Fall 9-2011

Reactive Oxygen Species Modulation of the Mu-Opioid Receptor

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By

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Submitted in partial fulfillment of the requirements for the
degree of Doctor of Philosophy in Molecular Bioscience from the
Department of Biological Sciences of Seton Hall University
September, 2011

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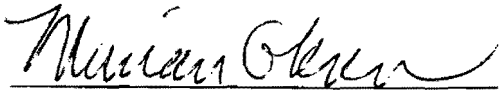

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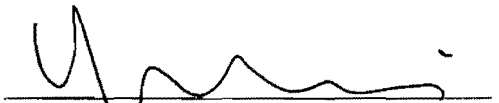

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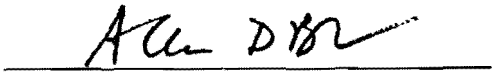

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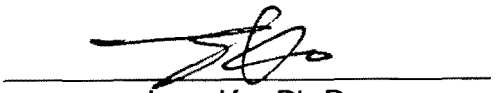

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Acknowledgements

First and foremost, I am heartily thankful to my mentor, Dr. Sulie L. Chang, whose encouragement, guidance and support from the initial to the final level enabled me to complete this project. I would also like to extend my sincere thanks to members of my dissertation committee for their time and effort put forth to the review of this work.

Also, many thanks to the faculty members of the Department of Biological Sciences who over the years provided many interesting lectures that ultimately inspired me to pursue my scientific curiosity.

I would also like to thank past and present members of the Institute of NeuroImmune Pharmacology who provided much needed encouragement and support.

I would also like to acknowledge my family for their sacrifices and the one above us all, the omnipresent God, for answering my prayers and for giving me the strength to plod, I thank you Lord.

This project was supported, in part, by the National Institutes of Health/National Institute on Drug Abuse (R01 DA007058, R21 DA019836 and K02 DA016149 to SLC).

-Erik F. Langsdorf

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Abstract

One aspect of this project set out to examine the effects of methamphetamine (METH) on the accumulation of cellular reactive oxygen species (ROS) and the expression of the mu-opioid receptor (MOR) in a dopaminergic cell line, SH-SY5Y, in order to delineate a possible role for ROS signaling in the coupling of dopaminergic and opioidergic pathways in neuronal cells. Prior evidence indicates neuronal cells treated with METH accumulate dopamine in the synaptic cleft which can lead to increased levels of ROS. Uncovering a role for ROS in increasing MOR expression in response to METH may provide a therapeutic target aimed at reducing the already established phenomenon of METH cross-sensitization to morphine, two drugs with high potential for abuse.

A second aspect of this project investigated the mechanism by which lipopolysaccharide (LPS) exposure alters MOR expression in immune and neuronal cells. Previous research notes the exposure to LPS increases the production of ROS in murine macrophages. Also, activation of MOR effects functions associated with the immune response. Therefore, uncovering a role for ROS in increasing MOR expression in immune and neuronal cell types could provide additional insights in how to address problems associated with LPS-induced inflammatory response and the subsequent acceleration of sepsis seen with morphine treatment.

The data presented here show that METH's effect on MOR expression is dependent upon sub-lethal levels of intracellular ROS increased by METH treatment, which suggests a possible coupling of METH- and opiate-mediated intracellular signaling. In addition, work from the second portion of this project found that LPS stimulated the intracellular ROS accumulation 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 TPA-HL-60 secreted TNF-α and GM-CSF. These data indicate 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.

I.

Introduction

i. Preface.

Reactive oxygen species (ROS) are highly reactive molecules mainly produced during the transfer of electrons through the electron transport chain and secreted by activated macrophage and neutrophils (McCord and Fridovich, 1978; Bast and Goris, 1989; Bayir, 2005). ROS participate in a multitude of signal transduction pathways as secondary messengers, amplifying extracellular signals originating at the cell surface. As secondary messengers, ROS signaling initiates cell differentiation, proliferation, and migration (Sauer et al., 2001; Van der Goes et al., 2001). Experimental evidence indicates that ROS can activate transcription factors that regulate the expression of genes involved in the inflammatory response (Conner and Grisham, 1996; Fialkow et al., 2007). Also, the activation of mitogen-activated protein kinases (MAP kinases) and Janus kinase / signal transducers and activators of transcription (JAK/STATs) are known to be affected by ROS (Hancock et al., 2001). Aside from their cell signaling actions, ROS play an important a role in host defense whereby acting as chemical agents that participate in the destruction of pathogens (McRipley and Sbarra, 1967). However, when cells are unable to maintain redox homeostasis, leading to an increase in intracellular ROS levels, oxidative stress can occur. The consequence of oxidative stress is often

associated with tissue injury and cellular damage (Conner and Grisham, 1996; Hancock et al., 2001).

The mu-opioid receptor (MOR) is a G protein-coupled receptor (GPCR) widely known to elicit euphoria and analgesia when activated by morphine (Trescot et al., 2008a). The anti-nociception properties of morphine are of benefit to patients suffering from cancer, burns, and both acute and chronic pain; however, continuous intake of morphine can lead to tolerance and addiction (Trescot et al., 2008b).

Experimental evidence points to the cross-sensitization of drugs of abuse, such as cocaine, alcohol, and nicotine, to morphine (Lett, 1989; Vezina and Stewart, 1990; Cador et al., 1995; McDaid et al., 2005). The cross-sensitization of drugs of abuse, resultant to the convergence of the catecholamine and opioid signaling pathways, implies that sensitization to a particular drug can be further enhanced with repeated intermittent exposure to a different drug of abuse; thus, essentially increasing the pleasurable effects of the drugs as well as increasing drug seeking behaviors (McDaid et al., 2005; Nestler, 2005; Stefano et al., 2007).

Activation of MOR also affects functions associated with the immune response (Bryant and Roudebush, 1990; Taub et al., 1991; Bidlack, 2000; Coussons-Read and Giese, 2001). Chronic exposure to morphine and subsequent activation of MOR suppresses the inflammatory response (Chang

et al., 1998; Bidlack, 2000). In these instances, a dysfunctional inflammatory response, consequential to activation of MOR, decreases the immune system's ability to mount an appropriate response to pathogenic microbial antigens, e.g. lipopolysaccharides (LPS) [Chang et al., 2001; Bidlack, 2000; Roy et al., 1998].

This dissertation project examines the direct and indirect effects of ROS on the modulation of MOR expression in neuronal and immune cell lines. Currently there are no reports in the scientific literature that indicate ROS signaling participates in modulating MOR expression; thus, uncovering a connection between ROS and MOR may provide additional insights into therapeutic interventions in opiate addiction and diseases linked to inflammation.

ii. Survey of literature.

The chemical nature of ROS, their source of production, and their effect on biological systems has been of interest to scientists for many years. Early reports recognized ROS for their role in mammalian host defense against pathogens; however, recent work has uncovered that ROS serve important functions as cell signaling molecules (Hancock et al., 2001). As the name implies, ROS are molecules that contain oxygen and some are more reactive than molecular oxygen due to varying reduced states. ROS present in biological systems include radicals such as superoxide and hydroxyl and the non-radicals hydrogen peroxide (H_2O_2), peroxynitrite and hypochlorous acid

(Hancock et al., 2001; Bayir, 2005; Fialkow et al., 2007) [Fig. 1]. The radical ROS are highly reactive and are capable of reacting with other molecules or radicals in an attempt to achieve a more stable electron configuration (Wu and Cederbaum, 2003). Of the three radical ROS noted above, hydroxyl radicals are perhaps the most reactive of these species and are thought to be the most reactive chemicals found in biological systems. Hydroxyl radicals are unstable, due to the free unpaired electron, and rapidly combine with target molecules residing in close proximity, which can lead to peroxidation of lipids and proteins (Fialkow et al., 2007). Superoxides are unstable but not as reactive as hydroxyl radicals and often quickly combine to form H_2O_2. The non-radical, H_2O_2 is not as reactive as the radical ROS but has been shown to inactivate enzymes by the oxidation of thiol groups (Bayir, 2005; Fialkow et al., 2007). Peroxynitrites are somewhat stable compared to the other non-radical ROS; however, at physiological pH peroxynitrites become protonated and rapidly breakdown to nitrates and hydroxyl radicals (Conner and Grisham, 1996). Hypochlorous acid, considered a highly reactive non-radical, is able to alter lipids and proteins and as a result modifies membrane architecture leading to cell lysis (Spickett et al., 2000). In addition, hypochlorous acid reacts indiscriminately with a wide variety of biomolecules including pyridine nucleotides and amino acids, which are essential building blocks of important cellular macromolecules (Conner and Grisham, 1996).

Radical ROS-

Superoxide ($O_2^{-\cdot}$): $NADPH + O_2 \rightarrow NADP^+ + O_2^{-\cdot}$

Hydroxyl (OH·): $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\cdot + OH^-$

Non-radical ROS-

Hydrogen peroxide (H_2O_2): $2\ O_2^{-\cdot} + 2\ H^+ \rightarrow H_2O_2 + O_2$

Hypochlorous acid (HOCl): $H_2O_2 + Cl^- + H^+ \rightarrow HOCl + H_2O$

Peroxynitrite ($ONOO^-$): $O_2^{-\cdot} + NO^{\cdot} \rightarrow ONOO^-$

Figure 1. Chemistry of ROS. Superoxides are produced during electron transfer through the electron transport chain and NADPH oxidase. Hydroxyl radicals are produced via the Haber-Weiss reaction when in the presence of transition metals such as Fe(II) or Cu(I). Hydrogen peroxide molecules are mainly produced during the dismutation of superoxides at low pH, or by the enzymatic activity of superoxide dismutases. Neutrophil and macrophage myeloperoxidases' catalyze the formation of hypochlorous acid, which in turn can react with superoxides to produce hydroxyl radicals. The formation of peroxynitrites occurs when superoxides react with nitric oxide, which are produced from nitric oxide synthases (NOS) and xanthine oxidoreductase (XOR) (Hancock et al., 2001; Bayir, 2005).

