Synergistic antinociceptive effects of alfentanil and propofol in the formalin test

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Abstract. The present study was conducted to determine the combined analgesic effect of alfentanil and propofol in the formalin test. Diluted formalin was injected into the dorsal surface of the right hind paw in rats. Nociceptive behavior was determined by counting the number of flinches of the injected paw for 1 h after injection; a reduction in formalin-induced flinching was interpreted as an antinociceptive effect. Isobolographic analysis was used to determine the type of antinociceptive interaction (additivity, antagonism or synergism). Extracellular signal-regulated kinase (ERK) and c-fos protein levels were also detected by western blot analysis to determine the potential mechanisms of the synergistic effect. Alfentanil, propofol or an alfentanil-propofol combination had an antinociceptive effect in the formalin test. The median effective dose (ED₅₀), value of the individual drug was also obtained. The derived theoretical ED₅₀ for the antinociceptive effect (4.36 mg/kg) was different from the observed experimental ED₅₀ value (2.51 mg/kg). The interaction between alfentanil and propofol produced the antinociceptive effect was synergistic according to isobolographic analysis. Furthermore, the combination of alfentanil and propofol treatments may produce synergistically antinociceptive effects by inhibiting the phosphorylation of ERK1/2 and decreasing the expression of c-fos in the spinal cord. These results demonstrated that combined treatment, with alfentanil and propofol, produced synergistic antinociceptive effects in the formalin test and may have therapeutic potential for the treatment of acute pain.

Introduction

Combinations of drugs that have similar effects have been employed clinically. Of the three types of drug interactions that can occur (synergism, additivity and antagonism), synergism is preferred as it allows lower doses of each drug to be used, which reduces the risk of any potential side effects is reduced (1). Clinically, combinations of opioid analgesics and intravenous anesthetics are used to establish balanced anesthe-sia and to reduce side effects, including earlier recovery and less postoperative nausea and vomiting. Opioids have long been used for the treatment of pain and are one of the most commonly prescribed drugs for pain management. They act through three receptors, termed μ, κ and δ opioid receptors. Opioid receptors are enabled by endogenously produced peptides, such as morphine, and opioid drugs, including fentanyl. Of these, the μ opioid receptor has an important role as the mediator of the majority of the effects of most clinically used opioids (2). Alfentanil is a short-acting synthetic opioid analgesic drug that targets μ opioid receptors and is ~4-fold faster than fentanyl in terms of the onset of effects (3). Opioid agonists are effective analgesic drugs and are irreplaceable in the treatment of inflammatory and severe pain (4).

Propofol is a widely used intravenous general anesthetic. It is reported to function at a specific site in γ-aminobutyric acid-A receptors (GABA_A,R) and functions as a positive allosteric modulator or an agonist (5). It is indistinct from the analgesic actions of propofol despite its anesthetic effects. Gilron et al (6) reported that propofol reduced hind paw formalin-induced expression of fos-like immunoreactivity in spinal neurons. The results of the study indicated the analgesic effect of propofol. Our previous research demonstrated the analgesic effects of propofol in several animal models (7). Sun et al (8) previously demonstrated the peripheral antinociceptive effect of propofol in an inflammatory pain model.

Extracellular signal-regulated kinase (ERK) is a mitogen-activated protein kinase (MAPK) subfamily member. It is activated in spinal dorsal horn neurons in response to injury and inflammation-induced hyperalgesia of the...
Formalin solution (5%; 50 µl) into the right hind paw, 24 rats were used as an experimental model of nociception and was performed as Formalin test. Sigma-Aldrich (Merck Millipore, Darmstadt, Germany). Unless otherwise stated, all other chemicals were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). C-fos antibody (cat. no. 2250; 1:1,000) were purchased from the desired concentrations of the drug. Rabbit p44 MAPK [phosphorylated ERK1,2 (pERK); Tyr204] antibody (cat. no. 4376; 1:1,000) and rabbit c-fos antibody (cat. no. 2250; 1:1,000) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Unless otherwise stated, all other chemicals were purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany).

