CHANGES IN THE STRUCTURE OF RAT MUCOUS GLYCOPROTEIN AND PROSTAGLANDIN CYTOPROTECTION

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Using a mucolytic agent, *N*-acetyl-L-cysteine (NAC), the structure of rat gastric mucous glycoprotein (GP) and its participation in prostaglandin cytoprotection were studied. Treatment of the undegraded native mucous GP with 20% NAC resulted in a marked reduction in molecular weight as obtained in the case of treatment with 0.5 M β -mercaptoethanol. The amount of GP remaining in the gastric tissue, including the mucous layer after 3 h-incubation was defined as an indication of the mucous adhesiveness to the gastric mucosal surface. The adhesiveness was markedly decreased by the *in vitro* or *in vivo* treatment with NAC. The cytoprotective effect of prostaglandin E_2 was significantly reduced by pretreatment with NAC. Elution profiles of mucous GP on Sepharose CL-2B showed a good correlation between decrease in the amount of the undegraded native mucous GP and the severity of gastric damage. In addition, the collapse of the native mucous GP into its subunits resulted in a decrease in the adhesiveness. These observations suggest that the maintenance of the native mucous GP is an essential factor for prostaglandin cytoprotection.

Keywords — *N*-acetyl-L-cysteine; prostaglandin E_2 ; cytoprotection; mucus; mucous glycoprotein; ethanol-induced damage

INTRODUCTION

Mucous gel adhering to the gastric mucosal surface plays an important role in mucosal protection.^{1,2)} Gastric mucous glycoproteins (GPs) have been isolated from hogs and humans, and the polymeric structure and gel-forming properties have been investigated.³⁻⁶⁾ Proteolysis or reduction with thiol reagents splits the undegraded native GP into subunits, with a concomitant loss of the viscous and gel-forming properties of the native GP.⁶⁻⁸⁾ Recent studies have shown that the gastric mucosal epithelium secretes HCO_3^- to neutralize the H^+ -diffusion through the mucus.^{9,10)} It has been suggested that the gastric mucus could support a significant pH gradient and retard the transfer of H^+ .¹¹⁾

Prostaglandins (PGs) protect the stomach against various damaging agents.¹²⁾ This property of PGs has been termed "cytoprotection" and is independent of acid inhibition. There have been numerous reports on the mechanisms of prostaglandin cytoprotection. Gastric mucus is thought to be one possible factor by which PGs protect the gastric mucosa.^{13,14)} It was reported

that the methyl analog of prostaglandin E_2 (PGE_2) increases the gastric mucus content in rats¹⁵⁾ and dogs.¹⁶⁾ Moreover, PGE_2 stimulates the incorporation of labelled fucose and glucosamine into gastric mucous GPs, *in vivo* in the rat.¹⁷⁾ We also observed the stimulatory effect of PGE_2 on gastric macromolecular GP biosynthesis.¹⁸⁾ However, this effect could not be regarded as the main mechanism involved in cytoprotection. We also suggested the importance of mucous adhesiveness to the gastric surface epithelium.¹⁹⁾

In the present work, we directed attention to the structure of gastric mucous GP in rats and its participation in prostaglandin cytoprotection, using a mucolytic agent, *N*-acetyl-L-cysteine (NAC).

MATERIALS AND METHODS

Animals — Male Wistar rats weighing 180—200 g were fasted for 24 h prior to the experiments. During the last 18 h, water was also withheld.

Induction of Ethanol-Induced Gastric Lesions and Treatment with *N*-Acetyl-L-cysteine — 1) *In Vitro* Treatment with NAC: PGE_2 (Sigma

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FIG. 1. Treatment with NAC in Vitro or in Vivo

Chemical, 0.1 mg/5% ethanol ml/kg) was given orally 30 min before the administration of 1 ml of absolute ethanol. These animals were killed 1 h after being given absolute ethanol and the stomachs were excised. Gastric mucosal lesion index was expressed as the following arbitrary scale: 0, no damage; 1, pin-point erosions; 2, 1–5 small erosions (< 2 mm); 3, > 5 small erosions or 1–5 large erosions (> 2 mm); 4, > 5 large erosions. The glandular stomachs were then exposed to 20 ml of 20% (w/v) NAC (Wako Pure Chemical) and left at room temperature for 5 min (Fig. 1). The stomachs of the control group were exposed to NaCl solution of the same osmotic pressure as 20% NAC.

