HHS Public Access

Author manuscript

J Perinatol. Author manuscript; available in PMC 2013 November 01.

Published in final edited form as:

J Perinatol. 2013 May ; 33(5): 374–382. doi:10.1038/jp.2012.124.

Opioids and clonidine modulate cytokine production and opioid receptor expression in neonatal immune cells

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Abstract

Objective—Opioids and clonidine, used in for sedation, analgesia and control of opioid withdrawal in neonates, directly or indirectly activate opioid receptors expressed in immune cells. Therefore, our objective is to study how clinically relevant concentrations of different opioids and clonidine change cytokine levels in cultured whole blood from preterm and full-term infants.

Study design—Using blood from preterm (≤ 30 weeks gestational age, n=7) and full-term (≥37 weeks GA, n=19) infants, we investigated the changes in cytokine profile (IL-1β, IL-6, IL-8, IL-10, IL-12p70, and TNF-α), cyclic adenosine monophosphate (cAMP) levels and μ-, δ-, and κ-opioid receptor (OPR) gene and protein expression following *in-vitro* exposure to morphine, methadone, fentanyl, or clonidine at increasing concentrations ranging from 0 to 1 mM.

Results—Following LPS activation, IL-10 levels were 146-fold greater in cultured blood from full-term than from preterm infants. Morphine and methadone, but not fentanyl, at $>10^{-5}$M decreased all tested cytokines except IL-8. In contrast, clonidine at $<10^{-9}$M increased IL-6, while at $>10^{-5}$M increased IL-1β and decreased TNF-α levels. All cytokine changes followed the same patterns in preterm and full-term infant cultured blood and matched increases in cAMP levels. All three μ-, δ- and κ-OPR genes were expressed in mononuclear cells from preterm and full-term infants. Morphine, methadone and clonidine, but not fentanyl, at $>10^{-5}$M decreased the expression of μ-OPR, but not δ- or κ-OPRs.

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CONFLICT OF INTEREST: None

Conclusion—Generalized cytokine suppression along with downregulation of μ-OPR expression observed in neonatal mononuclear cells exposed to morphine and methadone at clinically relevant concentrations contrast with the modest effects observed with fentanyl and clonidine. Therefore, we speculate that fentanyl and clonidine may be safer therapeutic choices for sedation and control of opioid withdrawal and pain in neonates.

Keywords

morphine; methadone; fentanyl; clonidine; inflammation; preterm infant; full-term infant

INTRODUCTION

Sick preterm and full-term infants are exposed to high doses of opioids in the neonatal intensive care unit (NICU) to provide sedation, analgesia and to treat neonatal abstinence syndrome (NAS). Clonidine, an α_2-adrenergic receptor agonist is broadly used to provide sedation and analgesia during anesthesia induction in children [1, 2, 3], and is also safe and effective in the treatment of NAS [4]. The similarities between the therapeutic effects of opioids and clonidine are suggestive of functional cross-talk between their receptors, opioid (OPR) and α_2-adrenergic, respectively. Evidence of this cross-talk includes that morphine also binds to α_2-adrenergic receptors [5], while clonidine indirectly activates OPRs via the induction of endogenous opiates [6]. This may explain the prevention of the cardiovascular effects of clonidine by both yohimbine (α_2-adrenergic receptor antagonist) and naloxone (non-specific OPR antagonist)[6, 7, 8]. Therefore, it is likely that opioids and clonidine share many other effects because the expression of classical OPRs (μ-, δ- and κ-) is not limited to neurons but they are also expressed in many adult immune cells from different species [9, 10, 11, 12, 13, 14]. Many components of the immune response are modulated by opioids[15] increasing the risk for sepsis in patients chronically exposed to these drugs [16]. We speculate that opioids, and perhaps clonidine, may also change immune responses in immune cells from neonates, a population highly susceptible to infections.

Compared to adults, the acquired immune response of preterm and full-term infants is deficient due to lower T-cell proliferation, greater CD4+/CD8+ ratio and lower natural killer cell activity [17]. Thus, the non-specific innate immune response becomes the primary mechanism to control infections in neonates. However, innate immunity in neonates is adapted to *in-utero* life, as shown by the significantly lower production of cytokines in response to Toll-like receptor (TLR)4 [18] and TLR2 activation [19], hypogammaglobulinemia, and decreased complement levels and opsonization [20], all of which are required to prevent excessive inflammation *in-utero*. Most of these adaptations disappear by full-term gestational age [21, 22, 23, 24], a time when neonatal innate response becomes similar to that of adults.

In many adult animal models and humans, opioid exposure down-regulates cytokine production [11, 25, 26, 27, 28, 29] and, in turn, modulates the expression of OPRs as demonstrated by decreased expression of μ-OPRs in IL-6 knockout mice [30], and by enhanced OPR expression in response to increased levels of IL-6, TNF-α, and IL-1β [31, 32, 33, 34]. Many similar effects have also been reported for clonidine [35, 36, 37, 38].

Because cytokine production is a crucial component of the innate immune response which is essential in neonates, the potential down-regulation of cytokine production by neonatal immune cells is of clinical significance in the NICU setting. Therefore, we hypothesized that opioids (morphine, methadone, and fentanyl) and clonidine, at clinically relevant concentrations, down-regulate cytokine production and decreases OPRs expression in immune cells of cultured blood from neonates. Here, we show for the first time the comparative detailed profile of cytokine modulation that follows the exposure to opioids and clonidine in immune cells from preterm and full-term infants and the respective changes in OPRs expression.

