A Novel Approach for the Control of Inflammatory Pain: Prostaglandin E2 Complexation by Randomly Methylated β-Cyclodextrins

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BACKGROUND: Inhibitors of cyclooxygenase, which block the formation of prostaglandin (PG) E2, are the standard treatment of inflammatory pain. These drugs, however, have serious gastrointestinal, renal, and cardiovascular side effects that limit their clinical use. Cyclodextrins are neutral glucose oligomers that form a hydrophilic outer and a hydrophobic interior cavity used to carry hydrophilic substances. Methyl-β-cyclodextrins are used currently in several drugs as enhancers and also to deliver PGs. We therefore hypothesized that randomly methylated β-cyclodextrins (RAMEB) could be used for pain treatment.

METHODS: An in silico screening for important inflammatory mediators (eg, PGE2, substance P, bradykinin, and calcitonin gene-related peptide) was performed to predict the probability of these molecules binding to RAMEB. Thereafter, a comprehensive in vitro study investigated the complexation affinity of the best target toward RAMEB or its RAMEB-fraction L (FL) using capillary electrophoresis.

Wistar rats were injected intraplantarly with complete Freund's adjuvant (CFA) for 96 hours to induce inflammatory hyperalgesia. Subsequently, rats were treated intraplantarly or intravenously either with RAMEB or RAMEB FL and compared with the respective controls. Parecoxib was used as positive control. Mechanical (paw pressure threshold, PPT) and thermal (paw withdrawal latency) nociceptive thresholds were determined before injection and at the indicated time points thereafter. Paw tissue was collected after treatments, and PGE2 and PGD2 contents were measured. Analysis of variance was used for data analysis followed by appropriate post hoc comparisons.

RESULTS: In silico screening indicated that PGE2, with the highest affinity, was the best candidate for RAMEB binding. Likewise, in capillary electrophoresis experiments, RAMEB had a high affinity to form inclusion complexes with the PGE2 (stability constant [*K*], 360 1/M; 95% confidence interval [C]: 347.58–372.42 M^{-1}). Local treatment with RAMEB alleviated CFA-induced mechanical (PPT: 76.25 g; 95% CI: 56.24–96.25 g) and thermal hyperalgesia (PPT: 8.50 seconds; 95% CI: 6.76–10.23 seconds). Moreover, a systemic administration of RAMEB decreased CFA-induced mechanical (PPT: 126.66 g; 95% CI: 114.54–138.77 g) and thermal hyperalgesia (paw withdrawal latency: 11.47 seconds; 95% CI: 9.26–13.68 seconds). RAMEB FL resulted in greater in vitro PGE2-binding capacity and decreased PG content as well as hyperalgesia in vivo to a similar extent. Motor activity of the rats was not altered by RAMEB or RAMEB FL.

CONCLUSIONS: Capture of PGs by cyclodextrins could be a novel and innovative tool for the treatment of inflammatory pain and bypassing some unwanted side effects of cyclooxygenase inhibitors. (Anesth Analg 2016;XXX:00–00)

Inflammatory pain is elicited by proalgesic mediators, for example, proinflammatory cytokines, prostaglandins (PG), and bradykinin (BRK).[1] PGs are a group of lipid compounds that are derived enzymatically from fatty acids.

In the cell, arachidonic acid is transformed enzymatically by cyclooxygenase (COX) into PGH2. Subsequently, PGH2 is converted by microsomal PGE synthase-1 into PGE2. PGE2 is one of the most important mediators in inflammatory pain. Inhibition of COX by nonsteroidal anti-inflammatory drugs or by selective COX-2 inhibitors suppresses the formation of PG and attenuates inflammatory pain; however, their therapeutic benefit is limited by gastrointestinal (eg, ulceration), renal (ie, renal failure), and cardiovascular (eg, cardiac insufficiency or myocardial infarction) side effects as well as impairment of the coagulation system (ie, postoperative bleeding).[2] PGE2 sensitizes primary afferent nociceptors via binding to prostanoid receptors (EP2 and EP4) in the peripheral as well as in the central nervous system.[3] Pronociceptive effects of PGE2 are mediated via adenyl cyclase-cAMP-protein kinase A and include sensitization of transient receptor potential V1 (TRPV1), purinergic P2X3 receptors, or voltage-gated calcium or sodium channels in nociceptive neurons.

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Intraplantar injection of heat-inactivated *Mycobacterium butyricum* in oily solution (complete Freund's adjuvant, CFA) elicits inflammatory hyperalgesia, migration of leukocytes into the tissue, and increased spontaneous activity of nociceptive Aδ and C nerve fibers.[4,5] COX-2 is expressed in the inflamed tissue by activated leukocytes as well as in neurons. In this context, monocytes/macrophages seem to be very important because predominant recruitment of monocytes via the chemokine CCL2 provokes hyperalgesia dependent on the formation of PGs via COX-2.[6]

Cyclodextrins are neutral glucose oligomers of truncated cone shape or toroids. They possess a hydrophilic exterior surface and a hydrophobic interior cavity, enabling them to form host–guest inclusion complexes with a wide range of pharmaceuticals in aqueous solution.[7] The drug carrier family of cyclic oligosaccharides is composed of 5 or more α-D-glucopyranoside units linked 1–4, as in amylose. Typical cyclodextrins contain 6, 7, or 8 glucose units and are denoted α-, β-, and γ-cyclodextrins, respectively. A great number of chemically modified cyclodextrins have been prepared to enhance the inclusion capacity and the physicochemical properties. The driving forces to form inclusion complexes are electrostatic, van der Waals, and hydrophobic interactions as well as hydrogen bonding.[8] β-cyclodextrins can form molecular complexes with numerous compounds, including PGEs.[9] The Food and Drug Administration generally recognizes cyclodextrins as safe when used to increase bioavailability of drugs.

Here, we screened different proalgesic molecules in silico for the complexation with randomly methylated β-cyclodextrin (RAMEB) and found that PGE2 binds RAMEB with greater affinity than other molecules. We therefore hypothesized that cyclodextrins capture PGE2 at the site of inflammation and elicit antinociception with similar efficacy as COX inhibitors. Antinociception by COX-2 inhibitor (parecoxib) was compared with RAMEB via local or systemic delivery. Treatment was investigated in PGE2-induced as well as local inflammatory (ie, CFA-induced) hyperalgesia in Wistar rats. Specifically, RAMEB fractions with increased efficacy of PGE2 binding were produced and analyzed for in vivo capture of PGE2 at the site of inflammation as well as for antinociceptive effects to mechanical and thermal stimuli.

MATERIAL AND METHODS

Reagents

PGE2 applied during the experiments was obtained from Sanofi-Aventis, Frankfurt, Germany. Cyclodextrins used in the experiments were products of Cyclolab Ltd., Budapest, Hungary, or purchased from other suppliers as follows:

- RAMEB: commercial randomly methylated β-cyclodextrin (Cawasol WP 1,8 by Wacker Chemie AG, Munich, Germany) average degree of substitution (DS): 12.5.
- RAMEB FL/FM/FH: specific fractions of commercial randomly methylated β-cyclodextrin (Cawasol WP 1,8 by Wacker Chemie AG) enriched in RAMEB cyclodextrins of relatively lower (DS~10.5), medium (DS~12), and higher DS (DS~13; prepared by CycloLab), respectively.

Reagents and solvents used during sample and buffer preparation were purchased from commercial suppliers and used without further purification.

