Pyrogen – prostaglandin coupling in the pathogenesis of fever: evidence against a role for nitric oxide

Jane Redford, Isis Bishai, and Flavio Coceani

Abstract: There is much debate on the mechanism by which blood-borne pyrogens trigger prostaglandin E_2 (PGE_2) synthesis in brain and fever. This investigation was undertaken to determine whether nitric oxide qualifies as a signal transducer for pyrogens at the interface between blood and brain. Experiments were carried out in vitro and in vivo using, respectively, preparations of cerebral tissue and microvessels from the rat, and the conscious, chronically instrumented cat. In vitro preparations produced PGE_2 and its production increased during a 30-min treatment with interleukin 1 (brain tissue) or endotoxin (microvessels). In addition, both pyrogens increased cyclic GMP levels in cerebral microvessels. In both brain tissue and microvessels, N^G-nitro-L-arginine had no effect on basal PGE_2 release, while it curtailed the pyrogen-stimulated release. The same treatment reduced the cyclic GMP accumulation brought about by pyrogens in the microvessels. Conversely, in the conscious cat, inhibitors of nitric oxide synthesis (N^G-monomethyl-L-arginine, N^G-nitro-L-arginine) had no effect on fever and the concomitant elevation of PGE_2 in cerebrospinal fluid, regardless of the pyrogen used (endotoxin, interleukin 1) and the route of administration (intravenous, intracerebroventricular). We conclude that nitric oxide may serve as a pyrogen mediator in brain. This mediator function, however, is seemingly not important in the development of fever.

Key words: pyrogen, fever mechanism, nitric oxide, prostaglandin E_2, blood–brain barrier.

Résumé : Le mécanisme par lequel les pyrogènes diffusés par le sang déclenchent la fièvre et la synthèse de la prostaglandine E_2 (PGE_2) dans le cerveau fait l'objet d'un débat intense. Cette étude a eu pour but de déterminer si le monoxyde d'azote peut être considéré comme un transducteur de signal pour les pyrogènes à l'interface sang–cerveau. On a effectué des expériences in vitro et in vivo, en utilisant respectivement des préparations de tissu cérébral et de microvaisseaux de rat et le chat chroniquement instrumenté, conscient. Les préparations in vitro ont produit des PGE_2 dont le taux s'est accru durant un traitement de 30 min avec l'interleukine 1 (tissu cérébral) ou une endotoxine (microvaisseaux). Les deux pyrogènes ont augmenté les taux de GMP cyclique dans les microvaisseaux cérébraux. Dans les microvaisseaux et le tissu cérébral, la N^G-nitro-L-arginine n'a pas eu d'effet sur la libération basale de PGE_2, alors qu'elle a réduit la libération stimulée par les pyrogènes. Le même traitement a réduit l'accumulation de GMP cyclique induite par les pyrogènes dans les microvaisseaux. À l'opposé, chez le chat conscient, les inhibiteurs de la synthèse du monoxyde d'azote (N^G-monométhyl-L-arginine, N^G-nitro-L-arginine) n'ont pas eu d'effet sur la fièvre et l'augmentation concomitante de PGE_2 dans le liquide céphalo-rachidien, peu importe le pyrogène (endotoxine, interleukine 1) et la voie d'administration (intraveineuse, intracérébroventriculaire) utilisés. Nous concluons que le monoxyde d'azote pourrait servir de médiateur pyrogène dans le cerveau. Toutefois, cette fonction médiatrice ne semble pas jouer un rôle important dans le développement de la fièvre.

Mots clés : pyrogène, mécanisme de la fièvre, monoxyde d'azote, prostaglandine E_2, barrière hémato-encéphalique.
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Introduction

Fever to exogenous pyrogens (e.g., bacterial endotoxin) is thought to develop in a series of steps, starting with the release of several pyrogenic peptides (i.e., cytokines) into the circulation, among which interleukin 1 (IL-1) and interleukin 6 (IL-6) appear most important, and ending with the action of a particular prostaglandin, prostaglandin E_2 (PGE_2), on thermoregulatory pathways in the hypothalamus (Dinarello 1991; Coceani 1991). Although this scheme is well accepted,

there is much debate on the mechanism by which blood-borne cytokines activate PGE_2 synthesis in brain. Considering the structural impermeability of cerebral capillaries to peptides and the low efficiency of any transport system for the cytokines across the capillary wall (Banks et al. 1991; Luheshi et al. 1994), it has been proposed that circulating pyrogens act outside the blood–brain barrier in one of the circumventricular organs (Stitt 1986), from where a signal is conveyed to structures inside the barrier resulting in the accelerated formation of certain cytokines (IL-6 and perhaps IL-1) (Klir et al. 1993, 1994; Roth et al. 1993; Coceani et al. 1993) and PGE_2 (Sirko et al. 1989). Considerable evidence implicates the organum vasculosum laminae terminalis (OVLT) as a target (Stitt 1986; see Coceani 1991). Alternatively, it has been suggested that cerebral capillaries have a transducing function by releasing signaling substances (i.e., PGE_2 and cytokines) from their antiluminal side following the action of pyrogens on the luminal side (Bishai et al. 1987; Schindler et al. 1990; Coceani et al. 1993). Our work, however, argues against the vasculature being a source of PGE_2, at least in response to circulating IL-1 and IL-6 (Bishai et al. 1987; I. Bishai and F. Coceani, unpublished data). The identification of endothelium-derived relaxing factor (EDRF) as nitric oxide (NO), with the subsequent realization that this agent is involved not only in vasoregulation but also in brain function (Bluthé et al. 1992; Kumar et al. 1993; Calapai et al. 1994; Horn et al. 1994; Kapás et al. 1994) as a mediator or modulator of synaptic events (Prast and Philippu 1992; Rettori et al. 1992; Costa et al. 1993; Lorrain and Hull 1993; Hawkins et al. 1994), provides a new perspective. Specifically, it raises the prospect that NO diffusing from the cerebral capillary wall (Kilbourn and Belloni 1990) may act as the coupling agent between pyrogens in the circulation and the PGE_2 synthetic system in brain. Several facts support this possibility. First, NO is formed in the endothelium of cerebral vessels (Kilbourn and Belloni 1990) and, in its role as a vasodilator, the compound is released towards the antiluminal surface. Secondly, pyrogens stimulate NO formation in blood vessels and their action can be rapid (Salvemini et al. 1989). Germane to the latter finding are data pointing to the involvement of NO synthase in the fast reaction of cells to challenges (Nathan and Xie 1994). Thirdly, NO is a potent activator of cyclooxygenase (Salvemini et al. 1993) and may, accordingly, promote PGE_2 synthesis in tissues, including brain (Rettori et al. 1992). Significantly, NO is effective on both isoforms, constitutive and inducible, of cyclooxygenase (Salvemini et al. 1993). Lastly, it may not be fortuitous that fever is associated with local vasodilatation in the hypothalamus (Rawlins et al. 1973).

