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Morphine Activates Toll-like Receptor 4 (TLR4) in Endothelial Cells

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ABSTRACT

Endothelial cells are especially important within the central nervous system as they line capillaries and form the blood-brain barrier. Moreover, they are the first cell type within the CNS to be exposed to foreign pathogens and drugs in the bloodstream. In other cell types in the CNS, activation of Toll-like Receptor 4 (TLR4) results in activation of transcription factors such as NF-κB and AP-1, both known to induce the production of pro-inflammatory cytokines. The expression of pro-inflammatory cytokines sensitizes neurons, thus decreasing pain thresholds and prolonging pain. Interestingly, recent research demonstrates that opioids (analgesics prescribed for chronic pain) not only bind the µ-opioid receptor to confer pain relief, but also activate TLR4. It is thought that TLR4 activation, and subsequent release of pro-inflammatory cytokines, contributes to the negative effects of tolerance, dependence, and withdrawal associated with morphine use. Because of the dearth of research surrounding opioids and their effect on this cell type, this study attempted to characterize TLR4 activation in endothelial cells. Endothelial cells were isolated from the brain and spinal cord and incubated with various doses of (+)-morphine. Results indicate that TLR4 is indeed activated by (+)-morphine, producing PGE₂ and increasing the mRNA of IκB, CD14, and TLR4 at 100 µM (+) morphine 24 h after incubation. The pro-inflammatory response induced by 100 µM (+)-morphine can be blocked with both PI3K and IκB-α inhibitors, significantly reducing NF-κB activation and PGE₂ production. Furthermore, signal transduction occurs in a MyD88-dependent fashion as CLI-095, a TLR4 signaling inhibitor that blocks the MyD88-independent pathway, had no significant effect on mRNA expression or PGE₂ production. These data suggest that (+)-morphine induces TLR4 activation in endothelial cells and, in concert with other cell types in the central nervous system, may contribute to the negative effects of morphine use.
INTRODUCTION

Pain management remains a significant health concern, especially for those who suffer from chronic pain. Chronic pain, or pain that persists beyond the natural course of healing, affects over five million people in the United States (Tawfik et al., 2007) and costs over $100 billion annually in medical expenses and lost productivity (Department of Health and Human Services, 1998; Stewart et al., 2003). Despite advances in pharmacotherapeutics, nearly two-thirds of chronic pain patients receive little to no relief from current treatments (Sindrup and Jensen, 1999; Collins et al., 2000; McQuay et al., 1996). The most effective class of analgesics today is opioids, primarily morphine. Unfortunately, opioids are associated with negative side effects such as dependence, tolerance, and subsequent abuse (Compton and Volkow, 2006; Manchikanti, 2006).

Much research has focused on the mechanisms underlying opioid tolerance and dependence, and how preventing these side effects can be used to optimize the analgesic effects of morphine. One realm of study investigates cells involved in innate immunity, particularly endothelial cells. Endothelial cells, which line capillaries, are important in facilitating transport. The endothelium is especially important around the central nervous system as it comprises the blood-brain barrier (Galley and Webster, 2004) and therefore is the first cell type within the CNS to be exposed to foreign pathogens. Endothelial cells are in contact with and respond to substances within the blood, and can be activated in an immune-like response against a harmful foreign pathogen (Galley and Webster, 2004). In the last decade, research has found that endothelial cells express Toll-like Receptor 4 (TLR4), a transmembrane protein that recognizes foreign pathogens (Gangloff et al. 2003). In other cell types in the central nervous system, activation of TLR4 results in activation of transcription factors such as NF-κB and AP-1, both
known to induce the production of pro-inflammatory cytokines (Malcangio et al., 1996; Mika, 2008; Obreja et al., 2002). The expression of pro-inflammatory cytokines sensitzes neurons, thus decreasing the pain threshold and prolonging pain (Malcangio et al., 1996; Mika, 2008; Obreja et al., 2002). While it is known that TLR4 recognizes bacteria, viruses, and fungi (Hameed et al., 2010), recent research of TLR4 in microglia, the immunocompetent cells within the central nervous system, shows that this receptor also binds morphine (Hutchinson et al., 2007; Watkins et al., 2009). Interestingly, in addition to binding opioid receptors to confer pain-reducing effects, morphine also activates microglia. This activation has been identified to occur in acute and chronic morphine and other opioid administration both within the spinal cord and within the brain (Song and Zhao, 2001; Hutchinson et al., 2007). The microglial activation and subsequent pro-inflammatory cytokine release sensitize neurons and diminish the analgesic effects of morphine, a mechanism that is thought to be a key contributor to the negative effects of opioids (Hutchinson et al., 2007; Watkins et al., 2009).

During the past decade, studies documenting TLR4 expression in endothelial cells indicate that they too may play a role in pro-inflammatory cytokine expression (Jou et al., 2006; Tang et al., 2007). While it is known that TLR4 receptors on endothelial cells respond to some TLR4 ligands, such as lipopolysaccharide (Laflamme and Rivest, 2001), there is less research surrounding TLR4 activation and morphine in this cell type. Given the relatively recent understanding of TLR4 and its connection to endothelial cells, there is great potential for clinically relevant findings. As such, this study will characterize TLR4 activation in response to morphine in endothelial cells as well as explore the effects of TLR4 inhibitors on this activation.
BACKGROUND

Given the important functions of the nervous system, the immune system has many specialized cells to protect the brain, nerves, and cerebrospinal fluid. Among these cell types are endothelial cells, which are part of the innate (non-specific) immune system. Endothelial cells are in contact with and respond to substances within the blood, and can be activated in an immune response against a harmful foreign pathogen (Galley and Webster, 2004). Endothelial cells create capillaries and are necessary in facilitating transport; the endothelium is especially important around the central nervous system as it comprises the blood-brain barrier (Galley and Webster, 2004). Similar to microglia and astrocytes, endothelial cells also express TLR4 (Faure et al., 1999, Jou et al. 2006; Tang et al. 2007), which can recognize potentially harmful substances and activate an immune-like response (Tanga, Nutile-McMenemy, & DeLeo, 2005; Wang et al., 2010). This immune response includes the secretion of pro-inflammatory cytokines, which recruit neutrophils to the site of injury and mediate the healing process (Martin and Roy, 2010).

Toll-like Receptor 4

The Toll-like receptor 4 (TLR4) is a transmembrane protein and part of pathogen-associated molecular pattern receptors that identify and mount a response to exogenous threats. In other words, these TLRs recognize patterns indicative of bacteria, fungi, and viruses that are potentially harmful and trigger an inflammatory signaling cascade to attract other immunocompetent cells to combat the foreign substance (Hameed et al., 2010). TLR4, in particular, responds to components of the cell wall of gram-negative bacteria (Gangloff et al., 2003) such as lipopolysaccharide (LPS) (Shimazu et al., 1999; Poltorak et al., 1998; Lehnardt et al., 2003; Hoshino et al., 1999). Additionally, TLR4 is important for recognizing endogenous
danger signals from injured or dying cells, including heat shock proteins, plasma proteins, and extracellular matrix degradation products (Tanga et al., 2005; Kim et al., 2007).

