Inhibition of TNF-α production contributes to the attenuation of LPS-induced hypophagia by pentoxifylline

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Porter, M. H., B. J. Hrupka, G. Altreuther, M. Arnold, and W. Langhans. Inhibition of TNF-α production contributes to the attenuation of LPS-induced hypophagia by pentoxifylline. Am J Physiol Regulatory Integrative Comp Physiol 279: R2113–R2120, 2000.—Cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) are assumed to mediate anorexia during bacterial infections. To improve our understanding of the role that these two cytokines serve in mediating infection during anorexia, we investigated the ability of pentoxifylline (PTX), a potent inhibitor of TNF-α production, to block the anorectic effects of the bacterial products lipopolysaccharide (LPS) and muramyl dipeptide (MDP) in rats. Intraperitoneally injected PTX (100 mg/kg body wt) completely eliminated the anorectic effect of intraperitoneally injection LPS (100 μg/kg body wt) and attenuated the anorectic effect of a higher dose of intraperitoneally injected LPS (250 μg/kg body wt). Concurrently, PTX pretreatment suppressed low-dose LPS-induced TNF-α production by more than 95% and IL-1β production 39%, as measured by ELISA. Similarly, high-dose LPS-induced TNF-α production was reduced by 90%. PTX administration also attenuated the tolerance that is normally observed with a second injection of LPS. In addition, PTX pretreatment attenuated the hypophagic effect of intraperitoneally injected MDP (2 mg/kg body wt) but had no effect on the anorectic response to intraperitoneally injected recombinant human TNF-α (150 μg/kg body wt). The results suggest that suppression of TNF-α production is sufficient to attenuate LPS- and MDP-induced anorexia. This is consistent with the hypothesis that TNF-α plays a major role in the anorexia associated with bacterial infection.

lipopolysaccharide; muramyl dipeptide; interleukin-1β; cytokines; tolerance; food intake

ACUTE BACTERIAL INFECTIONS and other pathophysiological processes are associated with anorexia. Anorexia is also observed after parenteral administration of lipopolysaccharides (LPS), which are the major constituents of the outer cell wall of gram-negative bacteria, and muramyl dipeptide (MDP), which is the minimal immunologically active structure of gram-positive bacterial cell walls. Administration of either LPS or MDP results in the expression of cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) (28, 39). These cytokines act at central (and perhaps peripheral) target sites to reduce food intake through complex mechanisms (16, 21, 30) and are believed to mediate LPS- and MDP-induced anorexia. Cytokine interaction in anorexia during bacterial infections is supported by the observation that TNF-α and IL-1β have a synergistic effect in inducing anorexia (36, 37). Several studies have used cytokine-receptor antagonists and transgenic animals to examine the role of cytokines in infection-induced anorexia (e.g., 5, 14, 15, 24) with inconclusive results.

Another way of studying cytokine mediation of LPS- and MDP-induced anorexia is to inhibit the endogenous synthesis of TNF-α or IL-1β in LPS- and MDP-treated animals. The nonspecific phosphodiesterase (PDE) inhibitor pentoxifylline (PTX) is known to inhibit TNF-α production after LPS stimulation both in vivo and in vitro (3, 9, 35). In the present study, we examined the ability of PTX to inhibit the anorexia associated with the intraperitoneal injection of LPS in rats. In addition, the production of TNF-α and IL-1β was quantified after LPS administration under the same conditions in PTX-pretreated rats to determine if there was any relationship between the suppressive effects of PTX on cytokine production and a possible attenuation of LPS-induced hypophagia. The possibility that PTX inhibition of cytokine synthesis might alter the development of tolerance to LPS-induced hypophagia was also investigated. In a second experiment, the ability of PTX to attenuate the anorexia in response to MDP administration was evaluated. Finally, we studied the ability of PTX to inhibit the hypophagia associated with exogenously administered TNF-α to provide evidence that blockade of TNF-α synthesis is the critical mechanism in a possible effect of PTX on LPS-induced anorexia.

