Dose-dependent enhancement or inhibition of mucin synthesis in isolated colonic mucous cells provoked by nitric oxide

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— Article —

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Abstract   Background/Aims: We investigated relation the dual effects (cytoprotection and cytopathy) of nitric oxide and mucin synthesis on the colonic mucosal defense using isolated guinea pig colonic cells.

Methods: The dualproperty of nitric oxide was shown by MTT assay and DNA fragmentation assay using two nitric oxide-generating drugs (NOC-12 and SNAP). Mucin biosynthesis was measured by ^{3}H-glucosamin uptake after addition various concentrations of NOC-12 and SNAP, cGMP analogue or cGMP inhibitor.

Results: At low concentrations of NOC-12 or SNAP, no cytopathy occurred, and the mucin biosynthesis was increased. Mucin biosynthesis was also increased by the addition of cGMP analogue, while mucin biosynthesis diminished when the cGMP concentration was decreased by a specific guanylate cyclase inhibitor. In contrast, at high concentrations of NOC-12 or SNAP, cytopathy including apoptosis occurred and mucin biosynthesis activity significantly decreased.

Conclusion: Nitric oxide plays a concentration-dependent role in the regulation of colonic mucin synthesis: low concentrations of nitric oxide cause cytoprotection by an increased mucin synthesis, while high nitric oxide doses are cytopathic by a decreased mucin synthesis. These opposite action of nitric oxide on mucin synthesis may give a better understanding why nitric oxide has dual properties to wards colonic mucosal defense.

Key words — colonic mucin, mucin synthesis, colonic mucosal defense, isolated colonic mucous cell, nitric oxide

Introduction

Mucin, a high-molecular-weight glycoprotein, is the principal component of the mucus that protects the colonic mucosal membrane from bacterial invasion and irritation by various substances. Mucosal defense mechanisms in the colon have not been fully elucidated, but the mucosal microcirculation and secretion of mucin are believed to be important, as they are in the stomach [1-4]. Nitric oxide (NO), a regulator of various physiologic functions [5], has a protective effect on the mucous membrane of the digestive tract [6-10], but also has been implicated in development of mucosal injury in ulcerative colitis and Crohn's disease [11-16]. Complex networks of various other interacting factors that are involved in

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mucosal defenses, including the microcirculatory, autonomic, and endocrine responses, tend to obscure the mechanisms of NO action in vivo. We therefore used primary cultures of isolated colonic mucosal cells where factors affecting defenses and injury in the mucosa can be studied individually to investigate direct effects of NO on cytopathy and mucin biosynthesis.

Materials and Methods

1) Isolation and NO exposure of colonic mucosal cells.

As previously described [17], the colonic mucosa of a male guinea pig was removed, scraped, and minced into small pieces. Preparations were digested twice at 37 ℃ for 20 minutes in Hank's balanced salt solution (HBSS; GIBCO, Grand Island, NY) containing 0.15 mg/ml of collagenase (Sigma Chemical, St. Louis, MO) and 3 mg/ml of Dispase (Godo-Shusei, Tokyo, Japan) in a water bath with agitation. After centrifugation, the pellet was reincubated under the same conditions in HBSS containing 1 mM EDTA (Sigma) for 5 minutes. The final cell suspension was placed in the wells of a 24-well tissue culture plate in a mixture of RPMI1640 tissue culture medium (GIBCO) with 10% heat-inactivated fetal bovine serum (FBS; Sigma) and antibiotics including 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml fungizone (GIBCO). The cells obtained from colonic mucosa were suspended in 40% Percoll (Pharmacia, Uppsala, Sweden). After centrifugation at 1,500 rpm for 20 minutes, three layers were separated and each of these fractions was collected. Cell viability was evaluated by exclusion of 0.4% trypan blue (Sigma). Hematoxylin and eosin (H and E), periodic acid-Schiff (PAS), and cytokeratin (CAM5.2; Becton Dickinson, San Jose, CA) stains were performed for each cell fraction immediately after separation to identify with mucous cells, and check the contamination of polymorphonuclear leukocytes and macrophages. We used two NO-generating drugs; 1-hydroxy-2-oxo-3-(N-ethyl-3-aminoethyl)-3-ethyl-1-triazene (NOC-12; Wako, Tokyo, Japan) and S-Nitroso-N-acetylpenicillamine (SNAP; BIOMOL Research Laboratories, Polymouth Meeting, PA). Cells were incubated with various concentrations of NOC-12(10^{-7} to 10^{-3} M) or SNAP (10^{-5} to 10^{-3} M). Details of this incubation are described with the individual analysis below.

