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Characteristics of thermoregulatory and febrile responses in mice deficient in prostaglandin EP_1 and EP_3 receptors

Takakazu Oka*¶, Kae Oka*, Takuya Kobayashi†, Yukihiko Sugimoto‡, Atsushi Ichikawa‡, Fumitaka Ushikubi§, Shuh Narumiya† and Clifford B. Saper*

*Department of Neurology, Beth Israel Deaconess Medical Center and Program in Neuroscience and Harvard Medical School, Boston, MA 02115, USA, †Department of Pharmacology, Faculty of Medicine, Kyoto University, Kyoto 606-8315, Japan, ‡Department of Physiological Chemistry, Kyoto University Faculty of Pharmaceutical Sciences, Kyoto 606-8501, Japan, §Department of Pharmacology, Asahikawa Medical School, Asahikawa 078-8307, Japan and ¶Division of Psychosomatic Medicine, Department of Neurology, University of Occupational and Environmental Health, Iseigaoka 1-1, Yahatanishi-ku, Kitakyushu, 807-8555, Japan

Previous studies have disagreed about whether prostaglandin EP_1 or EP_3 receptors are critical for producing febrile responses. We therefore injected lipopolysaccharide (LPS) at a variety doses (1 $\mu g\ kg^{-1}$–1 $mg\ kg^{-1}$) intraperitoneally (I.P.) into wild-type (WT) mice and mice lacking the EP_1 or the EP_3 receptors and measured changes in core temperature (T_c) by using telemetry. In WT mice, I.P. injection of LPS at 10 $\mu g\ kg^{-1}$ increased T_c about 1 °C, peaking 2 h after injection. At 100 $\mu g\ kg^{-1}$, LPS increased T_c, peaking 5–8 h after injection. LPS at 1 $mg\ kg^{-1}$ decreased T_c, reaching a nadir at 5–8 h after injection. In EP_1 receptor knockout (KO) mice injected with 10 $\mu g\ kg^{-1}$ LPS, only the initial (< 40 min) increase in T_c was lacking; with 100 $\mu g\ kg^{-1}$ LPS the mice showed no febrile response. In EP_3 receptor KO mice, LPS decreased T_c in a dose- and time-dependent manner. Furthermore, in EP_3 receptor KO mice subcutaneous injection of turpentine did not induce fever. Both EP_1 and EP_3 receptor KO mice showed a normal circadian cycle of T_c and brief hyperthermia following psychological stress (cage-exchange stress and buddy-removal stress). The present study suggests that both the EP_1 and the EP_3 receptors play a role in fever induced by systemic inflammation but neither EP receptor is involved in the circadian rise in T_c or psychological stress-induced hyperthermia in mice.

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Corresponding author C. B. Saper: Department of Neurology, Beth Israel Deaconess Medical Center and Program in Neuroscience and Harvard Medical School, Boston, MA 02115, USA. Email: csaper@caregroup.harvard.edu

*a,b*Prostaglandin E_2 (PGE_2) is a principal mediator of fever (Blatteis & Sehic, 1997). For example, systemic administration of lipopolysaccharide (LPS), a bacterial endotoxin, is thought to produce fever by inducing cyclooxygenase-2, a rate limiting enzyme for PGE_2 synthesis, by both venular endothelial cells (Yamagata *et al.* 2001; Ek *et al.* 2001; Schiltz & Sawchenko, 2002) and perivascular microglial cells (Elmquist *et al.* 1997; Schiltz & Sawchenko, 2002). PGE_2 released into the brain may act on the neurons expressing E type prostaglandin (EP) receptors in the anteromedial preoptic area of the hypothalamus (POA) (Scammell *et al.* 1996) thus producing fever. There are four subtypes of EP receptors: EP_1, EP_2, EP_3 and EP_4 (Coleman *et al.* 1994). Among the four receptor subtypes, EP_1, EP_3 and EP_4 receptors have been demonstrated within the rat POA (Oka *et al.* 2000), suggesting their possible role in febrile response. Previous pharmacological studies suggested the involvement of both EP_1 and EP_3 receptors in fever in rats, whereas EP_4 agonists caused only hypothermia (Oka & Hori, 1994; Oka *et al.* 1997, 1998; Oka & Saper, 2003). In mice, however, intravenous injection of LPS failed to produce fever in animals lacking the EP_3, but not animals lacking the EP_1 or EP_4 receptor genes (Ushikubi *et al.* 1998). Although this finding suggests that EP_3 receptors play a crucial role in fever in mice, the study by Ushikubi and colleagues looked only at a limited time frame (1 h after administration) and a single fixed dose of LPS (10 $mg\ kg^{-1}$), raising the question of whether other EP receptors may play a role under different conditions.

In rats, systemic administration of LPS is known to induce monophasic fever, multiphasic fever, or hypothermia depending on the dose, the route of administration, and the ambient temperature (Romanovsky *et al.* 1998*a,b*) and each phase is thought to be mediated by different neural mechanisms (Romanovsky, 2000; Szekely *et al.* 2000). Fever responses to LPS have not been documented as extensively in mice as in rats (Wang *et al.* 1997; Leon *et al.* 1997; Kozak *et al.* 1998; Li *et al.* 1999). However, the available data (Leon *et al.* 1997; Kozak *et al.* 1998) indicate that the dose of LPS is important, as LPS at 50–100 $\mu g\ kg^{-1}$ induces fever whereas 2.5 $mg\ kg^{-1}$ induces hypothermia. Therefore, we decided to document the changes in core body temperature (T_c) after LPS administration at a variety of doses in wild-type (WT) mice. Then, to test the

roles of the different EP receptors in producing fever, we assessed the changes in T_c induced by LPS telemetrically over a > 10 h period under unrestrained and awake conditions in mice lacking the EP_1 and EP_3 receptors.

We then did parallel experiments in mice in which fever was induced by local inflammation, which may be mediated by different afferent neural pathways (Goldbach *et al.* 1997; Gourine *et al.* 2001) and cytokines (Leon *et al.* 1996, 1997) from systemic inflammation. Furthermore, we assessed the role of EP_1 and EP_3 receptors in circadian body temperature and psychological stress-induced hyperthermia as well because drugs that inhibit cyclooxygenase (COX) synthesis have been reported to attenuate the circadian rise in body temperature (Scales & Kluger, 1987) and open-field stress-induced hyperthermia (Singer *et al.* 1986; Kluger *et al.* 1987). These findings suggest the involvement of prostaglandin synthesis in circadian rhythms of body temperature and psychological stress-induced hyperthermia. However, to date it is not known which EP receptors might mediate such thermoregulatory responses.

Thus, the present study was undertaken to determine the role of EP_1 and EP_3 receptors in (1) circadian changes in body temperature, (2) various phases of LPS-induced fever, (3) local inflammation-induced fever, and (4) psychological stress-induced hyperthermia using mice lacking the EP_1 and EP_3 receptor genes.

METHODS

Animals

Male C57BL/6 strain mice (24–33 g) (SLC, Inc., Shizuoka, Japan) were used. Mice lacking either the EP_1 or EP_3 receptor genes were generated as reported previously (Ushikubi *et al.* 1998) and were backcrossed to the C57BL/6 strain for five generations. Homozygote and wild-type mice of the second and third generation from this strain were used. To determine the genotype of each mouse, PCR analysis was performed on DNA extracted from the tails of neonates as described previously (Ushikubi *et al.* 1998). Mice were housed in a light- (12 h on/off; lights on at 7.00 h) and temperature- (22–24 °C) controlled and specific pathogen-free facility, with food and water available *ad libitum*. All experiments were approved by the Harvard Medical School and Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committees.

Surgery and monitoring T_c

Surgery for implanting telemetry transmitters was done under anaesthesia with chloral hydrate (350 mg kg^{-1}, I.P.). Using aseptic techniques, a temperature transmitter (TA-F20, Data Sciences International, Saint Paul, MN, USA) was placed in the peritoneal cavity via a midline incision. After surgery, mice were housed in a cage (dimensions: 27 × 17 × 13 cm) individually in a sound-attenuated room and were handled daily (5–7 days) to minimize stress during the actual injection procedures. T_c was monitored telemetrically, starting at least 24 h before drug injection to assess baseline T_c. The T_c signals were received by an antenna below the mice cage and relayed to a signal processor (Data Quest III System, Mini Mitter Co., Sun River, OR, USA) connected to a Compaq computer. T_c was monitored every 1 min.

Experiment 1. Circadian changes in T_c

Seven days after surgery, T_c was monitored for 2 days. After observation, these mice were used for the following experiments.

Experiment 2. Effects of intraperitoneal (I.P.) injection of LPS on T_c

Two groups of mice were injected intraperitoneally with LPS from *Salmonella typhimurium* (1 μg kg^{-1}, 10 μg kg^{-1}, 100 μg kg^{-1}, or 1 mg kg^{-1} in 0.15 ml) (Sigma, St Louis, MO, USA; lot 23H4047) or 0.15 ml of pyrogen-free 0.9% saline (PFS) (Sigma). LPS was dissolved in PFS. Injections were given between 9.00 h and 9.10 h. The dose of LPS was determined based upon an earlier study (Romanovsky *et al.* 1996). As the LPS at 0.1 μg kg^{-1} did not induce significant changes in T_c in the pilot study, only the results of LPS at 1 μg kg^{-1}–1 mg kg^{-1} are shown.

Experiment 3. Effects of subcutaneous (S.C.) injection of turpentine on T_c

Two groups of mice were injected subcutaneously with 0.15 ml of turpentine oil (Spectrum Quality Products, Inc., New Brunswick, NJ, USA) or PFS into the left thigh. Injections were given between 9.00 h and 9.10 h.

Experiment 4. Effects of cage-exchange stress on T_c

Cage-exchange stress was evoked by exchanging the home cages of two mice. The control mice were just replaced in the same cages. This experiment was done between 11.00 h and 14.00 h when the circadian changes in T_c were minimal.

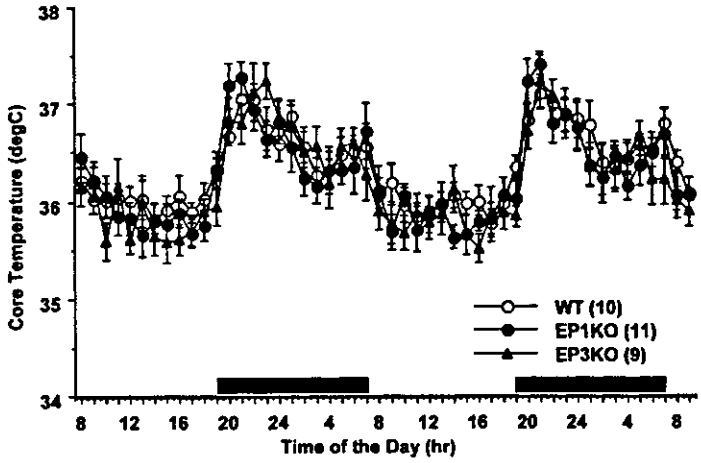

Figure 1. Circadian changes in T_c in WT (○), EP_1 receptor KO (●) and EP3 receptor KO (▲) mice

Each point represents mean ± S.E.M.
n = number of animals. Bar shows dark period. There was no significant difference among the three groups.