In Silico Screening Analysis

The 2-dimensional/3-dimensional chemical structures for the PGE2 (CID: 5280360) and BRK (CID: 439201) molecules were obtained from the PubChem database and prepared for molecular docking. The 3-dimensional chemical structures for the nerve growth factor (NGF, PDB ID: 4XPJ) and substance P (SP, PDB ID: 4HOM) structures were downloaded from the Protein Data Bank.[10,11] The homology model of calcitonin gene-related peptide (CGRP) was generated with the SWISS-MODEL web server.[12] The β-CD structure (ID: 1JL8) was obtained from the Protein Data Bank[13] and used as a template for the construction of RAMEB. The rigid-flexible molecular docking was performed by the Molecular Operating Environment docking algorithm.[14] The London-free energy of binding (ΔG_{bind}) was used as a scoring function to identify more favorable docked poses and correctly estimate the binding affinity of the hyperalgesic substances to RAMEB. The ΔG_{bind} value was calculated according to the following equation:

$$\Delta G_{bind} = c + E_{flex} + \sum_{h-bonds} C_{HB} f_{HB} + \sum_{atoms(i)} \Delta D_i,$$

where c represents the average gain/loss of rotational and translational entropy; E_{flex} is the energy because of the loss of flexibility of the peptide calculated from its topology; C_{HB} is the energy of an ideal hydrogen bond f_{HB} measures geometric imperfections of hydrogen bonds; and D_i is the desolvation energy of atom i. The ΔG_{bind} value was set to −10 kcal/mol as a threshold to discriminate the RAMEB binders from the RAMEB nonbinders.[14]

Capillary Electrophoresis

The crucial requirement of application of capillary electrophoresis in binding analysis is that at least one of the interacting species has to carry a charge. In our experiments (at pH 6.1), PGE2 was negative, whereas all the cyclodextrin derivatives were neutral.

Capillary electrophoresis was used to investigate the complexation affinity of PGE2 toward a variety of methylated cyclodextrin derivatives differing in number of glucose units and substitution pattern of the cyclodextrin scaffold. Capillary electrophoresis experiments were performed on a 7100 Capillary Electrophoresis instrument (Agilent Technologies, Waldbronn, Germany), equipped with a photodiode array detector and the ChemStation software for data handling. Complexation behavior was investigated in uncoated fused silica capillaries of 25/33.5 or 50/58.5 cm (effective/total length) at 25°C using direct ultraviolet detection at 200 nm. Along with the ultraviolet traces, the current and the voltage were monitored. Samples were run in triplicate; they were injected at 50 mbar for 4 seconds and +30 kV voltage was applied. 30 mM phosphate buffer was used (pH set to 6.1 with solid TRIS) as background electrolyte after preliminary optimization. In pilot experiment, each cyclodextrin was added at various concentrations (0-5-10-15 mM) to prepare the electrophoresis

buffer solutions. Capillaries were rinsed with the following sequence between runs: 1 minute water, 1 minute 0.1 N NaOH, 1 minute water, and 4 minutes background electrolyte. Conditioning of new capillaries was conducted by flushing with 1 M NaOH for 30 minutes followed by 0.1 M NaOH and buffer for 10 minutes each.

PGE2 stock solution was prepared by weighing approximately 1 mg of solid PGE2 and dissolved in the 1:1 mixture of ethanol and MQ grade water to obtain a solution of 1 mg/mL. The PGE2 stock solution was kept in a freezer. To obtain PGE2 sample solution, PGE2 stock solution was diluted 20-fold with MQ grade water and spiked with 0.1 v/v% DMSO. This served as an electroosmotic flow (EOF) marker.

Determination of the Complex Stability Constants

The effective electrophoretic mobility, μ_{eff}, of a charged molecule is a function of the charge-to-size ratio and the viscosity of the electrophoresis media. In practical terms, the effective electrophoretic mobility can be obtained from the following equation:

$$\mu_{eff} = \frac{l_c l_d}{U}\left(\frac{1}{t} - \frac{1}{t_0}\right),$$

where l_c is the total length of the capillary, l_d is the length of the capillary to the detector, U is the applied voltage, and t and t_0 are the peak appearance times of the analyte and the EOF, respectively.[15] The presence of additives such as cyclodextrins in the electrophoresis buffer changes the viscosity of the media, which, in turn, will affect the effective electrophoretic mobility. The effective mobility of the guest is influenced by the concentration of cyclodextrin, as shown in the following equation:

$$\mu_{eff} = \frac{\mu_{free} + \mu_{cplx} K[CD]}{1 + K[CD]},$$

where μ_{eff} is the ligand effective mobility at the actual cyclodextrin concentration, and μ_{free} and μ_{cplx} are the effective mobility of the free and complexed ligand. Based on the equation, the stability constants were determined with the x-reciprocal method[16]:

$$\frac{\mu_{eff} - \mu_{free}}{[CD]} = -K\left(\mu_{eff} - \mu_{free}\right) + K\left(\mu_{cplx} - \mu_{free}\right)$$

Animals and Treatment

Animal protocols were approved by the local animal care committees (Regierung von Unterfranken, Würzburg, Germany) and are in accordance with the International Association for the Study of Pain.[17] Male Wistar rats weighing 180 to 220 g were treated as described herein under brief isoflurane anesthesia. At the end of the experiment, animals were sacrificed by the intracardial injection of a solution of T61 (embutramide, mebezonium, and tetracaine) under isoflurane anesthesia according to national guidelines.

Inflammatory hyperalgesia was induced by intraplantar injection of 150 µL CFA (Calbiochem/Merck, Darmstadt, Germany) in the right hindpaw.[18,19] RAMEB or RAMEB fractions were either injected intraplantarly or intravenously (IV) under isoflurane anesthesia. In a pilot experiment, the most effective dose was determined. For intraplantar and IV injection, 4 mg and 20 mg were used per rat, respectively. As a standard treatment for the relief of hyperalgesia, parecoxib, a COX-2 inhibitor, was given in doses established previously.[6,20] For intraplantar injection, 0.2 to 1 mg/rat parecoxib and for IV injection, 5 mg/rat diluted in 0.9% saline were used.

Nociceptive Thresholds

Mechanical nociceptive thresholds were determined with the paw pressure algesiometer (modified Randall–Selitto test; Ugo Basile, Comerio, Italy) as described previously.[21] Pressure was applied with a blunt piston onto the dorsal surface of the hindpaw. The paw pressure threshold (PPT; weight, g) was defined as the pressure required to elicit paw withdrawal. The average of 3 measurements was calculated. Treatments were randomized and blinded. A decrease in the PPT by 20 g was interpreted as hyperalgesia, whereas an increase in the PPT by 20 g was interpreted as antinociception.

Thermal nociceptive thresholds were measured by the modified Hargreaves test as previously described.[21] An electronic timer measured the latency (time, second) required to elicit paw withdrawal (paw withdrawal latency [PWL]; IITC Inc./Life Science, Woodland Hills, CA) after application of radiant heat to the plantar surface of a hindpaw from underneath the glass floor using a high-intensity light bulb. The stimulus intensity was adjusted to 20 seconds for the PWL in noninflamed paws, and a cutoff of 30 seconds was set to avoid tissue damage. The average of 2 measurements taken with 20-second intervals was calculated. A decrease in PWL by 2 seconds was interpreted as hyperalgesia, whereas an increase by 2 seconds in PWL was interpreted as antinociception.

Enzyme-Linked Immunosorbent Essay (ELISA)

For PG analysis, subcutaneous paw tissue was obtained and minced in cold lysis buffer as described previously.[21,22] Homogenates were frozen at −80°C, and before ELISA, they were thawed overnight at 4°C. For PGD2 measurements, the samples were treated with methoxyamine (MOX) hydrochloride, which converts PGD2 into PGD2-MOX, preventing its further chemical degradation. After centrifugation, supernatants were harvested, and PGE2 and PGD2-MOX were measured by commercially available kits according to the manufacturers' instructions (PGE2 ELISA kit: R&D Systems, Minneapolis, MN; PGD2-MOX, Cayman Chemical Company, Ann Arbor, MI).