The experiments were approved by the Animal Research Committee of Osaka Medical College, and the animals were cared for according to the guidelines of this committee.

2) Cytopathy according to MTT assay.

To each well in 96-well microplates, 10^4 cells in 200 μl of RPMI1640 containing NOC-12 or SNAP at various concentrations (10^{-7} to 10^{-3} M) were added and cultured at 37 ℃ for 24 hours. To 100 μl of the supernatant, 3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyl tetrozolium bromide solution (MTT, Sigma) was added to result in a final MTT concentration of 25 mg/dl, and supernatants were incubated for 4 hours at 37 ℃. After adding 0.04 N HCl and isopropanol, absorbance was measured at 590 nm using EIA autoreader (Model 2550; BIO-RAD, Hercules, CA).

3) DNA fragmentation assay.

After adding 10^{-6} M 5-bromodeoxyuridine (BrdU) to 2x10^5 isolated cells/ml, the cells were cultured at 37 ℃ for 12 hours. To 10^5 BrdU-labeled cells/ml, each concentration of NOC-12 (10^{-7} to 10^{-3} M) was added for a 24-hour incubation at 37 ℃. Fragmented DNA then was measured by an enzyme-linked immunosorbent assay (ELISA; Boerringer Mannheim, Germany). Optical density was measured at a wavelength of 370 nm using an EIA autoreader.

4) Measurement of mucin biosynthesis.

This procedure also has been described previously [17,18]. Briefly, 1 μ Ci of ^{3}H-glucosamine was added to the media of cells in culture that then were incubated

at 37℃ for 1 hour, followed by addition of NOC-12 (10^{-7} to 10^{-3} M) or SNAP (10^{-5} to 10^{-3} M), the cGMP analogue dibutyryl cGMP (DBcGMP, 5 x 10^{-4} M and 10^{-3} M; Sigma), an inhibitor of soluble guanylate cyclase 1H-1,2,4-oxadiazole-4,3-a-quinoxaline-1-one (ODQ, 10^{-6} M; Sigma), or phosphate-buffered saline (PBS; control). Cultures then were incubated for an additional 24 hours. Media and cells were removed together, sonicated, and ultracentrifugated. To the supernatant a mixture of 2% phosphotungustic acid (PTA)/20% trichloroacetic acid (TCA) was added, and [^{3}H] activity was counted using a liquid scintillation counter. The [^{3}H] activity in test cultures was expressed as a ratio (%) relative to activity in control (PBS) cultures as a measure of mucin biosynthesis.

5) Measurement of intracellular cGMP.

After adding 5 x 10^{-5} M dipyridamole alone, 5 x 10^{-5} M dipyridamole was added together with 10^{-6} M NOC-12 and/or 10^{-6} M ODQ, and cultures were incubated for 30 minutes. Then 70% ethanol was added to stop the intracellular reaction, and the supernatant was collected. After concentrating and drying this supernatant in a nitrogen evaporator, the residue was dissolved in 1 ml of distilled water and measured using a cGMP enzyme immunoassay (EIA) kit (Amersham Japan, Tokyo, Japan).

6) Glycoconjugate expression according enzyme-linked lectin assay.