Experiment 5. Effects of buddy-removal stress on T_c

This stress model is a modified form of the putative 'anticipatory anxiety stress-induced hyperthermia' model of Zethof and colleagues (Zethof *et al.* 1994, 1995). Five days after surgery, mice (24–25 g) were returned to the cages in which they had previously been housed in a group (n = 5 per cage; 1 operated, 4 unoperated). Three to five days after group housing, each of the four unoperated mice were removed, one by one, every 2 min. This experiment was done between 11.00 h and 14.00 h.

Data analysis

The values are presented as means ± S.E.M. Significant differences were assessed by one-way analysis of variance followed by Dunnett's test or Student's t test for unmatched data. A difference was considered to be significant if $P < 0.05$.

RESULTS

Diurnal changes in T_c

Wild-type (WT), EP_1 receptor KO, and EP_3 receptor KO mice showed nearly identical diurnal changes in T_c, i.e. increased T_c during the dark period. The T_c did not differ among the three groups at any time point (Fig. 1).

Dose-dependent effect of I.P. injection of LPS on T_c

The initial T_c at time zero did not differ in any group. Intraperitoneal injection of 0.9% saline induced a transient increase in T_c of about 1 °C at 20 min after injection, which then decreased to the initial T_c within 60 min in all mice. T_c increased again at the beginning of the dark period (600 min after injection) in all mice.

LPS at 1 μg kg^{-1} (Fig. 2) induced two small peaks in T_c in the WT mice, at 100–160 min and 220–240 min after injection. In the EP_1 receptor KO mice there was a similar response, with the largest increase (1.1 ± 0.2 °C) 120 min after injection. However, in the EP_3 receptor KO mice the increase in T_c seen 20 min after injection was significantly blunted and the subsequent elevations in T_c did not occur.

LPS at 10 μg kg^{-1} (Fig. 3) caused an elevation of T_c in the WT mice at 60–260 min after injection, with the peak (1.0 ± 0.2 °C) 120 min after injection. In the EP_1 receptor KO mice the early rise in T_c (20–40 min after injection) was blunted, but this was followed by an increase of about 1 °C in T_c that peaked 120–140 min after injection. By contrast, the EP_3 receptor KO mice showed a blunting of the initial hyperthermia, followed by a hypothermic response with a decrease in T_c of about 1.0 °C from 40–140 min after injection.

At 100 μg kg^{-1}, LPS (Fig. 4) caused an increase in T_c in the WT mice from 260 to 600 min after injection, with a peak of 1.0 ± 0.2 °C 380 min after injection. In the EP_1 receptor KO mice T_c did not differ from the control saline-treated animals, whereas the EP_3 receptor KO mice demonstrated a blunting of the initial hyperthermia and then a subsequent profound fall in T_c of about 2.0 °C from 40 to 540 min after injection

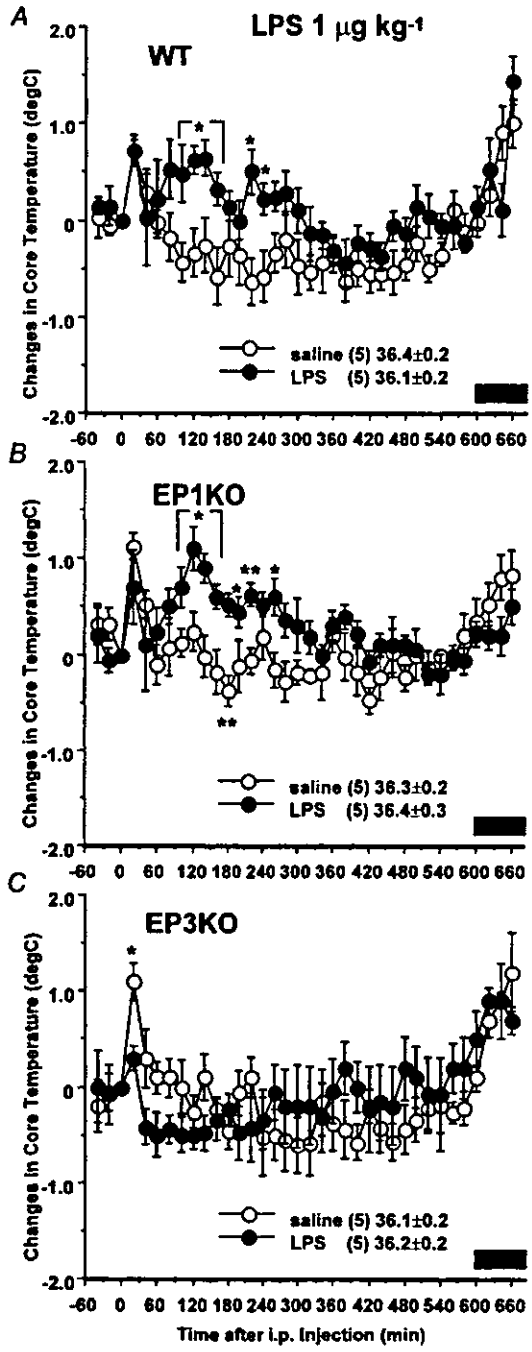

Figure 2. Effects of I.P. injection of LPS at 1 μg kg^{-1} on T_c in WT (*A*), EP1 receptor KO (*B*) and EP3 receptor KO (*C*) mice

Mice were injected with LPS (●) or 0.9 % saline (○) at time zero. The data are expressed as differences from the T_c at time zero, which is shown in the figure. Each point represents mean ± S.E.M. n = number of animals. Bar shows dark period. Symbols represent level of significance when compared with 0.9 % saline-injected control at each time point. *$P < 0.05$; **$P < 0.01$.

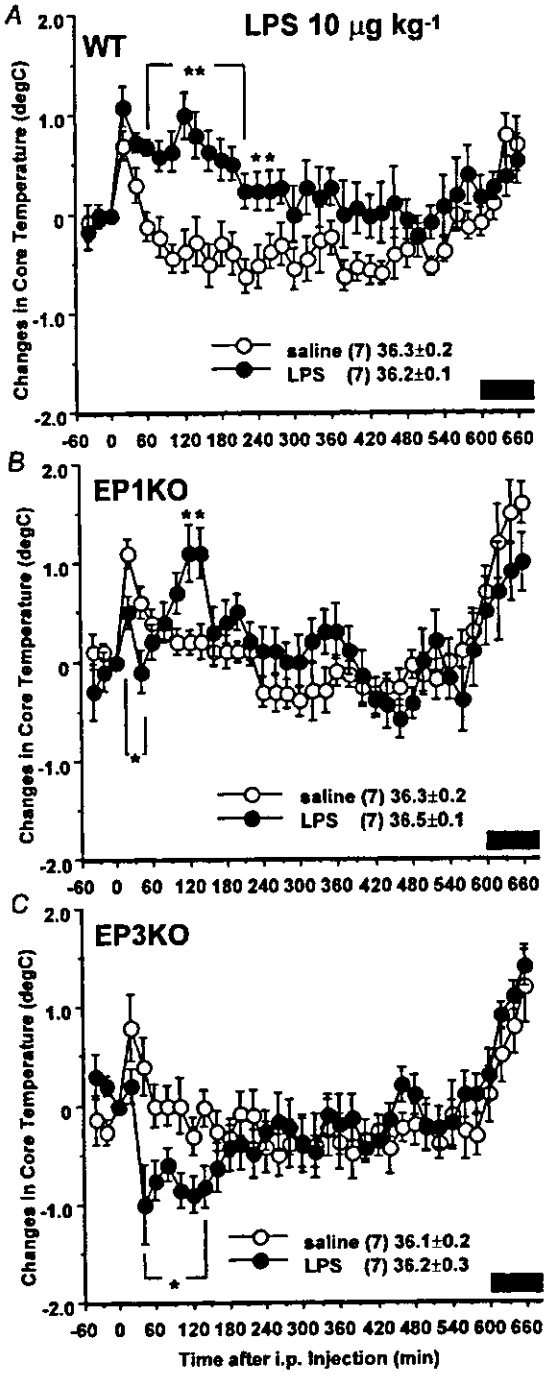

Figure 3. Effects of I.P. injection of LPS at 10 μg kg^{-1} on T_c in WT (*A*), EP$_1$ receptor KO (*B*) and EP$_3$ receptor KO (*C*) mice

Mice were injected with LPS (●) or 0.9 % saline (○) at time zero. The data are expressed as differences from the T_c at time zero, which is shown in the figure. Each point represents mean ± S.E.M. n = number of animals. Bar shows dark period. Symbols represent level of significance when compared with 0.9 % saline-injected control at each time point. *$P < 0.05$; **$P < 0.01$.

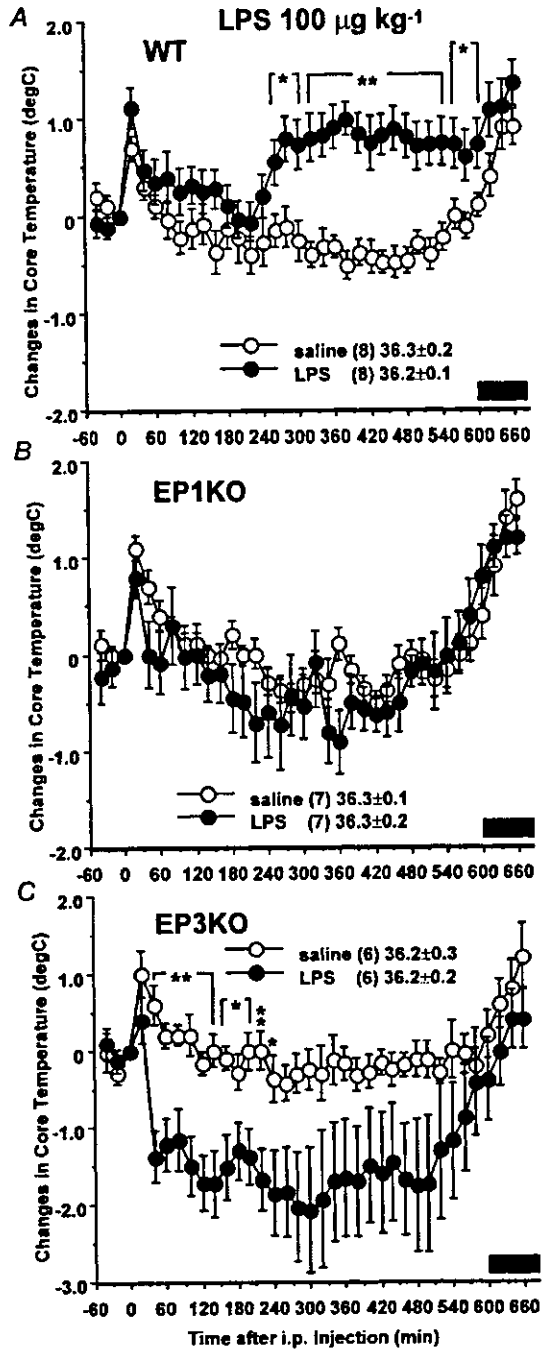

Figure 4. Effects of I.P. injection of LPS at 100 μg kg^{-1} on T_c in WT (*A*), EP$_1$ receptor KO (*B*) and EP$_3$ receptor KO (*C*) mice

Mice were injected with LPS (●) or 0.9 % saline (○) at time zero. The data are expressed as differences from the T_c at time zero, which is shown in the figure. Each point represents mean ± S.E.M. n = number of animals. Bar shows dark period. Symbols represent level of significance when compared with 0.9 % saline-injected control at each time point. *$P < 0.05$; **$P < 0.01$.