Several enzymatic processes generate ROS in mammalian cells. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidoreductase (XOR), and myeloperoxidase (MPO) are the best characterized catalysts that contribute to ROS production (Bayir, 2005). LPS, tumor necrosis factor-α (TNF- α), interleukin-1β (IL-1 β), and interleukin-6 (IL-6) activate NADPH oxidase in immune cells. These stimuli, along with NADPH supplied electrons, allow for the reduction of oxygen by the multiple-subunit complex NADPH oxidase (Bayir, 2005). Similarly, XOR generates superoxide and can be up-regulated by hypoxia, LPS, TNF- α, IL-1 β, and IL-6. Neutrophils and macrophages use MPO to catalyze the oxidation of chloride to hypochlorous acid (HOCl) in the presence of H_2O_2. In addition to the enzymes noted above, mitochondria convert up to 2% of cellular oxygen to superoxide when the cytochrome oxidase complex prematurely releases oxygen subsequent to receiving electrons from the cytochrome bc1 complex (Konstantinov et al., 1987; Fialkow et al., 2007).

ROS engage in gene regulation and participate as second messengers in signal transduction pathways in a variety of cell types. Hancock *et al.* (2001) highlight that ROS are ideal signaling molecules since their production can be stimulated and their signal can be rapidly removed. ROS have been shown to activate redox-sensitive transcription factors and coordinate distinct biological responses. For example, low oxidative stress can induce the expression of genes coding for antioxidant enzymes by activating the transcription factor Nrf2.

In addition, oxidative stress at an intermediate level can lead to the initiation of an immune response through the activation of NF-κB and AP-1 transcription factors. Finally, it has been shown that high levels of ROS can disrupt the flow of electrons through the electron transport chain and as a consequence lead to the damage of mitochondria, which can ultimately result in up-regulation of genes involved in programmed cell death (Gloire et al., 2006). Several reports have shown that signal transduction pathways involving the mitogen-activated protein kinases (MAP kinases) are activated by the non-radical ROS, H_2O_2 (Hancock et al., 2001). Fialkow et al. (1994) showed that treatment of neutrophils with H_2O_2 activated MAP kinase kinase (MEK), an upstream activator of MAP kinase, and inhibited the cysteine-containing protein tyrosine phosphatase, CD45. Because of the combination of ROS activation of MEK and inhibition of CD45, it was suggested that MAP kinases, exposed to oxidant, would have extended periods of activation (Fialkow et al., 2007). Guyton et al. (1996) used primary smooth muscle cells, mouse embryonic fibroblasts (NIH 3T3), human cervical cancer cells (HeLa), rat fibroblasts (Rat 1), and rat pheochromocytoma (PC12) cell lines to show that H_2O_2 activated ERK2 via RAt Sarcoma protein (Ras). The consequence of ERK2 activation by H_2O_2 seems to be related to cell survival since dominant negative Ras PC12 mutant resulted in increased sensitivity to H_2O_2 (Guyton et al., 1996). In addition to the MAP kinase pathway proteins, H_2O_2 also activates the JAK/STAT pathway. Simon et al. (1998) showed, using carcinoma cells (A-431) and Rat-1 cells, that

H_2O_2 activates STATs and involves JAK2 and TYK2 kinases. The authors further suggest that downstream affects of H_2O_2 include activation of STATs, which participate in the regulation of cell growth , ROS clearance, and apoptosis (Simon et al., 1998).

ROS play important roles in cellular communication, yet early research on ROS primarily focused on their microbiocidal properties and their role in human host defense (McRipley and Sbarra, 1967). For example, *in vitro* studies using XOR and acetaldehyde as a substrate along with chelated iron (Fe^{2+}-EDTA) indicated that this biochemical reaction produced lethal levels of ROS capable of killing pathogenic *Escherichia coli, Staphylococcus aureus, Listeria monocytogenes* and *Salmonella typhimurium* (Yamada et al., 1987). In these studies, inclusion of Fe^{2+}-EDTA increased the lethality of XOR metabolism by increasing the rate of production and accumulation of hydroxyl radicals similar to *in vivo* levels; therefore, the authors summarized that XOR-Fe^{2+}-EDTA was a relevant model of *in vivo* ROS production (Yamada et al., 1987). Work by Lindgren et al. (2004) also showed ROS are important metabolites involved in host defense against the facultative intracellular bacterium, *Francisella tularensis.* In their studies, mice that were $p47^{-/-}$ (p47 is a subunit of NADPH oxidase) were more susceptible to *F. tularensis* infection with higher numbers of bacteria present in the spleen and liver compared to wild-type mice (Lindgren et al., 2004). Furthermore, splenocytes harvested

from wild-type mice, during periods of increased morbidity, produced significantly higher levels of ROS than the $p47^{-/-}$ mice (Lindgren et al., 2004).

Although ROS serve important functions in cell communication, participate in the activation of signal transduction pathways and transcription factors; as well as, assisting in the clearance of pathogens via their microbiocidal properties, ROS present at high levels can lead to cellular damage. Such situations arise when factors increase ROS production or decrease the cellular antioxidant function thereby offsetting redox homoeostasis[the balance between ROS production and removal] (Bayir, 2005). Both intracellular and extracellular ROS levels are maintained at non-lethal levels by superoxide dismutases, catalases, and an intracellular thiol-reducing buffer consisting of glutathione and thioredoxion (Nakamura et al., 1997; Gamaley and Klyubin, 1999).

When cells are unable to maintain redox homeostasis, the intracellular ROS levels can persist and eventually damage proteins, DNA, and lipid membranes and initiate apoptosis (Conner and Grisham, 1996). Proteins, in the form of enzymes, carry out crucial functions in cell metabolism. The oxidation of enzymes at cysteine, methionine, and histidine residues, by hydroxyl radicals, can modify protein conformation and negatively affect function. Lipids serve a structurally supportive role isolating intracellular organelles and cytosolic reactions from the extracellular environment. Hydroxyl attack of phospholipids can result in the peroxidation of many

polyunsaturated fatty acid molecules creating a disruption of the plasma membrane (Wu and Cederbaum, 2003). ROS are a major source of DNA damage, causing strand breaks and mutations resulting from nucleotide deletions. Changes in DNA sequences that outpace DNA repair mechanisms potentially have permanent consequences with detrimental effects for the cell (Wu and Cederbaum, 2003).

In humans, MORs are distributed throughout the central and peripheral nervous system, on spinal cord projections, as well as in the brainstem, midbrain and cortex (Stein et al., 2003). Opioid receptors have also been identified on the peripheral processes of sensory neurons with pharmacologically similar effects to MORs found in the brain (Stein et al., 2003). Molecular evidence also points to the presence of MORs on various immune cells. MOR mRNA has been identified from human T- and B- cells, monocytes, macrophages, and granulocytes suggesting a role for the opioid pathway in the immune response (Chuang et al., 1995).

The sequence analysis of the cloned MOR places it in the GPCR receptor family. MORs consist of seven transmembrane domains, an extracellular N-terminus with intracellular C-terminus and three intracellular and extracellular loops (Law et al., 1999). Aside from morphine, MOR agonists include enkephalins, beta-endorphins, dynorphin A, and endomorphin-1 and 2 (Trescot et al., 2008a; Trigo et al., 2010). Interestingly, morphine, is widely excepted to be an exogenous MOR agonist, has recently been shown to be

synthesized by the human neuroblastoma cell line, SH-SY5Y (Boettcher et al., 2005). The activation of MOR leads to inhibition of cAMP and N- and P/Q-type Ca2+ channels; as well as, the stimulation of K^+ channels (Law et al., 2000; Trigo et al., 2010). The consequence of which, leads to physiological effects such as euphoria, reduced blood pressure, decreased respiration, and decreased neuron excitability (Trigo et al., 2010).

Methamphetamine (METH), a psychostimulant drug with a high potential for abuse, has been shown to cause damage to dopaminergic terminals in various regions of the brain (Yamamoto and Zhu, 1998; Kita et al., 2003; Mark et al., 2004). The mechanism of METH-induced neurotoxicity has long been thought to involve increased levels of dopamine, itself a mild neurotoxin, and dopamine-derived reactive oxygen species [ROS] (Filloux and Townsend, 1993; Riddle et al., 2006; Thomas et al., 2009). METH competes with dopamine for active sites on the dopamine transporter (DAT). The binding of METH to DAT allows for the diffusion of METH into the neuronal axon and reversal of DAT, essentially preventing dopamine re-uptake (Yamamoto et al., 2010). The absence of dopamine re-uptake allows for dopamine to accumulate within the synaptic cleft, in these instances dopamine in the presence of oxygen begins to undergo enzymatic and auto-oxidation thereby increasing the levels of ROS (Yamamoto and Bankson, 2005). As noted earlier, the generation of ROS is a consequence of cellular metabolism; however, an imbalance in ROS

homoeostasis caused by METH shifts the cytoplasm to a more oxidative state which can lead to the onset of oxidative stress (Yamamoto and Bankson, 2005). Oxidative stress, in turn, has been shown to lead to cellular apoptosis and tissue necrosis. For example, METH-treated dopaminergic neuronal SH-SY5Y cells undergo mitochondrial membrane depolarization, increased ROS production, and apoptosis (Wu et al., 2007a).

