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Cyclooxygenase-1 or -2—which one mediates lipopolysaccharide-induced hypothermia?

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Steiner AA, Hunter JC, Phipps SM, Nucci TB, Oliveira DL, Roberts JL, Scheck AC, Simmons DL, Romanovsky AA. Cyclooxygenase-1 or -2—which one mediates lipopolysaccharide-induced hypothermia? *Am J Physiol Regul Integr Comp Physiol* 297: R485–R494, 2009. First published June 10, 2009; doi:10.1152/ajpregu.91026.2008.—Systemic inflammation is associated with either fever or hypothermia. Fever, a response to mild systemic inflammation, is mediated by cyclooxygenase (COX)-2 and not by COX-1. However, it is still disputed whether COX-2, COX-1, neither, or both mediate(s) responses to severe systemic inflammation, and, in particular, the hypothermic response. We compared the effects of SC-236 (COX-2 inhibitor) and SC-560 (COX-1 inhibitor) on the deep body temperature (T_b) of rats injected with a lower (10 μg/kg iv) or higher (1,000 μg/kg iv) dose of LPS at different ambient temperatures (T_as). At a neutral T_a (30°C), the rats responded to LPS with a polyphasic fever (lower dose) or a brief hypothermia followed by fever (higher dose). SC-236 (2.5 mg/kg iv) blocked the fever induced by either LPS dose, whereas SC-560 (5 mg/kg iv) altered neither the febrile response to the lower LPS dose nor the fever component of the response to the higher dose. However, SC-560 blocked the initial hypothermia caused by the higher LPS dose. At a subneutral T_a (22°C), the rats responded to LPS with early (70–90 min, nadir) dose-dependent hypothermia. The hypothermic response to either dose was enhanced by SC-236 but blocked by SC-560. The hypothermic response to the higher LPS dose was associated with a fall in arterial blood pressure. This hypotensive response was attenuated by either SC-236 or SC-560. At the onset of LPS-induced hypothermia and hypotension, the functional activity of the COX-1 pathway (COX-1-mediated PGE_2 synthesis ex vivo) increased in the spleen but not liver, lung, kidney, or brain. The expression of splenic COX-1 was unaffected by LPS. We conclude that COX-1, but not COX-2, mediates LPS hypothermia, and that both COX isoforms are required for LPS hypotension.

body temperature; thermoregulation; fever; inflammation

SO STRONGLY IS SYSTEMIC INFLAMMATION associated with changes in deep body temperature (T_b) that every clinical definition of the systemic inflammatory response syndrome includes a change in T_b (11, 48). Whereas the majority (~90%) of patients with systemic inflammation have an increased T_b, a number of them (~10%) have a lowered T_b (6, 17). Thermoregulatory manifestations are also present in animal models of systemic inflammation. In a rat model of systemic inflammation induced by bacterial LPS, the pattern of T_b change depends on the ambient temperature (T_a) and the LPS dose. At a neutral or supraneutral T_a (warm environment), fever is the prevailing response; the fever is monophasic when the dose of LPS is low (just suprathreshold), but it turns polyphasic as the dose increases (66, 68, 69, 71, 79); a mild hypothermia may precede the polyphasic fever when the dose of LPS is high (68). At a subneutral T_a (cool environment), hypothermia followed by fever is the predominant response; the magnitude of the hypothermia increases along with the LPS dose (9, 52, 70). Our studies show that LPS-induced fever and hypothermia are both physiological responses brought about by brain-driven changes in thermoeffector activity (3, 4, 70). Whereas the biological value of fever is thought to be related to its immunostimulant and antibacterial effects (43), the biological value of hypothermia may be related to energy conservation when inflammation is severe enough to compromise tissue perfusion or threaten energy reserves (72, 82).

There is no doubt that cyclooxygenase (COX) plays a critical role in the genesis of fever by catalyzing the conversion of arachidonic acid to prostaglandin (PG) H_2, the immediate precursor of febrigenic PGE_2 (10, 35, 55, 66). Clinical fevers (5) and all phases of experimental, LPS-induced fever (13, 49, 81, 83, 90) are thought to be mediated by COX-2, the inducible isoform, and not by COX-1, the predominantly constitutive isoform. An involvement of COX in LPS-induced hypothermia has also been suggested (7, 19, 86), and it is believed to be associated with the formation of potentially cryogenic PGD_2 from COX-derived PGH_2 (86). However, studies of the COX isoforms involved in LPS hypothermia have yielded contradictory results. Dogan et al. (20) and Akarsu and Mamuk (2) reported a suppression of LPS hypothermia by a preferential (valeryl-salicylate) or selective (SC-560) COX-1 inhibitor in rats, thus suggesting that this response is mediated by COX-1. Zhang et al. (90) reported the opposite, hypothermia-enhancing, effect of SC-560 in the rat, thus suggesting that products of COX-1 inhibit LPS hypothermia in the same species. Further complicating the picture, Dogan et al. (20, 21) and Zhang et al. (90) found that a preferential (nimesulide) or selective (SC-236) inhibitor of COX-2 attenuated LPS hypothermia in rats, thus suggesting mediation by COX-2.

The present study was conducted to clarify which COX isoform, if any, mediates hypothermia in systemic inflammation. We compared the effects of SC-560 and SC-236 on the T_b responses of rats to different doses of LPS at different T_as. Because T_b responses to high doses of LPS are associated with hypotension (46, 62, 70), arterial blood pressure was monitored in a subset of experiments. As a follow up to our finding that

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the COX-1 inhibitor, but not the COX-2 inhibitor, blocked LPS-induced hypothermia, we measured the expression of COX-1 at the mRNA and protein levels and the functional activity of the COX-1 pathway at the onset of the LPS-induced responses.

