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Prostaglandin E_2-synthesizing enzymes in fever: differential transcriptional regulation

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Ivanov, Andrei I., Ralph S. Pero, Adrienne C. Scheck, and Andrej A. Romanovsky. Prostaglandin E_2-synthesizing enzymes in fever: differential transcriptional regulation. *Am J Physiol Regul Integr Comp Physiol* 283: R1104–R1117, 2002. First published July 25, 2002; 10.1152/ajpregu.00347.2002.—The febrile response to lipopolysaccharide (LPS) consists of three phases (*phases I–III*), all requiring de novo synthesis of prostaglandin (PG) E_2. The major mechanism for activation of PGE_2-synthesizing enzymes is transcriptional upregulation. The triphasic febrile response of Wistar-Kyoto rats to intravenous LPS (50 μg/kg) was studied. Using real-time RT-PCR, the expression of seven PGE_2-synthesizing enzymes in the LPS-processing organs (liver and lungs) and the brain "febrigenic center" (hypothalamus) was quantified. *Phase I* involved transcriptional upregulation of the functionally coupled cyclooxygenase (COX)-2 and microsomal (m) PGE synthase (PGES) in the liver and lungs. *Phase II* entailed robust upregulation of all enzymes of the major inflammatory pathway, i.e., secretory (s) phospholipase (PL) A_2-IIA → COX-2 → mPGES, in both the periphery and brain. *Phase III* was accompanied by the induction of cytosolic (c) PLA_2-α in the hypothalamus, further upregulation of $sPLA_2$-IIA and mPGES in the hypothalamus and liver, and a decrease in the expression of COX-1 and COX-2 in all tissues studied. Neither $sPLA_2$-V nor cPGES was induced by LPS. The high magnitude of upregulation of mPGES and $sPLA_2$-IIA (1,257-fold and 133-fold, respectively) makes these enzymes attractive targets for anti-inflammatory therapy.

cyclooxygenases; phospholipases; terminal prostaglandin E synthases; lipopolysaccharide; febrile phases

BACTERIAL LIPOPOLYSACCHARIDE (LPS, endotoxin) is widely used to study the febrile and other innate defense responses to gram-negative infection. An intriguing feature of the febrile response to a single bolus injection of LPS is that this response is brought about by several sequential bursts in the activity of thermoregulatory effectors (59). LPS fever consists of several rises in body temperature, so-called febrile phases, each having its own thermoregulatory mechanism (84). At least two febrile phases were identified in numerous studies in several species, including humans (for review, see Refs. 53, 60). However, additional, subsequent phases were likely missed in some of these studies due to a short observation period (usually 3–4 h), no measures of the thermoeffector activity recorded, and/or other methodological factors (for review, see Ref. 58). At least three febrile phases (*phases I–III*) have been identified in the rat (58, 59) and mouse (48), the species commonly used in research of inflammation and sepsis. Different febrile phases are accompanied by different sickness symptoms (e.g., hyperalgesia or allodynia at *phase I* vs. hypoalgesia at *phase II*; Ref. 57) and are thought to represent different strategies of adaptation to infection (60). Several authors attempted to explain the genesis of the polyphasic febrile response by sequential actions of different pyrogenic mediators, but no consensus has been reached (53).

Prostaglandin (PG) E_2 has long been known as the principal mediator of the febrile response (2) and the major therapeutic target for antipyretic therapy (37). All three phases of LPS fever are mediated by PG receptors (48, 80), and the entire febrile course requires de novo synthesis of PGE_2. Indeed, each phase of the biphasic LPS fever in rabbits coincides with a distinct rise in the blood level of PGE_2; both rises in body temperature and both rises in PGE_2 concentration can be blocked by an inhibitor of PG synthesis (61). Similarly, the two phases of LPS fever in guinea pigs are accompanied by two rises in PGE_2 concentration in the hypothalamic microdialysate; both febrile phases and both PGE_2 rises can be blocked by an inhibitor of PG synthesis (68). A blockade of PGE_2 synthesis in rats also leads to attenuation of the entire course of the febrile response to LPS (66). That de novo synthesis of PGE_2 is required throughout the entire febrile course agrees with both the short duration of the febrigenic action of PGE_2 (e.g., Ref. 76) and the rapidity of its inactivation (24).

PGE_2 synthesis occurs in three steps (Fig. 1). First, arachidonic acid (AA) is released from membrane phospholipids via action of phospholipase (PL) A_2 (43, 44, 72). Second, AA is converted to PGG_2 and then to PGH_2 by cyclooxygenase (COX) (71, 72). Third, PGH_2 is

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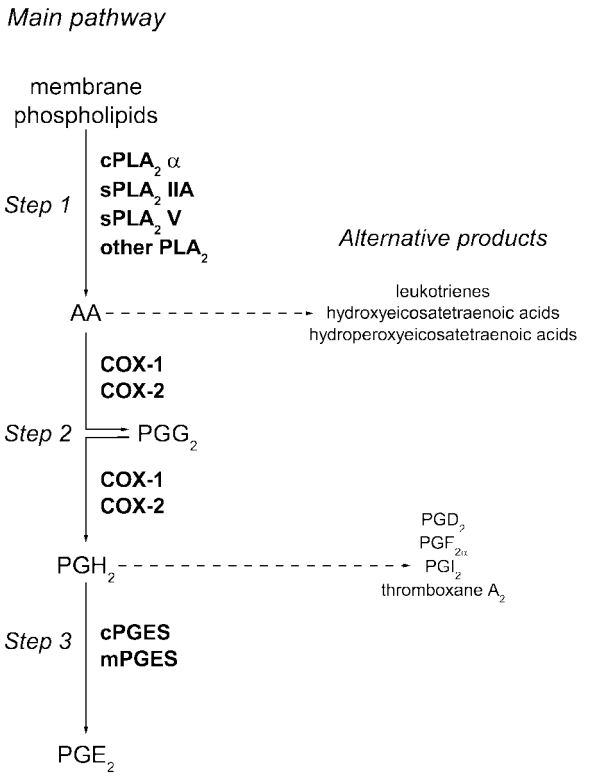

Fig. 1. The prostaglandin (PG) E_2-synthesizing cascade. Substrates and products are shown in regular font, enzymes in bold, and explanatory signs in italics. $cPLA_2$ and $sPLA_2$, cytosolic and secretory phospholipase A_2, respectively; COX-1 and COX-2, cyclooxygenase-1 and -2, respectively; cPGES and mPGES, cytosolic and microsomal PGE synthase, respectively.

isomerized to PGE_2 by a terminal PGE synthase (PGES) (72, 79). Each step of this cascade can be catalyzed by several nonhomologous enzymes and/or multiple isoforms of the same protein. Thus the mammalian PLA_2 family consists of more than 12 members, including cytosolic (c) PLA_2-α and secretory (s) PLA_2-IIA and -V (25, 43, 44). Two COX isoforms, i.e., COX-1 and COX-2, have been identified (71). The $PGH_2 \rightarrow PGE_2$ isomerizing activity is attributed to several proteins (79). The main cPGES is known as p23 protein (78). The main microsomal (m) PGES is a member of the so-called "membrane-associated proteins involved in eicosanoid and glutathione metabolism" superfamily (28, 29).

Among the proteins catalyzing PGE_2 synthesis, there are several groups of functionally coupled enzymes: COX-1 → cPGES (78), COX-2 → mPGES (45), and possibly $sPLA_2$ (-IIA and -V) → COX-2 (44, 47, 67). Functional coupling plays an important role in the selective distribution of AA and its metabolites between the PGE_2 pathway vs. several alternative eicosanoid pathways (Fig. 1), as well as between individual isoforms within the PGE_2 pathway (45, 47, 67). Under basal conditions, the rate of PGE_2 synthesis in various cells is low; it is limited by both the release of AA by PLA_2 and the consumption of AA by alternative pathways (Fig. 1, Ref. 72). However, inflammatory stimuli cause a robust activation of all three steps of PGE_2 synthesis, as well as a preferential redistribution of AA and its metabolites toward the PGE_2 pathway (17, 39).