The specific objective of our study was to ascertain whether cerebrovascular NO lends itself to an intermediary role in the febrile response to pyrogens. With the approach used, however, it was also possible to examine whether NO formed within the tissue of the brain has an alternative, or supplementary, role in fever. The latter question seemed important because neurally derived NO has been implicated in other actions of pyrogens (Bluthé et al. 1992; Calapai et al. 1994). Experiments were carried out with the rat in vitro, using brain tissue and microvessel preparations, and with the cat in vivo. The rat was used instead of the cat for the in vitro experiments in view of the number of animals required for each protocol and after noting that the central sequence of fever (Stitt 1986; Coceani 1991) and pyrogen effect on PGE_2 release from cerebral microvessels (Bishai et al. 1987; I. Bishai and F. Coceani, unpublished data) are comparable in the two species. The rat, on the other hand, was not suitable for cerebrospinal fluid (CSF) sampling (see Coceani et al. 1983). In either study, endotoxin and IL-1 were used as pyrogens. IL-6 was not tested because it is inactive on the NO synthase (Ikeda et al. 1994). While our study was in progress, several reports have appeared dealing with the same problem (Amir et al. 1991; Rothwell et al. 1993; Kapás et al. 1994; Gourine 1994). However, findings are inconclusive, because NO has been implicated at different steps in the fever process as a messenger of pyrogen (Rothwell et al. 1993) and PGE_2 (Amir et al. 1991) action, or has even been assigned an antipyretic rather than a propyretic function (Gourine 1994). Furthermore, among the pyrogens examined, endotoxin, but not IL-1, has been linked with NO (Rothwell et al. 1993; Kapás et al. 1994).

Materials and methods

Materials

Recombinant human interleukin 1β (hIL-1β) (courtesy of Upjohn Co., Kalamazoo, Mich.) and recombinant rat IL-1β (rIL-1β) (courtesy of DuPont Merck Pharmaceutical Co., Wilmington, Del.) were expressed in *Escherichia coli*. Their specific activity was, respectively, $2-4 \times 10^7$ and 2×10^7 U/mg (both standardized in the murine thymocyte assay). Recombinant proteins lost their pyrogenicity after heating at 70°C for 30 min. Endotoxin (lipopolysaccharide W, *E. coli*) and bovine serum albumin (BSA, Cohn fraction V) were purchased, respectively, from Difco (Detroit, Mich.) and Sigma (St. Louis, Mo.). Doses of pyrogens were selected from previous work in vivo (Coceani et al. 1988) and with isolated cerebral microvessels (Bishai et al. 1987) as well as from preliminary tests with brain minces.

The following compounds were used: L-arginine monohydrochloride (Eastman, Rochester, N.Y.); 3-isobutyl-1-methyl xanthine (IBMX; Sigma Chemical Co.); indomethacin (Sigma); N^G-monomethyl-L-arginine acetate (L-NMMA; courtesy of Dr. S. Moncada, Wellcome Research Lab., Beckenham, Kent); NADPH (Sigma); N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME; Sigma); and sodium nitroprusside (SNP; Sigma). Indomethacin is a cyclooxygenase inhibitor, while the remaining agents served to manipulate the NO – cyclic GMP system by promoting (L-arginine, NADPH) or inhibiting (L-NMMA, L-NAME) the synthesis of NO, by generating NO (SNP), and by interfering with the breakdown of cyclic GMP (IBMX).

Indomethacin was dissolved in distilled ethanol (10 mg/mL) prior to preparation of the final solution in an appropriate aqueous medium. All remaining compounds dissolved readily in aqueous media, and solutions were prepared as required on the day of the experiment. Precautions were taken to protect SNP solutions from light.

Unbuffered, artificial CSF (Elliott solution; see Elliott and Jasper 1949) was obtained from the pharmacy of our hospital. Other solutions were prepared with deionized, glass-distilled water and were filtered through a 0.22-μm pore Millex-GV membrane (Millipore, Mississauga, Ont.).

Glassware, syringes, solutions, and accessories for both in vitro and in vivo experiments were sterile and pyrogen-free.

Experimental procedure in vitro

Experiments were performed in pathogen-free, adult male rats (body weight, 150–300 g) of the Wistar strain. Animals were decapitated under halothane (2-bromo-2-chloro-1,1,1-trifluoroethane; Halocarbon Lab., River Edge, N.J.) anesthesia and their forebrain was rapidly removed for preparation of a cerebrocortical tissue mince or an enriched microvessel fraction. Specimens were freed of the pial membrane and, depending on the protocol, they were processed individually (preparation of tissue minces) or after pooling (preparation of microvessel fractions). Control experiments with the brain mince confirmed the presence of a functional cyclooxygenase system, which was liable to inhibition by indomethacin (I. Bishai and F. Coceani, unpublished data).