The oxidation of dopamine, however, is not the only source of METH-induced ROS production, nor is it an effect exclusive to neurons. *In vitro* studies have shown that the METH-induced production of ROS also occurs in astrocytes, a cell type not involved in dopamine anabolism (Abramov et al., 2005; Nikolova et al., 2005). Similar to the findings in METH-treated SH-SY5Y cells, evidence indicates that METH impairs mitochondrial function and increases ROS levels in murine astrocytes (Lau et al., 2000). It seems reasonable to speculate that ROS produced by astrocyes may impact cellular functions of neurons in close proximity.

Evidence from Raut et al. (2006) suggested that MOR function is decreased by oxidative stress (Riddle et al., 2006). However, Raut et al. (2007) indicated the possibility that a multi-factoral component was responsible for the decreased function of the MOR, not oxidative stress alone (Raut et al., 2007). Although high levels of oxidative stress decrease MOR function, there is evidence that suggests intermediate levels of ROS may in fact increase MOR

expression and function. Intermediate levels of ROS initiate the inflammatory response and increase the activation of NFκB (Gloire et al., 2006). Transcriptional activation of NFκB by hydrogen peroxide, itself a ROS, has been reported in SH-SY5Y cells (Larouche et al., 2008). Also, NFκB binding sites have been identified in the MOR promoter region (Kraus et al., 2003). Furthermore, amphetamine, a metabolite of METH, increases the localization of MOR mRNA in the rat striatum (Vecchiola et al., 1999). Collectively, these studies lead to the speculation that ROS may in fact lead to increased MOR expression through the activation of the transcription factor NFκB. Therefore METH treatment could possibly lead to an increase in MOR expression as a result of ROS accumulation and be associated with activation of transcription factors with binding site located on the MOR promoter region. This phenomenon may extend to other molecules, other than METH, that lead to increases in ROS accumulation and activation of transcription factors such as NFκB. One such molecule could be LPS.

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 S, 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. Annually, there are one million deaths from sepsis worldwide, and approximately 25-30% of the cases are due to gram-negative bacterial infection (Rumpa S, 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. 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). LPS induced levels of pro-inflammatory cytokines activate the HPA and lead to the secretion of corticotrophin-releasing hormone (CRH) and eventual production of cortisol which in turn promotes the inhibition of pro-inflammatory cytokines. 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 (Chang et al., 1998), 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 1988, Vidal *et al.* 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; Chang et al., 1996; Chang et al., 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; Chang et al., 2001; Chen et al., 2005).

As noted previously, ROS are highly reactive molecules produced during cellular respiration (Hancock et al., 2001). However, disease and stress can alter a cell's ability to effectively regulate ROS levels (Wu and Cederbaum, 2003). Interestingly, evidence from experimental and clinical studies correlate development of sepsis with ROS production, the depletion of antioxidants such

as vitamin C, E and A, and glutathione, and the accumulation of markers of oxidative stress, e.g. lipid peroxidation (Bayir, 2005). In addition, the 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.

iii. Project summary

One aspect of this project involved an *in vitro* study to examine the effects of METH on the accumulation of cellular ROS and the expression of the MOR in a dopaminergic cell line, SH-SY5Y, in order to delineate a possible role for ROS signaling in the coupling of dopaminergic and opioidergic pathways in neuronal cells.

A second aspect of this project involved an *in vitro* study to investigate the mechanism by which LPS exposure alters MOR expression in immune and neuronal cells. Specifically, the effects of LPS-induced ROS accumulation on MOR expression was examined in TPA-differentiated HL-60 (TPA-HL-60) macrophage-like cells (Rovera et al., 1979; Kowalski and Denhardt, 1989). Also, conditioned medium (CM) was assayed from 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. MOR expression was examined in SH-SY5Y neuroblastoma cells (Ciccarone et al., 1989) cultured in CM from the LPS-treated TPA-HL-60 cells.

II.

Materials and Methods

i. Cell culture.

The human SH-SY5Y neuroblastoma cell line, a gift from R. Ross, (Fordham University, New York), was grown in MEM+F12 medium supplemented with 10% fetal bovine serum (FBS), 100 U penicillin, and 100 µg/mL streptomycin (Gibco, Invitrogen Corp. Grand Island, NY). 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. Experimental 12- or 96-well plates (BD Biosciences, VWR, West Chester, PA) were seeded with SH-SY5Y cells at $1x10^5$ cells/mL in 1 mL/well in the 12-well plate and 0.1 mL/well in the 96-well plate, respectively, and cultured for 24 h prior to treatment. Experimental 12-well plates were seeded with HL-60 cells at $5x10^5$ cells/mL in 1 mL/well.

In this project, 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). The differentiation of TPA-HL-60 cells into macrophage like cells can be identified by the change from a suspension culture (HL-60) to an adherent culture [TPA-HL-60] (Rovera et al., 1979). Therefore, prior to the initiation any TPA-HL-60 experiential

treatment the shift from suspension culture to an adherent culture was monitored by phase contrast microscopy. 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 (Rovera et al., 1979; Kowalski and Denhardt, 1989). Stock solutions of TPA were dissolved in 100% ethanol to a concentration of 16 μM and diluted 1000-fold in medium. SH-SY5Y, HL-60, and TPA-HL-60 cells were maintained in a 5% CO_2 humidified incubator at 37° C.

ii. METH cell culture treatment.

METH (Sigma-Aldrich, St. Louis, MO) and hydrogen peroxide [H_2O_2] (Sigma-Aldrich, St. Louis, MO) stock solutions were prepared in 0.9 % saline, filter sterilized (0.2 μm filters, Pall Life Sciences, Ann Arbor, MI), and the cells were treated with medium containing METH at a final concentration of 0 to 6.8 mM or H_2O_2 at a final concentration of 0 to 400 μM.

For ROS scavenging experiments, cells were pre-treated with medium containing 100 μM vitamin E (Sigma-Aldrich, St. Louis, MO) for 3 h (Wu et al., 2007), and then treated with medium containing METH. Medium containing saline at a volume equivalent to the treatment served as the control in all of the related experiments.

iii. LPS cell culture treatment.

Stock solutions of lipopolysaccharide from *E. coli* strain 055:B5 (LPS, Sigma-Aldrich, St. Louis, MO) were prepared in 0.9% saline to a concentration of 10 mg/mL and filter sterilized (0.2 μm filters). TPA-HL-60 cells were treated with medium containing LPS at a final concentration of 0.125 to 0.500 mg/mL.

For ROS scavenging experiments, TPA-HL-60 cells were pre-treated with medium containing 100 μM vitamin E for 3 h and then treated with medium containing LPS. Where appropriate, saline at a volume equivalent to the treatment served as the control in all of the related experiments. Unless noted otherwise, in CM experiments, TPA-HL-60 supernatants were pooled, filter sterilized (0.2 μm filters), 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.

iv. Measurement of cell viability.

The alamarBlue assay detects metabolically active cells with an oxidation-reduction indicator, and has been previously used for assessing viability of neuronal and non-neuronal cells (White et al., 1996; Magliaro and Saldanha, 2009). Cell viability experiments were performed in 96-well plates. After treatment, 10 μl of alamarBlue (Invitrogen Corp., Grand Island, NY) was

added to each well. The cells were then incubated at 37 °C, 5% CO_2 for 2 h. Fluorescence was measured using a SpectraMax Gemini EM (Molecular Devices, Sunnyvale, CA) with excitation at 530 nm and emission fluorescence detected at 590 nm. The percent control was calculated as: 590 nm test reagent/590 nm saline control multiplied by 100.

v. Measurement of intracellular ROS.

Dihydrorhodamine 123 (DHR123) is an indicator of hydrogen peroxide, hypochlorous acid, and peroxynitrite anion (Crow, 1997; McBride et al., 1999; Li et al., 2001). Non-fluorescent DHR123 passively diffuses across cell membranes where it becomes oxidized to form fluorescent rhodamie123 in the presence of H_2O_2 and intracellular peroxidases. Rhodamine 123 is retained in the intracellular space and effectively measures the intracellular levels of ROS (Henderson and Chappell, 1993). Stock solutions of DHR123 were reconstituted in DMSO (Sigma-Aldrich, St. Louis, MO) to a concentration of 10 mM. Intracellular ROS levels in SH-SY5Y and TPA-HL-60 cells cultivated in 12-well plates were determined by confocal laser scanning microscopy (CLSM). After treatment, the medium was replaced with fresh medium containing 20 μM DHR123 (Sigma-Aldrich, St. Louis, MO), an indicator of ROS, and incubated for 30 min (Henderson and Chappell, 1993). The medium was then replaced with fresh medium alone, and the presence of ROS was detected with a FluoView

FV1000 CLSM (Olympus, Center Valley, PA) at 200X 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 mean fluorescence of 5 randomly selected cell clusters from 3 views, from 3 wells per treatment as follows:

cell cluster ROI (Σ avg. $CHS1^{1,2...5}$*$area^{1,2...5}$/ Σ area) – background ROI (Σ avg. $CHS1^{1,2...5}$*$area^{1,2...5}$/ Σ area), where ROI refers to the region of interest and avg. CHS1 refers to the average fluorescence of the pixilated ROI. All fluoresce levels were calculated by the FluoView FV1000 software.

For ROS measurements performed using TPA-HL-60 cells, the changes in intracellular ROS levels were calculated as the percent mean fluorescence of 10 randomly selected cell clusters from 3 views from 3 wells per treatment.

vi. Extraction and isolation of total RNA.