RotaRod

Wistar rats were placed on a RotaRod treadmill (RotaRod; Ugo Basile, Varese, Italy) rotating at accelerating mode from 5 to 50 rounds per minute over a cutoff time of 999 seconds. Rats underwent 3 training trials before testing. Performance time in seconds was recorded until the rat failed to stay on the RotaRod. The maximum performance time of 5

consecutive measurements was analyzed per session, with an intertrial interval of 5 minutes. Data were collected at the best defined time point obtained by PPT after compound administration. Bupivacaine (500 μL 0.5%) was peristaltically injected as a positive control.

Statistical Analysis

Data are expressed as mean ± SEM. In our experiments, results from several treatment groups, which received different doses of a drug, were compared with each other with the null hypothesis that there are no differences among the groups. To compare the different treatments, we used a 1-way analysis of variance (ANOVA) with Bonferroni correction as a post hoc measure. The interactions between the treatments and the time points were evaluated with the 2-way ANOVA with Bonferroni correction as a post hoc. When animals were exposed to repeated measurements, then repeated-measures ANOVA was performed. In our experiments, rats were examined at up to 7 time points and 3 to 4 doses of drugs (ie, before and 5 to 6 time points after treatment with the null hypothesis that there are no changes in PPT or PWL over time, ie, time, dose, and interaction do not affect treatment). Differences were considered significant if $P < .05$. In case of not normally distributed data, the test was performed on ranks. All analyses were conducted with the Statistical Package Sigma Stat version 12.5 (Systat Software Inc, San Jose, CA).

RESULTS

Screening of Proalgesic Mediators and Binding Affinity for RAMEB

To identify hyperalgesic proteins/peptides and lipids that bind to RAMEB, a virtual screening was performed that included BRK, SP, NGF, CGRP, and PGE2. We performed the rigid-flexible molecular docking using the CGRP and NGF molecules with high molecular weight as receptors and RAMEB as a ligand. On the other hand, the PGE2, BRK, and SP were used as ligands for RAMEB because of their low molecular weight. Figure 1A shows that PGE2 was predicted to bind with a minimal ΔG_{bind} value (ΔG_{bind}= −12.57 kcal/mol), indicating the greatest binding affinity to the RAMEB cavity. SP, NGF, and CGRP were predicted

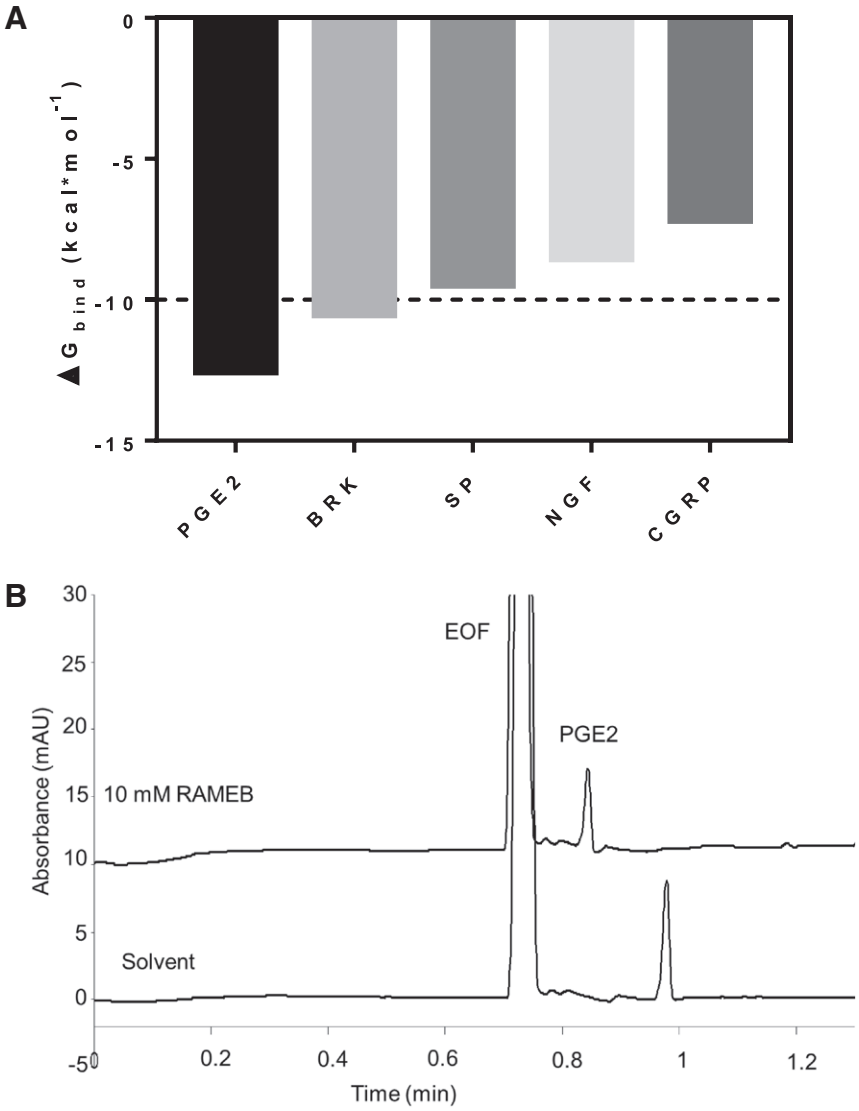

Figure 1. Predicted binding affinities and formation of stable complex of prostaglandin (PG)E2 to randomly methylated β-cyclodextrin (RAMEB) in vitro. A, Molecular docking of substance P (SP), calcitonin gene-related peptide (CGRP), bradykinin (BRK), or PGE2 with RAMEB was performed with the Molecular Operating Environment docking algorithm, and the London-free energy of binding was used as a scoring function. The threshold (= −10 kcal/mol) is depicted with a dashed line. B, The electrophoretic mobility of PGE2 was determined in the presence of RAMEB or solvent control. Complexation induced a significant shift of the electroosmotic flow (EOF).

as not binding RAMEB very well, with the exception of BRK (ΔG_{bind}= −10.54 kcal/mol) binding with lower affinity.

On the basis of these screening data and the literature, we proposed that PGE2 could be the target for complexation by β-cyclodextrins.[9] In the capillary electrophoresis, increasing the concentrations of RAMEB resulted in the shortening of migration times for the PGE2 peaks because of the complexation. Similarly, complexation yielded increasing migration time of the EOF marker because of the increasing viscosity of the buffer. Representative traces of migration times of PGE2 in the presence or absence of 10 mM RAMEB are shown (Figure 1B).

Reversal of PGE2-Induced Hyperalgesia

To study the pharmacologic potential of RAMEB as an analgesic agent in vivo, we established a model of PGE2-induced mechanical hyperalgesia. Intraplantar injection of PGE2 dose-dependently evoked hyperalgesia with a peak at 60 minutes (PPT: 56.66 g; 95% confidence interval [CI]: 55.10–58.22 g) and lasting up to 180 minutes (Figure 2A). No change of nociceptive thresholds was observed in the contralateral paw supporting the locally restricted action. On the basis of these results, we used 200 ng PGE2 in subsequent experiments. A similar time course was seen for thermal hyperalgesia (PWL: 6.93 seconds; 95% CI: 6.83–7.03 seconds). PGE2 significantly reduced the thermal nociceptive thresholds longer up to 240 minutes again with maximal hyperalgesia after 60 minutes (Figure 2B). Next, we tested the antinociceptive effect of β-cyclodextrins in PGE2-induced hyperalgesia. Optimal doses of locally and systemically injected RAMEB were established in previous dose-finding experiments (data not shown; Figure 4). Local and systemic RAMEB application completely reversed mechanical (PPT: 70.27 g; 95% CI: 63.50–77.04 g and PPT: 73.05 g; 95% CI: 60.23–85.88 g, respectively) and thermal hyperalgesia (PWL: 10.04 seconds; 95% CI: 9.17–11.64 seconds and PWL: 11.11 seconds; 95% CI: 9.75– 12.46 seconds, respectively; Figure 2C–F). Nociceptive thresholds in the contralateral paw were not significantly changed even in rats treated systemically with RAMEB. In conclusion, RAMEB can antagonize the pronociceptive effects of PGE2 in vivo and reverse hyperalgesia.