Brain tissue

The forebrain was washed with ice-cold Krebs solution (118 mM NaCl, 4.7 mM KCl, 3.5 mM $CaCl_2$, 0.4 mM $MgSO_4$, 1 mM KH_2PO_4, 11 mM dextrose, 23 mM $NaHCO_3$ gassed with 5% CO_2 in O_2; pH 7.4), and the gray matter from both hemispheres was chopped into fine pieces on a cooled glass plate. Aliquots of the cerebrocortical mince were then incubated (15 min, 37°C) with shaking in oxygenated Krebs medium (mean 2.2, range 0.7–6 mg protein/mL; 1 mL volume) to remove any prostaglandin generated during tissue processing. At the end of this period, the suspension was centrifuged (2000 × *g*) for 5 min and the resulting supernatant was discarded. Afterwards, the tissue pellet was suspended in 1 mL of oxygenated, normal Krebs or in Krebs with L-arginine and NADPH (100 μM for both) added (NO-sustaining medium), and was incubated again for 30 min at 37°C. Test compounds were added to the medium from the start of the preincubation (L-NAME) or only during the incubation (pyrogens, SNP), and the entire procedure was carried out in sterile polypropylene tubes. After completing the incubation, the content of tubes was frozen quickly in an acetone – solid CO_2 bath for storage at −80°C. Within 2 weeks, samples were thawed and centrifuged (8000 × *g*, 15 min) at 4°C, and the supernatant was saved for measurement of PGE_2. With each brain, PGE_2 formation was studied under control conditions and during treatment, and in each case incubations were performed in triplicate.

Cerebral microvessels

Microvessels were isolated as reported previously with the cat (Bishai et al. 1987), except that rats were killed by decapitation instead of exsanguination. Briefly, the forebrain of 5–7 rats was chopped into small pieces and homogenized in 10 volumes of Ringer–Hepes buffer (147 mM NaCl, 4 mM KCl, 3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5.5 mM dextrose, 15 mM Hepes; pH 7.4) containing 1% (w/v) BSA. The homogenate was then passed through a series of Nitex meshes (Kressilk and Thompson, Montréal, Que.) and the filtrate was centrifuged at 1000 × *g* for 15 min. The resulting pellet was suspended in Ringer–Hepes buffer containing 25% instead of 1% BSA and centrifuged at 6000 × *g* for 20 min. While the pellet was saved, the floating cap, consisting of myelin and brain neuropile constituents, was redispersed and centrifuged for a second time. The combined pellets were suspended in Ringer–Hepes buffer, passed through a glass-bead (250 μm diameter) column (12 × 15 mm), and the column was then washed with 200 mL of the same buffer. Afterwards, the glass beads were placed into buffer with gentle agitation to free the adhering microvessels. Once the beads had settled, the fluid was aspirated off and the microvessels were recovered by centrifugation (8000 × *g* for 10 min). The yield was 0.2–0.5 mg microvessel protein/g wet weight brain. In agreement with previous work (Bishai et al. 1987), microvessel preparations were made of capillary segments of variable length and few arterioles with their capillary ramifications. However, unlike the cat specimens, in which blood vessels were patent, in the rat the vessel lumen was often occupied by erythrocytes. The use of decapitation, and not exsanguination, to kill the rats likely accounts for this difference. On light microscopy, microvessel preparations were free of identifiable neurons and glia. In addition, their purity was confirmed in preliminary experiments by demonstrating enrichment of alkaline phosphatase (10-fold) and γ-glutamyltranspeptidase (24-fold) (see Bishai et al. 1987). The whole isolation procedure was carried out at 4°C.

Freshly isolated microvessels were incubated (mean 0.6, range 0.3–1.1 mg protein/mL; 0.3 mL volume) in phosphate-buffered saline (PBS) or Ringer–Hepes buffer containing L-arginine (100 μM), NADPH (100 μM), and IBMX (1 mM) (NO – cyclic GMP sustaining medium). PBS was used to duplicate the conditions of previous work in the cat (Bishai et al. 1987). Incubations (30 min, 37°C) were carried out in the absence and presence of test compounds. Afterwards, the tubes were dipped in an acetone – solid CO_2 bath and the frozen content was stored at −80°C until analysis. For this purpose, samples were thawed and centrifuged (8000 × *g*, 15 min) at 4°C, and supernatant and particulate fractions were used to assay, respectively, PGE_2 and cyclic GMP.

Experimental procedure in vivo

Experiments were performed on adult cats of either sex (2.4–5.4 kg body weight). With a single exception, three animals were used with each experimental protocol, and protocols were often repeated twice or three times in the same animal, taking care to space individual tests appropriately in time (see below). Two animals were used in the remaining protocol (i.c.v. IL-1 with i.c.v. L-NMMA pretreatment; see below). Following a published procedure (Coceani et al. 1983), animals were cannulated inside the rostral part of the third ventricle and, when required, inside the external jugular vein as well. About 2 weeks were allowed for recovery, and experiments were started after wounds had healed and CSF flowed freely from the ventricle. During tests, cats were not anesthetized and were fitted with thermistor probes coupled to a telethermometer – chart recorder assembly for continuous monitoring of colonic and ear (dorsal surface) temperatures. Skin temperature provided an index of peripheral vasomotor tone. The behaviour of the cats was also noted, and respiratory frequency was counted at regular intervals. Before any intervention, animals were allowed to settle down and attain a steady body temperature (range, 37.5–39.2°C). Endotoxin and hIL-1β were injected i.v. as a bolus (endotoxin, 0.8 μg/kg; hIL-1β, 25 or 35 ng/kg). Likewise, a bolus was used for i.c.v. injections (endotoxin,

