Peptidoglycan Fragments Decrease Food Intake and Body Weight Gain in Rats

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Received 28 February 1994/Returned for modification 8 April 1994/Accepted 9 May 1994

We hypothesized that peptidoglycan (PG) fragments decrease appetite in rats. Male Lewis rats (150 g; $n \geq 7$) received intraperitoneal (i.p.) injections of purified soluble PG fragments that had been treated with polymyxin B-agarose to remove residual endotoxin. Food consumption and body weight gain were determined at intervals after injection. Single i.p. injections of macromolecular extensively *O*-acetylated PG (S-*O*-PG) and non-*O*-acetylated PG fragments (24 to 240 μ g/kg) reduced food intake and body weight gain in a dose-dependent fashion during the first 12 h after injection. Low-molecular-weight disaccharide peptide monomers with nonreducing 1,6-anhydro-*N*-acetylmuramic acid ends and muramyl dipeptide (MDP; 1.6 mg/kg) were also appetite and weight gain suppressants, albeit at least 10-fold less potent than S-*O*-PG; however, muramidase-derived monomers and peptide cross-linked dimers with reducing muramic acid ends were inactive. Appetite suppression was not limited to the Lewis rat strain since another strain, F344, exhibited similar decreases in food intake after injection of S-*O*-PG or MDP. Oral administration of MDP or S-*O*-PG, at concentrations 3 and 20 times higher, respectively, than those that were active i.p., failed to elicit a hypophagic response. We conclude that soluble PG fragments are potent suppressants of food consumption and body weight gain in rats and that, although macromolecular PG is more potent than low-molecular-weight fragments, neither *O*-acetylation nor glycosidic linkage of PG fragments is required for activity. We speculate that PG fragments may contribute to loss of appetite during bacterial illness.

It is universally recognized that classic features of infectious illness include fever, increased sleepiness, and decreased appetite. These symptoms, together with other manifestations of the acute-phase response, are attributed to the release of the host's endogenous inflammatory cytokines such as the interleukins and tumor necrosis factor (49). Indeed, interleukin-1 (IL-1), a well-known endogenous pyrogen (7, 11), and tumor necrosis factor, a putative pyrogen (8, 35) and cryogen (16), possess somnogenic and/or anorectic effects when administered parenterally (24, 31, 32, 36, 44, 52).

Lipopolysaccharide (LPS), a unique component of gram-negative outer membranes, and muramyl dipeptide (MDP; adjuvant peptide), the minimal structure in Freund's complete adjuvant that retains immunomodulatory activity (10) and a synthetic component derived from the peptidoglycan (PG) of bacterial cell walls, induce fever (9, 38), enhance slow-wave sleep (20, 22), and decrease food intake (26, 30, 53). Since LPS and MDP stimulate the release of cytokines both in vivo (4, 38) and in vitro (6, 9), it is presumed that these bacterial components modulate appetite, sleep, and fever by triggering the release of the host's endogenous cytokines, which, in turn, mediate the response (21).

We hypothesized that naturally occurring PG fragments released from bacterial cell walls might contribute to the decrease in food consumption observed during bacterial illness. Indeed, on the basis of in vitro studies, it is likely that a large variety of PG fragments are released from intact bacteria in vivo by the action of bacterial PG-degrading enzymes that function in cell division (40, 48) as well as by host-derived PG hydrolases, such as lysozyme (2, 43). These physiologically realistic PG fragments, composed of alternating units of *N*-

acetylglucosamine and *N*-acetylmuramic acid (NAM) with a short peptide attached to the lactyl group of the NAM residue, range in size from submonomeric particles of <500 Da to enormous soluble macromolecules of >10⁶ Da (43). PG fragments induce numerous biological effects (13, 17), including pyrogenicity (28, 29) and somnogenicity (19, 27). Furthermore, PG fragments stimulate the release of IL-1 in vitro (34); hence, we investigated the ability of purified, naturally occurring PG fragments to decrease appetite in a rat model.

MATERIALS AND METHODS

Animals and housing conditions. Male inbred Lewis and F344 rats, weighing 125 to 149 g, obtained from Harlan Sprague Dawley, Inc. (Indianapolis, Ind.), were housed individually in metabolic cages in a temperature-controlled room (72 $\pm$ 5°F [22.2 $\pm$ 1.5°C]) and kept on a 12-h light–12-h dark cycle with lights on from 0700 to 1900. Lewis rats were used in all experiments unless otherwise noted.

