Castor oil induces laxation and uterus contraction via ricinoleic acid activating prostaglandin EP₃ receptors

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Castor oil is one of the oldest drugs. When given orally, it has a laxative effect and induces labor in pregnant females. The effects of castor oil are mediated by ricinoleic acid, a hydroxylated fatty acid released from castor oil by intestinal lipases. Despite the wide-spread use of castor oil in conventional and folk medicine, the molecular mechanism by which ricinoleic acid acts remains unknown. Here we show that the EP₃ prostanoid receptor is specifically activated by ricinoleic acid and that it mediates the pharmacological effects of castor oil. In mice lacking EP₃ receptors, the laxative effect and the uterus contraction induced via ricinoleic acid are absent. Although a conditional deletion of the EP₃ receptor gene in intestinal epithelial cells did not affect castor oil-induced diarrhea, mice lacking EP₃ receptors only in smooth-muscle cells were unresponsive to this drug. Thus, the castor oil metabolite ricinoleic acid activates intestinal and uterine smooth-muscle cells via EP₃ prostanoid receptors. These findings identify the cellular and molecular mechanism underlying the pharmacological effects of castor oil and indicate a role of the EP₃ receptor as a target to induce laxative effects.

G-protein coupled receptor | peristalsis | Ricinus communis | PGE₂

Castor oil, also known as Oleum Palmae Christi, is obtained from the seeds of Ricinus communis and has been used therapeutically for centuries (1, 2), being first described in the Ebers papyrus of ancient Egypt more than 3,500 y ago (3). Castor oil is a triglyceride characterized by a high content of the hydroxylated unsaturated fatty acid ricinoleic acid [(9Z,12R)-12-hydroxyoctadec-9-enoic acid] (4). After oral ingestion of castor oil, ricinoleic acid is released by lipases in the intestinal lumen, and considerable amounts of ricinoleic acid are absorbed in the intestine (5, 6). The released ricinoleic acid induces a strong laxative effect (5, 7). There is also a well-documented labor-inducing effect of castor oil in pregnant females at term; however, use of this drug for labor induction is not recommended because of unwanted effects, such as nausea (8).

The mechanisms underlying the pharmacological effects of ricinoleic acid remain elusive. Castor oil is regarded as a stimulant and irritant laxative without known mechanism of action (9). Several studies have shown that relatively high concentrations of ricinoleic acid can cause ultrastructural alterations in the villous tips of the intestinal mucosa (10, 11). Given the high concentrations of ricinoleic acid used in these experiments, it is, however, not clear whether these unspecific morphological effects are relevant for the laxative effect of castor oil. In part, conflicting data have been published with regard to the ability of ricinoleic acid to induce procontractile effects on intestinal smooth muscle and to alter intestinal ion transport and water flux. Although some groups observed an inhibition of water and electrolyte absorption (12–14), others found an activation of ion secretory processes by ricinoleid acid (15). In addition to effects of ricinoleic acid on intestinal ion transport and water flux, evidence has been provided that ricinoleic acid can directly affect intestinal motility (16–19). Whether these effects are mediated by the enteric nervous system or are direct effects on intestinal smooth muscle remained unclear.

The present study was undertaken to elucidate the molecular mechanism underlying the biological effect of castor oil-derived ricinoleic acid. Based on cellular signaling studies and an siRNA screening approach, we identified prostaglandin E₂ receptors as targets of ricinoleic acid and show that the EP₃ receptor mediates the effects of castor oil on the motility of the uterus and the intestine.