2) *In Vivo* Treatment with NAC: As shown in Fig. 1, 1 ml of 20% NAC was given and 1 h later PGE_2 (0.1 mg/5% ethanol ml/kg) was given orally. The animals of the control group were given 1 ml of NaCl solution of the same osmotic pressure as 20% NAC. Thirty minutes after PGE_2 had been administered, 1 ml of absolute ethanol was given orally. The control group received 5% ethanol and distilled water instead of PGE_2 and absolute ethanol respectively, as shown in parentheses (Fig. 1). In case of analysis of the gastric juice, the pylorus was ligated 5 min before the oral administration of NAC. The animals were sacrificed 1 h after being given absolute ethanol and the stomachs were excised. Gastric mucosal damage was examined, as described above. The gastric juice was collected and centrifuged at $400 \times g$ for 10 min. The supernatant fluid was used for the following experiment.

Treatment of Macromolecular Mucous GP with β -Mercaptoethanol and NAC — Mucous GP extracted from the rat gastric tissue including the

mucous layer was fractionated on Bio Gel A-1.5 m. Macromolecular GP (peak I) was collected and further fractionated on Sepharose CL-2B. Peak I-1 in the void volume and peak I-2 (lower-molecular weight GP) were found. Each fraction was dialyzed against distilled water and lyophilized. The lyophilized GP fractions were dissolved in a solution of 0.5 M β -mercaptoethanol (Nakarai Chemicals) or 20% (w/v) NAC and incubated for 5 h at 37 °C. The incubation mixtures were then dialyzed, applied to a Sepharose CL-2B column and fractions collected with 50 mM Tris-HCl buffer pH 7.2.

Incubation of Gastric Tissue for Estimation of Adhesiveness — The amount of mucous GP remained in the gastric tissue after 3 h-incubation was defined as an indication of mucous adhesiveness. The wet weight of the rat glandular stomach was determined and the tissue was incubated for 3 h at 37 °C with shaking (25 rpm/min) in an incubator-water bath (R-100D; Taiyo Scientific Industrial Co., Ltd.), under 95% O_2 and 5% CO_2 in 20 ml of Krebs-Ringer bicarbonate buffer containing antibiotics (Penicillin G; Meiji Seika 65 U/ml, Streptomycin; Meiji Seika 50 $\mu\text{g}/\text{ml}$) pH 7.4.¹⁹⁾ After termination of the incubation, the medium was separated from the tissue including mucous layer (gastric tissue) by centrifugation at $1\,000 \times g$ for 10 min at 4 °C.

Extraction and Fractionation of Rat Gastric GP — Extraction of mucous GP was carried out as described.²⁰⁾ The tissue was homogenized in 2 ml of 50 mM Tris-HCl buffer containing 2% Triton X-100 pH 7.2. The homogenate was then centrifuged at $10\,000 \times g$ for 30 min at 4 °C, and the supernatant fluid was dialyzed against

distilled water at 4 °C. The dialyzed material was applied to a column of Bio Gel A-1.5 m (100–200 mesh, 1.4 × 55 cm; Bio Rad Laboratories) previously equilibrated with 50 mM Tris-HCl buffer pH 7.2 and the column washed with the same buffer. The gastric juice and the incubation medium concentrated by lyophilization were also applied to a Bio Gel A-1.5 m. The column was washed with 50 mM Tris-HCl

buffer pH 7.2. The hexose content of each fraction (2.3 ml) was determined by the phenol/H₂SO₄ method with galactose as the standard.²¹⁾ In other experiments, mucous GP was obtained by the same method as described above and fractionated on Bio Gel A-1.5 m. Macromolecular GP fraction (peak I) obtained by Bio Gel A-1.5 m was further fractionated on Sepharose CL-2B (1.4 × 60 cm; Pharmacia Fine Chemicals). The

FIG. 2. Sepharose CL-2B Gel Filtration of Native Rat Gastric Glycoprotein (A) and the Glycoprotein after Treatment with β -Mercaptoethanol or NAC (B)
(B): $\circ$ — $\circ$, before treatment; $\bullet$ - - - $\bullet$, after treatment.

column was washed with 50 mM Tris-HCl buffer pH 7.2. The hexose content of each fraction was determined.