This procedure has been described previously [17]. Briefly, 10^{-6} M NOC-12 was added to cell cultures which then were incubated for 3 hours. After cells were separated, sonicated, and ultracentrifugated, the supernatant was obtained. After transferring 200μl of a supernatant into each of 96 wells of an ELISA microplate and allowing plates to stand overnight at 37℃, 200μl of a solution of each of the individual biotinylated lectins adjusted to 400 ng/ml were added and allowed to react according to the avidin-biotin-peroxidase complex method (Vectastain

ABC kit; Vector Laboratories, Burlingamme, CA). Optical density was measured at a wavelength of 405 nm using an EIA autoreader. Four lectins were used in this study; the sugar residue-binding sites were galactose or Gal β 1, 3 Gal NAc for peanut agglutinin (PNA); fucose for Ulex europeus agglutinin-1 (UEA1); GalNAc at Dorichos biflorus agglutinin (DBA); and GlcNAc for wheat germ agglutinin (WGA).

7) Statistical analysis.

Results from five animals (four samples/animal) were expressed as mean ± standard error. A probability level of 0.05 or less was considered significant. The difference between groups was evaluated by using a one-way ANOVA. If ANOVA indicated a statistically significant difference, a multiple post hoc test was performed to determine the significance between the means.

Results

1) Isolated colonic mucosal cells.

Isolated cells were separated into three layers by the Percoll density gradient method. Cells in the middle layer showed immunostaining for cytokeratin; approximately 70% of these cells also showed PAS staining. Cells in this layer were identified with mucous cells. None of neutrophiles showed in this layer. Viability of these cells was approximately 90% immediately after separation and 80% after 24 hours in culture. Dead cells and a small number of epithelial cells were present in the first (upper) layer, and blood cell components and a small number of epithelial cells were observed in the third (lower) layer.

2) MTT assay.

Expressed as percentages relative to unexposed control group values (0.775±0.029), MTT assay results after 24 hours of incubation with 10^{-7} M, 10^{-6} M, and 10^{-5} M NOC-12 were 109.9±9.6%, 115.9±10.9%, and 101.2±3.2%, respectively (no significant

differences and thus no cytopathic effect). In contrast, 10^{-4} M and 10^{-3} M of NOC-12, resulted in significantly decreased values, expressed relative to control data as $92.2 \pm 1.7\%$ and $58.6 \pm 10.5\%$, indicating cytopathy (Fig. 1).
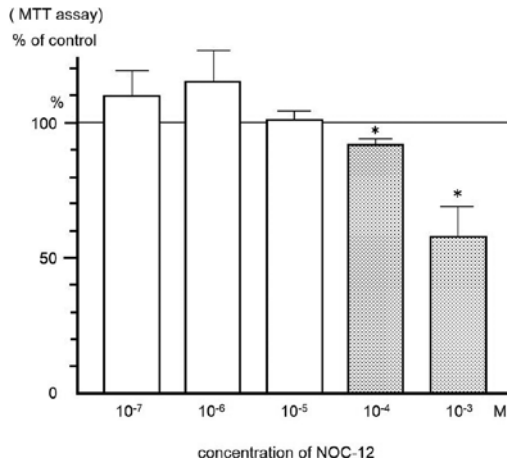
Figure 1. Cytopathy according to an MTT assay in cultured colonic mucosal cells after addition of various concentrations of a nitric oxide generators (NOC-12): No significant differences were seen in values after 24 hours of incubation with 10^{-7} M ($109.9\pm9.6\%$), 10^{-6} M ($115.9\pm10.9\%$), and 10^{-5} M ($101.2\pm3.2\%$) of NOC-12, no cytopathic effects were seen in low concentrations of NOC-12. In contrast, values of 10^{-4} M ($92.2\pm1.7\%$) and 10^{-3} M ($58.6\pm10.5\%$) NOC-12 were significantly decreased, indicating cytopathy in the control group. Values are expressed relation to those in cultures without the generators. * P<0.05, n=20.