**Formalin test.** The paw formalin test is a well-characterized experimental model of nociception and was performed as described previously (15). Following intraplantar injection of formalin solution (5%; 50 µl) into the right hind paw, 24 rats (n=6 per group) were placed in individual clear plastic cages (22x12x12 cm). Before the start of the experiments, the animals were acclimatized to the laboratory environment for at least one week. Flinching behavior was considered to be an expression of nociception. Time courses of antinociceptive responses resulting from the administration of different drugs were constructed by plotting the mean number of flinches as a function of time. The typical time course of the response to formalin is biphasic, with an early and short-lasting first phase followed, after a quiescent period, by a second, prolonged (tonic) phase. While phase I is considered to reflect acute nociceptive pain due to a direct stimulation of the nerve by formalin, phase II is attributed to the combination of ongoing inflammatory-associated afferent input from peripheral tissue and functional changes in the spinal dorsal horn (central sensitization) (16). Flinching was defined as rapid and brief withdrawal or flexing of the injected paw, lifting, licking and rubbing behavior. The time-response data were presented as the total number of flinches. To determine the ED50 values of each drug, the number of flinches was converted to the percentage of maximum possible effect (% MPE) according to the formula:

$$\text{MPE} (%) = 100 - \left( \frac{\text{Sum of flinching count with drug}}{\text{Sum of control flinching count}} \right) \times 100.$$
Temperature and incubated with the primary antibody specific for pERK1/2 or total ERK1/2 (1:1,000 dilution) and c-Fos (1:1,000 dilution). The membrane was washed with 0.05% TBS Tween buffer and incubated for 1 h with the secondary antibody conjugated with horseradish peroxidase (Goat anti-horseradish peroxidase; cat. no. 123-005-021; 1:1,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 h at room temperature and visualized in ECL solution (cat. no. 17050560; Bio-Rad Laboratories, Inc., Hercules, CA, USA), for 1 min, followed by film exposure for 1-10 min. The loading and blotting of equal amounts of proteins were verified by re-probing the membrane with antibody against β-actin (1:1,000; cat. no. sc-47778; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The intensity of each immunoblot assay band was quantified using a VersaDoc Imaging System (Bio-Rad Laboratories, Inc.). The experiments were repeated twice. Quantification of immunoreactivity corresponding to the total and phosphorylated bands was performed by densitometric analysis using Multi Gauge Version 3.0 (Fujifilm, Tokyo, Japan).

Statistical analysis. Results were presented as the mean ± standard error of the mean or as ED50 values with 95% CIs. The statistical significance of dose-responses was determined by one-way analysis of variance followed by the Tukey's post hoc test. Isobolographic calculations were performed by using the Pharm Tools Pro software (version no. 1.1.27, The McCary Group, Inc.). Statistical analysis of the isobolograms was performed as previously described (22) and differences between experimental and theoretical values were assessed by Student's unpaired t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Antinociceptive effect of alfentanil and propofol. Plantar injection of formalin produces nociceptive behavior, including flinches of the paw. Flinching was defined as rapid and brief withdrawal or flexing of the injected paw, lifting, licking and rubbing behavior. In the present study, the number of pain responses in 5 min intervals during phase I, for 20 min, and 10 min intervals during phase II, for 40 min, following formalin injection was recorded. saline-treated control rats exhibited discrete biphasic behavioral responses consisting of an early short-lasting response (phase I, 0-10 min post-injection), followed by a late, prolonged response (phase II, ~16-60 min post-injection). The duration of licking, lifting and rubbing were considered to be nociceptive behaviors in the formalin model. The mean number of flinches peaked around 0-10 min and 30-50 min after formalin intraplantar injection, which was followed by a gradual decline in all groups (Fig. 1A). Nociceptive behavior, the mean number of flinches, between saline and drug-treated groups was compared. There were no significant differences between any of the groups during phase II of the behavioral response. However, the amount of licking and lifting behavior was reduced in the alfentanil or propofol alone groups, and alfentanil-propofol combination group for the first 5 min after formalin injection, compared with the saline group during phase I (P<0.05; Fig. 1A). The total numbers of flinches during phase I and phase II following
formalin injection in the saline treatment group was 121.4±8.8 and 100.5±12.1, respectively. However, compared with the saline treatment group, the total number of flinches was significantly decreased in groups Alf (56.1±5.3), Pro (50.3±5.0) and Alf+Pro (33.2±4.6) during phase I (P<0.05; Fig. 1B). There were no significant differences between any of the groups during phase II.

