Administration of Endocannabinoids Prevents a Referred Hyperalgesia Associated with Inflammation of the Urinary Bladder


Background: Referred hyperalgesia to a somatopically appropriate superficial site is a cardinal symptom of visceral inflammatory pain and has been demonstrated after turpentine-induced urinary bladder inflammation in the rat. The authors examined the effect of the endocannabinoids anandamide and palmitoylethanolamide on the referred hyperalgesia associated with this model.

Methods: After measurement of baseline limb withdrawal latencies to a noxious heat stimulus, the bladders of 50 female Wistar rats were inflamed by intravesical administration of 0.5 ml 50% turpentine. Ten or 25 mg/kg of anandamide or palmitoylethanolamide or vehicle were administered immediately before introduction of turpentine. Antagonists to both the cannabinoid CB1 and CB2 receptors were coadministered with the higher dose of endocannabinoids. Latencies were recorded 2, 4, 6, 8, and 24 h after removal of turpentine. The difference between forelimb and hind limb withdrawal latencies was plotted against time, and areas under these curves were compared.

Results: Inflammation of the urinary bladder was associated with a relative thermal hyperalgesia referred to the hind limb. Anandamide and palmitoylethanolamide attenuated this referred hyperalgesia at doses of 10 and 25 mg/kg. The CB1 receptor antagonist SR141716A reduced the antihyperalgesic effect of anandamide, but the CB2 antagonist SR144528 did not. Coadministration of SR141716A with palmitoylethanolamide did not affect the antihyperalgesic effect but was reduced by SR144528.

Conclusions: Anandamide (via CB1 receptors) and palmitoylethanolamide (putatively via CB2 receptors) attenuated a referred hyperalgesia in a dose-dependent fashion. CB1 and CB2 receptors are strategically situated to influence the nerve growth factor–driven referred hyperalgesia associated with inflammation of the urinary bladder. These data implicate cannabinoids as a novel treatment for vesical pain.

There are fundamental clinical and physiologic differences between somatic and visceral pain.1 A classical feature of visceral injury is referred pain, whereby the sufferer localizes the origin of the pain to a distant superficial structure (muscle or skin or both) innervated by the same spinal nerve as the affected viscus. It has been suggested that the sensory thresholds in the dermatome–myotome to which the pain is referred are reduced so that hyperalgesia is evident. Several models and theories of referred pain have been described.1,2 Current evidence favors a “central” convergence-facilitation hypothesis whereby the afferent barrage from the affected viscus initiates a focus of sensitization in dorsal horn neurons that receive a convergent input from both visceral and somatic structures. Persistence of central plastic changes described in this theory also explains the clinical phenomenon of continued referred hyperalgesia after cessation of the noxious stimulus. For example, a referred hyperalgesia exhibited by patients with a ureteric calculus persists long after removal of the calculus.3

The neurotrophin nerve growth factor (NGF) plays a pivotal role in the development of visceral hyperalgesia, including referred hyperalgesia.4,5 The turpentine-inflamed rat urinary bladder is a well-established clinically relevant model of visceral hyperalgesia, and its features include (1) bladder hyper-reflexia,4,6 (2) sensitization of visceral primary afferents and segmental dorsal horn neurons,7,8 (3) recruitment of novel mechanoreceptive C fibers (silent afferents),8 (4) induction of the early immediate gene c-fos in the spinal cord,9 and (5) a referred (viscero-somatic) hyperalgesia.5 These manifestations of turpentine-induced bladder inflammation are NGF-dependent, and, of particular importance to this study, the referred hyperalgesia to the rat hind paw associated with bladder inflammation is attenuated by NGF sequestration and is reproduced by intravesical administration of NGF.10 Cannabinoid activity at both the neuronal CB1 receptor and the peripheral CB2 receptor expressed on mast cells could contribute. We have previously shown that exogenous administration of the endocannabinoids anandamide (an endogenous CB1 receptor agonist) and palmitoylethanolamide (a putative endogenous CB2 receptor agonist) reduce bladder hyper-reflexia, one of the NGF-dependent features of visceral hyperalgesia associated with this model.6 Therefore, we investigated the effect of anandamide and palmitoylethanolamide on the referred hyperalgesia associated with turpentine inflammation of the rat urinary bladder. Furthermore, to ascertain...
the contribution of cannabinoid receptor subtypes to the effects of cannabinoids, CB₁ and CB₂ receptor antagonists were used.

