activity and the expression of cNOS and NOS-2. The results revealed that the mucosal inflammatory responses to *H. pylori* lipopolysaccharide associated with up-regulation of caspase-3 activity were manifested in a massive induction in NOS-2 and a decline in cNOS, while the reduction in the extent of inflammatory changes achieved with sulglycotide treatment was also reflected in a decline in the mucosal expression of NOS-2, and up-regulation in cNOS expression. It is noteworthy that the enhanced expression of NOS-2 results in the formation of NO-related species which exert a direct inhibitory effect on NFkB causing transcriptional disturbances that lead to apoptosis, while the cNOS plays an active role in the inhibition of apoptogenic signals through S-nitrosylation of cysteine residue on the catalytic site of caspase-3 (29). Moreover, cNOS appears to be involved in the inhibition of the caspase enzymes through a cGMP mechanism, associated with phosphorylation-dephosphorylation events, that function on the level of caspase zymogen activation that requires cleavage adjacent to aspartates (12, 13). Thus, the observed suppression in cNOS and the induction of NOS-2 activity by *H. pylori* lipopolysaccharide may be an important factor in *H. pylori* cytotoxicity associated with mucosal inflammatory conditions that characterize gastritis in patients infected with this bacterium (2, 4).

While the exact molecular mechanism of the lipopolysaccharide-mediated events remains obscure, the available data point toward essential role of proteins of the NFκB family consisting of c-Rel, NFκB1, NFκB2, Rel A, and Rel B, and forming a variety of homo- and hetrodimers (12, 13, 32, 33). These proteins have been shown to be essential for the induction of NOS-2 gene expression in macrophages exposed to E. coli lipopolysaccharide as well as in astrocytes responding to challenge by lipopolysaccharide from S. typhimurium (12, 34). In the absence of challenge, NFκB exists in the cytoplasm in inactivated form through association with the inhibitory proteins, IκB (12, 13, 35). Following activation, the IκB proteins undergo phosphorylation and are selectively degraded, leading to the release of NFκB, its nuclear translocation, and binding to the affinity site in the promoters of inducible genes to activate the expression of NOS-2 and nitric oxide release (12, 34). The termination of NFκB activity occurs upon IκB resynthesis, which leads to dissociation of DNA-bound NFκB and its sequestration from the nuclear compartment and redistribution back into the cytoplasm (35).

The results of our study suggest that sulglycotide is capable to modulate the extent of mucosal inflammatory responses to *H. pylori* infection by countering the effects of the *H. pylori* lipopolysaccharide-mediated NFκB signaling pathway. These effects of sulglycotide are manifested in the interference with the events propagated by NOS-2 and caspase-3, and up-regulation of cNOS activity.

