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The Antinociceptive Effects of Local Injections of Propofol in Rats Are Mediated in Part by Cannabinoid CB1 and CB2 Receptors

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BACKGROUND: Propofol can inhibit fatty acid amidohydrolase, the enzyme responsible for the metabolism of anandamide (an endocannabinoid). To study the potential antinociceptive effect of propofol, we administered different doses (0.005, 0.05, 0.5, 5, and 500 μg) of the anesthetic in the hind paw of animals to determine an ED50. To further investigate the mechanisms by which propofol produced its antinociceptive effect, we used specific antagonists for the cannabinoid CB1 (AM251) and CB2 (AM630) receptors and measured fatty-acid amide/endocannabinoid (anandamide, 2-arachidonylglycerol, and palmitoylethanolamide) concentrations in skin paw tissues.

METHODS: Formalin tests were performed on 65 Wistar rats allocated to six different groups: 1) control (Intralipid™ 10%); 2) propofol (ED50 dose); 3) AM251; 4) AM251 / propofol; 5) AM630; 6) AM630 / propofol. Drugs were injected subcutaneously in the dorsal surface of the hind paw (50 μL) 15 min before 2.5% formalin injection into the same paw. Fatty-acid amide/endocannabinoid levels were measured by high performance liquid chromatography/mass spectrometry analysis.

RESULTS: Propofol produced a dose-dependent antinociceptive effect for the early and late phases of the formalin test with an ED50 of 0.08 ± 0.061 μg for the latter phase. This effect was antagonized by AM251 and AM630. It was locally mediated, since a higher dose of propofol given in the contralateral paw was not antinociceptive. Finally, only paw concentrations of palmitoylethanolamide were significantly increased.

CONCLUSION: In a test of inflammatory pain, locally injected propofol decreased pain behavior in a dose-dependent manner. This antinociceptive effect was mediated, in part, by CB1 and CB2 receptors.

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that would modulate the endocannabinoid system is beginning to attract more interest (11). For example, FAAH activity is also inhibited by nonsteroidal anti-inflammatory drugs (12,13), and we have shown that local administration of a combination of anandamide and nonsteroidal anti-inflammatory drugs produced antinociceptive effects in inflammatory (14) and neuropathic (15) pain models.

Therefore, the present study was designed to investigate the peripherally mediated antinociceptive effects of propofol in a test of acute and inflammatory pain. The mechanism by which propofol was able to produce its antinociceptive effect was also investigated by using specific antagonists for the cannabinoid CB₁ and CB₂ receptors, and by measuring fatty-acid amide/endocannabinoid paw skin concentrations.

METHODS

Animals

This research protocol was approved by the Animal Ethics Committee of the Université de Montréal and all procedures conformed to the guidelines of the Canadian Council for Animal Care. Sixty-nine male Wistar rats (Charles River, St. Constant, QC, Canada) weighing 180–220 g at the time of testing were housed in standard plastic cages with sawdust bedding in a climate-controlled room on a 12:12-h light–dark cycle. Animals were allowed free access to food pellets and water.

Drug Administration

Propofol 1% (2,6-diisopropylphenol) (Abbott Laboratories Ltd., St. Laurent, QC, Canada) was further dissolved in Intralipid® 10% (Pharmacia & Upjohn Inc., Mississauga, ON, Canada). AM251 and AM630 are 306-fold (16) and 70- to 165-fold (17) selective for CB₁ and CB₂ receptor antagonists, respectively. AM251 and AM630 were dissolved in normal saline containing 8 and 2.5% dimethylsulfoxide (DMSO), respectively. AM251 and AM630 were purchased from Tocris (Ellisville, MO).

Formalin Test

The formalin test is a well-established model of persistent pain characterized by two phases. The first (early) phase is described as an acute activation of C-fibers and the second (late) phase as an inflammatory reaction in peripheral tissue (18). Rats were acclimatized to the testing environment (clear Plexiglas box 29 × 29 × 25 cm) during 15 min or until cessation of explorative behavior. Propofol (0.005, 0.08, 0.05, 0.5, 5, or 500 μg in 50 μL), AM251 (80 μg in 50 μL), and AM630 (25 μg in 50 μL) were injected subcutaneously (s.c.) in the dorsal surface of the right hind paw 15 min before the injection of 2.5% formalin (50 μL) next to the previous injection. After each injection, the rat was immediately put back in the observation chamber. To exclude an effect of propofol on pain behavior in the time interval (15 min) after its administration and formalin injection, experiments were performed in four rats to which 0.1 mL of propofol 1% (1 mg) was injected s.c. Behavior was subsequently observed for 30 min. Apart from the initial licking of the paw (lasting a few seconds) associated with the actual pain of injection (due to needle trauma), no animal displayed signs of discomfort at this very high dose of propofol.

