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A role for reactive oxygen species in endotoxin-induced elevation of MOR expression in the nervous and immune systems¹

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Abstract

This study examined the mechanism by which exposure to lipopolysaccharide (LPS) alters mu-opioid receptor (MOR) expression in immune and neuronal cells using an *in vitro* conditioned medium model system. We found that LPS stimulated the intracellular accumulation of reactive oxygen species (ROS) and MOR expression in macrophage-like TPA-HL-60 cells. Conditioned medium from the LPS-stimulated TPA-HL-60 cells increased MOR expression in SH-SY5Y cells, a neuronal cell model, through actions mediated by TNF- α and GM-CSF. These data suggest that the endotoxin, LPS, modulates MOR expression in nervous and immune cells via ROS signaling, and demonstrates the crosstalk that exists within the neuroimmune axis.

Keywords

lipopolysaccharide; reactive oxygen species; mu-opioid receptor

1. Introduction

Lipopolysaccharide (LPS), an outer-membrane component of gram-negative bacteria, is a well characterized endotoxin that activates the immune system, and, in particular, induces inflammation (Dunn, 1991; Lopez-Bojorquez et al., 2004; Parrillo et al., 1990; Raetz and Whitfield, 2002; Rumpa, 2010). In some cases, endotoxemia progresses to severe sepsis, resulting in multiple organ dysfunction, septic shock, and death (Lopez-Bojorquez et al., 2004; Qu et al., 2009). Morbidity associated with severe sepsis is high. There are one million deaths from sepsis worldwide, and approximately 25-30% of the cases are due to gram-negative bacterial infection (Rumpa, 2010).

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The host-mediated response to endotoxemia involves the secretion of inflammatory cytokines and mediators as well as the activation of the coagulation and complement cascades (Andreasen et al., 2008; Dunn, 1991; Lopez-Bojorquez et al., 2004). The increased levels of circulating inflammatory cytokines resulting from LPS endotoxemia exacerbate systemic inflammation. Our previous studies showed that the levels of the pro-inflammatory cytokines, TNF- α , IL-1 β , and IL-6, are elevated in both the serum and brain of rats treated systemically with LPS (Chen et al., 2005; Ocasio et al., 2004). Other studies have reported an increase in the secretion of IL-1 β and TNF- α from macrophages following LPS treatment (Evans et al., 1991; Hsu and Wen, 2002). Our previous findings also indicated that LPS couples the immune and nervous systems via actions mediated by pro-inflammatory cytokines on the hypothalamic-pituitary-adrenal (HPA) axis and that such cross-talk is necessary in order to maintain homeostasis in response to infection (Chang et al., 1998).

Inflammatory cytokines can modulate the expression of the mu-opioid receptor (MOR) in both neuronal and immune cells. In 1998, we reported that co-treatment with IL-1 α and IL-1 β increases MOR expression in microvascular endothelial cells (Vidal et al., 1998). IL-6 increases MOR expression and MOR binding in SH-SY5Y neuroblastoma cells (Borner et al., 2004), and TNF- α increases MOR expression in human T lymphocytes, Raji B cells, U937 monocytes, primary human polymorphonuclear leukocytes, and mature dendritic cells (Kraus et al., 2003).

The activation of the opioidergic pathway via the MOR leads to suppression of the immune response (Gaveriaux-Ruff et al., 1998; Wang et al., 2002). Chronic administration of morphine, a MOR agonist, desensitizes the pro-inflammatory cytokine-mediated effects on the HPA axis and deregulates the immune response in rats (Chang et al., 2001; Chang et al., 1995; Chang et al., 1996; Chen et al., 2005). In addition, deregulation of immune responses by exogenous opioids leads to many of the complications associated with LPS-induced endotoxic shock (Chang et al., 2001; Chang et al., 1998; Chen et al., 2005).

Reactive oxygen species (ROS) are highly reactive molecules produced during cellular respiration (Bast and Goris, 1989; Bayir, 2005; McCord and Fridovich, 1978). Both intracellular and extracellular ROS are maintained at non-lethal levels by superoxide dismutases, catalases, and a thiol-reducing buffer consisting of glutathione and thioredoxin (Gamaley and Klyubin, 1999; Nakamura et al., 1997). However, disease and stress can alter a cell's ability to effectively regulate ROS. Elevated levels of ROS can damage proteins, DNA, RNA, and cell membranes by hydroxyl radical attack, and induce apoptosis (Bast and Goris, 1989; Machlin and Bendich, 1987; McCord and Fridovich, 1978). Exposure to LPS increases the production of ROS in murine macrophages (Hsu and Wen, 2002; Kim et al., 2004), and the accumulation of ROS is a promoting factor in the development of sepsis in rats (Bayir, 2005). Thus, ROS appears to play a key role in the LPS-induced inflammatory response and the subsequent incidence of sepsis.

In this study, an *in vitro* conditioned medium (CM) model was used to investigate the mechanism by which LPS exposure alters MOR expression in immune and neuronal cells. Specifically, we examined the effects LPS-induced ROS accumulation on MOR expression in TPA-differentiated HL-60 (TPA-HL-60) macrophage-like cells (Kowalski and Denhardt, 1989; Rovera et al., 1979). We also assayed the conditioned medium from the LPS-treated TPA-HL-60 cells for TNF- α , GM-CSF, IL-1 β , IL-8, IL-10, IL-12p70, IL-2, IL-6, and INF γ to determine if LPS-induced ROS had a modulating effect on the cytokine secretion profile. We then evaluated MOR expression in SH-SY5Y neuroblastoma cells (Ciccarone et al., 1989) cultured in CM from the LPS-treated TPA-HL-60 cells. We have previously used undifferentiated SH-SY5Y cells to study MOR expression (Yu et al., 2003; Zadina et al., 1993; Zadina et al., 1994), and others have reported the use of SH-SY5Y as a neuronal cell

model since it presents several neuronal markers, including tyrosine and dopamine- β -hydroxylase activity, uptake of norepinephrine, and core M_r 68,000 and M_r 150,000 neurofilament proteins (Ciccarone et al., 1989; Gao et al., 2001; Ruffels et al., 2004; Wu et al., 2007). Our findings indicate that ROS plays a key role in LPS-induced modulation of MOR expression in both neuronal and immune cells.