Results

In a screen for potential receptor-mediated effects using a library of biologically active lipids, we observed a Ca²⁺ transient after exposure of various cell types to ricinoleic acid. The response was strongest in the human megakaryocyte leukemia cell line MEG-01 (Fig. 1A). This effect was dose-dependent with an EC₅₀ of 5 μM (Fig. 1B) and could be blocked by pretreatment of cells with pertussis toxin (Fig. 1A). The biologically inactive trans isomer of ricinoleic acid, ricinelaic acid [(9E,12R)-12-hydroxyoctadec-9-enoic acid], as well as the nonhydroxylated homolog, oleic acid [(9Z)-octadec-9-enoic acid], were without effect (Fig. 1B). These data suggest that ricinoleic acid can specifically activate a G protein-coupled receptor (GPCR). To identify a putative GPCR activated by ricinoleic acid, we screened a small interfering RNA (siRNA) library targeting all known and predicted nonolfactory human GPCRs for its ability to interfere with activation of MEG-01 cells by ricinoleic acid. Fig. 1C shows that siRNAs pools directed against mRNAs encoding EP₃ and EP₄ (20–22) strongly reduced ricinoleic acid effects in MEG-01 cells. We verified that EP₃ and EP₄ receptors are expressed in MEG-01 cells and that prostaglandin E₂ (PGE₂) has effects comparable to ricinoleic acid in these cells (Fig. S1). Consistent with a role of EP₃ and EP₄ receptors in mediating cellular effects of ricinoleic acid, the selective antagonists of EP₃ and EP₄ receptors, L-798,106 and L-161,982, respectively, at maximally active concentrations inhibited ricinoleic acid- and PGE₂-induced calcium mobilization in MEG-01 cells (Fig. 1D). EP₃/PGE₂-mediated effects of ricinoleic acid were not because of formation of PGE₂ in response to ricinoleic acid (Fig. S2A and B). Consistent with this finding, ricinoleic acid effects were not affected by inhibition of cyclooxygenase (COX)-1 and COX-2 (Fig. S2C).

To further characterize the effects of ricinoleic acid on prostanoid receptors, we heterologously expressed prostanoid receptors together with the promiscuous G protein α-subunit Gα₁₅.

Author contributions: S.T. and S.O. designed research; S.T., T.F.A., and M.D. performed research; R.M.N. contributed new reagents/analytic tools; S.T., T.F.A., M.D., and S.O. analyzed data; and S.O. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1201627109/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1201627109

PNAS  |  June 5, 2012  |  vol. 109  |  no. 23  |  9179–9184
isomer ricinelaidic acid was inactive (Fig. S3A). Whereas ricinoleic acid was about one order-of-magnitude less potent than PGE2, the efficacy of ricinoleic acid to activate EP3 and EP4 receptors was comparable with that of PGE2 (Fig. 1E). Ricinoleic acid also activated murine EP3 and EP4 receptors (Fig. S3B).

None of the other prostanoid receptors, including IP, DP1, DP2, FP, and TP were activated by ricinoleic acid (Fig. 1F). In contrast to oleic acid, ricinoleic acid was able to displace 3H-PGE2 from EP3 receptors expressed in CHO cells with an IC50 of 500 nM, but ricinelaidic acid hardly competed with PGE2 for binding (Fig. 1G). Taken together, these data show that ricinoleic acid is a selective agonist of EP3 and EP4 receptors.

We then analyzed mice lacking EP3 or EP4 receptors (25–27) to test whether these receptors play a role in ricinoleic acid-induced pharmacological effects in vivo. Mice lacking either EP3 (Ptger3<sup>−/−</sup>; EP<sub>3</sub><sup>−/−</sup>) or EP4 (Ptger4<sup>−/−</sup>; EP<sub>4</sub><sup>−/−</sup>) showed normal intestinal transit time (Fig. 2A). When given castor oil, wild-type mice responded with a strong diarrhea, starting about 30 min after application. The laxative effect lasted for about 2 h. Interestingly, EP3 receptor-deficient mice were completely unresponsive, whereas mice lacking EP4 receptors responded like wild-type mice (Fig. 2B). Similarly, ricinoleic acid given orally also induced a strong laxative effect, which was abrogated in mice lacking the EP1 receptor (Fig. 2C). Mice lacking EP3 receptor were, however, indistinguishable from wild-type mice with regard to the effect of other laxatives, such as 5-hydroxytryptophan or polyethylene glycol (PEG3000) (Fig. 2D). Because ricinoleic acid has been reported to induce formation of PGE2 in the mammalian intestine (28), we tested the effect of COX inhibition on ricinoleic acid-induced diarrhea. Mice treated with COX-1 and COX-2 inhibitors responded normally to castor oil (Fig. 2E). Thus, ricinoleic acid-induced laxative effects are not a result of formation of prostanooids but are the result of a direct activation of EP3 receptors by ricinoleic acid.

