# Modulation of Small Intestinal Nitric Oxide Synthase by Gum Arabic

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**Preceding studies have revealed that gum arabic (GA), a natural proteoglycan (≥250,000 Da), has proabsorptive properties—as shown by increased sodium and water absorption—in normal rats, and especially in two animal models of diarrhea. Because nitric oxide (NO) metabolism is linked to gastrointestinal physiology, the goals of this study were to determine whether GA modulated NO and to determine intestinal function *in vivo* when NO production was enhanced by L-arginine (Arg), added at either 1 or 20 m*M*. Mechanistically, the goal was also to determine whether GA was a NO scavenger and a small intestinal NO synthase (NOS) inhibitor. Using a glucose–electrolyte solution in rat jejunal perfusions we found that GA at ±10 μ*M* (2.5 g/l) decreased nitrite and nitrate formation, tending to normalize water, sodium, and glucose absorption when modified by Arg addition. *In vitro* tests, with oxyhemoglobin as a marker, showed that GA at ≥5 μ*M* scavenged NO. For GA effects on NOS, small intestinal homogenate supernatants (10,000 *g*) from frozen tissues of either adult or 2-day-old rats were incubated for 1 hour at 37°C in the presence of 2 m*M* Arg and increasing GA concentrations (0–100 μ*M*). GA produced a concentration-dependent inhibition of NOS, reaching approximately 31% inhibition with 5 μ*M* GA and up to 51% with 50 μ*M* GA. GA at 100 μ*M* produced no further inhibition. The data indicate that GA, in addition to its ability to remove NO diffused into the intestinal lumen, may also partially inhibit intestinal NOS and thus modulate intestinal absorption through these mechanisms. Use of GA as a food additive may help in restoring or improving small intestinal function in conditions where functional damage has occurred.** Exp Biol Med 229:895–901, 2004

**Key words:** gum arabic; nitric oxide; intestinal absorption; nitric oxide synthase

The small intestine is the major site of nutrient and electrolyte absorption in the gastrointestinal tract, a function regulated by multiple mechanisms at the cellular and molecular levels (1). We have previously focused our attention on various types of soluble fiber as potential modifiers of the absorptive process. Prominent among the fibers was gum arabic (GA), a nonviscous, negatively charged natural product with moderate emulsifying properties (2, 3). Chemically, GA is a large-molecular weight proteoglycan complex containing pentoses, hexoses, and uronic acids (4). Preceding studies have revealed that the physiological properties of GA as a proabsorptive agent are demonstrable by changes of several parameters, including increased sodium uptake (5, 6) in normal rats and water and sodium absorption in two animal models of intestinal dysfunction (7), as well as a facilitation of recovery from diarrhea induced by cathartic agents (8). Furthermore, GA reduced chloride and sodium secretion in rats perfused under anesthesia with cholera toxin (9). Additional work indicated that GA enhanced diffusive mechanisms but had no effect on sodium-dependent carriers (10). Nitric oxide (NO) metabolism plays a critical role in the regulation of intestinal function (11–13). NO is a gas that also has the properties of a free radical and that is generated from L-arginine (Arg) by NO synthase (NOS), an enzyme with several isoforms (14, 15). In rat intestine, NOS I (neuronal NOS; nNOS) predominates and is located in the myenteric plexus (16, 17). NOS III (endothelial NOS; eNOS) is largely in endothelial and smooth muscle cells of blood vessels. Both are considered the constitutive (cNOS) isoforms and maintain unaltered activity (18). A third inducible form (iNOS, NOS II) is activated by cytokines and other signaling mechanisms. We have earlier shown dose-dependent effects of Arg on intestinal absorption and secretion (19). NO alters intestinal muscular contractility and motility, as well as water and electrolyte absorption and secretion through modulation of mucosal cGMP and cAMP levels (20). Earlier work indicated that the efficacy of GA might derive, at least in part, from regulation of NO-dependent gating of the basolateral membrane potassium channel (21). However, given that GA is essentially not

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absorbed at all or is degraded in the upper gastrointestinal tract, the possible linkage between the presence of GA and physiologic effects might be exerted via a purely physical mechanism, such as NO gas adsorption or scavenging as it diffuses into the lumen from its site of synthesis in the enterocyte. The persistence of NO intracellularly and in a complex milieu such as that found in the intestinal lumen is of the order of seconds (15), as it is rapidly oxidized to nitrite and, subsequently, to nitrate. However, the apical mucosa sites where NO may be generated by NOS might also be allosterically modulated by a macromolecule such as GA. The objectives of the study were to determine whether the capability of GA to modify absorption was effected by regulating NO concentration *in situ* and *in vivo*, acting as a NO scavenger or as a NOS inhibitor, under conditions of moderate or high rate of NO production. This was achieved by varying Arg concentration. In addition, we applied *in vitro* techniques to evaluate possible physicochemical modes of GA action.

