Morphine-induced Nitric Oxide Production in PC12 Cells

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Abstract

Background: The role of nitric oxide (NO) in many well-known effects of morphine is well defined. NO is involved in the signaling pathway of the N-methyl-D-aspartate (NMDA) receptor, which is proposed to mediate some of morphine’s effects. This research studies the effect of morphine and NMDA on lipopolysaccharide (LPS)-stimulated NO production by clonal rat pheochromocytoma (PC12) cells.

Methods: We used the Griess reaction to measure NO concentrations in cell culture medium.

Results: PC12 cells that were incubated for 24 h with varying concentrations of morphine (0.1, 1, 10, 100, and 1000 µM) plus LPS (1 µg/ml) did not significantly alter the concentration of NO in the medium. However, NO production increased when cells were treated for both 48 h with 100 and 1000 µM morphine and for 72 h with 10,100, and 1000 µM of morphine. After 72 h, 1 µM naloxone significantly decreased NO concentration. Naloxone, at doses of 0.1, 1, and 10 µM prevented NO production by 1000 µM of morphine. NMDA (0.1, 1, and 10 µM) did not alter NO concentrations after 24, 48 or 72 h. Morphine (1 µM)-induced NO production was inhibited by 10 µM NMDA after 48 h. Inhibition of NO was also noted with 1 and 10 µM concentrations of morphine after 72 h.

Conclusion: Chronic treatment of PC12 cells with morphine significantly increases LPS-stimulated NO production via naloxone-sensitive receptors. The cells seem to release endogenous morphine in medium. NMDA does not affect NO production, which may be due to the lack of functional NMDA receptor expression in PC12 cells.

Keywords: Morphine, Nitric oxide, NMDA, PC12 cells

Introduction

Nitric oxide (NO) is a small, gaseous molecule which functions as a neuronal messenger that diffuses easily across neuronal membranes. Nitric oxide synthase (NOS) acts on L-arginine to produce NO and L-citrulline. There are two forms of NOS, constitutive (cNOS) and inducible (iNOS). iNOS is expressed when it is induced by bacterial lipopolysaccharide (LPS) or some proinflammatory cytokines.

Opioid drugs like morphine exert many of their well-known effects via the NO pathway. It has been shown that morphine upregulates NOS biosynthesis and affects its function by activating opioid receptors. NO can activate the enzyme guanylyl cyclase and generate cyclic GMP (cGMP) from guanosine triphosphate (GTP). In vitro studies have shown that morphine is able to increase cGMP concentration. The NO-cGMP pathway may be involved in morphine-induced analgesia since administration of NOS inhibitors like Nω-nitro-L-arginine (L-NNA) prevent morphine analgesia.

It has been shown that NOS inhibitors can prevent the development of morphine tolerance or block morphine-induced CPP. The N-methyl-D-aspartate (NMDA) receptor is an important excitatory amino acid receptor involved in many neuronal functions like synaptic plasticity and neuronal development. However, over-activation of NMDA receptors can result in neuronal excitotoxicity and cell death. These ionotropic receptors are members of the glutamate receptor family and are highly permeable to Ca2+. Influx of calcium through the activated NMDA receptor channel leads to various actions, of which some occur through the initiation of NO synthesis followed by continuous NO emission. In fact, in some areas of the central nervous system, NMDA receptors are co-localized in neurons that contain NOS. Studies have indicated that NMDA receptor antagonists, similar to NOS inhibitors, can prevent some effects of morphine. For example, morphine-induced tolerance, analgesia and withdrawal can be inhibited by administration of NMDA receptor antagonists. Acquisition of morphine-induced CPP has been shown to be blocked by the NMDA receptor antagonist memantine.

As mentioned above, the relation between NO production and morphine effects and also between NMDA receptors and morphine have been indirectly evaluated by researchers. However, studies investigating the direct interaction between morphine, NMDA and NO production in cell culture systems are limited. Clonal rat pheochromocytoma (PC12) cells derived from chromaffin cells of the adrenal medulla are extensively used as a model system to study neurons as they show a number of neuronal characteristics. Therefore, we have selected the PC12 cell line to study the effects of morphine and NMDA on LPS-stimulated NO production by these cells. This study has also evaluated the direct interaction between morphine and NMDA in PC12 cells with regards to the NO pathway.
Materials and Methods

Reagents
Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Invitrogen. LPS, NMDA, naloxone hydrochloride, sulfanilamide and N-1-naphtylethylenediamine dihydrochloride were purchased from Sigma-Aldrich Co. (UK). Morphine sulfate was purchased from Temad Co. (Tehran, Iran).

Cell culture and sample treatment
PC12 cells were purchased from Pasteur Institute (Tehran, Iran). Cell culture medium consisted of DMEM (4.5 g/l) that contained 10% (v/v) heat-inactivated FBS, penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml). Cells were maintained at 37°C in a humidified atmosphere (90%) with 5% CO2. After overnight incubation (which lead to 40% – 50% confluence),20 cells were stimulated with LPS (1 μg/ml) and simultaneously treated with different doses of morphine, naloxone and NMDA alone (experiments 1, 2, and 3) or in combination (experiments 4, 5, and 6) for 24, 48, and 72 h. For each experiment, there was a control sample with equal volumes of medium that underwent no treatment.