3) DNA fragmentation assay.

Cellular DNA fragmentation rates 24 hours after NOC-12 addition again expressed as a percentage of control values (0.314 ± 0.028), were $119.8 \pm 11.5\%$ at 10^{-7} M, $138.5 \pm 14.3\%$ at 10^{-6} M, and $151.5 \pm 18.9\%$ at 10^{-5} M (no significant differences from controls). In contrast, at high concentrations of 10^{-4} M ($183.2 \pm 23.9\%$) and 10^{-3} M ($261.9 \pm 39.9\%$), the DNA fragmentation rate was significantly increased (Fig. 2).
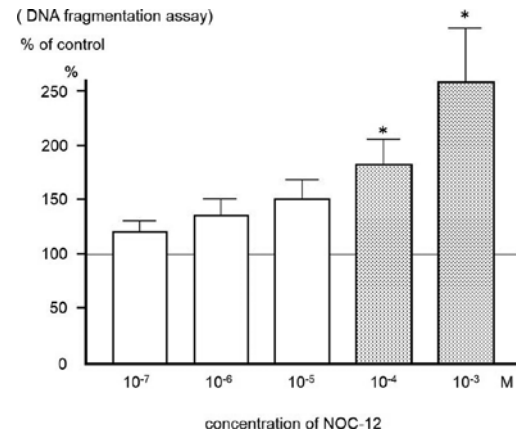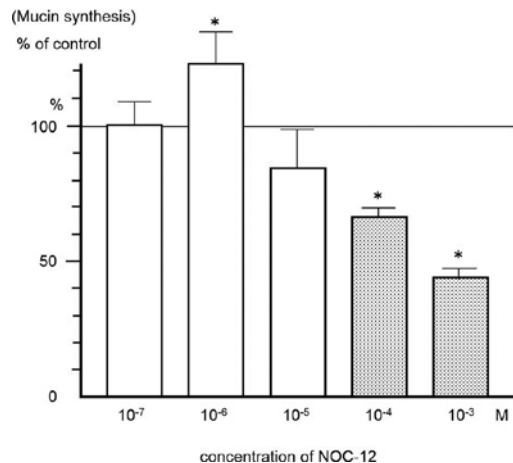

Figure 2. DNA fragmentation related to apoptosis in cultured colonic mucosal cells after addition of various concentrations of a nitric oxide generators (NOC-12): Values 24 hours after NOC-12 addition showed a concentration-dependent increase at 10^{-7} M ($119.8\pm11.5\%$), 10^{-6} M ($138.5\pm14.3\%$), and 10^{-5} M ($151.5\pm18.9\%$), especially a significant increase at 10^{-4} M ($183.2\pm23.9\%$) and 10^{-3} M ($261.9\pm39.9\%$) in the control group. Values are expressed as percentages relative to unexposed control group values. * P<0.05, n=20.

4) Mucin biosynthesis.

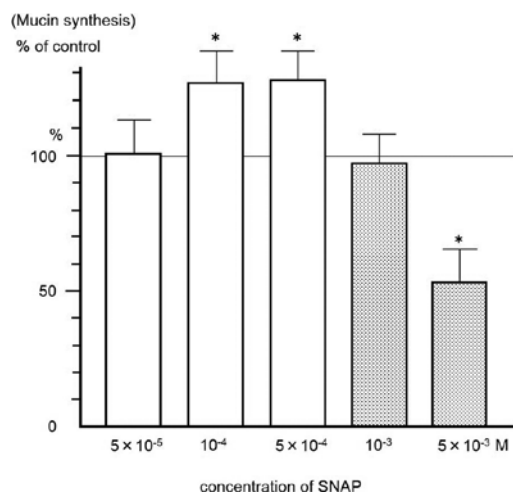Mucin biosynthetic activity (^{3}H-glucosamin uptake) 24 hours after addition 10^{-6} M of NOC-12 was $122.0 \pm 11.4\%$ of control activity ($1,958 \pm 230$ cpm), showing a significant increase beyond synthesis in the control group, while no significant differences were seen at 10^{-7} M ($101.2 \pm 8.2\%$) or 10^{-5} M ($84.2 \pm 12.2\%$). In contrast, at 10^{-4} M ($66.0 \pm 3.2\%$) and 10^{-3} M ($44.0 \pm 4.2\%$), mucin biosynthetic activity showed a significant, concentration-dependent decrease relative to synthesis in the control group (Fig.3).