## Materials and Methods

**Overview.** Three techniques were used in the study: intestinal perfusions to evaluate the capacity of GA as an absorption modulator under conditions of moderate or increased NO synthesis, assessment of GA ability to scavenge NO chemically produced *in vitro*, and valuation of GA as a possible NOS inhibitor.

**Intestinal Perfusions.** The jejunal perfusion procedure has been described in previous studies (7, 22). Briefly, male, 60- to 80-g Sprague-Dawley rats (Taconic, Inc., Germantown, NY), acclimatized in the animal facility for at least 48 hours, were fasted overnight and anesthetized with urethane (1.3 g/kg, intraperitoneally). An intestinal segment between 20 and 30 cm long immediately distal to the ligament of Treitz was cannulated, rinsed with saline, and perfused via a peristaltic pump (Model 1203, Harvard Instruments, Holliston, MA) for 2.5 hours at 10–12 ml/hour with solutions containing 90 m*M* sodium chloride, 111 m*M* glucose, 10 m*M* trisodium citrate, and either a low or high concentration of added Arg (1 m*M* or 20 m*M*, respectively). Both types of solutions contained either 0 or 2.5 g/l (±10 μ*M*) of GA. The controls were correspondingly either GA-free or had 2.5 g/l GA. Effluents of the first hour of perfusion were discarded while the system reached steady state. Afterward, five 15-minute fractions were collected for analysis, as described below. Results for the 15-minute fractions collected for every animal were averaged to subsequently calculate fluid and solute transport rates for each treatment. At the end of the perfusion, the rats were euthanized by exsanguination and the perfused segment was severed, weighed fluid-free, and measured stretched with a 3 g weight. If not otherwise indicated, chemicals were purchased from Sigma Chemical Corp. (St. Louis, MO). Tritiated water (2 μCi/l = 74 kBq/l; New England Nuclear, Boston, MA) was added to all solutions to quantify water influx based on the disappearance of the label.

Net water absorption was obtained from weight differences per unit of time between solutions entering and leaving the perfused intestinal segment. Water efflux, or secretion, was calculated by difference between influx and net water absorption. Sodium was assayed by atomic absorption spectrophotometry (SpectrAA 10; Varian Instruments, Sunnyvale, CA). Beta emission of tritium was quantitated with a β-scintillation counter (Tri-Carb 1900TR, Packard Instrument Co., Meriden, CT) calibrated with external standards. Nitrite and nitrate were assayed together, as the stability of the nitrite as the initial oxidation product of NO is questionable and is rapidly converted to nitrate (23). The analytical test was carried out following reduction of nitrate to nitrite with copperized cadmium. A nitrate standard (100 μ*M*) was similarly treated. An aliquot of the effluent perfusate, diluted if necessary, was mixed with Griess reagent and the color developed read at 540 nm in a spectrophotometer (Spectronic model 21D, Milton Roy, Rochester, NY). Nitrite concentrations were calculated from a regression curve of standards. Absorption rates were expressed as n*M* (or μl)/minute × cm. Figures and tables present the data as mean ± SEM. The protocols were approved by the institutional animal care and utilization committee.

***In vitro* NO Scavenging Test.** The ability of GA to alter the electron donor capacity of NO was tested *in vitro* using a NO chemical generator based on the reaction $2\ NO_2^- + 2\ I^- + 4\ H^+ \rightarrow 2\ NO + I_2 + 2\ H_2O$, which is pH and concentration dependent. The presence of NO was assessed by the formation of methemoglobin (MetHb) from oxyhemoglobin ($HbO_2$), following spectroscopically the decrease of the $HbO_2$ characteristic absorption peak at 576 nm for 2 minutes. GA was included in the reaction mixture at increasing concentrations. A human red cell hemolysate was used as the $HbO_2$ source to an initial absorbance of ± 0.150. The conditions were optimized with a 1.0 m*M* Hepes-Na buffer at pH 8.0 and with iodide at 2 m*M*. The reaction was initiated by the addition of sodium nitrite to a 2-m*M* final concentration. Under these conditions $HbO_2$ nearly disappeared in 2 minutes.