MATERIALS AND METHODS

Patients and specimens

This study complied with the Guidelines for Human Experimentation from the U.S. Department of Health and Human Services and received approval from The Johns Hopkins Institutional Review Board. Parents provided informed consent prior to inclusion in study.

Peripheral blood (0.2 to 0.4 mL) was collected from preterm infants (≤ 30 weeks gestational age), and cord blood (1 mL) was collected from full-term infants (≥ 37 weeks gestational age). Since peripheral blood sampling was consider an invasive procedure in healthy full-term neonates, we were required to use cord blood for our experiments, a source of blood that was for the most part not available in the preterm group due to size of the umbilical vessels and the acuity of the cases. We excluded infants with known genetic disorders, major respiratory or cardiovascular anomalies, intrauterine growth restriction or small for gestational age, suspected prenatal viral infections, clinical or histological (placental pathology) chorioamnionitis, confirmed sepsis (positive cerebral spinal fluid, urine or blood culture) or suspected sepsis (extension of initial antibiotic course to more than 48h) and confirmed prenatal drug exposure to cocaine, opioids or benzodiazepines by history or urine toxicological screens at any time during pregnancy and/or at delivery. All mothers of preterm infants included in the study received at least one dose of betamethasone prior to delivery which may have buffered the cytokine response observed in our experiments.

Whole blood was diluted at 1:10 (v:v) with 4X RPMI 1640 media containing 8% (v/v) ΔH human AB serum, penicillin/streptomycin (400 IU/ml/400 ug/mL), and 8 mM L-glutamine (Sigma-Aldrich, St. Louis, MO), and plated in 200 μL aliquots on a 48-well plate. Cultured whole blood was used to mimic the response of the mixture of immune cells observed *in-vivo* as described previously [39]. In the same plate, separate diluted whole blood aliquots were treated with morphine sulfate (Baxter, Deerfield, IL), fentanyl citrate (Hospira, Inc., Lake Forest, IL), methadone hydrochloride (Xanodyne Pharmaceuticals, Inc., Newport, KY; racemic mixture of R and S enantiomers), or clonidine hydrochloride (Sigma-Aldrich) at concentrations ranging from 0 (baseline) to 0.01 mM (fentanyl) or 1 mM (morphine, methadone, and clonidine) for 2h at 37°C and 5% CO_2, followed by blood activation with lipopolysaccharide (LPS; 100 ng/mL; Sigma-Aldrich) for 18h in culture (modified from [25]). Opioids and clonidine concentrations chosen for *in-vitro* testing were calculated based on clinically relevant blood concentrations reported in the literature [40, 41, 42, 43]. Concentrations of 10^{-5} to 10^{-3}M were clinically relevant for morphine and methadone, while concentrations

of 10^{-8} to 10^{-7}M were for fentanyl and clonidine. These calculated relevant molar concentrations may be underestimating actual *in-vivo* concentrations due to factors such as fluid restriction, hyperbilirubinemia, hypoalbuminemia, liver failure and drug interactions affecting sick neonates in the NICU. This is particularly true with respect to morphine and methadone, increasing the likelihood of achieving concentrations as high as 10^{-3}M [44, 45]. The concentration of LPS used for our experiments (100 ng/mL) was previously proven to produce significant TLR4 activation without decreasing cell survival [46] and has been used by others in similar models [47]. Collected supernatants were stored at -20°C for cytokine analysis performed at the University of Cincinnati, Laboratory of Immunobiology. Following supernatant collection, mononuclear cells (MNC) were isolated by adherence to plastic wells as previously described [47]. Ammonium-Chloride-Potassium lysis buffer (Quality Biological, Inc, Gaithersburg, MD) and Trypan Blue Exclusion (Gibco, Grand Island, NY) methods were used for cell count and to determine viability of MNC. MNC were then used to determine total cAMP concentrations and OPR expression profile.

ELISA for determination of cytokine and total cAMP concentrations

Pro-inflammatory IL-1β, IL-6, IL-8, IL-12p70 and TNF-α, and anti-inflammatory, IL-10, cytokine concentrations were measured (ELISA) using LINCOplex™ Multiplex kits (Millipore, Billerica, MA), following manufacturer's protocol, and concentrations were calculated using Luminex™ detection system (Millipore). The lowest dilution on the standard curve (0.64 pg/mL) was considered the lower limit of detection. Values below the lower limit of detection were calculated by maximum-likelihood estimation only if they represented <50% of all the measurements. In contrast, if >50% of all the measurements were below lower limit of detection, those cytokines were considered non-detectable.

Total cAMP concentrations were measured by non-acetylation procedure using the Amersham cAMP EIA System (GE Healthcare, Little Chalfont, Buckinghamshire, UK), as directed by the manufacturer. Results were reported as percentage change from baseline (no treatment, 0 mM) and standardized by mg of protein measured by Bradford Assay [48].