The PGE_2-synthesizing enzymes are activated via two major mechanisms, transcriptional upregulation and posttranslational modification (1, 25, 44, 45, 71). The existence of binding sites for a variety of proinflammatory transcription factors (e.g., activity enhancer-binding protein 2, nuclear factor-κB, and cAMP response element-binding protein) in the 5′-flanking region of $cPLA_2$-α, $sPLA_2$-IIA and -V (1, 43, 44), and COX-2 (71) genes suggests functional importance of transcriptional upregulation in inflammation. Indeed, LPS and proinflammatory cytokines, such as IL-1β and TNF-α, have been shown to induce massive release of PGE_2 via transcriptional upregulation of PLA_2 (1, 44), COX-2 (45, 71, 75), and/or mPGES (45, 73, 75) in vitro. Transcriptional upregulation of $sPLA_2$-IIA, COX-2, and mPGES has also been demonstrated in several in vivo studies of LPS shock (see, e.g., Refs. 21, 36, 46, 63).

In the present work, we studied the transcriptional upregulation of PGE_2-synthesizing enzymes during the triphasic febrile response of rats to a mild dose of LPS. We addressed the most intriguing question about pathogenesis of fever: how can the same process, synthesis of PGE_2, bring about three different febrile phases? We hypothesized that each phase (and each underlying burst of PGE_2 synthesis) involves transcriptional upregulation of different enzymes, possibly in different tissues. To test this hypothesis, we studied seven members of the PGE_2-synthesizing cascade: $cPLA_2$-α, $sPLA_2$-IIA, $sPLA_2$-V, COX-1, COX-2, cPGES, and mPGES. These enzymes represent two functionally coupled groups [viz., COX-1 → cPGES and $sPLA_2$ (-IIA and -V) → COX-2 → mPGES], as well as $cPLA_2$-α, an important catalyst of AA release in various in vitro models (25, 44, 62). Using real-time RT-PCR, we quantified the expression of these seven genes in the two principal LPS-processing organs (the liver and lung) and in the key brain structure for febrigenesis (the hypothalamus). Preliminary results of this study have been reported elsewhere (27).

MATERIALS AND METHODS

Animals

Fifty-three 10-wk-old male inbred Wistar-Kyoto rats (Harlan Sprague Dawley, Indianapolis, IN) were used. Initially, the rats were housed three per standard "shoebox"; after surgery, they were caged individually. The cages were kept in a rack equipped with a Smart Bio-Pack ventilation system (model SB4100) and Thermo-Pak temperature control system (model TP2000; Allentown Caging Equipment, Allentown, NJ); the temperature of the incoming air was maintained at 28°C. Thermal neutrality inside the rats' home cages and in the experimental setup (see below) was verified by the absence of marked tail-skin vasoconstriction and vasodilation (55). The cage space was enriched with artificial "rat holes" (cylindrical confiners made of stainless steel wire). In addition to spending time in the confiners voluntarily, the rats were systematically habituated to them (5 training sessions, 4–5 h each). The same confiners were used later in

experiments. Well-adapted, confined rats exhibit neither of stress hyperthermia nor any other signs of stress (59). Food (Teklad Rodent Diet "W" 8604, Harlan Teklad, Madison, WI) and water were available ad libitum. The room was on a light-dark cycle of 12:12 h (lights on at 7:00 AM). The experiments began between 8:00 and 9:00 AM. The protocols were approved by the Institutional Animal Care and Use Committee.

Surgery

For intravenous delivery of LPS, each animal was subjected to chronic jugular catheterization. Under intraperitoneal ketamine-xylazine-acepromazine (55.6, 5.5, and 1.1 mg/kg, respectively) anesthesia and antibiotic protection (enrofloxacin, 12 mg/kg sc), the rat was placed on an operating board, and a 1-cm longitudinal incision was made on the ventral surface of the neck, 1 cm to the right of the trachea. The muscles were retracted, and the right jugular vein was exposed and freed from its surrounding connective tissue. A silicone catheter (ID 0.5 mm, OD 0.9 mm) containing heparinized (50 U/ml) pyrogen-free saline was passed into the vena cava superior through the jugular vein. The 15-cm free end of the catheter was knotted, pulled under the skin to the nape, and exteriorized. The wound on the ventral surface of the neck was sutured, and the rat was allowed to recover for 2 days. The day after surgery, the catheter was flushed with heparinized saline.

Protocols

Experiment 1. This experiment was performed to determine the dynamics of the febrile response to LPS in the inbred Wistar-Kyoto strain. Colonic temperature (T_c) was used as an index of body core temperature. The rats were placed in confiners. Copper-constantan thermocouples were inserted 9 cm past the anus. The thermocouples were connected to a data logger (model AI-24, Dianachart, Rockaway, NJ) and then to a personal computer. Thereafter, the animals were transferred to a climatic chamber (Forma Scientific, Marietta, OH) set to 30°C (the midpoint of the thermoneutral zone for Wistar rats; see Ref. 55) and 50% relative humidity. The exteriorized portions of the jugular catheters were pulled through a wall port and connected to syringes filled with either *Escherichia coli* 0111:B4 LPS (Sigma Chemical, St. Louis, MO, 50 μg/ml) or saline. After a 1-h stabilization period, the animals were injected with either LPS (50 μg/kg) or saline (1 ml/kg). Their T_c was measured from 1 h before to 7 h after the injection. The T_c curves were processed (see *Data Processing and Analysis*) to determine the time points corresponding to the maximal thermoeffector activity at each of the three febrile phases. These points appeared to be 34 min (*phase I*), 94 min (*phase II*), and 296 min (*phase III*) post-LPS. At these time points, samples of the liver, lung, and hypothalamus were harvested in *experiment 2* for RNA isolation and analysis.

Experiment 2. Seven groups of rats were prepared exactly as for *experiment 1*, except that no thermocouples were inserted. Three groups of rats received LPS (50 μg/kg); their tissues were harvested 34, 94, or 296 min after the injection. Another three groups received saline; their tissues were harvested at the same time points. The remaining group of animals received no injection and served as an untreated control; their tissues were harvested at the point corresponding to the time of LPS or saline injection in the other six groups (*time 0*). This design allowed us to express the responses of LPS- and saline-treated rats relative to the untreated controls and thus to account for potential circadian dynamics in PG synthesis in afebrile rats (64). For tissue harvesting, each rat was anesthetized with intravenous ketamine-xylazine-acepromazine (5.6, 0.6, and 0.1 mg/kg, respectively). To immediately stop RNA degradation, the anesthetized animal was perfused through the left ventricle (right atrium cut) with 30 ml of saline followed by 30 ml of an RNA-preserving solution, RNAlater (Ambion, Austin, TX), diluted twofold with saline. Samples of the liver (~300 mg) and right lung (~150 mg) were collected rapidly and snap frozen in liquid nitrogen. The anesthetized animal was then decapitated, its brain was removed, and the entire hypothalamus (~80 mg) was dissected and frozen. All samples were stored at −80°C.

RNA Isolation and RT-PCR

Isolation. Total RNA was isolated from the liver and lung samples using Qiagen RNAeasy kits (Qiagen, Valencia, CA) according to manufacturer's instructions, with subsequent precipitation by 5 M LiCl at 4°C overnight. The pellets were washed twice with ice-cold 70% ethanol, air-dried, and resuspended in RNase-free water. Thereafter, the RNA samples were treated with DNase I (DNA-Free kit, Ambion), aliquoted, and frozen at −80°C. Because the yield of RNA from brain tissue is low, the isolation procedure was modified for the hypothalamic samples. Specifically, DNase digestion of hypothalamic RNA was incorporated into the Qiagen RNeasy isolation protocol and was performed using the Qiagen RNase-free DNase set. The LiCl precipitation step was skipped, and the isolated RNA was concentrated using Savant SpeedVac concentrator (model DNA-120, Savant Instruments, Farmingdale, NY). The purity of RNA was assessed by measuring the ratio of absorption at 260 nm to that at 280 nm (52) using a DU-70 spectrophotometer (Beckman Instruments, Fullerton, CA). For all samples, this ratio was >1.9. Integrity of the isolated RNA was verified by the consistent presence of two sharp bands (corresponding to 28S and 18S rRNA) in agarose/formaldehyde gels electrophoregrams stained with SYBR Gold (Molecular Probes, Eugene, OR) (3). The amount of RNA was quantified by absorption at 260 nm (52).