5 ng; hIL-1β, 20 ng). L-NMMA was given i.v. (5 mg/kg) or i.c.v. (25 μg) as a bolus 10 min prior to the administration of pyrogen. Intravenous administration of L-NAME consisted instead of either a bolus (5 mg/kg) or continuous infusion (1 mg $\cdot$ kg^{-1} $\cdot$ min^{-1} for 60 min). The timing of L-NAME bolus injection was the same as for L-NMMA, but L-NAME infusion was started 30 min prior to pyrogen injection. The dose of NO synthesis inhibitors was selected from data in the literature (Bellan et al. 1991; Ross et al. 1991; Wang et al. 1993) and from preliminary tests proving that the drugs were well tolerated by the conscious cat. When using the i.c.v. route, pyrogens and L-NMMA were dissolved in Elliott solution and were injected in 10-μL (endotoxin) or 100-μL (IL-1, L-NMMA) volumes. Intravenous injections were made instead in saline. CSF was collected at a rate approximating the rate of production (0.13−0.30 mL in 7−13 min), and the interval between samples varied from 0.5 to 4 h, depending on the protocol. In those instances in which animals showed a rise in body temperature, collections were made before the injection of pyrogen, once or twice through fever uprise, and immediately after fever had reached a peak. Additional samples were obtained at times during the latent period of the fever or at the very start of the fever (30 min from pyrogen administration; all samples were pooled in the latent-period group) and at 1-h intervals in the course of a sustained fever. When animals did not develop fever, CSF was collected at intervals corresponding chronologically to various stages of the fever response. In any experiment, however, the number of CSF collections did not exceed five. Samples were collected into an ice-cold vial containing 2 μg indomethacin and were subsequently stored at −20°C until analysis. At least 5 days elapsed between experiments in the same animal; however, with successive intravenous injections of endotoxin, the minimum interval was increased to 2 weeks to avoid tolerance. With all animals, the location of the ventricular implant was confirmed by examining the distribution of a dye in the ventricular system (Coceani et al. 1983).

All animal procedures were approved by the Animal Care Committee of The Hospital for Sick Children.

Analytical methods

PGE_2 measurement

PGE_2 was assayed directly in CSF samples and the supernatant fraction from tissue incubates (brain, microvessels) using a radioimmunoassay kit (DuPont, Boston, Mass.) with ^{125}I-labeled ligand. The assay procedure has been validated elsewhere for CSF (Coceani et al. 1988; Sirko et al. 1989), and control tests have confirmed this validation for tissue incubates. The limit of detection was 0.25 pg/tube, except for a few instances in which it was higher (1 pg).

Cyclic GMP measurement

Cyclic GMP was measured in the particulate fraction from microvessel incubates using a radioimmunoassay kit (^{125}I-labeled ligand; DuPont). For this purpose, specimens were dispersed in 6% trichloroacetic acid and sonicated (Brenson model W-140, 60−90 s at 20 kHz) at 4°C. The homogenate was left on ice for 1 h and then centrifuged (3000 $\times$ g for 15−20 min). The resulting supernatant was extracted four times with three volumes of water − saturated diethyl ether, and after discarding the ether phase, the water phase was lyophilized. The dry residue was reconstituted in 0.1−0.2 mL of sodium acetate buffer (50 mM, pH 6.2) and aliquots of this solution were treated with a mixture of acetic anhydride and triethylamine (1:2 v/v) before the assay. Cyclic GMP standard was also acetylated for preparation of a reference curve. Extraction and radioimmunoassay procedures were validated by spiking with cyclic GMP (unlabeled or tritium labeled) either the buffer or samples with a known nucleotide content. Recovery of added standard was quantitative and, consequently, assay values are given without correction. The limit of detection was 0.8 pg/tube.

Protein measurement

Protein content in each tissue fraction was measured according to Lowry et al. (1951).

Analysis of data

Deviations of body temperature from baseline are given as the maximal charge (ΔT) or the thermal response index (TRI, degrees $\times$ hours) for the number of hours indicated by the subscript.

Data are expressed as means $\pm$ SEM and n refers to the number of experiments both in vitro and in vivo. Each experiment in vitro included control and treatment incubations from a single brain (cerebrocortical mince preparation) or pooled brains (cerebral microvessel preparation) (see above). With brain tissue, incubations were carried out in triplicate and results were averaged prior to statistical analysis. Likewise, each value for the microvessels is the average of two or three incubations. In certain cases a measurement was missing owing to some technical failure and, accordingly, n values are given for each variable.

Statistical comparison of two means was done using Student's t test for paired or unpaired observations. Multiple comparisons were made with an analysis of variance (ANOVA) and Duncan's multiple-range test. Differences are considered significant for $p < 0.05$.

Results

PGE_2 formation by cerebrocortical tissue: effect of pyrogens and NO

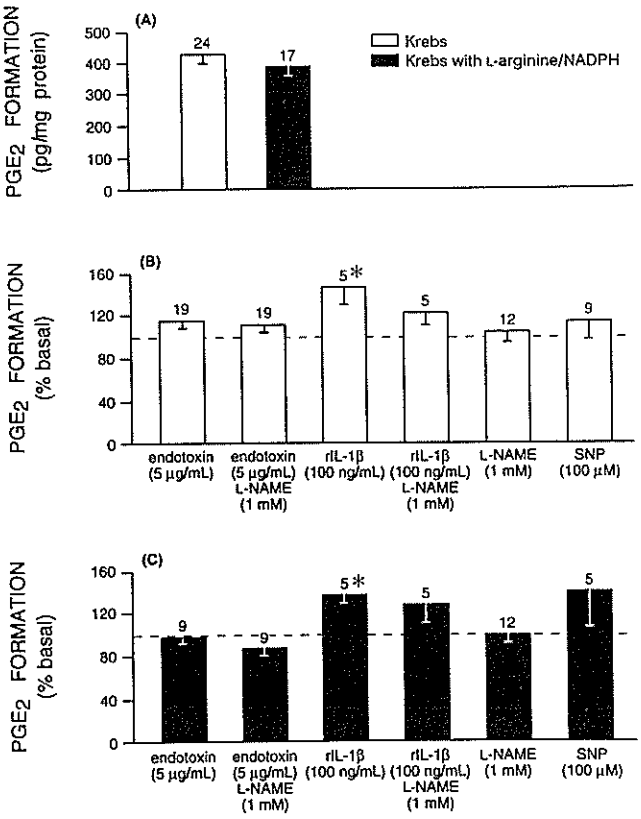
The basal formation of PGE_2 remained the same, regardless of whether the tissue was incubated in normal medium or a modified medium favoring NO synthesis (NO-sustaining medium, see Materials and methods) (Fig. 1A). Accordingly, L-NAME (1 mM) was ineffective under either condition (Figs. 1B and 1C). rIL-1β (100 ng/mL) moderately increased PGE_2 formation in both groups of tissues, and the elevation was reversed, in part, by L-NAME (Figs. 1B and 1C). No such response was noted with endotoxin (5 μg/mL), and PGE_2 activation did not attain significance (normal medium, Fig. 1B) or was absent altogether (modifed medium, Fig. 1C). Likewise, PGE_2 formation was inconsistently changed by SNP (100 μM), and stimulation was evident only in some of the experiments (Figs. 1B and 1C).