**NOS Assay.** Small intestinal segments obtained from either adult male rats or from 2-day-old rats of both sexes and kept at –20°C for no more than 2 weeks, were cut in small pieces and homogenized in a 1:5 ratio with a Tenbroek tissue grinder in a protease inhibitor–containing buffer (24). Cells and debris were separated at 600 *g* for 15 minutes at 4°C and were further centrifuged at 10,000 *g* for 15 minutes. The resultant supernatant was used for the assay. The incubation mixture contained 250 μ*M* calcium chloride, 10 μ*M* magnesium chloride, 200 U/ml calmodulin, 10 μ*M* flavin adenine dinucleotide (FAD), 20 μ*M* tetrahydropteridine, 10 μ*M* flavin mononucleotide (FMN), 400 μ*M* reduce nicotinamide adenine dinucleotide (NADPH), and 300 m*M* Hepes-NaOH pH 7.4 buffer. The

reaction was started by addition of the substrate Arg to a final concentration of 2 m*M*, including as a marker ±16 μCi/l (592 kBq/l) of [UL]-$^{14}$C-L-Arg. L-valine was also added at 1 m*M* as an arginase inhibitor (25). For each sample, separate 13 × 100 mm (or 12 × 75 mm) tubes were set up as reaction and blank; both were kept in ice-cold water until starting the incubation. Each tube contained 0.500 ml of the incubation mixture, 0.400 ml of sample, and 0.100 ml of substrate. The reaction was stopped after 1 hour of incubation at 37°C, with an excess of 67 m*M* acetate buffer pH 5.5 containing 6 m*M* ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) (quench buffer), and centrifuged at 600 *g* for 15 minutes. Blank tubes were inactivated at zero time with 2.000 ml of quench buffer.

Citrulline was separated from Arg by passage of the supernatant through a 6 × 40-mm AG 50W-X8-Na resin column (Bio-Rad Laboratories, Hercules, CA) that retained Arg. The resin was poured as a slurry prepared in 4.5-g batches of wet resin shaken with 6 ml of quench buffer. After passing the clear supernatant through the column, each one was washed with 2.000 ml deionized water and all effluents were collected, mixed, and counted for β-emission after addition of a water-compatible scintillation fluid. Under the assay conditions, total NOS was measured.

If the volumes indicated above are used, calculations are as follows:

$$\frac{(\text{DPM Sample} - \text{DPM Blank}) \times 2,000 \times 5}{\text{DPM } ^{14}\text{C-Arg per } 0.100 \text{ ml} \times 0.4} = \text{n}M/\text{hour} \times \text{g tissue}.$$

This assumes that the original tissue was homogenized in a 1:5 ratio. Another value might be applicable. Alternatively, for expressing data in reference to protein content:

$$\frac{(\text{DPM Sample} - \text{DPM Blank}) \times 2,000}{\text{DPM } ^{14}\text{C-Arg-Val per } 0.100 \text{ ml} \times 0.4 \times \text{protein [mg/ml]}} = \text{n}M/\text{h} \times \text{mg protein}.$$

NOS inhibition studies by GA were performed on adult rat intestinal homogenate supernatants by determining the activity of the enzyme with increasing concentrations of GA in the presence of two concentrations of either 2 m*M* or 0.5 m*M* Arg (26). For molarity calculations, GA was assigned a molecular weight of 250,000 Da (2–5).

**Statistical Analysis.** Comparisons among groups were by one-way analysis of variance and post hoc Tukey's test. If data were not normally distributed, the Kruskal-Wallis test of analysis of variance on ranks was carried out, with multiple comparisons versus a control group (27). A computer program (SigmaStat, Jandel Corp., San Rafael, CA) was used for these calculations. The threshold of significance was 0.05.

