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Short communication

Inhibitory effect of fentanyl citrate on the release of endothlin-1 induced by bradykinin in melanoma cells

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ABSTRACT

Background: Our previous study showed that the μ-opioid receptor agonist fentanyl citrate inhibits endothelin-1-and bradykinin-mediated pain responses in mice orthotopically inoculated with melanoma cells. We also demonstrated that bradykinin induces endothelin-1 secretion in melanoma cells. However, the analgesic mechanisms of fentanyl citrate remain unclear. Thus, the present study was conducted to determine whether fentanyl citrate affects bradykinin-induced endothelin-1 secretion in B16-BL6 melanoma cells.
Methods: The amount of endothelin-1 in the culture medium was measured using an enzyme immunoassay. The expression of endothelin-1, kinin B_2 receptors, and μ-opioid receptors in B16-BL/6 melanoma cells was determined using immunocytochemistry.
Results: Fentanyl citrate inhibited bradykinin-induced endothelin-1 secretion. The inhibitory effect of fentanyl citrate on the secretion of endothelin-1 was attenuated by the μ-opioid receptor antagonist naloxone methiodide. The immunoreactivities of endothelin-1, kinin B_2 receptors, and μ-opioid receptors in B16-BL6 melanoma cells were observed.
Conclusion: These results suggest that fentanyl citrate regulates bradykinin-induced endothelin-1 secretion through μ-opioid receptors in melanoma cells.
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Introduction

Fentanyl is a μ-opioid receptor agonist [1] that is widely used for pain relief in cancer patients [2]. Intravenous injection of fentanyl citrate inhibits pain responses (such as spontaneous licking and mechanical allodynia) in mice orthotopically inoculated with melanoma cells, and this analgesic effect is due to its peripheral action [3]. Therefore, it is suggested that systemic fentanyl citrate acts not only in the central nervous system but also in peripheral tissues. However, the mechanisms of the peripheral analgesic effect of fentanyl citrate remain unclear.

Mice given orthotopic inoculation of melanoma cells (B16-BL6 melanoma cells) show spontaneous licking behaviour through kinin receptors (B_1 and B_2) [4] and endothelin ET_A receptors [5]. Tumor cells produce several pain-related factors, such as bradykinin and endothelin-1 [6]. B16-BL6 melanoma cells also produce bradykinin [4], its related peptides [4], and endothelin-1 [5]. In addition, bradykinin increases the secretion of endothelin-1 through kinin B_2 receptors, but not kinin B_1 receptors, in B16-BL6 melanoma cells [7]. These findings suggest that bradykinin-induced endothelin-1 secretion is involved in the peripheral mechanisms of cancer pain. In this study, therefore, we investigated whether fentanyl citrate inhibits bradykinin-induced endothelin-1 secretion in B16-BL6 melanoma cells.

Materials and methods

Cell culture

B16-BL/6 melanoma cells, derived from the C57BL/6 mouse, were cultured in Eagle's minimum essential medium (Wako Pure Chemical Industries Ltd., Osaka, Japan) containing 10% foetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2 in a 24-well plate. Cells were used at less than 90% confluence.

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Reagents

Bradykinin was purchased from Peptide Institute, Inc. (Osaka, Japan). Fentanyl citrate and naloxone methiodide were purchased from Daiichi Sankyo Propharma Co., Ltd. (Tokyo, Japan) and Sigma-Aldrich (St. Louis, MO, USA), respectively. Fentanyl citrate and naloxone methiodide were applied 10 and 20 min before the application of bradykinin, respectively.

Enzyme immunoassay for determining endothelin-1

The culture medium of melanoma cells was collected 30 min after the application of bradykinin. The melanoma cells remaining in the wells were used for the determination of protein concentration. Endothelin-1 secretion in the medium was determined using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA). Protein was extracted from the cultured cells with 1% triton X-100 solution and protein concentration was determined with a protein assay reagent (Bio Rad, Hercules, CA, USA). The secretion of endothelin-1 was normalized with protein extracted from cells.

Immunocytochemistry

B16-BL6 cells cultured in a glass-bottom culture dish were fixed with 4% paraformaldehyde. After washing with phosphate-buffered saline (PBS) three times, the cells were treated with PBS containing 0.3% triton X-100 and 1% foetal bovine serum. The cells were incubated with anti-bradykinin B_2 receptor antibody (1:100; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-endothelin antibody (1:100; ABBIOTEC, San Diego, CA, USA), or anti-μ-opioid receptor antibody (1:100; Santa Cruz Biotechnology) at 4 °C overnight. The cells were washed with PBS and were reacted with anti-rabbit or anti-goat IgG antibody conjugated with Alexa 488 (Molecular Probes, Eugene, OR, USA). The signal was detected using a fluorescent microscope (Olympus Corporation, Tokyo, Japan).

Data processing

The data represent as means and standard error of the mean (SEM). Statistical significance was analysed using one-way analysis of variance followed by a *post-hoc* Student-Newman-Keuls test. A *p*-value of less than 0.05 was considered statistically significant.