Nociceptive behavior was observed with the help of a mirror angled at 45° below the observation chamber. Observation of the animal’s behavior was made in consecutive 5-min periods for 60 min after formalin administration. In each 5-min period, the total time the animal spent in three different behavioral categories was recorded: 1) the injected paw has little or no weight placed on it; 2) the injected paw is raised; 3) the injected paw is licked, shaken, or bitten. Nociceptive behavior was quantified using the composite pain score-weighted scores technique [1] calculated for the first (0–15 min) and second (15–50 min) phase of the behavioral response (19). The area under the curve (AUC) which corresponds to composite pain score-weighted scores technique [2] × time (min) was calculated for the acute phase (0–15 min) and the inflammatory phase (15–50 min) using the trapezoidal rule.

Protocol

The experiments were conducted in a randomized and blinded manner by the same experimenter. In a first study, the dose–response curves for propofol were determined using the data from the first and second phases. In a second study, the antinociceptive effects of propofol (at ED₅₀ dose) were studied in the absence or presence of cannabinoid antagonists: AM251 (80 μg) (14,15,20) and AM630 (25 μg) (14,15,20). For the first two studies (n = 4 for each group), the tested drugs were dissolved in the same total volume (50 μL) and administered in the right hind paw. Preliminary experiments in the formalin test (n = 3) have shown that there was no difference in pain behavior between Intralipid 10%, 0.9% NaCl in water, and 0.9% NaCl in water with 8% DMSO (data not shown). Therefore, Intralipid 10% was used as the control solution.

Finally, to exclude any possible systemic effect of the drugs, 500 μg of propofol was administered s.c. on the dorsal surface of the contralateral (left hind paw) or ipsilateral paw (n = 4 per group).

At the end of the formalin test (60 min after its injection), the skin and tissues of the dorsal surface of the right hind paw were removed, flash frozen in liquid nitrogen, and stored at −80°C until used for measurement of fatty-acid ethanalamides/endocannabinoids as described below.

High Performance Liquid Chromatography/Mass Spectrometry Analysis

Synthesis of [²H₄]-Labeled Standards

Standard [²H₄]-labeled fatty-acid ethanalamides were synthesized by the reaction of the corresponding
fatty acyl chlorides with [3H4]-labeled ethanolamine. Fatty acyl chlorides (purchased from Nu-Check Prep, Elysian, MN) were dissolved in dichloromethane (10 mg/mL) and allowed to react with 1 equivalent of [3H4]-labeled ethanolamine (purchased from Cambridge Isotope Laboratories, Andover, MA) for 15 min at 0–4°C. The reaction was stopped by adding purified water. After vigorous stirring and phase separation, the upper aqueous phase was discarded and the organic phase was washed twice with water to remove unreacted ethanolamine. The reaction resulted in quantitative formation of [3H4]-labeled fatty-acid ethanolamides, which were concentrated to dryness under a stream of N2 and reconstituted in chloroform at a concentration of 20 mM. Fatty-acid ethanolamide solutions were stored at −20°C until use. Identity and chemical purity (>99.9%) of the synthesized fatty-acid ethanolamides were determined by thin-layer chromatography high performance liquid chromatography/mass spectrometry (HPLC/MS). 2-AG and [3H8]2-AG were purchased from Cayman Scientific (Ann Arbor, MI).

**Tissue Preparation**

Skin tissue was diced with scissors and incubated in 2 mL of chloroform containing 25 pmol of [3H4]-anandamide, 25 pmol of [3H4]-2-AG, and 500 pmol of [3H4]-labeled-palmitoylethanolamid (PEA) overnight at 4°C with shaking. The tissue was then homogenized and lipids were extracted with 2 mL of methanol and 2.25 mL of saturated sodium chloride solution (1 M). The organic layer was removed, evaporated to dryness under N2, reconstituted in a mixture of chloroform/methanol (1:1, 100 μL), and transferred to 2.0 mL screw top vials with 0.1 mL glass inserts to be injected into the HPLC/MS.

**HPLC/MS Analysis**

Fatty-acid ethanolamides were quantified using an isotope dilution HPLC/MS assay in positive ionization mode (21).

**Paw Edema**

At the end of the formalin test, paw edema was measured at the base of the right hind paw using a digital micrometer (Mitutoyo Corporation, USA) with an instrumental error of ± (maximum measuring length/75) μm and a resolution of 0.001 mm (22).