METHODS

Animals

The study was conducted in male Wistar rats (Harlan, Indianapolis, IN) that weighed 290–380 g at the time of experiments. Initially, the rats were housed three per standard cage; after surgery, they were housed 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. Standard rat chow and tap water were available ad libitum. The room was on a 12:12-h light-dark cycle (lights on at 7:00 AM). 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 being located in the confiners (7 daily training sessions, 4 h each). The same confiners were used later in the experiments. Rodents are readily adaptable to confinement to an extent that habituated rodents respond to it with neither stress fever (71) nor other signs of stress (1, 32, 56, 78). Each rat was used in an experiment once and euthanized with sodium pentobarbital (100 mg/kg iv) immediately thereafter. All procedures were conducted under protocols approved by the St. Joseph's Hospital and Medical Center's Animal Care and Use Committee.

Surgical Preparation

Four days before an experiment, every rat was implanted with an intravenous catheter; some rats were also implanted with intra-arterial catheters. The procedures were performed under ketamine-xylazine-acepromazine (55.6, 5.5, and 1.1 mg/kg ip, respectively) anesthesia and antibiotic (enrofloxacin, 1.1 mg/kg sc) protection. During surgery, a rat was maintained on a board warmed to 37°C by a Deltaphase isothermal pad (Braintree Scientific, Braintree, MA).

For venous catheterization, a small longitudinal incision was made on the left ventral surface of the neck. The left jugular vein was exposed, freed from its surrounding connective tissue, and ligated. A silicone catheter (ID 0.5 mm, OD 0.9 mm) filled with heparinized (10 U/ml) saline was passed into the superior vena cava through the jugular vein and secured in place with ligatures. The free end of the catheter was knotted, tunneled under the skin to the nape, and exteriorized. The skin was sutured.

For arterial catheterization, the right ventral surface of the neck was incised, and the right carotid artery was isolated and clamped by a microclip. The tip of a PE-50 catheter (ID 0.6 mm, OD 1.0 mm) filled with heparinized saline was placed into the artery, the clip was removed, and the catheter was moved toward the aorta. The catheter was secured in place with ligatures. The free end of the catheter was heat-sealed and exteriorized at the nape. The skin was sutured.

To prevent postsurgical hypothermia, the animals were allowed to recover from anesthesia in an environmental chamber (model 3940; Forma Scientific, Marietta, OH) set to 28.0°C. The intravenous catheters were flushed with heparinized saline every other day; the intra-arterial catheters were flushed daily.

Experimental Setup

On the day of the experiment, each rat was placed in a confiner. For measurement of colonic temperature (an index of T_b), a copper-constantan thermocouple was inserted in the colon, 10 cm beyond the anal sphincter. The thermocouple was fixed to the base of the tail with adhesive tape and plugged into a data logger (Cole-Parmer, Vernon Hills, IL), which conveyed the data to a personal computer. The rat was transferred to an environmental chamber (Forma Scientific) set to either a neutral (30.0°C) or subneutral (22.0°C) T_a (67). The venous catheter was extended with a length of PE-50 tubing filled with saline, and the extension was passed through a wall port and connected to a syringe filled with the drug of interest. This setup permits intravenous drug administration without disturbing a rat and without causing a marked stress response that often presents a major limitation in thermoregulation experiments (69, 73).

If present, the arterial catheter was used for recording arterial pressure. A PE-50 tubing extension of the arterial catheter was passed through a wall port and connected to a differential pressure transducer (Columbus Instruments, Columbus, OH). The analog output of the transducer was converted by the Datamax logger interface (Columbus Instruments) into a digital signal, which was fed into a personal computer. The pulsatile arterial pressure data were collected and processed using the Datamax software (Columbus Instruments). Mean arterial pressure was calculated from a time integral of the pulsatile pressure.

Drug Administration

COX inhibitors. Selective COX-1 and COX-2 inhibitors (SC-560 and SC-236, respectively) were gifts from Pfizer (Groton, CT). In vitro studies demonstrate that SC-560 is ~700 times more potent to inhibit COX-1 than COX-2 (76), whereas SC-236 is ~1,800 times more potent to inhibit COX-2 than COX-1 (65). SC-560 and SC-236 were dissolved in ethanol to a final concentration of 16 and 8 mg/ml, respectively. These solutions were aliquoted and stored at −80°C until the day of the experiment. On the day of the experiment, an aliquot was warmed to room temperature, and infused intravenously at a rate of 31 μl/kg/min for 10 min. Control rats were infused with the vehicle at the same low rate. This infusion protocol produced neither hemolysis (determined based the color of the plasma) nor other signs of ethanol toxicity. The doses of SC-560 and SC-236 delivered over the 10-min infusion were 5 and 2.5 mg/kg, respectively. At these in-vivo doses, SC-560 maximally inhibits COX-1 without affecting COX-2, whereas SC-236 maximally inhibits COX-2 without affecting COX-1 (31, 53, 54).

LPS. E. coli 0111:B4 LPS was purchased from Sigma-Aldrich (St. Louis, MO). A stock suspension of LPS (5 mg/ml) in pyrogen-free saline was stored at −20°C. On the day of the experiment, the stock was diluted to a final concentration of either 10 or 1,000 μg/ml. The diluted LPS suspension or saline was bolus injected (1 ml/kg) through the extension of the venous catheter 20 min after completion of the 10-min-long infusion of SC-560, SC-236, or their vehicle. The resultant doses of LPS (10 or 1,000 μg/kg iv) have been repeatedly shown to cause a mild polyphasic fever (the lower dose) or a brief hypothermia followed by fever (the higher dose) at a neutral T_a, whereas they cause a dose-dependent hypothermia at a subneutral T_a (68–71, 79).