Methods

Animal Maintenance and Instrumentation

All experiments conformed to British Home Office regulations (Home Office, Animals Procedures Section, London, United Kingdom). Experiments were performed on 50 female Wistar rats (mean weight, 219.5 g; range, 190–260 g) that were housed according to national guidelines with a 12-h light–dark cycle and food and water ad libitum. Room temperature and humidity were controlled at a constant level during all experiments, which were performed at similar times in the light–dark cycle. For urethral cannulation and bladder inflammation, anaesthesia was induced and maintained by inhalation of isoflurane (1–2%) in 50:50 oxygen and nitrous oxide. A sterile, lubricated nylon catheter (1.02-mm OD; Portex, Hythe, Kent, United Kingdom 200/300/030) was introduced into the bladder transurethrally. An unobstructed flow of clear urine was obtained before installation of turpentine.

Bladder Inflammation

Turpentine (0.5 ml 50% in olive oil) was instilled via the urethral catheter into the urinary bladder and left in situ for 1 h before removal by the same route. This provokes a sterile bladder inflammation within 1 h that persists for at least 48 h.² Twenty animals were randomly assigned to one of four groups (each group, n = 5) that received either 25 mg/kg anandamide or equivalent volume of soya emulsion vehicle control or 25 mg/kg palmitoylethanolamide or equivalent volume of dimethyl sulphoxide (DMSO): saline (2:3) vehicle control by the intraperitoneal route. Two further groups (each n = 5) received 10 mg/kg of either anandamide or palmitoylethanolamide also by the intraperitoneal route. A further 20 animals were randomized into four more groups (each group, n = 5). One group received 25 mg/kg anandamide coadministered with 1 mg/kg of the CB₁ antagonist (SR141716A), and the other received the same dose of anandamide coadministered with 1 mg/kg of the CB₂ receptor antagonist (SR144528). Similarly, 25 mg/kg palmitoylethanolamide and 1 mg/kg of SR141716A or SR144528 were coadministered to the two remaining groups.

Thermal Sensory Threshold Assessment

Limb withdrawal latencies to a noxious thermal stimulus were measured to demonstrate thermal hyperalgesia. The plantar surface of the hind paw was used to assess alteration of somatic thresholds after visceral inflammation because its spinal innervation corresponds to an input at the L₄–L₅ level and shares a common root with vesical innervation (L₅–S₁). The latency of limb withdrawal was measured using a Harclereve’s device (Ugo Basile, Comerio, Varese, Italy). This device applies a beam of infrared radiation at a wavelength of 50 nm onto the plantar surface of the paw. To prevent tissue damage, the maximum temperature delivered is 46°C, and the system cuts out automatically after 21.4 s. The system automatically cuts out when the animal withdraws its limb, and a latency in seconds is displayed. The animals were initially acclimatized to the testing environment (Plexiglas box, 23 × 18 × 14 cm, mounted on an infrared lucent dry glass pane) before baseline limb withdrawal latencies of both the hind and forepaws were measured. For these and all subsequent measurements, the mean of three tests was taken as the withdrawal latency. There was a delay of at least 1 min before retesting the same animal and 3 min between testing on the same paw. Contemporaneous measurement of both forelimb and hind limb withdrawal latencies provided an intraanimal internal control to negate any obfuscating factors affecting sensory thresholds such as systemic effects of inflammation or persistent effects of anesthesia. Further latencies were measured at 2, 4, 6, 8, and 24 h after removal of turpentine from the bladder, and were all made by a single, blinded observer. After completion of the final test, the animals were humanely culled by cervical spine dislocation.