Isobolographic analysis for drug combination. In phase I, alfentanil, propofol and alfentanil-propofol combination groups led to a dose-dependent antinociceptive effect. Linear regressions for alfentanil (Y=186.0X-24.7; R^2=0.9861), propofol (Y=153.7X-10.92; R^2=0.9728) and co-administration (Y=91.97X+8.55; R^2=0.9962) were calculated by plotting MPE (%) against the log dose (Fig. 2). The ED_{50} of individual administration of alfentanil and propofol were 25.3±2.21 µg/kg and 8.7±1.344 mg/kg, respectively (Table I). The isobologram was constructed by connecting the ED_{50} of alfentanil on the abscissa with the ED_{50} of propofol on the ordinate to obtain the additive line (Fig. 3). For the drug combination, the ED_{50 mix} and the 95% CI of the mixture were computed by linear regression of the log dose-response curve. The ED_{50 mix} and ED_{50 add} were plotted in the isobologram (Fig. 3). The fixed drug-dose ratio based on mass quantity for alfentanil and propofol is 1:344. The total ED_{50 mix} for the alfentanil-propofol combination is 2.51±0.56 mg/kg (Table I), representing 7.27 µg/kg alfentanil and 2.50 mg/kg propofol. By isobolographic analysis, the ED_{50 add}=4.36 mg/kg ([0.5xED_{50} alfentanil] + [0.5xED_{50} propofol]) (Table I), representing 12.64 µg/kg alfentanil and 4.35 mg/kg propofol. The ED_{50 mix} was <ED_{50 add} (Table I). The γ value was 0.57, which suggests a synergistic interaction between alfentanil and propofol during phase I of the formalin test (Table I).
Table I. ED$_{50}$ and interaction index of alfentanil, propofol, and alfentanil-propofol combination in the formalin test in rats.

<table>
<thead>
<tr>
<th>Drug group</th>
<th>ED$_{50}$ (confidence limits)</th>
<th>ED$_{50}$ add</th>
<th>ED$_{50}$ mix</th>
<th>γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alfentanil (µg/kg)</td>
<td>25.3±2.21 (23.11-27.51)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Propofol (mg/kg)</td>
<td>8.73±1.34 (7.36-10.04)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Alfentanil + propofol (mg/kg)</td>
<td>NA</td>
<td>4.36</td>
<td>2.51±0.56$^a$</td>
<td>0.57</td>
</tr>
</tbody>
</table>

$^a$P=0.03 between ED$_{50}$ add and ED$_{50}$ mix, in corresponding nociceptive model indicates a synergistic interaction. ED$_{50}$ values were obtained following intravenous drug/drug combination administration in the formalin test. ED$_{50}$=Effective dose required to produce 50% antinociceptive activity. γ=ED$_{50}$ alfentanil combined with propofol/ED$_{50}$ alfentanil given alone + ED$_{50}$ propofol combined with propofol/ED$_{50}$ propofol given alone. Values >1 indicate additive interaction, values >1 indicate an antagonistic interaction and values <1 indicate a synergistic interaction. ED$_{50}$, median effective dose; SEM, standard error of the mean; ED$_{50}$ add, theoretical additive ED$_{50}$ for drug mixture; ED$_{50}$ mix, experimental ED$_{50}$ for drug mixture; γ, interaction index; NA, not applicable.

Figure 3. Isobologram for alfentanil-propofol combination treatment in the formalin test. The ED$_{50}$ values for each drug are plotted at the axes. The straight line that connects each ED$_{50}$ value is the theoretical additive line and the point highlighted on this line is the ED$_{50}$ add. There is a significant difference (P=0.02) between the ED$_{50}$ add and the ED$_{50}$ mix, calculated by Student’s t-test, indicating a synergistic drug interaction between alfentanil and propofol when co-administered. ED$_{50}$ median effective dose; ED$_{50}$ add, theoretical additive ED$_{50}$ for drug mixture; ED$_{50}$ mix, experimental ED$_{50}$ add.