Products of NOC-12 formed upon release of NO during an incubation without cells with NOC-12 at concentrations (10^{-7} to 10^{-3} M) at 37℃ for 24 hours were obtained and added to cultured cells under the same conditions. When mucin biosynthetic activity was measured, the products caused no significant difference from control activity at any concentration.

Mucin biosynthetic activity after addition 5 x 10^{-5} M, 10^{-4} M, 5 x 10^{-4} M, 10^{-3} M, and 5 x 10^{-3} M of SNAP were $102.5 \pm 15.6\%$, $126.8 \pm 13.0\%$, $127.0 \pm 12.0\%$, $98.1 \pm 11.5\%$, and $52.0 \pm 12.4\%$, respectively, showing a significant increase in 10^{-4} M and 5 x 10^{-4} M of SNAP beyond synthesis in the control group (Fig.4).

Figure 3. Mucin synthesis in cultured colonic mucosal cells after addition of various concentrations of a nitric oxide generators (NOC-12). Mucin biosynthetic activity 24 hours after addition 10^{-6} M of NOC-12 was 122.0±11.4%, showing a significant increase. In contrast, at 10^{-5} M (84.2±12.2%), 10^{-4} M (66.0±3.2%) and 10^{-3} M (44.0±4.2%), mucin biosynthetic activity showed a significant, concentration-dependent decrease. Values are expressed relative to those in cultures without the generators, as percent of control. * P<0.05, n=20.


Figure 4. Mucin synthesis in cultured colonic mucosal cells after addition of various concentrations of a nitric oxide generators (SNAP). Mucin biosynthetic activity after addition SNAP were showing a significant increase in 10^{-4} M (126.8±13.0%) and 5 x 10^{-4} M(127.0±12.0%), a significant decrease in 5 x 10^{-3} M (52.0±12.4%) of SNAP. Values are expressed as a ratio (percentage) relative to those in unexposed control cultures. * P<0.05, n=20.

5) Intracellular cGMP.

When cells were treated with 5 x 10^{-5} M of dipyridamole alone, the cGMP concentration was 3.7 ± 0.3 pmol/well. When both 5 x 10^{-5} M dipyridamole and 10^{-6} M NOC-12 were added to wells, the cGMP

concentration significantly increased to 5.9 ± 1.2 pmol/well. The guanylate cyclase inhibitor, ODQ, at 10^{-6} M decreased to 2.4 ± 0.5 pmol/well of cGMP concentration, and prevented increases in cGMP concentration (2.6 ± 0.8) in response to 10^{-6} M NOC-12 (Fig.5).
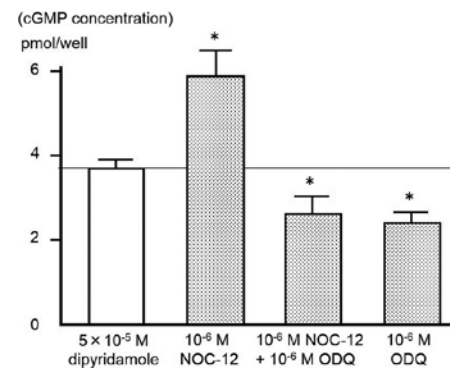
Figure 5. Intracellular cyclic GMP concentration in cultured colonic mucosal cells under various conditions. The cGMP concentration treated with 5 x 10^{-5} M of dipyridamole alone 6was 3.7±0.3 pmol/well. The cGMP value significantly increased to 5.9±1.2 pmol/well adding both 5 x 10^{-5} M dipyridamole and 10^{-6} M NOC-12. 10^{-6} M ODQ decreased to 2.4±0.5 pmol/well of cGMP concentration, and prevented increases in cGMP concentration (2.6±0.8) in response to 10^{-6} M NOC-12. NOC-12 : a nitric oxide generators; ODQ : a guanylate cyclase inhibitors. * P<0.05, n=20.