Total RNA was extracted and isolated using TRIzol or TRIreagent in accordance with the manufacturer's protocol (Ambion, Invitrogen Corp., Grand Island, NY). Spent culture media was aspirated by vacuum and 0.5 mL of TRIzol/TRIreagent was added to each well. Cells were incubated for 5 min at 15 to 30 °C. Cell lysate from two wells per treatment were combined in order to increase the total mass of RNA collected. Phase separation was performed

with the addition of 0.2 mL of chloroform (Sigma-Aldrich, St. Louis, MO) per 1.0 mL TRIzol/TRIreagent. Samples were shaken by hand for 15 sec and incubated at 15 to 30 °C for 3 min. Samples were then centrifuged (Eppendorf 5400, Westbury, NY) at 12,000 x g for 15 min in a 4 °C cold box. The upper layer aqueous phase containing the total RNA was then transferred to a fresh 1.5 mL microcentrifuge tube. Total RNA was precipitated from the aqueous phase with 0.5 mL 2-propanol (Sigma-Aldrich, St. Louis, MO) per 1.0 mL TRIzol/TRIreagent. Samples were gently vortexed for 5 sec, kept at 15 to 30 °C for 10 min and centrifuged at 12,000 x g for 10 min in a 4 °C refrigerator. After centrifugation, 2-propanol was discarded and the total RNA pellet was washed with 1.0 mL of 75% ethanol (Sigma-Aldrich, St. Louis, MO) solution prepared with di-ethyl-propyl carbonate (DEPC) in water (Ambion, Austin, TX). After 75% ethanol addition, samples were gently vortexed for 5 sec and centrifuged at 7,400 x g for 5 min. After centrifugation, the 75% ethanol solution was discarded the total RNA pellets were allowed to air dry for 20 min at at 15 to 30 °C. The total RNA pellets were then dissolved in 20 µl of DEPC water and measured at 260 and 280 nm using the ND-1000 (NanoDrop Technologies, Wilmington, DE).

vii. Synthesis of cDNA.

cDNA was prepared from 1 to 5 µg of SH-SY5Y and TPA-HL-60, respectively, total RNA in 20 µl containing 1X first strand buffer, 10 mM dithiothreitol (DTT), 0.25 mM 2'-deoxynucleoside 5' triphosphate (dNTPs), 0.015 µg/mL random primers, and 15 U/µl moloney murine leukemia virus (M-MLV) (Invitrogen Corp., Grand Island, NY). All reactions were prepared on wet ice. Negative control reactions, absent of the reverse transcriptase M-MLV, were prepared in parallel and supplemented M-MLV with DEPC water. 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. Reactions were stored at -80 °C.

viii. Measurement of gene expression.

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. Glyceraldehyde 3-phosphate dehydrogenase (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 Intergrated DNA Technologies (Coralville, IA).

ix. PCR Data Analysis.

The changes in MOR expression between treatment groups compared to control groups were determined by real-time PCR. Real-time PCR cycle threshold values (Ct), from triplicate real-time PCRs per treatment, were obtained from the ABI 7900 HT Fast Real Time PCR System. First, ΔCt values were calculated as follows: Average ΔCt MOR value – average ΔCt GAPDH value. Next, ΔΔCt values were determined using the equation: Average $\Delta Ct_{treatment} - \Delta Ct_{control}$. Fold, changes in MOR expression, normalized to control, were than calculated using the $2^{-\Delta\Delta Ct}$ equation (Livak and Schmittgen, 2001). In all experiments, GAPDH was used as an internal control to normalize any differences in total RNA additions in the reverse transcription reactions.

x. Measurement of cytokines.

Cytokines secreted by TPA-HL-60 cells were measured using a 96-well human pro-inflammatory cytokine tissue culture kit with slight modification to the manufacturer's procedure (Meso Scale Discovery, Gaithersberg, MD). After treatment, supernatants were centrifuged at 12,000 x 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, Gaithersberg, MD). Calibrator solutions were diluted in RPMI 1640 medium supplemented with 20% FBS, 100 U penicillin, and 100 µg/mL streptomycin, which were the medium conditions used in the cultivation of TPA-HL-60 cells, 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.

xi. 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 conditioned medium (CM) was centrifuged over

a YM-3 kDa centrifugal column filter according to the manufacture's protocol (Millipore, Billerica, MA), for 90 min at 2800 rpm using a Sorvall 6000D (Newton,CT) centrifuge. 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 filtered. The CM was then overlaid onto sub-confluent SH-SY5Y cells for 24 h. The use of YM-3 kDa centrifugal device in the separation of molecules with based on molecular weight, according to the recommendations established by the manufacture (Miilipore, Billerica, MA), recovers 90 to 100% of molecules with molecular weights greater than 5 kDa in the retenate.

xii. 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.

xiii. Statistical analysis.

Data in this study are represented as the mean ± SD. The number of replicates per treatment group used in each experiment is noted in the figure legends. Differences among treatment groups were analyzed by a one-way ANOVA, followed by a Tukey's post hoc test or a two-way ANOVA, followed by a Bonferroni's Multiple Comparison Test. Unless noted otherwise, all experiments were performed at least three times.

III.

Results

i. Effects of METH and H_2O_2 on SH-SY5Y cell viability.

Numerous studies have indicated a correlation between increased ROS levels and decreased cell viability (Trachootham et al., 2008). Therefore, in order to maximize the possibility of correlating changes in ROS accumulation with changes in MOR expression in viable populations of cells, subsequent experiments were performed with sub-lethal concentrations of METH and H_2O_2, i.e., less than a 50% reduction in cell viability. The lethal dosages of METH and H_2O_2 were determined using the alamarBlue assay. SH-SY5Y cells were treated with various concentrations of METH (0.85 to 6.8 mM) or H_2O_2 (25 to 400 μM) for 24 h and 48 h (Figure 2A, B). After 24 h of treatment, there was a significant decrease in cell viability with the highest METH dosage tested. After 48 h of treatment, viability was reduced to 75% with 0.85 mM METH and to 60% with 1.7 mM METH. SH-SY5Y treated for 24 h with concentrations of 100 μM H_2O_2 and above had significantly reduced cell viability from 55% to 20%.

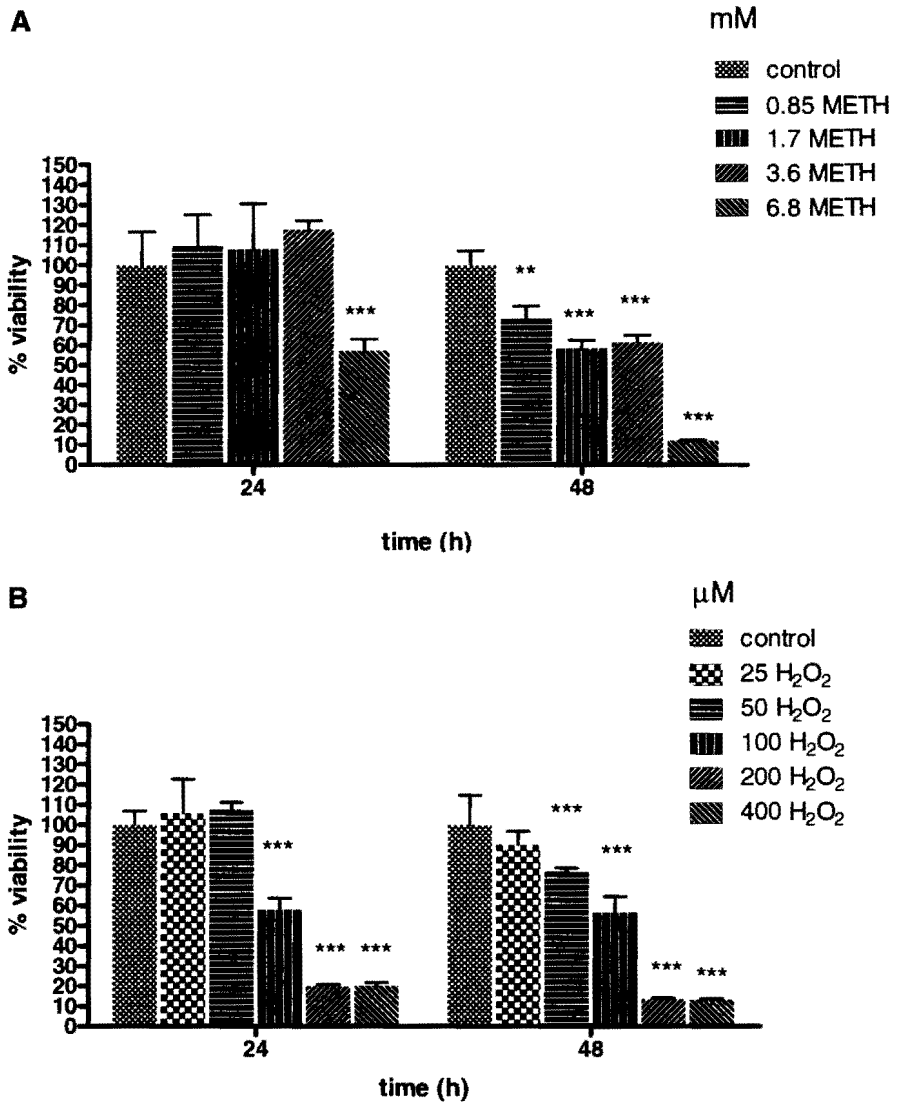

Figure 2. METH and H_2O_2 cellular toxicity. (A) SH-SY5Y cells were treated with saline (control), 0.85, 1.7, 3.4, or 6.8 mM METH for 24 or 48 h. (B) SH-SY5Y cells were treated with saline (control), 25, 50, 100, 200, or 400 μM H_2O_2 for 24 or 48 h. The alamarBlue assay was used to determine cell viability (indicated as % control). Values are the means ± SD of four replicates per treatment. Bonferroni's Multiple Comparison Test was used to determine significant differences. $^{*}p < 0.05$, $^{**}p < 0.01$.$^{**}p < 0.01$ and $^{***}p < 0.001$ compared to controls at the same time point.

ii. Effect of METH on intracellular accumulation of ROS.