All drugs were dissolved in phosphate buffered saline and then diluted with the medium accordingly to reach the intended drug concentration.

Measurement of NO production
Using the Griess reaction, we measured the accumulated nitrite in the culture medium as an indicator of NO production.21 Briefly, 50 μl of cell culture medium was mixed with an equal volume of 1% (w/v) sulfanilamide [in 5% (v/v) phosphoric acid] and 0.1% (w/v) N-1-naphthylethylenediamine dihydrochloride, and incubated at room temperature for 10 min. The absorbance at 548 nm was measured in a microplate reader. Fresh culture medium was used as the blank in all experiments. Sodium nitrite serial dilution standard curve was used to calculate concentrations in the samples. All samples were analyzed in triplicate.

Data analysis
Data are reported as mean ± SEM. Differences among treatment groups were tested using one-way ANOVA followed by Tukey’s test. P < 0.05 was considered statistically significant.

Results
Figure 1 shows NO concentration amounts (μM) in the culture medium of PC12 cells that contained LPS (1 μg/ml) plus different morphine concentrations (0.1, 1, 10, 100, and 1000 μM) for 24, 48, and 72 h incubation times. After 24h incubation with different morphine doses, the NO concentration was not significantly altered in the medium when compared with the control sample. However, morphine concentrations of 100 and 1000 μM significantly increased NO concentration after 48 and 72 h incubation with PC12 cells (P < 0.001). The 10 μM morphine concentration after a 72 h incubation period also significantly increased NO production (P < 0.01).

Figure 2 represents the effects of different naloxone concentrations (0.1, 1, and 10 μM) on NO production by PC12 cells in the presence of LPS (1 μg/ml) for incubation periods of 24, 48, and 72 h. There were no significant changes in NO concentration after the 24 and 48 h incubation periods of PC12 cells with different naloxone concentrations. However, NO concentration significantly decreased when cells were treated with a naloxone concentration of 1 μM for 72 h (P < 0.05).

Figure 3 shows the NO concentrations in culture medium when PC12 cells were treated with different NMDA concentrations (0.1, 1, and 10 μM) plus LPS (1 μg/ml) for 24, 48, and 72 h on the amount of NO in cell culture medium. NO concentration (μM) was measured by the Griess reaction. Data are presented as mean±SEM of 3 samples. *P<0.05 different from the respective control group.

1, and 10 μM) plus LPS (1 μg/ml) for 24, 48, and 72 h. None of the applied concentrations were able to significantly alter NO production at any of the incubation times (24, 48, and 72 h).

Figure 4 shows the effect of a fixed concentration of morphine (1000 μM) alone or in combination with different naloxone concentrations (0.1, 1, and 10 μM) in medium that contained LPS (1 μg/ml) for 24, 48, and 72 h on the amount of NO in cell culture medium. NO concentration (μM) was measured by the Griess reaction. Data are presented as mean±SEM of 3 samples. ***P<0.001 different from the respective control group.

Figure 5 shows the effect of a fixed concentration of morphine (0.1 μM) alone or in combination with different NMDA concentrations (0.1, 1, and 10 μM) in medium that contained LPS (1 μg/ml) for 24, 48, and 72 h on the amount of NO in cell culture medium. NO concentration (μM) was measured by the Griess reaction. Data are presented as mean±SEM of 3 samples. *P<0.05, **P<0.01, ***P<0.001 different from the respective control group.

Figure 6 shows the effect of a fixed concentration of morphine (1 μM) alone or in combination with different NMDA concentrations (0.1, 1, and 10 μM) in medium that contained LPS (1 μg/ml) after 24, 48, and 72 h. The data indicated that all naloxone concentrations significantly prevented NO production by this concentration of morphine after 72 h (P < 0.001). However, the same effect was not observed with any naloxone concentration after 24 or 48 h.

In Figure 5, the effect of a fixed morphine concentration (0.1 μM)
alone or in combination with different NMDA concentrations (0.1, 1, and 10 μM) on NO concentration in the medium that contained LPS (1 μg/ml) after 24, 48, and 72 h of incubation is seen. None of the NMDA concentrations significantly altered NO production by this concentration of morphine at any of the incubation times. Figure 6 shows the effect of a fixed morphine concentration (1 μM) alone or in combination with different NMDA concentrations (0.1, 1, and 10 μM) on NO production in the medium that contained LPS (1 μg/ml) after 24, 48, and 72 h incubation. The data indicated that NMDA concentrations did not change NO production by 1 μM morphine after 24 h incubation. However, after 48 h, NMDA (10 μM) significantly prevented NO production by morphine \( (P < 0.05) \). NO concentration was also significantly reduced by the NMDA concentrations of 1 μM \((P < 0.01)\) and 10 μM \((P < 0.001)\) after 72 h incubation.

