Involvement of Central Endothelin Receptors in Neonatal Morphine Withdrawal

BHAGYA L. PUPPALA,* SHAIFALI BHALLA,† GEORGE MATWYSHYN,† AND ANIL GULATI†,[1]

*Department of Pediatrics and Neonatology, Advocate Lutheran General Children's Hospital, Park Ridge, Illinois 60068; and †Department of Biopharmaceutical Sciences, University of Illinois at Chicago, Chicago, Illinois 60612

The involvement of central endothelin (ET) receptors in neonatal morphine tolerance has been demonstrated. The present study investigates the role of central ET receptors in morphine withdrawal in neonatal rats. The aim was to determine whether activation of G-proteins coupled to opioid and ET receptors by morphine and various ET receptor modulators is affected during morphine withdrawal in neonatal rats. Pregnant female rats were rendered tolerant to morphine by chronic exposure to morphine pellets during 7 days. On Day 8, pellets were removed and rats were allowed to undergo withdrawal for 24 hrs. Rat pups were delivered by cesarean section. G-protein stimulation induced by morphine; ET-1; the ET_A receptor antagonist, BMS182874; and the ET_B receptor agonist, IRL1620, were determined in the brain of neonatal rats undergoing morphine withdrawal by [^{35}S]GTPγS binding assay. Morphine produced higher ($P < 0.05$) maximal stimulation of G-protein in the morphine-withdrawal group (83.60%) compared with the placebo group (66.81%). ET-1–induced G-protein stimulation was also altered, and the median effective concentration (EC_{50}) during morphine withdrawal (170.60 n*M*) was significantly higher than placebo (62.5 n*M*; $P < 0.05$). The maximal stimulation induced by the ET_A receptor antagonist, BMS182874, in the morphine-withdrawal group (86.07%; EC_{50} = 31.25 n*M*) was significantly higher than in the placebo group (EC_{50} > 1000 n*M*). The ET_B agonist, IRL1620, induced G-protein stimulation was similar in placebo (73.43%, EC_{50} = 13.26 n*M*) and morphine-withdrawal groups (75.08%, EC_{50} = 11.70 n*M*), respectively. To our knowledge, this is the first report indicating involvement of central ET_A receptors in neonatal morphine withdrawal. Exp Biol Med 231:1157–1160, 2006

Key words: morphine; BMS182874 (5-[dimethylamino]-*N*-[3,4-dimethyl-5-isoxazolyd]-1-naphthalenesulfonamide); IRL1620 (*N*-Suc-[Glu^{9},Ala^{11,15}]ET-1[8–21]); [^{35}S]GTPγS-binding withdrawal; endothelin; neonatal rats; central nervous system

Introduction

Because of several reported benefits to neonatal behavior and outcomes from opioid-based analgesia and anesthesia (1–3), opioids are widely used in neonatal pain management. However, chronic use of opioid analgesics results in tolerance and dependence. Iatrogenic opioid dependence was first reported in infants receiving fentanyl during extracorporeal membrane oxygenation (4). Chronic exposure of the fetus during maternal opiate abuse also leads to severe neurologic and behavioral changes. Abrupt cessation of opiates leads to severe withdrawal syndrome, and management of these adverse effects may be a major challenge for the clinician. Recent animal studies have demonstrated that fetal rats and infant rat pups undergo opiate tolerance and physical dependence manifested as withdrawal if the dams are exposed to opiates during pregnancy (5–7).

Morphine and other opioids act by binding to and activating μ-, κ-, and δ-opioid receptors. Conformational changes in opioid receptors initiate signal transduction cascade because of activation of inhibitory G-proteins, $G_{i\alpha}$ and G_o (8–10). During opioid tolerance, opioid receptors are desensitized and there is an upregulation of second messengers, such as adenylate cyclase and cAMP (11). Chronic morphine treatment also results in functional uncoupling of μ-opioid receptors and G-proteins. We propose that endothelin (ET), an endogenous neuropeptide, may be an important factor in mediating opiate tolerance. We have demonstrated the involvement of central ET receptors in morphine analgesia and tolerance. In adult rats, it was found that ET_A receptor antagonists significantly potentiated morphine analgesia (12), and restored analgesic response of morphine during tolerance (13). Although peripheral administration of the ET_B receptor agonist, IRL1620, was shown to be involved in analgesia through opioid receptors, centrally administered ET_B receptor

The present study was funded by Chicago Labs Inc., Chicago, IL.