Histochemical Study — The rat stomach was fixed in 10% buffered formalin containing 1% cetyl pyridinium chloride. The fixed tissue was then embedded in paraffin, cut into 4 μ m sections, stained with periodic-acid-Schiff (PAS) using standard morphologic techniques and the stained sections were examined under a light microscope ($\times 200$ magnification).

Statistical Analysis — All data were expressed as the mean $\pm$ S.E. Statistical significance was evaluated by Student's *t*-test.

RESULTS

Effects of β -Mercaptoethanol and NAC on Structure of Macromolecular Mucous GP in Vitro

Reduction of the undegraded native GP (peak I-1) with 0.5 M β -mercaptoethanol resulted in a single included-peak on the Sepharose CL-2B (Fig. 2). The native GP from hog gastric mucosa gave an identical peak corresponding to the rat GP after reduction with 0.5 M β -mercaptoethanol (data not shown). Treatment of the native GP with 20% NAC gave an identical included-peak to the one seen with β -mercaptoethanol. When the lower-molecular

weight GP (peak I-2) was treated with β -mercaptoethanol or NAC, similar results were obtained.

Effect of NAC on Gastric Mucous GP in Vitro

Oral administration of absolute EtOH significantly decreased the GP content of peak I in the gastric tissue. The decrease was significantly prevented by pretreatment with PGE₂. The GP content of peak I was significantly decreased by the *in vitro* treatment with NAC in each group (Table I). In the low-molecular weight GP fraction (peak II and III) from the gastric tissue, no significant changes were observed. The adhesiveness of mucous GP to the gastric mucosal surface was estimated in an *in vitro* incubation system. After 3 h-incubation, a marked decrease in the GP content of peak I in the gastric tissue was observed in the NAC-treated group. The GP content of peak I was roughly 30% of that before incubation in the NAC-treated group. Thus, such a marked loss of gastric mucous GP into the incubation medium represents a marked decrease of the mucous adhesiveness. On the other hand, the GP content of peak I exceeded 70% in the control and (PGE₂ + EtOH)-treated group, after 3 h-incubation. The decrease in GP in the gastric tissue was also confirmed, as determined by the amount of GP in the medium after 3 h-

TABLE I. Effect of *in Vitro* Treatment with NAC on the Amount of Glycoprotein in the Gastric Tissue

Treatment	Hexose content (μ g/g tissue)		
	Peak I	Peak II	Peak III
Before incubation			
PGE ₂ + EtOH + NAC	695 $\pm$ 8	587 $\pm$ 31	140 $\pm$ 54
EtOH + NAC	462 $\pm$ 28	604 $\pm$ 28	143 $\pm$ 51
NAC	766 $\pm$ 40	591 $\pm$ 37	151 $\pm$ 18
Control	987 $\pm$ 37	619 $\pm$ 42	123 $\pm$ 21
EtOH	584 $\pm$ 14	597 $\pm$ 72	171 $\pm$ 28
PGE ₂ + EtOH	922 $\pm$ 48	500 $\pm$ 49	122 $\pm$ 13
After 3 h-incubation			
PGE ₂ + EtOH + NAC	208 $\pm$ 22 (30)	369 $\pm$ 18	71 $\pm$ 8
EtOH + NAC	148 $\pm$ 14 (32)	409 $\pm$ 55	86 $\pm$ 19
NAC	221 $\pm$ 17 (29)	431 $\pm$ 16	88 $\pm$ 5
Control	774 $\pm$ 66 (78)	506 $\pm$ 35	84 $\pm$ 19
EtOH	240 $\pm$ 37 (41)	394 $\pm$ 46	66 $\pm$ 29
PGE ₂ + EtOH	687 $\pm$ 15 (75)	414 $\pm$ 17	71 $\pm$ 7

Glycoprotein was fractionated on Bio Gel A-1.5 m. All values represents the mean $\pm$ S.E. (*n* = 3–4). Values in parentheses represent percent, before incubation. Significantly different. a) *p* < 0.05, b) *p* < 0.01, c) *p* < 0.001.