Materials

Anandamide and palmitoylethanolamide were purchased from Tocris Cookson (Bristol, Avon, United Kingdom). Anandamide was presented as a suspension in soya emulsion at a concentration of 10 mg/ml. Palmitoylethanolamide was dissolved in DMSO (Sigma, Poole, Dorset, United Kingdom) saline (2:3) to a concentration of 5 mg/ml. SR141716A was dissolved in DMSO saline (2:3) to a concentration of 1 mg/ml. SR144528 was also dissolved in DMSO and saline 2:3 to a concentration of 1 mg/ml.

Statistical Analysis

As previously reported, limb withdrawal latency was expressed as a percentage of the baseline value and as the difference between forelimb and hind limb. This measure of ΔThreshold was calculated by:

\[ Δ \text{Thresh} = \left( \frac{H_{\text{Thresh}}}{BH_{\text{Thresh}}} \times 100 \right) - \left( \frac{F_{\text{Thresh}}}{BF_{\text{Thresh}}} \times 100 \right) \]

where \( H_{\text{Thresh}} \) is the withdrawal latency of the hind limb, \( BH_{\text{Thresh}} \) is the baseline withdrawal latency of the hind limb, \( F_{\text{Thresh}} \) is the withdrawal latency of the forelimb, and \( BF_{\text{Thresh}} \) is the baseline withdrawal latency of the forelimb. The mean ΔThreshold was then plotted against time, with a negative difference indicating a relative hyperalgesia of the hind limb.
Changes in forelimb withdrawal latencies after treatment with endocannabinoids could potentially cause artefacts of ΔThreshold. Therefore, to examine changes, forelimb withdrawal latencies were compared with baseline (one-way analysis of variance [ANOVA], post hoc Dunnett test).

To assess effects across the total time period, the area under the curve (AUC) for each of the ΔThreshold against time curves for each animal in each group was calculated using the trapezium rule (Sigmaplot, Jandel Scientific Software, Version 4.01). Mean AUC values in each group were compared with control using ANOVA (post hoc Dunnett test; Sigmastat, Jandel Scientific, Version 2.0, San Rafael, CA).

Results

Referred Hyperalgesia after Turpentine Inflammation of the Rat Urinary Bladder

In the soya control group, a negative deflection consistent with a relative hyperalgesia of the hind limb was seen, and this effect is reduced by anandamide in a dose-related manner (fig. 1A). An example of derivation of ΔThreshold for the soya control group is given in table 1. Treatment with anandamide did not alter forelimb latencies significantly (ANOVA, Dunnett, P > 0.05). The mean AUC (derived from ΔThreshold agonist time curves) of both the anandamide treatment groups were significantly different from control (ANOVA, Dunnett, P < 0.05; fig. 1B). Similarly, plots of ΔThreshold against time demonstrate a negative deflection in the DMSO: saline control group that is attenuated by both 25 and 10 mg/kg palmitoylethanolamide (fig. 2A). Forelimb latencies after administration of palmitoylethanolamide were not changed from baseline (ANOVA, Dunnett, P > 0.05). Corresponding mean AUC values demonstrated both treatment groups were significantly different from control (ANOVA, Dunnett, P < 0.05; fig. 2B).

The effect of CB1 and CB2 receptor antagonists on the action of anandamide is shown in the ΔThreshold against time plot (fig. 3A). Analysis of the corresponding AUCs reveals the action of anandamide to be mediated via CB1 receptors (fig. 3B). The AUC after coadministration of anandamide with the CB1 receptor antagonist SR141716A is not significantly different from control (ANOVA, Dunnett, P > 0.05), whereas treatment with anandamide and the CB2 receptor antagonist SR144528 results in an AUC significantly different from control (ANOVA, Dunnett, P < 0.05). Concomitant administration of antagonists with 25 mg/kg palmitoylethanolamide indicates an action predominantly via CB2 receptor (figs. 4A and 4B). The AUC after coadministration of palmitoylethanolamide with SR141716A was significantly different from control (ANOVA, Dunnett, P < 0.05), whereas addition of SR144528 to 25 mg/kg palmitoylethanolamide resulted in an AUC not significantly different from control (ANOVA, Dunnett, P > 0.05).