Real Time qRT-PCR for determination of OPR gene expression

To determine changes in μ-, δ-, and κ-OPR gene expression, total RNA was extracted from MNC. PureLink™ Micro-to-midi Total RNA Purification System (Invitrogen, Carlsbad, CA) was used according to manufacturer's specifications. RNA was utilized to generate cDNA, using iScript cDNA synthesis kit (BioRad, Hercules, CA). Reverse transcription protocol included 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C. cDNA was then used to amplify the target genes by real time qRT-PCR using primers at 300 nM (**Table 1**). SYBR Green Supermix (BioRad) was used for signal detection by MyIQ PCR Thermocycler (BioRad). Two different amplification protocols were used for: 1) μ-OPR, 40 cycles of 1 min at 95°C, 1 min at 60°C, and 1.5 min at 72°C with 3 min for extension; and 2) δ- and κ-OPRs, 45 cycles of 1 min at 95°C, 1 min at 62°C, and 1.5 min at 72°C with 10 min for extension. The fold difference in gene expression was corrected to the reference gene, GAPDH, using the Pfaffl method [49]. Melting curves were used to ascertain single PCR products.

Western blot analysis for determination of OPR protein expression

Protein homogenates were obtained from MNC using ice-cold ethanol precipitation method, then reconstituted in 0.01M PBS (pH 7.4; Quality Biological, Inc., Gaithersburg, MD) and protein concentration was determined using the Bradford method [48]. Protein (20 μg) was diluted 2:1 (v/v) with loading buffer, containing 20% (w/v) glycerol, and loaded to 15% SDS-PAGE. Proteins were transferred to nitrocellulose membrane, stained with Ponceau S, blocked with 5% nonfat dry milk with 0.1% Tween 20 in Tris-buffered saline (pH 7.4, 50 mM), and incubated overnight at 4°C with rabbit monoclonal anti μ-OPR antibody (Abcam, Cambridge, MA; 1:3,000), rabbit polyclonal anti κ-OPR antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 1:500) or mouse monoclonal anti β-actin antibody (Sigma-Aldrich; 1:10,000). The membrane was then washed with milk, exposed to goat anti-rabbit or anti-mouse antibodies (Bio-Rad) at 1:10,000 for 1h and then developed with enhanced chemiluminescence using SuperSignal kit (Thermo Scientific, Rockford, IL). Films were scanned using Adobe Photoshop (Adobe Systems Inc., San Jose, CA), and optical density (OD) was determined with IP Lab Gel H software (Signal Analytics, Vienna, VA) adjusting for background. β-actin was used for protein loading correction. Protein levels are expressed as arbitrary density units (ADU).

Statistical analysis

Because of the non-normal distribution of the data, nonparametric statistics using Wilcoxon and Mann-Whitney U tests was applied. Results are reported as median with interquartile range (IQR, 25^{th} to 75^{th} percentile) and represented as box-and-whisker plots with outliers (boxes symbolize IQR). Significance was assigned by $p < 0.05$ (vs. baseline) with multiple comparison correction using Bonferroni correction. All data presented as percentages do not require adjustments by white blood cell count (WBC) because ratios were calculated based on responses in the same blood, and since the correction factor (WBC) was in both the numerator and the denominator, it was cancelled from the equation. SPSS 18.0 software (SPSS Inc., Chicago, IL) was used for analysis and graphs.

RESULTS

Baseline cytokine concentrations

Blood samples were obtained from 7 preterm and 19 full-term infants of whom demographics and hematological parameters are detailed in **Table 2**. Diluted blood was plated, cultured for 2h (no opioids or clonidine), activated with LPS (100 ng/mL), and then incubated for 18 additional hours after which time cytokines levels were measured in supernatants to provide baseline levels. While IL-1β, IL-6, IL-10, and TNF-α concentrations were significantly greater in supernatants recovered from full-term rather than preterm cultured blood; following correction with white blood cell (WBC) count (x 1000 / mm^3), only IL-10 levels remained significantly greater in supernatants recovered for full-term blood (146-fold greater than preterm blood, $p<0.001$, **Table 3**).

Changes in cytokine and cAMP concentrations

Full-term group—Cytokine concentrations did not change following exposure to opioid concentrations from 10^{-11} to 10^{-7}M, although a ~25% increase in IL-8 levels was observed in response to morphine at 10^{-7} (median, $p<0.05$ vs. baseline). At greater concentrations, morphine and methadone (10^{-3}M) significantly decreased all measured cytokines except for IL-8 (**Fig.1A** and **1C**). Interestingly, fentanyl did not decrease cytokine levels at any concentration but it did increase IL-12p70 levels by a median of 70% (p=0.01 vs. baseline) at 10^{-5}M concentration (**Fig. 1B**). Clonidine, on the other hand, increased IL-6 concentrations by a median of 55% (IQR 22.7 to 131.4%, p=0.01 vs. baseline) at 10^{-11}M and 38% (IQR 25.6 to 101.2%, p=0.02 vs. baseline) at 10^{-9}M. The highest tested concentration of clonidine (10^{-3}M) increased IL-1β by 199% (IQR 55.7 to 480.2%, p=0.02 vs. baseline), and decreased TNF-α by 53% (32.6-63.3%, p=0.01 vs. baseline) and IL-12p70 by 92% (IQR 96.2 to 67.2%, p =0.01 vs. baseline) without modulating IL-6 or IL-8 levels (**Fig.1D**). The biological significance of changes reported for IL-12p70 is unknown due to the overall low baseline levels (shown in **Table 3**).

Morphine, methadone and clonidine treatment at 10^{-3}M modulated the cytokine profile and increased total cAMP levels in MNC isolated by adherence from full-term infants by 38.9% (17.5-214.6%, p=0.005), 55.8% (13.5-327.1%, p=0.005) and 39.5% (21.8–252.2%, p=0.005), respectively by treatment group (**Fig.2**). No changes in cAMP were observed following *in-vitro* exposure to fentanyl at 10^{-5}M (data not shown).

