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Effect of Protein Synthesis Inhibitors on Leukocytic Pyrogen-Induced *in Vitro* Hypothalamic Prostaglandin Production

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In order to study the antipyretic effect of inhibitors of protein synthesis, hypothalamic tissue was incubated *in vitro* under controlled conditions and the amount of prostaglandin E_2 (PGE_2) measured in the supernatant medium. Rabbit anterior hypothalamic tissue was incubated with purified human leukocytic pyrogen (LP) and after 60 minutes the supernatant fluid was assayed for PGE_2 by radioimmunoassay. Control tissue incubated with Eagle's medium (MEM) released elevated levels of PGE_2; however, the additon of polymyxin B (PmxB), a cationic antibiotic which blocks the activities of bacterial endotoxins, significantly reduced PGE_2. In addition, endotoxin added to MEM induced from the brain tissue PGE_2 production which could be reduced by the addition of PmxB. Thus, commercial culture media such as MEM may contain sufficient amounts of endotoxin to stimulate brain PGE_2 production *in vitro*. Purified human LP incubated with hypothalamic tissue in the presence of PmxB induced PGE_2 production in a dose-dependent fashion. This release could be reduced ($p < 0.001$) by the presence of either cycloheximide or puromycin during incubation with LP. The addition of these inhibitors to unstimulated hypothalamic tissue incubations did not reduce background levels of PGE_2. It is concluded that the antipyretic effect of protein synthesis inhibitors results in a specific decrease in LP-induced levels of PGE_2.

INTRODUCTION

Substantial evidence exists that leukocytic pyrogen (LP) produces fever by first stimulating the production of prostaglandin E (PGE) in the anterior hypothalamus [1,2,3]. Once released, PGE and possibly other cyclo-oxygenase products bring about a rise in the temperature set-point of the hypothalamus with the subsequent development of a fever by physiologic (e.g, shivering and vasoconstriction) and behavioral means [4]. Recently we have shown that purified LP induces the synthesis of PGE_2 from rabbit brain tissue *in vitro* [5]. This response is dose-related and can be eliminated by inactivation of LP with moderate heating. An advantage of this *in vitro* assay is that the effects of LP-induced PGE_2 synthesis as well as other neurochemical changes can be directly studied under controlled conditions.

In addition to inhibitors of cyclo-oxygenase such as aspirin and indomethacin, inhibitors of protein synthesis such as cycloheximide (Cyhx) and anisomycin have been shown to act as antipyretic agents [6]. Injection of Cyhx into rabbits prevents fever due to intravenous injection of endotoxin or LP [7,8,9]. The mechanism of this antipyresis,

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however, is uncertain. With this fact in mind, the current study was undertaken to examine the effects of protein synthesis inhibitors (e.g., puromycin and Cyhx) on the LP-induced production of PGE_2 from rabbit hypothalami. The results indicate that Cyhx and puromycin decrease LP-induced hypothalamic PGE_2 release. This response may be due to the ability of protein synthesis inhibitors to decrease the synthesis of enzyme(s) needed for prostaglandin synthesis.

METHODS AND MATERIALS

Purification of Human LP

Human LP was produced from plateletpheresis by-products rich in monocytes. The mononuclear cells were separated, suspended, and stimulated with heat-killed *Staphylococcus albus* as previously described [10]. The crude 24-hour supernatant was first fractionated using a Millipore Pellicon concentrating device (Millipore, Bedford, MA) which excluded molecules greater than 100 kD and concentrated molecules between 100 and 10 kD. This concentrate was then dialyzed against pH 7.4 phosphate-buffered saline and passed over an anti-human LP affinity column; the LP was eluted with citric acid as previously reported [11]. This material was then concentrated in 10 percent lysine HCl and chromatographed over Sephadex G-50 (fine), 170 × 5.0 cm in 0.15 M NaCl at 4°C. The 15 kD peak of pyrogen activity was isolated and concentrated in 3.5 kD cut-off dialysis bags submerged in polyethylene glycol, 8–10 kD. An aliquot of this material, when precipitated with trichloracetic acid and applied to 17 percent polyacrylamide gels in SDS, revealed a single band stained with Coomassie blue. LP intended for biological assay was not run on gels but assayed in New Zealand albino rabbits by intravenous injection. Rectal temperatures were recorded every minute using indwelling Yellow Spring thermistors (Yellow Spring Instruments, Yellow Spring, OH) on a Digistrip II constant recording device (Kaye Instruments, Bedford, MA). Each lot of purified LP was assayed in 2 kg rabbits, and peak fevers between 0.6 and 0.9°C were considered a rabbit pyrogen dose (RPD). Using this method, peak fever in 100 rabbits occurred at 46.9 ± 8.9 minutes (SD). Each lot of LP was aliquoted and kept frozen at −22°C until used.