Functional Activity of the COX-1 Pathway and COX-1 Expression

COX-1 pathway activity was assessed on the basis of the ex vivo production of PGE_2 that is blocked by SC-560. We selected the COX-1-mediated synthesis of PGE_2 as a measure of the functional activity of the COX-1 pathway because the immediate product of the reaction catalyzed by COX-1, PGH_2, is unstable. Among the multiple products synthesized in the next step (by several PGE, D, F, and I synthases and by thromboxane synthases), PGE_2 is reasonably stable and the most robustly produced during inflammation in a wide spectrum of organs and tissues throughout the body (35). Furthermore, at least in some situations, the critical, rate-limiting step of inflammation-associated PGE_2 synthesis seems to be the one catalyzed by COX and not the one catalyzed by terminal synthases (8). If one accepts that LPS-induced hypothermia is mediated by PGD_2 (which may not be the case; see Refs. 27, 44), an alternative approach

would be to use the COX-1-mediated PGD_2 synthesis as a measure of COX-1 pathway activity. However, PGD_2 is much less stable than PGE_2, whereas some more stable products of PGD_2, such as 15-deoxy-$\Delta^{12,14}$-PGJ_2, increase (rather than decrease) deep T_b in rats (A. A. Steiner, A. S. Dragic, J. Pan, A. A. Romanovsky; unpublished observation). Hence, the stability of PGE_2 and the robustness of its synthesis under inflammatory conditions provide a solid justification for the use of COX-1-mediated PGE_2 synthesis as a measure of COX-1 pathway activity. It should be understood, however, that this measure reflects the enzymatic activity not only of COX-1, but also that of several PGE terminal synthases, and depends both on how COX-1 is coupled with each synthase and on which enzyme in each COX-1-synthase pair catalyzes the critical step.

Tissues for the functional activity assay were harvested from rats 50 min after injection of LPS (1,000 μg/kg) or saline at a T_a of 22.0 °C. This time point corresponds to the maximum rate of fall in T_b during LPS hypothermia. At the time of tissue harvesting, rats were anesthetized intravenously with ketamine-xylazine-acepromazine (5.6, 0.6, and 0.1 mg/kg, respectively). Following transcardiac perfusion with 30 ml of saline (10 ml/min), the entire brain, right kidney, spleen, right lung, and the central lobe of the liver were collected. Each tissue was rinsed with PBS (0.01 M, pH 7.4) and transferred to a polypropylene conical tube. PBS was added to each tube to achieve a PBS:tissue ratio of 5:1 (wt:wt), and the tissue was then homogenized on ice using an ultrasonic cell disruptor. Aliquots of the homogenate were preincubated (25°C, 15 min) with or without SC-560 (0.6 μM). At this concentration, SC-560 inhibits COX-1 activity by >95% (76). Preincubation was followed by incubation (37°C, 10 min) of the homogenate with arachidonic acid (30 μM). Enzymatic reactions were stopped by heating the homogenate to 65°C for 5 min. The homogenate was then centrifuged (13,000 *g*, 10 min, 4°C), and the supernatant and pellet were stored separately at −80°C. The supernatant was assayed for PGE_2 by radioimmunoassay using a rabbit antibody raised against a PGE_2-albumin complex (Sigma-Aldrich) and for total protein by the Bradford method (Bio-Rad, Hercules, CA); the assays were conducted according to the manufacturers' instructions. The concentrations of PGE_2 and total protein were used to calculate the COX-1 pathway activity (PGE_2 concentration/protein concentration/duration of arachidonic acid incubation). The COX-1 pathway activity was calculated by subtracting the ex vivo activity of a sample incubated in the absence of SC-560 from the activity of the same sample incubated in the presence of SC-560.

In view of the fact that LPS increased the functional activity of the COX-1 pathway in spleen but not in other tissues (see RESULTS), the expression of COX-1 was measured in spleen samples only. The pellets from the samples processed for functional activity were subjected to Western blot analysis for determination of COX-1 protein content, as follows. The pellet was reconstituted by sonication in PBS containing the Complete EDTA-free Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN). As a positive control for detection of COX-1, rat platelets were obtained from platelet-rich plasma and sonicated like the pellets. The reconstituted pellet (35 μg total protein) or platelet lysate (35 μg total protein) was resolved by SDS-polyacrylamide gel electrophoresis, transferred to a polyvinylidene fluoride membrane, and probed with a rabbit anti-COX-1 polyclonal antibody (1:1,000; Cayman, Ann Arbor, MI) for 12 h at 4°C. Blots were then incubated with a horseradish peroxidase-conjugated secondary antibody (anti-rabbit IgG, 1:20,000; Sigma-Aldrich) for 1 h at room temperature. The blot was developed using the Western Lightning Chemiluminescence Reagent (Perkin Elmer, Boston, MA) and X-ray film (Kodak, Rochester, NY). The Western blots were analyzed by densitometry using Adobe Photoshop CS3 extended edition: after digital (TIF) images of the films were color inverted, the mean gray value of a box drawn around a band of interest was subtracted from the value of a same-size box drawn over the background; the resulting value (arbitrary units) represented the intensity of the band.