METH-induced neurotoxicity is thought to involve increased levels of dopamine, itself a mild neurotoxin, and dopamine-derived reactive oxygen species [ROS] (Filloux and Townsend, 1993; Riddle et al., 2006; Thomas et al., 2009). In this study, CLSM was used to examine intracellular accumulation of ROS in SH-SY5Y cells in order to further maximize the possibility of correlating changes in ROS accumulation with changes in MOR expression. The concentrations of METH used here were found to decrease SH-SY5Y cell viability. The ROS indicator, DHR123, can passively diffuse into cells where it undergoes oxidation in the presence of H_2O_2 and peroxidases to form fluorescent rhodamine 123. Once DHR123 enters the cell and becomes oxidized it cannot diffuse across the plasma membrane; essentially remaining trapped within the cell (Henderson and Chappell, 1993).

SH-SY5Y cells were treated with saline, 0.85 mM METH, or 1.7 mM METH for 48 h. ROS accumulation increased in the METH-treated cells in a dose- and time-dependent manner (Figure 3D, E). After 48 h, 0.85 mM METH increased ROS accumulation by 50% compared to the saline control, and 1.7 mM METH increased ROS levels to 150% above control level. In addition, distinct morphological changes in the form of cell body contraction were observed when the SH-SY5Y cells were treated with 1.7 mM METH compared to the control (Figure 3A, C).

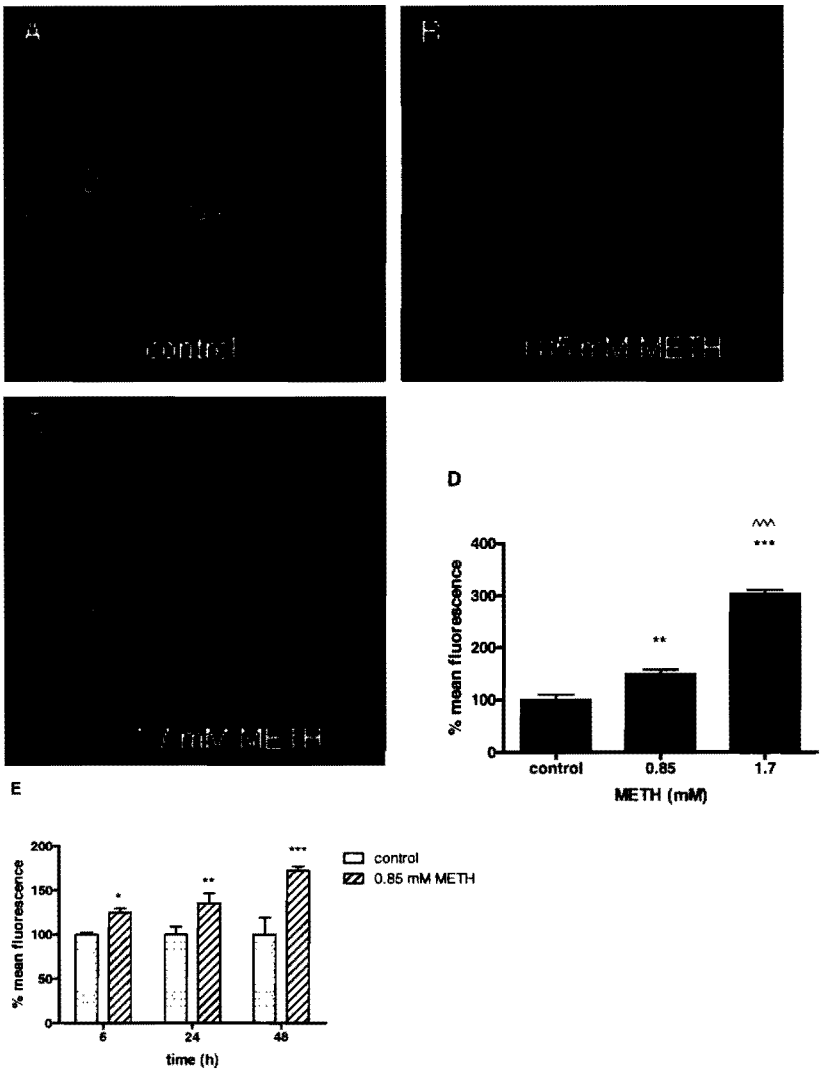

Figure 3. METH-induced intracellular accumulation of ROS. (A-C) Representative confocal images depicting ROS levels in cell cluster ROI and subtracted background ROI. Cell cluster ROI, circled in white, are numbered in red from 1 to 10 and background ROI, circled in blue, are numbered in red 11 to 20. SH-SY5Y cells were treated with (A, D) saline (control), (B, D) 0.85 mM METH, or (C, D) 1.7 mM METH for 48 h. (E) SH-SY5Y cells were treated with saline (control) or 0.85 mM METH for 6, 24, and 48 h. Relative levels of ROS (indicated as % mean fluorescence), were determined using DHR123 and confocal microscopy. Values are the means ± SD of three microscopic fields from three replicates per treatment. Tukey's post hoc test was used to determine statistically significant differences. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to controls. $^{\wedge\wedge}p < 0.001$, 1.7 mM METH compared to 0.85 mM METH.

iii. Effects of METH on SH-SY5Y MOR expression.

Initial experiments were performed to assess changes in the MOR expression under control conditions during the time points examined in this study. SH-SY5Y cells were treated with saline for 0, 6, 24 and 48 h. No significant changes in MOR expression were observed. The lack of any significant difference between the time points examined indicates that SH-SY5Y MOR expression remained stable from 0 to 48 h, under control (saline) conditions, post the 24 h recovery period after initial seeding. Therefore, subsequent time course experiments compared experimental treatments to the indicated control (Figure 4).

In order to identify a potential role for METH-induced ROS accumulation in MOR expression, measurement of MOR expression in the SH-SY5Y cells treated with METH, at concentrations corresponding with previously observed conditions where METH induced elevated intracellular levels of ROS, was performed. Real time PCR indicated MOR expression increased approximately 50% after treatment with either 0.85 mM or 1.7 mM METH (Figure 5A). Time-course experiments showed that the highest level of MOR expression was reached after 48 h with 0.85 mM METH (Figure 5B).

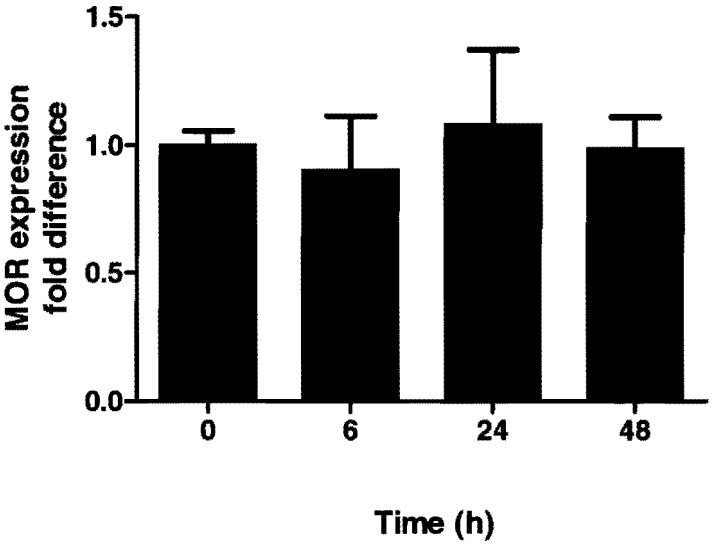

Figure 4. SH-SY5Y MOR expression time course. Total RNA from SH-SY5Y cells were harvested at 0, 6, 24, and 48 h. Real-time PCR was used to measure MOR and GAPDH amplification. Relative changes in MOR expression were determined using the $2^{-\Delta\Delta Ct}$ equation with GAPDH serving as the internal standard. The 0 h time point severed as the control. Tukey's post hoc test was used to determine statistically significant differences between time points. No statistically significant differences were detected between the time points analyzed.