While binding of LPS and related ligands triggers TLR4 signaling, signal transduction is also dependent upon recruitment of other extracellular proteins (Fig. 1). For example, the myeloid differentiation factor 2 (MD-2) is required to bind to TLR4 (Shimazu et al., 1999; Nagai et al., 2002) and this TLR4/MD-2 complex must join another complex to form a homodimer in the plasma membrane (Kobayashi et al., 2006; Prohinar et al., 2007; Laird et al., 2009). In most cell types, this homodimer must additionally bind to the GPI-anchored protein CD14 for signal transduction to occur (Wright et al., 1990; Haziot et al., 1988). However, in endothelial cells CD14 is not on the plasma membrane of the cell; rather, endothelial TLR4s interact with the soluble CD14 that flows in the bloodstream (Frey et al., 1992; Arditi et al., 1993; Haziot et al., 1993; Pugin et al., 1993; Galley and Webster, 2004). Then the resulting complex confers an intracellular signal via the Toll/IL-1 receptor (TIR) domain (Gangloff et al., 2003; Kim et al., 2007; Rittirsch et al., 2009) that either follows a MyD88-dependent or MyD88-independent pathway. MyD88 is a protein in the cytoplasm that similarly possesses a TIR domain, which interacts with the TIR domain of TLR4 (Horng et al. 2002; Laird et al., 2009). In the MyD88-dependent pathway, the resulting signal is then transduced to IL-1 receptor-associated kinases (IRAKs) that facilitate subsequent phosphorylations in the signaling cascade (Kawai et al., 1999; Kawai and Akira, 2007; Dauphine and Karsan, 2006). These phosphorylations ultimately activate the transcription factors NF-κB and AP-1, which trigger an innate immune response in the central nervous system (Laflamme and Rivest, 2001) and promote expression of pro-inflammatory cytokines such as IL-1β and IL-6 (Watkins and Maier, 2000; DeLeo et al., 2000; Tanga et al., 2005; Hutchinson et al., 2007). NF-κB is comprised of two subunits, p65 and p50.
and is inhibited by IκB-α (Greene et al., 1993). IκB-α is a cytoplasmic protein that binds the nuclear localization sequence of the p65 subunit such that NF-κB is not translocated to the nucleus (Greene et al., 1993). Upon phosphorylation, triggered by activated IRAKs, IκB-α dissociates from NF-κB and allows NF-κB to enter the nucleus and modulate transcription. For this study transcripts and proteins involved in the TLR4 signaling cascade, primarily TLR4, MD-2, CD14, IκB, NF-κB, and IL-1β, were measured to determine TLR4 activation.

**Fig. 1.** Signaling pathway of Toll-like Receptor 4 (TLR4)

TLR4 signal transduction requires recruitment of myeloid differentiation factor 2 (MD-2) and soluble CD14 that flows in the bloodstream. The resulting complex confers an intracellular signal via the Toll/IL-1 receptor (TIR) domain that either follows a MyD88-dependent or MyD88-independent pathway. In the MyD88-dependent pathway, the resulting signal is then transduced to IL-1 receptor-associated kinases (IRAKs) that facilitate subsequent phosphorylations. These phosphorylations ultimately cause IκB-α, a cytoplasmic protein that binds the nuclear localization sequence of the p65 subunit of NF-κB, to dissociate from NF-κB. Binding of TLR4 also activates the PI3K/Akt/NF-κB pathway. NF-κB then translocates to the nucleus to promote transcription of pro-inflammatory cytokines such as IL-1β and IL-6. TLR2 activity requires CD14 and contributes to NF-κB activation. TLR4 signaling also involves MAP kinase activity, which promotes COX2 transcription and ultimately the production of PGE2. (Gangloff et al., 2003; Shimazu et al., 1999; Zhang, 2011)
Another Toll-like receptor, TLR2, contributes to pro-inflammatory cytokine expression and is implicated in chronic morphine use (Zhang, 2011). While TLR4 was traditionally thought to bind exclusively to Gram-negative bacteria and TLR2 was thought to bind Gram-positive bacteria, research shows that both receptors bind many other ligands and often bind the same ligand with differing affinities (Dziarski et al., 2001; Muta and Takeshige, 2001). For example, both receptors can bind LPS, but TLR4 has a binding affinity that is approximately 100 times greater than TLR2 (Muta and Takeshige, 2001). Similar to TLR4, TLR2 recruits CD14 and can trigger the MyD88-dependent signaling pathway that ultimately increases expression of NF-κB, though it is unclear whether MD-2 is required (Dziarski et al., 2000; Dziarski et al., 2001; Yoshimura et al., 1999; Muta and Takeshige, 2001). Interestingly, in addition to promoting the anti-apoptotic PI3K/Akt/NF-κB pathway, TLR2 signaling also promotes apoptosis via factors such as FADD/p53 (Yin et al., 1999; Yin et al., 2006). It is thought that the apoptosis resulting from TLR2 activation may be the mechanism behind the decreased strength of the immune system and increased susceptibility to disease in chronic morphine use (Yin et al., 1999; Yin et al., 2006; Li et al., 2010). Moreover, inhibiting TLR2 expression in chronic morphine models decreased cell death (Li et al., 2010). With regards to TLR2 and pro-inflammatory cytokine expression, it has been shown that TLR2 is required for morphine-induced microglia activation, which results in increased inflammatory response (Zhang et al., 2011). This glial activation has already been implicated in morphine tolerance, dependence, and withdrawal (Peterson et al., 1998) therefore demonstrating the important role of TLR2 in morphine use. Given the similarities between TLR2 and TLR4 signaling pathways, TLR2 expression was also measured in this study.
The role of TLR4 in pain

The immune system was first implicated in morphine tolerance and dependence when it was observed that immunosuppressive treatments could alleviate withdrawal symptoms (Dafny et al., 1990). However, only recently has TLR4 activity on immunocompetent cells been connected to neuropathic pain (Malcangio et al., 1996; Mika, 2008; Obreja et al., 2002; Hutchinson et al., 2008). One of the clues linking TLR4 and pain was research showing that intrathecal administration of IL-1β induced allodynia and hyperalgesia (Malcangio et al., 1996; Mika, 2008; Obreja et al., 2002). Since IL-1β is a product of TLR4 signaling, studies further explored the role of TLR4 in neuropathic pain. One study demonstrated that TLR4 knockout mice showed decreased pro-inflammatory cytokine release and hypersensitivity (Tanga et al., 2005). The same researchers also found that TLR4 knockout mice and rats with intrathecal injections of TLR4 antisense oligodeoxynucleotide (to decrease spinal expression of TLR4) showed reduced allodynia and pro-inflammatory cytokine release after L5 nerve transection (Tanga et al., 2005). Following in this trend were results of experiments involving sciatic nerve chronic constriction injury (CCI), a rat model of partial nerve damage known to cause chronic pain in people and induce allodynia (where normally non-painful stimuli become painful) in rats. Rats experienced allodynia and pro-inflammatory cytokine release following the CCI surgery, both of which could be reversed via acute blockade of TLR4 (Hutchinson et al., 2007). Taken together, these studies illustrated the important function of TLR4 signaling in neuropathic pain, but they have focused primarily on glial cells. Since endothelial cells also express TLR4, it is important to assess their role in releasing sensitizing factors that promote neuropathic pain and decrease the analgesic effects of opioid treatment.
**Morphine and TLR4**

The traditional understanding of opioid-receptor interactions asserts that opioids interact primarily with opioid receptors. Morphine, for instance, binds to the $\mu$-opioid receptor on neurons to inhibit voltage-gated calcium channels, thus decreasing excitatory neurotransmission and promoting analgesia (Martin and Roy, 2010; Griffin, 2008). More specifically, the $\mu$-opioid receptor only interacts with the (-)-opioid stereoisomer in order to achieve analgesic effects; in fact, administration of the (+)-opioids has little to no analgesic effect (Wu et al., 2006).

Interestingly, it was not until research of the opioid receptor antagonist naloxone (used to treat opioid overdose) was conducted that alternative opioid-receptor interactions were conceived. A study examining the effects of naloxone on LPS-induced glial activation showed that pro-inflammatory cytokine release could be comparably mitigated by (-)-naloxone *as well as* (+)-naloxone (Liu et al., 2000). Because the $\mu$-opioid receptor only binds the (-)-isomer, these findings indicated that naloxone was binding a different receptor, which could bind both isomers and reverse LPS-induced activation. Subsequent research traced LPS-induced signaling and identified TLR4 as the other receptor that interacts with both (+) and (-)-opioid isomers (Wu et al., 2006; Hutchinson et al., 2008).

