Morphine treatment enhances extracellular ATP enzymolysis and adenosine generation in rat astrocytes

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Abstract: Recent studies have shown that astrocytes play important roles in ATP degradation and adenosine (a well known analgesic molecule) generation, which are closely related to pain signaling pathway. The aim of this study was to investigate whether morphine, a well known analgesic drug, could affect the speeds of ATP enzymolysis and adenosine generation in rat astrocytes. Intracellular calcium concentration ([Ca²⁺]) of astrocyte was measured by flow cytometry, and the time points that morphine exerted notable effects were determined for subsequent experiments. Cultured astrocytes were pre-incubated with morphine (1 μmol/L) and then were incubated with substrates, ATP and AMP, for 30 min. The speeds of ATP enzymolysis and adenosine generation were measured by high performance liquid chromatography (HPLC). The results showed that both 1.5 and 48 h of morphine pre-incubation induced maximal ATP enzymolysis speed in astrocytes among all the time points, and there was no statistical difference of ATP enzymolysis speed between morphine treatments for 1.5 and 48 h. As to adenosine, morphine pre-incubation for 1.5 h statistically increased adenosine generation, which was degraded from AMP, in cultured astrocytes compared with control group. However, no difference of adenosine generation was observed after 48 h of morphine pre-incubation. These results indicate that treatment of morphine in vitro dynamically changes the concentrations of ATP and adenosine in extracellular milieu of astrocytic cells. In addition, astrocyte can be regarded as at least one of the target cells of morphine to induce changes of ATP and adenosine levels in central nervous system.

Key words: morphine; ATP; adenosine; astrocytes
Enzymes responsible for ATP degradation and adenosine generation have been reported to be expressed in astrocytes\(^{[1,2]}\). These enzymes are called ecto-nucleotidases, by which ATP can be degraded to ADP, AMP and finally to adenosine. Notably, ATP and adenosine have different effects on pain transmission. Exogenous administration of ATP or its receptor agonists caused thermal hyperalgesia and mechanical allodynia \(^{[3-5]}\). On the contrast, both systemic and intrathecal injection of adenosine had antinociceptive effects in various pain models \(^{[6-8]}\). In addition, endogenous adenosine was considered to mediate analgesic effect of some therapies, such as acupuncture \(^{[9]}\).

Morphine, a well known analgesic drug, can directly affect astrocytes by binding to \(\mu\) or \(\delta\) receptors. Previous studies have shown that morphine modulates chemokine gene regulation in astrocytes \(^{[10]}\), alters astrocytes proliferation and differentiation \(^{[11]}\), and also regulates intracellular calcium concentration \((\text{[Ca}^{2+}\text{]}\)) \(^{[12,13]}\). Whether morphine treatment could alter the activities of ecto-nucleotidases to degrade ATP and produce adenosine in astrocytes remains unknown. In addition, the activities of ecto-nucleotidases involved in ATP degradation and adenosine generation in astrocytes were reported to be closely associated with calcium concentration \(^{[14]}\). Therefore measurement of calcium level was included to determine morphine treatment duration. In this study, we investigated the effects of both acute and chronic morphine treatment on the levels of extracellular ATP and adenosine respectively.

1 MATERIALS AND METHODS

1.1 Reagents

Dulbecco’s Modified Eagle’s Medium Nutrient Mixture F-12 HAM (DMEM/F12) and fetal bovine serum (FBS) were both purchased from Gibco Company (USA); Morphine hydrochloride was from Qinghai Pharmaceutical Factory (China); Fluo-3 AM was purchased from Biotium (USA); ATP, AMP and adenosine were all bought from Sigma.