TABLE II. *Effect of Pretreatment with NAC on Prostaglandin Cytoprotection in Rats*

Treatment	Lesion index
EtOH	3.0 ± 0.4
PGE ₂ + EtOH	0.2 ± 0.2 ^{a)}
NAC + PGE ₂ + EtOH	2.0 ± 0.4 ^{b)}

All values represents the mean ± S.E. (n = 5). Significantly different from EtOH. a) $p < 0.001$ Significantly different from PGE₂ + EtOH. b) $p < 0.01$.

TABLE III. *Effect of Oral Administration of NAC on the Amount of Glycoprotein in the Gastric Juice*

Treatment	Hexose content (μg/g tissue)		
	Peak I	Peak II	Peak III
NAC + PGE ₂ + EtOH	256 ± 29	142 ± 9	468 ± 22
NAC + EtOH	273 ± 10	115 ± 29	554 ± 19
NAC	307 ± 25	125 ± 1	563 ± 25
Control	245 ± 4	85 ± 12	245 ± 28
EtOH	258 ± 26	85 ± 8	310 ± 59
PGE ₂ + EtOH	226 ± 19	69 ± 5	269 ± 18

Glycoprotein was fractionated on Bio Gel A-1.5 m. All values represents the mean ± S.E. (n = 3–4). Significantly different. a) $p < 0.05$, b) $p < 0.01$.

TABLE IV. *Effect of Oral Administration of NAC on the Amount of Glycoprotein Fractionated on Bio Gel A-1.5 m in the Gastric Tissue*

Treatment	Hexose content (μg/g tissue)		
	Peak I	Peak II	Peak III
Before incubation			
NAC + PGE ₂ + EtOH	503 ± 44	514 ± 47	116 ± 21
NAC + EtOH	489 ± 64	516 ± 5	133 ± 18
NAC	497 ± 36	472 ± 9	140 ± 22
Control	721 ± 49	457 ± 27	126 ± 22
EtOH	593 ± 81	431 ± 49	196 ± 37
PGE ₂ + EtOH	696 ± 21	405 ± 48	133 ± 12
After 3 h-incubation			
NAC + PGE ₂ + EtOH	303 ± 9 (60)	455 ± 12	102 ± 7
NAC + EtOH	297 ± 20 (62)	455 ± 38	87 ± 16
NAC	313 ± 13 (63)	404 ± 35	90 ± 32
Control	618 ± 31 (86)	472 ± 27	76 ± 8
EtOH	450 ± 36 (75)	403 ± 23	82 ± 15
PGE ₂ + EtOH	598 ± 18 (85)	396 ± 50	110 ± 9

Glycoprotein was fractionated on Bio Gel A-1.5 m. All values represents the mean ± S.E. (n = 3–4). Values in parentheses represent percent, before incubation. Significantly different. a) $p < 0.05$, b) $p < 0.01$, c) $p < 0.001$.

incubation (data not shown).

Effect of NAC on the Cytoprotective Effect of PGE₂

As can be seen from Table II, PGE₂ markedly prevented the absolute EtOH-induced gastric le-

sions ($p < 0.001$). The cytoprotective effect of orally administered PGE₂ was significantly reduced by pretreatment with NAC ($p < 0.01$). The reduction in the cytoprotective effect was

FIG. 3. Bio Gel A-1.5 m Gel Filtration of an Extract from Rat Gastric Tissue Including Mucous Layer

also observed, when PGE_2 was administered intraperitoneally (data not shown). Severe hemorrhagic lesions occurred with oral administration of absolute EtOH. On the other hand, oral administration of NAC alone produced no hemorrhagic lesions in the gastric mucosa.

Effect of Oral Administration of NAC on GP Content in Gastric Tissue

Increase in the amounts of low-molecular weight GP (peak II and III) was observed by pretreatment with NAC, when the gastric juice was fractionated on Bio Gel A-1.5 m (Table III). In peak I, however, no significant changes were observed. The GP content of peak I in the tissue fraction was decreased by pretreatment with NAC (Table IV). Figure 3 shows typical elution profiles of GP in the tissue fraction on Bio Gel

A-1.5 m. After 3 h-incubation, the level of the GP content was decreased to approximately 60% of that seen before incubation in the NAC-treated group. On the other hand, it exceeded 80% in the control and in the ($\text{PGE}_2 + \text{EtOH}$)-treated group.