For quantification of COX-1 mRNA levels by quantitative real-time RT-PCR, spleen samples were collected (as described above) from a separate set of rats. In addition to samples from LPS- and saline-injected rats, samples from untreated rats were collected and used as reference samples for calculating relative expression values (see below). Total RNA was isolated from tissue samples using TRIzol (Invitrogen, Carlsbad, CA) and treated with Turbo DNA-free (Ambion, Austin, TX), as described in detail elsewhere (34). RNA integrity was determined by a 20100 Bioanalyzer (Agilent, Santa Clara, CA). Total RNA was reverse transcribed to cDNA by random hexamer priming using SuperScript III First-Strand Synthesis System (Invitrogen). For quantitative real-time PCR, a LightCycler (Roche Applied Science, Indianapolis, IN) was used. The concentration of double-stranded DNA amplicon was monitored using Light Cycler FastStart DNA Master Plus SYBR Green I (Roche Applied Science). Primers for COX-1 (gene of interest) were 5′-ACTGGAAACCCAGCACATTC (forward) and 5′-ACTCCTCCCTCCAGAAGAGC (reverse); annealing temperature was 62°C. Primers for β-actin (housekeeping gene) were 5′-CGAGTCCGCGTCCACCCGCGA (forward) and 5′-GACGACGAGCGCAGCGATATC (reverse); annealing temperature was 62°C. The relative expression *R* of the gene of interest was calculated according to the formula: $R_{i,t} = 2^{(Nh,t - Nh,c) - (Ni,t - Ni,c)}$, where *N* is the threshold cycle number, *i.e.*, the number of the amplification cycle in which fluorescence of a given sample becomes significantly different from the baseline signal (36). The indexes *i* and *h* refer to the gene of interest and housekeeping gene, respectively; the index *t* refers to individual samples from rats treated with either LPS or saline; and the index *c* refers to control samples (namely, samples pooled from untreated rats). This equation is based on the inverse proportionality between *N* and log_2C, where *C* is the initial template concentration in the PCR sample. The physical meaning of $R_{i,t}$ is the concentration of mRNA of interest (COX-1) in a sample from a treated (with LPS or saline) animal divided by the concentration of the same message in the simultaneously run untreated controls, in which each concentration is normalized for the concentration of a housekeeping mRNA (β-actin) in the same sample. Gene amplification was verified by running agarose gel electrophoresis of each amplicon obtained during the exponential phase of PCR amplification.

Statistical Analyses

The T_b and blood pressure responses were compared across treatments and time points by a two-way ANOVA. The data on COX-1 pathway activity were compared across treatments and organs by a two-way ANOVA. The COX-1 protein and mRNA levels in the spleen were compared across treatments by Student's *t*-test. All analyses were performed using Statistica Advanced 8.0 (StatSoft, Tulsa, OK). The data are reported as means ± SE.

RESULTS

LPS-induced hypothermia is blocked by SC-560 but enhanced by SC-236. We studied the effects of a COX-1 inhibitor (SC-560), a COX-2 inhibitor (SC-236), or their vehicle on the thermoregulatory responses of rats injected with a lower dose of LPS (10 μg/kg), a higher dose of LPS (1,000 μg/kg), or saline at a neutral (30°C) or subneutral (22°C) T_a. Baseline T_b ranged from 37.5 to 38.5°C; values near the upper end of the range were recorded at the neutral T_a, whereas values near the lower end of the range were recorded at the subneutral T_a. Regardless of T_a, no thermoregulatory response was observed in the saline-treated rats pretreated with SC-560, SC-236, or their vehicle (Figs. 1 and 2). At the neutral T_a (Fig. 1), the vehicle-pretreated rats responded to the lower dose of LPS with a typical polyphasic fever, which consisted of three consecutive T_b rises peaking at ~50, 120, and 300 min. All phases of

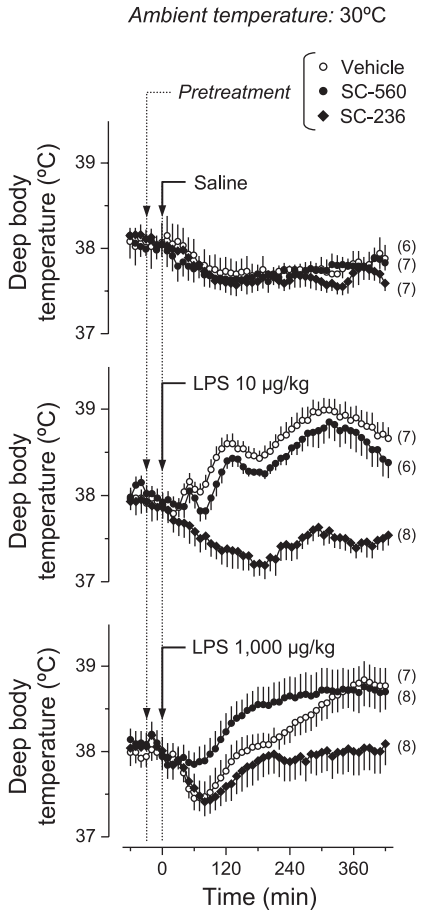

Fig. 1. Effects of COX-1 and COX-2 inhibitors on the thermal responses to LPS at a neutral ambient temperature (T_a). Rats kept at a T_a of 30°C were pretreated with a COX-1 inhibitor (SC-560; 5 mg/kg iv), a COX-2 inhibitor (SC-236; 2.5 mg/kg iv), or their vehicle before the intravenous administration of LPS (doses indicated) or saline. Arrowheads indicate the time at which pretreatment (10-min infusion) started; arrows indicate the time of LPS injection. The number of animals in each group (*n*) is indicated. Compared with the vehicle pretreatment, SC-236 blocked the febrile response to LPS ($P < 1.0 \times 10^{-5}$, 40–420 min for the lower LPS dose; $P < 2.2 \times 10-^{5}$, 160–420 min for the higher LPS dose), whereas SC-560 blocked the initial hypothermic response to the higher LPS dose ($P < 2.2 \times 10^{-5}$, 40–120 min).

the febrile response were abrogated by SC-236 ($P < 1.0 \times 10^{-5}$, 40–420 min), but none was affected by SC-560. At the same T_a, the vehicle-pretreated rats responded to the higher dose of LPS with a small drop in T_b followed by a long-lasting fever. SC-560 blocked the initial hypothermia ($P < 2.2 \times 10^{-5}$, 40–120 min), whereas SC-236 blocked the subsequent febrile response ($P < 2.2 \times 10^{-5}$, 160–420 min).

At the subneutral T_a (Fig. 2), hypothermia was the prevailing response to LPS. The hypothermic response was characterized by two subsequent drops in T_b: a prominent early drop (nadir at 70–90 min post-LPS) and a later drop (nadir at ~200 min) in T_b were consistently observed. The time course of the change in T_b was not dependent on the LPS dose, but the magnitude of the hypothermic response was greater at the higher dose. SC-560 largely attenuated the hypothermic responses to both doses of LPS ($P < 2.2 \times 10^{-5}$, 50–420 min for either dose). On the other hand, SC-236 exaggerated the hypothermic responses to LPS ($P < 2.2 \times 10^{-5}$, 80–420 min for the lower LPS dose, 130–420 min for the higher LPS dose).