[1] To whom correspondence should be addressed at Department of Biopharmaceutical Sciences (M/C 865), The University of Illinois at Chicago, 833 South Wood Street, Chicago, IL 60612. E-mail: gulati@uic.edu

Received September 9, 2005.
Accepted December 12, 2005.

1535-3702/06/2316-1157$15.00
Copyright © 2006 by the Society for Experimental Biology and Medicine

Table 1. EC_{50} Values for [^{35}S]GTPγS Binding Induced by Morphine and ET_A and ET_B Receptor Agonists and Antagonists in Normal Neonatal Rats (Placebo) and in Neonatal Rats After Morphine Withdrawal

	Treatment	
Drugs	Placebo withdrawal	Morphine withdrawal
Morphine	170.6 ± 0.1 n*M*	28.8 ± 0.2 n*M**
ET-1 (ET_A and ET_B agonist)	20.7 ± 0.1 n*M*	84.8 ± 0.1 n*M**
BMS182874 (ET_A antagonist)	>1000 n*M*	33.2 ± 3.3 n*M**
IRL1620 (ET_B agonist)	16.3 ± 0.1 n*M*	15.8 ± 0.1 n*M*

* $P < 0.05$ significantly different compared with corresponding morphine-naïve placebo-treated group.

agonists did not produce any effect on morphine analgesia (14). Studies conducted in neonatal rats showed involvement of ET receptors in morphine tolerance (15). We found that ET receptor antagonists did not act on opioid receptors directly, but modulated the action of morphine by acting through G-proteins (15). It was shown that chronic administration of morphine leads to modulation of G_i/G_o-coupled receptors. However, corresponding changes in the ET system and involvement of ET receptors in morphine withdrawal in the brain of neonatal rats is not known. Therefore, in the present study, we evaluated the effect of an ET_A receptor antagonist and an ET_B receptor agonist on G-protein stimulation in neonatal rats undergoing morphine withdrawal, using a [^{35}S]GTPγS-binding assay.

Materials and Methods

Neonatal rats harvested from pregnant female Sprague-Dawley rats (Harlan, Indianapolis, IN) rats at term (Day 22 of gestation) were randomly selected from each litter and used for [^{35}S]GTPγS binding. Studies were conducted according to guidelines established by Animal Care Committee of University of Illinois at Chicago.

Morphine and placebo pellets were obtained from the National Institute of Drug Abuse, Rockville, MD. Guanosine-5′-diphosphate (GDP) was dissolved in assay buffer (Sigma Aldrich, St. Louis, MO). [^{35}S]GTPγS (1000 Ci/mmol; Amersham Pharmacia Biotech, Piscataway, NJ) was diluted in assay buffer. Unlabeled guanosine-5′-*o*-(3-thio)triphosphate (GTPγS) was dissolved in assay buffer (Sigma Aldrich). Morphine sulfate (Mallinckrodt Chemical Co., St. Louis, MO) and IRL1620 (Sigma Aldrich) were dissolved in sterile saline. BMS182874 (Tocris Cookson Inc., Ellisville, MO) was dissolved in 20% dimethylsulfoxide. ET-1 (American Peptides Company Inc., Sunnyvale, CA) was dissolved in 0.1% bovine serum albumin. Dilutions of all drugs were prepared in assay buffer.

Pregnant female rats were rendered tolerant to morphine by a pellet implantation procedure (16). Rats were divided into two groups: group 1 received placebo pellets ($n = 4$); Group 2 received morphine pellets ($n = 4$). Each rat was subcutaneously implanted with six pellets during a 7-day period. One morphine pellet was implanted on Day 14 of gestation, two pellets on Day 16 of gestation, and three pellets on Day 18 of gestation. Control rats received placebo pellets containing the same excipients without morphine. On Day 21 of gestation, both placebo and morphine pellets were removed, the incision was closed with sterile wound clips, and rats were allowed to undergo withdrawal from morphine for 24 hrs. Pregnant females on Day 22 of gestation (at term) were anesthetized using 1% isoflurane anesthesia, and rat pups were delivered by cesarean section. Pups were sacrificed by cervical section immediately, the cerebellums were removed, and the brains were stored at −70°C until analysis. Neonatal rats from both groups were randomly selected and used for [^{35}S]GTPγS binding in neuronal membranes.