Once opioids were identified as TLR4 ligands, studies focused on how these interactions could be contributing to the molecular and physiological effects of opioid treatments. The hypothesis was that non-stereoselective binding of morphine to TLR4 activates the signaling cascade that results in pro-inflammatory cytokine expression, and this expression ultimately diminishes the analgesic effects of morphine (Milligan and Watkins, 2005; Watkins, Rice, and Maier, 2009). To explore this hypothesis, researchers studied the effects of (+)-naloxone on LPS-induced inflammation; as expected, naloxone acted as a noncompetitive antagonist of TLR4
and decreased the LPS-induced inflammatory effects (Liu et al., 2000; Hutchinson et al., 2009b). As mentioned earlier, TLR4 is already a significant player in CCI-induced allodynia, a state that could be reversed upon (+)-naloxone administration (Hutchinson et al., 2007). Additionally, potentiation of analgesia was observed when morphine and (+)-naloxone were co-administered as well as in related studies with morphine administration in TLR4 knockout mice (Buchanan et al., 2010; Hutchinson et al., 2009b). Overall, these studies indicate that (+)-opioid receptor antagonists have therapeutic potential because they can selectively reduce the inflammatory effects of the TLR4 pathway without interfering with the interactions at the opioid receptors (Hutchinson et al., 2007). Given the stereoselectivity of the µ-opioid receptor, (+)-morphine was used in this study so that TLR4 could exclusively be examined, absent of the confounding effects of (-)-morphine binding at the µ-opioid receptor.

Given that morphine-TLR4 interactions trigger pro-inflammatory cytokine release, which counteracts the analgesic effects of morphine, it is reasonable to infer that the TLR4 mechanism may be involved with morphine tolerance. Tolerance, or the need for larger doses to achieve the same effect, is one of the primary reasons physicians are reluctant to prescribe opioids for chronic treatments. Not only are larger doses of drug needed over time to alleviate pain, but also the higher doses can lead to serious addiction and abuse problems (Martin and Roy, 2010). Many studies show that chronic morphine increases glial activation and subsequent pro-inflammatory cytokine release; moreover, glial activation inhibitors restore the analgesic efficacy, therefore preventing the need to administer higher doses of morphine to achieve the same analgesic effects (Song and Zhao, 2001; Raghavendra et al., 2003; Mika et al., 2007). This glial activation can occur through trauma, injury, etc. or, in the case involving TLR4, exposure to LPS (Wu et al., 2006; Johnston and Westbrook, 2005). In other words, the antinociceptive
effects of morphine combined with the pronociceptive effects of glial activation result in net decrease in analgesic effect (Hutchinson et al., 2007). This transient reduction of analgesic effect produces acute tolerance that can, quite quickly, develop into serious analgesic tolerance as well as contribute to the allodynia and hyperalgesia associated with chronic morphine use (Hutchinson et al., 2007). The mitigation of analgesic effects via glial activation may also play a role in dependence and reward pathways, which makes TLR4 signaling and inflammation an even more compelling therapeutic target. Studies illustrate that administration of a glial activation inhibitor with morphine can reduce conditioned place preference, indicating that the rewarding aspects of morphine decrease and similarly reduces development of the associated dependency behaviors (Narita et al., 2006; Nakagawa and Satoh, 2004; Ozawa et al., 2001).

Once again, current studies provide intriguing evidence for the role of glial TLR4 in pain and opioid tolerance and dependence; however, research is lacking surrounding TLR4 activation in endothelial cells and how it may also contribute to negative effects of chronic opioid use. Moreover, because endothelial cells are the first cell type of the central nervous system to encounter intravenous opioids, it is important to characterize how these cells may respond.

**MATERIALS & METHODS**

*Endothelial Cell Isolation & Culture*

Endothelial cells were isolated from adult male Sprague-Dawley rats (300-375g, Harlan Laboratories, Madison, WI, USA). Rats were deeply anesthetized with isoflurane and then rapidly decapitated. The brain and spinal cord were dissected out and the meninges removed. The brain and spinal cord tissue was placed in 50 mL of ice-cold DMEM solution (Invitrogen, Carlsbad, CA, USA) supplemented with 100 μg/mL Penicillin-Streptomycin solution (Invitrogen), 50 μg/mL Gentamicin (Invitrogen), and 2 mM GlutaMAX-1 (Invitrogen). Under a
cell culture hood, the supplemented DMEM was removed and the tissue placed in a petri dish with 13.5 mL HBSS containing 200U/mL Collagenase II (Invitrogen) and 30U/mL DNase I (Sigma, St Louis, MO, USA). Using a sterile scalpel, the tissue was finely minced into small pieces and transferred to a 50 mL conical and incubated for 40 min at 37 °C. 10 mL of HBSS was added and the cell suspension centrifuged at 1000 x g for 8 min at 4°C. The supernatant was removed and the pellet resuspended in 30 mL of 20% BSA in DMEM and centrifuged at 1000 x g for 20 min at 4°C. The myelin layer lying at the surface was removed and 10 mL of HBSS containing 10 mg collagenase/dispase (Roche), and 300U DNase I stock (Sigma) was added to the pellet and incubated at 37°C for 30 min. 10 mL of HBSS (Ca²⁺ and Mg²⁺ free) was added and centrifuged at 700 x g for 6 min at 4°C. The supernatant was removed and the pellet resuspended in 2 mL of supplemented DMEM and gently layered over a 37% Percoll gradient containing 3.3% FBS. The Percoll gradients were centrifuged at 1000 x g for 10 min at 4°C (no brake or acceleration). At the interface of the gradient, the white layer containing the desired microvessel fragments was collected using a 1.5 inch 18G needle and added to 25 mL of cold supplemented DMEM. The solution was centrifuged at 1000 x g for 10 min at 4°C. The supernatant was removed and the remaining pellet resuspended in 48 mL warm DMEM (Invitrogen), containing 100 µg/mL Penicillin-Streptomycin solution, 50 µg/mL Gentamicin, 2 mM GlutaMAX-1, 20% FBS, 1 ng/mL basic fibroblast growth factor (bFGF), and 1 µg/mL puromycin. The cells were seeded into 24 well tissue culture plates previously coated with 8 µg fibronectin, 16 µg collagen type IV, and 8 µg collagen type I. Cells were incubated for 48 hours at 37 °C and 5% CO₂.

After the first 48-hour incubation period, the medium was removed and each well was washed twice with PBS (to remove the puromycin). Then, 1 mL of warm culture medium
without puromycin was added to each well. Every 2-3 days the medium was replaced until the cells were 60-80% confluent (about 7-8 days).

**RNA extraction, cDNA synthesis, and Reverse-Transcription PCR**

The RNA was extracted from the cells using TriZol (Invitrogen). 160 µL of chloroform was added to each sample. After 2 minutes of vortexing and 3 minutes of incubation at room temperature, the samples were centrifuged at 11900 x g for 15 minutes at 4°C. The aqueous phase was collected and 8 µL of glycogen was added to each sample and vortexed. Then, 400 µL of propanol was added to each sample, vortexed, and incubated at room temperature for 10 minutes. The samples were centrifuged at 11900 x g for 10 minutes at 4°C. Following this step, a pellet was visible and the supernatant was poured out and replaced with 1 mL of 75% ethanol (75% ethanol and 25% nuclease-free water). The samples were centrifuged at 7500 x g for 10 minutes at 4°C and the previous step was repeated (removing supernatant and replacing with 75% ethanol). After centrifuging again at 7500 x g for 10 minutes at 4°C, the samples dried for about 30 minutes. The pellets were then resuspended in 11 µL nuclease-free water (on ice) and transferred to thermal well 0.5 mL tubes.

