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Short communication

Magnitude of tolerance to fentanyl is independent of μ-opioid receptor density

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Abstract

The effect of a μ-opioid receptor irreversible antagonist on the development of tolerance to fentanyl was determined in mice. Mice were injected with saline or clocinnamox (3.2 mg/kg, i.p.) and 4 h later mice implanted s.c. with a placebo pellet or an osmotic minipump that infused fentanyl (0.165 mg/kg per day) for 3 days. Fentanyl pumps and placebo pellets were removed on the third day following implantation and 4 h later μ-opioid receptor saturation binding studies in whole brain ([^{3}H][D-Ala^2,MePhe^4,Gly-ol^5]enkephalin: DAMGO) or fentanyl analgesic dose–response studies (tailflick assay) were conducted. Fentanyl infusions and clocinnamox both significantly reduced the potency of fentanyl by 2.8- and 2.4-fold, respectively. When fentanyl and clocinnamox were administered together, a significant 5.0-fold reduction in fentanyl potency relative to the saline-placebo group was observed, which represents an additive effect of clocinnamox and fentanyl. The ED_{50} of fentanyl in clocinnamox-treated mice was shifted 2.1-fold by fentanyl infusion relative to the clocinnamox-placebo group. This is comparable to the 2.8-fold shift in the ED_{50} produced by fentanyl infusion in saline-treated mice. In binding studies, fentanyl produced a small (−9%) reduction in B_{max}, while clocinnamox significantly reduced (−41%) μ-opioid receptor density without altering affinity (K_d). In the clocinnamox-fentanyl group, there was a 50% reduction in B_{max}, which is similar to the additive effect observed in analgesia studies. These data indicate that changes in μ-opioid receptor density prior to the development of tolerance to fentanyl do not impact on the magnitude of tolerance.

Keywords: μ-Opioid receptor; Downregulation; Tolerance; Fentanyl; Clocinnamox; DAMGO ([D-Ala^2,MePhe^4,Gly(ol)^5]enkephalin); Analgesia

1. Introduction

Tolerance is a decrease in the analgesic potency of opioid agonists following prior treatment (Inturrisi, 1990). Although receptor theory predicts that a reduction in agonist potency can be accounted for by a decrease in receptor density (e.g., Kenakin, 1993), numerous studies indicate that receptor downregulation is not an essential factor for the development of tolerance (Loh et al., 1988; Puttfarcken and Cox, 1989; Tao et al., 1987; Yoburn et al., 1993). On the other hand, opioid agonists can produce downregulation under certain circumstances. For example, agonist-induced downregulation is dependent on intrinsic efficacy, dose, and treatment protocol (e.g., Yoburn et al., 1993).

Although receptor downregulation is not required for the development of tolerance, it may be an important determinant of the magnitude of tolerance. In a previous study (Lutfy and Yoburn, 1991), we found that an increase in receptor density did not alter the magnitude of tolerance to morphine. In an attempt to assess if a reduction in receptor density plays a role in tolerance, it is necessary to decrease receptor density prior to the development of tolerance. Therefore, in the present study, we used clocinnamox, a μ-opioid receptor irreversible antagonist which has been shown to selectively decrease μ-opioid receptor density without altering affinity (Aceto et al., 1989; Burke et al., 1994; Chan et al., 1995; Comer et al., 1992; Zernig et al., 1994), to decrease receptor density before inducing tolerance. Tolerance was produced by infusion of fentanyl, a relatively μ-selective opioid receptor agonist.

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2. Materials and methods

2.1. Subjects

Male, Swiss-Webster mice (22–24 g) obtained from Taconic Farms (Germantown, NY, USA) were used throughout. The animals were maintained 5–10 per cage with free access to food and water and housed for at least 24 h prior to experimentation.

2.2. Procedure

Mice were injected i.p. with saline or clocinnamox (3.2 mg/kg). Four hours post-injection mice were implanted s.c. with an inert placebo pellet or an osmotic minipump containing fentanyl (0.165 mg/kg per day). Three days following implantation, pumps or pellets were removed, mice were weighed and a baseline tailflick latency determined, and 4 h later fentanyl dose–response studies were conducted using the tailflick assay (see below). Other mice were killed 4 h after the removal of osmotic minipump or pellet, whole brain removed and binding studies were conducted (see below).

2.3. Analgesia assay

Analgesia (antinociception) was determined using the tailflick assay in which a beam of light was focused on the dorsal tail surface approximately 2 cm from the tip of the tail. The intensity of the light was adjusted so that baseline flick latencies determined prior to fentanyl administration were 2–4 s. If a mouse failed to flick by 10 s following fentanyl administration, the test was terminated and mice were defined as analgesic. Mice were tested for analgesia 15 min following fentanyl. All testing was conducted in a blind manner.