2. Materials and methods

2.1. Cell culture and treatments

Human HL-60 promyelocytic leukemic cells (ATCC, Manassas, VA) were grown in RPMI 1640 medium supplemented with 20% FBS, 100 U penicillin, and 100 μ g/mL streptomycin (Gibco, Invitrogen Corp., Grand Island, NY). Experimental 12-well plates (BD Biosciences, VWR, West Chester, PA) were seeded with HL-60 cells at 5×10^5 cells/mL in 1 mL/well. In this study, HL-60 cells were differentiated with 12-O-tetradecanoylphorbol-13-acetate (TPA) into macrophage-like cells (TPA-HL-60) over a period of 48 h (Sigma-Aldrich, St. Louis, MO). Stock solutions of TPA were dissolved in 100% ethanol to a concentration of 16 μ M and diluted 1000-fold in medium. Previous studies have shown greater than 95% of HL-60 cells differentiate to macrophage-like cells after a 48-h treatment with 16 nM TPA (Kowalski and Denhardt, 1989; Rovera et al., 1979). After TPA differentiation of HL-60 cells, the medium containing TPA was removed and the TPA-HL-60 cells were rinsed 3 times with 1 mL of phosphate-buffered saline solution (PBS) [Invitrogen, Grand Island, NY]. Human SH-SY5Y neuroblastoma cells, a gift from R. Ross (Fordham University, New York, NY), were grown in MEM + F12 medium supplemented with 10% FBS, 100 U penicillin, and 100 μ g/mL streptomycin (Gibco, Invitrogen Corp., Grand Island, NY). SH-SY5Y cells were seeded at 1×10^5 cells/mL in 1 mL/well. TPA-HL-60 and SH-SY5Y cells were maintained in a 5% humidified incubator at 37° C.

A stock solution of lipopolysaccharide from *E. coli* 055:B5 (LPS, Sigma-Aldrich, St. Louis, MO) was prepared in 0.9% saline. TPA-HL-60 cells were treated with medium containing LPS at a final concentration of 0 to 0.500 mg/mL. For ROS scavenging experiments, TPA-HL-60 cells were pre-treated with medium containing 100 μ M vitamin E (Sigma-Aldrich, St. Louis, MO) for 3 h, and then treated with medium containing LPS. Unless noted otherwise, in CM experiments, TPA-HL-60 supernatants were pooled, filter sterilized (0.2 μ m filters, Pall Life Sciences, Ann Arbor, MI), and immediately overlaid onto SH-SY5Y cells. SH-SY5Y cells used in CM experiments were grown for a period of 72 h prior to TPA-HL-60-CM treatment.

2.2. Measurement of intracellular ROS

Intracellular ROS levels in TPA-HL-60 cells cultivated in 12-well plates were determined by confocal laser scanning microscopy (CLSM). After LPS treatment, the medium was replaced with fresh medium containing 20 μ M dihydrorhodamine123 (DHR123) [Sigma-Aldrich, St. Louis, MO], and incubated for 30 min (Henderson and Chappell, 1993). DHR123 is an indicator of hydrogen peroxide, hypochlorous acid, and peroxynitrite anion (Crow, 1997; McBride et al., 1999; Radi et al., 2001). All treatment groups examined in CLSM experiments were loaded with DHR123 in medium. After incubation with DHR123, the medium containing DHR123 was removed and replaced with fresh medium alone. The loading and removal of all treatment groups with DHR123 prior to CLSM controls for any potential differences in ROS levels due to loading or leaking of DHR123. The medium was then replaced with fresh medium alone, and ROS levels were measured with a FluoView FV1000 CLSM (Olympus, Center Valley, PA) at 200 $\times$ magnification. Laser transmissivity was set to 20%; the cells were excited at 488 nm, and fluorescence emission was detected at 520 nm. Changes in intracellular ROS levels were calculated as the percent control from the

mean fluorescence intensity of 10 randomly selected cell clusters from 3 views from 3 wells per treatment as follows:

$$\text{cell cluster ROI} \left(\sum \text{avg. CHS1}^{1,2,\dots,10} * \text{area}^{1,2,\dots,10} / \sum \text{area} \right) - \text{background ROI} \left(\sum \text{avg. CHS1}^{1,2,\dots,10} * \text{area}^{1,2,\dots,10} / \sum \text{area} \right)$$

where ROI refers to the region of interest and avg. CHS1 refers to the average fluorescence of the pixilated ROI.

2.3. Measurement of gene expression

Total RNA was extracted and isolated using TRIzol or TRIreagent in accordance with the manufacturer's protocol (Ambion, Invitrogen Corp., Grand Island, NY). cDNA was prepared from 1 µg of total RNA in 20 µl containing 1X first strand buffer, 10 mM DTT, 0.25 mM dNTPs, 0.015 µg/mL random primers, and 15 U/µl M-MLV (Invitrogen Corp., Grand Island, NY). Reverse transcription reactions were incubated in a GeneAmp 2400 Thermocycler (Eppendorf, Westbury, NY) for 1 h at 37° C, followed by 10 min at 67° C.