Production of PGE_2 by Rabbit Brain Tissue

For each experiment, a rabbit was sacrificed by rapid CO_2 asphyxiation. Within one minute, the spinal cord was severed and the brain was removed from the rostral to frontal end, severing the pituitary stalk. The anterior hypothalamus was excised and quickly placed in ice-cold Eagle's minimal essential medium (MEM, Microbiological Associates, Walkerville, MD, containing 0.01 M HEPES buffer, 100 units penicillin G/ml, and 100 μg streptomycin/ml) as previously described [5]. The optic chiasm was dissected away and discarded. The hypothalamic tissue was then weighed, minced into 0.5 mm^3 sections, and suspended in MEM at a concentration of 10 mg/ml. 1.0 ml aliquots of the suspension were then added to 12 × 75 mm polypropylene tubes (Falcon Plastics, Oxnard, CA). The aliquots were incubated for 30 minutes in a 37°C shaking water bath. After this time, 3 ml of warm MEM were added to each tube and the suspensions then centrifuged at low speed (5 *g*) for five minutes. The supernatant fluid was discarded and 1 ml of fresh MEM, containing appropriate dilutions of various materials (that is, LP, endotoxin, or protein synthesis inhibitors), was added to duplicate tubes. The suspensions were then reincubated in the shaking water bath for an additional 60 minutes. Supernatants from these incubations were obtained by

low-speed centrifugation and immediately frozen in dry ice and stored at −70°C until assayed. Supernatants from each tube were assayed in duplicate for PGE_2 with a radioimmunoassay kit containing a specific antibody for PGE_2 (Seragen, Boston, MA), thus yielding four data points for each experimental incubation condition. The means of the duplicate PGE_2 assay results were used to calculate the mean for each duplicate incubation. Results are expressed as pg of PGE_2/mg of tissue ± SEM. The data analysis utilized the paired student's *t*-test.

Reagents

Cycloheximide was purchased from Boehringer-Mannheim and dissolved in MEM at a stock solution of 500 μg/ml and kept at −70°C. This was diluted in MEM to final concentrations and added to brain minces. Puromycin was obtained from Sigma and dissolved in MEM as described above for Cyhx. Polymyxin B was purchased from Pfizer, dissolved in pyrogen-free water, and stored at 1 mg/ml at 4°C. Endotoxin (*E. coli* RE-2) was obtained from the Bureau of Biologics (Bethesda, MD). For endotoxin levels, the limulus amebocyte lysate test was used (Associates of Cape Cod, Woods Hole, MA).

RESULTS

The Effect of Polymyxin B on Hypothalamic PGE_2 Production Induced by Endotoxin or LP