Results

Effects of fentanyl citrate on bradykinin-induced endothelin-1 secretion in B16-BL6 melanoma cells

Endothlin-1 was spontaneously secreted from cultured B16-BL6 melanoma cells (Fig. 1). Bradykinin (100 nM) significantly increased the secretion of endothelin-1, compared with non-treated cells: (endothelin-1 from non-treated cells) 0.33 ± 0.04 pg/mg protein, (endothelin-1 from cells treated with bradykinin) 0.56 ± 0.03 pg/mg protein (Fig. 1). Bradykinin-induced endothelin-1 secretion was inhibited dose-dependently by fentanyl citrate (0.02 and 20 μg/ml), compared with endothelin-1 secretion in cells treated with bradykinin alone: (endotelin-1 from cells treated with fentanyl citrate (0.02 μg/ml) and bradykinin) 0.49 ± 0.03 pg/mg protein, (endotelin-1 from cells treated with fentanyl citrate (0.2 μg/ml) and bradykinin) 0.34 ± 0.02 pg/mg protein, (endotelin-1 from cells treated with fentanyl citrate (2 μg/ml) and bradykinin) 0.32 ± 0.03 pg/mg protein, (endotelin-1 from cells treated with fentanyl citrate (20 μg/ml) and bradykinin) 0.31 ± 0.05 pg/mg protein (Fig. 1).

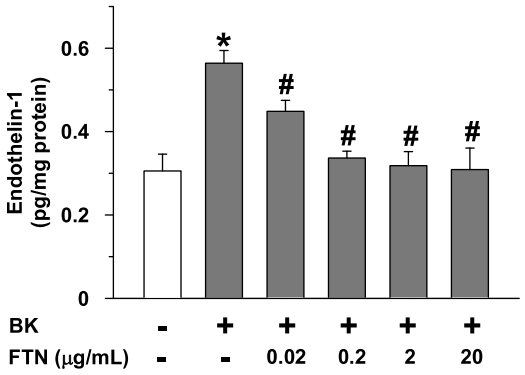

Fig. 1. Effects of fentanyl citrate on bradykinin-induced endothelin-1 secretion in B16-BL6 melanoma cells. The cells were incubated with (closed columns) or without (open columns) 100 nM bradykinin (BK). Fentanyl citrate (FTN) was applied 10 min before BK. Endothelin-1 secretion in the culture medium, which was collected 30 min after incubation with BK, was determined by enzyme immunoassay. Data are presented as mean ± standard error of the mean (n = 5–6). $^{*}p < 0.05$ as compared with the control (non-treated cells), $^{\#}p < 0.05$ as compared with BK only (Student-Newman-Keuls test).

Effects of naloxone methiodide on the inhibitory effect of fentanyl citrate on bradykinin-induced endothelin-1 secretion in B16-BL6 melanoma cells

Bradykinin (100 nM) increased significantly endothelin-1 secretion, compared with compared with non-treated cells: (endothelin-1 from non-treated cells) 0.33 ± 0.053 pg/mg protein, (endothelin-1 from cells treated with bradykinin) 0.74 ± 0.09 pg/mg protein (Fig. 2). The bradykinin-induced endothelin-1 secretion was inhibited significantly by fentanyl citrate (20 μg/ml): (endothelin-1 from cells treated with fentanyl citrate (20 μg/ml) and bradykinin) 0.23 ± 0.05 pg/mg protein (Fig. 2). The inhibitory effect of fentanyl citrate (20 μg/ml) was attenuated markedly by naloxone methiodide (40 μg/ml), but not naloxone methiodide (20 μg/ml): (endothelin-1 from cells treated with naloxone methiodide (20 μg/ml), fentanyl citrate (20 μg/ml) and bradykinin) 0.32 ± 0.07 pg/mg protein, (endothelin-1 from cells treated with naloxone methiodide (40 μg/ml), fentanyl citrate (20 μg/ml) and bradykinin) 0.58 ± 0.07 pg/mg protein (Fig. 2).

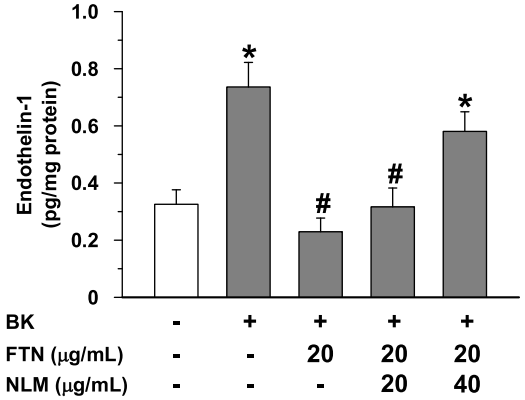

Fig. 2. Effects of naloxone methiodide against the inhibitory effect of fentanyl citrate on bradykinin-induced endothelin-1 secretion in B16-BL6 melanoma cells. The cells were incubated with (closed columns) or without (open columns) 100 nM bradykinin (BK). Fentanyl citrate (FTN) and naloxone methiodide (NLM) were applied 10 and 20 min before the administration of BK, respectively. Endothelin-1 secretion in the culture medium, which was collected 30 min after the application of BK, was determined by enzyme immunoassay. Data are presented as the mean ± standard error of the mean (n = 4–5). $^{*}p < 0.05$ as compared with the control (non-treated cells), $^{\#}p < 0.05$ as compared with BK only Student-Newman-Keuls test).

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