Preterm group—Only two concentrations, 10^{-5} and 10^{-3}M, were tested for morphine, methadone and clonidine due to the small blood sample volume permitted from preterm infants. Similarly, fentanyl was not tested since the pattern of cytokine change was very limited in our previous experiments using blood from full-term infants (described above). Cytokine concentrations in supernatants of cultured diluted peripheral blood from preterm infants showed a similar pattern of changes compared to those observed experiments using cord blood from full term infants: morphine and methadone at 10^{-3}M decreased all measured cytokines (**Fig.3A** and **3B**), while clonidine at 10^{-3}M, increased IL-1β by median of 108% (IQR 80.2 to 132.6%, p=0.01), and decreased TNF-α by 53% (33.7-59.8%, p=0.01; **Fig.3C**). As shown in **Table 3**, IL-12p70 expression was not detected.

MNC viability following 18 h exposure to opioids or clonidine at 10^{-3}M was evaluated in both study groups and no difference was found between groups or treatments with an average viability of 95 ± 3%.

Changes in OPR expression

Gene expression—All of the three major classical OPR genes (μ-OPR1, δ-OPR1 and κ-OPR1) were expressed in MNC from preterm and full-term infants isolated by adherence. While morphine (10^{-5}M) did not modulate μ-OPR1 gene expression, higher concentrations (10^{-3}M) decreased gene expression by a median of 35% (IQR 16-53%, p=0.01 vs. baseline) and 20% (10-60%, p=0.02 vs. baseline) in MNC from both preterm and full-term infants, respectively. μ-OPR1 gene expression, in MNC from preterm and full-term infants, was also inhibited by methadone at 10^{-5}M by a median of 57% (IQR 37-71%, p=0.04 vs. baseline)

and 20% (10-60%, p=0.02 vs. baseline), respectively; and at 10^{-3}M by 44% (38-76%, p=0.04) and 40% (36-50%, p=0.02), respectively. In contrast, clonidine (10^{-5}M) significantly increased μ-OPR1 gene expression by over 80% (median), while higher concentrations (10^{-3}M) inhibited gene expression by over 30% in both preterm and full-term MNC (p≤0.02 for both groups vs. baseline; **Fig. 4A**). Fentanyl, only tested in MNC from full-term infants, did not modify μ-OPR1 expression (data not shown).

High concentrations of morphine, methadone or clonidine did not modulate the gene expression of δ-OPR1 (data not shown). In contrast, morphine and clonidine at 10^{-5}M inhibited κ-OPR1 gene expression by a median of 32% (IQR 15-35%, p=0.01vs. baseline) and 48% (25-70%, p=0.02), respectively in MNC from preterm infants; and by 80% (64-88%, p=0.02) and 82% (59-92%, p=0.02), respectively in those from full-term infants (**Fig 4B**).

Protein expression: For those OPRs with modified gene expression following opioid or clonidine exposure, we determined the effect on protein expression using Western blot. Due to sample volume limitations with preterm infants, studies for OPR protein expression were only performed in MNC from full-term infants. Generally, the effect of the drugs at the different concentrations altered gene and protein expression to the same degree. For example, in response to concentrations of 10^{-5}M of morphine and methadone, μ-OPR1 protein expression was decreased by a median of 44.2% (IQR 32.2-56.3%, p=0.02 vs. baseline) and 15.1% (3-35.4%, p=0.02), respectively. At higher doses (10^{-3}M), only methadone significantly decreased μ-OPR1 protein expression by a median of 52.1% (IQR 43.6-57.7%, p=0.03 vs. baseline). While clonidine at 10^{-5}M increased μ-OPR1 gene expression, protein levels for μ-OPR1 did not differ from control levels. However, at 10^{-3}M clonidine did decrease μ-OPR1 protein expression by a median of 14.5% (IQR 7.9-43.8%, p=0.02 vs. baseline; **Fig. 4C**), as it did for gene expression. Fentanyl did not change μ-OPR1 protein expression Unlike the results for gene expression, κ-OPR1 protein levels were unchanged following treatment with either opioids or clonidine at any tested concentrations (**Fig. 4D**).

DISCUSSION

Opioids, such as morphine, methadone, and fentanyl, are widely used in neonates for the treatment of NAS, pain, and agitation. Clonidine, used for anesthesia induction in children [1, 2, 3], has been recently proposed as a new adjunct therapy for NAS [4]. While opioid exposure inhibits cytokine production by adult immune cells [25, 26, 27], ours is the first systematic evaluation of the effects of opioids and clonidine on neonatal immune cells. Using cultured blood from preterm and full-term infants to mimic the *in-vivo* response generated by the mixture of different immune cells, we show that: i) all OPRs receptors are expressed in neonatal MNC; ii) the kind and concentration of analgesic/sedative agent can differentially modulate the level of gene and protein expression of OPRs, the level of total cAMP, and the production of cytokines; and iii) these effects were for the most part similar between cells from preterm and full-term infants. Specifically, we find that clinically relevant concentrations (10^{-3}M) of morphine and methadone decrease μ-OPR1 gene and protein expression, while increasing cAMP accumulation and broadly decreasing overall

cytokine production. Conversely, fentanyl does not modulate cytokine production at any clinically relevant concentration and clonidine has less of an effect on OPR expression and cytokine production than methadone or morphine. These data suggest that concentrations of morphine and methadone that are translatable to the clinical setting in the treatment of sick infants in the NICU may be decreasing properties of the innate immune response in neonates, such as cytokine production.