The relative changes in SH-SY5Y MOR expression were determined using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). Real-time PCR was performed using 1 µl of cDNA as the template in 20 µl containing 1X Universal PCR Master Mix, 0.4 µM probe, and 0.4 µM of both sense and antisense primers in a 7900 HT Fast Real Time PCR System (Applied Biosystems Inc., Foster City, CA) using the following cycling parameters: 2 min at 50° C, 10 min at 95° C, 40 cycles for 15 s at 95° C, and 1 min at 60° C. MOR cDNA was amplified using a TaqMan probe: 5' /56-FAM/CTT-GCG-CCT-CAA-GAG-TGT-CCG-CA/3BHQ_1/-3'; sense primer 5-TAC-CGT-GTG-CTA-TGG-ACT-GAT-3; and antisense primer 5-ATG-ATG-ACG-TAA-ATG-TGA-ATG-3. GAPDH cDNA was used as an internal control and amplified using the TaqMan probe: 5' -56FAM/CCC-CAC-TGC-CAA-CGT-GTC-AGT-G/3BHQ-3'; sense primer 5' -GGA-AGC-TCA-CTG-GCA-TGG-C-3'; and antisense primer 5' -TAG-ACG-GCA-GGT-CAG-GTC-CA-3'. Probes and primers used in this study were synthesized by Integrated DNA Technologies, Coralville, IA.

2.4. Measurement of pro-inflammatory cytokines

Inflammatory cytokines secreted by TPA-HL-60 cells were measured using a 96-well human inflammatory cytokine tissue culture kit with slight modification to the manufacturer's procedure (Meso Scale Discovery, Gaithersburg, MD). The kit selected for this study was used to assay for TNF-α, GM-CSF, IL-1β, IL-8, IL-10, IL-12p70, IL-2, IL-6, and INFγ. After treatment, supernatants were centrifuged at 12,000 $\times g$ for 1 min in order to remove cellular debris. Culture filtrates were then stored at -80° C until the assays were performed. Assay wells were initially blocked with 1% (w/v) milk for 1 h at room temperature (RT) on a plate shaker at a speed setting of 5 (Lab-line Instruments, Inc. Melrose Park, IL). Measurement of electrochemiluminescent signal intensity was determined on the SECTOR 2400 instrument (Meso Scale Discovery, Gaithersburg, MD). Calibrator solutions were diluted in RPMI 1640 medium supplemented with 20% FBS, 100 U penicillin, and 100 µg/mL streptomycin in a concentration range of 10,000 to 2.4 pg/mL. Background signal (medium alone) was subtracted, and a linear regression model was used to fit the data.

2.5. Filtration of TPA-HL-60 CM

TPA-HL-60 cells were incubated with and without LPS (0.5 mg/mL) for 24 h. After LPS treatment, the CM was centrifuged for 90 min at 1,635 $\times g$ using a Sorvall 6000D centrifuge

(Sorvall, Newton, CT). At the end of centrifugation, the column filtrate was collected and the retentate was re-suspended in RPMI 1640 medium supplemented with 20% FBS, 100 U penicillin, and 100 µg/mL streptomycin to the original volume. The CM was then overlaid onto sub-confluent SH-SY5Y cells for 24 h.

2.6. Neutralization of TNF- α and GM-CSF

TPA-HL-60 cells were grown with and without LPS (0.5 mg/mL) for 24 h. After LPS treatment, TPA-HL-60 supernatants were pooled, filter sterilized, and treated with either anti-TNF- α (10 µg/mL), anti-GM-CSF (2 µg/mL), or both anti-TNF- α and anti-GM-CSF for 1 h at 37° C (Gomes et al., 2005). After neutralization, the CM was overlaid onto actively growing SH-SY5Y cells for 24 h.

2.7. Statistical analysis

Data in this study are represented as the mean $\pm$ SD. Differences among treatment groups were analyzed by a one-way ANOVA, followed by a Tukey's post hoc test or a Bonferroni's Multiple Comparison Test.

3. Results

3.1 Effect of LPS on ROS accumulation in TPA-HL-60 cells

HL-60 cells, differentiated for 48 h with 16 nM TPA (TPA-HL-60 cells), were treated with 0.125, 0.250, or 0.500 LPS for 24 h. Increased ROS production and intracellular accumulation of ROS have been reported in rat cardiomyocytes (Yuan et al., 2009) and rat microglia (Wang et al., 2004) using these concentrations of LPS. After LPS treatment, 20 µM of the ROS indicator, DHR123, was added for 30 min. DHR123 passively diffuses into cells where it undergoes oxidation to form the fluorophore, rhodamine 123 (Henderson and Chappell, 1993). CLSM was used to examine intracellular accumulation of ROS in the TPA-HL-60 cells. Saline solution (0.9% w/v), added at an equivalent volume, served as a vehicle control. Representative confocal images of TPA-HL-60 cells treated with LPS, then DHR123, in order to assess intracellular ROS accumulation are shown in Fig. 1A. TPA-HL-60 cells treated with 0.250 and 0.500 mg/mL LPS significantly increased intracellular ROS when compared to the vehicle control (Fig. 1B).

3.2. Effect of Vitamin E (VE) on LPS-induced ROS accumulation in TPA-HL-60 cells

To further investigate if ROS accumulation in TPA-HL-60 cells was directly related to LPS treatment, we then determined whether pre-treatment with the anti-oxidant, Vitamin E (VE), could block the LPS-induced accumulation of ROS in the TPA-HL-60 cells. The concentration of VE used in this study to block the accumulation of ROS is in the concentration range reported by Wu et al. (2007). Figure 2A shows representative confocal images of TPA-HL-60 cells treated with 0.500 mg/mL LPS alone (LPS), 100 µM VE alone for 3 h (VE), or pre-treated with 100 µM VE for 3 h prior to treatment with 0.500 mg/mL LPS (VE+LPS). As expected, LPS treatment alone significantly increased ROS by approximately 60% when compared to the vehicle control (0.9% saline + 0.1% ethanol) [Fig. 2B]. There was no significant difference in ROS in the cells treated with VE alone compared to control. ROS accumulation was inhibited in the cells treated with VE+LPS compared to LPS alone (Fig. 2B).