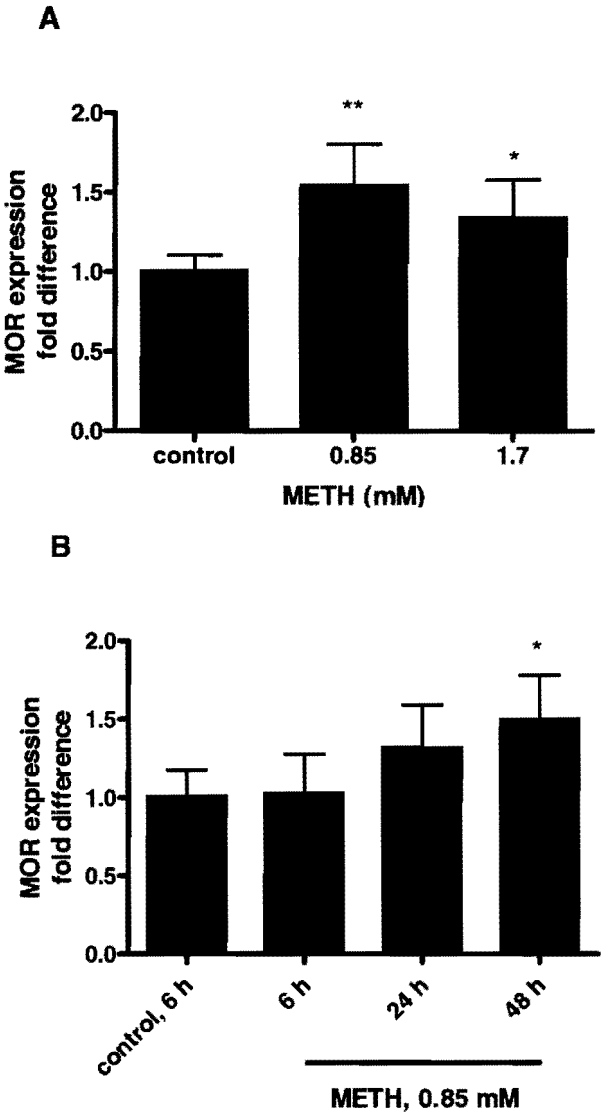

Figure 5. METH-induced MOR expression. (A) SH-SY5Y cells were treated with saline (control), 0.85, or 1.7 mM METH for 48 h. (B) SH-SY5Y cells were treated with saline (control) or 0.85 mM METH for 6, 24, and 48 h. Real-time PCR was used to measure MOR and GAPDH amplification. Relative changes in MOR expression were determined using the $2^{-\Delta\Delta Ct}$ equation with GAPDH serving as the internal standard. Values are the means ± SD of six replicates per treatment. Tukey's post hoc test was used to determine statistically significant differences. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to controls.

iv. Effects of H_2O_2 on SH-SY5Y MOR expression.

In this study, changes in ROS levels were assessed using DHR123, which indirectly measures the ROS H_2O_2 through the emitted fluorescence of rhodamine 123. Since increased levels of H_2O_2 were detected in response to METH treatment, MOR expression was measured, using real time PCR, in SH-SY5Y cells containing sub-lethal levels of H_2O_2 to see if there was a similar increase in MOR expression as observed with METH. All three concentrations of H_2O_2 tested significantly increased MOR expression by 50-60% above the control; however, none of the concentrations tested produced a dose-dependent effect on MOR expression (Figure 6A). This suggests that the concentrations of H_2O_2 saturated the cellular transcriptional response with regard to MOR expression. Time-course experiments showed that the highest level of MOR expression was reached 24 h after treatment with 20 μM H_2O_2 (Figure 6B).

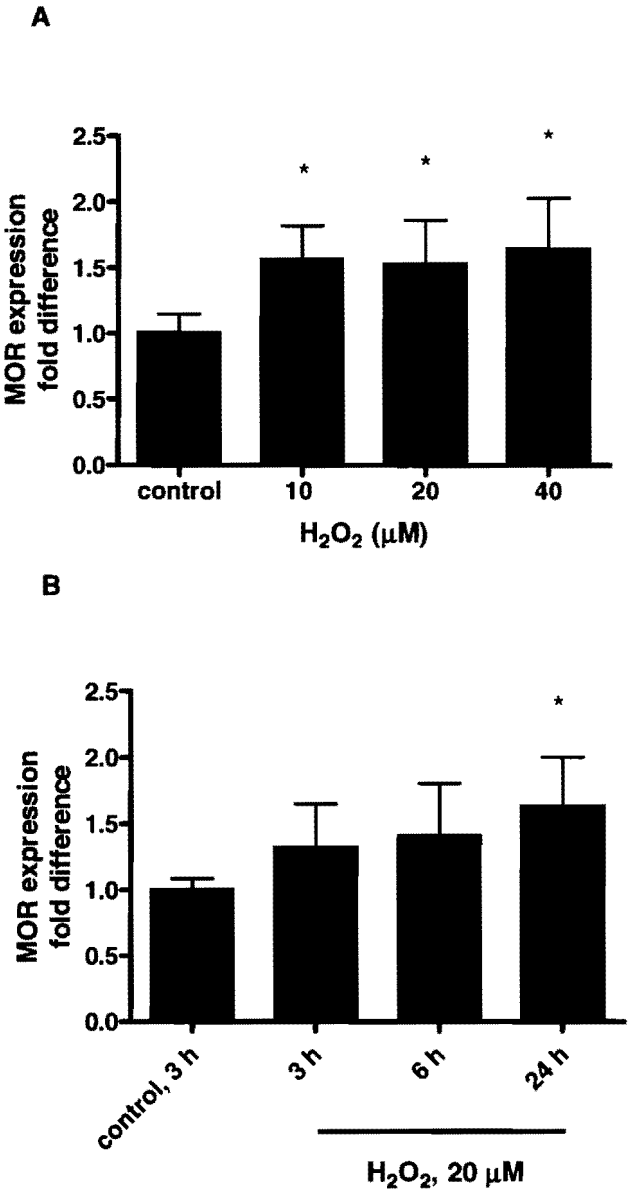

Figure 6. H_2O_2-induced MOR expression. (A) SH-SY5Y cells were treated with saline (control), or 10, 20, or 40 μM H_2O_2 for 24 h. (B) SH-SY5Y cells were treated with saline (control) or 20 μM H_2O_2 for 3, 6, or 24 h. Real time-PCR was used to measure MOR and GAPDH amplification. Relative changes in MOR expression (fold difference) were determined using the $2^{-\Delta\Delta Ct}$ equation with GAPDH serving as the internal standard. Values are the means ± SD of six replicates per treatment. Tukey's post hoc test was used to determine statistically significant differences between time points. $^*p < 0.05$ compared to controls.

v. Effect of vitamin E on METH-induced ROS accumulation in SH-SY5Y and SH-SY5Y MOR expression.

The observed H_2O_2-induced increase in MOR expression supported our hypothesis that METH-induced ROS serves as a mechanism for increased expression of the MOR. To test this hypothesis further, SH-SY5Y cells were pre-treated with the antioxidant, vitamin E, for 3 h and then treated with 0.85 mM METH. Vitamin E is a commonly know anti-oxidant and has been shown in previous studies using SH-SY5Y to block the accumulation of ROS (Wu et al., 2007). Using 100 μM vitamin E, METH-induced ROS levels were decreased to those similar to the control. Vitamin E (10 μM) was able to partially attenuate the METH-induced increase in ROS accumulation to 35% below the METH treatment group (Figure 7).

In MOR expression experiments, 100 μM vitamin E was able to attenuate the METH-induced increase in MOR expression to 25% above the control value. This level was not significantly higher than the control, but was significantly lower than with METH treatment. 10 μM vitamin E was able to partially attenuate the METH-induced increase in MOR expression to 33% above the control value (Figure 8).

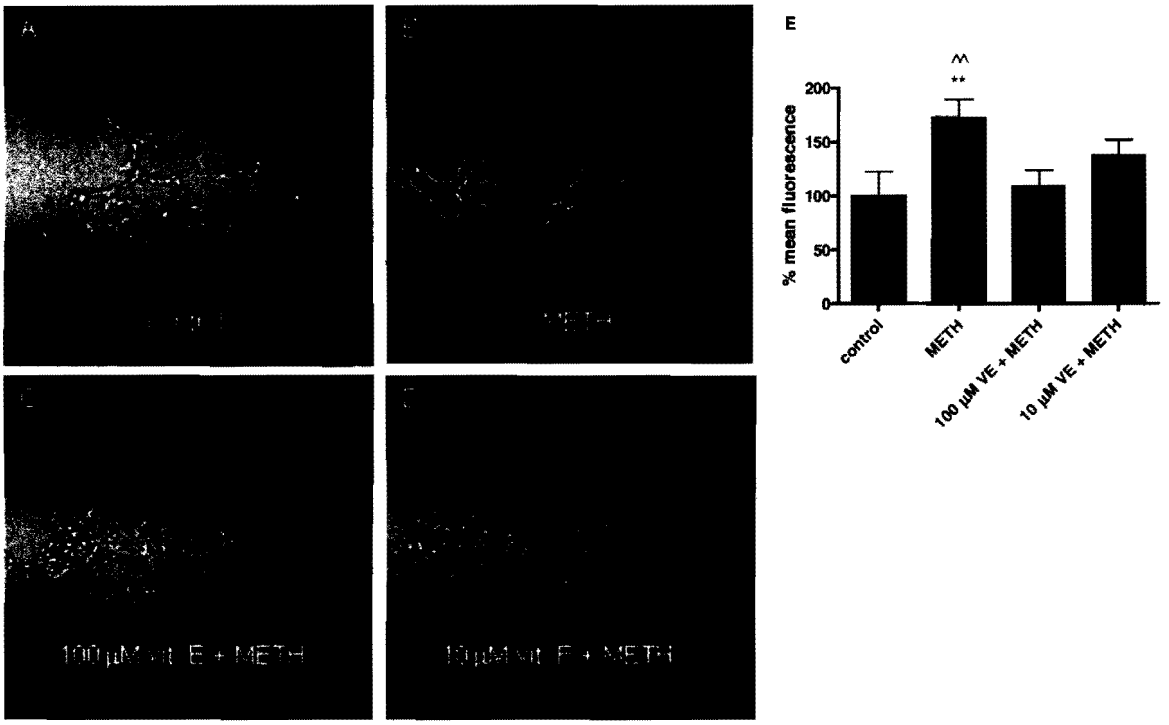

Figure 7. Vitamin E effect on METH-induced ROS accumulation. (A-D) Representative confocal images depicting ROS levels in cell cluster ROI and subtracted background ROI. SH-SY5Y cells were treated with saline (control), 0.85 mM METH alone, 100 µM vitamin E + 0.85 mM M ETH, or 10 µM vitamin E + 0.85 mM METH for 48 h. Relative levels of ROS, (indicated as % mean fluorescence), were determined using DHR123 and confocal microscopy. Values are the means ± SD of three microscopic fields from three replicates per treatment. Tukey's post hoc test was used to determine statistically significant differences between time points. $^{**}p < 0.01$ compared to the control. ^^p<0.01, METH alone compared to 100 µM vitamin E + METH.