Experimental protocol. Rats were provided premeasured powdered Teklad 4% Mouse/Rat Diet (Madison, Wis.) and water daily and allowed to eat and drink ad libitum. Plastic weigh boats were hung beneath the food jars to collect any spillage. Just prior to the onset of darkness each evening, between 1700 and 1900, any food spillage in the weigh boats was combined with the powdered rat diet in food jars, weighed, refilled, and reweighed. Each rat was also weighed at this time. The net 12-h (overnight) and 24-h food consumption and animal body weight gain were determined daily. The rats were acclimated to diet, handling, and maintenance conditions for at least 2 days prior to administration of PG or other test substances and divided into experimental and control groups of at least seven rats per group. On test days, a purified bacterial cell wall test substance suspended in saline (or saline alone) was administered by intraperitoneal (i.p.) injection of a 500- μ l bolus just after the animal was weighed. In some

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experiments, the test substance or saline was administered intragastrically as a 1-ml bolus by use of a stainless-steel biomedical animal feeding needle (Popper and Sons, Inc., New Hyde Park, N.J.) introduced through the oral cavity.

Other laboratories have assessed the hypophagic effects of various agents during daytime rather than nighttime hours by fasting experimental animals or by reversing the light-dark cycles of the animals. We believe our experimental model, in which bacterial cell wall components are administered just prior to the onset of darkness, the time during which rats consume approximately 85% of their diet, is more physiologically realistic since it permits access to food and water ad libitum and maintains the normal circadian rhythms of the rats.

Preparation of bacterial cell wall components. Soluble *O*-acetylated peptidoglycan (S-*O*-PG) and non-*O*-acetylated peptidoglycan (S-non-*O*-PG) fragments were obtained by sonication of intact PG purified from *Neisseria gonorrhoeae* FA19 and RD5, respectively, as described previously (41). Monomeric and dimeric PG fragments containing reducing NAM ends were purified as described previously (41) from *Chalaropsis* B muramidase digests of gonococcal PG and referred to as *Chalaropsis* monomer and *Chalaropsis* dimer, respectively. Nonreducing, 1,6-anhydro-NAM-containing disaccharide peptide monomers (anhydro monomer) were purified from *Escherichia coli* endopeptidase-transglycosylase digests of intact strain RD5 PG as described previously (41). MDP (adjuvant peptide) and *E. coli* O111:B4 LPS were purchased from Sigma Chemical Co. (St. Louis, Mo.).

PG fragments were treated with polymyxin B-agarose (Sigma) prior to injection to reduce residual endotoxin (41). The concentration of residual endotoxin in PG preparations was determined by *Limulus* amoebocyte lysate assay (Associates of Cape Cod, Inc., Woods Hole, Mass.) as described in the manufacturer's instructions.

All test substances were suspended in pyrogen-free water (Abbott Laboratories, Chicago, Ill.) and stored at -20°C , with the exception of the S-*O*-PG and S-non-*O*-PG, which were stored at 4°C . Just prior to injection, the test substances were suspended to the appropriate concentration in 0.9% NaCl (Abbott).

Data analysis. Data are presented as the mean ($\pm$ standard deviation) 12- or 24-h food consumption or 24-h body weight gain of groups of at least seven rats. In some experiments, data are presented as the percent decrease in food intake or body weight gain which is defined as follows: $[1 - (\text{mean food intake or body weight gain of test group} / \text{mean food intake or body weight gain of control group})] \times 100$. Group means were compared by using a one-way analysis of variance, and when statistically significant differences were detected, multiple comparisons were performed by using either the Student-Newman-Keuls or Dunnett's test; a P of <0.05 was considered significant. All statistical functions were performed by using Sigmastat software (Jandel Scientific, San Rafael, Calif.).