OPRs are coupled to protein G_i/G_o and their activation by low concentrations of opiates decreases the accumulation of cAMP, a critical second messenger that when inhibited leads to up-regulation of several cytokines [50]. Morphine at low concentrations enhances, while at high concentrations reduces, LPS-induced Nuclear Factor-kappa B (NF-κB) in adult immune cells, suggesting that opioids affect cytokine production at the transcriptional level and its direction is concentration dependent [27, 51]. Chronic administration or high concentrations of opioids is associated with increased cAMP (i.e., adenylyl cyclase superactivation), which acts to inhibit NF-κB and cytokine production [52]. Neither clonidine nor opioids have been explored with respect to their modulation of cAMP and their resulting effects on cytokine production *in-vivo* or *in-vitro* in neonates until now. Here, we suggest that the same principles for OPR activation in MNC from adults may apply to those from preterm and full-term infants. We demonstrate that, in newborns, cAMP production is increased in MNC exposed to morphine, methadone or clonidine at 10^{-3}M, explaining the significant down-regulation of pro-inflammatory (TNF-α, IL-1β, and IL-6) and anti-inflammatory (IL-10) cytokine production. This generalized cytokine suppression has unknown repercussions in the neonatal innate immune response and the risk for sepsis; however, since *in-utero* adaptations persist in preterm infants and resolve by full-term gestational age [21, 22, 23, 24], we can speculate that these effects may have a greater relevance in the sick preterm infant in the NICU. Furthermore, the cytokine responses observed in culture blood from preterm infants may be buffered by the use of perinatal steroids, suggesting that the real effects could be even greater. Our data also show that μ-OPR1 expression is down-regulated following exposure to 10^{-3}M concentrations of morphine and methadone, but not fentanyl, which was also reported in the central nervous system linked to high doses and development of opioid tolerance [53, 54]. We speculate that down-regulation of μ-OPR1 expression occurs in response to the decrease in cytokine production as suggested by the attenuated μ-OPR expression reported in IL-6 knockout mice [30] and the enhanced expression in response to increase IL-6, TNF-α, and IL-1β [31, 32, 33, 34].

All tested opioids, morphine, methadone and fentanyl, bind to all classical OPRs (μ-, δ-, κ-) but their potency at each receptor site depends on their biochemical composition. For instance, fentanyl binds more avidly than morphine to μ-OPRs (subtype 1 and 2), while morphine binds more avidly than fentanyl to κ- and δ-OPRs [55], which may explain the lack of cytokine changes seen with fentanyl exposure in our experiments. Furthermore, while μ-OPR may be the primary site for the effects seen in the central nervous system with morphine treatment, it may not be the sole site inducing changes in cytokine expression by immune cells in neonates reported above [56, 57, 58, 59] and perhaps the activation of κ- and δ-OPRs have a greater influence in those changes. In that sense, based on our results, we may argue both ways, that: i) κ- and δ-OPR overstimulation with high concentrations of

morphine and methadone may inhibit overall cytokine production by neonatal immune cells by adenylyl cyclase superactivation (as described above), secondarily decreasing μ-OPR expression in MNC and inducing further predominance of κ- and δ-OPRs (decreased competitive binding to μ-OPR), or ii) high morphine and methadone concentrations, may produce primarily a decline in μ-OPR expression at the transcriptional level, inducing a decrease in cytokine expression by a greater predominance of κ- and δ-OPR activation. Because, in our experiments, fentanyl, a more potent μ-OPR agonist, does not modulate cytokine production or OPR expression, we speculate that the effect of morphine and methadone in neonatal immune cells is linked to κ- and δ-OPRs via the potential mechanisms explained above. Although fentanyl exposure does inhibit IL-1β and TNF-α in rodent models [60] and in adult human whole blood experiments [61, 62], this effect is diminished after 3 days of exposure [63], perhaps related to a decline in μ-OPR expression. Therefore, we speculate that lower μ-OPR expression in immune cells from neonates versus those from adults may explain the difference in response to fentanyl observed in our experiments, a speculation that we cannot confirm with these experiments since an adult group was not included.

Clonidine, an α_2-adrenergic receptor agonist, prevents the immunomodulatory effects reported with morphine withdrawal in animals [8, 64]. In human adult whole blood and in cord blood MNC, clonidine also stimulates production of the anti-inflammatory cytokine, IL-10, while decreasing the production of the pro-inflammatory cytokine, TNF-α [7, 38]. It appears from our data that in MNC from newborns (full-term cord blood or preterm peripheral blood), clonidine does not modify the production of IL-10 but it does decrease TNF-α, confirming previous studies [7, 65]. In neonatal immune cells, the mechanism underlying these changes is still unknown; however, since cross-talk between α_2-adrenergic receptors and OPRs is evident [6, 7, 8], we postulate that the changes in cytokine expression is, at least in part, indirectly linked to activation of OPRs, as suggested by the increase cAMP accumulation which was also observed with clonidine treatment (**Fig 2**).