Following RNA extraction the Superscript II First-Strand Synthesis System (Invitrogen) was used to reverse transcribe RNA into cDNA. For cDNA synthesis, 5 ng/µL of random primer and 1 mM dNTP mix (Invitrogen) was added to each sample and incubated for 5 minutes at 65°C. The samples were immediately transferred to ice and a 6 µL mixture of 5X buffer (Invitrogen) and 10 mM dithiothreitol was added to each sample; the samples were incubated for 2 minutes at 25°C. Then, 200U of SuperScript II (Invitrogen) was added to each sample and incubated for 10 minutes at 25°C, 50 minutes at 42°C, and 15 minutes at 25°C. The samples were then stored at -20°C until further use.
The quantification of mRNA involves amplifying cDNA using Real-Time PCR machines. A 26 µL mixture of Quantitect SYBR Green PCR Master Mix (Qiagen, Valencia, CA, USA), 10 nM fluorescein, 500 nM forward and reverse primers (Invitrogen), and nuclease-free water, and 1 µL of sample cDNA was added to each well in a 96-well PCR plate (Bio-Rad, Hercules, CA). To minimize error, cDNA was added in duplicate and the resulting average was used for statistical analysis. Primers for the following genes were used: GAPDH, TLR4, CD14, MD-2, TLR2, IκB, and IL-1β. After 40 cycles (of 15 seconds at 94°C, 30 seconds at 55–60°C, and 30 seconds at 72°C) on the MyiQ Real-Time PCR machine (Bio-Rad), the threshold cycle data (CT-number of cycles for sample to amplify) was collected.

**In-Cell Western Assay**

The In-Cell Western assay was used to measure cellular protein expression in the endothelial cells. The supernatants were removed immediately after the corresponding drug incubation time and the cells fixed in 1 mL/well of 4% paraformaldehyde for 20 minutes at room temperature. The paraformaldehyde was removed and the cells were permeabilized with 1 mL/well of 1X PBS with 0.1% Triton X-100 (Li-Cor, Lincoln, NE, USA) for five minutes, at room temperature on a gentle rocker. The cells were washed 5 times with 1X PBS with 0.1% Triton X-100. 1 mL of Odyssey Blocking Buffer (Li-Cor) was added to each well to block non-specific binding sites and incubated on a rocker for 90 min at room temperature. After incubation, 1 mL of the primary antibody (diluted in Odyssey Blocking Buffer) was added to all wells except the control well (1 mL of Odyssey Blocking Buffer only). The antibodies used in this study were MD-2 (1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA), CD14 (1:100 dilution, Abcam, San Francisco, CA, USA), and the p65 subunit of NF-κB (1:500 dilution, Millipore, Billerica, MA, USA). Cells were incubated at 4°C overnight with gentle
rocking. The cells were then washed five times with 1 mL of 1X PBS + 0.1% Tween-20. 500 µL of secondary antibody only (1:800 dilution in Odyssey Blocking Buffer) was added to the control well and protected from the light. Then a 1:2000 dilution of DRAQ5 (Li-Cor) was added to the secondary antibody solution and 500 µL of this solution was added to all remaining wells. DRAQ5 is a fluorescent stain in the 700 nm channel that binds DNA in live or fixed cells; this stain is used to normalize the antibody signal (in the 800 nm channel) to the number of cells in each well. The cells were incubated for one hour at room temperature, protected from the light, with gentle rocking. The secondary antibody solution was removed and each well was washed five times with 1 mL of 1X PBS + 0.1% Tween-20 Solution (Li-Cor). Following the final wash, each plate was tapped to dry. The dry plates were scanned by the Odyssey Infrared Imaging System in the 700 nm (DRAQ5) and 800 nm (antibody) channels. Using the measurements of the control well to define background, non-specific antibody binding, the integrated intensity for the 700 nm and 800 nm channels was calculated.

**PGE₂ Competitive Immunoassay**

Prostaglandin E₂ (PGE₂), which is produced by cyclooxygenase 2 (COX2), is also a product of TLR4 signaling. Unlike IL-1β, PGE₂ is synthesized independently of NF-κB, though it does rely on PI3K (Hernandez et al., 2010; Rodríguez-Barbero et al., 2009). Since PGE₂ expression increases with TLR4 signaling, measuring PGE₂ is another way in which TLR4 activation can be quantified. The concentration of PGE₂ in supernatants was determined by using a PGE₂ competitive immunoassay kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to manufacturer’s protocol.
**Drug Administration**

*Experiment I: (+)-morphine timecourse*

In order to ascertain the effect of (+)-morphine at different time points and doses, the cells were incubated with 100 µM, 10 µM, 1 µM, and 0 µM doses of (+)-morphine (supplied by Dr. Kenner Rice, National Institute on Drug Abuse, Bethesda, MD, USA) for either 2, 4, or 24 hours. (+)-morphine was used so that the effect of opioid activity on TLR4 could exclusively be examined in the absence of the confounding effects of µ-opioid receptor binding. A limulus amebocyte lysate (LAL) test, which measures the presence of endotoxins, determined that there were no detectable levels of endotoxin in (+)-morphine. The morphine was dissolved in sterile water and stored at 4°C.

*Experiment II: Blockade of TLR4 signaling*

In order to identify the intracellular pathways involved following TLR4 activation by (+)-morphine, the TLR4 pathway was blocked at known key mediators. The three inhibitors, CLI-095 (Invivogen), Wortmannin (Invivogen), and Bay11-7082 (Invivogen), were dissolved in 100% DMSO and stored at -20°C. CLI-095 prevents TLR4 signaling by blocking the dimerization of receptors at the plasma membrane in a MyD88-independent fashion. Cells were incubated with CLI-095 two hours prior to administration of 100 µM (+)-morphine. Wortmannin inhibits PI3K, which is needed to activate the transcription factor NF-κB; cells were incubated with Wortmannin for one-hour before administration of 100 µM (+)-morphine. Another set of cells were incubated for 30 minutes before 100 µM (+)-morphine with Bay11-7082, which prevents phosphorylation of IκB-α such that NF-κB cannot be localized to the nucleus. Each drug was administered at 10 µM, 1 µM, 0.1 µM, and 0 µM doses in the presence
and absence of (+)-morphine. Twenty-four hours after (+)-morphine administration, supernatants were collected and cells processed for RT-PCR.

**Statistical analysis**

PCR data was normalized by converting CT values to percent of GAPDH (housekeeping gene) and by excluding the maximum (ΔCT) value. Within each dose and time group for all results, outliers that were three standard deviations from the mean were excluded from statistical analysis. To compare doses at different times, all mRNA and protein results were converted to percent of vehicle measures. All data were analyzed using one-way analysis of variance (ANOVA) comparing variance across drug dose. For experiment I each time interval was analyzed separately.

**RESULTS**

*Upregulation of IκB and IL-1β mRNA with (+)-morphine administration*

IL-1β is one of the final outputs of the TLR4 pathway and is one of many pro-inflammatory cytokines that contributes to morphine tolerance and dependence. Increased IL-1β mRNA would demonstrate that (+)-morphine administration triggers increased transcription of this pro-inflammatory cytokine. IL-1β mRNA (Fig. 2A) increased significantly (F_{3,17} = 6.42, P < 0.05) at 2 h with the 100 µM dose when compared to vehicle and decreased over time.

IκB is an inhibitory kinase that, when phosphorylated, is cleaved from the transcription factor NF-κB. This cleaving process exposes the nuclear localization signal on NF-κB, allowing NF-κB to translocate to the nucleus and promote transcription of pro-inflammatory cytokines. An increase in IκB mRNA provides an indirect measure of increased NF-κB activity. IκB mRNA (Fig. 2B) was significantly greater (F_{3,20} = 120.17, P < 0.001) than vehicle at the 100 µM (+)-morphine dose at 2 h and 24 h, but not at 4 h (F_{3,20} = 120.17, P > 0.05).
Upregulation of Toll-like receptor mRNA with (+)-morphine administration

If (+)-morphine binds TLR4, it is important to measure whether TLR4 transcription is altered in response to (+)-morphine administration. Due to TLR4 activation, there may be increased TLR4 transcription to replace receptors that are ligand-bound and internalized or to increase receptors on the plasma membrane. TLR4 mRNA (Fig. 3A) did not show significant increases at 2 h or 4 h (F(3,20)= 5.50, P > 0.05). At 24 h, TLR4 mRNA was significantly greater at the 100 μM (+)-morphine dose compared to vehicle (F(3,20)= 5.50, P < 0.05).