3.3. Effect of LPS on MOR expression in TPA-HL-60 cells

TPA-HL-60 cells were treated with 0.500 mg/mL LPS alone (LPS) for 24 h, or pretreated with 100 µM VE for 3 h prior to LPS treatment (VE+LPS). Relative real-time PCR showed that LPS at that concentration increased MOR expression by approximately 50% compared

to control (Fig. 3). VE alone did not have a significant effect on MOR expression; however, VE pre-treatment (VE+LPS) attenuated LPS-induced MOR expression compared to LPS alone (Fig. 3).

3.4. Effect of TPA-HL-60 conditioned medium (CM) on MOR expression in SH-SY5Y cells

We next used relative real-time PCR to examine if LPS-induced ROS accumulation in the TPA-HL-60 cells would have an effect on MOR expression in a neuronal cell line (SH-SY5Y). The CM from the TPA-HL-60 cells treated with LPS alone, VE alone, or VE+LPS was overlaid onto actively growing SH-SY5Y cells for 24 h. CM from the LPS-treated (CM, LPS) TPA-HL-60 cells significantly increased MOR expression in the SH-SY5Y cells by approximately 50% when compared to the vehicle control (Fig. 4), whereas VE treatment alone (CM, VE) had no significant effect on MOR expression compared to control. MOR expression in SH-SY5Y cells incubated with CM from the VE+LPS treatment group (CM, VE+LPS) was not significantly different from the CM, LPS group (Fig. 4). Worth noting, initial pilot experiments were performed to examine the effect of LPS on MOR expression in SH-SY5Y cells. We found that LPS at 0.500 mg/mL did not significantly change the level of MOR expression in SH-SY5Y cells when compared to vehicle control (data not shown).

3.5. Effect of LPS retentate and filtrate CM on MOR expression in SH-SY5Y cells

We next used an ultrafiltration process to determine if the increased MOR expression in the SH-SY5Y cells incubated with CM from LPS-treated TPA-HL-60 cells was a cytokine mediated event. Cytokines that are known to modulate MOR expression are larger than 3 kDa; therefore, ultrafiltration serves as an effective method for collection of CM void of cytokine mediators (Curfs et al., 1997). TPA-HL-60 cells were treated with 0.500 mg/mL LPS for 24 h. CM from both the vehicle control (0.9% saline) and LPS-treated cultures were separated into retentate (molecules > 3 kDa) and filtrate (molecules < 3 kDa) fractions. These fractions were then overlaid onto actively growing SH-SY5Y cells for 24 h. There was a significant 40% increase in SH-SY5Y MOR expression in the CM, LPS retentate compared to the CM, vehicle control retentate. However, the CM, LPS filtrate did not significantly affect SH-SY5Y MOR expression when compared to the CM, vehicle control filtrate CM (Fig. 5).

3.6. Effects of LPS on TPA-HL-60 pro-inflammatory cytokine secretion

The presence of pro-inflammatory cytokines in the CM from the TPA-HL-60 cells was determined using a sandwich immunoassay in conjunction with an electrochemiluminescent compound. TPA-HL-60 cells treated for 24 h with 0.500 mg/mL LPS exhibited increased IL-1 β , IL-2, IL-6, IL-10, IL-12p70, IFN- γ , TNF- α (Fig. 6A), and GM-CSF (Fig. 6B) secretion, but decreased IL-8 secretion (Table 1) compared to vehicle control. Pre-treatment of TPA-HL-60 cells with VE (VE+LPS) for 3 h significantly attenuated the LPS-induced secretion of both TNF- α and GM-CSF (Figs. 6A and 6B) compared to LPS treatment alone. CM from the VE alone treatment significantly increased IL-8 and IL-12p70 levels when compared to the vehicle control (Table 1).

3.7. Effect of TNF- α and GM-CSF neutralization in TPA-HL-60 CM on SH-SY5Y MOR expression

We next determined the effects of neutralization of the TNF- α and GM-CSF present in TPA-HL-60 CM on MOR expression in SH-SY5Y cells. In these experiments, anti-TNF- α , anti-GM-CSF, or both were added to the CM from the TPA-HL-60 cells, with and without LPS treatment, for 1 h at 37° C prior to being overlaid onto actively growing SH-SY5Y cells.

As expected, CM from the LPS-stimulated TPA-HL-60 cells significantly increased MOR expression in the SH-SY5Y cells (Fig. 7A, B, C). SH-SY5Y MOR expression was significantly increased by approximately 50% following neutralization of GM-CSF present in the CM from both the vehicle control (vehicle control+anti-GM-CSF) and LPS treatment (LPS+anti-GM-CSF) groups compared to vehicle control alone (Fig. 7A). However, neutralization of TNF- α had no effect on SH-SY5Y MOR expression in the vehicle control +anti-TNF- α group, and only a slight, non-significant increase in MOR expression in the LPS+anti-TNF- α group (Fig. 7B). Simultaneous neutralization of the GM-CSF and TNF- α present in the TPA-HL-60 CM significantly increased SH-SY5Y MOR expression by 60% in the vehicle control-anti-GM-CSF+anti-TNF- α group and by 50% in the LPS-anti-GM-CSF+anti-TNF- α group when compared to the vehicle control (Fig. 7C).

4. Discussion

In this study, we found that LPS stimulated the intracellular accumulation of ROS and the expression of the MOR in TPA-HL-60 cells, a macrophage cell model, and that CM from those cultures significantly increased MOR expression in SH-SY5Y cells, a neuronal cell model. These findings suggest that an indirect ROS signaling mechanism could be responsible, at least in part, for the modulation of LPS-stimulated MOR expression in SH-SY5Y cells.

Cytokines have been shown to regulate MOR expression (Borner et al., 2004; Chang et al., 1998; Kraus, 2009; Kraus et al., 2003; Wei and Loh, 2002). Our lab previously demonstrated that co-treatment of microvascular endothelial cells with IL-1 α and IL-1 β increases MOR expression (Vidal et al., 1998), and that IL-1 is the cytokine responsible for LPS-induced up-regulation of MOR expression in the rat mesentery (Chang et al., 2001). In this study, our data indicated that cytokines present in the CM from LPS-treated TPA-HL-60 cells are involved in the modulation of MOR mRNA expression in the SH-SY5Y cells. However, it remains to be determined whether the increases in MOR mRNA observed in this study correlate to changes at either the translational or functional level.