Finally, the 1 mg kg^{-1} dose of LPS (Fig. 5) caused a biphasic hypothermic response in the WT mice, with a fall in T_c of about 1.5 °C from 40 to 80 min after injection, and a second phase with more profound hypothermia from 180 to 500 min, with the maximal decrease (−4.0 ± 0.9 °C) at 320 min after injection. In the EP_1 receptor KO mice, the first phase of hypothermia was absent, and the second phase was similar if less intense than in the WT animals, reaching a nadir (−3.5 ± 1.0 °C) 280 min after injection. The EP_3 receptor KO mice, by comparison, showed a much more intense triphasic hypothermia, with a decrease in T_c of about 2 °C during the first 2 h, followed by a profound fall to a maximal decrease of −6.8 ± 1.2 °C at 360 min after injection. The T_c then increased by 4–5 °C by 540 min after injection, but remained about 2 °C below baseline for the remainder of the 12 h period of observation.

In summary, the EP_3 receptor KO mice failed to show a hyperthermic response to LPS at any dose, but rather demonstrated only hypothermic responses that became more profound and more prolonged as the dose of LPS increased. The EP_1 receptor KO mice had a more complex response, which varied at different dosages of LPS. At 1 μg kg^{-1} of LPS, their fever curve was very similar to WT animals. However at the 10 μg kg^{-1} dose the hyperthermia was briefer, and at 100 μg kg^{-1} there was no fever response at all. The 1 mg kg^{-1} dose caused a hypothermic response that was similar to, but less intense than that seen in WT mice.

Effect of s.c. injection of turpentine on T_c

Following turpentine injection, the WT and EP_1 receptor KO mice showed nearly identical responses, with higher T_c than saline-injected animals at the end of the first light period (from 9 to 10 h after injection) and during the second light period (from 24 to 33 h), but lower T_c during the second dark period (from 38 to 44 h). In contrast, in the EP_3 receptor KO mice, the turpentine-injected group showed reduced T_c 1 h after injection and then during the dark periods for the next 2 days (Fig. 6).

Effect of cage-exchange stress on T_c

Cage-exchange stress induced about a 2 °C increase in T_c within 20 min, gradually falling to an increase of about 1 °C at the end of the second hour when compared with control animals in the WT, EP_1 receptor KO, and EP_3 receptor KO mice. The degree of the maximal increase did not differ among the three groups (Fig. 7).

Effect of buddy-removal stress on T_c

In the WT mice, successive removal of four cage mates induced a 1.3 ± 0.1 °C increase in T_c in the remaining mouse within 20 min (Fig. 8*A*). Both the EP_1 receptor KO and EP_3 receptor KO mice showed a similar increase in T_c and the maximal T_c did not differ among the three groups (Fig. 8*B*).

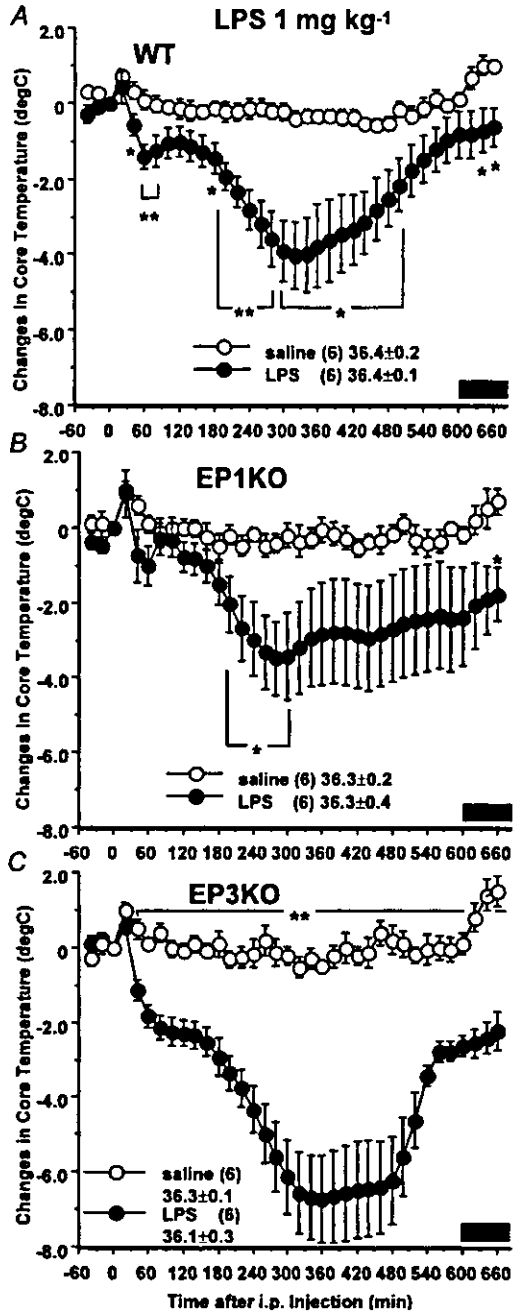

Figure 5. Effects of i.p. injection of LPS at 1 mg kg^{-1} on T_c in WT (*A*), EP_1 receptor KO (*B*) and EP_3 receptor KO (*C*) mice

Mice were injected with LPS (●) or 0.9 % saline (○) at time zero. The data are expressed as differences from the T_c at time zero, which is shown in the figure. Each point represents mean ± S.E.M. n = number of animals. Bar shows dark period. Symbols represent level of significance when compared with 0.9 % saline-injected control at each time point. $^*P < 0.05$; $^{**}P < 0.01$.

DISCUSSION

The present study demonstrated that (1) diurnal changes in T_c of both EP_1 receptor KO and EP_3 receptor KO mice are not different from those of WT mice, (2) EP_3 receptor KO mice do not produce febrile responses but in fact are hypothermic after either I.P. injection of LPS or S.C. injection of turpentine, whereas, in the EP_1 receptor KO

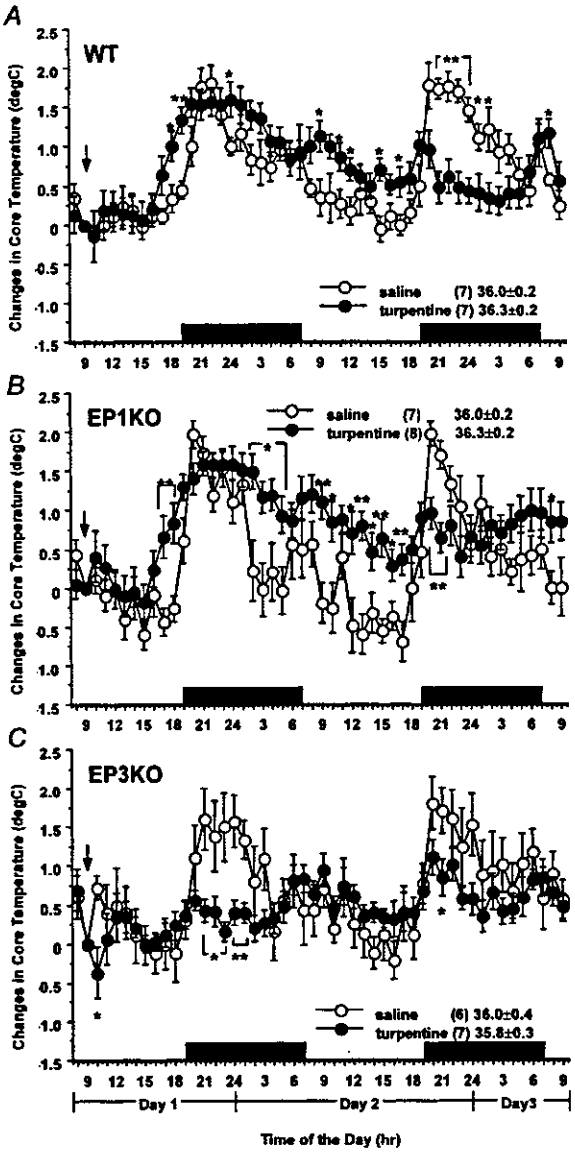

Figure 6. Effects of S.C. injection of turpentine on T_c in WT (*A*), EP_1 receptor KO (*B*) and EP_3 receptor KO (*C*) mice

Mice were injected with turpentine (●) or 0.9 % saline (○) at 9.00 h (arrows). The data are expressed as differences from the T_c at time zero, which is shown in the figure. Each point represents mean ± S.E.M. n = number of animals. Bar shows dark period. Symbols represent level of significance when compared with 0.9 % saline-injected control at each time point. $^*P < 0.05$; $^{**}P < 0.01$.

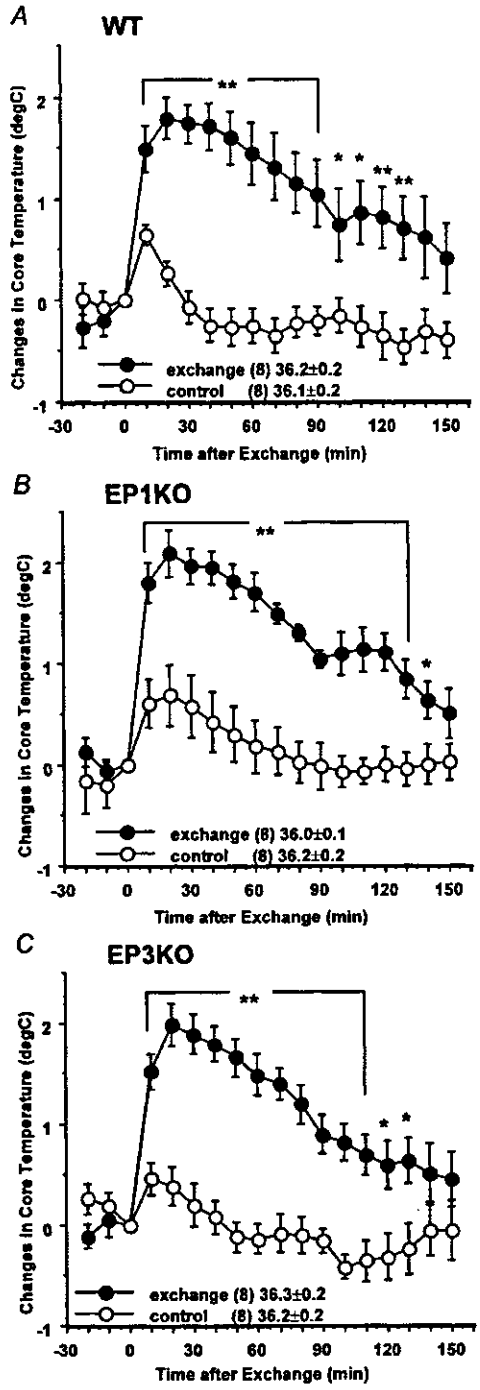

Figure 7. Effects of cage-exchange stress on T_c in WT (*A*), EP_1 receptor KO (*B*) and EP_3 receptor KO (*C*) mice

Cages of two mice were exchanged (●), or mice were removed and replaced in the same cages (○) as a control at time zero. The data are expressed as differences from the T_c at time zero, which is shown in the figure. Each point represents mean ± S.E.M. n = number of animals. Symbols represent level of significance when compared with control at each time point. $^*P < 0.05$; $^{**}P < 0.01$.

mice, LPS-induced fever is partially attenuated, (3) both EP_1 receptor KO mice and EP_3 receptor KO mice demonstrate hyperthermia due to cage-exchange stress and buddy-removal stress that is identical to WT mice. These findings suggest a crucial role for EP_3 receptors in causing fever at low dosages of LPS (1 and 10 $\mu g\ kg^{-1}$) and in the first 36 h after turpentine injection, and in limiting or preventing hypothermia at higher dosages of LPS (100 and 1000 $\mu g\ kg^{-1}$) and between 36 and 48 h after turpentine injection. EP_3 receptors, however, are not involved in the circadian rhythm of T_c or in psychological stress-induced hyperthermia in mice. EP_1 receptors, on the other hand, play a more subtle role in the pathogenesis of fever. They are particularly important in the late phase of fever (240–600 min) after intermediate (100 $\mu g\ kg^{-1}$) doses of LPS, but do not seem to be important for changes in T_c during local inflammation or stress, nor in circadian rhythms of T_c.

