A New Side-effect of Sufentanil: Increased Monocyte-endothelial Adhesion

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Abstract

**Background:** Opioids have been identified by the World Health Organization to be ‘indispensable for the relief of pain and suffering’. Side-effects, such as nausea, vomiting, postoperative delirium, and effects on breathing, of opioids have been well investigated; however, the influence of opioids on monocyte-endothelial adherence has never been reported. Therefore, we explored the effects of representative opioids, fentanyl, sufentanil, and remifentanil, on monocyte-endothelial adherence and the underlying mechanisms.

**Methods:** We built a cell adhesion model with U937 monocytes and human umbilical vein endothelial cells (HUVECs). Two kinds of connexin43 (Cx43) channel inhibitors, 18-α-GA and Gap 27, were used to alter Cx43 channel function in U937 monocytes and HUVECs, respectively, to determine the effects of Cx43 channels on U937-HUVEC adhesion. Subsequently, the effects of fentanyl, sufentanil and remifentanil on Cx43 channel function and U937-HUVEC adhesion were explored.

**Results:** When fentanyl, sufentanil and remifentanil acted on monocytes or endothelial cells, their effects on monocyte-endothelial adherence differed. When acting on U937 monocytes, sufentanil significantly increased U937-HUVEC adhesion via the inhibition of Cx43 channels in U937 monocytes (the mechanism was related to Cx43 channels modulating ATP release), while fentanyl and remifentanil did not have these influences. Although sufentanil could also inhibit Cx43 channel function in HUVECs, it had no effect on ATP release from HUVECs or U937-HUVEC adhesion.

**Conclusions:** We demonstrated that sufentanil application increases monocyte-endothelial adherence via the inhibition of ATP release mediated by Cx43 channels in monocytes. This side-effect of sufentanil should be considered seriously by clinicians.

Background

Opioids have been identified by World Health Organization to be ‘indispensable for the relief of pain and suffering’ [1, 2]. Opioids act on opioid receptors to produce morphine-like effects and are commonly used for the control of clinical pain in patients with cancer or undergoing surgery. These patients can experience pain from spending long periods in the supine position and are prone to hemodynamic changes. Under these circumstances, monocytes flowing in blood vessels easily adhere to inflamed or damaged vascular endothelial cells [3]. Monocyte-endothelial adherence plays an important part in the initial stages of inflammatory vascular diseases [4]. Adherent monocytes not only damage the vascular endothelium directly but also cause the release of inflammatory factors and chemoattractants indirectly. This process is continuously self-reinforcing, eventually resulting in vascular damage and deterioration, thrombosis formation, and even development of atherosclerosis. Therefore, we believe that monocyte-endothelial adherence is a pre-requisite for vascular damage [5].

In contemporary society, opioids are not only extensively used for the control of cancer pain [6] but also widely used in anaesthesia and postoperative analgesia for surgical patients [7]. In studies on the side-
effects of opioids, researchers pay extensive attention common symptoms such as nausea, vomiting, postoperative delirium, and breathing effects; however, the effect of opioids on monocyte-endothelial adherence has never been reported [8-10].

Connexin43 (Cx43), which belongs to a transmembrane protein family known as connexin, has been reported to be associated with monocyte-endothelial adherence [5, 11]. Cx43 forms gap junction channels that mediate cytosolic signalling molecules movement between neighbouring cells. The gap junctions are composed of two hemichannels, which dock together end-to-end [12]. Hemichannels can exist unopposed in plasma membranes and have various functions, such as ATP release [13]. Furthermore, extracellular ATP can be rapidly metabolized to adenosine (ADO), which has well-known anti-inflammatory effects that decrease monocyte-endothelial adherence via A2B receptors [14].

Therefore, for the first time, we investigated the effects of fentanyl, sufentanil and remifentanil (as representative opioids) on monocyte-endothelial adherence, as well as the underlying mechanisms. We determined whether these opioids influence monocyte-endothelial adherence via ATP release mediated by Cx43 channels. We found that sufentanil, but not fentanyl or remifentanil, enhances monocyte-endothelial adherence via the inhibition of Cx43 channel function on monocytes. This side-effect of sufentanil should be considered seriously by clinicians. We believe that these results provide new insights into the rational use of narcotic drugs.

Methods

Cell cultures

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Medical Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University.