Endotoxin induces the release of LP both *in vitro* and *in vivo*. In additon, there are several experiments which suggest that endotoxin can also act directly on the brain and induce fever; recently endotoxin has been shown to stimulate brain cells to synthesize PGE_2 *in vitro* [5]. Since endotoxins are seemingly ubiquitous contaminants of many laboratory reagents, it was not surprising to detect significant levels of endotoxin in the MEM used in these studies. Endotoxin levels from 0.1–1 ng/ml were measured in six different lots of MEM. Polymyxin B, a positively charged antibiotic, has been shown to block or inhibit many of the multiple biological activities of endotoxins, including the induction of LP *in vitro* and *in vivo* [12,13]. Sections of hypothalami were incubated with either MEM alone, MEM containing 15 μg/ml of PmxB, MEM to which endotoxin had been added (30 pg/ml), or MEM containing both endotoxin and PmxB. As shown in Fig. 1, background levels of PGE_2 were elevated in control incubations of hypothalamic tissue in MEM. However, PmxB added to the MEM resulted in significantly lower levels of PGE_2. As an additional test, known amounts of endotoxin were added to MEM both in the absence and the presence of PmxB. As shown in Fig. 1, PmxB inhibits this endotoxin-induced release of PGE_2 from rabbit brain tissue.

The next experiments involved the dose-response effects of human LP in inducing PGE_2 from rabbit hypothalami in the presence of these same concentrations of PmxB. As shown in Fig. 2, PmxB had no effect on LP-induced PGE_2 production. In these experiments, four different lots of human LP purified by the same methods were used. In each case the activity of the LP in producing fever in the rabbit following intravenous injections was compared to the induction of PGE_2 *in vitro*. As shown, 10^{-3} RPD induced significant PGE_2 production ($p < 0.05$).

Effects of Protein Synthesis Inhibitors on Release of PGE_2

Anterior hypothalamic tissue was incubated with LP and cycloheximide (0.2 μg/ml). Following the preincubation, both LP and Cyhx were added within one minute

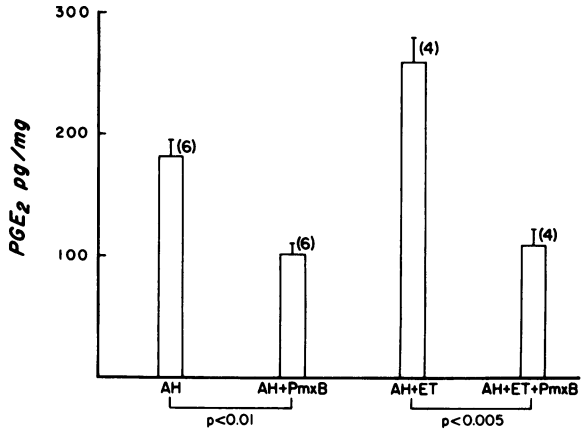

FIG. 1. Effects of various agents on the release of PGE_2 from rabbit anterior hypothalamic tissue. First bar (anterior hypothalamus, **AH**) is control value for MEM only. Polymyxin (10 units/ml—second bar) was present during preincubation and standard incubation at 15 μg/ml. Endotoxin (30 pg/ml) was added to stimulate PGE_2 release (third bar). AH was incubated with both endotoxin and PmxB (fourth bar). Results are ±SEM and numbers in parentheses represent the number of supernatants tested.

of each other at room temperature. These incubations were then returned to the water bath for 60 minutes as described in Methods. The results as depicted in Fig. 3 show that Cyhx does not inhibit the background release of PGE_2 from brain tissue ($p > 0.2$). On the other hand, Cyhx significantly reduces the release of PGE_2 from hypothalamic tissue incubated with purified human LP ($p < 0.001$). The ability of Cyhx to reduce LP-induced PGE_2 production was consistently observed in all experiments using all lots of LP.

In order to further clarify whether Cyhx was having a toxic effect on the brain cells or interfering with cell metabolism, an additional series of experiments was carried out in which the effect of Cyhx on PGE_2 production during the preincubation was studied. Cyhx was added to brain tissue either both during and after the preincubation step or only after the preincubation. Regardless of when Cyhx was added to the tissue, background levels of PGE_2 were not significantly reduced (Fig. 4, $p > 0.1$). Moreover,