Reverse transcription. Total RNA was reverse transcribed to cDNA by random hexamer priming using GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA) and SuperScript II RT (Life Technologies, Rockville, MD). Sixteen RNA samples were run together in each RT-PCR: two from each of the three LPS-treated groups; two from each of the three saline-treated groups; two from the untreated group; and two additional samples (1 from a saline- and the other from LPS-treated group) that were run with no RT added (so-called "−RT" controls). All samples were selected randomly. All liver and lung RNA samples were 2 μg each; all hypothalamic samples were 1 μg each. The reaction volume was 20 μl. The whole study consisted of three independent runs of RT-PCR in duplicates.

PCR. A temperature cycler (LightCycler, Roche Molecular Biochemicals, Indianapolis, IN) was used. The concentration of double-stranded DNA after each cycle of amplification was monitored by SYBR Green I fluorescence (5, 15). Specific PCR primers that were used to amplify the PGE_2-synthesizing genes and GAPDH, a housekeeping gene, are listed in Table 1. Those primers that were designed by the authors were based on the coding sequences of rat genes (with the exception of cPGES, for which the mouse gene was used) deposited in GenBank. All PCR samples were prepared by mixing 2 μl of cDNA, 2 μl of 25 mM $MgCl_2$, 0.5 μl of a stock solution of the forward primer (10 mM), 0.5 μl of a stock solution of the

Table 1. *Primers for RT-PCR*

Gene	Primers	Reference	Amplicon Size, bp	Annealing Temperature, °C
$cPLA_2$	F:5′-GCACATAATAGTGGAACACC R:5′-ACACAGTGCCATGCTGAACC	this study	410	58
$sPLA_2$ IIA	F:5′-ATCCCATCCAAGAGAGCTGAC R:5′-TGTTTCCGGCAGGAGTCCTG	47	326	62
$sPLA_2$ V	F:5′-CCATCCTTCCTGTGTTGCAGC R:5′-ACCAATCAGTGCCATCCTTAGG	this study	311	62
$sPLA_2$ IIA	F:5′-ATCCCATCCAAGAGAGCTGAC R:5′-TGTTTCCGGCAGGAGTCCTG	47	326	62
$sPLA_2$ V	F:5′-CCATCCTTCCTGTGTTGCAGC R:5′-ACCAATCAGTGCCATCCTTAGG	this study	311	62
COX-1	F:5′-TAAGTACCAGGTGCTGGATGG R:5′-GGTTTCCCCTATAAGGATGAG	10	244	58
COX-2	F:5′-TACAAGCAGTGGCAAAGGCC R:5′-CAGTATTGAGGAGAACAGATGGG	10	283	62
cPGES	F:5′-ACCATGCAGCCTGCTTCTGC R:5′-CATGACTGGCCGGATTCTCC	this study	240	59
mPGES	F:5′-ATCAAGATGTACGCGGTGGCT R:5′-CACTTCCCAGAGGATCTGTA	this study	375	60
GAPDH	F:5′-AGACAGCCGCATCTTCTTGT R:5′-CCACAGTCTTCTGAGTGGCA	47	587	58

$cPLA_2$ and $sPLA_2$, cytosolic and secretory phospholipase A_2, respectively; COX, cyclooxygenase; cPGES and mPGES, cytosolic and microsomal PGE synthase, respectively; F, forward; R, reverse.

reverse primer (10 mM), 2 μl of LightCycler DNA Master SYBR Green I solution (Roche), and 13 μl of sterile water. The PCR reaction consisted of a predenaturation step (95°C, 30 s) and 25–40 cycles of amplification. Each cycle included denaturation (95°C, 1 s), annealing (the gene-specific temperature from Table 1, 10 s), and elongation (72°C, 12 s for GAPDH, 10 s for all other genes).

Verification of PCR specificity and identification of the amplicons. Specificity of amplification was verified by the monophasic character of the melting curve generated for each amplification product by the LightCycler at the end of PCR (15). It was further confirmed by running agarose gel electrophoresis of each amplicon and obtaining a single band of the expected size (Table 1). For each gene of interest in each tissue, the obtained LightCycler PCR products were independently identified by sequencing. First, the products were purified with a QIAquick PCR purification kit (Qiagen) and concentrated in a Savant SpeedVac. Then a unidirectional sequencing reaction was performed on an ABI 377 automated DNA sequencer (Applied Biosystems, Foster City, CA) using an ABI Prism BigDye terminator cycle sequencing kit according to manufacturer's instructions. Finally, the sequences were compared with entries in GenBank using the BLAST program. For each product, the correspondence to the targeted gene was confirmed.

Data Processing and Analysis

Selection of time points for tissue harvesting. The ultimate goal was to harvest the tissues at three time points corresponding to three peaks of biochemical changes that drive thermoeffectors at the three febrile phases. It is not T_c per se, but rather its velocity (first derivative), that is proportional to the rate of change of the total heat content in the body (the sum of heat loss and heat production), which is determined by the activity of thermoregulatory effectors. For this reason, the time points for tissue harvesting were identified as the three local maxima of the T_c velocity as a function of time. The T_c curves were averaged across the subjects; the resultant curve was smoothed; and its first derivative was computed using Microsoft Excel. The three points were identified as 34 min (*phase I*), 94 min (*phase II*), and 296 min post-LPS (*phase III*).

Quantification of PCR products. The relative expression R of each gene of interest was calculated as follows

$$R_{i,t} = 2^{(N_{h,j} - N_{h,c}) - (N_{i,t} - N_{i,c})} \quad (1)$$

where N is the threshold cycle number, i.e., the number of the amplification cycle in which the fluorescence of a given sample becomes significantly different from the baseline signal. The indices i and h refer to the gene of interest (a PGE_2-synthesizing gene) and the housekeeping gene (GAPDH), respectively. The index t refers to a sample from a treated (with either LPS or saline) animal. The index c refers to samples from the two untreated controls run in the same RT-PCR (the variables $N_{i,c}$ and $N_{h,c}$ were the means for the 2 controls in $\log_2$ scale). *Equation 1* is based on the inverse proportionality between N and $\log_2 C$, where C is the initial template concentration in the PCR sample (5, 15). Hence the physical meaning of $R_{i,t}$ is the concentration of mRNA of interest in a sample from a treated animal divided by the concentration of the same message in the simultaneously run samples from untreated controls, where each concentration is normalized for the concentration of GAPDH message in the same sample. For all PGE_2-synthesizing genes in all tissues, the threshold cycle number N was generated by LightCycler software. The only exception was mPGES message in liver samples from untreated and saline-treated rats. In these samples, mPGES mRNA was undetectable and only the primer dimer was amplified; the threshold cycle for the primer dimer was reached before that for mPGES mRNA. For statistical purposes, the value of N for the primer dimer was accepted as that for mPGES in untreated and saline-treated liver samples. Such an assumption is conservative and would result in underestimating R values for mPGES in all liver samples from LPS-treated animals. For each gene of interest in each tissue sample, the relative expression R was measured in duplicate.

Statistical analysis. The T_c responses and the relative expression data for each gene in each tissue were compared across treatments (LPS vs. saline) and time points (3 phases)

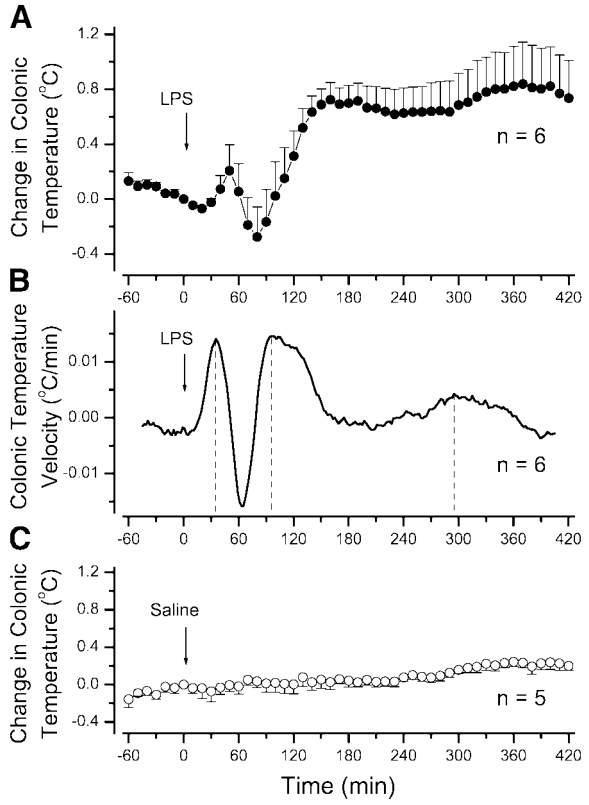

Fig. 2. The temperature responses of inbred Wistar-Kyoto rats to an intravenous injection of either lipopolysaccharide (LPS; 50 μg/kg) or saline at *time 0* (arrow). Baseline colonic temperatures in the LPS- and saline-treated groups were identical (37.6 ± 0.1°C). *A* and *C*: colonic temperature. *B*: velocity of colonic temperature. The times corresponding to the local maxima of the velocity (34, 74, and 296 min) are marked with dashed lines. These time points were selected for tissue harvesting (see *Data Processing and Analysis*).

by two-way ANOVA followed by Newman-Keuls post hoc test using Statistica AX99 (StatSoft, Tulsa, OK). It appeared that all comparisons made were characterized by a level of statistical significance either >99.6% ($P < 3.9 \times 10^{-3}$) or <94.9% ($P > 5.1 \times 10^{-2}$); the differences revealed are reported in the text and figures as highly significant or insignificant, respectively. Among the highly significant differences, 15 were characterized by the probability of null hypothesis $P < 1.0 \times 10^{-8}$. All data are presented as means ± SE.