6) Effect of a cGMP analogue and a guanylate cyclase inhibitor on mucin biosynthesis.

Mucin biosynthetic activity of cells treated with 5 x 10^{-4} M and 10^{-3} M DBcGMP was significantly increased (116.3 ± 5.4% and 120.9 ± 9.7% of control activity; 1,833 ± 196 cpm, respectively). When 10^{-6} M ODQ alone was added instead, mucin biosynthetic activity was 91.2 ± 6.1% of control synthesis and 93.6 ± 8.7% in response to 10^{-6} M NOC-12 (Fig.6).
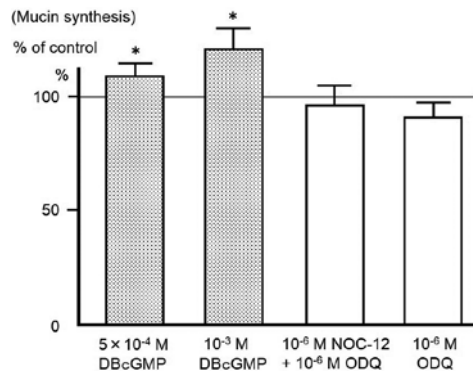
Figure 6. Mucin synthesis in cultured colonic mucosal cells after addition of DBcGMP, NOC-12, and ODQ. Mucin biosynthetic activity of cells treated with 5×10^{-4} M and 10^{-3} M DBcGMP was significantly increased (116.3±5.4% and 120.9 ±9.7%). Mucin biosynthetic activity was 91.2±6.1% at 10^{-6} M ODQ alone and 93.6±8.7% in response to 10^{-6} M NOC-12 with 10^{-6} M ODQ. DBcGMP : a cGMP analogue; NOC-12 : a nitric oxide generators; ODQ : a guanilate cyclse inhibitors. $* P<0.05$, n=20.

7) Intracellular glycoconjugate expression after NOC-12 stimulation.

Lectin binding activity for PNA, UEA1, DBA, and WGA in normal colonic mucin of control group was 0.018 ± 0.004, 0.036 ± 0.003, 0.328 ± 0.053, and 0.564 ± 0.063, respectively. The ratio of PNA lectin-binding mucin in cells after addition of 10^{-6} M NOC-12 to that in the control group was $101.6 \pm 12.1\%$. The ratios of UEA1, DBA, and WGA binding mucin were $92.8 \pm 5.2\%$, $95.5 \pm 7.3\%$, and $106.0 \pm 6.0\%$, respectively. None of these represented significant differences from binding by control cells. In contrast, while mucin was decreased in cells treated with 10^{-3} M NOC-12, the relative amount of PNA-binding mucin was significantly greater then in control cells ($121.3 \pm 11.6\%$).