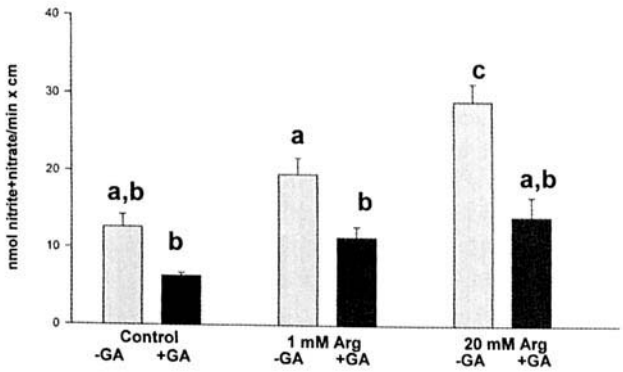


**Figure 1.** Concentration of nitrite plus nitrate released into the lumen of rats perfused under basal conditions (Control) in the absence of gum arabic (–GA) or the presence of 10 μ*M* (2.5 g/l) GA, (+GA), and similarly during jejunal perfusions in the presence of 1 m*M* Arg or 20 m*M* Arg. The bars represent the means and the SEM. Data not sharing a superindex are different at the 0.05 level. The number of rats in each group is the same as that in Table 1.

## Results

**Intestinal Perfusion Experiments.** The appearance of nitrite and nitrate in the lumen was related to the concentration of Arg added to the solutions (Fig. 1). Although the presence of 1 m*M* Arg was not sufficient to provide a clear differentiation from the baseline omitting Arg, addition of 20 m*M* Arg increased the concentration of the NO oxidation products in the effluents. GA had the uniform effect of reducing nitrite and nitrate concentration to about half that measured in its absence, resulting in luminal levels that were indistinguishable from each other.

Sodium data (Fig. 2) revealed that addition of GA to the perfusing control solution did not modify absorption. However, the presence of 1 m*M* Arg in the medium induced an increase in sodium removal rates from the intestinal lumen; GA canceled this effect, returning absorption rates to baseline. In contrast, inclusion of 20 m*M* Arg in the solution without GA produced lower absorption rates than

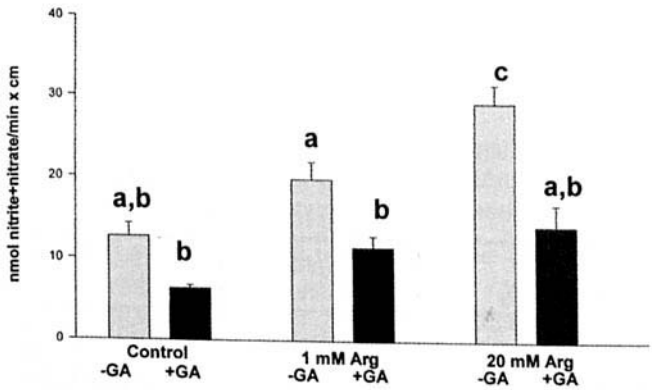


**Figure 2.** Rates of sodium absorption in the course of small intestinal perfusions under conditions described in the text and in the legend of Figure 1. The significance of differences among groups is indicated by not having a common letter indicator on the respective columns. Other characteristics of the graph are indicated in the preceding legend.

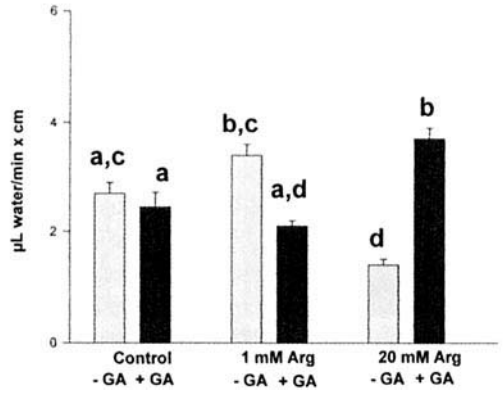


**Figure 3.** Net water absorption rates obtained in the perfusion experiments described in the Materials and Methods section. The values are derived from the difference in fluid weights entering and leaving the perfused segment, with appropriate adjustments to the actual pumping rates and length of the perfused intestinal segment. Significance of differences is coded as indicated in the legend of Figure 1.