RESULTS

Thermal Responses

Fever has not been studied in inbred Wistar-Kyoto rats before; the present data show that febrile responsiveness of these rats is somewhat lower than that of outbred rats of several common strains (59). The inbred Wistar-Kyoto rats responded to LPS (50 μg/kg iv) with three subsequent rises in T_c peaking at ~50, 160, and 370 min postinjection (Fig. 2*A*). The triphasic pattern of the febrile response was more obvious from the T_c velocity plot (Fig. 2*B*); the same plot was used to determine the time points for tissue harvesting. In contrast to LPS, the injection of saline caused practically no changes in T_c (Fig. 2*C*); the difference in the T_c curves between the LPS- and saline-treated rats was highly significant.

Gene Expression in the Liver

In this organ, transcripts of all genes of interest were readily detectable under all conditions, except for mPGES transcript in the untreated and saline-treated groups (see Fig. 3 for electrophoregram and Figs. 4–6 for quantitative results). In the saline-treated animals, no significant differences between different time points were found for any gene. Although hepatic COX-2 showed a tendency for downregulation in saline-treated animals (a ~70% decrease in the transcript concentration at *phases II* and *III*), this effect was not significant. Compared with the values of expression in the saline-treated rats, the following genes were significantly upregulated in the LPS-treated animals: $sPLA_2$-IIA (~5-fold at *phase II* and 9-fold at *phase III*; Fig. 4), COX-2 (~17-, 42-, and 18-fold at *phases I–III,* respectively; Fig. 5), and mPGES (~18-, 279-, and 1,257-fold at the 3 subsequent phases, Fig. 6). Two other genes were significantly downregulated at *phase III*: $cPLA_2$-α (~3-fold, Fig. 4) and COX-1 (7-fold, Fig. 5). The two remaining genes, $sPLA_2$-V (Fig. 4) and cPGES (Fig. 6), were unaffected by LPS.

Gene Expression in the Lungs

Transcripts of all genes of interest were detectable in the lung samples (Figs. 7–10). In the saline-treated

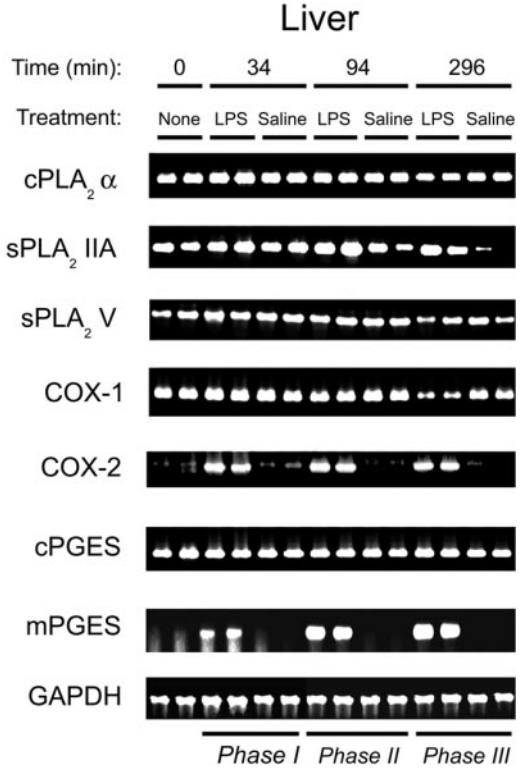

Fig. 3. Expression of genes encoding PGE_2-synthesizing enzymes in the liver (14 representative samples). The LightCycler PCR reactions were stopped at the exponential phase of amplification. Amplicons were separated by 1.5% agarose gel electrophoresis and visualized by SYBR Gold nucleic acid stain.

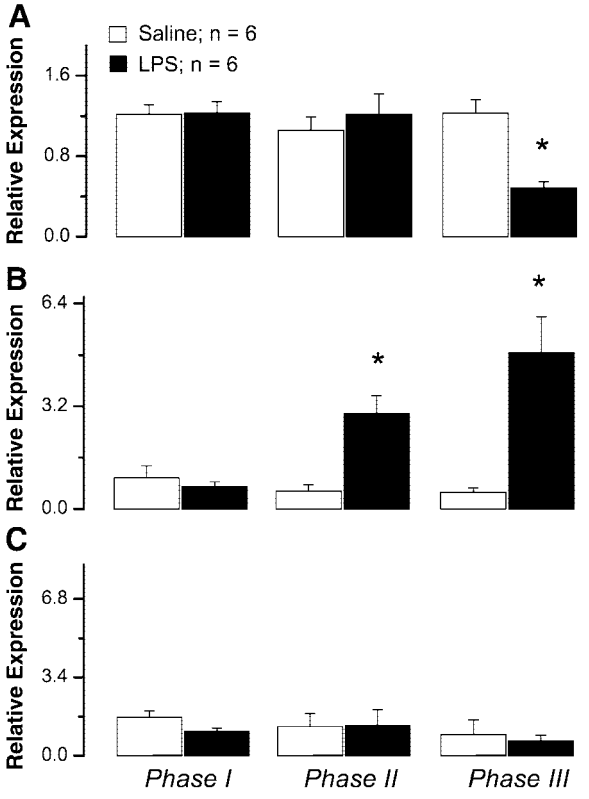

Fig. 4. Quantitative effects of LPS (50 μg/kg iv) and saline on the relative expression of PLA_2 genes in the liver. *A*: cPLA$_2$-α. *B*: sPLA$_2$-IIA. *C*: sPLA$_2$-V. Each datum is the concentration of the gene-specific mRNA in a tissue sample collected at a given time point from an experimental (treated with either LPS or saline) rat divided by the concentration of the same message in a sample harvested from an untreated control at *time 0*. These ratios were calculated for samples amplified in the same RT-PCR reaction. To equalize cDNA content in different samples, the ratios were normalized for the concentration of GAPDH message (see *Data Processing and Analysis*). $^*P < 3.9 \times 10^{-3}$.

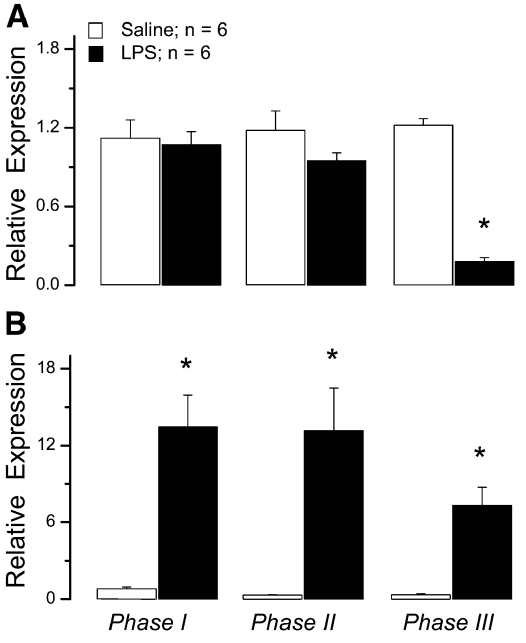

Fig. 5. Effects of LPS and saline on the relative expression of COX in the liver. *A*: COX-1. *B*: COX-2. $^*P < 3.9 \times 10^{-3}$.

animals, no significant differences between different time points were found for any gene. The effects of LPS on expression of all genes in the lungs were similar, but not identical, to those in the liver. Compared with the values of expression in the saline-treated rats, the following genes were significantly upregulated in the LPS-treated animals: sPLA$_2$-IIA (~9-fold at *phase II* and 7-fold at *phase III*; Fig. 8), COX-2 (~5-fold at *phase I* and 15-fold at *phase II*; Fig. 9), and mPGES (~3-, 33-, and 18-fold at *phases I–III*, respectively; Fig. 10). In contrast to COX-2, COX-1 was significantly downregulated in fever (3-fold at *phase III*, Fig. 9). At *phase III*, pulmonary sPLA$_2$-V was downregulated (2-fold, Fig. 8), whereas cPLA$_2$-α showed a tendency for downregulation (the effect was proven insignificant by the post hoc analysis, Fig. 8). The remaining gene, cPGES (Fig. 11), was unaffected by LPS.