[^{35}S]GTPγS binding was performed according to the procedure described earlier (17), using approximately 100 μg protein in each sample. The total volume in each tube was 0.5 ml, containing 0.35 ml of homogenate, various concentrations of drugs (morphine, ET-1, BMS182874, and IRL1620), 30 μ*M* GDP, 100 n*M* [^{35}S]GTPγS, and assay buffer. The concentration range for morphine, ET-1, BMS182874, and IRL1620 was 0.98–1000 n*M*. Nonspecific binding was measured using 10 μ*M* unlabeled GTPγS. Specific binding was expressed as femtomoles per milligram of protein (mean ± SEM).

Data was analyzed by one-way analysis of variance (ANOVA) followed by a Bonferroni test. A level of $P < 0.05$ was considered significant.

Results

Morphine and ET-1 produced concentration-dependent increases in [^{35}S]GTPγS binding. Basal GTP binding was in the range of 0.14 ± 0.01 to 0.89 ± 0.40 fmol/mg protein. Basal binding was statistically similar ($P > 0.05$) in placebo and morphine-withdrawal groups. Maximal GTP binding in the morphine-withdrawal group (83.60 ± 5.77%) was significantly higher ($P < 0.05$) as compared with the placebo group (66.81 ± 1.51%). An median effective concentration (EC_{50}) value for morphine-stimulated GTP binding in morphine-withdrawal group was 28.78 ± 0.17 n*M* (Table 1), which was significantly lower than the EC_{50} value in the placebo group (170.60 ± 0.10 n*M*). ET-1–

induced GTP binding was attenuated in the morphine-withdrawal group; however, at concentrations greater than the EC_{50} value, the G-protein activation was increased. Maximal stimulation in the placebo and morphine-withdrawal group was 74.88 ± 1.50% and 87.16 ± 4.83%, respectively. The EC_{50} value in the morphine-withdrawal group was 84.79 ± 0.04 n*M*. This was significantly higher than the EC_{50} value in the placebo group (20.66 ± 0.12 n*M*; Table 1).

The maximal GTP binding with the ET_A receptor antagonist, BMS182874, in the placebo group was 6.71 ± 1.51%. This indicates that BMS182874 did not stimulate G-proteins in the placebo group. In the morphine-withdrawal group, BMS182874 produced a maximal stimulation of 79.90 ± 8.22%, which was significantly higher ($P < 0.05$) than in the placebo group. The EC_{50} value for BMS182874-stimulated [^{35}S]GTPγS binding in the placebo group was greater than 1000 n*M*, whereas the EC_{50} value in the morphine-withdrawal group (33.20 ± 3.29 n*M*) was significantly lower ($P < 0.05$) compared with the placebo group. The ET_B receptor agonist, IRL1620, produced a maximal stimulation of 73.43 ± 8.89% in the placebo group, which was similar ($P > 0.05$) to that of the morphine-withdrawal group (75.08 ± 5.41%). The EC_{50} values for IRL1620-stimulated [^{35}S]GTPγS binding in the placebo (16.34 ± 0.12 n*M*) and morphine-withdrawal (15.80 ± 0.11 n*M*) groups were similar (Table 1).

Discussion

Mechanisms involved in withdrawal after chronic opioid administration are extremely complex, involving changes in opiate signal transduction and interactions between opiate and nonopiate systems (18). In the present study, we investigated the effect of morphine withdrawal G-protein activation of opioid and ET receptors in the brain of neonatal rats.

Studies show that infant rat pups and fetal rats experience opiate tolerance if dams are exposed to opiates during pregnancy (19). We found that morphine-induced stimulation of GTP binding was higher, whereas ET-1–induced stimulation of GTP binding was lower in neonatal rats undergoing morphine withdrawal compared with the placebo group. These findings clearly implicate a role of central ET receptors in morphine withdrawal in neonatal rats. It was further found that the ET_A receptor antagonist, BMS182874, did not affect GTP binding in normal rats, but significantly increased GTP binding in neonatal rats undergoing morphine withdrawal. Our results indicate that these changes taking place in G-protein–coupling mechanisms are restored by ET_A receptor antagonists.

Although opioid receptor and ET receptor coupling to G-proteins is affected in a similar manner in morphine tolerance, the present findings suggest that opioid receptors and ET receptors are affected differently during withdrawal. The development of opioid dependence and withdrawal may involve complex interactions between several neurotransmitter systems having opposing actions on the G-protein system (20). Neonates have been found to have significantly different characteristics of receptors and concentration of neurotransmitters compared with adults, and extensive postnatal developmental changes take place in the central nervous system. Based on the findings of the present study, we speculate that ET_A receptors may play a role in morphine withdrawal in neonatal rats. It is conduced that ET_A receptor antagonists restore coupling of G-proteins to opioid receptors, which may play an important part in opiate tolerance and withdrawal.