1.2 Astrocytes culture

Cultures of rat cortical astrocytes were prepared from newborn (1–2 d) Sprague-Dawley rats according to the method of McCarthy and de Vellis \(^{[15]}\) with a modification. Briefly, rat pups were killed by cervical dislocation and whole brains were removed. Cerebral cortices were isolated, mechanically dissociated, trypsinized, triturated and centrifuged at 400 g for 5 min. The resulting pellet was resuspended in DMEM/F12 containing 10% FBS and antibiotics, and then plated in Nunc flasks. At confluence (7–9 d in culture), the flasks were shaken vigorously at 250 r/min for 16 h to remove neurons, microglia and oligodendrocytes. Astrocytes were trypsinized and seeded onto 12- or 24-well plates.

1.3 Calcium measurement

\([\text{Ca}^{2+}]\) was measured to determine morphine treatment duration using Fluo-3 AM staining detected by flow cytometer (FCM, BENKMAN-COULTER company, USA). Cultured astrocytes plated on 12-well plates were incubated with morphine (1 \(\mu\)mol/L) for 0, 1.5, 6, 24, 48 and 72 h, respectively. Then they were loaded with Fluo-3 AM (5 \(\mu\)mol/L) at 37°C for 30 min without light, then washed 3 times and centrifuged to get the cell pellet. \([\text{Ca}^{2+}]\) was measured by using FCM (exciting wavelength: 488 nm; emitting wavelength: 525 nm). These measurements were conducted in at least three independent experiments, in triplicates. The data were expressed as the percentage over control level.

1.4 Ecto-nucleotidases assay

Cultured astrocytes in 24-well plates were incubated with morphine (1 \(\mu\)mol/L, diluted with culture media) for 1.5 and 48 h, and then washed three times. The reaction was started by the addition of 200 mL incubation medium prepared with HEPES buffer (2 mmol/L CaCl\(_2\), 120 mmol/L NaCl, 5 mmol/L KCl, 10 mmol/L glucose, 20 mmol/L HEPES, and pH 7.4). For AMP incubation, MgCl\(_2\) (2 mmol/L) was used instead of CaCl\(_2\) \(^{[16]}\). Final concentrations of ATP and AMP were both 100 mmol/L, ensuring enough substrates to evaluate the enzyme activity. After incubation at 37°C for 30 min, the medium were collected and placed on dry ice instantly, then stored at \(-80°C\) until measurement. The chromatographic conditions were as described previously with some modifications \(^{[17]}\). A Hypersil C18 column (4.6 mm\(\times\)300 mm, 5 \(\mu\)m, Merk) was used, and KH\(_2\)PO\(_4\) (50 mmol/L) solution was used as the mobile phase at a rate of 0.5 mL/min. 20 \(\mu\)L of standards or samples were injected into HP1100 pump (ESA) to measure absorbance at wavelength of 254 nm. Retention times were assessed by using nucleotide standards. The non-enzymatic hydrolysis of ATP and AMP was consistently less than 5%. If cells were incubated without the addition of nucleotides, the culture medium would not present any detectable peaks. The activities were determined in at least three independent experiments, in triplicates. Specific activity was expressed as nmol/min per \(1\times10^6\) cells.
1.5 Data analysis and statistics
All data were presented as means±SEM. Significant differences were analyzed with one-way ANOVA and following LSD test using the software SPSS (version 13.0 for Windows). \( P<0.05 \) was defined as statistically significant.

2 RESULTS
2.1 \([\text{Ca}^{2+}]_i\) remarkably changed under 1.5 and 48 h of morphine incubation
In order to determine suitable time points, we used flow cytometry to measure the levels of cytoplasmic calcium. Consistent with previous findings\cite{12,13}, morphine treatment firstly up-regulated the level of \([\text{Ca}^{2+}]_i\), and then down-regulated it. As shown in Fig.1, the calcium concentration increased by \((15±6)\%\) and decreased by \((19±4)\%\) after morphine incubation for 1.5 h and 48 h respectively, and the changes of \([\text{Ca}^{2+}]_i\) at the time points of 1.5 and 48 h were most significant compared with the other time points (1.5 h vs 6 h, 48 h vs 72 h, \(n=9, P<0.05\)). Therefore both the two time points were selected for the following experiments.