Discussion

These data confirm previous findings demonstrating the development of a thermal hyperalgesia referred to the plantar surface of the hind paw after turpentine-induced inflammation of the rat urinary bladder. This was attenuated in a dose-dependent fashion by anandamide acting via the CB1 receptor and by a CB2 receptor-mediated effect of palmitoylethanolamide. The efficacy of these endocannabinoids in this model of visceral referred hyperalgesia suggests a role for cannabinoid agonists in the clinical management of referred hyperalgesia secondary to visceral inflammation. Moreover, the
referred hyperalgesia of this model has been previously shown to be a NGF-dependent phenomenon, and these data further highlight a cannabinoid role in the attenuation of NGF-driven inflammatory processes.

Nerve Growth Factor Is Pivotal in Plastic Changes after Visceral Inflammation

Nerve growth factor plays a pivotal role in this model of persistent visceral pain and in its clinical counterparts. First, not only is NGF protein and mRNA upregulated in animal models of bladder inflammation and in certain types of human cystitis, but the high-affinity receptor for NGF (tyrosine kinase A) is present on nearly all pelvic afferent neurons supplying the bladder. This particular capacity to respond to NGF suggests that NGF may be of special importance in visceral hyperalgesia. Second, sequestration of NGF by a tyrosine kinase A-immunoglobulin G fusion molecule attenuates manifestations of turpentine-induced inflammation of the urinary bladder, such as bladder hyper-reflexia and referred hyperalgesia. Finally, many of the features of turpentine-induced visceral hyperalgesia are reproduced by intravesicular administration of exogenous NGF, including bladder hyper-reflexia, sensitization of visceral primary afferents, and referred hyperalgesia.

Plasticity and Pathways Involved in a Visceral Referred Hyperalgesia

Inflammation has profound effects on both the peripheral and central nociceptive processing of sensory information, which contributes to the spinal plasticity important in the development of referred hyperalgesia. For example, clinical human studies describe a renal calculus-induced referred hyperalgesia. Persistence of spinal plastic changes is demonstrated by continuing hyperalgesia even after stone destruction.

Other studies examining neurogenic plasma extravasation in appropriate areas of expected referred uterine pain after mustard oil–induced uterine inflammation also implicate mechanisms of referred hyperalgesia. Afferent–afferent interaction in the spinal cord or a sympathetic reflex have been postulated, yet data suggest that both may be important. First, after bladder inflammation, the early immediate gene, c-fos, is expressed in several areas of the spinal cord (indicative of areas of visceral afferent input), including the superficial dorsal horn, the deep lateral dorsal horn and lamina X. The latter two areas also contain preganglionic autonomic neurons and interneurons. Incidentally, after nociceptive input from somatic structures, Fos is expressed in similar areas also containing preganglionic autonomic neurons and interneurons. The latter two areas also contain preganglionic autonomic neurons and interneurons. Incidentally, after nociceptive input from somatic structures, Fos is expressed in similar areas also containing preganglionic autonomic neurons and interneurons.

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Targets of CB₁ Receptor–Mediated Attenuation of Referred Hyperalgesia

Peripheral. Peripheral analgesic actions of cannabinoids have been demonstrated in somatic inflammatory pain models, yet a specific peripheral site of visceral analgesia has not been clarified. However, there is pharmacologic evidence that the CB₁ receptor is present in visceral structures, notably the urinary bladder, and the gene encoding CB₁ is present in NGF-dependent primary afferent neurons that make up the majority of visceral primary afferent input. Furthermore, not only are CB₁ receptor binding sites anterogradely transported out to the periphery, but mRNA for the CB₁ receptor has been detected in the sensory cell bodies of the dorsal root ganglion. CB₁-mediated reduction of visceral afferent input presents a potent "upstream" site to attenuate the development of spinal plastic changes leading to referred hyperalgesia, but also presents a site of therapeutic action remote from the brain site responsible for unwanted psychotropic side effects.