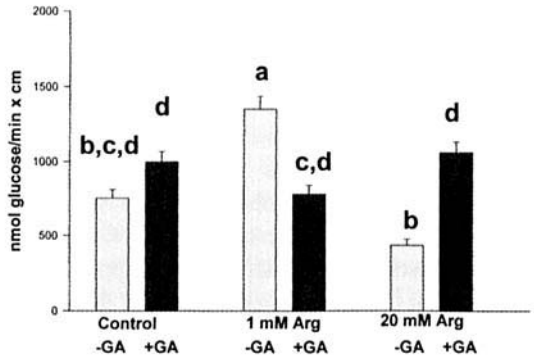


**Figure 4.** Glucose absorption in the jejunal segment perfused under conditions described in the text. Presence or absence of gum arabic (GA) is denoted by –GA and +GA, respectively, and the concentration of Arg is indicated below each pair of bars. The coding of the statistical evaluation of the data is given in Figure 1 and in Table 1.

solutions with 1 m*M* Arg, but they were not distinguishable from the control –GA. The presence of GA in the control solution sufficed to make it different from the absorption-depressing effect produced by the high concentration of Arg. Addition of GA to the 20-m*M* Arg preparation trebled the absorption rates of sodium so that values exceeded those of the baseline control –GA.

Net water absorption presented a comparable picture to sodium. No effects could be attributed to GA in the control solution (Fig. 3). For this parameter, inclusion of 1 m*M* Arg, in the absence of GA, was not sufficient to cause an increase in absorption, but the addition of GA to the same perfusate produced a decrease of the absorption rates in reference to the –GA, although the values remained comparable to those of control solutions. Arg at 20 m*M* concentration caused a significant decline over all preceding values. This effect was clearly overcome, and a large increase was observed, when 10 µ*M* (2.5 g/l) GA was present in the perfusing solution. As shown in the data for unidirectional fluxes, the stimulatory effects of 1 m*M* Arg and the negative effects of 20 m*M* Arg were also apparent in water influx values (Table 1). In contrast, the presence of either 1 or 20 m*M* Arg had no effect on water efflux. The presence of GA at the high Arg concentration did not further modify fluid effusion.

As expected, the pattern of glucose absorption followed closely that of sodium (Fig. 4). Multiple comparisons revealed that addition of GA in any of the three types of solutions resulted in indistinguishable absorption rates that were, in turn, similar to those of the control –GA.

***In vitro* NO Scavenging by GA.** The baseline conditions of the assay resulted in a rapid disappearance of $HbO_2$ with virtual completion by 2 minutes (Fig. 5). In the presence of increasing GA concentrations a greater proportion of $HbO_2$ remained unchanged, to an extent >80%, with 5.0 g/l (20 µ*M*) GA, indicating a diminished

**Table 1.** Unidirectional Water Fluxes in Rats During Jejunal Perfusions at Two Levels of L-Arginine (Arg) in the Presence or Absence of Gum Arabic (GA)[a]

| Treatment (*n*) | Water Influx µl/minute × cm | Water Efflux µl/minute × cm |
|---|---|---|
| Control (6) | 6.56 ± 0.11[a] | 3.71 ± 0.17[a,d] |
| + GA (9) | 6.65 ± 0.26[a] | 4.20 ± 0.12[a,c] |
| 1 m*M* Arg (12) | 7.63 ± 0.19[c] | 4.14 ± 0.24[a,c] |
| 1 m*M* Arg + GA (11) | 6.44 ± 0.15[a] | 4.35 ± 0.28[a] |
| 20 m*M* Arg (8) | 4.36 ± 0.24[b] | 2.95 ± 0.09[b,d] |
| 20 m*M* Arg + GA (11) | 6.99 ± 0.16[a,c] | 3.38 ± 0.18[b,c,d] |

[a] Data are means ± SEM. Values in the same column not sharing a superscript are different ($P < 0.05$).