LPS-Induced Hypotension Is Blocked by SC-560 or SC-236

Because the hypothermic response to LPS has been reported to be associated with hypotension (46, 70), we studied the effects of SC-560 and SC-236 on the blood pressure changes caused by the higher dose of LPS. This experiment was performed at the subneutral T_a (22°C), because the effects of SC-560 and SC-236 on LPS hypothermia were most manifest at this T_a (Fig. 2). Baseline mean arterial pressure was ~130 mmHg, a value similar to those recorded by others in rats exposed to a subneutral T_a (15, 64). No significant change in mean arterial pressure was observed in the saline-treated rats pretreated with SC-560, SC-236, or their vehicle (Fig. 3). Injection of LPS (1,000 μg/kg) to the vehicle-pretreated rats evoked a decrease (~30 mmHg) in blood pressure. The blood pressure reached a nadir at ~80 min after LPS injection, corresponding in time to the first phase of the hypothermic response. LPS-induced hypotension was largely attenuated by pretreatment with either SC-560 ($P < 2.2 \times 10^{-5}$, 50–170 min) or SC-236 ($P < 1.2 \times 10^{-4}$, 50–120 min), despite the

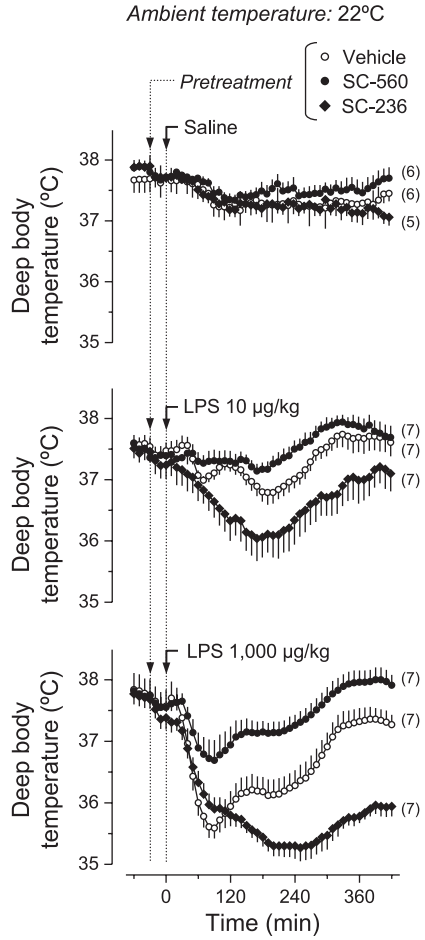

Fig. 2. Effects of COX-1 and COX-2 inhibitors on the thermal responses to LPS at a subneutral T_a. Rats kept at a T_a of 22°C were pretreated with a COX-1 inhibitor (SC-560, 5 mg/kg iv), a COX-2 inhibitor (SC-236, 2.5 mg/kg iv), or their vehicle before the intravenous administration of LPS (doses indicated) or saline. Compared with the vehicle pretreatment, SC-560 blocked the hypothermic response to LPS ($P < 2.2 \times 10^{-5}$, 50–420 min for either LPS dose), whereas SC-236 enhanced the hypothermic response ($P < 2.2 \times 10^{-5}$, 80–420 min for the lower LPS dose, 130–420 min for the higher LPS dose).

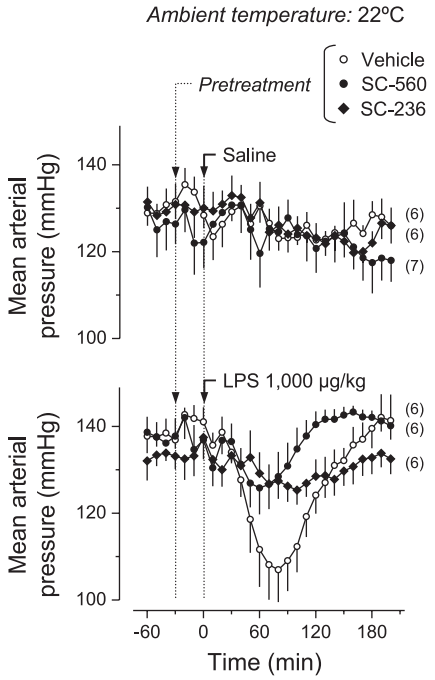

Fig. 3. Effects of COX-1 and COX-2 inhibitors on LPS-induced hypotension. Rats kept at a T_a of 22°C were pretreated with a COX-1 inhibitor (SC-560, 5 mg/kg iv), a COX-2 inhibitor (SC-236, 2.5 mg/kg iv), or their vehicle before the intravenous administration of LPS (dose indicated) or saline. LPS-induced hypotension was blocked by pretreatment with either SC-560 ($P < 2.2 \times 10^{-5}$, 50–170 min) or SC-236 ($P < 1.2 \times 10^{-4}$, 50–120 min), compared with the vehicle pretreatment.

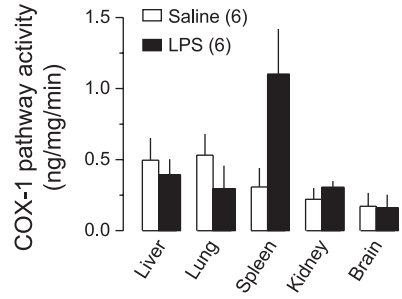

Fig. 4. Functional activity of the COX-1 pathway at the onset of LPS-induced hypothermia. The activity was measured in homogenates of tissues collected 50 min after intravenous administration of LPS (1,000 μg/kg) or saline at a subneutral T_a (22°C). The COX-1 pathway activity (ng/mg/min) was determined as a SC-560-sensitive portion of the ex vivo production of PGE_2 expressed as PGE_2 concentration (ng/ml) divided by the total protein concentration (mg/ml) and the duration of incubation with arachidonic acid (min). Compared with saline, LPS significantly increased the COX-1 pathway activity in the spleen ($P < 7.5 \times 10^{-3}$) but not in the liver, lung, kidney, or brain.

fact that these drugs had opposite effects on LPS-induced hypothermia.