Since it is possible that TLR2 is activated by (+)-morphine, TLR2 transcripts were measured as well. TLR2 mRNA (Fig. 3B) was significantly greater at the 100 μM (+)-morphine at 2 h (F(3,16)= 5.30, P < 0.05) and then significantly less at 4 h (F(3,20)= 21.59, P < 0.0001). It appears that the 100 μM (+)-morphine caused TLR2 mRNA to increase quickly at 2h and diminish, while TLR4 mRNA did not significantly increase until later at 24 h post drug administration.

Upregulation of CD14 and MD-2 mRNA with (+)-morphine administration

Two of the accessory proteins required to form the TLR complex are CD14 and MD-2; while both are required for TLR4 signaling, only CD14 is necessary for TLR2. While CD14 mRNA (Fig. 4A) was not significantly greater at 2 h and 4 h after drug administration, 100 μM (+)-morphine produced a significant increase (F(3,20)= 5.92, P < 0.05) in CD14 mRNA compared to vehicle. MD-2 mRNA (Fig. 4B) was significantly greater (F(3,20)= 7.83, P < 0.001) at 100 μM (+)-morphine compared to vehicle at 4 h and approached significance at 24 h (F(3,20)= 7.83, P = .080). The mRNA expression of both CD14 and MD-2 mirrors that of TLR4, which was significant at 100 μM (+)-morphine compared to vehicle at 24 hours.
Effects of (+)-morphine administration on protein expression

While measuring mRNA is important, it is equally vital to measure protein expression, as transcription does not always lead to translation. To ascertain whether changes in mRNA were reflected in protein, NF-κB (p65 subunit), CD14, and MD-2 was measured using an In-Cell Western assay. While NF-κB expression (Fig. 5A) at the 100 µM dose increased at 24 hours, it was not statistically significant. Of the three proteins analyzed, CD14 (Fig. 5B) was the only protein that significantly increased following (+)-morphine administration. At 24 h both 10 µM and 100 µM (+)-morphine significantly increased CD14 protein (F_{3,18} = 11.05, P < 0.01 and P < 0.001 respectively) compared to vehicle. However, CD14 protein at the 100 µM (+)-morphine dose was significantly downregulated (F_{3,18} = 190.10, P < 0.0001) at 4 h compared to vehicle. MD-2 protein levels (Fig. 5C) did not show any demonstrable trend at 2 h and 4 h and showed significant decreases at 100 µM and 10 µM (+)-morphine doses (F_{3,18} = 4.294, P < 0.05 and P < 0.05 respectively), which is contrary to the mRNA results showing that MD-2 transcripts were significantly higher at 4 h. While mRNA and protein trends matched, in general, for NF-κB and CD14, the same was not true for MD-2.

The effect (+)-morphine on prostaglandin E2 expression

Another downstream product, which is independent of NF-κB, of TLR4 signaling is expression of cyclooxygenase 2 (COX2), producing prostaglandin E2 (PGE2) (Hernandez et al., 2010). Moreover, in TLR4 deficient mice (TLR4/-), PGE2 expression is severely reduced (Hernandez et al., 2010). Consequently, measuring PGE2 is another way in which TLR4 activation can be quantified. PGE2 was measured at 100 µM, 10 µM, 1 µM, and 0 µM at 24 h (Fig. 6). While there was no significant difference at 10 µM and 1 µM doses of (+)-morphine, PGE2 expression was significantly higher than vehicle at 100 µM (+)-morphine (F_{3,38} = 4.80, P <
These results, in combination with mRNA and In-Cell Western assay data, showed 100 µM (+)-morphine to have the most significant effects. Thus 100 µM (+)-morphine was used for later experiments with TLR4 inhibitors, in order to better assess the effect of these inhibitors on the TLR4 pathway.

The effect of TLR4 signaling inhibitor and (+)-morphine on mRNA

The administration of a TLR4 signaling inhibitor (CLI-095) was used to determine whether disrupting TLR4 dimerization upstream would inhibit transcription of proteins downstream in the signaling cascade. Both IL-1β (Fig. 7A) and IκB mRNA (Fig. 7B) showed similar trends of increased expression at 10 µM CLI-095 + 100 µM (+)-morphine, but this relationship was not significant with respect to vehicle (F_{5,13} = 4.26 and F_{5,14} = 1.84 respectively, P > 0.05). IL-1β and IκB transcripts for 1 µM CLI-095 + 100 µM (+)-morphine and 0.1 µM CLI-095 + 100 µM (+)-morphine were comparable to vehicle. There was no demonstrable trend in TLR4 transcripts (Fig. 7C) at any dose of CLI-095 compared to vehicle. CD14 (Fig. 7D) showed slight downregulation of mRNA at 10 µM CLI-095 + 100 µM (+)-morphine and 1 µM CLI-095 + 100 µM (+)-morphine. However, the decrease was not significant (F_{5,18} = 2.70, P > 0.05). Similar to IL-1β and IκB, MD-2 mRNA (Fig. 7E) increased at 10 µM CLI-095 + 100 µM (+)-morphine; unlike IL-1β and IκB, though, mRNA levels were greater than vehicle at 1 µM CLI-095 + 100 µM (+)-morphine and 0.1 µM CLI-095 + 100 µM (+)-morphine. Once again, none of these changes were significant (F_{5,19} = 1.67, P > 0.05).

The effect of PI3K inhibitor and (+)-morphine on mRNA

The second TLR4 signaling inhibitor was Wortmannin, a PI3K inhibitor. PI3K is involved in phosphorylating IκB-α, which is needed to expose the nuclear localization sequence of NF-κB. Wortmannin blocks PI3K activity, thus preventing signaling through the
PI3K/Akt/NF-κB pathway. Since PI3K is also involved in other cell survival signaling pathways, it is reported to have both anti-inflammatory and pro-inflammatory effects (Laird et al., 2009). While all mRNA levels showed a trend of increased transcription at 100 μM (+)-morphine compared to vehicle, with the exception of CD14, none of these relationships were significant. IL-1β transcripts (Fig. 8A) were downregulated at the 1 μM CLI-095 + 100 μM (+)-morphine and 0.1 μM CLI-095 + 100 μM (+)-morphine; however, at the highest dose 10 μM CLI-095 + 100 μM (+)-morphine, IL-1β mRNA increased. IκB mRNA (Fig. 8B) similarly showed upregulation at 10 μM CLI-095 + 100 μM (+)-morphine, while other doses were comparable to 100 μM (+)-morphine levels. For both IL-1β and IκB, the changes in mRNA were not significant. Transcripts of TLR4 (Fig. 8C) and MD-2 (Fig. 8E) for all doses of Wortmannin + 100 μM (+)-morphine were comparable to 100 μM (+)-morphine, with no significant variations in mRNA. For CD14 (Fig. 8D), however, mRNA was significantly less at 10 μM CLI-095 + 100 μM (+)-morphine (F_{3,14} = 11.48, P < 0.001, P < 0.05), 1 μM CLI-095 + 100 μM (+)-morphine (P < 0.01), and 0.1 μM CLI-095 + 100 μM (+)-morphine (P < 0.01) compared to 100 μM (+)-morphine. Vehicle and 10 μM CLI-095 mRNA were also significant with respect to 100 μM (+)-morphine (P < 0.01 and P < 0.0001 respectively). Overall, while general trends showed increased transcription at 100 μM (+)-morphine compared to vehicle, there was no significant downregulation of transcripts with Wortmannin except for CD14.