PGE_2 formation and cyclic GMP accumulation in brain microvessels: effect of pyrogens and NO

PGE_2 formation

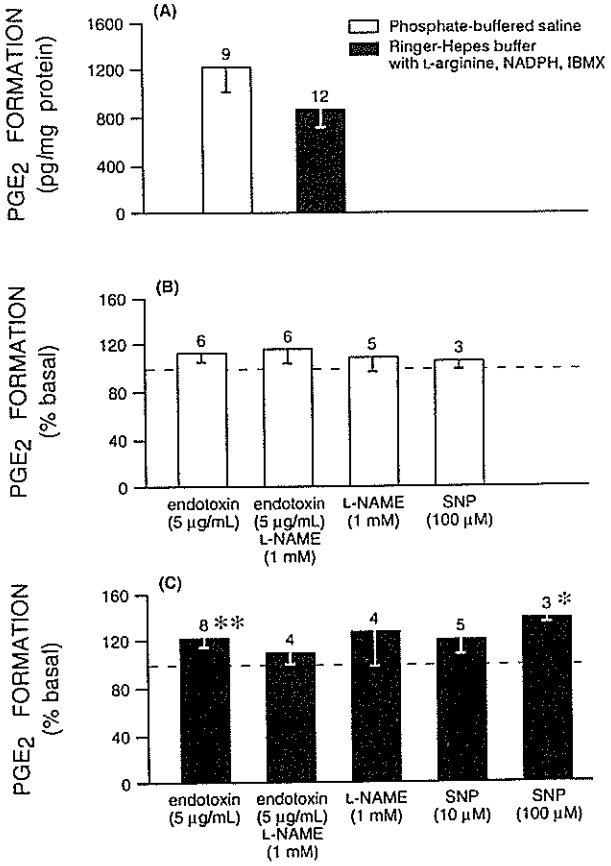
In the absence of pyrogens and SNP, the pattern of PGE_2

Fig. 1. Effect of pyrogens and SNP on PGE_2 formation in rat cerebrocortical tissue. Incubation was carried out in normal Krebs medium (open bars) or Krebs medium with L-arginine (100 μM) and NADPH (100 μM) added (NO-sustaining medium, see Materials and methods) (closed bars). Results, expressed as a percentage of basal release (see broken line), are mean ± SE for the number of experiments given above each bar. In this and the following two figures (i.e., in vitro experiments), a significant difference between control and treatment groups (calculated on the absolute values) is indicated with one ($p < 0.05$) or two ($p < 0.01$) asterisks, and a significant difference between controls in different media is indicated with one ($p < 0.05$) or two ($p < 0.01$) daggers. For abbreviations, see text.



release from intact microvessels was comparable with that observed with brain minces. Specifically, release values did not change appreciably during exposure to conditions promoting the generation of NO and accumulation of the target nucleotide (i.e., use of the NO − cyclic GMP sustaining medium, see Materials and methods) (Fig. 2A) or, conversely, resulting in inhibition of NO formation (L-NAME, 1 mM) (Figs. 2B and 2C). However, both endotoxin (5 μg/mL) and SNP (100 μM) moderately increased PGE_2 synthesis in vessels that had been suspended in the modified medium (Fig. 2C). Furthermore, endotoxin action was curtailed by L-NAME (Fig. 2C). PGE_2 was not measured in experiments with IL-1, because previous work with both feline (Bishai et al. 1987) and murine (I. Bishai and F. Coceani, unpublished data) cerebral microvessels had shown that synthesis of the compound was not affected by the treatment.

Fig. 2. Effect of endotoxin and SNP on PGE_2 formation in freshly isolated, intact cerebral microvessels in the rat. Incubation was carried out in PBS (open bars) or Ringer–Hepes buffer containing L-arginine (100 μM), NADPH (100 μM), and IBMX (1 mM) (NO − cyclic GMP sustaining medium, see Materials and methods) (closed bars). Results, expressed as a percentage of basal release (see broken line), are mean ± SE for the number of experiments given above each bar.



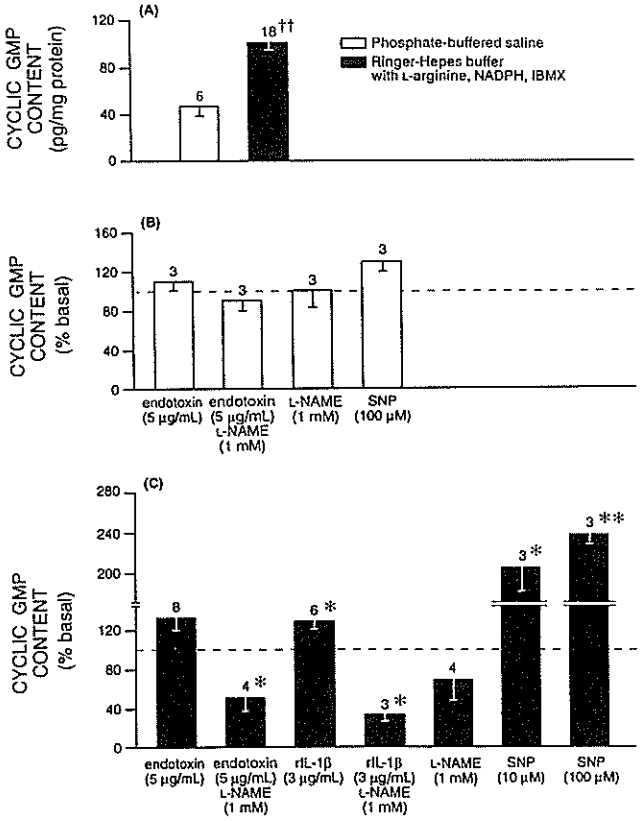
Cyclic GMP content

Unlike PGE_2 synthesis (Fig. 2A), cyclic GMP content of microvessels increased during incubation with the NO − cyclic GMP sustaining medium (Fig. 3A). In addition, in the same medium, endotoxin (5 μg/mL) and rIL-1β (3 μg/mL) promoted the accumulation of the nucleotide and, although to a lesser degree, they mimicked SNP (10 and 100 μM) (Fig. 3C). Conversely, endotoxin had no effect at all in the normal medium (Fig. 3B). Whether in response to endotoxin or rIL-1β, any increase in cyclic GMP over basal values was reversed by L-NAME (1 mM) (Fig. 3C).