Baseline T_c and circadian changes in T_c

In the present study, the baseline T_c of EP_1 and EP_3 receptor KO mice did not differ from WT mice, suggesting neither EP_1 nor EP_3 receptors are critical for maintaining normal body temperature. This finding is in agreement with a previous finding that antipyretic drugs failed to alter normal body temperature in rhesus monkeys (Barney & Elizondo, 1981). In rats, however, prostaglandin synthesis inhibitors such as indomethacin decreased T_c at night in spite of having little effect on T_c during the day (Scales & Kluger, 1987), suggesting a contribution of prostaglandins (PGs) to the circadian rise in T_c. If this is also the case in mice, a circadian rise in T_c must involve prostanoid receptors other than EP_1 or EP_3.

LPS fever and hypothermia

Systemic administration of LPS produces monophasic fever, multiphasic fever or hypothermia depending upon the dose in rats (Romanovsky *et al.* 1998*a*,*b*). LPS-induced multiphasic fever consists of at least three phases in rats with T_c peaking 1, 2 and 5 h after injection when animals are restrained and held at 30 °C (Romanovsky *et al.* 1998*b*). For unrestrained rats at 23 °C, the first peak is not seen (Elmquist *et al.* 1996). Different neural mechanisms are thought to be involved in each phase, i.e. capsaicin-sensitive afferent fibres (either non-vagal or both non-vagal and vagal) in the 1st phase and vagal efferent fibres in the 3rd phase (Romanovsky, 2000; Szekely *et al.* 2000). However, studies on the effects of LPS on T_c in mice have been more limited (Wang *et al.* 1997; Leon *et al.* 1997; Kozak *et al.* 1998; Li *et al.* 1999) and comparable data on the extensive dosing range described in rats (0.1 $\mu g\ kg^{-1}$–1 $mg\ kg^{-1}$) have not been reported (Romanovsky *et al.* 1996). Therefore, we injected a wide range of doses of LPS (0.1 $\mu g\ kg^{-1}$–1 $mg\ kg^{-1}$, I.P.) into unrestrained mice at 23 °C and found at least four phases of T_c response: a peak at 20 min which appears to be due to handling (because it is seen even after PFS injection); an elevation at 40–80 min (which was missing in EP_1 receptor KO mice and may correspond to the 1st phase in rats); and peaks at 2 h and 5–8 h post-injection in WT mice that may correspond to the 2nd and 3rd phases, respectively, in rats.

A previous study examining restrained mice at an ambient temperature of 25 °C showed that the fever peak 20 min after injecting 10 $mg\ kg^{-1}$ LPS intravenously was absent in the EP_3 receptor KO mice but not EP_1 receptor KO mice

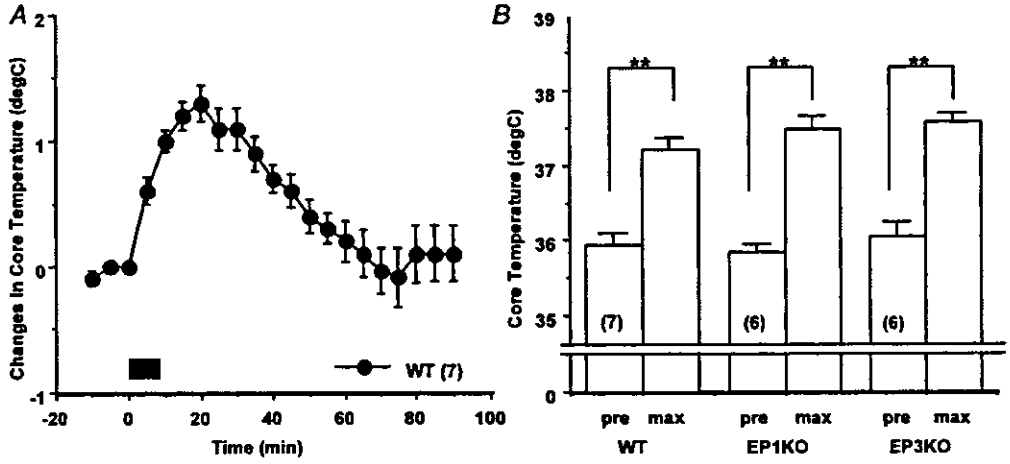

Figure 8. Effects of buddy-removal stress on T_c in WT, EP_1 receptor KO and EP_3 receptor KO mice

A, four out of five WT mice were removed from their home cage every 2 min, which started at time zero (bar). The time course of changes in T_c of the remained mouse is expressed as differences from the T_c at time zero. *B*, the maximal increases in T_c (max) induced by removing four mice from groups of five WT, EP_1 receptor KO and EP_3 receptor KO mice. Each point represents mean ± S.E.M. n = number of animals. Symbols adjacent to columns represent level of significance when compared with T_c at time zero (pre). $^*P < 0.05$; $^{**}P < 0.01$.

(Ushikubi *et al.* 1998). The EP_3 receptor KO animals showed normal thermogenic responses to being restrained (as in our stress-handling experiments). It is difficult to compare the previous experiments with the present ones, as in the current study the mice were handled briefly to inject LPS I.P. and the stress fever in our study coincided with the temperature elevation 20 min after LPS injection in the Ushikubi *et al.* study (1998). However, the EP_3 receptor KO mice in our study did have a lower T_c at 20 min after the 1, 10 or 100 $\mu g\ kg^{-1}$ LPS, compared with WT mice.

In addition, because the animals were studied at lower ambient temperature and for a longer period of time, the present study revealed that in the EP_3 receptor KO mice LPS decreased T_c in a dose-dependent manner and that LPS ($1\ mg\ kg^{-1}$) induced hypothermia that was more profound than that seen in WT and EP_1 receptor KO mice. These findings suggest that EP_3 receptors are involved in all phases of LPS-induced hyperthermia, and their absence unmasks a hypothermic process that predominates even at moderate dosages of LPS ($10\ \mu g\ kg^{-1}$). LPS-induced hypothermia is also attenuated by type 2 cyclooxygenase inhibitors, but exacerbated by type 1 inhibitors (Zhang *et al.* 2003). The prostanoid species, site of action, and prostanoid receptor type that produce the hypothermic response to LPS are not known. However, EP_3 receptors appear to have an inhibitory effect on LPS-induced hypothermia, and thus clearly are not involved in producing it.

In contrast, we found that, in EP_1 receptor KO mice, the second phase of fever at 40–80 min after $10\ \mu g\ kg^{-1}$ LPS was blunted and the 4th phase of fever at 240–600 min after $100\ \mu g\ kg^{-1}$ LPS was eliminated. Again, the present experiments are not directly comparable to those of Ushikubi *et al.* (1998) because the conditions were quite different and the late changes in T_c occurred at time points beyond the 1 h window used in their study. Previous studies in rats have shown that intracerebroventricular (I.C.V.) administration of an EP_1 receptor agonist caused a rapid-onset fever within 10–20 min that lasts for > 2 h (Oka *et al.* 2003). These observations are consistent with the action of PGE_2 on EP_1 receptors playing an important role in the early components of fever response to low doses of LPS, and in the late part of the response at higher dosages. EP_1 receptor KO animals also had a profound hypothemic response, similar to WT animals, at high dosages of LPS, indicating that the EP_1 receptors also are not necessary for this hypothermic action of LPS.

An alternative explanation for some of our findings might be a defect in prostaglandin synthesis, caused by the absence of either EP_1 or EP_3 receptors (e.g. if downstream signalling from either receptor influences PGE_2 synthesis). However, we did not measure prostaglanin levels in the brain, and hence this remains a subject for future investigation.

Turpentine-induced fever

Systemic inflammatory stimuli (e.g. systemic LPS injection) may cause fever by mechanisms that are different from those activated by local inflammation (e.g. s.c. injection of turpentine or intramuscular injection of LPS; see Goldbach *et al.* 1997; Gourine *et al.* 2001; Leon *et al.* 1996). For example, subdiaphragmatic vagotomy attenuates I.P. LPS-induced fever but not intramuscular LPS-induced fever in guinea-pigs (Goldbach *et al.* 1997). Destruction of capsaicin-sensitive neurons exaggerates I.P. LPS fever but not fever induced by local inflammation due to injection of Freund's incomplete adjuvant in rats (Gourine *et al.* 2001). Mice with IL-1 type I receptor deletion produce fever identical to WT mice after I.P. injection of LPS, but do not produce fever after s.c. turpentine (Leon *et al.* 1996).

The present study showed that the fever about 10 h after S.C. turpentine was blocked only in the EP_3 receptor KO mice. Thus, the EP_3 receptor may play a role in fever due to local inflammation. However, S.C. turpentine also suppressed the normal circadian rise in T_c during the first dark period after injection in EP_3 receptor KO mice. The mechanism for this response is not known but similar phenomena have also been seen in turpentine-injected IL-6 KO mice (Kozak *et al.* 1997).

Psychological stress-induced hyperthermia

Models of hyperthermia induced by psychological stress have two distinct mechanisms: some models are PG dependent and others are not (Oka *et al.* 2001). We, therefore, tested both types of models: the cage-exchange stress tested the PG-dependent model (Singer *et al.* 1986; Kluger *et al.* 1987; Morimoto *et al.* 1991) and the modified form of the putative 'anticipatory anxiety' stress (Zethof *et al.* 1995) tested the PG-independent model.

Previous studies showed that cage-exchange stress caused increased plasma PGE_2 levels in rats (Morimoto *et al.* 1991) and that the hyperthermia it induced was attenuated by COX inhibitors (Singer *et al.* 1986; Kluger *et al.* 1987; Morimoto *et al.* 1991). In contrast, hyperthermia in the anticipatory anxiety model was not blocked by COX inhibitors but was blocked by serotonin (5-HT) 1A receptor agonists in mice (Zethof *et al.* 1994, 1995). In both types of models, a central noradrenergic component was involved (Lecci *et al.* 1990; Nakamori *et al.* 1993; Soszynski *et al.* 1996).