The Functional Activity of the COX-1 Pathway (but not COX-1 Expression) Is Increased by LPS

The functional activity of the COX-1 pathway (COX-1-mediated PGE_2 synthesis ex vivo) was measured in tissues collected 50 min after administration of LPS (1,000 μg/kg) or saline at T_a of 22°C; this time corresponds to the onset of LPS-induced hypothermia (and also hypotension). In the controls (saline-treated rats), the COX-1 pathway was active in all organs investigated: liver, lung, spleen, kidney, and brain (Fig. 4). Compared with saline, LPS did not significantly change the activity of the COX-1 pathway in the liver, lung, kidney, or brain. However, it produced a 3.6-fold increase in the COX-1 pathway activity in the spleen ($P < 7.5 \times 10^{-3}$).

We next evaluated whether the increased activity of the COX-1 pathway in the spleen was associated with changes in the splenic expression of COX-1 measured at the mRNA and protein levels. For both COX-1 and β-actin, a single mRNA product of the expected size (196 bp for COX-1 and 106 bp for β-actin) was amplified (Fig. 5*A*). No product was amplified in the absence of primers or when water was used instead of RNA (data not shown). The spleen mRNA expression of COX-1 (relative to β-actin) did not differ ($P = 0.16$) between LPS- and saline-treated rats (Fig. 5*A*). The Western blot analysis of COX-1 revealed a protein with a molecular mass between 70 and 80 kDa in the Western blot membranes of all spleen samples (Fig. 5*B*). The same molecular mass protein was markedly expressed in platelets (positive control for COX-1), and no such protein was revealed when the Western blot membranes were incubated without the anti-COX-1 antibody (negative control; data not shown). The intensity of the COX-1 bands did not differ ($P = 0.69$) between samples obtained from the spleens of LPS- and saline-treated rats (Fig. 5*B*). Taken together, the data indicate that the expression of COX-1 in the spleen did not change at the onset of LPS hypothermia (and hypotension).

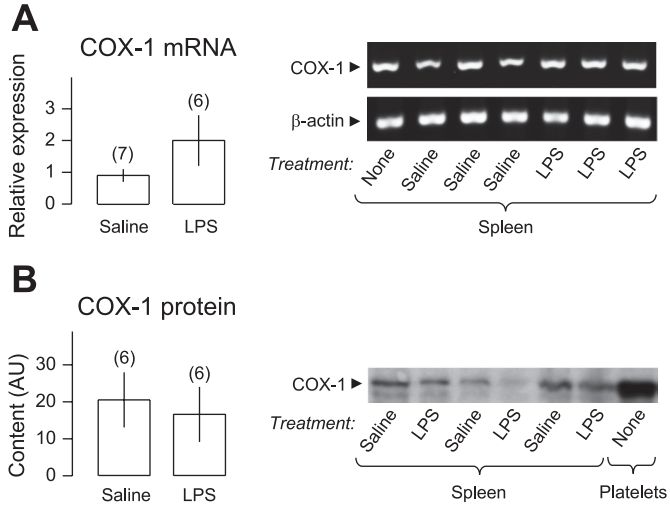

Fig. 5. Expression of COX-1 at the onset of LPS-induced hypothermia. COX-1 mRNA (*A*) and protein (*B*) were measured in spleen samples collected 50 min after intravenous administration of LPS (1,000 μg/kg) or saline at a subneutral T_a (22°C). *A*: mean relative expressions determined by quantitative real-time RT-PCR are shown on the left, whereas agarose-gel bands of amplicons obtained at the exponential phase of PCR amplification are shown on the right. *B*: mean contents (arbitrary units, AU) of COX-1 protein determined by densitometry of Western blot bands are shown on the left, whereas representative images of Western blot bands for spleen samples and platelet lysate (positive control) are shown on the right. Although β-actin expression was not examined in the Western blot analysis, it should be noted that an identical amount of total protein (35 μg) was loaded into each gel lane. Compared with saline, LPS did not increase the level of COX-1 mRNA or protein.

DISCUSSION

Effects of COX-1 and COX-2 Inhibitors on LPS-Induced Responses

The present study was carried out to clarify which isoform of COX mediates hypothermia in systemic inflammation. First, we evaluated the effects of SC-560 (COX-1 inhibitor) and SC-236 (COX-2 inhibitor) on the thermoregulatory responses to LPS. SC-236 blocked all phases of LPS-induced fever in experiments conducted at a neutral T_a, whereas it enhanced LPS-induced hypothermia at a subneutral T_a. These findings indicate that COX-2 not only mediates fever but also limits the hypothermic response to LPS. It is possible that COX-2-mediated production of the same febrigenic mediator, possibly PGE_2 (57), underlies the involvement of this enzyme in both the development of fever and the limitation of hypothermia. Indeed, the complexity of the thermoregulatory response to LPS is thought to result from a balance between a febrile component (driven by febrigenic mediators) and a hypothermic component (driven by cryogenic mediators) (42, 82, 84).