RESULTS

S-*O*-PG decreases food consumption and body weight gain in a dose-dependent fashion. Rats receiving a single i.p. injection of the highest concentration of S-*O*-PG tested (240 $\mu\text{g/kg}$ of body weight) exhibited a 40% decrease in food intake compared with the saline-injected control animals ($P < 0.05$) (Fig. 1A), whereas the group receiving the intermediate dose (24 $\mu\text{g/kg}$) decreased food intake by 15% ($P < 0.05$). The lowest concentration of S-*O*-PG tested (2.4 $\mu\text{g/kg}$) had no significant effect on food consumption. The hypophagic response of the rats to the highest concentration of PG was

FIG. 1. S-*O*-PG decreases food intake (A) and body weight gain (B) dose dependently in Lewis rats. Symbols: ▲, saline; —○—, 240 μg of S-*O*-PG per kg; —○—, 24 μg of S-*O*-PG per kg; ···○···, 2.4 μg of S-*O*-PG per kg. The error bars indicate standard deviations for a group of nine rats. The arrow indicates the day of injection. *, $P < 0.05$ versus saline controls.

significantly greater than that to the intermediate concentration ($P < 0.05$).

The hypophagic response to PG occurred within the first 12 h after injection and was apparently of short duration since the PG-injected animals consumed food in amounts indistinguishable from control animals during the daylight phase, 12 to 24 h postinjection (data not shown), and the ensuing 12-h dark phase (Fig. 1A).

The effect of PG on food consumption was, as expected, accompanied by a corresponding dose-dependent decrease in body weight gain. S-*O*-PG (240 and 24 $\mu\text{g/kg}$) decreased body weight gain in rats by 64 and 35%, respectively, compared with the saline-injected controls ($P < 0.05$) (Fig. 1B), whereas the lowest dose tested (2.4 $\mu\text{g/kg}$) had no significant effect. Data presented in Fig. 1 are from one experiment, but a dose-dependent suppression of food intake and weight gain was confirmed in each of nine experiments using 240 $\mu\text{g/kg}$ and four experiments using 24 $\mu\text{g/kg}$.

To eliminate the possibility that the S-*O*-PG-mediated suppression of food intake was due to residual contaminating endotoxin, we determined the minimum hypophagic dose of the frequently employed smooth-LPS standard from *E. coli* O111:B4 and compared this with the level of endotoxin detected in the S-*O*-PG preparations by the *Limulus* amoebocyte

FIG. 2. LPS decreases food intake dose dependently in Lewis rats. Symbols: ▲, saline; —■—, 10 µg of LPS per kg; ---■---, 2.5 µg of LPS per kg; ···■···, 0.6 µg of LPS per kg. The error bars indicate standard deviations for a group of nine rats. The arrow indicates the day of injection. *, $P < 0.05$ versus saline controls.

cyte lysate assay. In our experience, the lowest dose of LPS which significantly decreased food consumption was 10 µg/kg ($P < 0.05$ versus saline control) (Fig. 2). The amount of *Limulus* assay-reactive material in preparations of S-O-PG was equivalent to a concentration of 1 ng of endotoxin per 15 µg of S-O-PG or less. It should be emphasized that this figure probably represents a gross overestimation of endotoxin contamination since S-O-PG itself is positive in the *Limulus* assay (18). In fact, the *Limulus* assay reactivity of other S-O-PG preparations is reduced by as much as 90% upon digestion of the glycan backbone with PG-specific muramidases (41). Thus, even if all *Limulus*-reactive material in the S-O-PG preparations was due to contaminating LPS, the level of LPS would be less than 1/1,000 of the amount necessary for LPS alone to decrease appetite.

Rats do not compensate for PG-mediated hypophagia. To determine if rats compensate for decreased caloric intake by overeating subsequent to PG-induced hypophagia, food consumption was monitored for 10 days after injection of S-O-PG (240 µg/kg) in one experiment. As in previous experiments, PG-injected rats decreased food intake immediately after administration but resumed normal food intake by 24 h postinjection. At no time during the 10-day observation period did PG-treated rats consume more than the saline-injected rats (Fig. 3); hence, rats did not compensate for lost calories by rebound hyperphagia.