To further analyze the role of EP3 receptors in the pharmacological effects of castor oil, we performed in vitro experiments on isolated small intestine. In flux measurements on intestinal mucosa using the Ussing chamber, we did not observe any effect of ricinoleic acid, but carbachol induced a strong secretory effect (Fig. 3A and B). However, myographic analysis of ileal segments of the small intestine from wild-type mice showed an increase in contractile activity in response to castor oil (Fig. 3C and D), an effect insensitive to inhibition of COX-1 and COX-2 (Fig. S4). Thus, myographic analysis of ileal segments, the small intestine of EP3<sup>−/−</sup> mice did not respond to ricinoleic acid (Fig. 3E and F) but showed a similar response as intestine from wild-type and EP<sub>3</sub><sup>−/−</sup> mice to the muscarinic receptor agonist carbachol (Fig. 3D, F, H, and I).

Expression of EP3 receptors has been reported in the intestine as well as in the uterus, the major sites of ricinoleic acid effects (29). In the mammalian intestine, EP3 receptors have been shown to be expressed in epithelial cells, enteric ganglia cells, immune cells, as well as in longitudinal but not circular smooth-muscle layers (22, 29–31). Using EP3 receptor-deficient mice that express β-galactosidase instead of EP3 (25), we could verify expression of EP3 in some epithelial cells, as well as in the longitudinal smooth-muscle layer of the intestine (Fig. S5). To test which cell type in the intestine mediates EP3 receptor-dependent laxative effects of ricinoleic acid, we mated mice carrying a conditional allele of the EP3 receptor (Ptger3<sup>fl/fl</sup>) (27) with mice expressing the recombinase Cre, either under the control of the villin promoter (32) (villin-Cre) or the smooth-muscle myosin heavy-chain promoter (SMMHC-CreERT<sub>2</sub>) (33) to specifically induce EP3 receptor-deficiency in epithelial cells or in smooth-muscle cells, respectively. The response to castor oil given orally was indistinguishable between wild-type, SMMHC-CreERT<sub>2</sub>-villin-Cre, and villin-Cre:Ptger3<sup>fl/fl</sup> mice (villin-Cre:EP<sub>3</sub><sup>fl/fl</sup>).
other stimuli, like 5-hydroxytryptophan and PEG3000, were not affected by smooth muscle-specific EP3 deficiency, indicating that these animals were responsive to diarrhea-inducing agents (Fig. 4B and Fig. S6). In addition, we verified absence of EP3 encoding mRNA in the intestinal smooth-muscle layer of smooth muscle-specific EP3 knockout (Fig. S7A), and we found no difference in basal intestinal function of smooth muscle-specific EP2 receptor-deficient mice compared with wild-type animals (Fig. S7B). As expected from the in vivo experiments, intestinal segments from smooth muscle-specific EP3 receptor-deficient mice were not contracted by ricinoleic acid but still responded to carbachol (Fig. 4 C–F). Thus, EP3 receptors on intestinal smooth-muscle cells mediate the laxative effects of ricinoleic acid released from castor oil.