Discussion

At the low NOC-12 concentrations that did not show a cytopathic effect by DNA fragmentation assay, mucin biosynthesis tended to increase; at the higher concentrations that show cytopathy including apoptosis by a decreased, mucin biosynthesis significantly and concentration-dependently. The small amount of NO generated by a low concentration of NOC-12 would be protective because of increased mucin biosynthesis. However, the cytopathy induced at high concentrations, apparently caused reduction in mucin biosynthesis. In normal colonic mucosa inducible NO synthesis (iNOS) is not expressed, but iNOS is expressed strongly in inflammatory cells such as macrophages and polymorphonuclear leukocytes that infiltrate tissues in ulcerative colitis and Crohn's disease [19,20]. The large amount of NO generated by this iNOS decreases mucin biosynthesis by cells, attenuating the mucous barrier overlying the epithelial surface and thus exacerbating mucosal injury. While metabolites of NOC-12 apart from NO were not measured in our study, they were shown not to affect mucin biosynthesis in the present experiments. NO may impair ribonucleotide reductase required for DNA synthesis or activate apoptosis-inducing genes by activating IL-1β conversion-inhibiting enzyme-like protease and NF-κB [21,22]. Within the mitochondria, aconitase and the enzymes of the electron transfer system are considered to be inhibited by reaction with NO, which would impair ATP synthesis, including cytopathy [23]. Further investigation is necessary.

NO diffuses through the cell membrane to act directly on target molecules within cells. Guanylate cyclase, which produced cGMP, is considered a representative target molecule [9,24]. NO/cGMP protects endothelial cells from damage in the pulmonary artery [25]. In the stomach NO has been shown to facilitate mucin secretion via cGMP [6,17,26]. In colonic mucoepithelial cells, NO has been found to be involved in electrolyte transport and mucin secretion [27-30]. The effects of NO-generating drugs eliciated mucin exocytotic response through a cGMP-dependent and a cGMP-independent pathway, respectively, in human colonic mucus-secreting cell line [31]. The cGMP level was increased by NO-donor (10^{-4} M SNAP), decreased by specific guanylate cyclase inhibitor (10^{-6} M ODQ) in rat colonic single smooth muscle cells [32]. When our

cell cultures were treated with 10^{-6} M NOC-12 or 10^{-4} M SNAP, mucin biosynthesis was significantly increased and the intracellular cGMP concentration also was elevated. Furthermore, mucin biosynthetic activity was significantly increased by treatment with a membrane-permeable cGMP analogue, while mucin biosynthesis diminished when the cGMP concentration was decreased by 10^{-6} M ODQ. These findings suggest that mucin biosynthesis elevation by NO at low concentrations involves a cascade mechanism including intracellular cGMP. NO/cGMP is considered to have opposing effects, cytoprotection by an increased mucin biosynthesis and cytopathy by a decreased mucin biosynthesis, on the colonic mucous cells.

Not only the amount but also the composition of mucin is altered in colon cancer and inflammatory intestinal diseases [33,34]. Glycoprotein, the principal component of mucin, is an o-glycosylated core protein bearing five types of carbohydrate residues at the sugar chain terminals. While mucin synthesis was increased by 10^{-6} M NOC-12, the carbohydrate structures of mucin glycoproteins by enzyme-linked lectin assay were not altered. However, treatment with 10^{-4} M NOC-12 not only decreased mucin synthesis but also significantly increased the relative amount of PNA-binding mucin. PNA lectin binds specifically to Gal β 1, 3 Gal NAc in sugar residues in glycoproteins, showing affinity for sugar residues adjacent to sialic acid. While PNA-binding mucin is not observed in normal colonic mucosa, binding is intense in colonic specimens with cancer, premalignant lesions, or chronic inflammation [34]. Similar decreases in mucin synthesis and relative increases in PNA-binding mucin have been found in gastric mucosal injury induced by ethanol or nonsteroidal anti-inflammatory drugs. PNA binding mucin including sialic acid is high viscosity, has protective effect of epithelial mucosa.

In a cytopathic state reducing the mucin biosynthetic activity, viscous mucus containing PNA-binding mucin shows a compensatory relative increase that tends to protect the colonic mucosa. Both quantity and quality of mucin appear to be important in the mucosal protective and injurious effects of NO. Our findings of cytoprotection and cytopathic effects by NO might give useful speculation for the future therapy such as free radical scavenger for ulcerative colitis.

Acknowledgments

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