As most 5-HT and noradrenaline neurons exhibit EP_3 receptor-like immunoreactivity (Nakamura *et al.* 2001), we hypothesized that either or both models might be attenuated in the EP_3 receptor KO mice. However, EP_3 receptor KO mice responded in both protocols with a hyperthermia identical to WT and EP_1 receptor KO mice. Thus, the present findings suggest that the EP_3 or EP_1 receptors are not involved in psychological stress-induced hyperthermia, at least in these two models, and that the mechanism of psychological stress-induced hyperthermia is different from that of immune challenge-induced fever.

Localization of EP receptors involved in producing fever

Our results show that both the EP_1 and EP_3 receptors appear to play a role in producing different phases of fever. However, it is not clear where in the body the receptors are located that produce these responses.

It is known, for example that injection of E-type PGs into the anterior tip of the preoptic area near the wall of the third ventricle can induce fever responses (Williams *et al.* 1977; Stitt, 1991; Scammell *et al.* 1996) and that injection of a cyclooxygenase inhibitor into this region attenuates intravenous LPS fever responses (Scammell *et al.* 1998). Both EP_1 and EP_3 receptors are located in the paramedian preoptic region and so could mediate the fever response at that level.

However, neither preoptic COX inhibitor injections nor lesions entirely prevent fever responses (Lipton & Trzcinka, 1976; Matsumura *et al.* 1998). Considering these observations, one may speculate that EP receptors at other levels of the nervous system and perhaps some peripheral sites may also contribute to the response. For example, EP_3 receptors have been found in the raphe pallidus region (Ek *et al.* 2000; Nakamura *et al.* 2001), which regulates the sympathetic response in brown adipose tissue during fever responses (Morrison, 1999). EP_3 receptor mRNA or immunoreactivity has also been found on vagal afferents in the nucleus of the solitary tract and in the intermediolateral cell column in the spinal cord (Nakamura *et al.* 2000; Ek *et al.* 2000). It is also possible that EP_3 receptors may be found on peripheral cell types involved in preventing the profound hypothermia that accompanies injection of large (1 mg kg^{-1}) doses of LPS in mice at 23 °C. For example, there is evidence from injection of antibodies against TNFα that much of the hypothermic response is due to the action of TNFα, presumably causing peripheral systemic vasodilatation that is not centrally mediated (Kozak *et al.* 1995). If the white blood cells and macrophages secreting TNFα have EP_3 receptors that limit their response, the absence of the EP_3 receptor could result in a more profound hypothermic response to LPS.

We have not yet performed similar studies in mice with deletion of the EP_2 or EP_4 receptor genes. It would be interesting to test fever responses in EP_4 receptor KO mice because most of the POA and paraventricular hypothalamic neurons that show Fos expression after LPS injection also express EP_4 receptors (Oka *et al.* 2000). Lesions of the paraventricular nucleus do attenuate fever responses, but lesions of the region containing the POA cells that express EP_4 receptors do not (Lu *et al.* 2000, 2001). In addition, intracerebroventricular injection of an EP_4 receptor agonist decreases the T_c in rats (Oka *et al.* 2003), suggesting that EP_4 receptors in the brain may be involved in hypothermic but not hyperthermic responses to LPS.

A key focus for future studies will be on the locations of the different EP receptors that are involved in causing fever responses. As opposed to the constitutive knockout animals that we used, in which a gene is absent throughout development and in every body tissue, it will be useful to have animals with conditional knockouts or knockins of EP receptors. Such animals will provide important opportunities to establish the location of sites where the EP receptors play a role in the complex process that regulates T_c after administration of LPS.

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18

Insight into Prostanoid Functions: Lessons from Receptor-knockout Mice

Yukihiko Sugimoto, Shuh Narumiya and Atsushi Ichikawa

Kyoto University, Kyoto, Japan

PROSTANOID RECEPTORS

Prostaglandins (PGs) and thromboxanes (TXs) are the eicosanoids synthesized via the cyclooxygenase (COX) pathway. The collective term for this family of eicosanoids is "the prostanoids". Prostanoids are synthesized in a variety of cells in response to various physiological and pathological stimuli, and are then quickly released from the cells and act as local hormones in the vicinity of their production site to maintain local homeostasis (Halushka *et al* 1989). Prostanoids exert a wide variety of actions in the body, which are mediated by specific receptors on the plasma membranes of target cells. Prostanoid receptors were initially characterized pharmacologically in several bioassay systems, including contraction–relaxation assays on various smooth muscles and the aggregation of platelets. These receptors are classified into five basic types, termed DP, EP, FP, IP and TP, on the basis of their sensitivity to the five primary prostanoids, PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2, respectively. Furthermore, EP is subdivided into four subtypes, EP_1, EP_2, EP_3 and EP_4, on the basis of their responses to various agonists and antagonists (Kennedy *et al* 1982; Coleman *et al* 1990).

The prostanoid receptors have also been characterized biochemically using radioactive specific ligands (Coleman *et al* 1994b). Biochemical studies showed that the actions of prostanoids are mediated by G proteins, and the ligand-binding properties indicated that a variety of prostanoids cross-react with each receptor, suggesting the structural similarity of the receptors. It has been reported repeatedly that the actions of prostanoids are associated with changes in second messenger levels. Some prostanoid actions had been noticed to be associated with changes in cyclic AMP (cAMP) levels, phosphatidylinositol turnover or free calcium ion concentrations in the cell. However, none of the receptors had been isolated and cloned until the TXA_2 receptor, TP, was purified from human blood platelets in 1989 (Ushikubi *et al* 1989) and its cDNA cloned in 1991 (Hirata *et al* 1991). These studies revealed that the TP was a G-protein-coupled, rhodopsin-type receptor with seven transmembrane domains. Homology screening of mouse cDNA libraries subsequently identified the structures of all of the eight types and subtypes of the prostanoid receptors. These receptors have been expressed and their ligand-binding properties and signal transduction mechanisms have been examined in homogenous receptor populations in heterologous expression systems. In addition, the tissue and cell distributions of the receptors have been studied by Northern blot and *in situ* hybridization analyses of their mRNA expression. The correlation of this knowledge with the findings that have accumulated from pharmacological studies, using COX inhibitors and various prostanoid analogues with agonistic and antagonistic activities, have helped to define the actions of each type of receptor (Coleman *et al* 1990) as well as helped to reveal novel actions of these receptors. The accumulated knowledge from these analyses on the structure, pharmacological and biochemical properties and cellular distribution of the prostanoid receptor molecules have been described elsewhere (Narumiya *et al* 1999; Sugimoto *et al* 2000) and some of them are summarized in Table 18.1.

In recent years, the method of inactivating the function of a gene specifically and completely in mice has become a routine procedure. Gene disruption by the creation of a targeting vector and its introduction into embryonic stem cells was established in the 1980s (Doetschman *et al* 1987; Capecchi 1989). To date, targeted gene disruptions have been reported, not only in the enzymes engaged in prostanoid synthesis but also in the prostanoid receptors. Mice deficient in each prostanoid receptor have been generated, and initial analyses of the EP_1- (Ushikubi *et al* 1998), EP_2- (Kennedy *et al* 1999; Tilley *et al* 1999; Hizaki *et al* 1999), EP_3- (Ushikubi *et al* 1998), EP_4- (Nguyen *et al* 1997; Segi *et al* 1998), DP- (Matsuoka *et al* 2000), FP- (Sugimoto *et al* 1997), IP- (Murata *et al* 1997) and TP- (Thomas *et al* 1998) deficient mice have been reported. Such progress in strategy has enabled us to confirm the existing knowledge from pharmacological and biochemical analyses, to uncover novel prostanoid functions, and to answer questions that otherwise could not have been addressed. This section summarizes the phenotypes observed in prostanoid receptor-deficient mice compared with those observed in mice with altered prostanoid synthesis (Table 18.2), and presents various important insights into the mechanisms of the physiological actions of the prostanoids via their receptors.

MICE DEFICIENT IN EACH EP SUBTYPE (EP_1, EP_2, EP_3 AND EP_4)

EP_4 was the most recent of the subtypes to be pharmacologically identified, having been identified in 1994 (Coleman *et al* 1994a), but this receptor subtype is thought to be responsible for many of the actions of the PGE_2, such as the dilatation of smooth muscles, inhibition of immune responses and regulation of mucus secretion (Coleman *et al* 1990). Since both EP_2 and EP_4 are coupled to the stimulation of adenylate cyclase, the two receptors have been

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Table 18.1 Properties of the mouse prostanoid receptors[1]

Receptor type	K_d, nM (radioligand)	Rank order of binding affinity[2]	Signalling	Gene locus (mouse/human)	Alternatively spliced isoforms
EP_1	21 ($[^3H]PGE_2$)	$PGE_2 > iloprost > PGE_1$	$[Ca^{2+}]\uparrow$	8/19p13.1	2 (Rat)[3]
EP_2	27 ($[^3H]PGE_2$)	$PGE_2 = PGE_1 >$ butaprost	cAMP↑	14/14q22	None
EP_3	3 ($[^3H]PGE_2$)	$PGE_2 = PGE_1 >$ iloprost	cAMP↓ $[Ca^{2+}]\uparrow$	3/1p31.2	3 (Mouse)[3] 7 (Human) 4 (Bovine)[3]
EP_4	11 ($[^3H]PGE_2$)	$PGE_2 = PGE_1$	cAMP↑	15/5p13.1	None
DP	40 ($[^3H]PGD_2$)	$PGD_2 >$ BW245C	cAMP↑	14/14q21.3	None
FP	1.3 ($[^3H]PGF_{2\alpha}$)	$PGF_{2\alpha} > PGD_2$	$[Ca^{2+}]\uparrow$	3/1p31.1	2 (Ovine)
IP	4.5 ($[^3H]$iloprost)	cicaprost > iloprost > PGE_1	cAMP↑ $[Ca^{2+}]\uparrow$	7/19q13.3	None
TP	3.3 ($[^3H]$S-145)	S-145 > STA_2 > U46619	$[Ca^{2+}]\uparrow$ cAMP↓	10/19p13.3	2 (Human)[3]

[1]References for cDNA cloning, chromosomal mapping and multiple receptor isoforms are summarized in previous reviews (Narumiya *et al* 1999; Sugimoto *et al* 2000).
[2]Basic prostanoids and their derivatives with low K_d values ($< 10^{-6}$ M) are indicated. Cicaprost and iloprost are stable IP agonists, STA_2 and U46619 are stable TP agonists, and S-145 is a stable TP antagonist. Butaprost and BW245C are selective EP_2 and DP agonists, respectively. For details of the binding characters, see Kiriyama *et al* (1997).
[3]Some alternatively spliced receptor isoforms have been found to differ in their signal transduction pathways.

suspected to function in a similar manner. However, a drastic induction of EP_2 gene expression in response to hormonal and proinflammatory stimuli, but not that of EP_4, has been identified in various kinds of cells, suggesting that the two receptors have rather different roles in various physiological processes (Sugimoto *et al* 2000). It is interesting in this respect to study the phenotypes that appear in the EP_2- and EP_4-knockout mice. In contrast, EP_1 and EP_3 have been shown to be coupled to an increase in intracellular CA^{2+} mobilization. It should be noted that EP_3 is the only prostanoid receptor that inhibits adenylate cyclase. To date, EP_3 has been shown to be involved in pyrogen-induced fever generation and in mucosal defence of the gastrointestinal tract. However, the roles of EP_1 in the body remain to be clarified.