REFERENCES

1. Hamlet, A., Thoreson, A. C., Nilsson, O., Svennerholm, A. M., and Olbe, L. (1999) *Gastroenterolology* **116,** 259–268.
2. Stolte, M., and Edit, S. (1996) *Scand. J. Gastroenterol.* **31**(Suppl 214), 13–16.
3. Rune, S.J. (1996) *Scand. J. Gastroenterol.* **31**(Suppl 214), 2–4.
4. El-Omar, E. M., Oien, K., El-Nujumi, A., Gillen, D., Wirz, A., Dahill, S., Williams, C., Ardill, J. E. S., and McColl, K. E. L. (1997) *Gastroenterology* **113,** 15–24.
5. Slomiany, B. L., Piotrowski, J., and Slomiany, A. (1997) *Arzniem. Forsch. Drug Res.* **47,** 475–482.
6. Piotrowski, J., Piotrowski, E., Skrodzka, D., Slomiany, A., and Slomiany B. L. (1997) *Scand. J. Gastroenterol.* **32,** 203–211.
7. Piotrowski, J. (1998) *J. Physiol. Pharmac.* **49,** 3–24.
8. Slomiany, B. L., Piotrowski, J., and Slomiany, A. (1998) *Biochem. Mol. Biol. Int.* **46,** 1063–1070.
9. Brzozowski, T., Konturek, P. C., Sliwowski, Z., Drozdowicz, D., Pajdo, R., Stachura, J., Hahn, E. G., and Konturek, S. J. (1997) *J. Physiol. Pharmac.* **48,** 699–717.
10. Slomiany, B. L., Piotrowski, J., and Slomiany, A. (1999) *Gen. Pharmac.* **30,** 434–442.
11. Pahan, K., Sheikh, F. G., Khan, M., Namboodiri, A. M. S., and Singh, I. (1998) *J. Biol. Chem.* **273,** 2591–2600.
12. Chen, C. C., Wang, J. K., Chen, W. C., and Lin, S. B. (1998) *J. Biol. Chem.* **273,** 19424–19430.
13. Choi, K. B., Wong, F., Harlan, J. H., Chaudhary, P. M., Hood, L., and Karsan, A. (1998) *J. Biol. Chem.* **273,** 20185–20188.
14. Mannick, J. B., Miao, Y. Q., and Stamler, J. S. (1997) *J. Biol. Chem.* **272,** 24125–24128.
15. Pahan, K., Raymond, J. R., and Singh, I. (1998) *J. Biol. Chem.* **274,** 7528–7536.
16. Mustafa, S. B., and Olson, M. S. (1998) *J. Biol. Chem.* **273,** 5073–5080.
17. Salvasen, G. S., and Dixit, V. M. (1997) *Cell* **91,** 443–446.
18. Thornberry, N. A., and Lazebnik, Y. (1998) *Science* **281,** 1312–1316.
19. Sekkai, D., Aillet, F., Israel, N., and Lepoivre, M. (1998) *J. Biol. Chem.* **273,** 3895–3900.
20. Moss, S. F., Calam, J., Agarawal, B., Wang, S., and Holt, P. R. (1996) *Gut* **38,** 498–501.
21. Price, A. B. (1991) *J. Gastroenterol. Hepatol.* **6,** 209–222.
22. Rauws, E. A. J., Langenberg, W., Houthoff, H., Zanen, M. C., and Tytgat, G. N. J. (1988) *Gastroenterology* **94,** 33–40.
23. Piotrowski, J., Skrodzka, D., Slomiany, A., and Slomiany, B. L. (1997) *Gen. Pharmac.* **28,** 705–708.
24. Psilogenis, M., Alberico, P., Bianchi, G., and Nazzari, M. (1991) *in* Drugs in Gastroenterology (Braga, P. D., Ed.), pp. 232–244, Raven Press, New York.
25. Slomiany, B. L., Murty, V. L. N., Piotrowski, J., and Slomiany, A. (1994) *Gen. Pharmac.* **25,** 833–841.

26. Slomiany, B. L., Piotrowski, J., and Slomiany, A. (1997) *Biochem. Mol. Biol. Int.* **42,** 155–161.
27. Kim, C. Y., Jung, H. C., Kim, J. M., Song, I. S., and Kim, C. Y. (1998) *Scand. J. Gastroenterol.* **33,** 1249–1255.
28. Moss, S. F., Legon, S., Davies, J., and Calam, J. (1994) *Gut* **35,** 1567–1570.
29. Seol, D. W., and Billiar, T. R. (1999) *J. Biol. Chem.* **274,** 2072–2076.
30. Kumar, S., and Colussi, P. A. (1999) *TIBS* **24,** 1–4.
31. Green, D. R., and Reed, J. C. (1998) *Science* **281,** 1309–1312.
32. Cheshire, J. L., Williams, B. R. G., and Baldwin, A. S. (1999) *J. Biol. Chem.* **274,** 4801–4806.
33. O'Connell, M. A., Bennett, B. L., Mercurio, F., Manning, A. M., and Mackman, N. (1998) *J. Biol. Chem.* **273,** 30410–30414.
34. Feinstein, D. L., Galea, E., Aquino, D. A., Li, G. C., Xu, H., and Reis, D. J. (1996) *J. Biol. Chem.* **271,** 17724–17732.
35. Han, Y., Meng, T., Murray, N. R., Fields, A. P., and Brasier, A. R. (1999) *J. Biol. Chem.* **274,** 939–947.