To test our hypothesis that ROS plays a role in the LPS-stimulated cytokine secretion in TPA-HL-60 cells, we examined the levels of pro-inflammatory cytokines secreted by TPA-HL-60 cells in response to LPS-induced ROS accumulation. Of the cytokines measured, both TNF- α and GM-CSF levels were found to be significantly decreased when ROS accumulation was blocked by the antioxidant, VE.

TNF- α appears to have a positive effect on SH-SY5Y MOR expression. TNF- α stimulates transcription initiation sites on the MOR gene (Borner et al., 2002), and TNF- α increases MOR expression in human T lymphocytes, Raji B cells, U937 monocytes, primary human polymorphonuclear leukocytes, and mature dendritic cells (Kraus et al., 2003).

GM-CSF down-regulates MOR expression in dendritic cells, which may involve inhibitory actions on IL-4 (Kraus et al., 2003). We observed an increase in MOR expression in response to the neutralization of GM-CSF in vehicle control+anti-GM-CSF and LPS+anti-GM-CSF treatment groups when compared to the vehicle control. These data suggest that only low levels of GM-CSF, present in the CM, are necessary to modulate MOR expression since the concentration of GM-CSF in the vehicle control was significantly less than that of the LPS treatment group (Table 1).

We also observed that neutralization of TNF- α in the LPS treatment group only partially attenuated MOR expression. This partial attenuation of MOR expression may be due to the effects of pro-inflammatory cytokines other than TNF- α in the up-regulation of the MOR. Interestingly, when both TNF- α and GM-CSF were neutralized simultaneously (LPS+anti

GM-CSF+anti TNF- α), MOR expression was not significantly decreased compared to the LPS treatment group. This indicates that GM-CSF's inhibitory effects on MOR expression only becomes apparent when cytokines capable of increasing MOR expression are neutralized, e.g., TNF- α . This again underscores the possibility that cytokines capable of increasing MOR expression can compensate for one another since the simultaneous neutralization of both GM-CSF and TNF- α (LPS+anti GM-CSF+anti TNF- α) did not significantly reduce MOR expression when compared to the LPS treatment group.

We hypothesized that, since morphine potentiates LPS cytotoxicity, activation of the opioid pathway by morphine in a clinical setting could cause an adverse physiological response, i.e., acceleration of sepsis to septic shock (Chang et al., 2001; Ocasio et al., 2004). The damaging consequences of endotoxin shock resulting from exposure to LPS, and the subsequent signaling actions mediated by ROS may, in fact, be exacerbated as a result of the immunosuppressive effects associated with MOR activation in neuronal and non-neuronal cells (Gaveriaux-Ruff, Matthes et al., 1998; Wang, Charboneau et al., 2002).

Our findings suggest that the LPS-induced ROS signaling that occurs in immune cells may indirectly regulate the opiodergic pathway by modulating MOR expression in neurons. Our data also indicate that ROS, produced in LPS challenged TPA-HL-60 cells, is involved in modulating the secretion of TNF- α and GM-CSF, two cytokines that have previously been shown to modulate MOR expression. This mechanism, i.e., LPS-induced ROS production coupled to cytokine secretion in immune cells, can impact molecular events in neurons, and highlights one possible way endotoxin exposure resulting from bacterial infection promotes an interaction between the nervous and immune systems.

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References

- Andreasen AS, Krabbe KS, Krogh-Madsen R, Taudorf S, Pedersen BK, Moller K. Human endotoxemia as a model of systemic inflammation. *Curr Med Chem*. 2008; 15:1697–1705. [PubMed: 18673219]
- Bast A, Goris RJ. Oxidative stress. *Biochemistry and human disease. Pharm Weekbl Sci*. 1989; 11:199–206. [PubMed: 2694090]
- Bayir H. Reactive oxygen species. *Crit Care Med*. 2005; 33:S498–501. [PubMed: 16340433]
- Borner C, Holtt V, Kraus J. Involvement of activator protein-1 in transcriptional regulation of the human mu-opioid receptor gene. *Mol Pharmacol*. 2002; 61:800–805. [PubMed: 11901219]
- Borner C, Kraus J, Schroder H, Ammer H, Holtt V. Transcriptional regulation of the human mu-opioid receptor gene by interleukin-6. *Mol Pharmacol*. 2004; 66:1719–1726. [PubMed: 15448191]
- Chang SL, Felix B, Jiang Y, Fiala M. Actions of endotoxin and morphine. *Adv Exp Med Biol*. 2001; 493:187–196. [PubMed: 11727765]
- Chang SL, Patel NA, Romero AA. Activation and desensitization of Fos immunoreactivity in the rat brain following ethanol administration. *Brain Res*. 1995; 679:89–98. [PubMed: 7648269]
- Chang SL, Patel NA, Romero AA, Thompson J, Zadina JE. FOS expression induced by interleukin-1 or acute morphine treatment in the rat hypothalamus is attenuated by chronic exposure to morphine. *Brain Res*. 1996; 736:227–236. [PubMed: 8930328]
- Chang SL, Wu GD, Patel NA, Vidal EL, Fiala M. The effects of interaction between morphine and interleukin-1 on the immune response. *Adv Exp Med Biol*. 1998; 437:67–72. [PubMed: 9666258]
- Chen R, Zhou H, Beltran J, Malellari L, Chang SL. Differential expression of cytokines in the brain and serum during endotoxin tolerance. *J Neuroimmunol*. 2005; 163:53–72. [PubMed: 15885308]