Spinal. Electrophysiologic and behavioral data support a spinal action of cannabinoid analgesia. Comparison of the structures associated with postulated mechanisms of referred hyperalgesia and recent detailed elucidation of CB₁ receptor expression in spinal cord may suggest a specific role for spinal cannabinoids in the modulation of visceral hyperalgesia. First, the CB₁ receptor is found in the same spinal lamina (lamina I) as part of the central input of NGF-dependent class of peptidergic primary afferent neurons that terminate in the hind limb. The CB₁ receptor is found in the same spinal lamina as part of the central input of NGF-dependent class of peptidergic primary afferent neurons that terminate in the hind limb.
laminae I and II outer. Second, anandamide has been shown to inhibit the release of calcitonin gene-related peptide from the central terminals of NGF-dependent primary afferent nociceptors. Third, CB₁ is expressed in the same spinal areas as convergent cells identified by Fos expression after both vesicular and somatic inflammation such as the superficial dorsal horn and lamina X. Fourth, the CB₁ receptor is expressed mainly on neurons intrinsic to the spinal cord, and these interneurons are also present in areas analogous to the sympathetic intermediolateral nucleus and sacral parasympathetic nucleus. Thus, agonist actions at the CB₁ receptor on spinal interneurons could inhibit the development of referred hyperalgesia (1) by attenuating central afferent input, (2) by direct modulation of convergent neurons, or (3) by inhibition of putative autonomic processes.

However, systemic administration of the endocannabinoids in this study can only lead to a speculative site of action, and given the existing evidence for supraspinal mechanisms of cannabinoid analgesia, a supraspinal action of anandamide cannot be discounted.

**Targets of CB₂ Receptor–Mediated Attenuation of Referred Hyperalgesia**

Agonists at the CB₂ receptor expressed on mast cells also provide a separate mechanism by which palmitoylethanolamide could attenuate the NGF-mediated processes that contribute to referred hyperalgesia. NGF provokes mast cell degranulation, which releases a number of inflammatory mediators, including more NGF, effectively amplifying the NGF signal. Indeed, the mast cell has been recognized as a key player in certain clinical inflammatory conditions of the urinary bladder. Facci et al. demonstrated the efficacy of palmitoylethanolamide in the prevention of mast cell degranulation putatively via the peripheral CB₂ receptor. However, although in this study and others the analgesic effects of palmitoylethanolamide are reversed by the CB₂ antagonist (SR144528), recent data questions whether palmitoylethanolamide binds to the CB₂ receptor with sufficient affinity to provoke agonist activity. Even if palmitoylethanolamide did not itself act at the CB₂ receptor, it could act as a CB₂-like receptor at which SR144528 also has antagonist activity. The label “CB₂-like receptor” has been coined by several investigators to account for the non-CB₁–non-CB₂ receptor–mediated actions of palmitoylethanolamide. Palmitoylethanolamide could also interfere with the breakdown of endogenous cannabinoids active at the CB₂ receptor in an “entourage effect.” These explanations could account for the observed attenuation of referred hyperalgesia by palmitoylethanolamide, which is reversed by SR144528.

The convergence facilitation theory proposes peripheral and spinal mechanisms thought to be important in the development of visceral referred hyperalgesia. Many of these processes involve NGF-driven elements. We have shown that anandamide (via CB₁ receptors) and palmitoylethanolamide (involving a CB₂ receptor–mediated process) attenuate this referred hyperalgesia. There is an anatomic basis for peripheral CB₁- and CB₂-mediated effects and a site for central CB₁ mediated actions both able to inhibit the processes necessary to incur a referred hyperalgesia. These data reinforce the analgesic action of cannabinoids, especially in the treatment of symptoms of visceral inflammatory hyperalgesia and propose cannabinoids, or compounds that inhibit degradation of endocannabinoids, as novel therapeutic strategies in visceral pain states.

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Fig. 4. (A) Plots of ΔThreshold against time after dimethyl sulphoxide (DMSO):saline control (filled circles), 25 mg/kg palmitoylethanolamide (PEA; open circles), coadministration of 25 mg/kg palmitoylethanolamide with 1 mg/kg SR141716A (filled triangles), or 1 mg/kg SR144528 (open triangles). (B) Corresponding mean areas under the curve (AUC) of ΔThreshold versus time plots. The AUCs for 25 mg/kg palmitoylethanolamide alone and when coadministered with 1 mg/kg SR141716A are both significantly different from DMSO:saline control (*each n = 5, ANOVA, Dunnett, P < 0.05).
References

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