2.2 More ATP was degraded under 1.5 and 48 h of morphine incubation
As shown in Fig. 2, control group with no morphine incubation degraded ATP at the speed of \((8.468±0.143)\) nmol/min per \(1\times10^6\) cells. After 1.5 h of morphine incubation, the speed of ATP enzymolysis increased to \((9.061±0.231)\) nmol/min per \(1\times10^6\) cells, which was significantly different from that in control group \((P<0.05)\). Moreover, 48 h of morphine pretreatment also increased the speed of ATP enzymolysis to \((9.366±0.109)\) nmol/min per \(1\times10^6\) cells, which was higher than that of control group \((P<0.05)\). However, there was no statistical difference in ATP enzymolysis between 1.5 h group and 48 h group \((P>0.05)\).

2.3 More adenosine was generated under 1.5 and 48 h of morphine incubation
Figure 3 is the statistical histograms of adenosine

![Fig. 1 Effect of morphine treatment on intracellular calcium concentration ([Ca]$^{2+}$) in rat cortical astrocytes detected by flow cytometer. Morphine (1 μmol/L) firstly up-regulated [Ca]$^{2+}$, and then down-regulated it in cultured astrocytes. Means±SEM, \(n=9, *P<0.05\) vs 6 h of morphine incubation, $^*P<0.05$ vs 72 h of morphine incubation.](image1)

![Fig. 2. Effect of morphine treatment on enzymolysis speed of extracellular ATP in rat cortical astrocytes detected by chromatography. Both 1.5 and 48 h of morphine (1 μmol/L) incubation enhanced the speed of ATP enzymolysis. However, there was no statistical difference in ATP enzymolysis between 1.5 h and 48 h groups. Means±SEM, \(n=9, *P<0.05\) vs control (0 h).](image2)

![Fig. 3. Effect of morphine treatment on generation speed of extracellular adenosine in rat cortical astrocytes detected by chromatography. Morphine (1 μmol/L) incubation for 1.5 h enhanced the speed of adenosine generation from AMP, while 48 h of morphine treatment had no effect on adenosine generation compared with control group. Means±SEM, \(n=9, *P<0.05\) vs control (0 h), $^*P<0.05$ vs 48 h of morphine incubation.](image3)
generation in different groups. Control group (0 h) generated adenosine at the speed of (1.899±0.249) nmol/min per 1×10⁶ cells. Morphine incubation for 1.5 h increased the speed of adenosine production to (2.485±0.188) nmol/min per 1×10⁶ cells, which was significantly higher than that of control group (P<0.05). However, the speed of adenosine production after 48 h of morphine incubation was (1.909±0.165) nmol/min per 1×10⁶ cells, showing no difference from that of control group (P>0.05).

3 DISCUSSION

In the present study, we demonstrated that acute morphine treatment induced adenosine generation in extracellular milieu of astrocytes. This result was consistent with previous reports that the antinociceptive effects of opioids were partly mediated by endogenous adenosine. Morphine acted on opioid receptors to release adenosine which subsequently bound with its receptors to produce analgesia[18]. On the contrary, intrathecal adenosine which subsequently bound with its receptors produced analgesia whereas adenosine alleviated pain [3,6-8], we assume that increased adenosine level and decreased ATP level might contribute to the analgesic effect of morphine, which was consistent with the phenomenon that acute morphine treatment alleviated pain effectively, whereas chronic administration of morphine would produce decreased antinociception effect. Furthermore, the substantial changes of ATP and adenosine concentration indicate that the activities or expressions of ecto-nucleotidases expressed on astrocytes responsible for ATP enzymolysis and adenosine generation are significantly influenced by morphine treatment. However, the mechanisms of morphine affecting levels of ATP and adenosine need further study.

REFERENCES