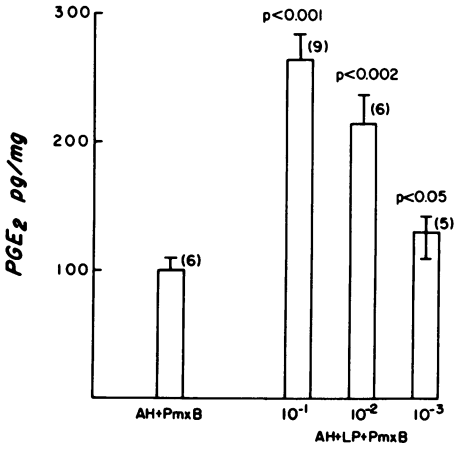

FIG. 2. Effects of PmxB on the LP-induced release of PGE_2. PmxB (10 units/ml) does not block LP-induced release of PGE_2 from rabbit hypothalamic tissue. Concentrations of LP (represented by numbers underneath the bars) are in rabbit pyrogenic doses (see text, Methods). Statistical differences are between experimentals and controls.

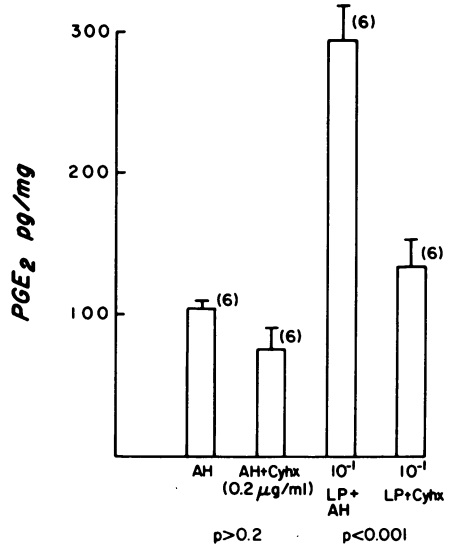

FIG. 3. Cycloheximide effects on the LP-induced release of PGE_2. Cycloheximide (Cyhx, 0.2 μg/ml) does not reduce background release of PGE_2 from rabbit hypothalamic tissue (first bar versus second bar) but does reduce the amount of PGE_2 released by tissue incubated with LP (third bar versus fourth bar).

Cyhx reduced LP-induced release of PGE_2 by similar amounts regardless of whether the protein synthesis inhibitor was added at the same time as the LP or was present before and after the addition of LP (Fig. 5).

Cycloheximide has been described as toxic in that when given systemically it can interfere with the ability of rats and rabbits to maintain normal body temperature [14,9,15]. Although our experimental design does not reflect possible peripheral effects of Cyhx, we used another inhibitor of protein synthesis, puromycin. Incubation of hypothalamic tissue with this drug (2 or 10 μg/ml) did not significantly affect the background release of PGE_2 ($p > 0.2$, Fig. 6). These concentrations of puromycin, like Cyhx, are consistent with the concentrations used in several cell culture systems to block protein synthesis in a non-toxic, reversible system [16]. Similar to the results

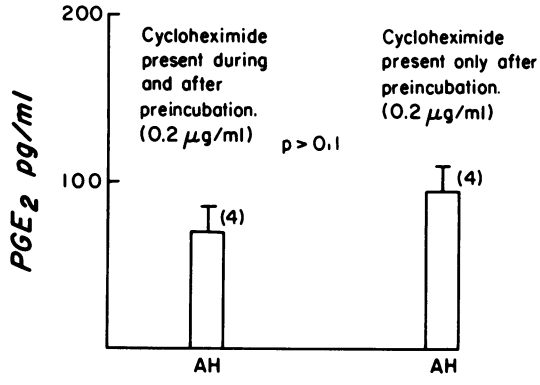

FIG. 4. The effect of Cyhx added to hypothalamic cultures without LP. Cyhx (0.2 μg/ml) was added during preincubation and during the standard incubation (first bar) or only during the standard incubation (second bar). The amount of PGE_2 from rabbit hypothalamic tissue was measured following the standard incubation period.