In another experiment, macromolecular GP (peak I) of the gastric tissue obtained by Bio Gel A-1.5 m was further fractionated on Sepharose CL-2B and typical elution profiles are shown in Fig. 4. Oral administration of absolute EtOH caused a significant decrease in the amount of the native macromolecular GP (peak I). The marked decrease ($p < 0.01$) in GP content of peak I-1 caused by EtOH was significantly prevented ($p < 0.05$) by pretreatment with PGE_2 (0.1 mg/kg, *p.o.*) (Table V). However, when

FIG. 4. Sepharose CL-2B Gel Filtration of Macromolecular Weight Gastric Glycoprotein (Peak I) Isolated by Bio Gel A-1.5 m

NAC was given orally before the administration of PGE_2 , the amount of GP of peak I-1 was significantly decreased ($p < 0.05$). After 3 h-incubation, the level of the GP content was 82% of that before incubation in the control and was 72% in the ($\text{PGE}_2 + \text{EtOH}$)-treated group. This level decreased to about 60% in the other group. With the lower-molecular weight GP (peak I-2a and I-2b), although no significant changes were observed between each group before incubation, a 3 h-incubation revealed significant changes. The level of the GP content was about 70% of that seen before incubation in the control

and ($\text{PGE}_2 + \text{EtOH}$)-treated group. However, it decreased to 31–47% in the other group. The decrease in GP content of the lower-molecular weight GP fraction (peak I-2a and I-2b) during the incubation period was much more extensive than that of the native macromolecular GP fraction (peak I-1).

Histochemical Study

Figure 5 shows the histologic evidence of the gastric mucosa. In the intact rats, PAS staining positive substances were distributed on the mucosal surface epithelium and in the neck of the gland of the mucosa (A). In the EtOH-

TABLE V. Effect of Oral Administration of NAC on the Amount of Glycoprotein Fractionated on Sepharose CL-2B in the Gastric Tissue

Treatment	Peak I-1	Hexose content ($\mu\text{g/g}$ tissue)	
		Peak II-2	Peak I-2b
Before incubation			
NAC + PGE ₂ + EtOH	196 $\pm$ 23	236 $\pm$ 54	256 $\pm$ 31
NAC + EtOH	184 $\pm$ 23	208 $\pm$ 39	238 $\pm$ 40
NAC	174 $\pm$ 20	221 $\pm$ 20	257 $\pm$ 24
Control	333 $\pm$ 38	226 $\pm$ 32	306 $\pm$ 16
EtOH	194 $\pm$ 14	248 $\pm$ 18	262 $\pm$ 27
PGE ₂ + EtOH	330 $\pm$ 49	196 $\pm$ 8	315 $\pm$ 27
After 3 h-incubation			
NAC + PGE ₂ + EtOH	120 $\pm$ 10 (61)	78 $\pm$ 9 (33)	116 $\pm$ 12 (45)
NAC + EtOH	111 $\pm$ 13 (60)	87 $\pm$ 13 (42)	113 $\pm$ 11 (47)
NAC	107 $\pm$ 9 (61)	93 $\pm$ 12 (42)	121 $\pm$ 9 (47)
Control	273 $\pm$ 33 (82)	156 $\pm$ 28 (69)	217 $\pm$ 14 (71)
EtOH	122 $\pm$ 10 (63)	76 $\pm$ 4 (31)	102 $\pm$ 7 (39)
PGE ₂ + EtOH	239 $\pm$ 23 (72)	132 $\pm$ 15 (67)	234 $\pm$ 19 (74)

Glycoprotein was fractionated on Bio Gel A-1.5 m. All values represents the mean $\pm$ S.E. ($n = 5$). Values in parentheses represent percent, before incubation. Significantly different. a) $p < 0.05$, b) $p < 0.01$, c) $p < 0.001$.

treated rats there were deep necrotic lesions and an extensive desquamation of the gastric surface epithelial cell layer. Edema of the submucosa and leukocyte accumulation were also observed. PAS staining positive substances were markedly reduced (B). PGE₂ prevented the reduction in PAS staining substances caused by EtOH (C). PAS staining substances on the surface epithelium were destroyed and the amounts reduced by NAC alone. However, there were no hemorrhagic lesions (D). Treatment with NAC before PGE₂ led to an extensive desquamation, as was observed in the EtOH-treated rats (E).