Pyrogen fever: effect of NO synthesis inhibitors

In agreement with previous work (Coceani et al. 1988), PGE_2 levels in the CSF ranged between 6 and 100 pg/mL (mean, 46 pg/mL; 51 experiments) prior to any treatment. Only occasionally did basal levels exceed this range (mean, 154 pg/mL; range, 105–330 pg/mL; 9 experiments) and, in nearly all cases, the finding could be linked to the location of the sampling cannula inside the third ventricle, specifi-

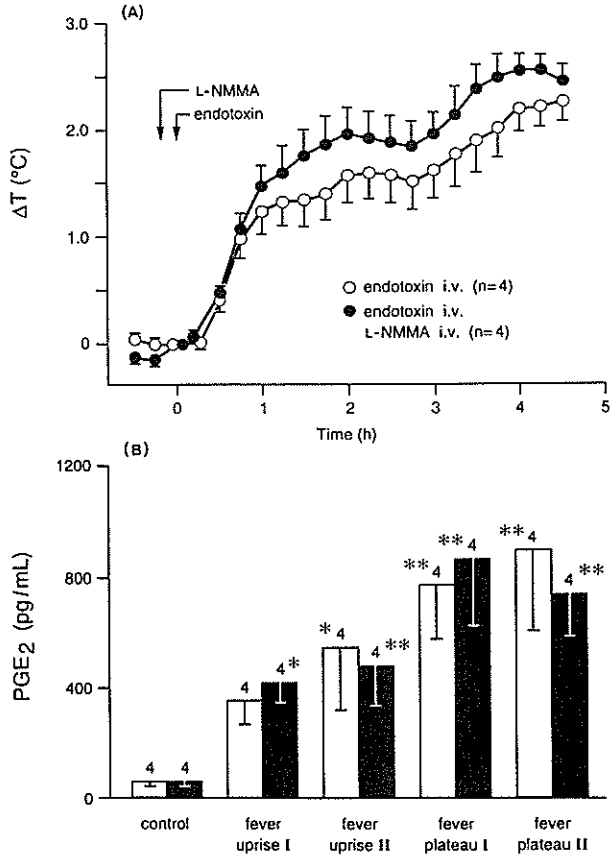
Fig. 3. Effect of pyrogens and SNP on cyclic GMP content of freshly isolated, intact cerebral microvessels in the rat. Incubation was carried out in PBS (open bars) or Ringer–Hepes buffer containing L-arginine (100 μM), NADPH (100 μM), and IBMX (1 mM) (NO – cyclic GMP sustaining medium, see Materials and methods) (closed bars). Note that the response to endotoxin in the modified medium (Fig. 3C) is close to significance (t value, 2.25, when a value of 2.36 is required for $p < 0.05$). Results, expressed as a percentage of basal release (see broken line), are mean ± SE for the number of experiments given above each bar.



cally its proximity to the anterior recess. Intravenous administration of NO synthesis inhibitors, either as a bolus (L-NMMA, 5 mg/kg; 3 experiments) or continuous infusion (L-NAME, 1 $mg \cdot kg^{-1} \cdot min^{-1}$ for 60 min; 2 experiments), had no effect on body temperature ($\Delta T \leq 0.2$°C) and CSF PGE_2 (85 ± 12 pg/mL) over a 4-h observation period. Likewise, respiratory rate and general behaviour remained normal, the only sign of treatment being a transient shaking of the head following the injection of L-NMMA. No change at all in the condition of the animals was noted after i.c.v. L-NMMA (25 μg), and both body temperature and PGE_2 levels remained within, or rose marginally above, the normal limits ($\Delta T \leq 0.4$°C; PGE_2, 160 ± 65 pg/mL; 3 experiments).

Pyrogens, given i.v. as a bolus, elicited a typical febrile response and the expected elevation in CSF PGE_2 (see Coceani et al. 1988). With endotoxin, the rise in body temperature was gradual and showed a biphasic pattern (Fig. 4A). hIL-1β produced a faster fever that tended to subside after reaching the peak (Fig. 5A). The two pyrogens also differed in potency, with endotoxin being more effective than hIL-1β

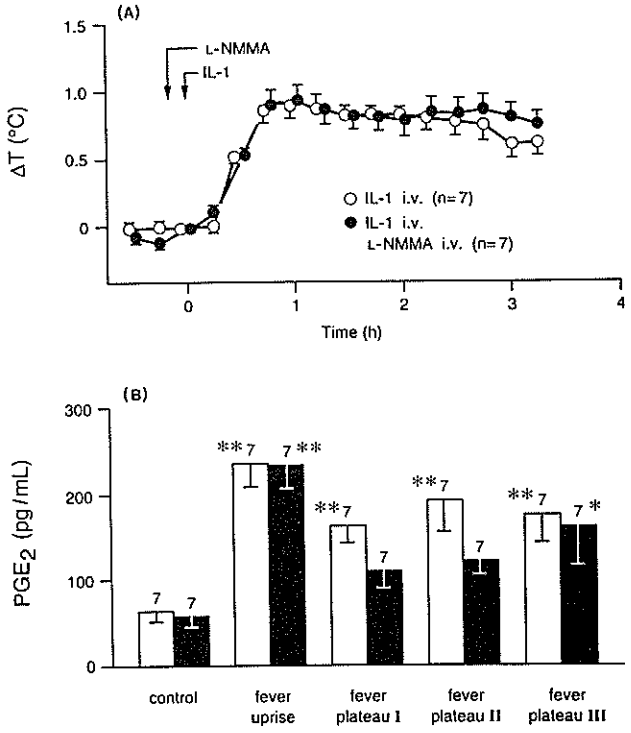
Fig. 4. Conscious cat. Rise in (A) body temperature and (B) CSF content of PGE_2 caused by i.v. bolus injection of endotoxin (0.8 μg/kg) before (open sysmbols and bars) and after (closed symbols and bars) treatment with i.v. L-NMMA. Number of experiments appears with the temperature recording and above each set of CSF values. Fever curves are not significantly different by ANOVA and by comparing the thermal response index (TRI_4 5.9 ± 0.7 (endotoxin) vs. 7.0 ± 0.5 (endotoxin and L-NMMA)). In this and the following figures, a significant difference in PGE_2 values relative to control is indicated with one ($p < 0.05$) or two ($p < 0.01$) asterisks.