The translation of the concentrations of opioids and clonidine that induce decreases in cytokine production and μ-OPR1 expression *in-vitro* to those relevant in the NICU setting is cumbersome; however, there are a few previous studies that may put these results in perspective. With respect to morphine, when used for post-operative analgesia in full-term infants, serum concentrations of 10 and 32 ng/dl, are achieved with doses as small as 5 and 10 μg/k/h, respectively [40, 44], suggesting that changes in dosages can produce exponential increases in serum concentrations in neonates. Since morphine is adjusted to response in the NICU, doses may be higher than 200 μg/k every 2-4h, suggesting that serum concentrations of 10^{-5} and 10^{-3}M are not unlikely. Furthermore, lower volume of distribution (fluid restriction or increased interstitial edema), hyperbilirubinemia, hypoalbuminemia, liver failure, lower clearance of the drug and drug interactions in sick neonates may further increase the risk of greater serum morphine concentrations [44]. These factors also affect methadone blood concentrations which may vary up to 40- fold despite the same dosage schedule, explaining the equally variable therapeutic responses [45, 66, 67]. In neonates exposed *in-utero* to methadone, withdrawal symptoms appear when blood concentrations fall below 88 ng/dl [41], perhaps representing the threshold for NAS treatment (10^{-5}M);

however, based on the many factors described above levels of 10^{-3}M may still be clinically relevant. Fentanyl potency is 100-fold greater than that of morphine, suggesting that concentrations of 10^{-5}M represent concentrations as high as 10^{-3}M of morphine. In infants undergoing cardiac surgery, plasma fentanyl levels are 15 ng/ml (10^{-7}M) during IV infusion at 18 μg/k/h [42]; however, this rate is significantly greater than that used for sedation in the NICU. Lastly, the pharmacokinetics of clonidine during different therapeutic paradigms for NAS treatment in neonates demonstrate that the clearance of the drug improves rapidly during the second week of life with levels that can be as high as ~1.5 ng/mL (10^{-8} M) at doses of 2 μg/ k/ dose q4h [43]. Therefore, clinically relevant concentrations for morphine and methadone are in the range of 10^{-5} to 10^{-3}M, while for fentanyl and clonidine are in the range of 10^{-8} to 10^{-7}M, suggesting that fentanyl and clonidine may have a safer immunomodulatory profile compared to morphine and methadone.

Opioids are titrated to effect for many clinical uses in neonates including treatment of pain, control of NAS, and induction of anesthesia. Clonidine is used as an effective adjunct therapy for NAS and significantly decreases the duration of opioid exposure without negative impact on cardiovascular outcomes [4]. While further studies must be conducted to examine the immunomodulatory effects of opioids *in-vivo*, our study using neonatal cultured blood shows significant inhibition of cytokine release at clinically relevant concentrations of morphine and methadone. Our results also suggest that fentanyl and clonidine may be safer alternatives in neonates, since their modulation of cytokine production and OPRs is limited. Sick neonates have significant deficiencies in acquired immunity and adaptations of innate immunity, which increase their baseline risk for developing infections in the NICU setting. Therefore, a greater understanding of the potential immunosuppressant effects of perinatal opioid exposure is imperative for judicious use of these drugs in the NICU.

ACKNOWLEDGMENT

The authors gratefully acknowledge Dr. Frances Northington and Dr. Robert Bennett for their support; Mrs. Debra Flock and Ms. Ariel Mason for their administrative assistance and the obstetric team at Johns Hopkins Hospital for their kind support with patient enrollment. We are also indebted to our patients and their families for their willingness to participate in this study.

FINANCIAL SUPPORT: National Institute for Drug Abuse (NIDA) R25DA021630 (E.B.G).

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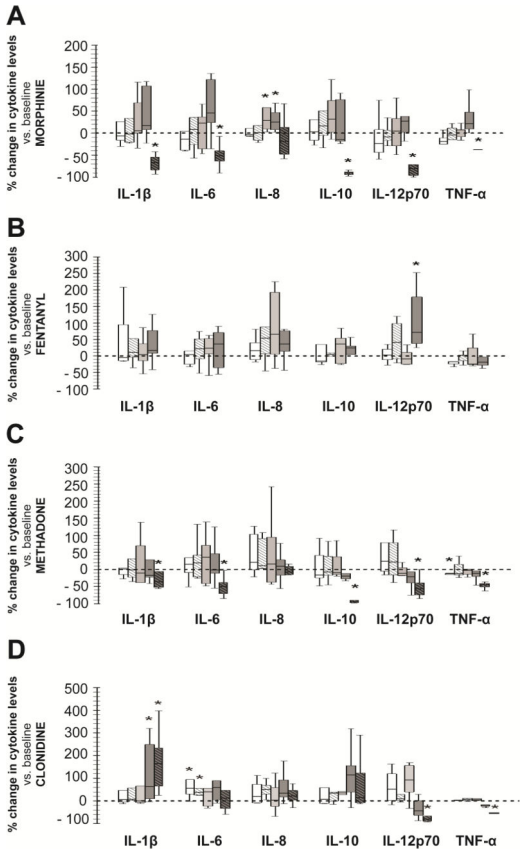

Figure 1.

Percent change in cytokine levels in supernatants of cultured blood from full-term infants following exposure to morphine **(A)**, fentanyl **(B)**, methadone **(C)**, and clonidine **(D).** Concentrations were: 10^{-11}M (white), 10^{-9}M (hashed-white), 10^{-7}M (light grey), 10^{-5}M (dark grey), and 10^{-3}M (hashed-black). Changes represented as box-and-whisker plot, boxes represent IQR and median (solid line inside the boxes). Reference line at 0 represents baseline (0 M). *, p≤0.01 (vs. baseline, Wilcoxon test); n=7.