*The effect of IκB-α inhibitor and (+)-morphine on mRNA*

Compared to CLI-095 and Wortmannin, the IκB-α inhibitor Bay11-7082 works much further downstream in the TLR4 signaling pathway. By blocking the phosphorylation of IκB-α, Bay11-7082 allows IκB-α to hide the nuclear localization sequence of NF-κB, thus preventing NF-κB from translocating to the nucleus. With the exception of IL-1β, all the other protein
transcripts were significantly downregulated at 10 μM drug + 100 μM (+)-morphine compared to 100 μM (+)-morphine. IL-1β (Fig. 9A) showed increased mRNA at all doses compared to 100 μM (+)-morphine, though none were significantly greater. IκB mRNA (Fig. 9B), on the other hand, was significantly less at 1 μM drug + 100 μM (+)-morphine (F$_{5,13}$= 17.32, P < 0.05) and 10 μM drug + 100 μM (+)-morphine (P < 0.001) compared to 100 μM (+)-morphine. Vehicle and 10 μM Bay11-7082 mRNA were also significantly less with respect to 100 μM (+)-morphine (P < 0.001 and P < 0.001 respectively). TLR4 transcripts (Fig. 9C) were similarly downregulated at 1 μM drug + 100 μM (+)-morphine (F$_{5,15}$= 120.17, P < 0.05) and 10 μM drug + 100 μM (+)-morphine (P < 0.001) compared to 100 μM (+)-morphine. While CD14 transcripts (Fig. 9D) were not significantly less at 1 μM drug + 100 μM (+)-morphine, there was significant downregulation at the higher dose 10 μM drug + 100 μM (+)-morphine (F$_{5,15}$= 29.70, P < 0.0001) with respect to 100 μM (+)-morphine. Similar to CD14, MD-2 mRNA (Fig. 9E) was only downregulated at 10 μM drug + 100 μM (+)-morphine (F$_{5,14}$= 8.06, P < 0.05), 10 μM Bay11-7082 (P < 0.05), and vehicle (P < 0.05). The overall trend demonstrated significant downregulation of all mRNA (except IL-1β) at 10 μM drug + 100 μM (+)-morphine and at 1 μM drug + 100 μM (+)-morphine for TLR4 and IκB.

The effect of TLR4 inhibitors and (+)-morphine on prostaglandin E$_2$ expression

In order to determine whether the various TLR4 inhibitors had any effect on PGE$_2$ synthesis, PGE$_2$ was measured in the supernatant. In response to the TLR4 inhibitor (Fig. 10A) there were no significant variations with respect to 100 μM (+)-morphine, although PGE$_2$ levels were higher for 100 μM (+)-morphine compared to vehicle and all other doses of CLI-095 were comparable to vehicle. For the PI3K inhibitor (Fig. 10B), on the other hand, PGE$_2$ expression at all doses was significantly less compared to 100 μM (+)-morphine; this is consistent with
previous research stating that while PGE\(_2\) production is independent of NF-κB, it still requires PI3K (Rodriguez-Barbero \textit{et al.}, 2006). PGE\(_2\) is significantly less at 10 \(\mu\)M Wortmannin + 100 \(\mu\)M (+)-morphine (\(F_{5,20}= 70.12, P < 0.01\)), 1 \(\mu\)M Wortmannin + 100 \(\mu\)M (+)-morphine (\(P < 0.01\)), 0.1 \(\mu\)M Wortmannin + 100 \(\mu\)M (+)-morphine (\(P < 0.01\)), 10 \(\mu\)M Wortmannin (\(P < 0.05\)), and vehicle (\(P < 0.01\)). PGE\(_2\) levels for the IκB-α inhibitor (Fig. 10C) show a dose dependent decrease with respect to 100 \(\mu\)M (+)-morphine though, similar to TLR4 inhibitor results, there is no significant variation. Overall, the three TLR4 inhibitors demonstrate a downregulation of PGE\(_2\) with respect to 100 \(\mu\)M (+)-morphine, but only the PI3K inhibitor exhibits significant decreases in expression.

**DISCUSSION**

Analysis of the time course following (+) morphine administration in endothelial cells suggests that TLR4 is activated by (+)-morphine, and TLR2 may be activated as well. While TLR2 transcripts significantly increased at 2 h, TLR4 transcripts did not increase significantly until 24 h. Interpreting the increase in TLR2 transcripts as receptor activation is consistent with \textit{in vivo} studies that found increased TLR2 transcripts in the brain and CNS microvasculature upon LPS injection (Laflamme \textit{et al.}, 2008). Moreover, the upregulation of TLR2 mRNA was transient, occurring as quickly as 30 min after LPS exposure and diminishing at 3-6 hours—a trend that is mirrored in this study, where TLR2 transcripts increased at 2 h and decreased significantly at 4 h and 24 h. The increase in TLR4 transcripts, however, is more controversial. In peripheral vascular endothelial cells upregulation of TLR4 mRNA is observed upon LPS exposure (Zeuke \textit{et al.}, 2002; Faure \textit{et al.}, 2001), demonstrating that an increase in mRNA signifies receptor activation. In rat brains, however, TLR4 transcripts are markedly downregulated in response to LPS challenge (Laflamme and Rivest, 2001). Because CNS
endothelial cells are at the interface of the cardiovascular system and nervous system, it is unclear whether upregulation of TLR4 transcripts signify activation. At the very least TLR4 upregulation coincides with increased CD14 and MD-2 mRNA levels, perhaps indicating that there is TLR4 signaling is occurring at 24 hours.

Interestingly, the IL-1β mRNA amplification does not align with TLR4, but rather with TLR2. Because IL-1β is also a product of TLR2 signaling, these results suggest that the initial increase of IL-1β transcripts may be due to TLR2 activation as opposed to TLR4. However, there is the possibility that other downstream products, such as PGE₂, are synthesized in response to TLR4 activation. Since CD14 and MD-2 transcripts do not increase until after 2 h, it is most likely that TLR2 uses existing CD14 proteins and confers an MD-2 independent signal (since MD-2 is not required for TLR2). The results show that CD14 and MD-2 transcription increases after TLR2 activation but at the same time as TLR4 transcription increases, suggesting that these accessory proteins are upregulated to accommodate increased TLR4 signaling.

The timecourse mRNA results are not completely reflected in the In-Cell Western protein data. The increase in NF-κB (p65 subunit) protein at 100 µM (+)-morphine does mimic an increase in IκB mRNA at the same dose, but the protein levels are not significant. CD14 protein expression aligns perfectly with mRNA results, showing a significant increase at 24 h at the 100 µM (+)-morphine dose. The clear and very significant increase in CD14 protein at 100 and 10 µM (+)-morphine at 24 h indicates that much more CD14 is needed for the cells. These results are consistent with previous research that shows robust increases in CD14 upon exposure to LPS (Laflamme and Rivest, 2001). Since endothelial cells produce soluble CD14 rather than membrane CD14, it is likely that cells must produce much more CD14 mRNA and protein to compensate for protein lost in the supernatant. In other words, when CD14 is not specifically
targeted to the membrane, it may show a more robust increase in transcription and translation compared to other membrane-bound proteins in the signaling pathway that are not lost to supernatants in vitro or the bloodstream in vivo. MD-2 protein showed no demonstrable trend, which could indicate that MD-2 transcripts are degraded prior to translation. Another possibility is that the binding of antibody to MD-2 is affected by changes depending on the association of MD-2 to TLR4 versus the plasma membrane; more research is warranted to explore the role of MD-2 protein.