METHODS

Animals and housing conditions. Adult male, Sprague-Dawley rats (Charles River Laboratories, Sulzfeld, Germany) were used. They were individually housed in temperature-controlled (22 ± 0.5°C) colony rooms in stainless steel wire-bottom drawer cages. The rats were kept on an artificial 12:12-h light-dark cycle with the lights on from 2200 to 1000 and fed a ground rat chow diet (Nafag, Gossau, Switzerland).
Food intake was measured by manually weighing (±0.1 g) the feeding cups at 2, 4, 6, 8, 10, 12, and 24 h after injections. Spillage, collected on paper spread beneath the cages, was also measured. Food and tap water were available ad libitum. Before experiments, the rats were adapted to diet and experimental conditions for at least 2 wk. Each experiment was performed with a different group of rats. All procedures and protocols were approved by the Kanton of Zurich’s Animal Use and Care Committee.

Test procedures. On test days, groups of rats were matched for food intake and body weight during the preceding dark phase. In each study, all rats received a single intraperitoneal injection (pretreatment) of drug or corresponding vehicle solution followed 1 h later by another intraperitoneal injection (treatment) of drug or corresponding vehicle solution. Treatment injections were administered ~15 min before the onset of the dark cycle. Drug solutions were freshly prepared before injections.

For all feeding experiments, the pretreatment injections consisted of either 100 mg/kg body wt PTX (Sigma, dissolved in sterile isotonic saline) delivered in 0.1 ml/100 g rat or isotonic, pyrogen-free saline (NaCl). The treatment injection was either LPS (from *Escheria coli* serotype 0111:B4, Sigma L-2630, dissolved in sterile, isotonic saline), MDP (adjuvant peptide, Sigma, no. A-9519, dissolved in sterile, isotonic saline), or recombinant human TNF-α (rhTNF-α; Endotec, discarded, solved in a buffer consisting of sterile, filtered PBS containing 0.1% pyrogen-free BSA). Control rats received an equivalent volume of the appropriate vehicle. The dose of PTX was determined from previous experiments examining its ability to attenuate TNF-α production in LPS-treated animals (3, 29). The doses of LPS, MDP, and TNF-α were selected on the basis of previous studies comparing the effectiveness of these compounds to suppress food intake in rats (17, 31).

Experiment 1. The effect of PTX pretreatment on the anorectic response to LPS (100 μg/kg body wt, delivered in 0.1 ml/100 g body wt) was tested in 28 rats (mean body wt 252 g) that were distributed into four groups (*n* = 7). The four groups were as follows: PTX pretreatment followed by LPS treatment (PTX-LPS), NaCl pretreatment followed by LPS treatment (NaCl-LPS), NaCl pretreatment followed by NaCl treatment (NaCl-NaCl), and PTX pretreatment followed by NaCl treatment (PTX-NaCl). Food intake was measured as previously described.

A previous study in this laboratory demonstrated that repeated exposure to LPS results in the rapid development of tolerance to the anorectic effect of LPS (31). Therefore, 24 h after the completion of the first study, the same rats as in the first trial received a second injection of LPS (100 μg/kg body wt) or vehicle to examine the effect of PTX on the induction of tolerance by LPS.

Experiment 2. The effect of PTX pretreatment on the anorectic response to MDP (2 mg/kg body wt, delivered in 0.1 ml/100 g rat) was tested in 28 rats (mean body wt 288 g) that were distributed into four groups (*n* = 7) as explained in the first experiment, except that MDP was used instead of LPS. Food intake was then measured as previously described. A second injection of MDP was not given because MDP does not lose its anorectic effect with repetitive intraperitoneal injections (17).

Experiment 3. The effect of PTX pretreatment on the feeding response to rhTNF-α (150 μg/kg body wt, delivered in 0.1 ml/100 g rat) was tested in 28 rats (mean body wt 280 g) that were distributed into four groups (PTX-TNF, NaCl-TNF, NaCl-vehicle (Veh), PTX-Veh, *n* = 7 each) as described.

Experiment 4. The rhTNF-α (150 μg/kg body wt) administered in experiment 3 suppressed feeding more than the LPS (100 μg/kg body wt) administered in experiment 1. Therefore, we performed a fourth experiment to assess the ability of PTX (100 mg/kg body wt) to inhibit the hypophagia in response to a higher dose of LPS (250 μg/kg body wt) that would more closely resemble the hypophagia observed with rhTNF-α. The experiment was performed with 28 rats (mean body wt 252 g) in the same manner as experiment 1, except for the higher dose of LPS used, and the second injection of LPS was not administered to examine the effect on tolerance.