2.4. Cumulative dose–response protocol

A cumulative dose–response protocol was used for all studies. All mice ($n = 8$/group/treatment) in a treatment group were injected s.c. with a starting dose of fentanyl (0.01 mg/kg) and tested for analgesia 15 min later. All mice that were not analgesic were given a second dose of fentanyl within 5 min of testing and then tested for analgesia again 15 min later. This cumulative dose–response protocol was continued until 100% of mice were analgesic (cumulative fentanyl dose range: 0.01–0.355 mg/kg). The actual fentanyl doses used in the cumulative dose–response protocol were determined in a previous study (Duttaroy and Yoburn, 1995). Each mouse was tested in only one dose–response study.

2.5. Brain opioid receptor binding

Binding studies were performed as described by Yoburn et al. (1989a). Mice ($n = 2$/group/treatment) were killed and whole brain rapidly removed, weighed and then homogenized in 80 volumes of ice-cold 50 mM Tris buffer (pH 7.4). Homogenates were then centrifuged at 15 000 rpm for 15 min at 4°C, the supernatant discarded and the pellet resuspended in buffer and centrifuged again. The pellet was resuspended in buffer and incubated in a shaking water bath for 30 min at 25°C. Homogenates were centrifuged a third time and finally resuspended in 20–80 volumes of 50 mM phosphate buffer (pH 7.2). An aliquot (200 μl) of homogenate was then assayed in triplicate in tubes containing 0.04–5.0 nM [^{3}H][D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (DAMGO). Nonspecific binding was determined in the presence of 1000 nM levorphanol. Homogenates were incubated for 90 min at 25°C. Incubation was terminated by the addition of ice-cold phosphate buffer and the samples were filtered over GF/B glass fiber filters with a cell harvester. Filters were washed three times with cold buffer, transferred to vials with scintillation cocktail and then counted in a liquid scintillation spectrophotometer. Counts per minute (cpm) were converted to desintegrations per minute (dpm) using the external standard method. Specific binding was the difference between binding determined in the absence of cold ligand and the presence of cold ligand. Protein was determined using a microassay technique based on the method of Bradford (1976) using reagent purchased from Bio-Rad (Richmond, CA, USA).

2.6. Drugs and drug administration

Fentanyl citrate was obtained from Sigma Chemical (St. Louis, MO, USA). Clocinnamox was generously supplied by Dr. James Woods (Department of Pharmacology, University of Michigan, Ann Arbor, MI, USA) and Dr. John Lewis (School of Chemistry, University of Bristol, Bristol, UK). The inert placebo pellets were obtained from Research Triangle Institute (Research Triangle Park, NC, USA) through the Research Technology Branch of the National Institute on Drug Abuse. Fentanyl citrate was dissolved in 0.9% saline and doses are expressed as free base. Clocinnamox was dissolved in dH_2O and doses expressed as the salt. All saline, fentanyl and clocinnamox injection volumes were 10 ml/kg. Pellets and osmotic minipumps (ALZET Model 2001, 1 μl/h; Alza, Palo Alto, CA, USA) were implanted s.c. at the nape of the neck. Pumps and pellets were implanted and removed while mice were lightly anesthetized with halothane/oxygen.

2.7. Data analysis

ED_{50} values were determined using Probit analysis (Finney, 1973) and were analyzed by Kruskal-Wallis ANOVA (analysis of variance) and post-hoc Newman-Keuls test. Saturation binding studies were evaluated using nonlinear regression to estimate B_{max} and K_d values. All

Table 1
Effect of clocinnamox and chronic fentanyl treatment on fentanyl analgesia and μ-opioid receptor binding

Treatment	ED_{50} (mg/kg)	Shift in ED_{50} (relative to saline-placebo)	Shift in ED_{50} (relative to clocinnamox-placebo)	B_{max} (fmol/mg protein)	Percent change in B_{max}
Saline-placebo	0.025 ± 0.003	–	–	161 ± 9	–
Saline-fentanyl	0.070 ± 0.035	2.8 [a]	–	147 ± 7	−9
Clocinnamox-placebo	0.059 ± 0.034	2.4 [a]	–	95 ± 23	−41 [a]
Clocinnamox-fentanyl	0.124 ± 0.039	5.0 [a]	2.1 [b]	81 ± 13	−50 [a]

Mice were injected i.p. with saline or clocinnamox (3.2 mg/kg) and 4 h later implanted s.c. with placebo or osmotic minipumps that infused fentanyl (0.165 mg/kg per day) for 3 days. Pumps or pellets were removed on the third day following implantation and 4 h later saturation binding studies ([^{3}H]DAMGO) or fentanyl analgesia dose-response studies were conducted. Data are means (±S.E.M.) of 4 independent binding and dose–response experiments. [a] Significantly different ($P < 0.05$) from saline-placebo. [b] Significantly different ($P < 0.05$) from clocinnamox-placebo.

binding data were best fit by a one-site model. Cumulated binding data were analyzed by ANOVA and post-hoc tests using Bonferroni's method.