Given the labor-inducing effects of castor oil and the expression of EP3 in the myometrium (7, 8, 34, 35) (Fig. S5C), we measured the effect of ricinoleic acid on contractility in nonpregnant and pregnant uteri. Whereas nonpregnant and pregnant wild-type uteri showed a strong increase in magnitude and frequency of contractions when exposed to ricinoleic acid (Fig. 5 A, E, F, J, and K), uteri from EP3 receptor-deficient mice did not respond at all (Fig. 5 C, E, H, J, and K). Both wild-type and EP3-deficient uteri from nonpregnant and pregnant mice contracted when exposed to carbachol (Fig. 5 B, D, E, G, I, J, and K).

**Discussion**

Castor oil, a natural triglyceride containing mainly ricinoleic acid, has a long history as a remedy because of its various biological effects, including an increase in propulsive intestinal motility. Based on the observation that ricinoleic acid, which is released from castor oil by intestinal lipases, induces calcium transients in various cells, and by using a siRNA screen against all nonolfactory GPCRs, we found that ricinoleic acid is a selective agonist of EP3 and EP4 receptors. Using mice with constitutive and conditional EP3 or EP4 receptor deficiency, we show that the pharmacological effects of castor oil are mediated by activation of EP3 receptors on smooth-muscle cells. This discovery provides the long-sought mechanism of action of one of the oldest drugs, which is still used in conventional, alternative, and folk medicine. The unexpected, highly specific mechanism of action may promote a reevaluation of the medical use of castor oil and suggests novel approaches to increase intestinal motility.

Consistent with a specific mechanism, the pharmacological activity of ricinoleic acid shows a strong dependency on the structure of the drug as the trans isomer ricinoleic acid, as well as the nonhydroxylated fatty acid oleic acid are without effect (7). This structure-activity relationship can also be found with regard to the ability of ricinoleic acid and related fatty acids to activate the EP3 and EP4 receptor. Although both PGE2 and ricinoleic acid are unsaturated and hydroxylated fatty acids, the ability of ricinoleic acid to act as an EP3/EP4 receptor agonist was not foreseeable with the currently available tools. Given the high specificity of ricinoleic acid for EP3 and EP4 receptors, it may be of interest to further explore the potential of hydroxylated fatty acids to specifically activate prostanoid receptors.

PGE2 and EP receptors have been implicated in the regulation of intestinal and uterine functions (30, 34). Both, pharmacological and molecular biology studies showed the presence of EP3 receptors in the pregnant uterus, and activation of EP3 receptors has been demonstrated to evoke contraction of uterine smooth muscle (35, 36). In fact, the PGE2 and PGE3 analogs misoprostol and sulprostone can be used to induce labor (37). In the small intestine, EP3 receptors are expressed in smooth-muscle cells, neurons, as well as in some epithelial cells. Although the procontractile effect of PGE2 on isolated intestinal smooth muscle involves EP3, EP4, and FP receptors (38), it has been unclear which receptor is responsible for the increase in peristalsis induced by PGE3 (39). Based on in vitro studies using other stimuli, like 5-hydroxytryptophan and PEG3000, were not affected by smooth muscle-specific EP3 deficiency, indicating that these animals were responsive to diarrhea-inducing agents (Fig. 4B and Fig. S6). In addition, we verified absence of EP3 encoding mRNA in the intestinal smooth-muscle layer of smooth muscle-specific EP3 knockout (Fig. S7A), and we found no difference in basal intestinal function of smooth muscle-specific EP2 receptor-deficient mice compared with wild-type animals (Fig. S7B). As expected from the in vivo experiments, intestinal segments from smooth muscle-specific EP3 receptor-deficient mice were not contracted by ricinoleic acid but still responded to carbachol (Fig. 4 C–F). Thus, EP3 receptors on intestinal smooth-muscle cells mediate the laxative effects of ricinoleic acid released from castor oil.

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cells endogenously or heterologously expressing prostanoid receptors, as well as on the use of inducible and tissue-specific EP3 receptor-deficient mice, our data clearly indicate that the EP3 receptor on intestinal smooth-muscle cells mediates the laxative effect of ricinoleic acid and, therefore, is a major prostanoid receptor in the intestine mediating propulsive effects on gut motility.