**Statistical Analysis**

Pain behavior for each treatment group was expressed as mean ± se. The dose–response curve for propofol was determined using ALLFIT software (23). In the second study, the antinociceptive effects of propofol in the absence or presence of cannabinoid antagonists were assessed for significance using factorial experimental design (24). To compare ipsi- versus contralateral paw injections of the drugs, an analysis of variance adapted for factorial experimental design was used. The different components of the total variation were settled a priori using multiple regression analysis (25). The critical level of significance was set at 5% (P < 0.05).

**RESULTS**

**Antinociceptive Effect of Propofol and Implication of Cannabinoid Receptors**

Propofol decreased pain behavior in the formalin test with an ED50 (± se) of 0.015 μg ± 0.0161 and 0.084 μg ± 0.0611 for the early and late phases, respectively (Figs. 1A and B). Pain behavior after injection of propofol in the contralateral hind paw was not statistically different when compared with the control (Intralipid 10%) group [F(1,6) = 0.42 and 0.72 for the first and second phases, respectively] (Fig. 2).

When given locally (dorsal surface of the paw) at ED50 dose, propofol produced a significant antinociceptive effect when compared with the control group either for the early (Figs. 3A and B) [F(1,18) = 9.17, P < 0.01] or late (Figs. 3A and C) phase [F(1,18) = 11.65, P < 0.005] of the formalin test. This antinociceptive effect was inhibited by AM251 and AM630, since there was no statistical difference between those antagonists given alone compared with their combination with propofol for the first [F(1,18) = 1.87 and 1.46] or second phase [F(1,18) = 0.21 and 0.95], respectively (Figs. 3A–C).
Paw Edema
Edema of the injected paw differed significantly among the groups \(F(5,18) = 19.53, P < 0.001\): AM251 \(F(1,18) = 37.99, P < 0.001\) and AM630 \(F(1,18) = 35.09, P < 0.001\) when given alone significantly reduced paw edema (Fig. 3D).

Concentration of Fatty-Acid Ethanolamides/Endocannabinoids After Peripheral Administration of Propofol
The amounts of anandamide and 2-AG in paw tissues were not significantly higher when propofol (0.05 \(\mu\)g) was given locally compared with the control group (Intralipid 10%) (Figs. 4A and B) \(F(1,12) = 1.90\) and 1.94, respectively). However, the amount of PEA in paw tissues was significantly higher when propofol (0.05 \(\mu\)g) was given locally compared with the control group (Fig. 4C) \(F(1,12) = 6.68, P < 0.025\).

DISCUSSION
In this study, we have demonstrated that propofol, when given in the periphery (hind paw), produced a dose-dependent antinociceptive effect. This effect is locally mediated, since a high dose of propofol given in the contralateral paw did not reduce pain behavior. We also observed a slight increase in the concentration of anandamide, 2-AG, and PEA in paw skin tissues, which was only significant for PEA when propofol was injected locally at ED\(_{50}\) doses. We suggest that the antinociceptive effect of propofol was mediated, in part, by cannabinoid CB\(_1\) and CB\(_2\) receptors, since antagonists to these receptors (AM251 and AM630, respectively) completely inhibited the antinociceptive effect of propofol when administered together. However, the dose dependency of propofol long existed when the data from tissue levels of endocannabinoids and PEA was considered. In our opinion, propofol’s exact mechanism of action involves much more than a simple tissue increase in anandamide levels. This could have been expected, as many local factors can interact to modulate anandamide levels. For example, FAAH and cyclooxygenase-2 enzymes are present locally and anandamide is a potential target for them. Furthermore, 2-AG, PEA, and other fatty-acid amides are present locally and their metabolism (by FAAH in particular) is highly complex, and could also explain different levels of anandamide.

The action of propofol at the spinal level has been demonstrated in animal models using different modalities (noxious heat, mechanical stimulus) \((2,5)\), but evidence for the peripheral antinociceptive effect of propofol has only recently been demonstrated using a bee venom-induced inflammatory pain state \((6)\). However, in this latter study only a single dose of propofol was used, and moreover, it was dissolved in DMSO. In some studies DMSO has been shown to produce analgesia \((26,27)\). Therefore, the antinociceptive effect of propofol reported in the study by Sun et al. \((6)\) may
not have been optimal, requiring confirmation. Intralipid 10%, which has the same composition as propofol 1%, was used in the present study to avoid this bias. Furthermore, it was shown that a dose of 0.08 μg reduced pain behavior by 50%, which constitutes a dose six times inferior to the one used by Sun et al. (6).