Of the three pharmacological TLR4 inhibitors tested, the IκB-α (BAY11-7082) inhibitor proved most effective. The TLR4 inhibitor (CLI-095) and PI3K inhibitor (Wortmannin) did not show significant downregulation of transcripts compared to the 100 µM (+)-morphine positive control. In fact, the 10 µM Wortmannin + 100 µM (+)-morphine demonstrated the opposite effect. Since the literature shows that PI3K can confer both proinflammatory and anti-inflammatory effects, it is possible that the low doses of Wortmannin (1 µM and 0.1 µM) block proinflammatory effects while high doses (10 µM) promote inflammatory effects. Because the IκB-α inhibitor is further downstream and prevents nuclear localization of NF-κB, it perhaps has a more direct effect than the TLR4 or PI3K inhibitors. There may be compensatory mechanisms further upstream that prevent TLR4 or PI3K inhibitors from effectively downregulating TLR4, MD-2, CD14, and IL-1β transcripts. For example, while the TLR4 signaling inhibitor CLI-095 does block TLR4, it does so in a MyD88-independent manner. Previous studies have showed that CLI-095 potently inhibits NF-κB activation induced by LPS in cells transiently expressing TLR4, MD-2, and CD14; however, it does not affect NF-κB activation that is mediated by MyD88 (Kawamoto et al., 2008). Thus the lack of trends in the TLR4-signalling inhibitor data supports the idea that (+)-morphine induced TLR4 signaling is MyD88-dependent. To better
assess the downstream effects of blocking TLR4, other TLR4 inhibitors including naltrexone and LPS-RS will be investigated in the future.

The IκB-α inhibitor demonstrated significant downregulation of all mRNA (except IL-1β) at 10 μM drug + 100 μM (+)-morphine and at 1 μM drug + 100 μM (+)-morphine for TLR4 and IκB. The fact that IL-1β mRNA data does not demonstrate the same trends as the other proteins is actually consistent with the time course data (Experiment I). Recall that IL-1β was significantly upregulated at 2 h at 100 μM (+)-morphine compared to vehicle, but that IL-1β levels diminished greatly at 4 h and 24 h. Thus it is no surprise that IL-1β does not exhibit the same trends in response to an IκB-α inhibitor at 24 h. This data further suggests that there may be another product downstream of NF-κB that is produced at 24 h. One such product could be PGE₂, which was downregulated (albeit not significantly) with respect to 100 μM (+)-morphine in a dose-dependent manner with the IκB-α inhibitor. While the TLR4 inhibitor also did not demonstrate significant downregulation compared to 100 μM (+)-morphine, there was decreased mRNA comparatively. The PI3K inhibitor, in fact, had the most significant effect on PGE₂, with significant (P < 0.01) decreases at all drug+100 μM (+)-morphine doses. These data are consistent with the fact that PGE₂ can be produced independent of NF-κB, but is dependent on PI3K.

While this study characterized the effects of different doses of (+)-morphine at different times on TLR4, as well as the effects of three specific TLR4 inhibitors, more research is certainly needed. Specifically, it would be beneficial to explore other possible downstream products of TLR4 signaling. This research suggests that TLR4 may be promoting expression of another cytokine or protein other than IL-1β. In addition, experimenting with a MyD88-inhibitor or other TLR4 signaling inhibitors that are MyD88-dependent would be useful in determining
whether TLR4 signaling can be more effectively blocked upstream. It would be also be beneficial to investigate TLR2 further to determine whether this receptor may be responsible for the deleterious effects of morphine use. In the end, examining how morphine activates TLRs and modulates pro-inflammatory cytokine release at the cellular level contributes to a greater understanding of how morphine treatment affects the body.

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FIGURES

Figure 1. Signaling pathway of Toll-like Receptor 4 (TLR4)

TLR4 signaling requires recruitment of myeloid differentiation factor 2 (MD-2) and soluble CD14. The resulting complex confers an intracellular signal via the Toll/IL-1 receptor (TIR) domain that either follows a MyD88-dependent or MyD88-independent pathway. In the MyD88-dependent pathway, the signal is transduced to IL-1 receptor-associated kinases (IRAKs) that facilitate subsequent phosphorylations. These phosphorylations cause IκB-α, a cytoplasmic protein that binds the nuclear localization sequence of NF-κB (p65 subunit), to dissociate from NF-κB. Binding of TLR4 also activates the PI3K/Akt/NF-κB pathway. NF-κB translocates to the nucleus to promote transcription of pro-inflammatory cytokines such as IL-1β and IL-6.

TLR2 activity requires CD14 and contributes to NF-κB activation. TLR4 signaling also involves MAP kinase activity, which promotes COX2 transcription and ultimately the production of PGE2. (Gangloff et al., 2003; Shimazu et al., 1999; Zhang, 2011)

Figure 2: The effect of 100 μM, 10 μM, 1 μM, and 0 μM doses of (+)-morphine at 2, 4, and 24 h on mRNA expression

mRNA was measured by extracting RNA, synthesizing cDNA, and comparing RT-PCR threshold data to GAPDH housekeeping gene. All groups had n=6.

A. IL-1β mRNA was significantly upregulated in a dose dependent manner at 2 h (F_{3,17}= 6.423, P < 0.05) and then decreased significantly (P < 0.05) over time.

B. IκB mRNA was significantly upregulated in a dose-dependent manner at 2 h (F_{3,20}= 120.17, P < 0.001) and 24 h (F_{3,20}= 120.17, P < 0.0001).

*P < 0.05, ***P < 0.001, ****P < 0.0001 with respect to vehicle
**Figure 3:** The effect of 100 µM, 10 µM, 1 µM, and 0 µM doses of (+)-morphine at 2, 4, and 24 h on mRNA expression

mRNA was measured by extracting RNA, synthesizing cDNA, and comparing RT-PCR threshold data to GAPDH housekeeping gene. All groups had n=6.

A. TLR4 mRNA was not significantly different with respect to vehicle at 2 h or 4 h, but was significantly upregulated at 24 h ($F_{3,20} = 5.501$, $P < 0.05$).

B. TLR2 mRNA was significantly upregulated at 2 h ($P < 0.05$) and significantly downregulated at 4 h ($F_{3,20} = 21.59$, $P < 0.0001$).

* $P < 0.05$, **** $P < 0.0001$ with respect to vehicle

**Figure 4:** The effect of 100 µM, 10 µM, 1 µM, and 0 µM doses of (+)-morphine at 2, 4, and 24 h on mRNA expression

mRNA was measured by extracting RNA, synthesizing cDNA, and comparing RT-PCR threshold data to GAPDH housekeeping gene. All groups had n=6.

A. CD14 mRNA was not significantly different with respect to vehicle at 2 h or 4 h, but was significantly upregulated at 100 µM (+)-morphine at 24 h ($F_{3,20} = 5.924$, $P < 0.05$).

B. MD-2 mRNA was significantly upregulated at 100 µM (+)-morphine at 4 h ($F_{3,20} = 7.831$, $P < 0.001$) and approached significance at 24 h ($F_{3,20} = 7.831$, $P = 0.0796$).

* $P < 0.05$, *** $P < 0.001$ with respect to vehicle
**Figure 5:** The effect of 100 µM, 10 µM, 1 µM, and 0 µM doses of (+)-morphine at 2, 4, and 24 h on protein expression

Protein expression was measured using an In-Cell Western assay. All (+)-morphine groups had n=6 and vehicle had n=4.

A. NF-κB (p65 subunit) was not significant, though the increase at 24 h reflects similar increases in mRNA.

B. CD14 protein was significantly less (F_{3,18} = 190.10, P < 0.0001) at the 100 µM (+)-morphine dose compared to vehicle at 4 h, whereas both the 100 µM and 10 µM protein levels were significantly higher (F_{3,18} = 11.05, P < 0.01 and P < 0.001, respectively) than vehicle at 24 h.

C. MD-2 protein was significantly less at 100 µM and 10 µM (+)-morphine doses (F_{3,18} = 4.294, P < 0.05 and P < 0.05 respectively).