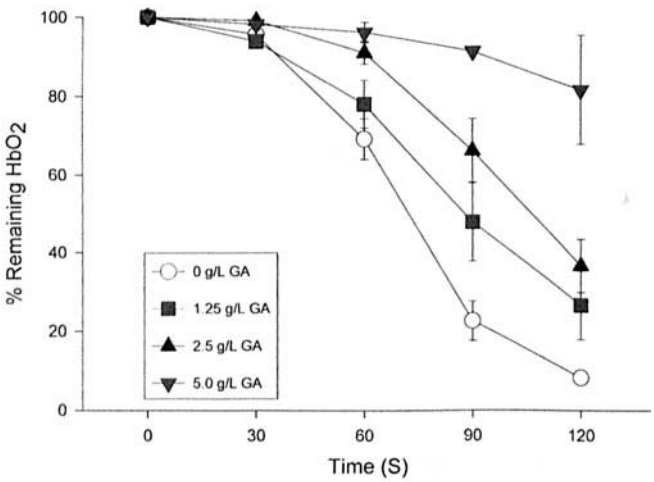


**Figure 5.** *In vitro* tests of the NO scavenging properties of gum arabic (GA). The plots represent the percentage of oxyhemoglobin ($HbO_2$) remaining at successive time points in the absence or presence of increasing concentrations of GA: 1.25 g/l (5 µ*M*), 2.5 g/l (10 µ*M*), and 5.0 g/l (20 µ*M*). After 60 seconds, the values for 2.5 and 5.0 g/l GA are significantly different ($P < 0.05$) from those of the control (0 g/l GA). The bars represent the SEM for five experiments at each GA concentration.

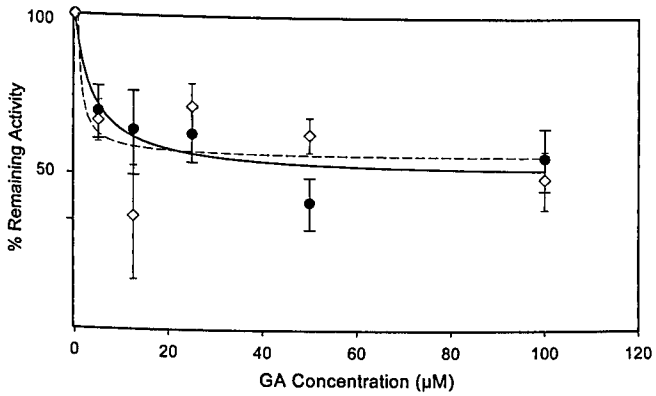


**Figure 6.** Plot of the relative nitric oxide synthase inhibition by gum arabic (GA) at increasing concentrations. The closed circles represent data obtained with frozen tissues of adult rats, processed as indicated in the text. The open diamonds correspond to values obtained with frozen small intestine of 2 day-old rat pups. The full and dashed lines represent a quadratic conformation of the respective sources of data, as described above. The dispersion bars denote the SEM. Values are the means of 4 experiments in each series.

presence of free NO in the medium and, hence, a greater preservation of the $HbO_2$ marker and less MetHb formed.

***In vitro* Inhibition of NOS by GA.** We obtained similar curves representing the remaining activity when the enzyme was tested with intestinal mucosa preparations from either frozen adult rat or 2-day-old pups' small intestinal mucosa (Fig. 6). An inverse second-order regression curve fitted both sets of data, with $r^2 = 0.711$ ($P < 0.01$) for the adult tissue material and 0.772 for the 2-day-old samples ($P < 0.01$). Application of classical enzyme kinetics treatment to the adult rat specimen data revealed that GA exerted a competitive type of inhibition with a $K_i$ graphically estimated to be 113 μ*M* (approximately 28 g/l GA).

## Discussion

The overall effect of GA in the *in vivo* perfusion experiments presented here was to act as a moderator of physiologic responses to the addition of the NO precursor, Arg, in the intestinal absorptive process. The results obtained *in vivo* were also consistent with the ability of GA to scavenge chemically generated NO and to inhibit NOS under *in vitro* conditions.

An unequivocal decrease in nitrite and nitrate concentration was found in the intestinal lumen perfused with solutions containing 10 μ*M* (2.5 g/l) of GA. This occurred when either 1 m*M* or 20 m*M* Arg was added; this decrease was compatible with GA having a scavenging effect that removed NO from immediate oxidation to nitrite and nitrate. Although the concentration of nitrite and nitrate measured in the lumen was not proportional to the concentration of the NO precursor, Arg, the decline in the end products of NO metabolism resulting from the addition of GA was comparatively the same; that is, to approximately half that of the concentration found when GA was omitted. This result is also consistent with the extent of NOS inhibition by GA obtained in the *in vitro* experiments that showed an inhibition plateau at approximately 50% of the uninhibited intestinal mucosa activity. Therefore, both scavenging and NOS inhibition might be responsible for the observations.