TNF-α and IL-1β determination after LPS administration. Animals received either PTX- or NaCl-pretreatment injections followed by either LPS- or NaCl-treatment injections as previously described. Plasma samples were taken 5 min after the completion of the treatment injections, a time point at which both TNF-α and IL-1β plasma levels are elevated in response to LPS (1). Rats were anesthetized by intraperitoneal injection (1.00 ml/kg body wt) of a mixture of 80 mg/ml ketamine (Ketalar-100, Grubh), 20 mg/ml xylazine (Rompun, Bayer), and 0.05 mg/ml acepromazine (Sedaline, Chassot and Cie). A cutaneous incision on the midline of the upper abdomen was made that extended to the chest cavity, exposing the heart. Blood was aspirated by heart puncture with a needle and syringe. Blood was then placed in polypropylene test tubes treated with 40 μl EDTA solution and stored on ice until the end of the collection procedure. Samples were then centrifuged (1,500 g at 4°C for 10 min). Plasma was placed in microcentrifuge tubes for storage at −80°C until cytokine determination.

TNF-α and IL-1β analyses were performed for experiment 1 (low dose LPS: 100 μg/kg body wt), whereas only TNF-α was analyzed for experiment 4 (high dose LPS: 250 μg/kg body wt).

Biotak cellular communication assays (Amersham Life Science) were used for quantitative determination of rat TNF-α and IL-1β in plasma. These TNF-α and IL-1β assays are based on a solid-phase ELISA that uses an antibody for either rat TNF-α or rat IL-1β bound to the wells of a microtitre plate together with a biotinylated detection antibody to either rat TNF-α or rat IL-1β and streptavidin conjugated to horseradish peroxidase. Both assays are highly sensitive (TNF-α: <10 pg/ml, IL-1β: <8 pg/ml) and specific for either rat TNF-α or IL-1β.

Statistics. Differences between group means were tested using an analysis of variance appropriate for a 2 × 2 factorial arrangement of PTX and LPS followed by a modified *t*-test when appropriate. *P* values <0.05 were considered significant.

RESULTS

Pretreatment with PTX (as compared with NaCl pretreatment) clearly attenuated the hypophagia after intraperitoneal injection of 100 μg LPS/kg body wt (Fig. 1). At every time point measured, food intake was significantly lower in NaCl-LPS rats than in the rats of the other three groups. PTX pretreatment did not affect the food intake of NaCl-treated animals.

After the second injection of 100 μg LPS/kg body wt in PTX-pretreated rats (administered 48 h after the first injection of LPS), food intake started to decline compared with NaCl-LPS animals at 8 h. This difference reached statistical significance at 24 h (Fig. 2).

PTX pretreatment (when compared with NaCl) reduced LPS induction of TNF-α production by >95%
PTX pretreatment (when compared with NaCl pretreatment) again reduced LPS induction (250 μg/kg body wt) of TNF-α production significantly (P < 0.05; Fig. 8). TNF-α levels were miniscule in the NaCl-treatment groups and significantly lower than the TNF-α levels in LPS-treated animals (P < 0.05).

DISCUSSION

To our knowledge, this is the first study to demonstrate that the PDE inhibitor PTX is able to attenuate the anorexia in response to intraperitoneally administered LPS (100 μg/kg body wt; 250 μg/kg body wt) and MDP (2 mg/kg body wt) but is unable to inhibit the anorexia in response to intraperitoneal rhTNF-α (150 μg/kg body wt). PTX pretreatment also blocked TNF-α production in response to low (100 μg/kg body wt)- and high (250 μg/kg body wt)-dose LPS stimulation. Together, these findings support the hypothesis that endogenous TNF-α plays a major role in LPS-induced hypophagia. This interpretation is also consistent with a previous study from our laboratory (31) that showed that tolerance to the hypophagic effect of exogenous TNF-α is sufficient to eliminate LPS-induced hypophagia.