**Discussion**

In the present study we investigated the effects of morphine and NMDA on LPS-stimulated NO production by PC12 cells. It has been shown that LPS and cytokines are able to induce NOS at the transcriptional level and lead to NO production in the medium,\(^{22,23}\) a process that takes several hours.\(^{24}\) In vitro studies have indicated that LPS-induced NO production can be modulated by opioids.\(^{25,26}\) There are reports which claim that morphine may increase\(^{27}\) or decrease\(^{28}\) NO production in response to LPS. The conflicting results may be due to several factors, such as the type of studied cell, dose of morphine, or duration of exposure.\(^{28}\)

Our data indicated that acute treatment with different doses of morphine was not able to increase NO production in LPS-stimulated PC12 cells. However, after 48 and 72 h of exposure to morphine, NO production significantly increased in these cells. Our results have confirmed studies which claim that phenomenon such as tolerance and physical or psychological dependence observed after chronic or sustained exposure to opioids are critically dependent on NO production.\(^{28,30}\)

Molecular mechanisms that have been proposed for morphine-induced NO production may be the modulation of intracellular calcium currents by morphine which may activate Ca\(^{2+}\)/calmodulin-dependent NOS.\(^{31}\) It has been shown that chronic morphine exposure can lead to an increase in NOS expression\(^{32,33}\) or activity.\(^{34}\) NO production by morphine has been observed to occur both under basal and LPS-activated states.\(^{35,36}\) Our data have also indicated that naloxone is able to significantly prevent NO production after 72 h incubation of PC12 cells with morphine. This is also in agreement with previous findings that morphine-induced NO release is mediated through naloxone-sensitive receptors.\(^{35,36}\) It has been shown that PC12 cells express a functional mu opioid receptor protein;\(^{37}\) however, expression of kappa or delta opioid receptors is controversial and varies with the PC12 cell lines used.\(^{38,39}\)

In addition, our results showed that 72 h incubation with only naloxone (1μM) has significantly inhibited NO production in LPS-stimulated PC12 cells, which may propose the existence of an opioid-like compound in the culture medium. This may support previous findings which have indicated that PC12 cells have the ability to produce morphine chemically identical to the synthetic molecule.\(^{38}\) It seems that the inhibitory effect of naloxone on NO production is related to antagonizing the effect of morphine released by PC12 cells. Further studies are needed to prove this hypothesis.

The NMDA receptors are believed to be critically involved in excitatory synaptic transmission in the central nervous system; they have a major role in brain development and memory formation.\(^{41}\) However, a variety of neurological disorders are manifested when NMDA receptors are over-stimulated. In order to have a functional receptor, the NR1 subunit of the NMDA receptor must co-assemble with one or more NR2A-D and/or NR3A-B subunits.\(^{42}\) Our data have shown that PC12 cells treated with NMDA did not significantly induce NO production after 24, 48, or 72 h. These results do not confirm studies with other cell lines such as neuroblastoma SH-SY5Y cells, in which the activation of NMDA receptors leads to NOS activation and NO production.\(^{43}\) The reason for this discrepancy may be found in some reports indicating that PC12 cells express no or only negligible amounts of NMDA receptor protein when cultivated in an undifferentiated state (without nerve growth factor) despite the presence of mRNA-transcripts that correspond to some NMDA receptor subunits.\(^{44,45}\) Although different variants of PC12 cells have different profiles of NMDA receptor subunit expressions,\(^{46}\) it has been claimed that NMDA receptors are not found in significant levels in PC12 cells.\(^{46}\) This matter may lead to rapid degradation of unassembled NR1 subunits and lack of functional NMDA receptor production on the cell surface\(^{47}\) which may be an important factor that limits functional responses to NMDA in PC12 cells. Current flow recording in response to applied NMDA has been unsuccessful in PC12 cells.\(^{48}\)

Surprisingly, according to our data the same ineffective doses of NMDA are able to significantly decrease NO production by a chronic sub-effective dose of morphine (1 μM). This is difficult to explain as previous findings claim that NMDA receptors are not functionally expressed in PC12 cells (see above). Considering that the viability of cells were unaffected by the doses of drugs used (data not shown), other reasons come to mind. One possible explanation may be based upon previous data which has revealed that chronic morphine administration may affect NMDA receptor subunit expression.\(^{49}\) Whether this interaction happens in PC12 cells or not, needs to be further investigated.

In conclusion, chronic treatment of PC12 cells with morphine significantly increases LPS-stimulated NO production via naloxone-sensitive receptors. It seems that PC12 cells are able to synthesize and release endogenous morphine in the medium. Treatment of cells by NMDA itself did not affect NO production which may be related to the absence of functional NMDA receptor expression in PC12 cells. However, NMDA significantly decreased NO production when co-administered with morphine. The reason for the latter observation needs to be clarified in the future.

**References**