The fact that ricinoleic acid has been shown to release prostanoids from intestinal tissue under in vitro conditions (28) raises the question whether PGE2 acting on EP3 receptors contributes

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**Fig. 3.** Ricinoleic acid effects on the intestine in vitro. (A and B) Effect of 100 μM ricinoleic acid (RA) or 100 μM carbachol on short-circuit current (Isc) in the ileum (A) or the distal colon (B) of wild-type mice. (C–F) Effect of 100 μM ricinoleic acid (C, E, G, I) or 100 μM carbachol (D, F, H, I) on the contractile activity of a segment of the ileum from wild-type (C, D, I), EP3−/− (E, F, I), or EP3−/− mice (G, H, I). Panel I shows the average tension during 5 min after addition of vehicle (c), ricinoleic acid (RA), or carbachol (C) to the ileum. Shown are mean values ± SEM. *P ≤ 0.05, **P ≤ 0.01.

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**Fig. 4.** Effects of castor oil on the intestine are mediated by EP3 expressed by smooth-muscle cells. (A and B) Effect of 8 μL/g body weight castor oil (A) or 1 μg/g body weight 5-hydroxytryptophan intraperitoneally (B) on feces excretion in wild-type mice (WT), mice with smooth muscle-specific EP3 receptor deficiency (SMMHC-CreERT2;EP3flox/flox), mice with intestinal epithelial cell-specific EP3 receptor deficiency (villin-Cre;EP3flox/flox), or mice expressing Cre in smooth muscles or intestinal epithelial cells (SMMHC-CreERT2;EP3flox/flox and villin-Cre;EP3flox/flox, respectively). Shown is the excretion of feces during the indicated periods before and after application of the diarrhea-inducing agents. Shown are mean values ± SEM, n ≥ 6. **P ≤ 0.01 (compared with time period −1). (C–F) Effect of 100 μM ricinoleic acid (C and E) or 100 μM carbachol (D and F) on the contractile activity of segments of the ileum prepared from mice expressing the Cre recombinase in smooth muscle cells (SMMHC-CreERT2;EP3flox/flox) (C and D) or mice with smooth muscle-specific EP3 receptor deficiency (SMMHC-CreERT2;EP3flox/flox) (E and F). Arrows indicate time points of stimuli application. All animals carrying the SMMHC-CreER transgene had been induced by treatment with tamoxifen.
In animals with blocked COX-1 and COX-2 activity, castor oil- and ricinoleic acid-induced effects were not affected. Thus, PGE\(_2\), which may be formed in response to ricinoleic acid, is not involved in the pharmacological effects of castor oil-derived ricinoleic acid. In animals with blocked COX-1 and COX-2 activity, castor oil-induced laxation was not affected, showing the number of contractions/10 minutes of ricinoleic acid (RA), or carbachol (C) to uteri. Shown are mean values ± SEM. **P < 0.01; n.s., not significant [compared with respective control (C)].

**Methods.** Ricinoleic acid, ricinelaic acid, oleic acid, carbachol, 5-hydroxytryptophan, PEG3000, and pertussis toxin were from Sigma-Aldrich. PGE\(_2\), PGO\(_2\), N5398, L161,982, cilostazol, prostanycin and U46619 were from Cayman Chemical. FR122047 and L-798,106 were from Tocris. Fura-2/AM was from Invitrogen.

**Cell Transfection and Determination of [Ca\(^{2+}\)].** MEG-01 cells were seeded on a 96-well plate and transfected with indicated cDNAs or control DNA (50 ng/well) using FuGENE6 reagent (Roche Diagnostics), as previously described (42). Forty-five minutes after transfection, cells were loaded with 5 \( \mu \)M coelenterazine h (Invitrogen) in calcium-free HBSS containing 10 mM HEPES, pH 7.4, for 1 h at 37 °C. Cells were washed in HBSS containing 1.8 mM Ca\(^{2+}\) and were then transferred to an automated fluorometer plate reader (Flexstation-3; Molecular Devices). After ligand stimulation, calcium transients were recorded as relative fluorescence units (RFU) for 2 min, and the integrated area under the calcium transients was quantified by using SoftMaxPro (Molecular Devices) and expressed as area under the curve (AUC).