- Ciccarone V, Spengler BA, Meyers MB, Biedler JL, Ross RA. Phenotypic diversification in human neuroblastoma cells: expression of distinct neural crest lineages. *Cancer Res.* 1989; 49:219–225. [PubMed: 2535691]
- Crow JP. Dichlorodihydrofluorescein and dihydrorhodamine 123 are sensitive indicators of peroxynitrite in vitro: implications for intracellular measurement of reactive nitrogen and oxygen species. *Nitric Oxide.* 1997; 1:145–157. [PubMed: 9701053]
- Curfs JH, Meis JF, Hoogkamp-Korstanje JA. A primer on cytokines: sources, receptors, effects, and inducers. *Clin Microbiol Rev.* 1997; 10:742–780. [PubMed: 9336671]
- Dunn DL. Role of endotoxin and host cytokines in septic shock. *Chest.* 1991; 100:164S–168S. [PubMed: 1884642]
- Evans R, Kamdar SJ, Duffy TM. Tumor-derived products induce Il-1 a, Il-1 b, Tnf a, and Il-6 gene expression in murine macrophages: distinctions between tumor- and bacterial endotoxin-induced gene expression. *J Leukoc Biol.* 1991; 49:474–482. [PubMed: 2016568]
- Gamaley IA, Klyubin IV. Roles of reactive oxygen species: signaling and regulation of cellular functions. *Int Rev Cytol.* 1999; 188:203–255. [PubMed: 10208013]
- Gao Z, Huang K, Xu H. Protective effects of flavonoids in the roots of *Scutellaria baicalensis* Georgi against hydrogen peroxide-induced oxidative stress in HS-SY5Y cells. *Pharmacol Res.* 2001; 43:173–178. [PubMed: 11243719]
- Gaveriaux-Ruff C, Matthes HW, Peluso J, Kieffer BL. Abolition of morphine-immunosuppression in mice lacking the mu-opioid receptor gene. *Proc Natl Acad Sci U S A.* 1998; 95:6326–6330. [PubMed: 9600964]
- Gomes I, Mathur SK, Espenshade BM, Mori Y, Varga J, Ackerman SJ. Eosinophil-fibroblast interactions induce fibroblast IL-6 secretion and extracellular matrix gene expression: implications in fibrogenesis. *J Allergy Clin Immunol.* 2005; 116:796–804. [PubMed: 16210053]
- Henderson LM, Chappell JB. Dihydrorhodamine 123: a fluorescent probe for superoxide generation? *Eur J Biochem.* 1993; 217:973–980. [PubMed: 8223655]
- Hsu HY, Wen MH. Lipopolysaccharide-mediated reactive oxygen species and signal transduction in the regulation of interleukin-1 gene expression. *J Biol Chem.* 2002; 277:22131–22139. [PubMed: 11940570]
- Kim SH, Johnson VJ, Shin TY, Sharma RP. Selenium attenuates lipopolysaccharide-induced oxidative stress responses through modulation of p38 MAPK and NF-kappaB signaling pathways. *Exp Biol Med (Maywood).* 2004; 229:203–213. [PubMed: 14734799]
- Kowalski J, Denhardt DT. Regulation of the mRNA for monocyte-derived neutrophil-activating peptide in differentiating HL60 promyelocytes. *Mol Cell Biol.* 1989; 9:1946–1957. [PubMed: 2664463]
- Kraus J. Regulation of mu-opioid receptors by cytokines. *Front Biosci (Schol Ed).* 2009; 1:164–170. [PubMed: 19482692]
- Kraus J, Borner C, Giannini E, Holtt V. The role of nuclear factor kappaB in tumor necrosis factor-regulated transcription of the human mu-opioid receptor gene. *Mol Pharmacol.* 2003; 64:876–884. [PubMed: 14500744]
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* 2001; 25:402–408. [PubMed: 11846609]
- Lopez-Bojorquez LN, Dehesa AZ, Reyes-Teran G. Molecular mechanisms involved in the pathogenesis of septic shock. *Arch Med Res.* 2004; 35:465–479. [PubMed: 15631870]
- Machlin LJ, Bendich A. Free radical tissue damage: protective role of antioxidant nutrients. *Faseb J.* 1987; 1:441–445. [PubMed: 3315807]
- McBride AG, Borutaite V, Brown GC. Superoxide dismutase and hydrogen peroxide cause rapid nitric oxide breakdown, peroxynitrite production and subsequent cell death. *Biochim Biophys Acta.* 1999; 1454:275–288. [PubMed: 10452962]
- McCord JM, Fridovich I. The biology and pathology of oxygen radicals. *Ann Intern Med.* 1978; 89:122–127. [PubMed: 208444]
- Nakamura H, Nakamura K, Yodoi J. Redox regulation of cellular activation. *Annu Rev Immunol.* 1997; 15:351–369. [PubMed: 9143692]