*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 with respect to vehicle

**Figure 6:** The effect of 100 µM, 10 µM, 1 µM, and 0 µM doses (+)-morphine at 24 h on PGE_{2}

PGE_{2} was measured using a competitive immunoassay. All groups had n=6.

While there was no significant difference in protein expression at 10 µM and 1 µM doses of (+)-morphine, PGE_{2} expression was significantly higher than vehicle at 100 µM (+)-morphine (F_{3,38} = 4.797, P < .05).

*P < 0.05 with respect to vehicle
**Figure 7:** The effect of 10 µM, 1 µM, 0.1 µM doses of TLR4 inhibitor (CLI-095) with 100 µM (+)-morphine at 24 h on mRNA expression

mRNA was measured by extracting RNA, synthesizing cDNA, and comparing RT-PCR threshold data to GAPDH housekeeping gene. All groups had n=4.

**A.** IL-1β mRNA was greater at 10 µM CLI-095 + 100 µM (+)-morphine compared to 100 µM (+)-morphine, but not significantly. All other transcript levels were close to vehicle.

**B.** IκB mRNA was greater at 10 µM CLI-095 + 100 µM (+)-morphine compared to 100 µM (+)-morphine, but not significantly. All other transcript levels were close to vehicle.

**C.** TLR4 mRNA was greater at 10 µM CLI-095 + 100 µM (+)-morphine compared to 100 µM (+)-morphine, but not significantly. All other transcript levels were close to vehicle.

**D.** CD14 mRNA showed no demonstrable trend or significant variation between doses.

**E.** MD-2 mRNA was slightly greater at 10 µM CLI-095 + 100 µM (+)-morphine and 1 µM CLI-095 + 100 µM (+)-morphine compared to 100 µM (+)-morphine, but not significantly. All other transcript levels were close to vehicle.

**Figure 8:** The effect of 10 µM, 1 µM, 0.1 µM doses of PI3K inhibitor (Wortmannin) with 100 µM (+)-morphine at 24 h on mRNA expression

mRNA was measured by extracting RNA, synthesizing cDNA, and comparing RT-PCR threshold data to GAPDH housekeeping gene. All groups had n=4.

**A.** IL-1β mRNA was comparable across all doses and there were no significant variations with respect to 100 µM (+)-morphine.

**B.** IκB mRNA was upregulated at 10 µM CLI-095 + 100 µM (+)-morphine, however, there were no significant variations with respect to 100 µM (+)-morphine.
C. TLR4 mRNA showed no dose-dependent trend and transcript levels for all doses of Wortmannin + 100 µM (+)-morphine were comparable to 100 µM (+)-morphine.

D. CD14 mRNA was significantly less at 10 µM CLI-095 + 100 µM (+)-morphine (F_{3,14} = 11.48, P < 0.001), 1 µM CLI-095 + 100 µM (+)-morphine (F_{3,14} = 11.48, P < 0.01), and 0.1 µM CLI-095 + 100 µM (+)-morphine (F_{3,14} = 11.48, P < 0.01) compared to 100 µM (+)-morphine. Vehicle and 10 µM CLI-095 mRNA were also significantly less with respect to 100 µM (+)-morphine (F_{3,14} = 11.48, P < 0.01 and P < 0.0001, respectively).

E. MD-2 mRNA showed no dose-dependent trend and transcript levels for all doses of Wortmannin+ 100 µM (+)-morphine was comparable to 100 µM (+)-morphine.

**P < 0.01, ***P < 0.001, ****P < 0.0001 with respect to 100 µM (+)-morphine

**Figure 9**: The effect of 10 µM, 1 µM, 0.1 µM doses of IκB-α inhibitor (Bay11-7082) with 100 µM (+)-morphine at 24 h on mRNA expression mRNA was measured by extracting RNA, synthesizing cDNA, and comparing RT-PCR threshold data to GAPDH housekeeping gene. All groups had n=4.

A. IL-1β mRNA for all doses did not vary significantly with respect to 100 µM (+)-morphine, though an increase of transcripts was observed at 1 µM Bay11-7082+100 µM (+)-morphine.

B. IκB mRNA was significantly less compared to 100 µM (+)-morphine at 1 µM drug + 100 µM (+)-morphine (F_{5,13} = 17.32, P < 0.05) and 10 µM drug + 100 µM (+)-morphine (F_{5,13} = 17.32, P < 0.001). Vehicle and 10 µM Bay11-7082 mRNA were also significantly less (F_{5,13} = 17.32, P < 0.001 and P < 0.001 respectively).

C. TLR4 mRNA were significantly downregulated at 1 µM drug + 100 µM (+)-morphine (F_{5,15} = 120.17, P < 0.05) and 10 µM drug + 100 µM (+)-morphine (F_{5,15} = 120.17, P < 0.001) compared
to 100 µM (+)-morphine. Vehicle and 10 µM Bay11-7082 mRNA were also significantly less (F5,15= 120.17, P < 0.01 and P < 0.001 respectively).

**D.** CD14 mRNA with respect to 100 µM (+)-morphine was significantly less at 10 µM drug + 100 µM (+)-morphine (F5,15= 29.70, P < 0.0001), 10 µM Bay11-7082 (F5,15= 29.70, P < 0.0001), and vehicle (F5,15= 29.70, P < 0.001).

**E.** MD-2 mRNA with respect to 100 µM (+)-morphine was significantly less at 10 µM drug + 100 µM (+)-morphine (F5,14= 8.056, P < 0.05), 10 µM Bay11-7082 (F5,14= 8.056, P < 0.05), and vehicle (F5,14= 8.056, P < 0.05).

*P < 0.05, ***P < 0.001, ****P < 0.0001 with respect to 100 µM (+)-morphine

**Figure 10:** The effect of 10 µM, 1 µM, .1 µM doses of TLR4 inhibitors with 100 µM (+)-morphine at 24 h on PGE2 expression

PGE2 was measured using a competitive immunoassay. All groups had n=4.

**A.** There were no significant variations with respect to 100 µM (+)-morphine, although PGE2 levels were higher for 100 µM (+)-morphine compared to vehicle.

**B.** PGE2 is significantly less at 10 µM Wortmannin + 100 µM (+)-morphine (F5,20= 7.119, P < 0.01), 1 µM Wortmannin + 100 µM (+)-morphine (F5,20= 7.119, P < 0.01), .1 µM Wortmannin + 100 µM (+)-morphine (F5,20= 7.119, P < 0.01), 10 µM Wortmannin (F5,20= 7.119, P < 0.05), and vehicle (F5,20= 7.119, P < 0.01) compared to 100 µM (+)-morphine.

**C.** PGE2 levels for the IκB-α inhibitor (Bay11-7082) show a dose dependent decrease with vehicle significantly less than 100 µM (+)-morphine (F5,20= 3.55, P < 0.05).

*P < 0.05, **P < 0.01 with respect to 100 µM (+)-morphine

*P < 0.05, ***P < 0.001, ****P < 0.0001 with respect to 100 µM (+)-morphine
Figure 1
Figure 2

(A) IL-1β mRNA

(B) IκB mRNA
A  TLR4 mRNA

TLR4 mRNA relative to GAPDH (% control)

Time (hours)

- vehicle
- 10 μM (+)-morphine
- 1 μM (+)-morphine
- 100 μM (+)-morphine

B  TLR2 mRNA

TLR2 mRNA relative to GAPDH (% control)

Time (hours)

* 2
* 4
*** 24

Figure 3
Figure 4
Figure 5

A. NFκB (p65 subunit) protein

B. CD14 protein

C. MD-2 protein
Figure 6

(+)-morphine (PGE$_2$)

PGE$_2$ (pg/mL)

(+)-morphine dose (µM)

*
Figure 7
Figure 8
Figure 9
Figure 10
References