PTX pretreatment before low-dose LPS administration resulted in a significantly greater (P = 0.0023) suppression of TNF-α production (>95%) compared...
with the inhibition of IL-1β production (39%). TNF-α production in response to high-dose LPS administration was also significantly reduced (90%) by PTX pretreatment (IL-1β production was not measured in response to high-dose LPS stimulation). The decrease in IL-1β levels may be a direct result of TNF-α inhibition, because TNF-α stimulates IL-1β production (1). The literature is confusing concerning the ability of PTX to inhibit IL-1β production in models of bacterial infection. Depending on the experimental conditions, PTX has been shown to upregulate, downregulate, or have no effect on IL-1β production after administration of gram-negative or gram-positive bacterial products (8, 34, 38). Nevertheless, the present data do not allow for the exclusion of a role of IL-1β in LPS-induced anorexia. The results demonstrate, however, that without substantial TNF-α production, even high plasma levels of IL-1β (presumably ≥ the 61% of the NaCl-low-dose LPS group) are not sufficient to inhibit feeding to the same extent as in the presence of TNF-α. This observation is in agreement with other data suggesting a limited role for IL-1β alone in the anorexia of the LPS model of infection. For example, intraperitoneal or intracerebroventricularly administered IL-1β-receptor antagonist (IL-1ra) does not inhibit LPS-induced hypophagia (14). More recently, IL-1β-converting enzyme-deficient mice were shown to resist the anorectic effect of intracerebroventricularly but not intraperitoneally administered LPS (4). In addition, tolerance to the hypophagic effect of LPS is accompanied by the absence of an increase in serum TNF-α in response to subsequent LPS stimulation, whereas IL-1β production is not affected or even augmented (17, 33). Furthermore, sensitization to the anorectic effect of IL-1β after repeated administration does not alter the hypophagic effect of LPS on feeding (22), as would be expected if endogenous IL-1β mediates LPS-induced anorexia. The lack of IL-1β in transgenic mice also does not prevent the anorexia induced by LPS or influenza virus (15). It should be noted, however, that LPS also reduces food intake in TNF double-receptor knockout mice (24). Thus IL-1β and, in particular, TNF-α presumably play a substantial role in LPS-induced anorexia, which becomes evident when these cytokines or their actions are acutely antagonized. On the other hand, IL-1β and TNF-α are not necessary for LPS-induced hypophagia. Yet, it is reasonable to assume that transgenic animals do not discretely evaluate how the intact system works and are probably not the best models to investigate LPS-induced hypophagia, be-
cause the knockout strategy does not account for adaptive and compensatory mechanisms during development that may modify the connection between hypophagia and the knockout gene product (TNF-α or IL-1β). PTX pretreatment also partially inhibited the development of tolerance to low-dose LPS-induced hypophagia in the first experiment. This may be the result of PTX’s ability to block TNF-α expression after LPS stimulation. Studies indicate that LPS stimulation of cytokine production initiates mechanisms that lead to tolerance (11, 26). Thus by drastically attenuating TNF-α production after LPS administration, PTX may interfere with the initiation of tolerance.

Infections caused by gram-positive organisms, which contain sparse LPS, are also accompanied by elevated plasma levels of TNF-α and IL-1β (39). In agreement with results from a previous study in this laboratory (17), administration of 2 mg/kg body wt MDP significantly inhibited food intake in the second experiment. The novel finding in this study is that PTX pretreatment was sufficient to completely attenuate MDP-induced hypophagia. From this observation, it can be speculated that TNF-α production plays a significant role in MDP-induced hypophagia. Further studies are necessary to substantiate this possibility.

PTX blocks TNF-α production (in response to both gram-positive and gram-negative bacteria) by inhibiting the synthesis of TNF-α mRNA (10, 13, 41). In the third experiment, PTX did not alter the anorexia associated with administration of exogenous rhTNF-α. This indicates that PTX does not act downstream of TNF-α production and is therefore consistent with the hypothesis that PTX blocks LPS- and MDP-induced hypophagia by inhibiting the synthesis of endogenous TNF-α. The failure of PTX to attenuate the anorectic effect of TNF-α was not due to the magnitude of TNF-α’s effect, because PTX attenuated the even stronger anorexia induced by the high LPS dose in experiment 4.

Substances other than PTX also inhibit TNF-α production and attenuate LPS-induced hypophagia. For example, pretreatment with the calcium-channel blocker verapamil inhibits the hypophagic effect of LPS (20), suggesting a calcium-sensitive mechanism is involved. Similar to PTX, verapamil also suppresses both LPS-induced TNF-α and IL-1β increases in plasma (12) but does not attenuate the hypophagic effect of exogenously administered TNF-α (18).

Glucocorticoids also inhibit LPS- and MDP-induced hypophagia (16). Similar to PTX and verapamil, dexamethasone blocks TNF-α production (10, 13). Therefore, glucocorticoid treatment for the anorexia of acute bacterial infections may be beneficial. However, many of the side effects of glucocorticoids preclude their sustained administration (2).