O-Acetylation of PG is not required for hypophagic response. S-O-PG preparations, as employed in experiments discussed so far, possess *O*-acetyl derivatives on C-6 of about 50% of the NAM residues (51). This chemical configuration of PG has been associated with resistance to degradation by PG hydrolases (43, 51) and increased potency in some biological activities, including arthritogenicity (12, 42) and stimulation of IL-1 release (37); thus, we hypothesized that S-O-PG might be a more potent appetite suppressant than S-non-O-PG. The data suggested, however, that *O*-acetylation of PG is not necessary for hypophagia in rats (Table 1). At concentrations of 240 and 24 µg/kg, both S-O-PG and S-non-O-PG decreased food intake and body weight gain compared with saline controls ($P < 0.05$), but the effects of S-O-PG and S-non-O-PG

FIG. 3. S-O-PG-induced hypophagia is not followed by rebound hyperphagia. Symbols: ▲, saline; ○, 240 µg of S-O-PG per kg. The error bars indicate standard deviations for a group of nine rats. The arrow indicates the day of injection. *, $P < 0.05$ versus saline control.

did not differ significantly from each other. These results were confirmed in two additional experiments.

Low-molecular-weight PG fragments are appetite suppressants. The previous experiments, all utilizing soluble macromolecular PG fragments, support the hypothesis that PG contributes to the hypophagia frequently observed during bacterial illness; however, since many physiologically realistic PG fragments are of low molecular weight, we assessed the effects of dimeric, monomeric, and submonomeric PG fragments on food consumption and body weight gain. Reducing un-cross-linked disaccharide peptide monomers and peptide cross-linked dimers (1.6 mg/kg), prepared by using *Chalaropsis* B muramidase, had no effect on food consumption, whereas the 1,6-anhydro-NAM-containing monomers and MDP (1.6 mg/kg) both reduced food intake by approximately 25% compared with controls ($P < 0.05$) (Table 2). Similarly, PG dimers and monomers, prepared with *Chalaropsis* B muramidase, had no effect on body weight gain, while 1,6-anhydro-NAM-containing monomers and MDP decreased weight gain by greater than 50% ($P < 0.05$) (Table 2). Data presented are from one experiment; the differential ability of the PG fragments to reduce food consumption was confirmed in two additional experiments. Endotoxin contamination in 1,6-anhydro-NAM-containing monomer preparations was less than 1 ng/400 µg of monomer; in MDP preparations, contaminating endotoxin was present at less than the detection limit of 1 ng/2 mg of MDP. These concentrations of contaminating endotoxin are 2,500

TABLE 1. *O*-Acetylation of PG is not required for hypophagic effect

PG	Dose (µg/kg)	Effect of PG on ^a :	
		Food consumption (% decrease)	Wt gain (% decrease)
S-O-PG	240	28	66
S-non-O-PG	240	21	60
S-O-PG	24	16	40
S-non-O-PG	24	12	40

^a Determined 24 h after injection of PG fragments and expressed relative to saline controls; all decreases were significantly different from saline controls ($P < 0.05$).

TABLE 2. Effect of low-molecular-weight PG fragments on appetite and weight gain

PG fragment ^a	Effect of fragment on ^b :	
	Food consumption (% decrease)	Wt gain (% decrease)
MDP	23 ^c	54 ^c
Anhydro monomer	28 ^c	69 ^c
<i>Chalaropsis</i> monomer	4	13
<i>Chalaropsis</i> dimer	6	12

^a The dose of all low-molecular-weight PG fragments was 1.6 mg/kg, administered i.p.

^b Determined 12 h (food consumption) or 24 h (weight gain) after injection of PG fragments and expressed relative to saline-injected controls.

^c $P < 0.05$ versus saline controls.

and 12,500 times less, respectively, than the minimum hypophagic dose of LPS (Fig. 2).

PG is hypophagic in both F344 and Lewis rats. The F344 rat strain has been shown to be relatively resistant to PG-induced arthritis in an in vivo model in comparison with the Lewis rat strain (1, 55). Further, thioglycolate-elicited peritoneal macrophages from F344 rats produce significantly less IL-1 in vitro than do peritoneal macrophages from Lewis rats when stimulated by PG (37). Thus, we hypothesized that F344 rats might be relatively resistant to the hypophagic effects of PG. However, S-O-PG (240 and 24 μ g/kg) and MDP (1.6 mg/kg) decreased food consumption in both F344 and Lewis rats compared with saline controls ($P < 0.05$) (Table 3) and, at the doses tested, did so to similar extents. As expected, the decrease in food consumption in both rat strains was accompanied by a decrease in weight gain, in comparison with saline controls ($P < 0.05$) (Table 3).