Gene Expression in the Hypothalamus

All genes of interest were detectable from hypothalamic RNA of all animals (Figs. 11–14). Saline injection did not change the expression. LPS administration caused a highly significant upregulation of the expression of hypothalamic cPLA$_2$-α (~2-fold at *phase III*; Fig. 12), sPLA$_2$-IIA (27-fold at *phase II* and 133-fold at *phase III*; Fig. 12), COX-2 (3-, 12-, and 5-fold at the 3 consequent phases; Fig. 13), and mPGES (8-fold at *phase II* and 30-fold at *phase III*; Fig. 14).

DISCUSSION

Transcriptional Regulation of PLA$_2$ in Fever

Recent studies have demonstrated important roles for both intracellular (cPLA$_2$-α; Refs. 25, 62, 67) and

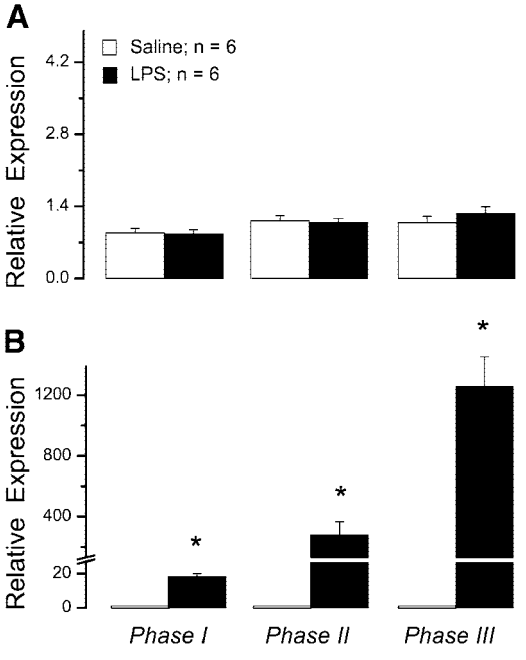

Fig. 6. Effects of LPS and saline on the relative expression of terminal PGES in the liver. *A*: cPGES. *B*: mPGES. $^*P < 3.9 \times 10^{-3}$.

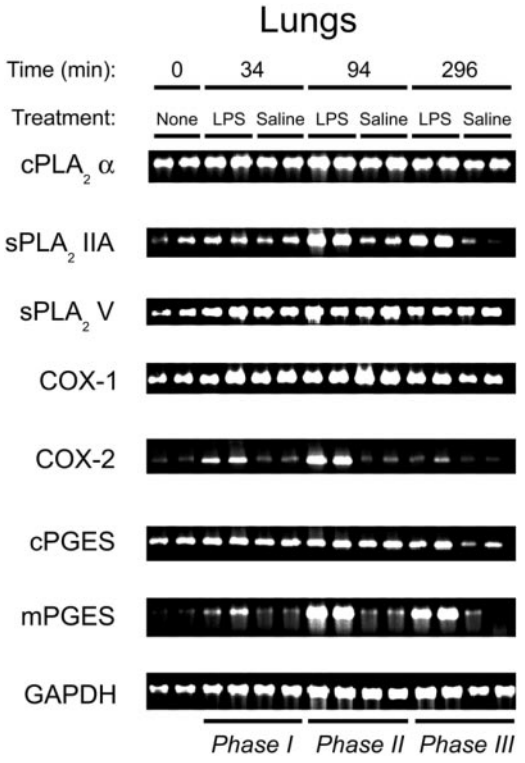

Fig. 7. Expression of genes encoding PGE_2-synthesizing enzymes in the lungs (electrophoregrams of 14 representative samples).

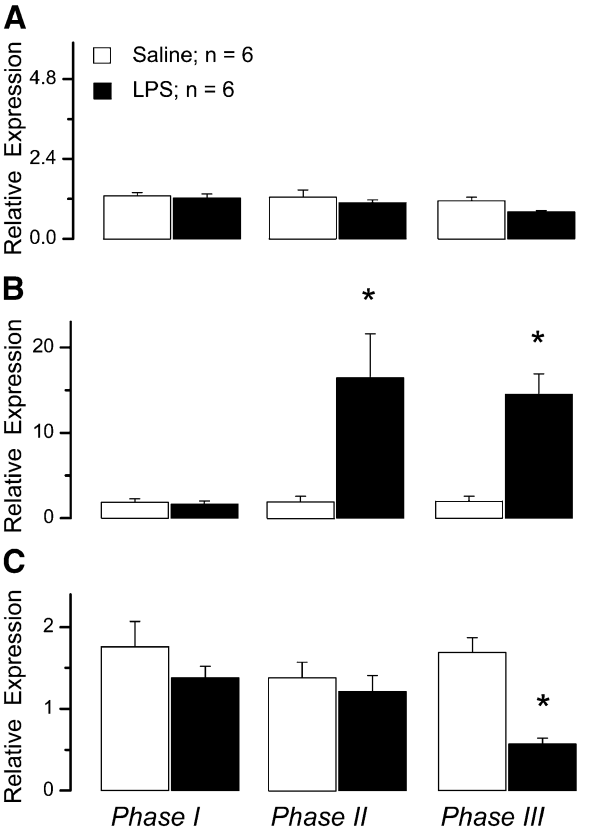

Fig. 8. Effects of LPS and saline on the relative expression of PLA_2 in the lungs. *A*: $cPLA_2$-α. *B*: $sPLA_2$-IIA. *C*: $sPLA_2$-V. $^*P < 3.9 \times 10^{-3}$.

extracellular ($sPLA_2$-IIA and -V; Refs. 43, 44, 67) PLA_2 for PGE_2 synthesis induced by a variety of inflammatory agents. The present study shows that the $sPLA_2$-IIA gene is the most sensitive to the transcription-inducing action of LPS among the three PLA_2 studied. Interestingly, upregulation of $sPLA_2$-IIA transcription was readily detectable in both the brain and periphery, but the magnitude of the upregulation was much higher in the hypothalamus (133-fold) than in the peripheral, LPS-processing organs (up to 9-fold). Upregulation of this gene by low doses of LPS in vivo has not been described previously. However, increased blood levels of $sPLA_2$-IIA and its transcriptional upregulation have been repeatedly found in various animal models of LPS shock and in septic patients (4, 67, 81). In the rat, large, shock-inducing doses of LPS (up to 10 mg/kg) induce upregulation of $sPLA_2$-IIA in a variety of peripheral organs (33, 46, 63, 81) and in the brain (33).

Unexpectedly, LPS failed to upregulate $sPLA_2$-IIA's homolog, $sPLA_2$-V, in the present work. The genes encoding $sPLA_2$-IIA and -V are located on the same chromosome; their transcriptional regulation is thought to be coordinated (11, 43); and their functional redundancy is evident in cell culture experiments (11, 44, 67). However, dissociation in the expression of $sPLA_2$-IIA and -V was found by Sawada et al. (63) in two in vivo models of LPS shock. Based on this observation and the present findings, the in vivo roles of $sPLA_2$-IIA and -V are different and possibly tissue specific.