at the dose employed. Changes in CSF PGE_2 paralleled those in body temperature with regard to both time course and magnitude (Figs. 4B and 5B). Pretreatment with i.v. L-NMMA had no significant effect on the fever to either pyrogen and the associated elevation in CSF PGE_2 (Figs. 4 and 5). Similarly i.v. L-NAME was ineffective against hIL-1, regardless of whether the inhibitor was given by bolus (TRI_3 3.3 ± 0.6 (hIL-1) vs. 2.6 ± 0.4 (hIL-1 and L-NAME); 3 experiments) or, in two animals, by continued infusion (TRI_3 2.6 (IL-1) vs. 2.5 (hIL-1 and L-NAME); 2 experiments).

Contrary to the finding with i.v. injections, endotoxin and hIL-1β produced febrile responses of a comparable pattern by the i.c.v. route (see Coceani et al. 1988). As shown in Figs. 6A and 7A, with either pyrogen body temperature started to rise after some delay and progressed gradually to a peak. Furthermore, the two agents were similarly effective in causing a sustained increase in the PGE_2 content of the

Fig. 5. Conscious cat. Rise in (A) body temperature and (B) CSF content of PGE_2 caused by i.v. bolus injection of hIL-1β (25 or 35 ng/kg depending on the animal) before (open symbols and bars) and after (closed symbols and bars) treatment with i.v. L-NMMA. Number of experiments appears with the temperature recording and above each set of CSF values. Fever curves are not significantly different by ANOVA and by comparing the thermal response index (TRI_3 2.3 ± 0.09 (hIL-1) vs. 2.1 ± 0.2 (hIL-1 and L-NMMA)).

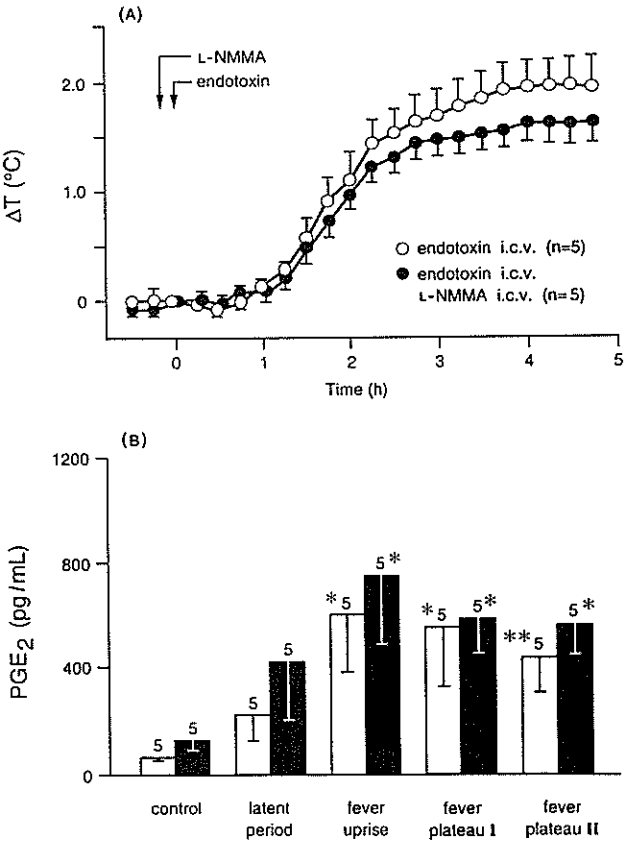


CSF (Figs. 6B and 7B). However, pretreatment with i.c.v. L-NMMA, as the i.v. pretreatment, did not attenuate the fever in any significant way, nor did it reverse the PGE_2 elevation at any stage of the fever (Figs. 6 and 7).

Discussion

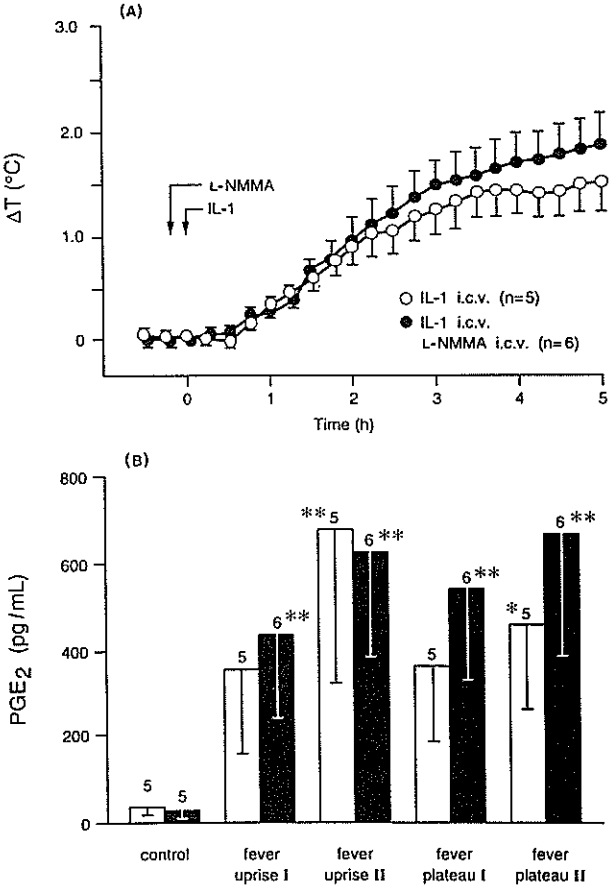
The present investigation shows that isolated cerebral microvessels, specifically capillaries, are able to generate NO and that formation of this agent may increase upon exposure to pyrogens. This conclusion is based on the demonstration that the guanylate cyclase – cyclic GMP system, a known target for NO action, is activated, albeit modestly, during pyrogen treatment and that acceleration of PGE_2 release induced by endotoxin is reversed in part by L-NAME. The latter finding suggests the existence of a NO link in the endotoxin-stimulated synthesis of PGE_2. Our findings extend previous work of Kilbourn and Belloni (1990) with cultured brain endothelial cells and indicate that NO activation brought about by pyrogens takes place over a time interval that is short enough to be relevant to the latency of onset of the fever. Therefore, NO diffusing from the abluminal surface of the endothelium could be involved not only in the regulation of muscle tone in resistance brain vessels, as it is conventionally thought, but also in signal transduction across the blood–brain barrier. With regard to the pathogenesis of