The data obtained for sodium, water, and glucose absorption presented common traits. This finding is not surprising given the sodium dependency of mediated glucose transport and the fluid drag effect of these solutes (28). However, under conditions of relatively high glucose concentration, as in this experiment, a linkage between glucose disappearance and water influx could also be expected because of transmembrane diffusion of molecules such as glucose, carried in the bulk phase when present in the intestinal lumen at >25–50 m*M* (29).

In general, we observed an absorption increase with 1 m*M* Arg and a return approximately to baseline levels by the addition of 10 μ*M* (2.5 g/l) GA. The increase in absorption under these conditions in the absence of GA, could be attributed to a somewhat greater generation of NO than under baseline conditions and its corresponding vasodilating effects (13, 15). An earlier study with basal solutions of slightly different composition showed that 1 and 2 m*M* Arg had this absorption-stimulating effect, which was to an extent dependent on the sodium concentration of the perfusing solution (16). When Arg was increased to 20 m*M*, water and sodium absorption decreased, although not below baseline levels. Histological evidence of vasodilation at 1 m*M* Arg and vasoconstriction with 20 m*M* Arg (16) are consistent with overproduction of NO in the latter and stimulation of cGMP and prostaglandin E2 (PGE2) production (30, 31). This bimodal action of NO has been well documented in other studies that revealed lower levels of secretion in isolated rabbit ileum (11) and amelioration of experimental colitis (32) with moderate NO generation. In contrast, NO overproduction has been linked to colonic electrolyte secretion (33).

Beyond earlier observational reports that showed that GA can enhance sodium and water absorption (6, 7), accelerate weight normalization of rats given several days of cathartic treatment (8), and diminish chloride and sodium secretion in rats exposed to cholera toxin *in situ* (9), we established that GA enhanced diffusion processes (10) and modified NO-dependent gating of the basolateral membrane potassium channel (17).

The evidence presented here reveals that GA does not totally obliterate the effects of excess NO at two levels of stimulation but tends to restore normalcy. This could be because of several possible reasons. GA can only exert its effects locally, as the size of the molecule (≥250,000 Da) prevents its uptake by either diffusion or mediated transport, and its possible endocytosis could only have limited effect. The likelihood of GA scavenging NO in the intestinal lumen is conceivable because of the high diffusibility of a small molecule such as NO (30 Da) and its rapid oxidation to nitrite and, further, to nitrate (14) in an environment not entirely deprived of oxygen. Although NOS is considered to be a cytosolic enzyme, there is as yet no direct evidence that

it is also bound to the apical membrane—a site where GA could inhibit NO synthesis.

This inhibitory effect might play a role under the conditions of the intestinal perfusions because it has been shown that NOS is quite physiologically active and mediates epithelial repair (34). Microscopical observations following perfusions (6, 7) reveal a modest degree of mucosal shedding, even at the low hydrostatic pressures used in this type of experiments; thus, any of the NOS isoforms released or leaked from the enterocytes could become inhibited by GA, as documented in the *in vitro* experiments. The asymptotic characteristics of the inhibitory effect are also consistent with earlier studies in which no additional proabsorptive action was observed with added GA up to 40 $\mu M$ (10 g/l) (6). This similarity is further substantiated by the estimated kinetic characteristics of the inhibitory effect of GA that result in a $K_i$ with a value $>100$ $\mu M$ ($>25$ g/l) GA. In view of this, it may be argued that, under the experimental conditions *in vivo*, the NO scavenging effect is more significant than NOS inhibition. This is further supported by our previous demonstration that zinc chelated with small-molecular weight molecules has comparable NO scavenging properties that can be associated with the potential enteroprotective effects of these complexes (35).

Whey proteins have recently been shown to form complex biomacromolecules, or coacervates, with GA at low pH (36). The conditions of the experiments in this are above the pH range at which such interactions may occur. However, if GA would be orally administered in the presence of milk proteins, coacervates might form and alter the physicochemical status of these proteins, with yet-to-be-determined consequences for the digestive process. GA has shown absorption enhancement effectiveness even with exposure to low pH, as evidenced in preliminary rat studies, where GA in test solutions was introduced via a stomach tube. This resulted in an increased zinc uptake over time (37). Future oral experiments should provide additional evidence regarding the nutritional and therapeutical potential of GA while considering its capacity to restore or improve small intestinal function

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