PG fragments do not suppress food intake after oral administration. We determined if PG fragments could decrease food consumption and body weight gain after oral administration. Doses of S-O-PG and MDP as high as 5.0 mg/kg had no effect on rat food intake or weight gain (data not shown) when administered intragastrically by using a stainless-steel feeding needle inserted through the oral cavity. However, the same preparations of S-O-PG and MDP at concentrations of 250 and 1.6 mg/kg, respectively, resulted in significant decreases in food consumption when injected i.p. just as other preparations (Fig. 1A and Table 2) had previously.

DISCUSSION

PG was once believed to be merely a biologically inert corset that determined cell shape and maintained bacterial integrity;

TABLE 3. PG decreases food intake and weight gain in Lewis and F344 rats

PG fragment (dose)	Rat strain	Effect of fragment on ^a :	
		Food consumption (% decrease)	Wt gain (% decrease)
S-O-PG (240 μ g/kg)	Lewis	27	73
	F344	32	85
S-O-PG (24 μ g/kg)	Lewis	15	32
	F344	15	40
MDP (1.6 mg/kg)	Lewis	21	54
	F344	19	64

^a Determined 24 h after injection of PG fragments and expressed relative to saline-injected controls; all decreases were significantly different from saline controls ($P < 0.05$).

however, it is now clear that PG is a potent biological effector both in vivo and in vitro (13). Within the last 20 years, it was noted that PG possesses other activities, e.g., pyrogenicity (28, 29) and somnogenicity (19, 27), suggesting that PG impacts central nervous system function in addition to its well-documented effects on the immune system. PG may evoke fever and sleep via an IL-1-mediated mechanism since IL-1 is known to be both pyrogenic (7) and somnogenic (24) and since PG induces release of IL-1 from macrophages (34) and glial cells (37). Herein, we report another bioactivity of PG, suppression of appetite, that is normally controlled centrally. The data suggest that S-O-PG decreases food intake and body weight gain in rats dose dependently after a single i.p. injection. The effect is of relatively short duration, evident during the first 12 h after injection but not thereafter. Rats do not compensate for loss of caloric intake by overeating once the hypophagic effect of PG subsides.

Chemical modification of the glycosidic PG backbone by O-acetylation of the C-6 of the NAM residues seems to confer resistance to enzymatic degradation of PG (43) and is associated with maximum potency of PG for some bioactivities (12, 42). Hence, we hypothesized that S-O-PG would be a more potent appetite suppressant than S-non-O-PG; however, both configurations of PG fragments decreased food intake to the same extent. One explanation is that the hypophagic effect of PG is initiated shortly after administration such that appetite suppression is signaled before PG degradation occurs. Alternatively, one might hypothesize that if the bioactive component of the PG preparations is a low-molecular-weight product of PG degradation, the S-non-O-PG would be more potent than the S-O-PG since it is more readily digested in vitro; however, our data do not support this theory either. In fact, further experiments exploiting our inventory of PG monomers and dimers argue that low-molecular-weight PG fragments are less potent than macromolecular PG on both a weight and a molar basis.

Only two of the four low-molecular-weight fragments tested display hypophagic potential in the rat. The 1,6-anhydro-NAM-containing monomers and MDP, both previously documented to be potent somnogens (23, 25), are effective appetite suppressants; hence, glycosidic linkage is not required for hypophagic effect. However, neither reducing un-cross-linked disaccharide peptide monomers, documented to be active somnogens (14, 19), arthritogens (12), and IL-1 inducers (37), nor peptide cross-linked dimers are active. It is not surprising that the latter do not suppress appetite since peptide cross-linked dimers have been inactive in all biological activities tested thus far, but it is presently unclear what accounts for the inactivity of reducing monomers in light of their biological potency in other systems. It does not seem likely that the reducing NAM itself confers inactivity since the identical structure is present in MDP and at the ends of S-O-PG.