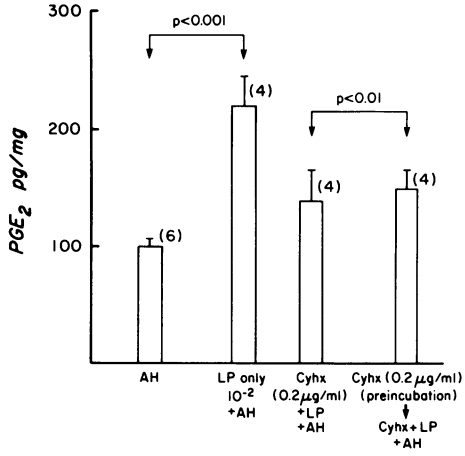

FIG. 5. The effect of Cyhx on LP-induced PGE_2 production. Cyhx was added either with LP or during both the preincubation and standard incubation with LP.

obtained using Cyhx, however, the additon of puromycin to brain tissue incubated with LP resulted in a 50 percent reduction in the amount of PGE_2 detected in the supernatant after 60 minutes (Fig. 6).

DISCUSSION

The initial results of these studies indicate that commercial media such as MEM probably contain sufficient levels of endotoxin to affect our experimental conditions by stimulating brain PGE_2 production *in vitro*. The addition of polymyxin B to the media eliminates or significantly reduces the ability of such contaminating endotoxins to induce PGE_2 release or production from rabbit hypothalamic tissue. The antibiotic, however, does not affect the ability of LP to induce the synthesis of PGE_2 from rabbit hypothalamic tissue, and these results are consistent with previous studies demonstrating that LP and endotoxin effects on *in vitro* PGE_2 production are separable [5]. Not all lots of MEM contain the same amounts of endotoxin, and thus background levels of PGE_2 induced by media alone may vary from experiment to experiment. This problem was overcome by including polymyxin B in the culture media of all subsequent incubations and provided the opportunity to carry out these studies without endotoxin contamination as a contributing factor. As shown by other investigators [12,13], the addition of this antibiotic to culture media is especially important when various agents are employed in pyrogen experiments for their ability to stimulate the production and release of LP. We can now add that production of PGE_2 from brain, whether *in vitro* or *in vivo,* can also be affected by contaminating endotoxins and that the inclusion of polymyxin B can block potential endotoxin effects.

Once we were able to obtain consistent results using PmxB in the hypothalamic incubations, studies were carried out in order to investigate the effect of the antipyretic protein synthesis inhibitors on brain PGE_2 production *in vitro*. Cycloheximide, a potent inhibitor of protein synthesis *in vitro,* has been used as a chemotherapeutic agent in certain cancers, and patients receiving the drug during a febrile illness undergo a dramatic reduction in fever within an hour following an infusion of the drug [6]. In addition, administration of Cyhx to animals receiving either endotoxin or LP eliminates or lowers their fevers [7,8,9]. Investigations have shown that this antipyresis is not due to Cyhx preventing pyrogens from entering the brain from the circulation since fevers produced by LP or endotoxin given intracerebroventricularly (ICV) can

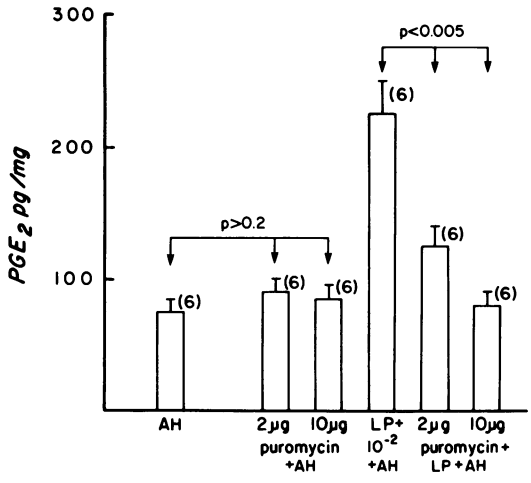

FIG. 6. Effect of puromycin on the release of PGE_2 from hypothalamic tissue. Puromycin was added to hypothalamic tissue alone or in the presence of LP. Concentrations of puromycin are shown in micrograms/ml.

still be inhibited by Cyhx [17,3]. Moreover, the doses of Cyhx which are antipyretic in rabbits given exogenous pyrogens are not sufficient to prevent the synthesis of LP *in vivo* [8]; however, this is in contrast to the fact that both Cyhx and puromycin block LP synthesis when added to phagocytes at the time of stimulation with exogenous pyrogens in vitro [16,18].