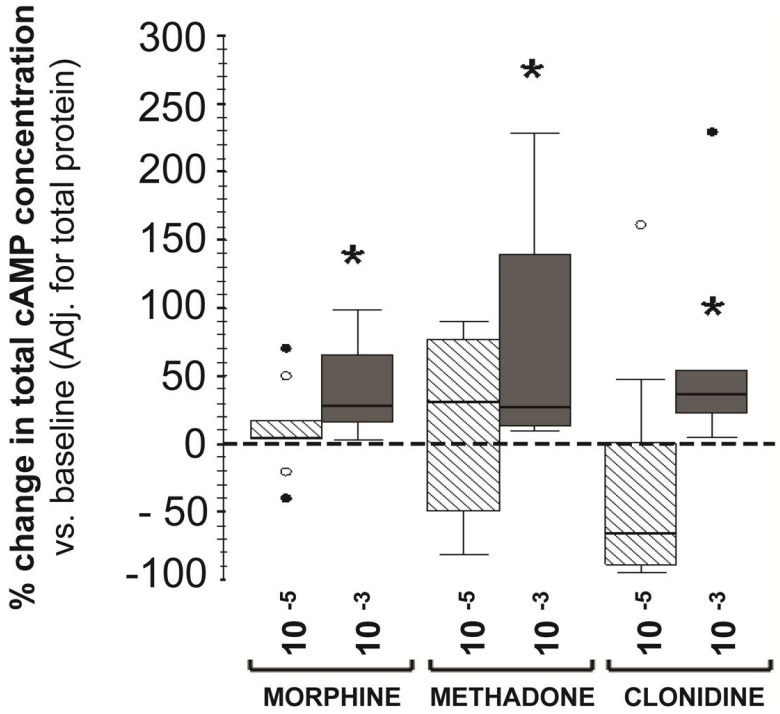

Figure 2.

Percent change in protein-adjusted total cAMP concentrations following treatment with morphine, methadone, and clonidine at concentrations of 10^{-5}M (hashed-white) and 10^{-3}M (dark grey). Changes presented as box-and-whisker plot. Reference line at 0 represents baseline condition (0 M). *, $p < 0.01$ (vs. baseline, Wilcoxon rank test). ○, outliers; ●, extremes; n=10 (full-term infants).

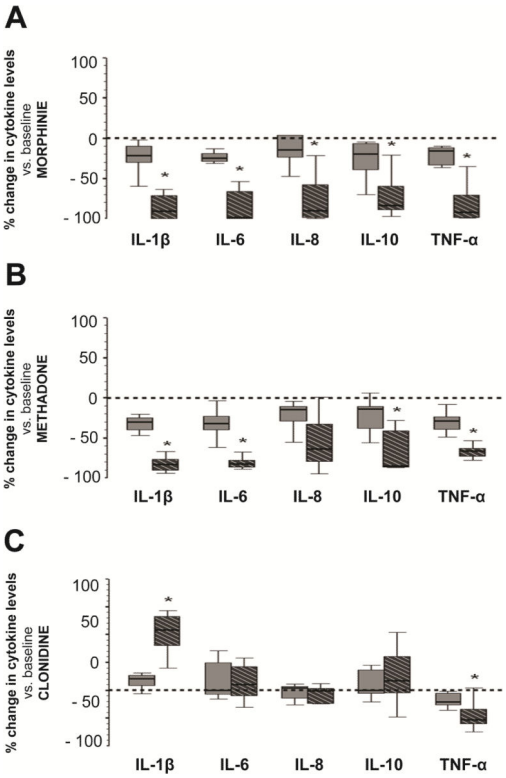

Figure 3.

Percent change in cytokine levels in supernatants of cultured blood cells from preterm infants following exposure to morphine **(A)**, methadone **(B)**, and clonidine **(C).** Concentrations included 10^{-5} M (dark grey) and 10^{-3} M (hashed-black). Changes represented as box-and-whisker plot, with boxes representing IQR and median (solid line in boxes). Reference line at 0 represents baseline condition (0 M). *, $p \leq 0.01$ (vs. baseline, Wilcoxon rank test); n=7.

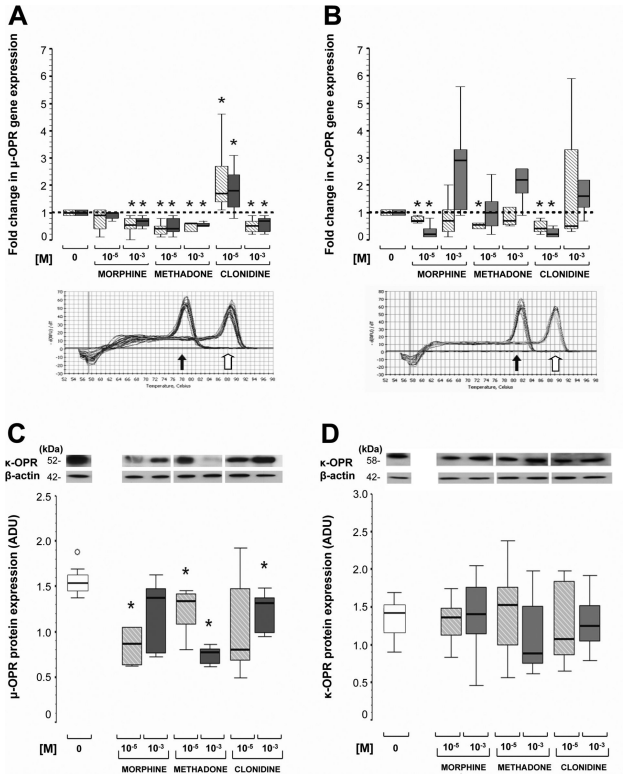

Figure 4.