The COX-1 inhibitor, SC-560, consistently blocked LPS hypothermia (but not fever) under all experimental conditions tested, thus indicating that COX-1 is required for the development of LPS hypothermia. The COX-1 product involved in LPS hypothermia has yet to be identified, but PGD_2 is a potential candidate, as it induces hypothermia when injected in rats either intracerebroventricularly (86) or intravenously: as an albumin complex (A. A. Steiner, A. S. Dragic, J. Pan, A. A. Romanovsky, unpublished observation) or as a hydroalcoholic solution (A. Garami, E. Pakai, A. A. Romanovsky; unpublished observation). It should be noted, however, that Krueger et al. (44) have reported that high doses of PGD_2 cause fever (rather than hypothermia) in rabbits; the same authors have suggested that effects of PGD_2 on thermoregulation (and sleep) may be species specific. While this study was in preparation, Gao et al. (27) reported that intracisternal PGD_2 did not cause hypothermia in rats and, in fact, caused a delayed fever, possibly by interfering with the transport of the febrigenic PGE_2 in the brain. Clearly, the search for the COX-1-derived mediator of hypothermia should continue.

In contradiction with the present findings are studies by Dogan et al. (20) and Zhang et al. (90), which reported that SC-236 blocked LPS hypothermia. In the study by Dogan et al. (20), such a blockade occurred only when the dose of SC-236 was substantially higher (40 mg/kg) than the doses (1–15 mg/kg) known to selectively block COX-2 in vivo (31, 53). At such a high dose, SC-236 might have inhibited COX-1, or it might have exerted COX-unrelated effects (39). Although the study by Zhang et al. (90) employed a lower dose of SC-236 (5 mg/kg), it was limited by marked stress hyperthermia following intraperitoneal drug administration, which overlapped substantially with the early hypothermic response to LPS, thus making the results difficult to interpret. Zhang et al. (90) reported another observation that seemingly contradicts the present results, i.e., that SC-560 might have prolonged LPS hypothermia. It should be considered, however, that Zhang et al. (90) used a low dose of LPS, which resulted in only minimal decreases in T_b (~0.5°C) in both SC-560-treated and SC-560-untrerated rats. In the present study, the usage of higher doses of LPS in a tightly controlled thermal environment allowed us to produce a much stronger hypothermic response (~2°C) and, therefore, to study effects on the hypothermic response at a higher "resolution."

Although we found opposite effects of SC-560 and SC-236 on LPS-induced hypothermia, both drugs attenuated LPS-induced hypotension. The fact that SC-236 enhanced hypothermia while blocking hypotension is of interest, as it is currently the strongest piece of evidence suggesting that hypothermia is not a consequence of hypotension-associated hypoperfusion. The present results are also the first demonstration that both COX isoforms are required for the hypotensive response to LPS to develop. With a single exception (33), all previous studies found that COX-2 is not required for the development of LPS hypotension (47, 87), and one study (87) found that COX-1 is also uninvolved in this response. However, it should be considered that all previous studies were performed in anesthetized rats receiving fluid resuscitation. Under these conditions, rats respond to high doses of LPS with a slowly developing, progressive fall in blood pressure (47, 87). The present study was conducted in unanesthetized rats not receiving fluid resuscitation, conditions under which rats respond to high doses of LPS with a rapid drop in blood pressure that corresponds in time to the first phase of the hypothermic response (70).

Functional Activity of the COX-1 Pathway During LPS-Induced Responses

The involvement of COX-2 in the LPS-induced responses is consistent with the expression of this isozyme being upregulated as early as 30 min after intravenous administration of LPS (34, 80). Expressional upregulation is widely accepted as a principal mechanism for activation of COX-2 in inflammation (35, 58). On the other hand, the molecular mechanisms underlying the involvement of COX-1 in LPS-induced responses are unknown. We now report that the functional activity of the COX-1 pathway (COX-1-mediated PGE_2 synthesis ex vivo) is increased in the spleen at the onset of LPS-induced hypothermia and hypotension. This increase in the activity of the COX-1 pathway occurred without upregulation of COX-1 mRNA or protein. Although the observed activation of the COX-1 pathway in the spleen does not imply that it is the splenic COX-1 that mediates LPS hypothermia (and hypotension), the association between COX-1 pathway activation in the spleen and LPS hypothermia should not be dismissed. Furthermore, our recent data (E. Pakai, A. Garami, T. B. Nucci, A. A. Romanovsky, unpublished data) show that LPS hypothermia is attenuated in splenectomized rats, thus suggesting a role for factors synthesized in the spleen.

A possible mechanism for COX-1 activation involves peroxide species, such as hydroperoxide and peroxynitrate. These species serve as activators for both COX isozymes by promoting the formation of a tyrosyl radical near the enzyme's active sites (51). Hence, it is possible that the increased production of hydroperoxide and peroxynitrate during inflammation (26, 63) promotes activation of COX. The fact that higher peroxide levels are required to activate COX-1 than COX-2 (14, 45) is consistent with the observation that the hypothermic response (dependent on COX-1) occurs in the most severe forms of systemic inflammation, e.g., in the response to high doses of LPS (9, 52, 70). It should also be noted that mechanisms

upstream or downstream from COX-1 may affect the activity of the COX-1 pathway, since these mechanisms determine substrate availability to COX-1 and the fate of the COX-1 product, respectively (see *Perspectives and Significance*).

A splicing variant of COX-1 has been identified in canine tissues (16). Originally named COX-3, this variant is now most commonly referred to as COX-1b. Might COX-1b have contributed to the increased COX-1 pathway activity found in the present study? At present, the answer to this question appears to be negative. Although a COX-1b transcript (which retains intron-1) has been found in rat tissues (40, 41), a functional COX-1b protein has not yet been identified in this species (77). The rat intron-1 (unlike the canine intron-1) contains an incomplete codon sequence consisting of 32 codons plus 2 extra nucleotides; the extra nucleotides are available to form a codon with the nucleotide of the exon that follows, thus shifting the reading frame of the exonic portion of the transcript (77).