- Ocasio FM, Jiang Y, House SD, Chang SL. Chronic morphine accelerates the progression of lipopolysaccharide-induced sepsis to septic shock. *J Neuroimmunol.* 2004; 149:90–100. [PubMed: 15020069]
- Parrillo JE, Parker MM, Natanson C, Suffredini AF, Danner RL, Cunnion RE, Ognibene FP. Septic shock in humans. Advances in the understanding of pathogenesis, cardiovascular dysfunction, and therapy. *Ann Intern Med.* 1990; 113:227–242. [PubMed: 2197912]
- Qu C, Guo S, Guo H, Wang C, Ma L, He Y, Wang L, Lou J, Wang X. Increased serum endotoxin and elevated CD14 and IL-1 β expression in a rat model of cerebrogenic multiple organ dysfunction syndrome. *Med Chem.* 2009; 5:462–467. [PubMed: 19534684]
- Radi R, Peluffo G, Alvarez MN, Naviliat M, Cayota A. Unraveling peroxynitrite formation in biological systems. *Free Radic Biol Med.* 2001; 30:463–488. [PubMed: 11182518]
- Raetz CR, Whitfield C. Lipopolysaccharide endotoxins. *Annu Rev Biochem.* 2002; 71:635–700. [PubMed: 12045108]
- Rovera G, Santoli D, Damsky C. Human promyelocytic leukemia cells in culture differentiate into macrophage-like cells when treated with a phorbol diester. *Proc Natl Acad Sci U S A.* 1979; 76:2779–2783. [PubMed: 288066]
- Ruffels J, Griffin M, Dickenson JM. Activation of ERK1/2, JNK and PKB by hydrogen peroxide in human SH-SY5Y neuroblastoma cells: role of ERK1/2 in H₂O₂-induced cell death. *Eur J Pharmacol.* 2004; 483:163–173. [PubMed: 14729104]
- Rumpa S, S. D, Chatterjee R, Kaur I. The Pathophysiology of Septic Shock. *International Journal of Pharma and Bio Sciences.* 2010; 1:1–10.
- Vidal EL, Patel NA, Wu G, Fiala M, Chang SL. Interleukin-1 induces the expression of mu opioid receptors in endothelial cells. *Immunopharmacology.* 1998; 38:261–266. [PubMed: 9506826]
- Wang J, Charboneau R, Balasubramanian S, Barke RA, Loh HH, Roy S. The immunosuppressive effects of chronic morphine treatment are partially dependent on corticosterone and mediated by the mu-opioid receptor. *J Leukoc Biol.* 2002; 71:782–790. [PubMed: 11994502]
- Wang T, Qin L, Liu B, Liu Y, Wilson B, Eling TE, Langenbach R, Taniura S, Hong JS. Role of reactive oxygen species in LPS-induced production of prostaglandin E₂ in microglia. *J Neurochem.* 2004; 88:939–947. [PubMed: 14756815]
- Wei LN, Loh HH. Regulation of opioid receptor expression. *Curr Opin Pharmacol.* 2002; 2:69–75. [PubMed: 11786311]
- Wu CW, Ping YH, Yen JC, Chang CY, Wang SF, Yeh CL, Chi CW, Lee HC. Enhanced oxidative stress and aberrant mitochondrial biogenesis in human neuroblastoma SH-SY5Y cells during methamphetamine induced apoptosis. *Toxicol Appl Pharmacol.* 2007; 220:243–251. [PubMed: 17350664]
- Yu X, Mao X, Blake AD, Li WX, Chang SL. Morphine and endomorphins differentially regulate micro-opioid receptor mRNA in SHSY-5Y human neuroblastoma cells. *J Pharmacol Exp Ther.* 2003; 306:447–454. [PubMed: 12754318]
- Yuan H, Perry CN, Huang C, Iwai-Kanai E, Carreira RS, Glembotski CC, Gottlieb RA. LPS-induced autophagy is mediated by oxidative signaling in cardiomyocytes and is associated with cytoprotection. *Am J Physiol Heart Circ Physiol.* 2009; 296:H470–479. [PubMed: 19098111]
- Zadina JE, Chang SL, Ge LJ, Kastin AJ. Mu opiate receptor down-regulation by morphine and up-regulation by naloxone in SH-SY5Y human neuroblastoma cells. *J Pharmacol Exp Ther.* 1993; 265:254–262. [PubMed: 8097244]
- Zadina JE, Harrison LM, Ge LJ, Kastin AJ, Chang SL. Differential regulation of mu and delta opiate receptors by morphine, selective agonists and antagonists and differentiating agents in SH-SY5Y human neuroblastoma cells. *J Pharmacol Exp Ther.* 1994; 270:1086–1096. [PubMed: 7932156]

Figure 1. Effect of LPS on reactive oxygen species (ROS) accumulation in TPA-HL-60 cells
 TPA-HL-60 cells were treated with 0.9% saline (vehicle control), 0.125 mg/mL LPS, 0.250 mg/mL LPS, or 0.500 mg/mL LPS for 24 h. (A) Representative confocal images demonstrating ROS levels in cell cluster region of interest (ROI) and subtracted background ROI. (B) Relative levels of ROS (indicated as % of control) were determined using DHR123 and confocal microscopy. Values are the means $\pm$ SD of three microscopic fields from three replicates per treatment in three different experiments. Significant differences between treatment groups were determined using a Tukey's post hoc test. * $p < 0.05$ compared to vehicle control.

Figure 2. Effect of Vitamin E (VE) on LPS-induced ROS accumulation in TPA-HL-60 cells
 TPA-HL-60 cells were treated with 0.9% saline (vehicle control), 0.500 mg/mL LPS alone (LPS), pre-treated with 100 μ M VE alone for 3 h (VE), or pre-treated with 100 μ M VE plus 0.500 mg/mL LPS (VE+LPS) for 24 h. (A) Representative confocal images demonstrating ROS levels in the cell cluster region of interest (ROI) and subtracted background ROI. (B) Relative levels of ROS (indicated as % of control) were determined using DHR123 and confocal microscopy. Significant differences between treatment groups were determined using a Tukey's post hoc test. ** $p < 0.01$ compared to vehicle control, ^ $p < 0.05$ compared to LPS alone.

Figure 3. Effect of LPS on TPA-HL-60 MOR expression

TPA-HL-60 cells were treated with 0.9% saline (vehicle control), 0.500 mg/mL LPS alone (LPS), 100 μ M vitamin E alone (VE), or 100 μ M VE plus 0.500 mg/mL LPS (VE+LPS) for 24 h. Relative changes in MOR expression (fold difference) were determined using the $2^{-\Delta\Delta C_t}$ equation, with GAPDH serving as the internal standard. Values are the means $\pm$ SD of six replicates per treatment from two different experiments. Significant differences between treatment groups were determined using a Tukey's post hoc test. ** $p < 0.01$ compared to vehicle control, ^^ $p < 0.01$ compared to LPS alone.