3. Results

Clocinnamox treatment did not significantly alter baseline tailflicks compared to saline-treated mice (data not shown). Fentanyl infusion (0.165 mg/kg per day) for 3 days produced a significant 2.8-fold shift in fentanyl's analgesic potency ($P < 0.05$) with respect to the saline-placebo group (Table 1). Clocinnamox alone (3.2 mg/kg) significantly reduced the analgesic potency of fentanyl by 2.4-fold ($P < 0.05$). When fentanyl and clocinnamox were given together, a significant 5.0-fold reduction in fentanyl potency was observed relative to the saline-placebo group, which represents an additive effect of clocinnamox and fentanyl. In addition, the ED_{50} of the clocinnamox-fentanyl group was significantly shifted ≈2.1-fold relative to the clocinnamox-placebo group. This change in potency is comparable to the change in the ED_{50} (i.e., ≈2.8-fold) for the saline-fentanyl-treated mice compared to the saline-placebo group.

Fentanyl produced a small (−9%), but not statistically significant, reduction in B_{max}, while clocinnamox-treated mice had a significant (−41%) reduction with respect to the saline-placebo group (Table 1). In the clocinnamox-fentanyl group, there was a 50% reduction in B_{max}, which represents an additive effect of clocinnamox and fentanyl alone. Fentanyl and clocinnamox treatment did not significantly ($P > 0.05$) alter receptor affinity (overall mean = 0.43 ± 0.08 nM).

4. Discussion

The results of this study suggest that the magnitude of tolerance to fentanyl is independent of opioid receptor density. Tolerance to fentanyl was observed in both control animals and in mice that had been treated with clocinnamox and consequently had a significant reduction (−41%) in μ-opioid receptors. Furthermore, the magnitude of tolerance was similar in both clocinnamox-treated and saline-treated groups, with the ED_{50} of fentanyl in the clocinnamox-fentanyl group shifted 2.1-fold relative to the clocinnamox-placebo group, and the ED_{50} in the saline-fentanyl group shifted 2.8-fold relative to saline-placebo. In addition, when fentanyl and clocinnamox were combined, a significant 5.0-fold reduction in fentanyl potency was observed demonstrating an additive effect of clocinnamox and fentanyl. If tolerance was dependent upon receptor density then one might expect a change in the magnitude of tolerance following clocinnamox treatment. Since the degree of tolerance following fentanyl infusion was comparable in the saline- and clocinnamox-treated groups, tolerance to fentanyl appears to be independent of receptor density. However, it is important to note that tolerance requires receptor occupancy and that the infusion dose of fentanyl in the present study may have been sufficient to occupy the minimal number of receptors to produce a 2–3-fold shift in fentanyl potency for both control and clocinnamox-treated mice. Thus, if a higher dose of fentanyl was infused, or a drug of lower intrinsic efficacy was employed, such that greater receptor occupancy was required, then opioid receptor alkylation may increase the magnitude of tolerance.

In binding studies, fentanyl alone produced a small (−9%) decrease in the B_{max}, while clocinnamox-treated mice showed a significant reduction (−41%) in receptor density. It is worth noting that μ-opioid receptor density was probably very low immediately following clocinnamox (Burke et al., 1994; Chan et al., 1995) and was in the process of recovery at the time of binding studies in the present experiment. In the clocinnamox-fentanyl group, there was a 50% reduction in B_{max}, which is an additive effect of clocinnamox and fentanyl alone. Although only a small decrease in B_{max} was produced by fentanyl (0.165 mg/kg per day), higher doses of fentanyl can be used for producing greater magnitude downregulation, as shown in earlier studies (Yoburn et al., 1993).

It might be suggested that receptors not affected by

clocinnamox (i.e., δ-, κ-opioid receptors) may have mediated some of the effect of fentanyl. While it is not possible to entirely rule out that suggestion, fentanyl has been shown to be more that 100-fold selective for μ-opioid receptors than δ- and κ-opioid receptors (Yoburn et al., 1995; France et al., 1995; Maguire et al., 1992). Thus, it is likely that the tolerance observed in the present study was mediated via μ-opioid receptors.

These data, in combination with an earlier report (Lutfy and Yoburn, 1991), raise the possibility that neither a decrease nor an increase in receptor density prior to the development of tolerance impacts on the magnitude of tolerance. The present results extend our observation that an increase in receptor density induced by chronic opioid antagonist treatment does not change the degree of tolerance to morphine (Lutfy and Yoburn, 1991). Furthermore, the degree of tolerance to morphine was similar in two strains of mice that differed in sensitivity to morphine and density of μ-opioid receptors (Yoburn et al., 1989b). Taken together, these data suggest that receptor density prior to the induction of tolerance may not affect the magnitude of tolerance, although it remains to be determined if this suggestion holds for very low intrinsic efficacy opioids or higher infusion doses of fentanyl. It is noteworthy that the development of tolerance itself does not require concurrent changes in opioid receptors since treatments that produce tolerance and dependence do not necessarily alter binding (e.g., Loh et al., 1988; Puttfarcken and Cox, 1989; Tao et al., 1987, 1990; Yoburn et al., 1993). In summary, then, the critical mechanisms that produce tolerance do not appear to rely upon receptor density prior to the development of tolerance, or changes in receptor density that occur as tolerance develops and appear to depend upon intracellular mechanisms distal to the receptor.

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