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

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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.


## 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 (Parrillo et al., 1990; Dunn, 1991; Raetz and Whitfield, 2002; Lopez-Bojorquez et al., 2004; Rumpa et al., 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 et al., 2010).

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 (Dunn, 1991; Lopez-Bojorquez et al., 2004; Andreasen et al., 2008). 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-1β, and IL-6, are elevated in both the serum and brain of rats treated systemically with LPS (Ocasio et al., 2004; Chen et al., 2005). Other studies have reported an increase in the secretion of IL-1β 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-1α and IL-1β 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., 1995, 1996, 2001; 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., 1998, 2001; Chen et al., 2005).

Reactive oxygen species (ROS) are highly reactive molecules produced during cellular respiration (McCord and Fridovich, 1978; Bast and Goris, 1989; Bayir, 2005). Both intracellular and extracellular ROS are maintained at non-lethal levels by superoxide dismutases, catalases, and a thiol-reducing buffer consisting of glutathione and thioredoxion (Nakamura et al., 1997; Gamaley and Klyubin, 1999). However, disease and stress can alter a cell's ability to effectively regulate ROS. Elevated

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levels of ROS can damage proteins, DNA, RNA, and cell membranes by hydroxyl radical attack, and induce apoptosis (McCord and Fridovich, 1978; Machlin and Bendich, 1987; Bast and Goris, 1989). 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 of LPS-induced ROS accumulation on MOR expression in TPA-differentiated HL-60 (TPA-HL-60) macrophage-like cells (Rovera et al., 1979; Kowalski and Denhardt, 1989). We also assayed the conditioned medium from the LPS-treated TPA-HL-60 cells for TNF-α, GM-CSF, IL-1β, IL-8, IL-10, IL-12p70, IL-2, IL-6, and INFγ 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 (Zadina et al., 1993, 1994; Yu et al., 2003), 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 μg/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 $5\times10^5$ 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 μM and diluted 1000-fold in medium. Previous studies have shown that greater than 95% of HL-60 cells differentiate to macrophage-like cells after a 48-h treatment with 16 nM TPA (Rovera et al., 1979; Kowalski and Denhardt, 1989). 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 μg/mL streptomycin (Gibco, Invitrogen Corp., Grand Island, NY). SH-SY5Y cells were seeded at $1\times10^5$ 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 *Escherichia 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 μM 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 μm 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 μM 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 control 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× 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:

$$\text{cell cluster ROI}\left(\sum\text{avg. CHS1}^{1,2\ldots10}*\text{area}^{1,2\ldots10}/\sum\text{area}\right) - \text{background ROI}\left(\sum\text{avg. CHS1}^{1,2\ldots10}*\text{area}^{1,2\ldots10}/\sum\text{area}\right)$$

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 $2^{-\Delta\Delta Ct}$ 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, Gaithersberg, 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 ×g