Expression of the third PLA_2 studied, $cPLA_2$-α, was also affected by LPS in a tissue-specific manner. This gene showed a tendency for downregulation in the lungs and was significantly downregulated in the liver at *phase III* of LPS fever, whereas it was upregulated at the same phase in the hypothalamus. No reports on

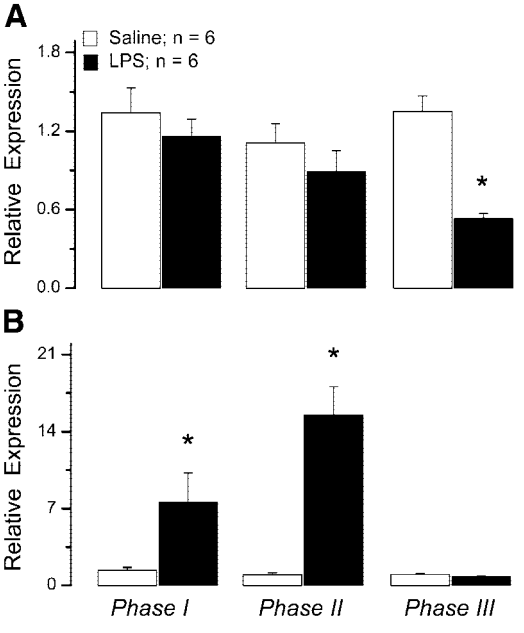

Fig. 9. Effects of LPS and saline on the relative expression of COX in the lungs. *A*: COX-1. *B*: COX-2. $^*P < 3.9 \times 10^{-3}$.

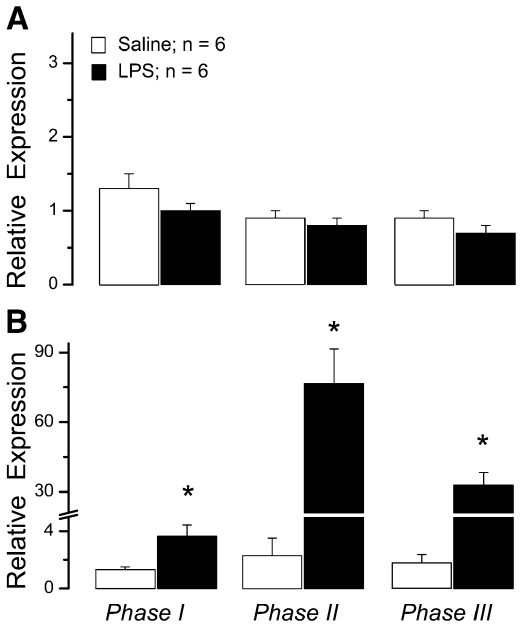

Fig. 10. Effects of LPS and saline on the relative expression of terminal PGES in the lungs. *A*: cPGES. *B*: mPGES. $*P < 3.9 \times 10^{-3}$.

regulation of $cPLA_2$-α expression by LPS in vivo were found in the literature, although increased mRNA level and activity of this enzyme were detected in leukocytes from septic patients (34).

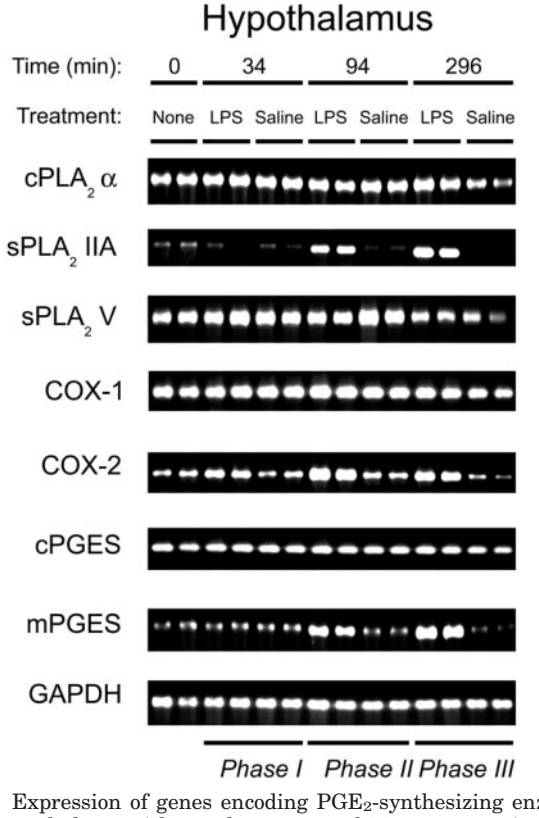

Fig. 11. Expression of genes encoding PGE_2-synthesizing enzymes in the hypothalamus (electrophoregrams of 14 representative samples).

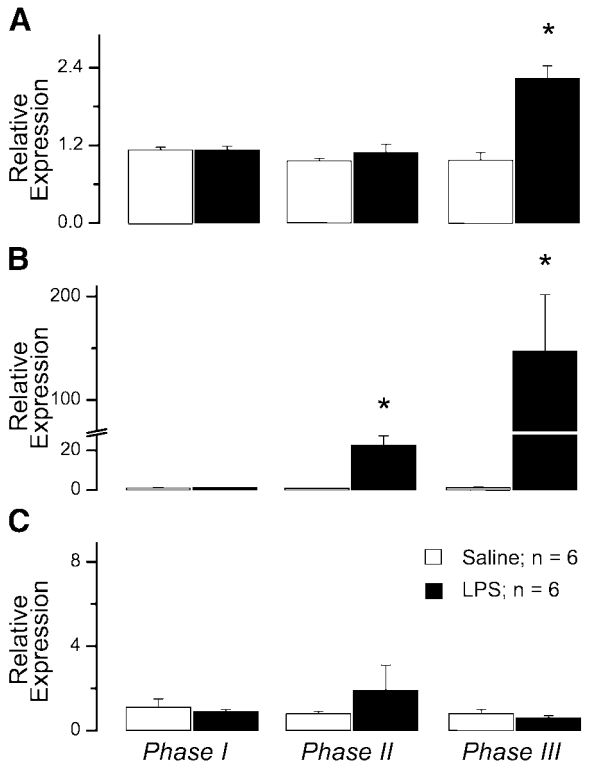

Fig. 12. Effects of LPS and saline on the relative expression of PLA_2 in the hypothalamus. *A*: $cPLA_2$-α. *B*: $sPLA_2$-IIA. *C*: $sPLA_2$-V. $*P < 3.9 \times 10^{-3}$.

Cyclooxygenases

In the present study, we found contrasting responses to LPS by the two COX isoforms: transcriptional upregulation of COX-2 and downregulation of COX-1.

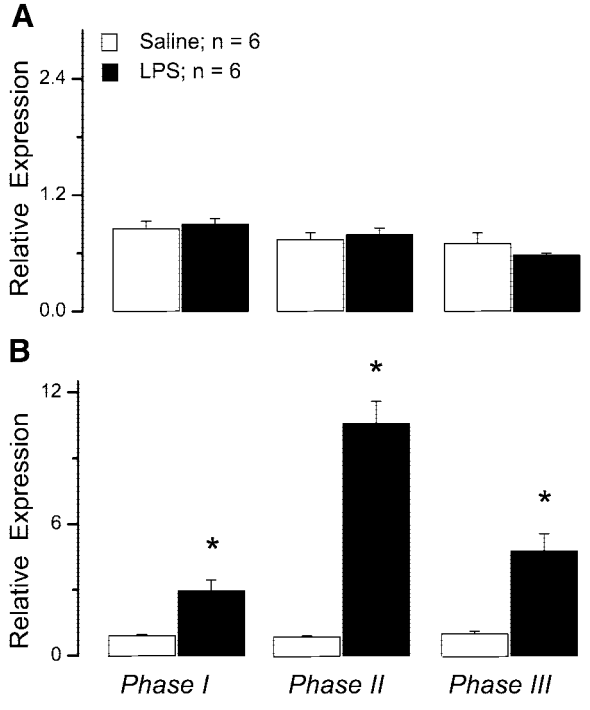

Fig. 13. Effects of LPS and saline on the relative expression of COX in the hypothalamus. *A*: COX-1. *B*: COX-2. $*P < 3.9 \times 10^{-3}$.