S-O-PG decreases food intake and weight gain in both the F344 and Lewis strains of rats. While F344 rats have been shown to be more resistant than Lewis rats to PG-induced arthritis in an in vivo model (1, 55), this difference is believed to be due to a defective hypothalamic-pituitary-adrenal (HPA) axis response to bacterial cell wall products and IL-1 in Lewis rats (50). Thus, resistance to cell wall-induced arthritis in F344 rats appears regulated by a functional negative feedback loop involving the HPA axis. On the basis of current data, it appears that the HPA axis may not play a prominent role in regulation of PG-induced hypophagia.

In addition to the difference in susceptibility to arthritis, supernatants from PG-stimulated, thioglycolate-elicited peritoneal macrophages from F344 rats are less potent than

supernatants from Lewis rat macrophages in stimulating thymocyte proliferation (37), an *in vitro* measure of IL-1 bioactivity. Others have shown that resident macrophages from Buffalo rats, another PG-induced arthritis-resistant strain, secrete less biologically active and immunoreactive IL-1 than resident macrophages from Lewis rats upon stimulation with PG (3); however, Lewis and Buffalo rat macrophages express equivalent levels of IL-1 mRNA, IL-1 receptor antagonist (IL-1ra) mRNA, and IL-1ra. Thus, the differential effects of PG on production of IL-1 from Lewis rat macrophages versus Buffalo rat macrophages is apparently due to a posttranscriptional mechanism. It is not known whether differences in levels of IL-1 mRNA, IL-1ra mRNA, or IL-1ra exist between PG-stimulated F344 and Lewis macrophages. Although more IL-1 bioactivity is detected in supernatants from PG-treated Lewis macrophages than from similarly treated F344 macrophages *in vitro*, we have not eliminated the possibility that IL-1 is involved in mediating the hypophagic effect of PG in rats, despite the observation that appetite appears to be suppressed to similar degrees in Lewis and F344 rats.

Both S-O-PG and MDP fail to elicit a hypophagic response in rats after oral administration, even at doses 20 and 3 times higher, respectively, than doses which are effective when administered *i.p.* MDP has been documented previously to increase slow-wave sleep in rats after oral administration of the same dose used in the appetite experiment (22); therefore, it seems that this route of administration does not destroy bioactivity of the molecule but rather may diminish its access to cells necessary for potentiation of appetite suppression.

We have discounted the possibility that the PG-mediated suppression of appetite is due to contaminating endotoxin by pretreating PG preparations with polymyxin B-agarose and demonstrating that the concentration of residual endotoxin is several orders of magnitude less than that which is required for LPS alone to decrease food intake. Our estimate of 10 µg/kg as the minimum hypophagic dose of LPS is within twofold of the minimum effective dose of LPS reported by others (30) and only about twice that of S-O-PG. The similar potencies of LPS and PG as appetite suppressants are striking since, in most other systems studied to date, LPS is by far the more potent. Obviously, various chemical and physical forms of LPS differ widely in biological potency (33), and some might be considerably more potent than standard O111:B4 LPS. However, it appears that appetite suppression may be one of the rare biological reactions in which PG and LPS have similar potencies.

It is interesting, and perhaps not merely coincidental, that three cardinal features of infection, *i.e.*, fever, enhanced sleep, and decreased appetite, are regulated in the central nervous system at the hypothalamus and involve serotonergic pathways (5, 54). It is known that the pyrogenic and somnogenic effects of MDP involve serotonin (38), and MDP has been shown to compete with serotonin for binding to a murine monocytic cell line (46, 47), brain cells (45), and myelin basic protein (39). However, it is unknown at this time if PG modulates appetite via a serotonergic mechanism.

We have not ruled out the possibility that the reason rats are hypophagic after PG administration is because they are merely sleeping more and, hence, eating less. In fact, PG may decrease appetite and increase sleepiness simultaneously via similar mechanisms. We hope to separate the hypophagic and somnogenic effects of PG in future experiments as has been done with tumor necrosis factor (15).

From the data presented, we conclude that PG decreases food intake and body weight gain in rats when administered *i.p.* and that neither *O*-acetylation nor glycosidic linkage of PG

fragments is required for activity. These data are consistent with the hypothesis that PG fragments may contribute to loss of appetite during bacterial illness.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI14826 from the National Institutes of Health.

The expert technical assistance of Lora English and Adam Dalheim is gratefully acknowledged.

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