The human umbilical vein endothelial cells (HUVECs) and U937 monocytes used in this study were both purchased from American Type Culture Collection (Manassas, VA, USA). HUVECs were cultured in human endothelial serum-free medium (Invitrogen, Carlsbad, CA, USA) with 20% fetal bovine serum (Invitrogen), 100 U/ml penicillin-streptomycin (Invitrogen), 100 μg/ml heparin (Sigma-Aldrich, St. Louis, MO, USA), and 150 μg/ml endothelial cell growth supplement (Becton, Dickinson and Company, Frankin Lakes, NJ, USA). U937 monocytes were cultured in RPMI1640 medium (Invitrogen), which contains 20% fetal bovine serum (Invitrogen) and 100 U/ml penicillin-streptomycin (Invitrogen). HUVECs and U937 monocytes were both cultured in an incubator (5% CO₂, 37°C, and 90% humidity) (Thermo Fisher Scientific, Waltham, MA, USA).

Cell counting kit-8 assay
Cell vitality was detected using cell counting kit-8 kit assays (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), according to the manufacturer’s instructions.

**Cell treatments**

According to the requirements of each experiment, HUVECs and U937 monocytes were pre-treated with different chemicals, including 18-α-GA (a connexin channel inhibitor; 50 μM, for 1 hour; Sigma-Aldrich) and Gap 27 (a connexin mimetic peptide that inhibits Cx43 channel function; 300 μM, for 1 hour; Sigma-Aldrich); α, β-methylene ADP (APCP; a CD73 inhibitor; 300 μM, for 1 hour; Sigma-Aldrich), exogenous ATP (200 μM, for 1 hour; Sigma-Aldrich), and ADO (100 μM, for 1 hour; Sigma-Aldrich); fentanyl (10 μg/ml, for 24 hours; Yichang Humanwell Pharmaceutical Co., LTD, Yichang, Hubei, China), sufentanil (25 ng/ml, for 24 hours; Yichang Humanwell Pharmaceutical Co., LTD), and remifentanil (50 ng/ml, for 24 hours; Yichang Humanwell Pharmaceutical Co., LTD).

**Adhesion assay**

U937-HUVECs adhesion was detected according to procedures described previous studies: U937 monocytes were first labelled with calcein-acetoxymethyl ester (5 μM, Invitrogen) for 30 min in the incubator. The labelled U937 monocytes were then washed twice and resuspended in a serum-free medium. The labelled U937 monocytes were counted and poured onto confluent HUVEC monolayers, which had been pre-treated for 12 hours with recombinant mouse tumour necrosis factor α (10 ng/mL; Peprotech, Rocky Hill, NJ, USA). The plates were incubated for 1 hour and then rinsed twice slightly with the serum-free medium. Adherent U937 monocytes remained on HUVECs and were counted with a fluorescence microscope (Olympus IX71, Tokyo, Japan). Eight different 200× visual fields in each well were selected for analysis [3].

**ATP and ADO release detection**

ATP release was detected with ATP bioluminescence assay kits (Sigma-Aldrich). The supernatants of HUVEC and U937 monocyte cultures were harvested on ice. One hundred microliters of supernatant were added to 100 μl of ATP assay mix solution in 96-well culture plates. The luminescence was read by a fluorospectrophotometer (Cary Eclipse, FL0811M005; bio/chemi-luminescence mode). The ADO content was detected using related ELISA kits (Xinyu Biotechnology, Shanghai, China), according to the manufacturer’s instructions [4].

**Protein detection**

Cx43 expression was detected with western blotting. Proteins samples were quantified with Pierce™ BCA Protein assay kits (Thermo Fisher Scientific, Inc.). The protein sample (25 μg) was added into SDS-PAGE,
and then transferred onto a polyvinylidene fluoride membrane. After blocking with 5% milk for 1 hour at room temperature, the membranes were incubated with Cx43 antibody overnight at 4°C (anti-Cx43; 1:3000; Cat: SAB4501174, Sigma-Aldrich) and anti-β-tubulin for 1 hour (1:10000; Cat: T4026, Sigma-Aldrich). Protein band sizes were estimated with Alpha View software (version number: 2.2.14407, Protein Simple, Santa Clara, CA, USA). The original blots were showed in Supplementary Figure 1.