Antinociception by a Systemically or Locally Injected COX-2 Inhibitor in Inflammation

Inflammatory hyperalgesia was evoked in Wistar rats by intraplantar injection of CFA. At 4 days of inflammation, the antinociceptive effect of a standard COX-2 inhibitor, parecoxib, was determined after IV injection (using doses established by others[6]) or as a dose–response experiment after intraplantar injection. Intraplantarly injected parecoxib dose-dependently reversed mechanical as well as thermal hyperalgesia in rats with 4 days of CFA inflammation with a maximal effect between 30 and 60 minutes (PPT: 82.08 g; 95% CI: 63.15–101.00 g and PWL: 14.35 seconds; 95% CI: 12.89–15.82 seconds; Figure 3A, B). In line with previous studies, the IV injected COX-2 inhibitor reversed both mechanical and thermal hyperalgesia (PPT: 145 g; 95% CI: 134.34–155.65 g and PWL: 13.88 seconds; 95% CI 10.77–16.99 seconds; Figure 3C, D).[20,23] No significant changes in nociceptive thresholds were observed in the contralateral non-inflamed paw (PPT: 74.6 ± 2.0 g, PWL 13.1 ± 0.7 seconds). Reversal of mechanical and thermal hyperalgesia lasted up to 2 hours.

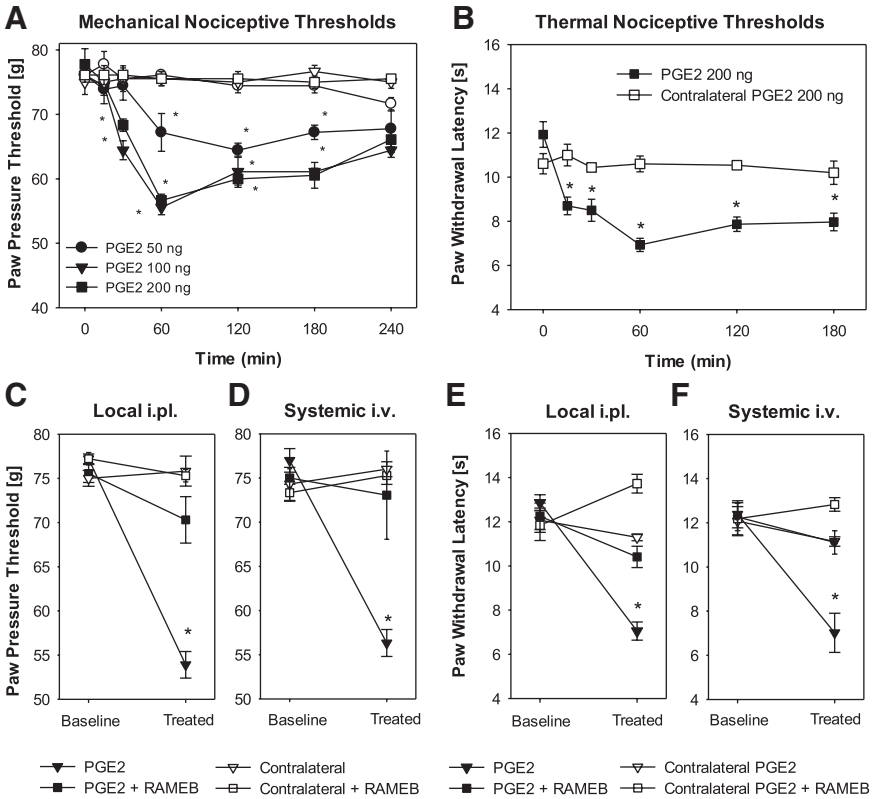

Figure 2. Randomly methylated β-cyclodextrin (RAMEB) reverses prostaglandin (PG)E2-induced hyperalgesia in vivo. A and B, Rats were intraplantarly injected with different doses of PGE2 (50 ng, black circles; 100 ng, black triangles; 200 ng, black squares). Mechanical (C) and thermal (D) nociceptive thresholds were obtained before injection (time point 0) and at the indicated time points thereafter. The dose-dependent decrease of mechanical nociceptive thresholds was maximal at 60 minutes postintraplantar PGE2. Contralateral paws are shown as control (white symbols). C–F, Rats were injected with the highest dose of PGE2 (200 ng) intraplantarly (black triangles). Mechanical (C, D) and thermal (E, F) nociceptive thresholds were measured before PGE2 injection (baseline) and at 60 minutes thereafter (treated). Groups of rats received intraplantar (C, E) or intravenous (D, F) injections of RAMEB (black squares; intraplantarly 4 mg or intravenously 20 mg RAMEB). Contralateral paws were used as controls (white triangles or squares; all n = 6, 2-way analysis of variance, Bonferroni post hoc correction, *P < .05). Data are presented as means ± SEM.