DISCUSSION

At least two factors are required for the mucous barrier to exert physiological effects: the thickness of the adherent mucous layer and the integrity of the structure. Data on the effect of various agents on mucous thickness and the structure of the mucous gel have been reported.^{6,22,23} When the undegraded native mucous GP from the hog stomach, which is MW 2×10^6 daltons, is treated with proteolytic enzymes or β -mercaptoethanol, there is a collapse of the GP structure into four equal sized subunits of MW 5×10^5 daltons.⁶⁻⁸ This is thought to be the result of breakage of the cova-

lent structure of the protein core of component GPs by proteolysis, or by splitting of the disulfide bridges.

In the present study, rat gastric mucous GP showed a similar profile on Sepharose CL-2B to that of hog mucous GPs. In addition, a marked reduction in the molecular weight of GP induced by β -mercaptoethanol was observed. Therefore, the rat gastric mucous GP has a molecular weight of about 2×10^6 daltons and is composed of GP subunits joined by disulfide bridges.²⁴ We also found that the collapse of mucous GP occurred after incubation of the undegraded mucous GP with 20% NAC, thereby indicating reductive cleavage of disulfide bridges. This may be attributed to a mucolytic action of NAC.

Moreover, we investigated the effect of NAC on the adhesiveness of mucous GP. When the gastric tissue was incubated, mucous GP on the gastric mucosal surface was gradually released into the incubation medium. The total GP content (tissue plus medium) in each fraction on Bio Gel A-1.5 m was unchanged at any of the incubation times tested, suggesting that mucous GP is released into the medium without degradation during the incubation period.¹⁹ Thus, the loss of gastric mucous GP into the medium rep-

FIG. 5. PAS Staining of the Rat Gastric Mucosa (A) intact group. (B) ethanol(EtOH)-treated group. (C) (PGE_2 + EtOH)-treated group. (D) NAC-treated group. (E) (NAC + PGE_2 + EtOH)-treated group ($\times 200$ magnification).

resents the decrease of mucus adhesiveness, and the adhesiveness of mucous GP can be estimated by comparing the ratio of the amount of GP remained in the tissue after 3 h-incubation to that before the incubation. In the previous study,¹⁹⁾ we found that the adhesiveness of macromolecular mucous GP fractionated Bio Gel A-1.5 m was markedly decreased by the oral administration of absolute ethanol and that the decrease could be significantly prevented by pretreatment with PGE_2 . The amount of the undegraded

native GP (peak I-1) and the degraded subunits (peak I-2a and I-2b) were measured by fractionating the macromolecular weight mucous GP on Sepharose CL-2B in the present study. The amount of the native GP was markedly decreased by oral administration of NAC. In addition, a 3 h-incubation of the gastric tissue showed a marked decrease in the mucous adhesiveness by treatment with NAC, both *in vitro* and *in vivo*. Our observations suggest that NAC affects the native mucous GP, in part, by split-

ting into lower-molecular weight GP subunits, which resulted in a decreased adhesiveness.

A previous study¹⁹⁾ suggested that the adhesiveness of mucous GP to the gastric mucosal surface was closely related to prostaglandin cytoprotection. However, it is not clear whether the maintenance of the mucous adhesiveness is responsible for prostaglandin cytoprotection. Zlotoff *et al.*²⁵⁾ reported a marked reduction in prostaglandin cytoprotection in rats with subsequent NAC treatment and suggested that the gastric protective effect of PGE₂ depends, in part, on the increased released of gastric mucus. To ascertain the role of mucous GP in cytoprotection, the gastric mucous GP was pretreated with NAC in the present study. The cytoprotective effect of PGE₂ was significantly reduced by pretreatment with NAC, in which case the adhesiveness was markedly decreased and the amount of the undegraded native GP was also significantly decreased. These results suggests the importance of mucous adhesiveness in prostaglandin cytoprotection. The present study demonstrated that there was good correlation between the decrease in the amount of the undegraded native GP and the severity of gastric damage, and that the collapse of mucous GP into subunits leads to the decrease in the adhesiveness. This would be related to the loss in the viscous and gel-forming properties of native mucous GP. These observations indicate that maintenance of the undegraded native GP is an essential factor in the cytoprotection seen with prostaglandin.

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