**Parachute dye-coupling assay**

Parachute dye-coupling assays were used to detect gap junction function in HUVECs. HUVECs were grown to confluence. Donor cells were first labelled with calcein-AM (5 μM) in the incubator for 30 minutes. According to a donor:receiver ratio of 1:150, donor cells were seeded onto receiver cells. After 4 hours, the results were observed with a fluorescence microscope (Olympus DP73, Tokyo, Japan). The average number of receiver cells around every donor cell was counted; this reflected the function of Cx43 channels [15].

**Statistical analysis**

Statistical analysis was performed using SPSS 15.0 software (SPSS, Inc., Chicago, IL, USA). Multiple comparisons among groups were performed using repeated-measures one-way analyses of variance, followed by Tukey post hoc comparisons.

**Results**

**Cx43 expressed on monocytes modulated U937-HUVECs adhesion via ATP release**

As previously reported, 18-α-GA (a Cx43 channel inhibitor) and Gap 27 (a connexin mimetic peptide) effectively attenuated Cx43 channel function [4, 16]. As shown in Figures 1a-c, when U937 monocytes were pre-treated with 18-α-GA or Gap 27 for 1 hour, there was no effect on U937 survival or Cx43 expression (Fig. 1a, b); however, U937-HUVECs adhesion was increased significantly (Fig. 1c), indicating that inhibiting the function of Cx43 channels in U937 monocytes resulted in monocyte-endothelial adherence deterioration.

Cx43 channels are known to be permeable to ATP [17], and ATP can be rapidly metabolized to ADO by extracellular enzymes. Additionally, ADO has well-known anti-inflammatory effects that decrease monocyte-endothelial adherence by interacting with A2B receptors [18]. Therefore, we investigated the involvement of ATP release from U937 monocytes via Cx43 channels in the regulation of U937-HUVECs adhesion. As shown in Figures 1d and e, the inhibition of Cx43 channels in U937 monocytes via 18-α-GA and Gap 27 administration attenuated ATP release from U937 monocytes, as well as the ADO content. Thus, important factors that resist monocyte-endothelial adherence were weakened.
Extracellular enzymes known to be involved in conversion of ATP to ADO are CD39 (converting ATP to AMP) and CD73 (converting AMP to ADO). This pathway is robust in endothelial cells. To confirm the function of ADO on U937-HUVEC adhesion, we used APCP (a competitive inhibitor of CD73) to inhibit the production of ADO from ATP \[19\]. APCP application on U937 monocytes resulted in the reduction of ADO, and subsequently caused a significant increase in U937-HUVEC adhesion (Fig. 1f, g). These results strongly suggested that ADO production from endogenously released ATP had a potent anti-adhesive effect. In order to confirm this conclusion, we supplied exogenous ATP and ADO to reverse the effects of 18-α-GA and Gap 27 on U937-HUVEC adhesion. As shown in Figure 1h, the application of exogenous ATP and ADO reduced the increase of U937-HUVEC adhesion provoked by 18-α-GA and Gap 27 administration, demonstrating the effects of ATP and ADO on monocyte-endothelial adherence from another viewpoint.

**Cx43 expressed on HUVECs had no effect on U937-HUVEC adhesion**

Cx43 is also expressed on HUVECs; therefore, we investigated the effects of Cx43 expressed on HUVECs on monocyte-endothelial adherence. As shown in Figures 2a-c, 18-α-GA and Gap 27 pre-treatment on HUVECs had no influence on HUVEC survival or Cx43 expression, but they attenuated dye coupling between HUVECs, indicating that Cx43 channel function was reduced. Although 18-α-GA and Gap 27 pre-treatment inhibited Cx43 channel function in HUVECs, both agents had no effect on U937-HUVEC adhesion (Fig. 2d). We speculated that this contradictory phenomenon was because the ATP or ADO released from HUVECs was not changed (Fig 2e, f). When we supplemented exogenous ATP and ADO, U937-HUVEC adhesion was decreased. The application of APCP on HUVECs caused an increase in U937-HUVEC adhesion, because APCP inhibited ADO production from ATP (Fig. 2g). These results suggest that Cx43 expressed on HUVECs did not modulate monocyte-endothelial adherence via ATP release.