Figure 4. Effect of TPA-HL-60 conditioned medium (CM) on SH-SY5Y MOR expression

Conditioned medium (CM) from TPA-HL-60 cells treated with 0.9% saline + 0.1% ethanol (CM, vehicle control), 0.500 mg/mL LPS alone (CM, LPS), 100 μ M vitamin E alone (CM, VE), or 100 μ M+0.500 mg/mL LPS (CM, VE+LPS) was overlaid onto SH-SY5Y cells for 24 h. Relative changes in MOR expression (fold difference) were determined using the $2^{-\Delta\Delta C_t}$ equation, with GAPDH serving as the internal standard. Values are the means $\pm$ SD of six replicates per treatment from three separate experiments. Significant differences between treatment groups were determined using a Tukey's post hoc test. * $p < 0.05$ compared to CM, vehicle control.

Figure 5. Effect of LPS retentate and filtrate CM on SH-SY5Y MOR expression

Conditioned medium (CM) from TPA-HL-60 cells treated with 0.9% saline + 0.1% ethanol (CM, vehicle control) or 0.500 mg/mL LPS alone (CM, LPS) was separated into retentate and filtrate fractions by centrifugal force ultrafiltration and overlaid onto SH-SY5Y cells. Relative changes in MOR expression (fold difference) were determined using the $2^{-\Delta\Delta C_t}$ equation, with GAPDH serving as the internal standard. Values are the means $\pm$ SD of six replicates per treatment from three separate experiments. Significant differences between (CM, vehicle control retentate), and (CM, LPS retentate), or (CM, vehicle control filtrate) and (CM, LPS filtrate) were determined by a Bonferroni's Multiple Comparison Test. * $p < 0.05$ compared to CM, vehicle control retentate.

Figure 6. Effects of LPS and VE pre-treatment on TPA-HL-60 pro-inflammatory cytokine secretion

(A-B) Conditioned medium (CM) from TPA-HL-60 cells treated with 0.9% saline + 0.1% ethanol (CM, vehicle control), 0.500 mg/mL LPS alone (CM, LPS), 100 μ M vitamin E alone (CM, VE), or 100 μ M + 0.500 mg/mL LPS (CM, VE+LPS) for 24 h was assayed for TNF- α and GM-CSF levels using a multiplex, electrochemiluminescent sandwich immunoassay. Values are the means $\pm$ SD of five replicates per treatment from four independent experiments. Significant differences between treatment groups were determined using a Tukey's post hoc test. *** p < 0.001 compared to CM, vehicle control, ^{AAA} p < 0.001 compared to CM, LPS.

Figure 7. Neutralization of TNF- α and GM-CSF present in TPA-HL-60 CM

TPA-HL-60 cells were treated with 0.9% saline (vehicle control) or 0.500 mg/mL LPS (LPS) for 24 h. Neutralizing antibodies to (A, C) GM-CSF (2 μ g/mL), and/or (B, C) TNF- α (10 μ g/mL) were added first to TPA-HL-60 CM from vehicle control and LPS treatment groups for 1 h at 37° C, and then overlaid onto SH-SY5Y cells for 24 h. Relative changes in SH-SY5Y MOR expression (fold difference) were determined using the $2^{-\Delta\Delta C_t}$ equation, with GAPDH serving as the internal standard. Values are the means $\pm$ SD of six replicates per treatment from two independent experiments. Significant differences between treatment groups were determined using a Tukey's post hoc test. * p < 0.05 and ** p < 0.01 compared to vehicle control.

Table I

Conditioned medium (CM) from TPA-HL-60 cells treated with 0.9% saline+0.1% ethanol (CM, vehicle control), or 0.500 mg/mL LPS (CM, LPS), or 100 μ M vitamin E (CM, VE), or 100 μ M+ 0.500 mg/mL LPS (CM, VE+LPS) for 24 h was assayed for IL-1 β , IL-2, IL-6, IL-8, IL-10, IL-12p70, INF γ , TNF- α , and GM-CSF levels. The concentrations of cytokines measured are expressed in units of pg/mL. Values are the means $\pm$ SD of five replicates per treatment from 4 independent experiments. Significant differences between treatment groups were determined using Tukey's post hoc test.

cytokine	CM, vehicle control	CM, LPS	CM, VE	CM, VE+LPS
IL-1b	240.90($\pm$ 29.82)	602.76($\pm$ 47.79) ***	238.86($\pm$ 21.27)ns	547.41($\pm$ 43.48) ***
IL-2	3.74($\pm$ 0.28)	7.27($\pm$ 0.32) ***	4.498($\pm$ 0.16)ns	7.64($\pm$ 1.45) ***
IL-6	345.22($\pm$ 26.98)	4917.04($\pm$ 272.38) ***	448.1($\pm$ 42.29)ns	4753.54($\pm$ 81.33) ***
IL-8	328.31($\pm$ 3.54)	102.49($\pm$ 5.79) ***	387.17($\pm$ 19.84) ***	108.96($\pm$ 1.57) ***
IL-10	49.60($\pm$ 1.90)	424.27($\pm$ 28.56) ***	46.22($\pm$ 3.02)ns	399.96($\pm$ 14.76) ***
IL-12p70	0.90($\pm$ 0.07)	2.49($\pm$ 0.11) ***	1.18($\pm$ 0.11) *	2.45($\pm$ 0.25) ***
INF γ	1.93($\pm$ 0.27)	5.20($\pm$ 0.43) **	2.46($\pm$ 0.62)ns	4.93($\pm$ 0.39) ***
TNF- α	9.98($\pm$ 1.55)	729.57($\pm$ 78.58) ***	13.35($\pm$ 1.38)ns	508.94($\pm$ 26.85) ***
GM-CSF	4.53($\pm$ 0.67)	142.36($\pm$ 12.72) ***	4.75($\pm$ 0.22)ns	116.06($\pm$ 3.52) ***

*
p < 0.05,

**
p < 0.01,

p < 0.001 compared to CM, vehicle control, ns, not significant when comparing CM, VE to CM vehicle control.