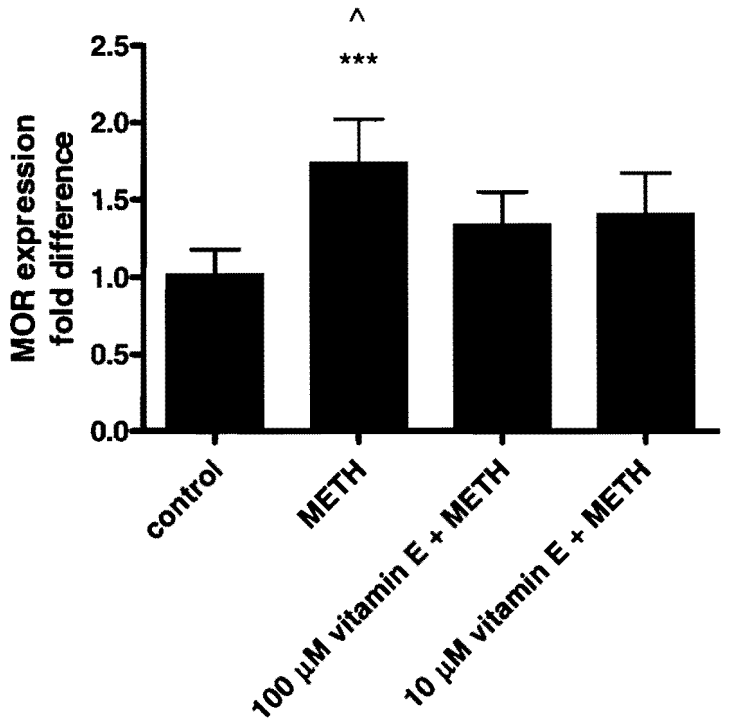

Figure 8. Vitamin E effects on SH-SY5Y MOR expression. SH-SY5Y cells were treated with saline (control), 0.85 mM METH, 100 µM vitamin E + 0.85 mM METH, or 10 µM vitamin E + 0.85 mM METH for 48 h. Real time-PCR was used to measure MOR and GAPDH amplification. Relative changes in MOR expression were determined using the $2^{-\Delta\Delta Ct}$ equation with GAPDH serving as the normalizing standard. Values are the means ± SD of three microscopic fields from six replicates per treatment. Tukey's post hoc test was used to determine statistically significant differences between time points. ***$p < 0.001$ compared to the control. ^$p < 0.05$ METH alone compared to 100 µM vitamin E + METH.

vi. Effect of LPS on TPA-HL-60 ROS accumulation.

Exposure to LPS increases the production of ROS in murine macrophages (Hsu and Wen, 2002; Kim et al., 2004). Therefore, in this study 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 mg/mL LPS for 24 h. Confirmation of differentiation was determined by the phenotypic shift from suspension culture to an adherent culture as observed using phase contrast microscopy. After LPS treatment, the cells were treated with 20 μM of the ROS indicator, DHR123, for 30 min. CLSM was used to examine changes in intracellular accumulation of ROS in the TPA-HL-60 cells resulting from the LPS treatment. Saline solution (0.9% w/v), added at an equivalent volume, served as a vehicle control. Figure 9A shows representative confocal images of TPA-HL-60 cells treated with LPS, then DHR123, in order to assess intracellular ROS accumulation. TPA-HL-60 cells treated with 0.250 and 0.500 mg/mL LPS significantly increased intracellular ROS when compared to the vehicle control (Figure 9B).

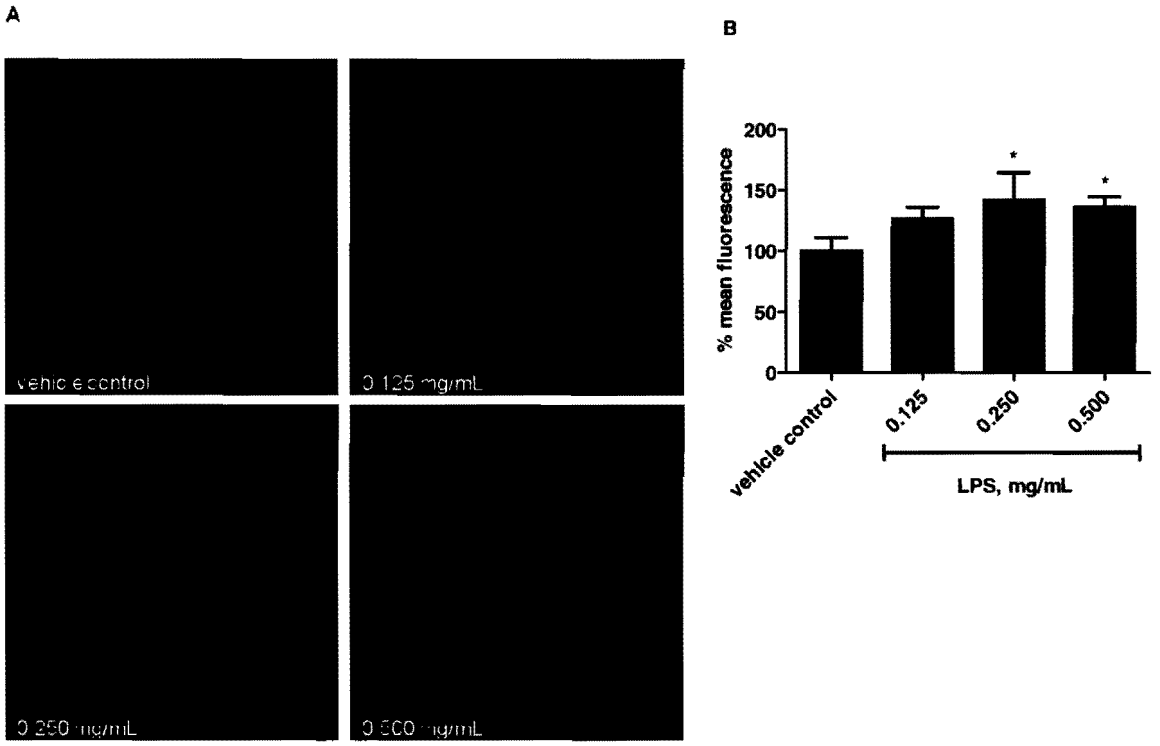

Figure 9. Effect of LPS on TPA-HL-60 reactive oxygen species (ROS) accumulation. TPA-HL-60 cells were treated with 0.9% saline (vehicle control), 0.125 mg/mL LPS, 0.250 mg/mL LPS, or 0.500 mg/mL LPS for 24 h. (A) Representative confocal images demonstrating ROS levels in cell cluster region of interest (ROI) and subtracted background ROI. (B) Relative levels of ROS (indicated as % mean fluorescence) were determined using DHR123 and confocal microscopy. Values are the means ± 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.

vii. 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. Figure 10A 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) [Figure 10B]. 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 by approximately 40% (Figure 10B).

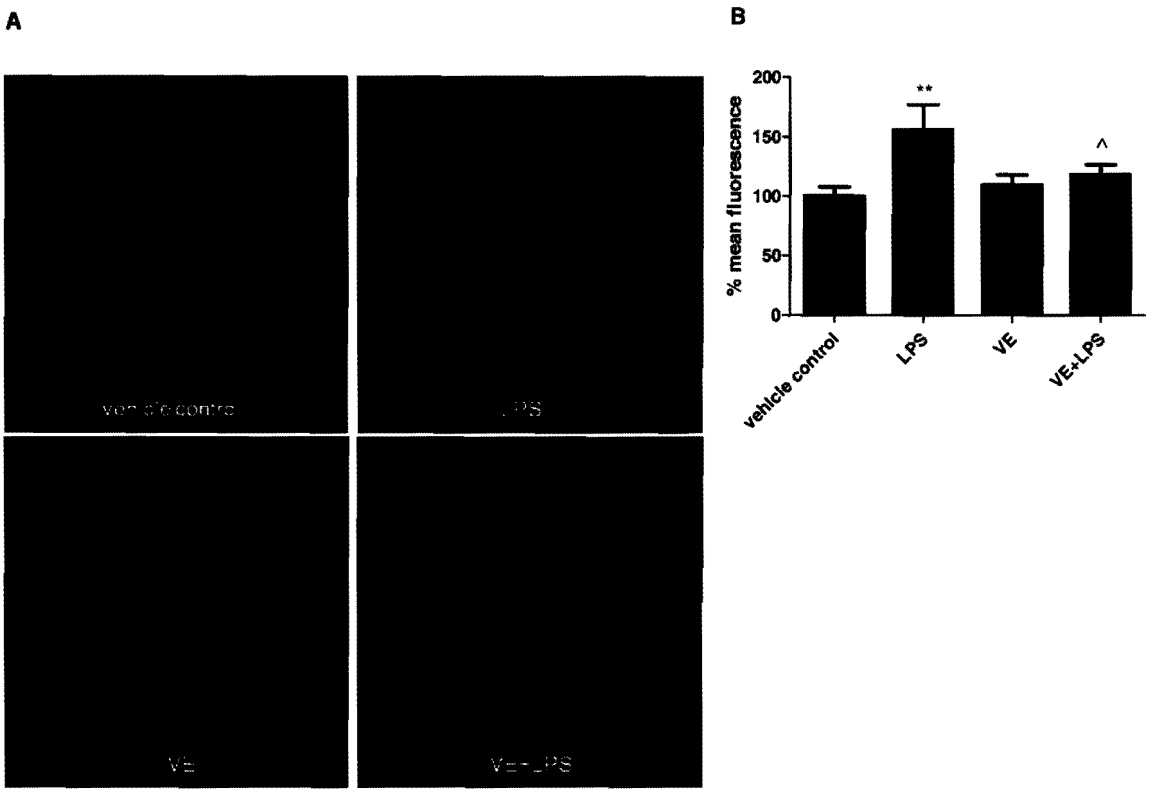

Figure 10. Effect of Vitamin E (VE) on LPS-induced ROS accumulation in TPA-HL-60 cells. TPA-HL-60 cells treated with 0.9% saline (vehicle control), 0.500 mg/mL LPS alone (LPS), pre-treated with 100 μM VE alone for 3 h (VE), or pre-treated with 100 μM VE plus 0.500 mg/mL LPS (VE+LPS) for 24 h. (A) Representative confocal images demonstrating ROS levels in the cell cluster region of interest (ROI) and subtracted background ROI. (B) Relative levels of ROS (indicated as % mean fluorescence) 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, $^{\wedge}p < 0.05$ compared to LPS alone.

viii. Effect of LPS on TPA-HL-60 MOR expression

In order to examine whether LPS induced ROS accumulation modulated TPA-HL-60 MOR expression, 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 (Figure 11). 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 (Figure 11).