**Discussion**

The major finding of the current study was that the combination of alfentanil and propofol led to synergistic antinociceptive effects in the formalin test. Subcutaneous hind paw injection of formalin triggers biphasic nociceptive response. While phase I is considered to reflect acute nociceptive pain due to direct stimulation of the nerve by formalin, phase II is attributed to the combination of ongoing inflammatory-associated afferent input from peripheral tissue and functional changes in the spinal dorsal horn (central sensitization) (24). Accordingly, the present study investigated the inhibitory effects of alfentanil or propofol on the number of flinches in a given time following formalin injection. Flinches were considered to indicate a nociceptive response. Injection of formalin led to persistent inflammatory pain throughout the test. The current study observed that nociceptive behavior during phase I, but not phase II, was reduced when treated with alfentanil, propofol or both. Furthermore, it was established that combined treatment with alfentanil and propofol led to synergistic antinociceptive effects. The present study calculated the ED$_{50}$ mix of the alfentanil-propofol combination in phase I of the formalin test. The ED$_{50}$ for treatment with alfentanil or propofol alone were calculated as 25.3±2.21 µg/kg and 8.7±1.344 mg/kg, respectively. The ED$_{50}$ mix was significantly less than their corresponding ED$_{50}$ add, and the calculated interaction index (γ) was <1. The results of this study initially demonstrated the synergistic interaction between alfentanil and propofol in the formalin test. Notably, the ED$_{50}$ of propofol in the formalin test was less than its ED$_{50}$ for clinical application, suggesting that the synergistic antinociceptive effect may potentially be beneficial for its use in clinic for pain treatment.
A previous study reported that propofol at sub-hypnotic dosage (0.25 mg/kg) reduced acute pain induced by argon laser stimulation in humans (24). Furthermore, a study that involved healthy volunteers suggested that propofol delivered intravenously, at 0.25 mg/kg followed by 25 µg/kg/min or more, led to a reduction in pain intensity (25). Additionally, another study demonstrated that intravenously administered propofol (0.25-0.5 mg/kg) depressed pain induced by tibial pressure algiesimetry in patients who had been through gynecologic surgery (26). However, one study concluded that propofol (0.5 mg/kg) did not affect thermal pain detection thresholds (27). In the current study, the ED₅₀ of propofol was calculated to be 8.7±1.344 mg/kg in a formalin test performed on rats. Propofol is a commonly used intravenous general anesthetic that acts on GABAₐR and enhances the action of GABA. It is indistinct from the analgesic actions of propofol despite its profound anesthetic effects. Goto et al (28) concluded that propofol had no effect on phase II nociceptive behavioral responses induced by formalin injection in the hind paw of rats. However, Gitron et al (6), reported that propofol reduced hind paw formalin-induced expression of fos-like immunoreactivity in spinal neurons. Their results indicated the analgesic effect of propofol. Antinociceptive effects of propofol have previously been reported in humans (24). It is suggested, based on in vivo studies, that propofol reduced pain in rats via spinal GABA₀Rs (29,30). Alfentanil is an analgesic that acts as an agonist of µ opioid receptors and it is used to relieve acute pain or the severe, chronic and disabling pain associated with terminal conditions, including cancer and degenerative conditions, which include rheumatoid arthritis. The analgesic effects of alfentanil and propofol are regulated through different receptors at the level of the spinal cord. However, the synergistic antinociceptive effects of their combined treatment and the underlying molecular mechanisms of alfentanil and propofol in acute nociceptive pain remain unclear. The current study demonstrated that the synergistic antinociceptive action of alfentanil and propofol in the formalin test is regulated by ERK₁/₂ and c-fos, as described above (Figs. 4 and 5) (9,10). Further studies are required to investigate the mechanism in further detail.
In summary, the present research suggests synergism between alfentanil and propofol. These results provide evidence for the potential benefits that the development of synergistic drug combinations of opioid analgesics with intravenous anesthetics may have if applied clinically. The combination of alfentanil with propofol may prove beneficial for the treatment of pain, including neuropathic or anti-inflammatory disease. Furthermore, the combination of alfentanil and propofol treatments may produce synergistically antinociceptive effects through inhibition of pERK1/2 and decreased expression of c-fos in the spinal cord.

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References