Ductus Arteriosus

In EP_4-deficient mice from an inbred 129 strain, the ductus arteriosus (DA) fails to close after birth, and this is followed by death in the early neonatal period. The DA is an arterial connection in the foetus that directs the blood to be oxygenated away from the pulmonary circulation and toward the placenta. Thus, in wild-type animals, the drop in PGE_2 that acts as a trigger for DA closure in the neonate is sensed through EP_4. It is worth noting that when the gene disruption occurs on a mixed genetic background a small percentage of mice survive, suggesting that alleles at other loci can provide an alternative mechanism for DA closure (Nguyen *et al* 1997; Segi *et al* 1998). Loftin *et al* (2001) examined this issue by using mice deficient in either or both COX isoforms. The absence of only COX-1 did not affect closure of the DA. However, 35% of COX-2-deficient mice die with a patent DA. The mortality and patent DA incidence due to the absence of COX-2 is significantly increased (79%) when one copy of the COX-1 gene is also inactivated. Furthermore, 100% of the mice deficient in both isoforms die with a patent DA. These results indicate the dominant contribution of COX-2 to DA closure, but this effect can be partly compensated by the COX-1 isoform.

Ovulation and Fertilization

Recent studies on mice deficient in COX-2 showed multiple failures in female reproduction, including ovulation and fertilization,

Table 18.2 Major phenotypes of mice deficient in the prostanoid receptors

Disrupted gene	Phenotypes	Gene disruption showing similar phenotypes
DP	Reduced allergic responses in ovalbumin-induced bronchial asthma	
EP_1	Decreased aberrant crypt foci formation in response to azoxymethane	COX-2(−/−)
EP_2	Impaired ovulation and fertilization	COX-2(−/−)
	Decreased intestinal polyp formation in $Apc^{\Delta 716}$ mice	COX-2(−/−), $cPLA_2$(−/−)
	Salt-sensitive hypertension	
	Impaired osteoclastogenesis *in vitro*	
EP_3	Impaired febrile response to pyrogens	COX-2(−/−)
	Impaired duodenal bicarbonate secretion	
	Increased bleeding tendency and decreased susceptibility to thromboembolism	
EP_4	Patent ductus arteriosus	COX-2(−/−)/COX-1(−/−), COX-2(−/−)
	Impaired mucosal integrity and enhanced immune response in colitis	COX-2(−/−), COX-1(−/−)
	Decreased inflammatory bone resorption	
FP	Loss of parturition	COX-1(−/−), $cPLA_2$(−/−)
IP	Thrombotic tendency	
	Decreased inflammatory swelling	
	Decreased acetic acid writhing	
TP	Bleeding tendency and resistance to thromboembolism	

suggesting that PGs play essential roles in multiple processes occurring during early pregnancy (Davis *et al* 1999; Dinchuck *et al* 1995; Lim *et al* 1997). Three groups independently generated mice lacking EP_2, which showed a failure during early pregnancy. Kennedy *et al* (1999) and Tilley *et al* (1999) found that EP_2-deficient female mice consistently deliver fewer pups than their wild-type counterparts irrespective of the genotypes of the mating males. They detected slightly impaired ovulation and a dramatic reduction in fertilization in EP_2-deficient mice and concluded that reproduction failures in Cox-2-deficient mice is at least partly due to the dysfunction of EP_2. Hizaki *et al* (1999) further found that this phenotype is due to impaired expansion of the cumulus oophorus. Since EP_2 and COX-2 are induced in the cumulus in response to gonadotropins, and since PGE_2 can induce cumulus expansion by elevating cAMP (Eppig 1981), the authors suggest that the PGE_2 and EP_2 system work as a positive-feedback loop to induce oophorus maturation required for fertilization during and after ovulation. Indeed, unovulated eggs remaining in the corpora lutea were observed at a higher frequency in EP_2-deficient mice. It is interesting in this respect that indomethacin treatment has been reported to result in the formation of luteinized unruptured follicles in humans (Priddy *et al* 1990).

Fever Generation

The E-type PG is a powerful inducer of fever when injected into the brain, and the level of PGE_2 increases in the preoptic area (POA) during lipopolysaccharide (LPS)-induced fever. In addition, indomethacin completely abolishes both the LPS-induced fever and the increased levels of PGE_2 in the POA (Kluger 1991; Saper and Breder 1994). The febrile responses to PGE_2, interleukin (IL)-1β and LPS in mice lacking EP_1, EP_2 and EP_4 were comparable to those in wild-types. The EP_3-deficient mice failed to show a febrile response to all of these stimuli (Ushikubi *et al* 1998). Thus, PGE_2 mediates fever generation in response to both exogenous and endogenous pyrogens by acting on EP_3. It has also been reported that COX-2-deficient mice also show impaired febrile responses, suggesting that COX-2 is involved in fever generation (Li *et al* 1999). Indeed, intravenous administration of IL-1β induces expression of both COX-2 and microsomal PGE synthase in endothelial cells of the brain microvessels (Ek *et al* 2001). The resultant PGE_2 appears to act on EP_3 in the POA, especially in the region surrounding the organum vasculosa lamina terminalis (OVLT), which is the most sensitive area of the brain for microinjected PGE_2 to produce fever (Elmquist *et al* 1997). In fact, the mRNA for EP_3 is particularly abundant in the regions surrounding the OVLT (Sugimoto *et al* 1994) and EP_3 immunoreactivity is also present in the cell bodies of these neurons, with a distribution pattern similar to that of EP_3 mRNA (Nakamura *et al* 1999). Thus, EP_3 expressed in the neurons surrounding the OVLT appear to work as an initial input of 'pyrogenic' PGE_2 to alter the set-point of thermal regulation.

Colorectal Cancer

COX-2 has been implicated in the progression of colorectal cancer. Supporting evidence comes from a study in which COX-2-deficient mice were crossed with mice with a truncated Apc gene ($Apc^{\Delta 716}$), used as a model of human familial adenomatous polyposis (Oshima *et al* 1996). The $Apc^{\Delta 716}$ heterozygous/COX-2-deficient mice have a dramatically reduced number and size of intestinal polyps. This provides direct genetic evidence for the role of COX-2 in tumorigenesis. Sonoshita *et al* (2001) reported that the homozygous disruption of EP_2 in Apc-knockout mice caused significant decreases in the number and size of the intestinal polyps, showing similar effects to those induced by the COX-2 gene disruption. Regarding the mechanism, the authors indicate that an increased cellular cAMP level through EP_2 signalling amplifies COX-2 expression and stimulates the expression of vascular endothelial growth factor in the polyp stroma. In a separate paper, carcinogen-induced formation of aberrant crypt foci, putative preneoplastic lesions of the colon was examined; foci formation was decreased in EP_1-deficient mice to ~60% of the level in wild-type mice (Watanabe *et al* 1999). Furthermore, partial reduction of foci formation was observed by the administration of an EP_1-antagonist in the diet of azoxymethane-treated wild-type mice. Similar treatment also reduced the number of polyps in Min mice, suggesting that PGE_2 contributes to carcinogen-induced colon foci formation through its action on EP_1. Thus, there is an apparent discrepancy regarding the identity of the EP subtypes acting in carcinogenesis, which awaits further study for clarification.

Gastrointestinal Functions

The current hypothesis regarding the medicinal usage of aspirin-like drugs is that the inhibition of COX-2 is responsible for their beneficial effects, whereas the inhibition of COX-1 is responsible for their adverse effects, the most common being gastric ulceration (Langenbach *et al* 1999). However, neither COX-1-deficient nor COX-2-deficient mice showed spontaneous ulcer formation, although gastric PG levels in COX-1 null mice were greatly reduced to levels observed following an ulcerative dose of indomethacin (Langenbach *et al* 1995; Moham *et al* 1995). Thus, elimination of COX-1-derived PGs alone was not sufficient to cause gastric ulcers. In contrast to the understanding of the contribution of the COX enzymes in the ulcerative process, which prostanoid receptor is involved in the protective actions against ulcerative stimuli is poorly understood. EP and other prostanoid receptor-knockout mice will be used to clarify this issue. Indeed, Takeuchi *et al* (1999) recently found that EP_3 but not EP_1 is involved in acid-induced duodenal bicarbonate secretion, which is physiologically important in the mucosal defence against acid injury.

Prostanoids, especially the E-type PG, have been suggested to contribute to mucosal defence in gastrointestinal inflammation, such as in inflammatory bowel disease. Indeed, genetic absence of COX-1 or COX-2 exacerbated the extent of dextran sodium sulphate (DSS)-induced colitis (Morteau *et al* 2000). This treatment increased intestinal PGE_2 production in a COX-2-dependent manner. Among the EP-deficient mice, only EP_4-deficient mice showed a greatly increased susceptibility to a low dose (3%) of DSS that caused only mild colonic injury in wild-type mice (Kabashima *et al* 2002). The phenotype was mimicked in wild-type mice by administration of an EP_4-selective antagonist. The EP_4 deficiency caused impaired mucosal defence and aggregation of neutrophils and lymphocytes in the colon. A high dose (7%) of DSS elicited severe colitis in wild-type mice, but an EP_4-agonist reversed these effects of DSS. An EP_4-antagonist suppressed recovery from colitis and induced significant proliferation of $CD4^+$ T cells. In the colon isolated from EP_4-deficient mice with DSS-induced colitis, gene expression of epidermal growth factor was reduced and the expression of chemoattractants increased, compared with wild-type mice treated with DSS. Thus, PGE_2 contributes to maintain intestinal homeostasis via EP_4 by promoting epithelial regeneration and also by inhibiting intestinal immune responses.

Vascular Homeostasis

PGE_2 also elicits contractile and/or relaxant responses of vascular smooth muscles *in vitro*. Kennedy *et al* (1999) administered PGE_2 and its analogues intravenously into wild-type and EP_2-deficient mice and examined their responses *in vivo*. Infusion of PGE_2 or an EP_2 agonist, butaprost, induces transient hypotension in wild-type mice. In EP_2-deficient mice, butaprost failed to elicit hypotension but, unexpectedly, PGE_2 evoked considerable hypertension. The authors discussed that the absence of EP_2 abolishes the ability of the mouse vasculature to vasodilate in response to PGE_2 and unmasks the contractile response via the vasoconstrictor PGE receptor(s). Moreover when fed on a high-salt diet, the EP_2-deficient mice develop significant hypertension, with a concomitant increase in urinary excretion of PGE_2. Thus, PGE_2 is produced in the body in response to a high-salt diet and works to negatively regulate blood pressure via the relaxant EP_2. Interestingly, the relative contribution of each EP subtype appears to be different between males and females (Audoly *et al* 1999). In females, EP_2 and EP_4 mediate the major portion of the vasodepressor response to PGE_2. In males, EP_2 plays only a modest role, and most of the vasodepressor effect is mediated by the phospholipase C-coupled EP_1. In addition, in male mice, EP_3 actively opposes the vasodepressor actions of PGE_2. Thus the haemodynamic actions of PGE_2 are mediated through complex interactions of several PGE receptors.