**Effects of fentanyl, sufentanil, and remifentanil on U937-HUVEC adhesion**

Fentanyl, sufentanil, and remifentanil are commonly used in the clinic for patients with cancer or undergoing surgery; however, their effects on monocyte-endothelial adherence are unknown. We found that the effects of these opioids on monocyte-endothelial adherence differed, depending on whether they acted on monocytes or endothelial cells. When acting on U937 monocytes, sufentanil significantly increased U937-HUVEC adhesion; in contrast, fentanyl and remifentanil did not have this effect (Fig. 3a). However, when acting on HUVECs, none of these analgesics influenced U937-HUVEC adhesion (Fig. 3b).

**Sufentanil attenuated Cx43 channel function in HUVECs but had no effect on ATP or ADO release**
U937-HUVECs adhesion is regulated by ATP release via Cx43 channels, as shown in Figure 1; the effects of fentanyl, sufentanil, and remifentanil on ATP release from HUVECs are shown in Figure 4. Although sufentanil inhibited Cx43 channel function in HUVECs (without affecting HUVEC survival or Cx43 expression) (Fig. 4a-c), it had no influence on ATP or ADO release from HUVECs (Fig. 4d, e). This might be the reason why sufentanil had no effect on U937-HUVECs adhesion when acting on HUVECs, even though it could inhibit Cx43 channel function on HUVECs. Fentanyl and remifentanil had no effects on Cx43 expression, Cx43 channel function in HUVECs, and ATP and ADO release (Fig. 4).

Sufentanil, but not fentanyl or remifentanil, attenuated ATP and ADO release from U937 monocytes, affecting U937-HUVEC adhesion

Figures 5a and b show that fentanyl, sufentanil, and remifentanil had no effects on U937 survival and Cx43 expression; however, sufentanil application obviously attenuated ATP and ADO release from U937 monocytes, while fentanyl and remifentanil did not show these effects (Fig. 5c, d).

The pre-treatment of U937 monocytes with exogenous ATP or ADO reversed the increase in U937-HUVEC adhesion induced by treatment with sufentanil (fentanyl and remifentanil themselves did not affect U937-HUVEC adhesion, but exogenous ATP and ADO also attenuated U937-HUVEC adhesion). When APCP was used to inhibit ADO production from ATP, the anti-adhesion effect of ATP disappeared (Fig. 5e-g), suggesting that ATP converting to ADO played an important part in the anti-adhesion effect.

Discussion

Opioids have been used for many years in clinical practice, especially for cancer pain management, anaesthesia, and postoperative analgesia [20, 21]. Studies on the side effects of opioids have mainly focused on nausea, vomiting, postoperative delirium, and breathing effects, and the effects of opioids on monocyte-endothelial adherence are unknown [22]. For the first time, we investigated the effects of representative opioids, including fentanyl, sufentanil, and remifentanil, on monocyte-endothelial adherence. The present study showed that when these three opioids acted on monocytes or endothelial cells, their effects on monocyte-endothelial adherence differed. When acting on U937 monocytes, sufentanil significantly increased U937-HUVEC adhesion by inhibiting Cx43 channels expressed on U937 monocytes (the mechanism of which was related to Cx43 channels modulating ATP release). In contrast, fentanyl and remifentanil had no influence on U937-HUVECs adhesion or ATP release. Although sufentanil could also inhibit Cx43 channel function in HUVECs, it had no effect on ATP release from HUVECs, as well as U937-HUVEC adhesion, further indicating that ATP release might be the main reason Cx43 channels can regulate U937-HUVECs adhesion.

Under normal physiological conditions, the interaction of flowing monocytes with vascular endothelial cells is minimal. However, circulating monocytes are prone to adherence to vascular endothelial cells
under pathological conditions, especially when the vascular endothelial cells are inflamed and damaged [23, 24]. There are numerous risk factors, such as surgical stimulation, inflammation and vascular injury induced by surgery, long durations in the supine position, and chemotherapies, that could result in monocyte-endothelial adherence in patients with cancers or undergoing surgery [25-27]. However, the effects of analgesics on monocyte-endothelial adherence have not been previously reported. Fentanyl, sufentanil and remifentanil are all widely used for different kinds of pain control, not only for surgical patients, but also for patients with cancer. Their side-effects on monocyte-endothelial adherence should be considered by clinicians.