Fig. 6. Conscious cat. Rise in (A) body temperature and (B) CSF content of PGE_2 caused by i.c.v. bolus injection of endotoxin (5 ng) before (open symbols and bars) and after (closed symbols and bars) treatment with i.c.v. L-NMMA. Number of experiments appears with the temperature recording and above each set of CSF values. Fever curves are not significantly different by ANOVA and by comparing the thermal response index (TRI_4 5.3 ± 0.6 (endotoxin) vs. 4.4 ± 0.2 (endotoxin and L-NMMA)). Note that basal content of PGE_2 (128 and 330 pg/mL) in two experiments of the treatment group greatly exceeds the others (72 ± 6 pg/mL), thus explaining the large error.



fever, NO would qualify for this messenger role better than PGE_2, since its synthesis in the cerebral microvasculature is liable to activation by both endotoxin and IL-1. In addition, our in vitro results suggest that NO could also act as a pyrogen mediator, specifically IL-1 mediator (see Figs. 1B and 1C), in the brain parenchyma. It follows that NO could be formed in the OVLT in response to blood–borne IL-1 and could subsequently seep beyond the glial limiting layer (i.e., the glial layer substituting the blood–brain barrier at that site, see Krisch et al. 1978) into the substance of the hypothalamus to initiate the sequence of events, PGE_2 activation in particular, leading to fever. Significant in this connection is the fact that OVLT neurons have the enzyme for NO synthesis (Jurzak et al. 1994) and that NO, notwithstanding the intrinsic lability, may diffuse unaltered through brain tissue (Wood and Garthwaite 1994). In brief, our data in vitro, coupled with data in the literature, indicate that NO would meet the requirements for a messenger function in brain and, specifically, would lend itself to the role of transducing agent

Fig. 7. Conscious cat. Rise in (A) body temperature and (B) CSF content of PGE_2 caused by i.c.v. bolus injection of hIL-1β (20 ng) before (open symbols and bars) and after (closed symbols and bars) treatment with i.c.v. L-NMMA. Number of experiments appears with the temperature recording and above each set of CSF values. Fever curves are not significantly different by ANOVA and by comparing the thermal response index (TRI_4 3.7 ± 0.8 (hIL-1) vs. 3.9 ± 0.8 (hIL-1 and L-NMMA)).



for pyrogens at the interface between blood and brain. However, despite its apparent soundness, this scheme is not borne out by the experiments in vivo since it was shown that arginine analogs have no effect on pyrogen fever. One may then conclude that NO, even if formed in the process of fever within the microvasculature and (or) the OVLT, may not reach the hypothalamic target(s) in sufficient amount for activation of PGE_2 synthesis. In this context, it must also be pointed out that any NO-induced PGE_2 release would occur primarily in proximity of the capillary wall, that is, at a location in the extracellular milieu from which the compound is most efficiently transported into the circulation (Bito 1987).

Although the original postulate on the role of NO could not be confirmed, two issues emerging from the present work warrant elaboration. They are (*i*) the functional relationship between the NO and PGE_2 systems in the cerebral capillary wall and brain parenchyma and (*ii*) the nature of the pyrogen–PGE_2 coupling at the blood–brain interface in light of the negative finding with NO.

Leaving aside the question of the participation of NO in fever, our study indicates that this agent may interact with PGE_2, although only under certain conditions. Specifically, in the absence of pyrogens, NO and PGE_2 appear to act independently in both the vasculature and brain parenchyma. Consistent with this arrangement is the finding that basal PGE_2 release does not rise in a modified medium in which NO synthesis is facilitated, nor does it abate when the normal medium is supplemented with L-NAME. Conversely, during pyrogen treatment and the attendant acceleration of PGE_2 synthesis, L-NAME becomes a PGE_2 inhibitor, implying that pyrogens can activate cyclooxygenase both directly and, to a limited degree, with the intermediacy of NO. The latter mechanism for PGE_2 activation, however, is seemingly not important in vivo, at least insofar as the genesis of fever is concerned. Whether important or not, the existence of an activity-related linkage between NO and PGE_2 has been reported by another group (Rettori et al. 1992) after comparing the effect of L-NMMA on the hypothalamic release of PGE_2 before and during norepinephrine stimulation.

While arguing against the role of the cerebral microvasculature as a source of pyrogen transduction products, such as NO (this paper) and PGE_2 (Bishai et al. 1987), our study reasserts the importance of a functional connection between the OVLT and the hypothalamic preoptic region in the fever process (see Stitt 1986). The precise nature of this connection remains to be defined, although conceptual considerations and experimental findings point to the involvement of a specific nerve tract (Coceani 1991). This neural mechanism would convey the signal for humoral changes inside the hypothalamus, specifically the rise in PGE_2 (Sirko et al. 1989) and IL-6 (Coceani et al. 1993; Klir et al. 1993), which are deemed important for the onset and progression of the fever.

In conclusion, our study could not confirm the involvement of NO as a pyrogen messenger at the interface between blood and brain. The concept that the OVLT plays a critical role in fever as both target and transducing structure for blood-borne pyrogens is further validated.

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