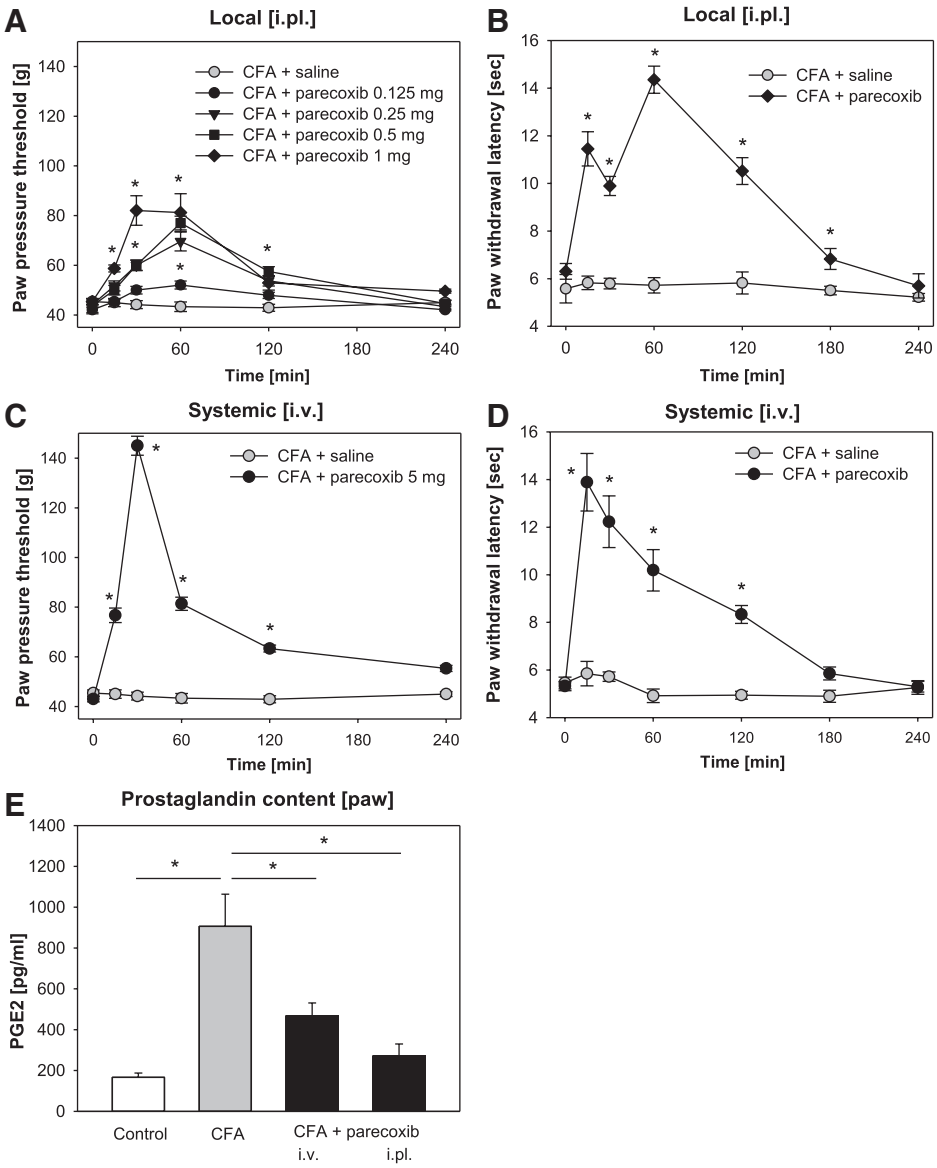

Figure 3. The cyclooxygenase (COX)-2 inhibitor parecoxib dose-dependently reduces hyperalgesia and prostaglandin E2 content in rats with complete Freund's adjuvant (CFA)-induced hindpaw inflammation. Rats were injected intraplantarly with CFA. At 96 hours of inflammation, rats were injected intraplantarly with different doses of parecoxib (A, B; 0.125 mg, black circles; 0.25 mg, black triangles; 0.5 mg, black squares; and 1 mg, black diamond's) or intravenously treated with 5 mg of parecoxib (C, D; black circles) and compared with the respective controls (saline intravenously or intraplantarly gray circles). Mechanical (A, C) and thermal (B, D) nociceptive thresholds were determined before injection (time point 0) and at the indicated time points thereafter (all n = 6, 2-way analysis of variance (ANOVA), Bonferroni post hoc correction, $*P < .05$). (E) Rats were injected as described previously, and PGE2 content was determined in inflamed paws by ELISA (n = 6, 1-way ANOVA, Bonferroni post hoc correction, $*P < .05$). Data are presented as means ± SEM.

In the next step, we examined PG content in inflamed paws (Figure 3E). CFA inflammation led to a 5-fold increase in PGE2 levels in the subcutaneous paw tissue (905.82 pg/mL; 95% CI: 403.92–1407.71 pg/mL). This was significantly reduced by IV (468.64 pg/mL; 95% CI: 270.29–667 pg/mL) and intraplantar parecoxib (271.89 pg/mL; 95% CI: 123.55–420.23 pg/mL).

Antinociceptive Efficacy of RAMEB in a Model of Inflammatory Hyperalgesia

In the model of CFA-induced hindpaw inflammation, an intraplantar injection of RAMEB significantly and dose-dependently reversed the mechanical hyperalgesia for 2 hours (Figure 4A). The maximal effect was seen after 45 minutes (PPT: 76.25 g; 95% CI: 56.24–96.25 g). Increasing RAMEB doses prolonged the effect to 3 hours. No change was observed in the contralateral noninflamed paw. The greatest dose of intraplantar RAMEB also reversed thermal hyperalgesia (PWL: 8.50 seconds; 95% CI: 6.76–10.23 seconds; Figure 4B) after 45 minutes. Systemic treatment with RAMEB via the IV route elicited a more pronounced and longer-lasting antinociceptive effect with a similar kinetic compared with intraplantarly injected RAMEB (PPT: 126.66 g; 95% CI: 114.54–138.77 g) and thermal hyperalgesia (PWL: 11.47 seconds; 95% CI: 9.26–13.68 seconds; Figure 4C, D). No changes in mechanical or thermal nociceptive thresholds were observed in the contralateral paw or after injection of solvent (data not shown).

Effect of Fractionation of RAMEB on Binding Affinity and PG Content

Although we did not observe toxic effects, methylated β-cyclodextrins can be cytotoxic in high concentrations because of perturbations of epithelial integrity and cholesterol depletion.[24] Therefore, we generated the fractions of RAMEB to find a fraction with greater binding affinity for PGE2 that could be used at a lower concentration and potentially be less toxic. These fractions were derived from commercial RAMEB by enrichment of low (RAMEB-FL), medium (RAMEB-FM), and highly (RAMEB-FH)

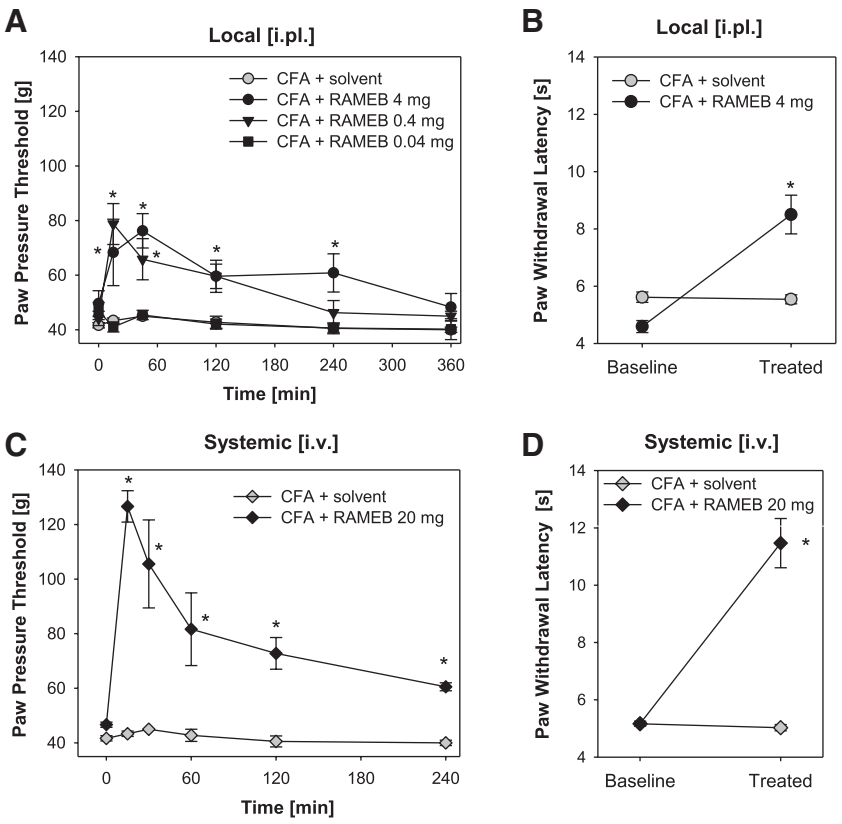

Figure 4. Randomly methylated β-cyclodextrin (RAMEB) decreases mechanical and thermal hyperalgesia in rats with complete Freund's adjuvant (CFA)-induced inflammation. Wistar rats were injected intraplantarly with CFA. (A–D) At 96 hours of inflammation, rats were treated with different doses of RAMEB intraplantarly (A, B; 0.04 mg, black triangles; 0.4 mg, black squares; and 4 mg black circles) or intravenously (20 mg; C, D; black diamonds). Mechanical (A, C) nociceptive thresholds were determined before injection (time point 0) and at the indicated time points thereafter. (B, D) Thermal nociceptive thresholds were determined before (time point 0) and at the time of peak effect (intraplantarly 45 minutes and intravenously 15 minutes). Solvent injected rats are shown as controls (gray circles intraplantarly and gray diamonds intravenously; n = 6, 2-way analysis of variance, Bonferroni post hoc correction, $*P < .05$). Data are presented as means ± SEM.

substituted cyclodextrin fractions, respectively. The approximate stability constants were determined on the basis of experiments at 2 cyclodextrin concentrations (5 and 15 mM) run in triplicate. Fraction RAMEB-FL had the lowest average DS and formed the most stable complexes (K: 580 M^{-1}; 95% CI: 565.10–594.90; Figure 5A). Local and systemic applications of all RAMEB fractions were examined in CFA inflammation. RAMEB-FL and RAMEB-FM significantly increased the mechanical nociceptive thresholds when no significant changes were observed after the injection of RAMEB-FH (Figure 5B, C). In line with the in vitro stability constants, RAMEB-FL demonstrated the most pronounced antinociceptive effect, especially after systemic injection, and this fraction was used in subsequent experiments.