For studies of heterologously expressed receptors, CHO-K1 cells stably expressing a calcium-sensitive bioluminescent fusion protein consisting of aequorin and green fluorescent protein (24) were seeded in 96-well plates and transfected with indicated cDNAs or control DNA (50 ng/well) using FuGENE6 reagent (Roche Diagnostics), as previously described (42). Two days after transfection, cells were loaded with 5 \( \mu \)M coelenterazine h (Invitrogen) in calcium-free HBSS containing 10 mM HEPES, pH 7.4, for 3.5 h at 37 °C. Forty-five minutes before experiments, the buffer was replaced with HBSS containing 1.8 mM Ca\(^{2+}\). Measurements were performed by using a luminometer plate reader (Luminoskan Ascent; Thermo Electron). The area under each calcium transient (measured from maximum calcium release to the baseline) was calculated using Ascent software (Thermo Electron) and expressed as AUC.

**siRNA Screening.** Four separate siRNAs of a siRNA library directed against 514 genes including 407 nonolfactory human GPCRs and 86 olfactory human GPCRs (Qiagen) targeting the same mRNA were pooled. MEG-01 cells were reverse-transfected with siRNA pools at a final concentration of 5 \( \mu \)M for each siRNA. Seventy-two hours later, cells were loaded with Fura-2/AM and ricinoleic acid-induced calcium transients were recorded and analyzed as described above. Ratios of AUC of ricinoleic acid induced calcium transients in cells transfected with siRNA pools targeting a particular GPCR and AUC of effects in cells transfected with scrambled siRNA were determined.

**RT-PCR.** RNA was isolated from MEG-01 cells with the Neasy Mini Kit (Qiagen). For reverse-transcription reaction, 1 \( \mu \)g total RNA was reverse-transcribed. cDNA synthesis was monitored by PCR of a 401-bp fragment of glyceraldehyde-3-phosphate dehydrogenase.

**Genetic Mouse Models.** EP3- and EP2-deficient mice have been described previously (25, 26) and were kept in the animal house of the Institute for Clinical Pharmacology of the J. W. Goethe University, Frankfurt. Ptger2lox/lox (Jackson Laboratories) were used for the conditional deletion experiment and also служит как источник библиотеки генов для глобального EP2-рецептора и EP2-рецептора. Мice inducible SMMHC-CreERT2 (Jackson Laboratories) were used for the conditional deletion experiment and also служит как источник библиотеки генов для глобального EP2-рецептора и EP2-рецептора.
were performed at least 7 d after the last injections. All animal experiments were approved by the Regierungspräsidium Karlsruhe and Darmstadt.

**Determination of Laxative Effects.** To measure the laxative effects of different substances, mice were starved 16 h before the experiments. On the day of the experiment, mice were treated with the indicated substances, and the bottom of the cages was covered with white tissue paper. The weight of shapeless and watery stools was determined hourly.

**Determination of Intestinal Transit Time.** Total intestinal transit time was measured as described previously (46). Briefly, mice were given carmine red solution orally (150 μl tap water containing 3 mg carmine red). Mice were returned to individual cages covered with white paper. The time taken until the excretion of red feces was measured.