It has been demonstrated that propofol increased the whole-brain content of anandamide (through inhibition of FAAH), which could explain its sedative properties, mediated by the cannabinoid CB1 receptor (7). However, propofol was a mild inhibitor of FAAH compared with strong inhibitors like URB532 and URB997 (28). This could explain the small increase in the concentration of anandamide in paw skin tissues reported in the present study. Furthermore, PEA is a fatty-acid amide that has both antiinflammatory and antinociceptive properties (29,30). PEA does not bind to cannabinoid receptors (31,32), although its effects are antagonized by cannabinoid CB2 receptor antagonists (33). Indeed, Calignano et al. (33) found that PEA inhibited both early and late phases of formalin-evoked pain behavior after intraplantar injection in mice. Furthermore, these authors showed that the analgesia produced by PEA was reversed by the CB2 receptor antagonist SR144528, but not by administration of the CB1 receptor antagonist SR141716A, nor by the opioid antagonist naloxone. Therefore, the significant local increase in PEA in the present study could explain why the antinociceptive properties of propofol were attenuated by a CB2 receptor antagonist. These findings corroborate the results, which suggest that the dose-dependent antinociceptive effects of propofol are mediated, in part, by CB1 and CB2 cannabinoid receptors.

Regarding paw tissue levels of endocannabinoids and PEA, no saline controls were tested. However, we have measured, in a previous study (34), fatty-acid amides, such as anandamide and PEA, in rat paw tissues. The measurements were performed using similar techniques, although obviously not at the same time, and therefore not under exactly the same conditions. However, the levels of anandamide and PEA after injection of 2.5% formalin were in the same magnitude as the one reported in this study with Intralipid 10%.

To further confirm the findings of the present study, it must be considered that cannabinoid CB1 and CB2 receptors are expressed in dorsal root ganglion, spinal cord, and also in peripheral tissues (9). We showed, using the Western blot technique, that the CB1 and CB2 receptor proteins are indeed present in peripheral tissues under normal conditions (naïve rats), and that their expression is increased in neuropathic conditions (9). Furthermore, FAAH activity is present in peripheral tissues (34,35) and it is therefore reasonable to propose that propofol inhibited FAAH activity, although weakly. Additionally, it was shown that FAAH knockout mice exhibit significant reduction in pain behavior in the formalin test, probably due to the local increase in anandamide concentrations (35). Finally, a direct action of propofol on cannabinoid receptors is very unlikely, as Patel et al.

Figure 3. Effects of AM251 and AM630 on propofol antinociceptive effects when administered at an ED50 dose into the hind paws 15 min before 2.5% formalin (50 μL) administration. (A) Antinociceptive effect of propofol. (B) Effects of cannabinoid antagonists on propofol antinociceptive effects for the first phase of the test (0–15 min). (C) Effects of cannabinoid antagonists on propofol antinociceptive effects for the late phase of the test (15–50 min). (D) Paw edema. Data are expressed as mean ± se (n = 4 per group). Area under the curve for the early phase, †P < 0.01 for propofol versus control (Intralipid 10%) group. Area under the curve for the late phase, #P < 0.005 for propofol versus control (Intralipid 10%) group. *Propofol versus control (Intralipid 10%) group, P < 0.01. ‡AM251 versus AM251 + propofol, P < 0.001. ∆AM630 versus AM630 + propofol, P < 0.001.
(7) have shown that propofol did not bind to CB₁ receptors, although binding to CB₂ receptors was not determined.

We cannot exclude the fact that other mechanisms of action may be involved in the attenuation of pain sensation by propofol. Indeed, the antinociceptive effects of propofol at high doses are difficult to explain by the sole involvement of the cannabinoid system. Furthermore, as other studies have shown, propofol can control pain by a central modulation through the opioid system (4) and/or by interacting with spinal γ-aminobutyric acid₆ receptors (3). Although, the action of propofol on neuronal cells is well documented, its involvement in glial cells seems controversial, since it had no effect on the production of nitric oxide or tumor necrosis factor-α from glial cells stimulated by lipopolysaccharide (36). Sun et al. (6) suggested that propofol could activate γ-aminobutyric acid₆ receptors and/or inhibit N-methyl-d-aspartate receptors although, to our knowledge, these experiments have not yet been performed. Finally, it is always possible that Intralipid interfered with the metabolism of fatty-acid amides, being itself a lipid-based compound.

The marked decrease in paw edema reported after local administration of both cannabinoid antagonists could be explained by the fact that these antagonists are dissolved in NaCl 0.9% which is better absorbed than Intralipid and/or propofol. Another explanation may be the involvement of a non-CB₁/non-CB₂ distinct cannabinoid receptor regulating cannabinoid-induced vasodilatation (11).

In conclusion, peripheral administration of propofol was associated with a dose-dependent decrease in pain behavior in animals subjected to inflammatory pain. This effect was locally mediated by action on cannabinoid CB₁ and CB₂ receptors and with the involvement of PEA, a fatty-acid ethanolamide. However, the exact contribution of these receptors, and of other mediators, to the antinociceptive effects of propofol merits further study.

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