Bone Remodelling

The E-type PG can also affect bone remodelling, in both bone formation and resorption. The bone resorptive activity of PGE_2 is associated with the occurrence of an increased number of osteoclasts. Sakuma *et al* (2000) and Miyaura *et al* (2000) reported impaired osteoclast formation in culture cells from EP_4-deficient mice. They found that osteoclast formation is most potently induced by analogues with EP_4-agonistic activity. Indeed, PGE_2-induced osteoclast formation was impaired in osteoblast cultures from the EP_4-deficient mice and osteoclast precursors from the spleen of wild-type mice. Suzawa *et al* (2000) further found that bone resorption by PGE_2 was greatly decreased in bone from EP_4-deficient mice, which showed an equal level of response to dibutyryl cAMP added to the culture as the bones from control mice. These studies clearly established the role of the EP_4 subtype of PGE receptors in PGE_2-mediated bone resorption. On the other hand, Li *et al* (2000) reported that the osteoclastogenic response to PGE_2 and other stimulants is reduced significantly in culture cells from EP_2-deficient mice. This apparent discrepancy may reflect redundant roles of the two relaxant PGE receptors. Sakuma and Miyaura *et al* found a small but significant PGE_2-dependent response in EP_4-deficient mice, and Li *et al* reported a further decrease in osteoclastogenesis when an EP_4-selective antagonist was added to EP_2-deficient cells.

Exogenous PGE_2 has been shown to induce not only bone resorption, but also bone formation. Yoshida *et al* (2002) examined the effects of PGE_2 infusion into the periosteal region of the femur for 6 weeks in wild-type or mice deficient in each EP. PGE_2 induced callus formation on the femur at the site of infusion in wild-type mice, but not in EP_4-deficient mice. Consistently, bone formation was induced in wild-type mice by infusion of an EP_4-selective agonist, but not by agonists specific for other EP subtypes. The EP_4-agonist completely blocked the bone loss induced by ovariectomy or immobilization, and restored the bone mass with an increased density of osteoblasts lining the bone surface. These results suggest that EP_4 is responsible for both bone resorption and bone formation induced by PGE_2 and that activation of EP_4 induces bone remodelling *in vivo*.

DP-KNOCKOUT MICE

Allergic Asthma

Allergic responses are often associated with an increase in prostanoid formation. PGD_2 is the major prostanoid generated by mast cells upon allergen challenge and is produced abundantly in allergic diseases such as asthma, allergic dermatitis and conjunctivitis. However, the specific role played by PGD_2 in allergy has been unclear. Matsuoka *et al* (2000) focused on the specific role of PGD_2 in allergy by subjecting DP-deficient mice to ovalbumin-induced allergic asthma. They found a marked reduction in airway inflammation, obstruction and hypersensitivity in DP-deficient animals, suggesting that PGD_2, acting via DP, works as a mediator of allergy. Interestingly, DP expression was seen in bronchiolar and alveolar epithelial cells only in antigen-challenged mice and not in mice immunized before the antigen challenge. On the contrary, Gavett *et al* (1999) reported that both COX-1- and COX-2-deficient mice showed enhanced allergic lung responses in a similar asthmatic model. This study by Gavett *et al* highlighted the beneficial aspects of prostanoids in an asthmatic model. The idea that prostanoids play protective roles in asthma was originally raised upon the finding that aspirin is not beneficial for allergy and can even cause asthmatic attacks in certain individuals. It is suspected that other prostanoids normally antagonize the action of PGD_2, resulting in aspirin treatment having complex effects on the disease pathway. Another factor of consideration in this issue is the existence of another PGD_2 receptor, $CRTH_2$, which is also a plasma membrane-type receptor but more closely related to the *N*-formyl peptide receptor superfamily than to the other prostanoid receptors (Hirai *et al* 2001). This receptor is expressed preferentially in Th2 cells and has been shown to mediate chemotactic movement in response to PGD_2. The exact roles of PGD_2 via this receptor in allergy remain to be clarified.

Sleep

PGD_2 is a potent endogenous sleep promoting substance in rats and other mammals including humans (Hayaishi *et al* 2000). PGD_2 infused into the subarachnoid space underlying the rostral basal forebrain was effective in inducing sleep. Mizoguchi *et al* (2001) infused PGD_2 into the lateral ventricle of wild-type and DP-deficient mice and determined the amounts of non-rapid eye movement (NREM) and rapid eye movement (REM) sleep. In wild-type mice, PGD_2 infusion significantly increased NREM sleep. In DP-deficient mice, however, the amount of neither NREM nor REM sleep was altered at all by PGD_2 infusion. Thus, PGD_2 predominantly increased NREM sleep in wild-type mice and DP is crucially involved in PGD_2-induced NREM sleep. The authors further demonstrated that the activation of DP elicited an increase in the extracellular adenosine content in the subarachnoid space of the rostral basal forebrain after PGD_2 infusion. The amount of PGD_2-induced sleep was reduced by pretreatment with an adenosine A_{2A} receptor-specific antagonist (Satoh *et al* 1996), while administration of an adenosine A_{2A} receptor-selective agonist into the subarachnoid space induced sleep (Satoh *et al* 1999). These results suggested that PGD_2-induced sleep is mediated by the adenosine A_{2A} receptor system.

FP-KNOCKOUT MICE

Luteolysis and Parturition

FP-deficient mice do not show any abnormalities during early pregnancy or any changes in the oestrous cycle (Sugimoto *et al*

1997). However, FP-deficient pregnant mice do not perform parturition, apparently due to the lack of labour. FP-deficient mice do not undergo parturition, even when given exogenous oxytocin, and show no prepartum decline in progesterone. A reduction in progesterone levels due to ovariectomy 24 h before term caused an upregulation of uterine receptors for oxytocin and normal parturition in the FP-deficient mice. These experiments indicate that the luteolytic action of $PGF_{2\alpha}$ is required in mice to diminish progesterone levels and thus permit the initiation of labour. Indeed, ovarian expression of 20α-hydroxysteroid dehydrogenase, a catabolic enzyme for progesterone, is absent in FP-deficient mice, while this enzyme is induced at the mRNA and protein levels on day 19 of pregnancy in wild-type mice (Stocco *et al* 2000). The luteolytic role for $PGF_{2\alpha}$ in the induction of labour in mice is also supported by the finding that mice lacking the gene encoding COX-1 also exhibited a similar parturition failure (Gross *et al* 1998). In these mice, production of $PGF_{2\alpha}$ in intrauterine tissues during late pregnancy is significantly reduced and the administration of $PGF_{2\alpha}$ on day 19 is able to restore normal parturition. In wild-type mice, the uterine expression of COX-1 mRNA gradually increases from day 15 of pregnancy, reaches maximal levels on day 17, and rapidly decreases after day 20, the day when parturition normally occurs. Tsuboi *et al* (2000) found that the uterine expression of COX-1 mRNA was still at high levels on day 20 in FP-deficient mice. This observation suggests that progesterone withdrawal serves as a negative feedback system for uterine COX-1 expression.

Considering the phenotypes of the EP_2- and FP-knockouts as well as the COX-2- and COX-1-knockouts, it can be concluded that prostanoids play essential roles in multiple processes in female reproduction. This is also supported by research on cytosolic phospholipase A_2 ($cPLA_2$), another key enzyme in prostanoid synthesis which catalyses cleavage of the eicosanoid precursor, arachidonic acid, from phospholipids. $cPLA_2$-deficient females have smaller litter sizes and delayed parturition, which are interpreted as phenotypes equivalent to those seen in EP_2-deficient and FP-deficient mice, respectively (Bonventre *et al* 1997; Uozumi *et al* 1997). Moreover, the administration of a progesterone receptor antagonist (RU-486) to mice at term to substitute for the luteolytic decline in progesterone corrected the defect in labour seen in the $cPLA_2$-deficient mice.

IP- AND TP-KNOCKOUT MICE

Inflammation and Pain

Vasodilation and pain sensation are two classic features of acute inflammation to which prostanoids appear to contribute. Aspirin-like drugs suppress these responses, and PGE_2 and PGI_2 can mimic these actions. Carrageenan-induced paw oedema and acetic acid-induced writhing are representative models for acute inflammation and pain, respectively. In IP-deficient mice, both responses are completely absent (Murata *et al* 1997). Thus, PGI_2, acting on IP, works as a physiological mediator of these responses. However, it remains to be seen whether PGI_2 and IP play important roles in other types of inflammation and pain. Regarding pain, PGs are involved not only in hyperalgesia, an increased sensitivity to a painful stimulus, but also in allodynia, a pain response to a usually non-painful stimulus (Malmberg and Yaksh 1992). The former is caused by sensitizing the free end of pain neurons at the site of peripheral inflammation, while the latter condition is frequently seen in neuropathic pain and is thought to occur in the spinal cord (Bley *et al* 1998). The circulating IL-1β cytokine, which originates at the site of peripheral injury and cannot pass the blood–brain barrier, induces both COX-2 and mPGES activities in cells lining the blood–brain barrier (Samad *et al* 2001). PGE_2 then enters the brain and cerebrospinal fluid and induces prostanoid receptor activation on neurons and microglia. This increases neuronal excitability and leads to non-painful stimuli becoming painful, basically converting a peripheral injury to a central pain response without nerve impulse transmission. At present, the possible involvement of EP_1, EP_3 and IP in pain has also been suggested (Minami *et al* 2001; Ueno *et al* 2001). Because the dorsal root ganglion expresses several types of prostanoid receptor mRNAs, including IP, EP_1, EP_3 and EP_2 (Oida *et al* 1995), the exact contribution of receptors other than IP to pain generation should be carefully determined.

Haemostasis

PGI_2 and TXA_2, produced abundantly by vascular endothelial cells and platelets, respectively, are a potent vasodilator and vasoconstrictor, respectively. Mice lacking IP are viable, normotensive and reproductive; however, their susceptibility to thrombosis is increased (Murata *et al* 1997). Their platelets no longer respond to the PGI_2 agonist cicaprost, neither does vascular smooth muscle relax upon this treatment, effectively demonstrating that a single IP subtype mediates both platelet and smooth muscle cell effects. The thrombotic tendencies of the IP-deficient mice were tested in a model of arterial thrombosis. IP-knockouts demonstrated more extensive thrombus formation than wild-type animals after injury induced by ferric chloride. These findings confirmed the long-proposed role of PGI_2 as an endogenous antithrombotic agent, and suggested that this antithrombotic system is activated in response to vascular injury to minimize its effects. TP-deficient mice showed an increased bleeding tendency and were resistant to cardiovascular shock induced by intravenous infusion of a TP agonist, U-46619, and arachidonic acid (Thomas *et al* 1998). Interestingly, endogenous PGE_2 is likely to contribute to platelet aggregation via EP_3; gene disruption of this PGE receptor resulted in an increased bleeding tendency and decreased susceptibility to thromboembolism (Ma *et al* 2001).

CONCLUDING REMARKS

Prostanoids have been suggested to have roles in many physiological processes, based on the various actions of aspirin-like drugs and exogenously added agonists. Until recently, however, the specific receptor involved in each process was unclear due to the failure to elucidate the molecular characteristics of each prostanoid receptor. The study of mice null for each prostanoid receptor has seen a remarkable development in the past several years. Some unexpected findings have raised many new questions about the traditional views based on the actions of aspirin-like drugs. However, post-genomic approaches such as SNP and expression profile analyses should reveal complete answers to those questions in the near future. For example, a polymorphic variation of the human TP gene has been identified and its connection to allergic diseases has been discussed (Unoki *et al* 2000). Thus, this exciting field of study will no doubt bring about many novel findings related not only to the prostanoids but also to the eicosanoids in the new millennium.