The mechanism for protein synthesis inhibitors acting as antipyretics remains unclear. Siegert et al. [8] have shown that Cyhx reduces fever without affecting cerebrospinal fluid levels of PGE_2. However, recent results have shown that Cyhx does not interfere with the production of fever caused by the administration of PGE_2 or arachidonic acid [17,19]. In addition, Cyhx inhibits the *in vitro* release of prostaglandins from mouse BALB/3T3 cells treated with methylcholanthrene [20], while various other protein synthesis inhibitors reduce the LP-induced release of PGE_2 from human dermal fibroblasts [21,22]. These later findings as well as those reported in this paper indicate that protein synthesis inhibitors may exert their antipyretic effect by interfering with the synthesis of PGE_2.

In the present studies, we consistently observed that hypothalamic tissue incubated with Cyhx and LP released significantly less amounts of PGE_2 than tissue incubated with LP alone. In addition, background levels of PGE_2 released by these tissues were unaffected by the presence of Cyhx. Similar results were obtained when another protein inhibitor, puromycin, was used. These data do not rule out the possibility that protein synthesis inhibitors are toxic to brain tissue and thereby decrease its ability to respond to LP; on the other hand, recent evidence shows that protein synthesis inhibitors are non-toxic to human dermal fibroblasts [21]. In these experiments, the ability of fibroblasts to release PGE_2 in response to LP was decreased by the presence of Cyhx; however, fibroblasts incubated for various time periods with Cyhx retained their viability and showed no decrease in background PGE_2 production. These results support the concept that inhibitors of protein synthesis block some synthetic step leading to LP-induced release of PGE_2 rather than simply injuring or killing the responding cells.

The synthetic step blocked by protein synthesis inhibitors is unknown. However, since fibroblasts synthesize PGE_2 in the presence of both arachidonic acid and protein synthesis inhibitors it is unlikely that inhibitors of protein synthesis interfere signifi-

cantly with the activity of cyclo-oxygenase (preliminary data and [21]). On the other hand, it may be that LP induces PGE_2 release by first activating a phosphodiesterase enzyme which cleaves diacylglycerol (DAG) from phosphotidylinositol [23]. The DAG so formed may have arachidonic acid on its 2-position, which in turn could be cleaved by a specific enzyme (a diacylglycerol lipase and/or a phosphatidic acid specific phospholipase-23). Protein synthesis inhibitors may therefore decrease the LP-induced release of PGE_2 by interfering with the synthesis of one or more of the enzymes involved in the release of arachidonic acid.

Another protein synthesis inhibitor, anisomycin, which is less toxic than Cyhx in that it does not interfere with normal thermoregulation of rats or rabbits [24], blocks or significantly reduces fever induced by either endotoxin or LP [25,26]. Unlike Cyhx, however, anisomycin also prevents fevers induced by PGE_2 [27]. In addition, after administration of endotoxin, anisomycin treatment results in an increased amount of PGE_2 in the cerebrospinal fluid [3]. It is unclear whether the same results would be obtained if fever were induced by an injection of LP instead of large intravenous doses of endotoxin which probably induce PGE_2 production from cells throughout the brain [5]. Recent results from this laboratory [22] and elsewhere [21] indicate that anisomycin inhibits the LP-induced release of PGE_2 from fibroblasts and may, therefore, also decrease the LP-induced release of PGE_2 from hypothalamic tissue. It is also possible that inhibitors of protein synthesis which are also antipyretics may not induce antipyresis by the same mechanism.

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