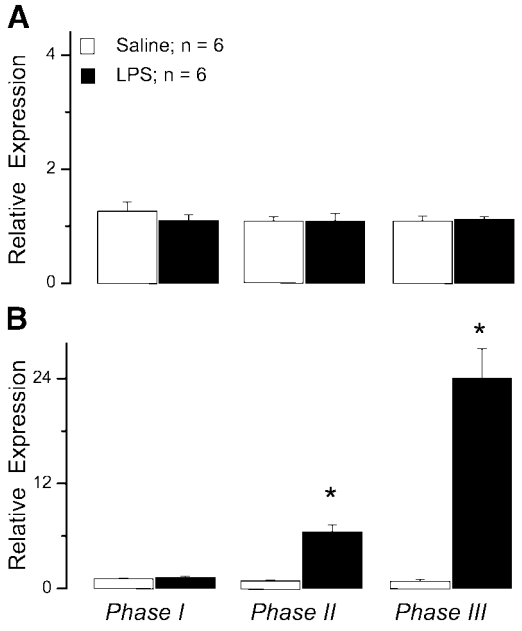

Fig. 14. Effects of LPS and saline on the relative expression of terminal PGES in the hypothalamus. *A*: cPGES. *B*: mPGES. $^*P < 3.9 \times 10^{-3}$.

Although the two isoforms of COX share ~60% of their sequences and catalyze the same reaction, they have different physiological functions and are differentially regulated. COX-1 is expressed constitutively in most tissues where it plays a wide range of housekeeping roles (71, 85). Although a low-magnitude (2- to 3-fold) induction of COX-1 by mitogens and growth factors was found in several in vitro studies (for review, see Ref. 85) and confirmed in LPS shock (83) and polymicrobial peritonitis (74) in vivo, this isoform is generally considered resistant to transcriptional upregulation (71, 85). In contrast, COX-2 transcript is barely detectable in most quiescent cells but robustly upregulated by a variety of stimuli, including LPS and proinflammatory cytokines (71, 85).

In this study, the expression of COX-2 mRNA was upregulated in the liver, lungs, and hypothalamus of the febrile rats, and the kinetics of this upregulation was remarkably fast. Already at *phase I* (34 min after LPS injection), the level of transcript was increased in all the tissues studied. Importantly, the *phase I*-associated responses of COX-2 genes were much stronger in the peripheral tissues (17-fold overexpression in the liver and 5-fold in the lungs) than in the brain (3-fold overexpression). In all the tissues, the induction peaked at *phase II* and declined at *phase III*. Although the rapid induction of COX-2 in the brain was observed in earlier in situ hybridization studies (9, 32, 51), this is the first report showing that this gene is rapidly upregulated in the periphery as well. Moreover, the upregulating effect of LPS on the COX-2 gene is greater in the peripheral LPS-processing organs than in the hypothalamus.

The cellular source of the LPS-induced COX-2 expression within different tissues is unclear. In vitro and ex vivo studies identified such an expression in Kupffer cells (but not hepatocytes) in the liver (6, 17) and in bronchial epitheliocytes, vascular myocytes, endotheliocytes, and alveolar macrophages in the lungs (21). Kupffer cells and alveolar macrophages are the same types of cells that, together with endotheliocytes, rapidly take up LPS from the circulation in vivo (22). Attempts to localize the LPS-induced expression of COX-2 in the brain produced conflicting results pointing at either endothelial cells (9, 32, 40) or perivascular microglia and meningial macrophages (20).

In contrast to COX-2, the expression of COX-1 was unchanged during *phases I-II* of LPS fever and was strongly suppressed in the liver (7-fold) and lungs (3-fold) at *phase III*. This finding agrees with data of Liu et al. (36) and Devaux et al. (16) showing downregulation of COX-1 gene in the liver, lungs, and heart of rats challenged with shock-inducing doses of LPS. In our study, the decrease of COX-1 mRNA to below its basal level occurred concurrently with the decline of COX-2 mRNA from the peak of its response at *phase II* to basal (lungs) or suprabasal (liver and hypothalamus) levels. This simultaneous suppression of both genes suggests a common mechanism for their transcriptional downregulation at the later stages of the febrile response. Such a mechanism may involve activation of the peroxisome proliferator-activated receptors, which are considered negative regulators of the inflammatory response (12). A potent endogenous agonist of these receptors, 15d-PGJ_2, was recently shown to suppress LPS-induced COX-2 expression in cultured macrophage-like cells (26). 15d-PGJ_2, a natural metabolite of PGD_2, is synthesized via a COX-dependent pathway and may, therefore, constitute a negative-feedback mechanism for COX expression (12).

Terminal PGE Synthases

Two terminal mPGES were studied in the present work, mPGES and cPGES. In all tissues studied, mPGES gene exhibited strong transcriptional upregulation during LPS fever. Yet, the dynamics of this response were tissue specific. In the liver and lungs, mPGES mRNA was already increased at *phase I*; in the hypothalamus, it was not induced until *phase II*. Furthermore, whereas the expression of hepatic and hypothalamic mPGES steadily increased throughout the febrile course, the level of the pulmonary transcript peaked at *phase II* and declined thereafter. A remarkable feature of the mPGES response was its magnitude: the expression of this gene was upregulated 1,257-fold (liver), 33-fold (lungs), or 30-fold (hypothalamus).

While this study was in progress, several groups reported upregulation of mPGES expression by IL-1β (18) or large (120–400 μg/kg) doses of LPS (38, 45, 86) in vivo. Murakami et al. (45) and Mancini et al. (38) found an LPS-induced increase in mPGES mRNA in the brain, lungs, heart, spleen, kidney, testis, stomach, and seminal vesicles in the rat. Yamagata et al. (86) reported an induction by LPS of mPGES transcript

throughout the entire rat brain. The authors found that the message was localized mostly in the veins and venular-type vessels, and the protein was abundant in the perinuclear envelope of endothelial cells, where mPGES was colocalized with COX-2 (86). Ek et al. (18) obtained similar results by finding co-induction of mPGES and COX-2 by IL-1β in brain vascular cells, presumably endotheliocytes and perivascular macrophages.

In contrast to mPGES, expression of cPGES was changed neither in the peripheral organs nor in the hypothalamus during LPS fever in the present study. This finding is in line with the results by Tanioka et al. (78), who failed to find any effect of LPS, IL-β, or TNF-α on the level of cPGES mRNA in cultured human and murine gliocytes, epitheliocytes, and fibroblasts. Furthermore, LPS did not affect the expression of cPGES in peripheral rat tissues 48 h postadministration (78). Therefore, it is likely that cPGES is not a subject of transcriptional regulation by inflammatory stimuli.

Functional Consideration

The present experiments identified three genes that are markedly upregulated during LPS-induced fever in all tissues studied: $sPLA_2$-IIA, COX-2, and mPGES. This finding agrees with earlier in vitro studies showing that an increase in the mRNA level for these enzymes by either transcriptional stimulation or transfection caused robust production of PGE_2 (39, 45, 67, 73, 75), whereas inactivation of the transcripts by antisense oligonucleotides suppressed PGE_2 synthesis in stimulated cells (44, 45, 71, 78). A crucial role in the genesis of LPS-induced fever has been established for one of these enzymes, COX-2, and proposed for another, $sPLA_2$. Several selective COX-2 inhibitors have been shown to either attenuate or completely block LPS fever in rats, pigs, monkeys, and humans (for review, see Ref. 69), whereas COX-2 (but not COX-1) knockout mice have been proven to lack the ability to mount a fever in response to LPS (35). Annexins I (lipocortin) and V, small anti-inflammatory proteins that inhibit $sPLA_2$ activity (30), have been shown to attenuate the febrile response in rodents (13, 50). However, the antipyretic action of annexins may also involve suppression of transcriptional upregulation of COX-2 and inducible nitric oxide synthase (41). No functional evidence supporting a role of mPGES in the febrile response has yet been obtained. However, the proposed coupling between sPLA and COX-2 (44, 47, 67) and between COX-2 and mPGES (45) along with the co-induction of $sPLA_2$-IIA, COX-2, and mPGES reported in the present work argue for the functional importance of all three enzymes in the genesis of fever.

It should be noted, however, that this study focused only on the transcriptional regulation and did not assess other regulatory mechanisms, such as modulation of mRNA stability and translational efficiency or post-translational modifications of the proteins (25, 44, 45, 71). It cannot be ruled out, therefore, that those genes that failed to respond to LPS with a strong transcriptional upregulation in the present study (e.g., $cPLA_2$-α, $sPLA_2$-V, and cPGES) are still involved in the febrile response. For example, the primary mechanism for $cPLA_2$-α activation is phosphorylation with a consequent translocation of the enzyme to the perinuclear envelope, which is rich with COX-2 and mPGES (25). Hence, the relatively small changes in the expression of $cPLA_2$-α observed in the present work do not contradict recent data showing a prominent role for this enzyme in the LPS- or cytokine-induced synthesis of PGE_2 (25, 62, 67).