For isometric tension recordings, 1-cm segments of μ receptor cDNA using Fugene 6 (Roche) transfection 7H mmol glucose. The solution was gassed with μ fl fl Formation. (i.e., the concentrations × is expressed as × and 95% (vol/vol) O (PGE μ 1 o f6 specific ME-01 cells were treated as μ receptor, and indicated concentrations receptor in binding buffer containing μ RNA was extracted from the μ The Ussing chamber experiments μ = μ were carried out in a bathing solution containing: 107 NaCl mmol fl L −1,4.5 KCl mmol L −1, 25 NaHCO₃ mmol L −1, 1.8 Na₂HPO₄ mmol L −1, 0.2 NaH₂PO₄ mmol L −1, 1.25 CaCl₂ mmol L −1, 1 MgSO₄ mmol L −1, 12.2 mmol L −1, 2·0·2N a H 1−1,4·5K C lm m o l 3−1,1·2N aH CO 2·1−1. The solution was gassed with carbogen [5% (vol/vol) CO₂ and 95% (vol/vol) O₂] at 37 °C and pH 7.4 (adjusted by NaHCO₃/HCl). For the experiments with colon, the serosa and muscularis propria were stripped away by hand to obtain a mucosa-submucosa preparation from the distal or proximal colon. All other intestinal segments were mounted as intact intestinal wall in the Ussing chamber. The tissue was fixed in a modified Ussing chamber bathed with a volume of 3.5 mL on each side of the mucosa. The tissue was incubated at 37 °C and short-circuited by a computer-controlled voltage-clamp device (Ingenieur Büro für Mess- und Datentechnik Müssler) with correction for solution resistance. Tissue conductance (Gt) was measured every minute by the voltage deviation induced by a current pulse (± 50 μA, duration 200 ms) under open-circuit conditions. Short-circuit current (Isc) was continuously recorded on a chart-recorder. Isc is expressed as μEq h −1 cm −2 (i.e., the flux of a monovalent ion per time and area, with 1 μEq h −1 cm −2 = 26.9 μA cm −2).

Radioligand Binding Assay. To measure the equilibrium binding of [5,6,8,11,12,14,15-3H(N)] prostaglandin E₂ (PGE₂) (180 Ci/mM; Perkin-Elmer), CHO-K1 were seeded in 24-well plates. Twenty-four hours later, they were transfected with a plasmid containing human EP₃ receptor cDNA using Fugene 6 (Roche) transfection reagent, according to the manufacturer’s instructions. Two days after the transfection, cells were rinsed once with ice-cold binding buffer (PBS + 0.5% fatty-acid free BSA) and competition binding assays were carried out by incubating CHO-K1 cells expressing human EP₃ receptor in binding buffer containing a concentration of 5 nM [3H]PGE₂ and indicated concentrations of unlabeled substances for 90 min at 4 °C. Binding was stopped by three washing steps with ice-cold binding buffer. Thereafter, cells were lysed in lysis buffer (0.1% Triton X-100, 2 N NaOH) and transferred to vials containing scintillation fluid (Ultima-Gold; Perkin-Elmer). Radioactivity was measured by a scintillation counter (Hidex 300SL).

RT-PCR and Quantitative PCR. RNA was extracted from the megakaryocyte leukemia cell line MEG-01 or the indicated organs with the RNasy Mini Kit (Qiagen). One microgram total RNA was reverse transcribed. cDNA synthesis was verified by PCR of a 401-bp fragment of glyceraldehyde-3-phosphate dehydrogenase. To quantify the expression of mouse EP₃ receptor, real-time PCRs were performed using LightCycler 480 II (Roche) on 100 ng cDNA from different organs by using EP₃ specific primers which also matched a specific fluorescent probe (Universal Probe Library, Roche). Mouse genomic DNA was used with the same set of primers and probes as a standard.