Fentanyl, sufentanil and remifentanil selectively target the µ-opioid receptor, which belongs to the G protein-coupled receptor family [28]. Conformational flexibility is one of the most essential characteristics of G protein-coupled receptors, which are involved in ligand recognition and subsequent activation or inactivation [29]. Compared to the other two opioids, sufentanil is the agonist with the highest affinity toward µ-opioid receptors. This may explain why sufentanil can interact with µ-opioid receptors and activate the downstream signalling pathways of G proteins, but fentanyl and remifentanil cannot [30, 31]. This might also be the reason why sufentanil could alter Cx43 channel function, while fentanyl and remifentanil did not have this kind of effect. Certainly, this hypothesis should be confirmed in future studies.

The current study revealed an interesting phenomenon: depending on whether Cx43 channel function in U937 monocytes or HUVECs was altered, its effects on ATP release were different. When Cx43 channel function in U937 monocytes was inhibited, ATP release was attenuated; in contrast, altering function of Cx43 channels expressed on HUVECs did not affect ATP release. As previously reported, Cx43 exist as unopposed hemichannels in plasma membranes of U937 monocytes, where they play a role in many different functions, such as ATP release [32]. In contrast, on HUVECs, Cx43 exist as integral channels between endothelial cells, mediating cytosolic signalling molecule movement between neighbouring cells, and ATP release is not integral to these Cx43 functions [33]. That might be the reason why the inhibitors, 18-α-GA and Gap 27, inhibited Cx43 channel function, but had no effect on ATP release in HUVECs (Fig. 2a-d).

The mechanism of ATP release mediated by Cx43 hemichannels has been well clarified. ATP is rapidly metabolized to ADO by extracellular enzymes; ADO has well-known anti-inflammatory effects that decrease monocyte-endothelial adherence through interaction with A2B receptors. The enzymes involved are CD39, which converts ATP to AMP, and CD73, which converts AMP to ADO. From the available evidence, this pathway, which protects against cell adhesion, is robust in endothelial cells [34, 35]. As previously reported, in addition to Cx43 channels, both connexin37 (Cx37) channels and pannexin1 are potential pathways for ATP release. However, among all connexin channels, homomeric Cx37 channels are the most size-restrictive in terms of being impermeable to Lucifer yellow, Alexa 488, and 6-carboxyfluorescein and are only weakly permeable to some smaller molecules, such as NBD-MTMA and Alexa 350 [36, 37]. Therefore, we favour the idea that ATP release from monocytes is via channels formed wholly and/or partially by Cx43 (homomeric Cx43 and/or heteromeric Cx43/Cx37 channels). All available
facts indicate that Cx43 channels expressed on monocytes play an important role in monocyte-endothelial adherence. The other potential pathway relevant to ATP release involves pannexin1, but there are still no reports regarding pannexin1 expression on monocytes [38]. Furthermore, 18-α-GA at a very low concentration, as used in the current study, has no effects on pannexin1 [39]. The analyses all suggest that the release of ATP from monocytes is rapidly and dynamically regulated by Cx43 channel function.

Conclusions

With the development of society, the consumption of opioids has tended to increase worldwide [40]. The side-effects of opioids on monocyte-endothelial adherence should be seriously taken into consideration by clinicians. The present results demonstrate that sufentanil application might increase U937-HUVEC adhesion by inhibiting ATP release mediated by Cx43 channels expressed on U937 monocytes. In contrast, fentanyl and remifentanil do not show these effects. These results might provide new insights into the rational use of opioids in the clinic.

Abbreviations

ADO, adenosine; APCP, α,β-methylene ADP; Cx37, connexin37; Cx43, connexin43; HUVEC, human umbilical vein endothelial cell

Declarations

Ethics approval and consent to participate:

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Medical Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University.

Consent for publication:

Not Applicable.

Availability of data and materials:

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests:

The authors declare that they have no competing interests.
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Authors' contributions:

All authors read and approved the final manuscript. Dongdong Yuan, Dezhao Liu and Guoliang Sun designed this study. Dongdong Yuan and Dezhao Liu wrote the manuscript together. Guoliang Sun revised the manuscript, Dongdong Yuan, Zhaowei Zou, Xianlong Li, Nan Chen and Na Guo performed all the experiments.

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