1. Anand KJ, Barton BA, McIntosh N, Lagercrantz H, Pelausa E, Young TE, Vasa R. Analgesia and sedation in preterm neonates who require ventilatory support: results from the NOPAIN trial. Neonatal Outcome and Prolonged Analgesia in Neonates. Arch Pediatr Adolesc Med 153: 331–338, 1999.
2. Anand KJ, Sippell WG, Aynsley-Green A. Randomised trial of fentanyl anaesthesia in preterm babies undergoing surgery: effects on the stress response. Lancet 1:62–66, 1987.
3. MacGregor R, Evans D, Sugden D, Gaussen T, Levene M. Outcome at 5–6 years of prematurely born children who received morphine as neonates. Arch Dis Child Fetal Neonatal Ed 79:F40–43, 1998.
4. Suresh S, Anand KJ. Opioid tolerance in neonates: a state-of-the-art review. Paediatr Anaesth 11:511–521, 2001.
5. Jones KL, Barr GA. Opiate withdrawal in the fetal rat: a behavioral profile. Pharmacol Biochem Behav 66:419–424, 2000.
6. Jones KL, Barr GA. Ontogeny of morphine withdrawal in the rat. Behav Neurosci 109:1189–1198, 1995.
7. Windh RT, Little PJ, Kuhn CM. The ontogeny of mu opiate tolerance and dependence in the rat: antinociceptive and biochemical studies. J Pharmacol Exp Ther 273:1361–1374, 1995.
8. Zhang J, Ferguson SS, Barak LS, Bodduluri SR, Laporte SA, Law PY, Caron MG. Role for G protein-coupled receptor kinase in agonist-specific regulation of mu-opioid receptor responsiveness. Proc Natl Acad Sci U S A 95:7157–7162, 1998.
9. Suresh S, Anand KJ. Opioid tolerance in neonates: mechanisms, diagnosis, assessment, and management. Semin Perinatol 22:425–433, 1998.
10. Liu JG, Anand KJ. Protein kinases modulate the cellular adaptations associated with opioid tolerance and dependence. Brain Res Brain Res Rev 38:1–19, 2001.
11. Avidor-Reiss T, Nevo I, Saya D, Bayewitch M, Vogel Z. Opiate-induced adenylyl cyclase superactivation is isozyme-specific. J Biol Chem 272:5040–5047, 1997.
12. Bhalla S, Matwyshyn G, Gulati A. Potentiation of morphine analgesia by BQ123, an endothelin antagonist. Peptides 23:1837–1845, 2002.
13. Bhalla S, Matwyshyn G, Gulati A. Endothelin receptor antagonists restore morphine analgesia in morphine tolerant rats. Peptides 24:553–561, 2003.
14. Bhalla S, Matwyshyn G, Gulati A. Central endothelin-B receptor stimulation does not affect morphine analgesia in rats. Pharmacology 72:20–25, 2004.
15. Puppala BL, Matwyshyn G, Bhalla S, Gulati A. Evidence that morphine tolerance may be regulated by endothelin in the neonatal rat. Biol Neonate 86:138–144, 2004.
16. Gulati A, Bhargava HN. Cerebral cortical 5-HT1 and 5-HT2 receptors of morphine tolerant-dependent rats. Neuropharmacology 27:1231–1237, 1988.
17. Narita M, Mizoguchi H, Nagase H, Suzuki T, Tseng LF. Up-regulation

of spinal mu-opioid receptor function to activate G-protein by chronic naloxone treatment. Brain Res 913:170–173, 2001.

18. Vaccarino AL, Kastin AJ. Endogenous opiates: 2000. Peptides 22: 2257–2328, 2001.
19. Barr GA, Zmitrovich A, Hamowy AS, Liu PY, Wang S, Hutchings DE. Neonatal withdrawal following pre- and postnatal exposure to methadone in the rat. Pharmacol Biochem Behav 60:97–104, 1998.
20. Basheer R, Yang J, Tempel A. Chronic prenatal morphine treatment decreases G alpha s mRNA levels in neonatal frontal cortex. Brain Res Dev Brain Res 70:145–148, 1992.