Study Limitations and Design Considerations

In the present study, baseline colonic temperature in confined rats ranged from 37.5 to 38.5°C. These values are higher (by ~0.5°C) than baseline values of abdominal temperature recorded telemetrically in freely moving rats (see, for example, Refs. 2 and 90). However, this expected difference likely stems from two factors (for detailed analysis, see Ref. 71). First, colonic temperature is one of the highest temperatures in the rat's body. It is 0.1–0.8°C higher than aortic temperature (22), and it is very likely higher than abdominal temperature, especially if the latter is measured near the abdominal wall (71). Second, the perception that "normal" values of abdominal temperature range from 37.0 to 37.5°C originated from experiments performed at room temperature (see, e.g., Refs. 2, 90), which are usually subneutral for rats (67). At a subneutral T_a, basal T_b of rats in our experiment rarely exceeded 38.0°C. Only when rats were exposed to a neutral T_a (30°C) did their basal T_a reach 38.5°C, an observation that agrees with the known influence of T_a on basal T_b (28, 67, 71, 73, 89). Nevertheless, the presence of a low-grade confinement stress in our experiments cannot be ruled out completely, especially because the rats had a somewhat elevated basal arterial pressure (~130 mmHg). One can argue, however, that the increased blood pressure, which was observed at a subneutral T_a only, was due to mild cold exposure (15, 64), rather than confinement, to which the animals were well adapted.

In spite of the fact that we carefully chose the doses of SC-560 and SC-236 so that each drug was administered at the minimal dose that has been demonstrated to maximally inhibit one COX isoform without affecting the other in vivo (31, 53, 54), the effectiveness of our approach is subject to limitations inherent to any pharmacological approach, including the possibility that a drug may produce COX-unrelated (off-target) effects (12). The limitations of pharmacological approaches can be offset by the use of complementary approaches such as genetically modified mice. Indeed, mice genetically deficient of COX-1 or COX-2 have been instrumental in studies that established the role of COX-2 in the mediation of fever (49, 83). However, the early hypothermia, which was observed in the present study and that has been reported to occur in response to LPS in rats (70, 79), rabbits (88), and chicken (18), does not occur in mice (73). The early (starts within minutes) hypothermic response of rats occurring at LPS doses of 10 μg/kg and higher clearly differs from the late (starts at 4 h or later) and prolonged (lasts 12 h or more) hypothermic response that develops in mice injected with extremely high (10,000 μg/kg and higher) doses of LPS (38, 73, 75). Hence, mice cannot be used to study the early LPS hypothermia that is common for other species. Another limitation of our study is that it is unknown to what extent the data obtained can be generalized beyond the experimental conditions used: an LPS model of systemic inflammation in the rat.

Conclusion

In conclusion, by conducting a differential analysis of the effects of two highly selective COX inhibitors in several models of LPS fever and hypothermia, the present study indicates that COX-1, and not COX-2, is the isoform that mediates the hypothermic response to LPS, at least in the rat. By investigating the functional activity of the COX-1 pathway (COX-1-mediated PGE_2 synthesis ex vivo) and COX-1 expression at the mRNA and protein levels, this study has also found that COX-1 pathway activity increases in the spleen at the onset of LPS hypothermia via mechanisms that do not involve transcriptional upregulation of COX-1. By evaluating the effects of COX inhibitors on blood pressure, this study has further shown that both COX isoforms are required for the hypotensive response to LPS to develop.

Perspectives and Significance

In the absence of transcriptional upregulation of COX-1 in the spleen, an increased splenic COX-1 pathway activity during LPS hypothermia may involve not only posttranslational activation of COX-1 (discussed above), but also the activation of mechanisms upstream or downstream from COX-1. The COX substrate, arachidonic acid, derives from the breakdown of membrane phospholipids by enzymes of the phospholipase (PL) A_2 superfamily (35, 58). It is generally accepted that certain PLA_2 enzymes may couple more favorably with one COX isoform than with the other. For example, cytosolic PLA_2-α colocalizes with COX-1, but not with COX-2, in the Golgi apparatus of activated epithelial cells in vitro (29). Therefore, the activation [possibly by phosphorylation (50)] of cytosolic PLA_2-α may direct arachidonic acid to COX-1 in preference to COX-2. The phosphorylated form of cytosolic PLA_2 has been detected in the lungs as early as 40 min after intravenous administration of LPS (80).

The mechanisms downstream from COX determine which bioactive prostanoids are formed (35, 85). Recent studies (23, 34, 74) have shown that microsomal PGE synthase-1 (mPGES-1) is the enzyme responsible for the conversion of PGH_2 into febrigenic PGE_2. This enzyme is functionally coupled with COX-2 in marked preference to COX-1, presumably because both COX-2 and mPGES-1 are primarily compartmentalized in the perinuclear envelope (60). Massive (more than 1,000 fold) transcriptional upregulation of mPGES-1 occurs in the course of LPS-induced fever (23, 34). The terminal synthases involved in the hypothermia of systemic inflammation remain to be identified, but given the ability of PGD_2 to cause hypothermia, at least according to some reports (86), it is reasonable to suspect that a PGD synthase (PGDS) may be involved. There are two known PGDS isoforms: lipocalin

PGDS (L-PGDS) and hematopoietic PGDS (H-PGDS) (58). Whereas the LPS-induced production of PGD_2 in neural tissue appears to be dependent on COX-2 and L-PGDS (30), the early production of PGD_2 by activated macrophages and mast cells seems to depend on COX-1 and H-PGDS (59, 61). Interestingly, large amounts of H-PGDS are present in the rat spleen (37), the organ in which the COX-1 pathway was activated at the onset of LPS hypothermia. Furthermore, Feleder et al. (24, 25) have recently shown that splenectomy or splenic vein ligation enhances LPS fever, and they have proposed that LPS causes the synthesis of a cryogenic lipid in the spleen. Our recent experiments (E. Pakai, A. Garami, T. B. Nucci, A. A. Romanovsky, unpublished data) further show that splenectomy attenuates LPS hypothermia. Whether the activation of a COX-1 → H-PGDS pathway in the spleen and the consequent production of PGD_2 or some other COX-1-mediated mechanism underlies LPS-induced hypothermia is a subject of future studies.

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