Eicosanoids are also implicated in the anorexia associated with the administration of bacterial products, because LPS and MDP hypophagia is inhibited by the antipyretic drug indomethacin (19, 20). It is unlikely

Fig. 5. Effect of PTX pretreatment (100 mg/kg body wt) on the hypophagic response to muramyl dipeptide (MDP; 2 mg/kg body wt). Each value represents the mean ± SE of 7 rats. *Values of the NaCl-MDP group are significantly lower than the values of the other 3 groups (P < 0.05, modified t-test after significant ANOVA).

Fig. 6. Effect of PTX pretreatment (100 mg/kg body wt) on the hypophagic response to recombinant human TNF-α (150 μg/kg body wt). Each value represents the mean ± SE of 7 rats. *Values of TNF-α treatment groups are significantly lower than values of vehicle-treatment groups (P < 0.05, modified t-test after significant ANOVA).
that indomethacin inhibits LPS- and MDP-induced anorexia through the same mechanism as PTX, because indomethacin actually increases rather than suppresses LPS-stimulated TNF-α production (27) under most circumstances.

The molecular mechanisms by which PTX and other compounds inhibit TNF-α synthesis remain unclear. PTX prevents the degradation of cAMP, leading to an increase in the intracellular concentration of cAMP, which suppresses TNF-α production (9, 13, 35, 41). Increased cAMP activates protein kinase A (PKA), which catalyzes the phosphorylation of proteins, altering their conformation and activity. The modes of action by which activation of the cAMP/PKA cascade affects molecular mechanisms responsible for TNF-α production (and, to a lesser extent, IL-1β production) remain unclear but are the focus of ongoing research (40). Perhaps increased PKA activity alters factors such as activation of phospholipase C, ceramide-activated protein kinase, protein kinase C, and protein tyrosine kinase, which are known to play a role in LPS induction of cytokine synthesis (32).

In conclusion, our results suggest that PTX, mainly through its ability to drastically inhibit TNF-α production, can either inhibit or significantly attenuate the anorexia associated with LPS and MDP administration. A role for IL-1β in LPS-induced hypophagia cannot be excluded due to the pleiotropic actions of cytokines. However, the data do indicate that without substantial TNF-α production, even IL-1β plasma levels at 61% (PTX-LPS) of control (NaCl-LPS) have absolutely no effect on feeding. This observation corresponds with other data that indicate that IL-1β is not necessary for LPS-induced hypophagia (14, 15, 22). On the contrary, our results suggest that inhibition of TNF-α by PTX is crucial for the elimination of LPS-induced anorexia. In addition, suppression of TNF-α production also appears capable of antagonizing MDP-induced hypophagia, despite evidence that these compounds (LPS and MDP) do not inhibit feeding through the same mechanisms (17).

**Perspectives**

PTX alone, or in combination with other agents, may prove beneficial in treating anorexia during acute and chronic disease. PTX is an established drug with no severe side effects (41) and may improve therapeutic strategies in a variety of clinical situations that involve TNF-α. Several disease models and clinical studies suggest a beneficial effect of TNF-α blockade by PTX administration. PTX was found to prevent mortality during endotoxic shock and to inhibit the inflammatory response. A role for IL-1β in LPS-induced hypophagia cannot be excluded due to the pleiotropic actions of cytokines. However, the data do indicate that without substantial TNF-α production, even IL-1β plasma levels at 61% (PTX-LPS) of control (NaCl-LPS) have absolutely no effect on feeding. This observation corresponds with other data that indicate that IL-1β is not necessary for LPS-induced hypophagia (14, 15, 22). On the contrary, our results suggest that inhibition of TNF-α by PTX is crucial for the elimination of LPS-induced anorexia. In addition, suppression of TNF-α production also appears capable of antagonizing MDP-induced hypophagia, despite evidence that these compounds (LPS and MDP) do not inhibit feeding through the same mechanisms (17).
action of TNF-α on neutrophil function (29). In rheumatoid arthritis, PTX treatment resulted in a significant improvement in 50% of patients (25). PTX decreases TNF-α mRNA accumulation and TNF-α production in human immunodeficiency virus (HIV)-infected patients (6) and decreases HIV replication (7). PTX is also beneficial in the treatment of premature infants with sepsis complicated by shock (23). Further studies are necessary to determine whether PTX administration can be beneficial in attenuating the anorexia associated with acute and chronic pathophysiological processes in humans.

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