Next, we assessed the influence of RAMEB or RAMEB-FL on the motor activity of the rats. The local anesthetic bupivacaine was used as positive control. Bupivacaine impaired motor function in the RotaRod test in rats (Figure 5D), whereas local application of RAMEB or RAMEB-FL into inflamed paw did not alter the performance time.

To analyze the effect of RAMEB and the fraction RAMEB-FL on tissue PG levels, PGE2 and PGD2 were quantified in inflamed paw tissue. CFA inflammation led to a 4-fold increase in PGE2 levels in the subcutaneous paw tissue (939.85 pg/mL; 95% CI: 585.00–1295.00 pg/mL). PGE2 levels were significantly reduced in the tissue of inflamed paws after treatment with intraplantarly (540.61 pg/mL; 95% CI: 398.00–684.00 pg/mL) and IV-injected RAMEB (528.81 pg/mL; 95% CI: 415.00–643.00 pg/mL), that is, 58.6% and 57.1%, respectively, and well as RAMEB-FL intraplantarly (625.22 pg/mL; 95% CI: 363.00–888.00 pg/mL), that is, 45.0% (Figure 6A). PGE2 levels remained unaltered after IV treatment with RAMEB-FH (data not shown). To assess the specificity of complexation in vivo, PGD2-MOX levels (chemically stabilized PGD2) in the subcutaneous tissue were measured (953.07 pg/mL; 95% CI: 660.27–1245.87 pg/mL; Figure 6B). PGD2 has only a minor role in the generation of hyperalgesia possibly via voltage-gated sodium channels.[25] PGD2 production was 23.51-fold increased at 96 hours of CFA inflammation. Treatment with intraplantar and IV injection of RAMEB reduced PGD2-MOX content (329.96 pg/mL; 95% CI: 213.98–445.94 pg/mL and 577.99 pg/mL; 95% CI: 411.11–744.86 pg/mL, respectively) in the paw, that is, 41.8% and 68.7%, respectively, and by 52.7% via RAMEB-FL intraplantarly (472.57 pg/mL; 95% CI: 318.87–626.28 pg/mL).

Antinociception by RAMEB-FL

Local and systemic applications of RAMEB-FL were examined in CFA inflammation (Figure 7). Intraplantar injection evoked maximal antinociception after 30 to 45 minutes and lasted up to 120 minutes (PPT: 81.38 g; 95% CI: 67.38–95.40 g; Figure 7A). Thermal hyperalgesia was relieved for 60 minutes (PWL: 11.24 seconds; 95% CI: 7.85–14.63 seconds; Figure 7B) and returned to baseline hyperalgesia after 120 minutes. Systemic application of RAMEB-FL was not more effective (PPT: 82.77 g; 95% CI: 68.28–97.27 g). The peak effect in PPT was observed after 15 minutes and lasted up to 45 minutes (Figure 7C). Thermal nociceptive thresholds were decreased (PWL: 13.50 seconds; 95% CI: 10.96–16.03

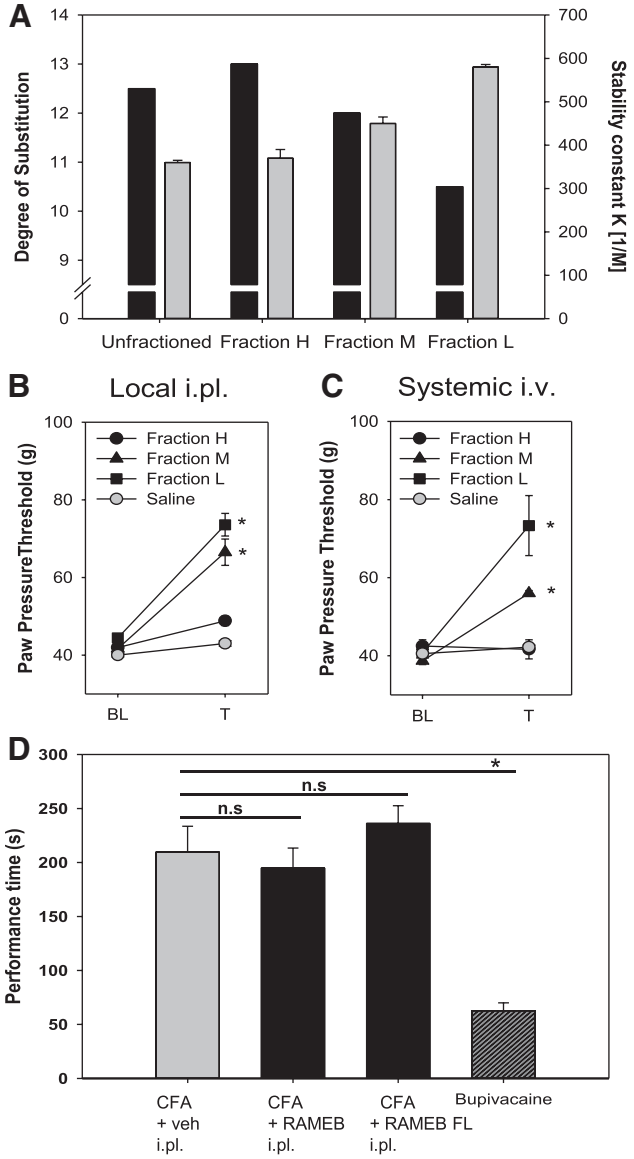

Figure 5. Subfractionation of randomly methylated β-cyclodextrin (RAMEB) improves prostaglandin (PG) complexation in vitro, differs in antinociceptive efficacy in vivo, and has no effect on motor performance. (A) Average stability constants (K) of complexes of PGE2 and RAMEB were determined by capillary electrophoresis in vitro. (B, C) Wistar rats were intraplantarly injected with complete Freund's adjuvant (CFA). At 96 hours of inflammation, rats were treated with subfractioned RAMEB with different degrees of substitution (FH, black circles; FM, black triangle; FL, black square; and saline, gray circle) intraplantarly (4 mg; B) or intravenously (20 mg; C). Mechanical nociceptive thresholds were determined before injection (time point 0) and at the time of maximum effect (intraplantarly 45 minutes, intravenously 15 min). D, Motor function was tested using a RotaRod before and 45 minutes after treatment with RAMEB or RAMEB-FL. Bupivacaine was used as positive control (n = 6, 2-way analysis of variance, Bonferroni post hoc correction, *P < .05). Data are presented as means ± SEM.