Fold change in μ- **(A)**, and κ- **(B)** OPR gene expression in MNC from preterm (hashed-white) and full-term (grey) infants in response to morphine, methadone, and clonidine at 10^{-5}M and 10^{-3}M. Protein expression of μ- **(C)** and κ- **(D)** OPR in MNC from full-term infants to similar treatments at 10^{-5}M (hashed-grey) and 10^{-3}M (dark grey). Changes represented as box-and-whisker plot, with boxes representing IQR and median (solid line in boxes). For gene expression **(A** and **B)**, reference line at 1 represents gene expression at baseline condition (0 M) and melting curves identified a single PCR product for each OPR (block arrow) along with GAPDH (housekeeping gene, white arrow). For protein expression (**C** and **D**), representative 15% SDS-PAGE immunoblots for respective OPRs with β-actin loading controls are also presented. *, $p < 0.05$ (vs. baseline, Wilcoxon rank test). ○, outliers, n=7/group.

TABLE 1

PRIMERS FOR REAL TIME qRT-PCR

Gene	Direction	Sequence	Base pair
OPRM1	**S**	5′- AGTTCTGTATCCCAACCTCTTCC -3′	60-bp
	AS	5′ - TCTGACGAATTCGAGTGGAG -3′	
OPRK1	**S**	5′ -GGAGAGAATAGTAGCTGTATGT-3′	120-bp
	AS	5′ -AGCAGTACCCTAAAATGATATT-3′	
OPRD1	**S**	5′ - TGCACCTGTGCATCGCGCTGA -3′	141-bp
	AS	5′ - GGCTGAAGCTGCTGGGGTC -3′	
GAPDH	**S**	5′ - AACAGCGACACCCACTCCTC -3′	258-bp
	AS	5′ - GGAGGGGAGATTCAGTGTGGT -3′	

AS, anti-sense; **OPRD1**, δ-opioid receptor; **OPRK1**, κ- opioid receptor; **OPRM1**, μ-opioid receptor; **S**, sense.

TABLE 2

CHARACTERIZATION OF STUDY GROUPS

		Preterm group	Full-term group
Demographic and perinatal variables		**n = 7**	**n = 19**
Gestational age (wk)	mean ± SD	26 5/7 ± 1.8	39 2/7 ± 1.0
Birth weight (g)	mean ± SD	865 ± 252	3165 ± 374
Gender	% male	(4) 57 %	(13) 68.4%
Race	% African American	(6) 86%	(12) 63.2%
1 minute APGAR score	median, IQR	5 (5 - 7)	8 (8 - 9)
5 minutes APGAR score	median, IQR	7 (7 - 8)	9 (9 - 9)
Delivery method	% cesarean section	(4) 57 %	(7) 36.8 %
Clinical choriamnionitis		(5)71 %	(0) 0 %
Pathological chorioamnionitis		(3) 43 %	(0) 0 %
Confirmed infection		(0) 0 %	(0) 0 %
Hematological parameters			
Hematocrit	mean ± SD	43 ± 6 %	49 ± 5 %
White blood cell count (per mm^3)	mean ± SD	8953 ± 3461	14233 ± 4168
Neutrophils	mean ± SD	52 ± 19 %	49 ± 13 %
Lymphocytes	mean ± SD	32 ± 17 %	41 ± 16 %
Monocytes	mean ± SD	9 ± 5 %	8 ± 0.5 %
Platelet count (× 1000 per mm^3)	mean ± SD	165 ± 72	265 ± 30

TABLE 3

BASELINE CYTOKINE CONCENTRATIONS POST- LPS ACTIVATION

Cytokines (pg/ml)		Preterm group n = 7		Full-term group n = 19		p-value	
Pro-inflammatory		**Absolute**	**WBC adjusted†**	**Absolute**	**WBC adjusted†**	**Absolute**	**WBC adjusted†**
IL-1β	**Mean**	40.5 1	2.89	138.7	8.88	0.03*	0.11
	25tile	17.13	1.72	116.5	6.84		
	75tile	53.04	13.7	188.0	17.57		
IL-6	**Mean**	317.15	24.56	2409.2	166.2	0.001*	0.07
	25tile	179.7	11.24	2197.2	118.3		
	75tile	941.2	204.2	3999.7	373.3		
IL-8	**Mean**	3000 . 5	433.1	5894.9	453.0	0.41	0.96
	25tile	1995.4	192.4	4775.4	404.2		
	75tile	9354.6	3697.5	7183.7	556.0		
IL-12p70	**Mean**	ND	ND	1.03	0.09	--	--
	25tile	--	--	0.49	0.03		
	75tile	--	--	1.81	0.12		
TNF-α	**Mean**	63 . 1	5.55	310.6	17.42	0.03*	0.25
	25tile	45.0	3.65	180.6	9.72		
	75tile	156.5	28.80	452.5	28.43		
Anti-inflammatory							
IL-10	**Mean**	7 . 7 1	0.55	647.8	80.57	<0.001*	<0.001*
	25tile	4.31	0.38	557.8	48.90		
	75tile	12.03	4.52	1158.0	149.5		

*$p < 0.05$, preterm vs. fullterm group (Mann Whitney U Test)

†Cytokine concentrations adjusted by WBC = [Absolute cytokine concentration (pg/ml) / WBC (× 1000 per mm^3)]