Determination of PGE₂ Formation. MEG-01 cells were treated as indicated. The supernatant was collected, and PGE₂ concentrations were determined with an ELISA kit according to the manufacturer’s instructions (Cayman Chemical). Total protein content of the cell was determined using the Bradford assay, and prostanoid concentrations per mg cellular protein were determined.
Fig. S1. Expression of EP3 and EP4 and effects of PGE2 in MEG-01 cells. (A) RT-PCR of cDNA from MEG-01 cells using EP3, EP2, EP3, EP4, or GAPDH-specific primers. (B) Effect of PGE2 at increasing concentrations on [Ca^{2+}] in MEG-01 cells. AUC, area under the curve.
Fig. S2. (A) Effect of buffer, ricinoleic acid (30 μM) and arachidonic acid (30 μM) given for the indicated time periods on the formation of PGE₂ in MEG-01 cells. Shown are mean values ± SEM, n ≥ 3. (B) Effect of cyclooxygenase (COX)-1 and COX-2 inhibitors NS398 and FR122047 on ricinoleic acid and arachidonic acid induced PGE₂ formation in MEG-01 cells. COX-1/2 inhibitors were given 30 min before ricinoleic acid and arachidonic acid. Shown are mean values ± SEM, n ≥ 3. (C) Effect of the COX-1 and COX-2 inhibitors NS398 and FR122047 on ricinoleic acid-induced increases in [Ca^{2+}]_i in MEG-01 cells. RFU, relative fluorescence units.
Fig. S3. Effect of ricinelaidic acid on human EP receptors heterologously expressed in CHO cells, and effect of ricinoleic acid on mouse EP3 and EP4 receptors. (A) Effect of 100 μM of ricinoleic acid or ricinelaidic acid on [Ca²⁺], in CHO-K1 cells expressing human EP3 or EP4 together with a promiscuous G protein α-subunit. (B) Effect of increasing concentrations of ricinoleic acid on [Ca²⁺], in CHO-K1 cells transfected with cDNAs encoding the murine EP4 receptor and two isoforms of the EP3 receptor (EP3-1/EP3a, EP3-2/EP3b) (1, 2) or transfected with an empty vector (mock) together with a Ca²⁺-sensitive bioluminescent fusion protein and a promiscuous G protein α-subunit G15. AUC, area under the curve. Shown are mean values ± SEM, n ≥ 6.

Fig. S4. Effect of COX-1/COX-2 inhibitors on ricinoleic acid-induced contraction of ileal segments of the small intestine. (A–C) Ileal segments of wild-type mice were pretreated with vehicle (control) or with 10 μM NS398 and 1 μM FR122047. Thereafter, ricinoleic acid (RA, 100 μM) was added and tension was recorded as described. Arrows indicate the time point of ricinoleic acid application. Shown are mean values ± SEM, n ≥ 3; *P ≤ 0.05.

Fig. S5. Expression of EP<sub>3</sub> receptors in intestine/uterus. EP<sub>3</sub>−/− animals were killed, the indicated organs were sectioned and stained for β-galactosidase activity, and counterstained with H&E. Shown are sections from the ileum (A and B) and colon (C and D) with mucosa and submucosa (A and C) as well as with the muscularis externa (B and D). The strongest staining was seen in the longitudinal layer of the muscularis externa. (E and F) Nonpregnant and pregnant mouse uterus showing strongest staining in the circular layer of the myometrium. [Scale bars, 50 μm (A and C), 25 μm (B and D), and 100 μm (E and F).]
Fig. S6. Effect of PEG3000 on feces formation in wild-type, SMMHC-Cre;EP<sub>3</sub><sup>+/+</sup> and SMMHC-Cre;EP<sub>3</sub><sup>fl/fl</sup> mice. Shown is the formation of stool 1 h before and at the indicated time periods (h) after oral application of PEG3000. Shown are mean values ± SEM, n ≥ 3.

Fig. S7. (A) Copy number of the cDNA encoding EP<sub>3</sub> receptor after reverse transcription of mRNA samples prepared from the ileum (Left) and kidney (Right) of wild-type, SMMHC-Cre;EP<sub>3</sub><sup>+/+</sup> or SMMHC-Cre;EP<sub>3</sub><sup>fl/fl</sup> mice. (B) Food intake per day, feces formation per day, and intestinal transit time in wild-type, SMMHC-Cre;EP<sub>3</sub><sup>+/+</sup> or SMMHC-Cre;EP<sub>3</sub><sup>fl/fl</sup> mice.