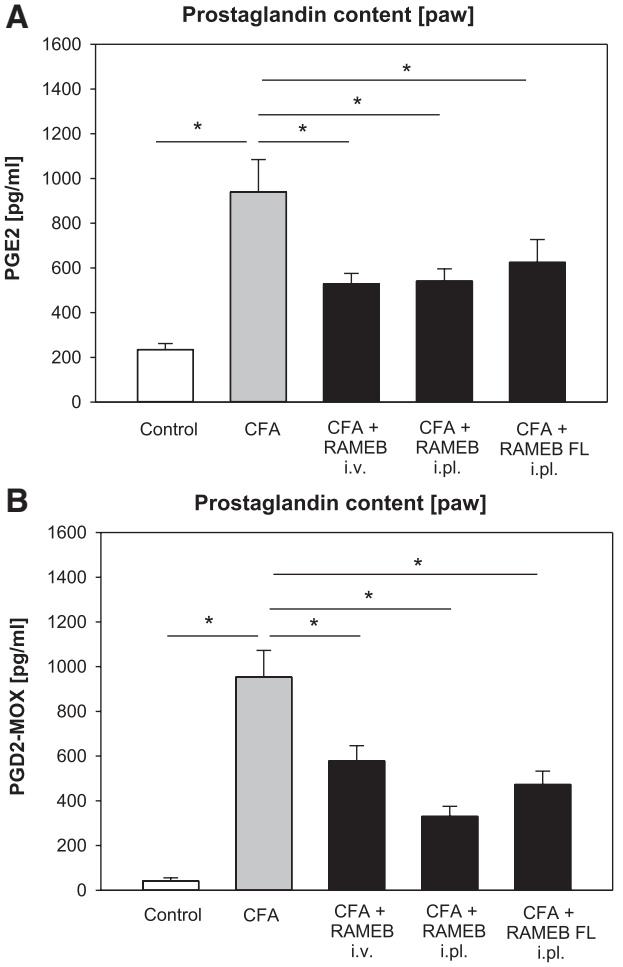

Figure 6. Subfractioned randomly methylated β-cyclodextrin (RAMEB) reduces prostaglandin content to a similar extent as unfractioned RAMEB. (A, B) Rats with 96 hours of complete Freund's adjuvant (CFA)-induced inflammation were treated with intraplantar or intravenous injection of unfractioned or fractioned (FL) RAMEB (black bars; uninjected paws, white bar; CFA only, gray bar). Inflamed paws were removed 45 minutes (intraplantar injection) or 15 minutes (intravenous injection) later and prostaglandin (PG) content (PGE2 and PGD2-MOX) was determined by enzyme-linked immunosorbent assay (n = 6–7, 1-way analysis of variance, Bonferroni post, *P < .05). Data are presented as means ± SEM.

seconds) similarly with a maximal effect after 15 minutes lasting up to 60 minutes (Figure 7D).

DISCUSSION

In this study, we demonstrate that RAMEB and its fraction RAMEB-FL elicit a potent antinociceptive effect similar to COX-2 inhibitors via systemic or local application without impairment of motor activity. Fractionation of RAMEB resulted in greater in vitro PGE2-binding capacity in the RAMEB-FL fraction. Antinociception was linked to a decrease in PGE2 and PGD2 concentration in the subcutaneous tissue. The decreased PG content in vivo was similar after treatment with COX-2 inhibitors, RAMEB, or RAMEB-FL.

PGs in Inflammatory Pain

PGs are formed by the 2 enzymes COX-1 and the inducible COX-2 in sensory neurons and non-neuronal cells in the periphery as well as in the spinal cord.[26,27] Enzyme expression of COX-2 is upregulated in inflammation both peripherally as well as centrally in neuronal as well as non-neuronal cells. Inhibition of COX at both peripheral and

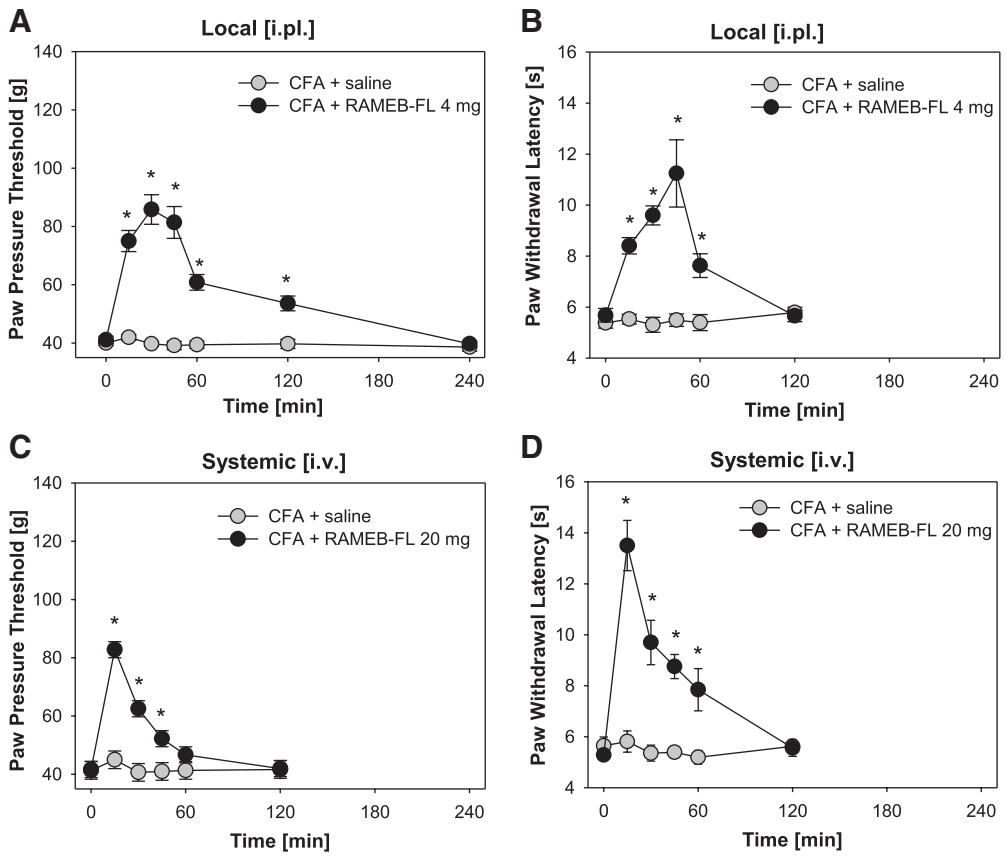

Figure 7. Subfractioned randomly methylated β-cyclodextrin (RAMEB) induces less effective antinociception in complete Freund's adjuvant (CFA)-induced inflammation. Inflammation was induced in the right hind paws of Wistar rats by CFA for 96 hours, followed by intraplantar (4 mg; A, B) or intravenous (20 mg; C, D) injection of the RAMEB fraction FL. The control group was treated with solvent only (gray circles). Mechanical (A, C) and thermal (B, D) nociceptive thresholds were measured before (time point 0) and at the indicated time points (n = 6, 2-way analysis of variance, Bonferroni post, $*P < .05$). Data are presented as means ± SEM.

central sides can contribute to analgesia. The relative contribution of both sides of action in the clinical context is still under investigation.

Induction of COX-2 in neural cells in the central nervous system seems to be a major contributor to inflammatory hyperalgesia. In accordance, systemic treatment with parecoxib as well as RAMEB resulted in more potent antinociception compared with a peripheral RAMEB or parecoxib application in CFA-induced hindpaw inflammation. The major peripheral effect of PGE2 is to sensitize the afferent neurons to noxious chemical, thermal, and mechanical stimuli. Indeed, capture of PGE2 in the paw tissue and a reduced concentration of PGE2 resulted in an increase in mechanical as well as thermal nociceptive thresholds. Other PGs also play a role in inflammatory pain. PGD2 might elicit hyperalgesia via voltage-gated sodium channels.[25]

PG–Cyclodextrin Complex Formation

The complex stability constants determined with capillary electrophoresis suggested that PGE2 is effectively captured by RAMEB. An upscaleable column chromatographic separation system was developed and optimized to separate RAMEB fractions of various average DS. The product was characterized in term of solid-state appearance by optical microscopy, solubility in water and common organic solvents, peak distribution and purity by high-performance liquid chromatography, average DS by nuclear magnetic resonance spectroscopy, and content of residual solvents by gas chromatography. These data were collected and reported in the Certificate of Analysis of the substance. The nuclear magnetic resonance analysis proved the concept suggested by the high-performance liquid chromatography, namely that the RAMEB fraction FL is enriched in cyclodextrin fractions of lower average DS and has an average DS of 10.4. The other tests revealed that the substance is a white amorphous powder, highly soluble in water, and methanol and that it contains residual solvent only at trace concentrations. A comprehensive in vitro study was performed to evaluate the interaction behavior of RAMEB fraction FL and PGE2 with the use of capillary electrophoresis. This series of experiments revealed that this special cyclodextrin derivative has high affinity to form inclusion complex with the PGE2 target molecule (~580 1/M).