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Uptake of histamine by mouse peritoneal macrophages and a macrophage cell line, RAW264.7

Satoshi Tanaka, Katsuya Deai, Mariko Inagaki, and Atsushi Ichikawa
Department of Physiological Chemistry, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan

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Tanaka, Satoshi, Katsuya Deai, Mariko Inagaki, and Atsushi Ichikawa. Uptake of histamine by mouse peritoneal macrophages and a macrophage cell line, RAW264.7. *Am J Physiol Cell Physiol* 285: C592–C598, 2003. First published April 30, 2003; 10.1152/ajpcell.00470.2002.—We have previously demonstrated that dietary histamine is accumulated in the spleens of L-histidine decarboxylase (HDC)-deficient mice, which lack endogenous histamine synthesis. To characterize the clearance system for dietary histamine in mice, we investigated the cell type and mechanism responsible for histamine uptake in the spleens of HDC-deficient mice. Immunohistochemical analyses using an antihistamine antibody indicated that a portion of the $CD14^+$ cells in the spleen is involved in histamine storage. Peritoneal macrophages obtained from Balb/c mice and a mouse macrophage cell line, RAW264.7, had potential for histamine uptake, which was characterized by a low affinity and high capacity for histamine. The histamine uptake by RAW264.7 cells was observed at physiological temperature and was potently inhibited by pyrilamine, chlorpromazine, quinidine, and chloroquine, moderately inhibited by N^{α}-methylhistamine, dopamine, and serotonin, and not affected by tetraethylammonium and 1-methyl-4-phenylpyridinium. Intracellular histamine was not metabolized in RAW264.7 cells and was released at physiological temperature in the absence of extracellular histamine. These results suggest that histamine uptake by macrophages may be involved in the clearance of histamine in the local histamine-enriched environment.

cation transporter; chlorpromazine; pyrilamine; quinidine

HISTAMINE HAS BEEN FOUND to exert its roles in a wide variety of physiological and pathological processes, such as inflammation, allergy, gastric acid secretion, and neurotransmission (1, 3, 18, 25). Because histamine is a potent mediator in these responses, it is very important to maintain local homeostasis by eliminating this histamine from the microenvironment. Expression of histamine-metabolizing enzymes, such as diamine oxidase (DAO, histaminase) and histamine *N*-methyl transferase (HMT), in some tissues contributes to the clearance of systemic histamine (13, 26). Another possible mechanism of histamine elimination is cellular uptake. However, no plasma membrane transporter specific for histamine has been identified and the characteristics of cellular histamine uptake are largely unknown. Recently, a family of organic cation transporters has been cloned (7), and some of them have been reported to be capable of histamine uptake (6). Little attention, however, was paid to this histamine uptake due to its relatively lower affinity compared with the other organic cations. Because the enzymes involved in histamine metabolism, such as DAO and HMT, and the putative transporters involved in histamine uptake have been found in limited types of tissues, it is possible that another system is involved in the local clearance of histamine.

We recently established an L-histidine decarboxylase (HDC)-deficient mouse strain, in which de novo synthesis of histamine is undetectable (12). However, a small but detectable amount of histamine was observed in some tissues of these HDC-deficient mice. Regarding the origin of this histamine, commercially available mouse diets were found to contain a trace amount of histamine, and some kinds of enterobacteria are known to produce histamine (16). Furthermore, we have recently observed that the histamine content in several tissues, such as brain, skin, stomach, and spleen, were significantly increased in the HDC-deficient mice when they were maintained on a histamine-enriched diet (11). These findings indicate that the HDC-deficient mouse strain is a good model for analyses of dietary histamine uptake without the possible interfering effects of endogenous histamine synthesis. Our purpose in this study is to identify and characterize the cellular uptake system for histamine.

MATERIALS AND METHODS

Materials. The following materials were purchased from the sources indicated: an antihistamine antibody from Sigma (St. Louis, MO), an anti-CD14 antibody from Pharmingen (San Diego, CA), an anti-heat shock cognate 70 (Hsc70) antibody from StressGen (Victoria, Canada), an Alexa 546-conjugated anti-rat IgG antibody, an Alexa 488-conjugated anti-rabbit IgG antibody, and an Alexa 488-conjugated anti-rat IgG antibody from Molecular Probes (Eugene, OR), a rhodamine-conjugated anti-rabbit IgG antibody from Leinco Technology, (Ballwin, MO), [^{3}H]histamine (23.3 Ci/mmol) from DuPont-New England Nuclear (Boston, MA), and dimaprit, N^{α}-methylhistamine, thioperamide, and clozapine from

Address for reprint requests and other correspondence: A. Ichikawa, Dept. of Physiological Chemistry, Graduate School of Pharmaceutical Sciences, Kyoto Univ., Sakyo-ku, Kyoto 606-8501, Japan (E-mail: aichikaw@pharm.kyot-u.ac.jpo).

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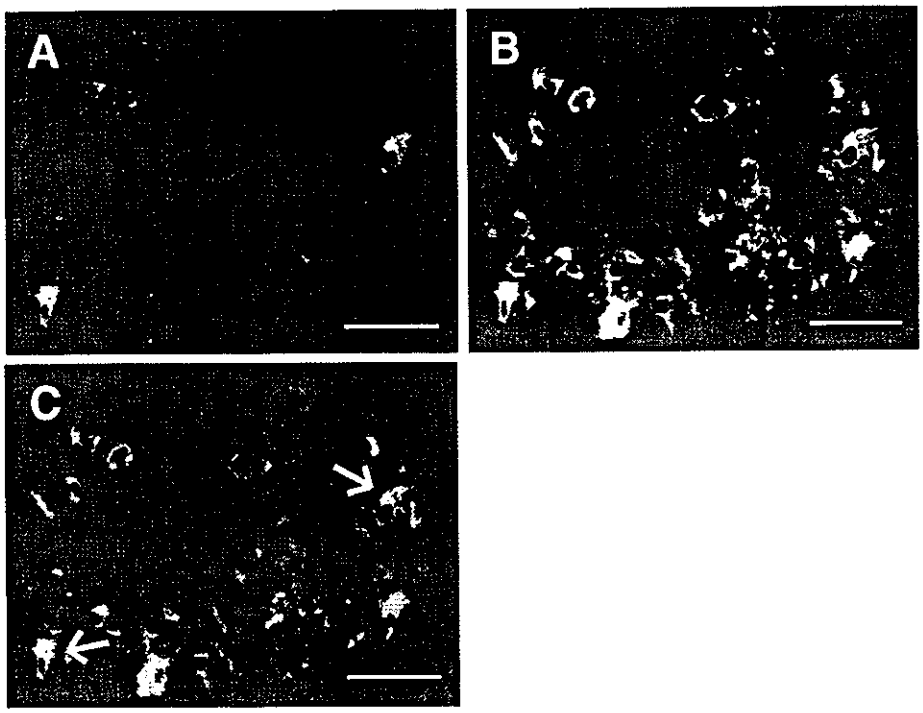

Fig. 1. Immunohistochemical analyses using an antihistamine antibody. A spleen from a male Balb/c mouse was collected and treated with Bouin's fixative for 24 h at 4°C. Cryostat sections (8 μm in thickness) were incubated with an antihistamine antibody (1:100) (*A*) and an anti-CD14 antibody (1:100) (*B*) for 1 day at 4°C. The sections were stained with an Alexa 488-conjugated anti-rabbit IgG antibody (1:100) and a rhodamine-conjugated anti-rat IgG antibody (1:100). Fluorescent images were obtained using a confocal microscope (MRC-1024, Bio-Rad Laboratories). A superimposed image is shown in *C*. The arrows indicate cells immunoreactive to both antibodies. Bars = 30 μm.

Uptake of histamine by peritoneal macrophages and a macrophage cell line, RAW264.7, during in vitro incubation. Because CD14$^+$ spleen cells were immunoreactive to the antihistamine antibody, we then measured histamine uptake by peritoneal macrophages of Balb/c mice. A significant uptake of histamine was observed at 37°C, although not at 4°C (Fig. 2*A*). The time course of histamine uptake was unchanged under

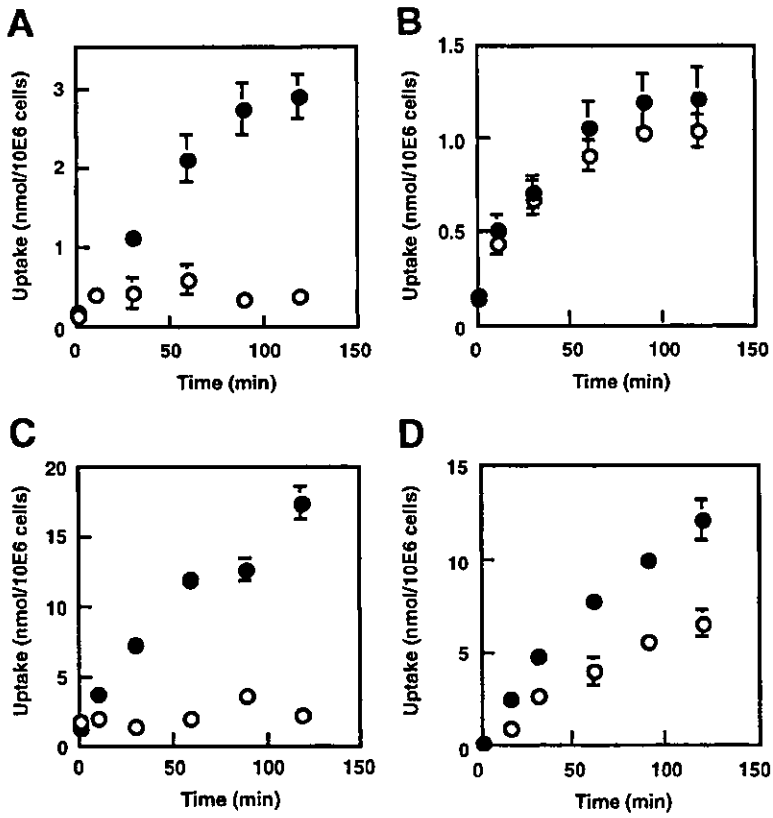

Fig. 2. Temperature and Na^+ dependency of histamine uptake by peritoneal macrophages and RAW264.7 cells. Histamine uptake in mouse peritoneal macrophages (*A* and *B*) and in RAW264.7 cells (*C* and *D*) was measured. Cells were incubated with [^{3}H]histamine (2.33 μCi/ml) in the presence of cold histamine (final concentration = 6 mM) for the periods indicated at 37°C, and then the uptake was measured using a liquid scintillation counter. (*A* and *C*). Uptake of [^{3}H]histamine was performed at 37°C (●) or at 4°C (○). *B* and *D*: uptake of [^{3}H]histamine was measured in KRH buffer in the presence (●) or absence (○) of Na^+. In the sodium-free condition, Na^+ in the Krebs-Ringer-HEPES (KRH) buffer was replaced with Li^+. The values are presented as means ± SE (n = 3).