The three time points selected for tissue harvesting might not have been ideal for assessing each of the 21 individual gene responses (7 enzymes × 3 tissues) studied. Fever involves not only the induction of PGE_2 synthesis by endogenous pyrogens, but also the induction of synthesis and/or release of multiple antipyretic substances, including vasopressin, melanocortins, glucocorticoids, anti-inflammatory cytokines, and even some products of AA (31, 87). Because these factors counteract the action of PGE_2, it is possible that neither the peaks of PGE_2 concentration in the blood nor the peaks of responses of individual PGE_2-synthesizing enzymes in different tissues strictly coincide with the peaks of the rate of change in body temperature. Hence, the precise quantitative dynamics of the PGE_2-synthesizing enzymes reported in this study should be viewed with caution.

Three Febrile Phases: Three Expressional Patterns of PGE_2-Synthesizing Enzymes

The present study identified three different patterns of gene expression corresponding to *phases I–III* of the febrile response to LPS (Fig. 15). At *phase I*, the most remarkable event was strong induction of the functional couple COX-2 → mPGES in the peripheral LPS-processing organs; we speculate that this febrile phase is triggered primarily by peripherally synthesized PGE_2. *Phase II* was characterized by robust transcriptional upregulation of the whole cascade, $sPLA_2$-IIA → COX-2 → mPGES, both in the periphery and in the brain. The most prominent events occurring at *phase III* were the induction of $cPLA_2$-α in the hypothalamus and further upregulation of $sPLA_2$-IIA and mPGES in the liver and hypothalamus. *Phase III* was also characterized by downregulation of $cPLA_2$-α and COX-1 in the liver; downregulation of $sPLA_2$-V and COX-1 in the lungs; a decline in COX-2 expression in all tissues studied; and a decrease of mPGES expression in the lungs. We speculate that both peripheral and central bursts of PGE_2 synthesis contribute to febrigenesis at *phase III*, and that the hypothalamic burst occurs primarily via PL-dependent mechanisms, whereas the hepatic burst is mPGES dependent.

Phase I. The peripheral origin of *phase I* is supported by several lines of evidence. This phase can be blocked by inhibition of PG synthesis in the periphery (but not in the brain) by nonselective COX inhibitors (42). It can also be blocked by elimination of peripheral macro-

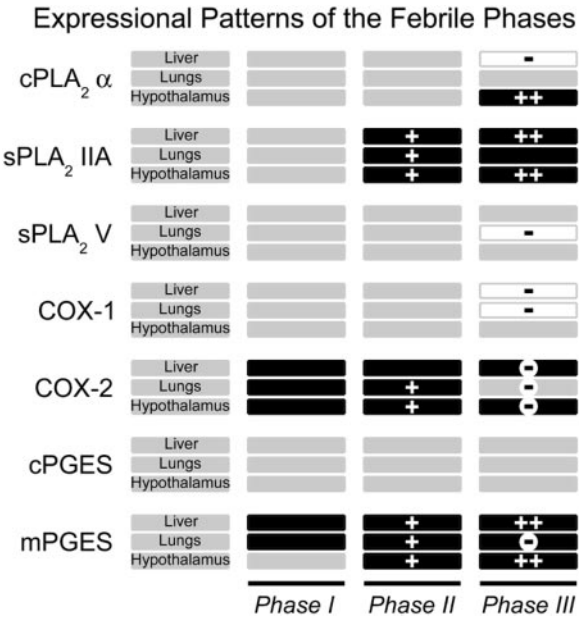

Fig. 15. Schematic summary of the results. Upregulation of a PGE_2-synthesizing gene in LPS-treated rats at a given febrile phase (compared with the corresponding saline-treated rats) is shown in black; downregulation is shown in white; gray represents no statistically significant changes. +, Significant upregulation of a gene at *phase II* compared with *phase I*; ++, significant upregulation at *phase III* compared with *phase II*; −, significant downregulation at *phase III* compared with *phase II*. Note that some genes (COX-2 in the liver and hypothalamus and mPGES in the lungs) are upregulated at *phase III* compared with their expression in saline-treated rats, but downregulated compared with their expression at *phase II*.

phages (important LPS-processing and PGE_2-synthesizing cells) or by selectively denervating the liver (the major harbor of macrophages in the body) (14, 70). The present data indicate that the early induction of PGE_2-synthesizing enzymes, COX-2 and mPGES, is most prominent in the liver, but also occurs in the lungs. Once synthesized in the LPS-processing organs, PGE_2 can trigger *phase I* by signaling the brain via either the humoral or the neural route. The humoral route implies carrier-mediated transport of PGE_2 with the circulation into the brain (54). The neural route of PGE_2 signaling is likely to involve capsaicin-sensitive sensory fibers (77), possibly within the vagus nerve (19, 56, 87). Vagal sensory neurons express PG receptors of the EP3 type (19), and the *phase I* of LPS fever does not occur in EP3 or EP1 receptor knockout mice (48, 80). The importance of the hepatic vagus for the genesis of the febrile response to small amounts of circulating LPS has been demonstrated (70), whereas an involvement of the pulmonary vagus has been hypothesized (19).

Phases II and III. A large body of experimental data connects the later stages of LPS fever with hypothalamic PGE_2. A peripheral administration of LPS activates PGE_2 synthesis in the anteroventral preoptic area of the hypothalamus (82). The preoptic hypothalamus is rich in PG receptors (49) and exceptionally sensitive to the febrigenic action of exogenous PGE_2 (65). Furthermore, microinjections of a COX inhibitor into the same area, but not neighboring structures, attenuate the fever response to intravenous LPS (66). Our data suggest that stimulation of hypothalamic PGE_2 synthesis by LPS occurs via transcriptional upregulation of the $sPLA_2$-IIA → COX-2 → mPGES cascade at *phase II* and via upregulation of $cPLA_2$-α, $sPLA_2$-IIA, and mPGES at *phase III*. Transcriptional upregulation of COX-2 and mPGES by LPS in the hypothalamic blood vessels at time points corresponding to *phases II* and *III* has been demonstrated (9, 32, 51, 86). The mechanisms of such upregulation remain speculative. Recent studies suggest an involvement of the LPS-induced circulating cytokines, TNF-α and IL-1β (7, 8, 18, 32). Peripheral administration of either TNF-α or IL-1β induces a marked fever (31, 87) and upregulates COX-2 and/or mPGES expression in the brain vasculature (7, 8, 18, 32). Based on the time courses of the plasma TNF-α and IL-1β responses to LPS (23), circulating TNF-α may play a role in the global upregulation of the $sPLA_2$-IIA → COX-2 → mPGES cascade during *phase II*, whereas IL-1β may stimulate expression of PGE_2-synthesizing genes in the hypothalamus during *phase III*.

Concluding Remarks

This is the first study of transcriptional regulation of the entire PGE_2-synthesizing cascade in an in vivo model of systemic inflammation, the febrile response of rats to a mild dose of intravenous LPS. We found that LPS fever is accompanied by upregulation of four PGE_2-synthesizing genes: mPGES, $sPLA_2$-IIA, COX-2, and $cPLA_2$-α (up to 1,257-, 133-, 42-, and 2-fold, respectively). The highest magnitude of upregulation makes mPGES and $sPLA_2$-IIA attractive targets for antipyretic/anti-inflammatory therapy

This is also the first report demonstrating the dynamics of the transcriptional regulation of PGE_2 synthesis by LPS in vivo: different homologs/isoforms of PGE_2-synthesizing enzymes are subsequently up- and downregulated in different tissues in the progression of the acute inflammatory response. The onset of the febrile response (*phase I*) involves the induction of a functional couple COX-2 → mPGES in the peripheral LPS-processing organs. *Phase II* entails robust upregulation of the major inflammation-associated pathway for PGE_2 synthesis, $sPLA_2$-IIA → COX-2 → mPGES, both in the periphery and in the brain. Induction of hypothalamic $cPLA_2$-α and further transcriptional upregulation of $sPLA_2$-IIA and mPGES in the hypothalamus and liver occur at *phase III*, while both COX-1 and COX-2 appear downregulated. The fact that mPGES and $sPLA_2$-IIA are strongly upregulated when expression of COX-2 already declines adds to their attractiveness as pharmacological targets.

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