Cyclodextrins and Their Mechanism of Antinociception

To explore the mechanisms of antinociception by RAMEB, we measured PGE2 in the peripheral inflamed paw tissue. Interestingly, both PGE2 and PGD2-MOX were reduced after RAMEB treatment. Therefore, the reduction of PGs seems to be the major antinociceptive mechanism.

However, other mechanisms are still possible. In inflammation, BRK is rapidly produced in plasma and tissues and involved in the initiation of pain as well as the development of hypersensitivity in inflamed or injured tissues.[28] In this study, the in silico analysis predicted the binding of BRK to RAMEB. A previous study showed that kinins are important for nociception associated with acute short-lasting inflammation (eg, visceral nociception in acid-induced writhing) and that nociception is significantly decreased in BRK receptor deficient mice.[28] In contrast, mechanical and thermal nociceptive thresholds did not differ between wild-type and BRK receptor-deficient mice in the model of CFA-induced inflammation demonstrating that BRK does not contribute to the inflammatory hyperalgesia in this model.[28] For these reasons, we did not include BRK in the investigation of the analgesic effect of RAMEB or RAMEB-FL.

Methyl-β-cyclodextrin can be used to deplete cholesterol, an essential component of lipid rafts.[29] In vitro treatment of dorsal root ganglion cells significantly reduces TRPV1-mediated capsaicin- and proton-activated currents.[30] Specifically, a decrease of the cholesterol content of a cell affects the ionic selectivity of TRPV1, suggesting that cholesterol depletion of the cell modulates the TRPV1 ion channel, which, in turn, may ameliorate pain and inflammation.[31] These effects may play an additional role in the analgesic effect of RAMEB.

Cyclodextrins in Pain Therapy

Cyclodextrins have been used in other systems for the treatment of different diseases, for example, in drug delivery of bisphosphonates for bone anabolism. PGEs are potent bone anabolic agents as demonstrated convincingly in vivo by both systemic and local delivery. An alendronate-β-cyclodextrin/PGE1 molecular complex can stimulate the strong local bone anabolic reaction.[32] PGE1 was released in vivo and in vitro to allow for this effect.

Furthermore, cyclodextrins are used to complex different analgesics like diclofenac or piroxicam. Hydroxypropyl-β-cyclodextrin-diclofenac administered IV can effectively treat acute moderate-to-severe pain after major surgery as shown in an open-label, multiday, repeated dose clinical trial.[33,34] Complexation improves pharmacokinetics and reduces side effects. Indeed, hydroxypropyl-β-cyclodextrin-diclofenac is better tolerated and has a faster onset of action. The exact mechanisms are not understood completely. One could also speculate that some of the analgesic efficacy in patients is because of PGE2 capture of the hydroxypropyl-β-cyclodextrin itself. On the basis of the available literature in these studies, a control using hydroxypropyl-β-cyclodextrin itself has not been explored. Similarly, piroxicam-β-cyclodextrin is as effective as standard piroxicam with a faster onset of action on the first day of treatment and less gastrointestinal mucosal toxicity than standard piroxicam.[35] It has to be examined in the future whether hydroxypropyl-β-cyclodextrin has an additive effect.

Similarly, hydroxypropyl-β-cyclodextrin can improve spinal opioid delivery. Spinal administration of opioids like morphine with hydroxypropyl-β-cyclodextrin in rats prolonged the duration of antinociception and reduced the incidence of catalepsy otherwise elicited by a supramaximal intrathecal dose of each of the opioids. The magnitude of the potentiating effect of hydroxypropyl-β-cyclodextrin on opioids was dependent on the concentration of the hydroxypropyl-β-cyclodextrin and varied with drug lipid partition coefficients.[36] In their study, no effect of hydroxypropyl-β-cyclodextrin was observed when administered spinally. In summary, complexation of analgesic drugs with cyclodextrins improves pharmacokinetics and possibly side effects. Based on our results, however, an analgesic effect of the cyclodextrin itself should be evaluated as well.

Toxicity of Cyclodextrins and Comparison With Standard Treatment

β-cyclodextrin itself can be toxic because of the several mechanisms including cholesterol depletion from the cell membrane. Toxicity of the unsubstituted β-cyclodextrin is because of the crystallization and concentration in the kidney. Therefore, attempts have been made to reduce the toxicity by using different substitutions. Hydroxypropyl-β-cyclodextrin is well tolerated in the animal species tested (rats, mice, and dogs), particularly when dosed orally, and shows only limited toxicity. Slight biochemical changes in body fluids are seen in studies with short duration, whereas studies with longer duration produced additional minor hematological changes but no histopathologic changes.[37] Methylated β-cyclodextrins effectively form complexes, and RAMEB is cytotoxic in high concentrations. For example, RAMEB produced an irreversible loss of cell layer barrier function in respiratory epithelial cells at ≥25 mM.[24] In vivo short-term exposure to inhaled hydroxypropyl-β-cyclodextrin, γ-cyclodextrin, and RAMEB solutions is nontoxic after the assessment of bronchoalveolar lavage, lung and kidney histology, bronchial responsiveness to methacholine, and blood urea.[38] RAMEB and its fractions are composite material—a mixture of a large number of isomers and molecules of different substitution (methylation) grades. The quantification of RAMEB therefore is a challenging task, and a measurement in this matter has not been established yet. A literature search and an inquiry to the supplier of RAMEB (Wacker Chemie AG Ltd., Germany) did not reveal any results supporting the hypothesis that RAMEB might be unstable in bloodstream.

In our study, we compared RAMEB and RAMEB fraction to a standard therapy using parecoxib,[20] because this COX inhibitor is specific for COX-2. Local and systemic treatment of RAMEB was similar to the potency of parecoxib. Systemic treatment was more effective possibly because of the spinal COX-2 inhibition or spinal reduction of PGE2. This clearly enhanced potency in different routes of administration was not any more observed when RAMEB fractions were used. In summary, RAMEB and RAMEB fractions are equally potent to COX-2 inhibitors but presumably lack gastrointestinal and cardiac toxicity.

CONCLUSIONS

RAMEB fractions with greater affinity to PGE2 effectively reduce inflammatory and PGE2-induced hyperalgesia comparable with the effect of established COX-2 inhibitors. Antinociception correlated with a selective reduction in PGE2 in the tissue when PGD2 remained unchanged. RAMEB fraction FL seems to be an interesting innovative candidate for local as well as systemic pain therapy. ■

DISCLOSURES

Name: Reine-Solange Sauer, PhD.
Contribution: This author designed, performed, and analyzed the pain behavior experiments and ELISA and participated in the manuscript draft.
Name: Heike Lydia Rittner, MD.
Contribution: This author designed the study, analyzed the data, and wrote the manuscript.
Name: Norbert Roewer, MD.
Contribution: This author designed the study, analyzed the data, and wrote the manuscript.
Name: Tamás Sohajda, PhD.
Contribution: This author performed cyclodextrin fractionation, capillary electrophoresis, and wrote the manuscript in part.
Name: Sergey Shityakov, MD, PhD.
Contribution: This author designed in silico analysis and contributed to the manuscript.
Name: Alexander Brack, MD.
Contribution: This author designed the study, analyzed the data, and wrote the manuscript.
Name: Jens-Albert Broscheit, MD.
Contribution: This author designed the experiments for cyclodextrin fractionation and partly wrote the manuscript.
This manuscript was handled by: Jianren Mao, MD.

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