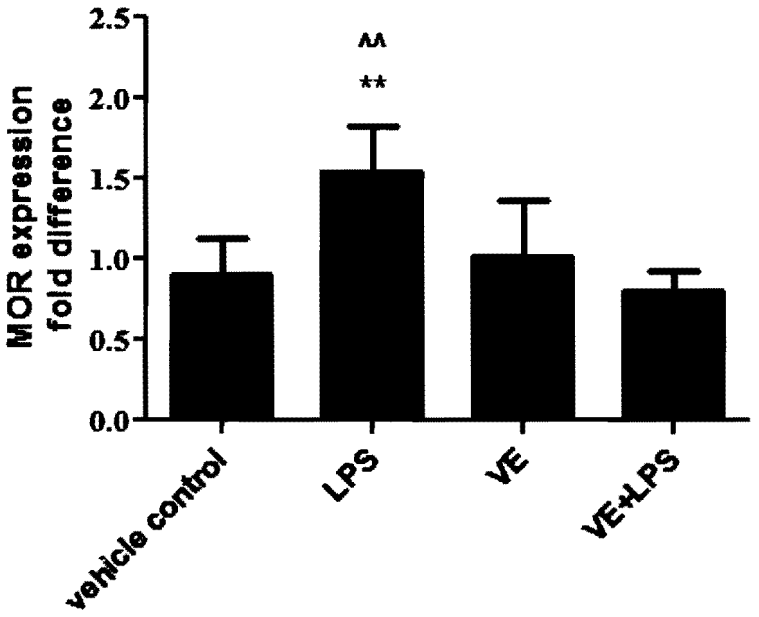

Figure 11. Effect of LPS on TPA-HL-60 MOR expression. TPA-HL-60 cells were treated with 0.9% saline (vehicle control), 0.500 mg/mL LPS alone (LPS), 100 μM vitamin E alone (VE), or 100 μM+ 0.500 mg/mL LPS (VE+LPS) for 24 h. Relative changes in MOR expression (fold difference) were determined using the $2^{-\Delta\Delta Ct}$ equation with GAPDH serving as the internal standard. Values are the means ± 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, ^^ $p < 0.01$ LPS compared to VE+LPS.

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

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 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. To note, the CM from LPS-treated TPA-HL-60 cells could contain ROS, produced by the TPA-HL-60, as well as, pro-inflammatory cytokines. 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 (Figure 12), 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 (Figure 12).

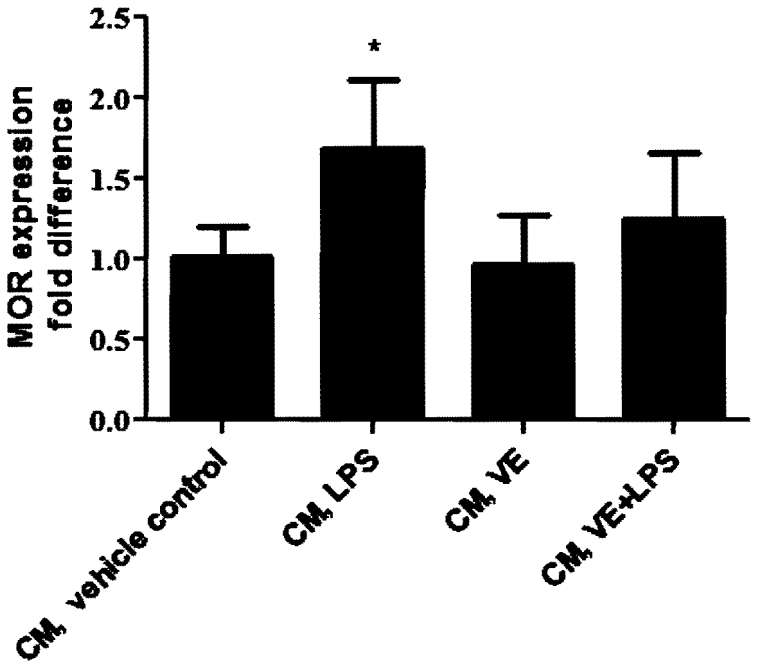

Figure 12. Effect of TPA-HL-60 conditioned medium (CM) on SH-SY5Y MOR expression. Conditioned medium (CM) from TPA-HL-60 cells treated with 0.9% saline + 0.1% ethanol (CM, vehicle control), 0.500 mg/mL LPS alone (CM, LPS), 100 μM vitamin E alone (CM, VE), or 100 μM+0.500 mg/mL LPS (CM, VE+LPS) was overlaid onto SH-SY5Y cells for 24 h. Relative changes in MOR expression (fold difference) were determined using the $2^{-\Delta\Delta Ct}$ equation, with GAPDH serving as the internal standard. Values are the means ± 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.

The direct effect of LPS on SH-SY5Y was examined to confirm if in fact that the CM from LPS treated TPA-HL-60 cells was responsible for the increase in SH-SY5Y MOR expression. The CM from the TPA-HL-60 cells treated with LPS or vehicle control and medium spiked with 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 60% when compared to the vehicle control. The medium spiked with LPS (M, LPS) had no significant effect on MOR expression compared to vehicle control (Figure 13). These results suggest that TPA-HL-60 cells, a macrophage like cell, treated with LPS, an endotoxin, is in fact effecting the expression of MOR in SH-SY5Y, a neuronal cell model, independent of LPS itself acting directly on SH-SY5Y.

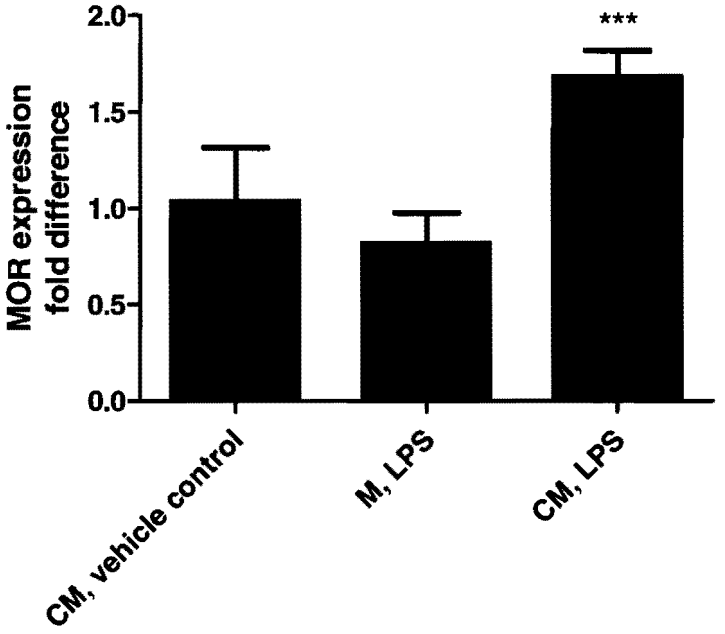

Figure 13. Effect of LPS on SH-SY5Y MOR expression. Conditioned medium (CM) from TPA-HL-60 cells treated with 0.9% saline + 0.1% ethanol (CM, vehicle control), 0.500 mg/mL LPS alone (CM, LPS), or medium spiked with 0.500 mg/mL LPS (M, LPS) was overlaid onto SH-SY5Y cells for 24 h. The M, LPS treatment was kept at same conditions as the CM, vehicle control and CM, LPS treatments to account for any possible degradation of LPS during the incubation period. Relative changes in MOR expression (fold difference) were determined using the $2^{-\Delta\Delta Ct}$ equation, with GAPDH serving as the internal standard. Values are the means ± SD of six replicates per treatment from three separate experiments. Significant differences between treatment groups were determined using a Tukey's post hoc test. ***$p < 0.001$ compared to CM, vehicle control.

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

We next used a centrifugal filtration 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, centrifugal filtration serves as an effective method for collection of CM void of cytokine mediators (Curfs et al., 1997). The use of YM-3 kDa centrifugal device in the separation of molecules with based on molecular weight, according to the recommendations established by the manufacture (Miilipore, Billerica, MA), recovers 90 to 100% of molecules with molecular weights greater than 5 kDa in the retenate. 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 (Figure 14). These results suggest that molecules less than 3 kDa, e.g. ROS, where not causing an increase in SH-SY5Y MOR expression, but suggesting the molecules responsible, e.g. cytokine, were greater than 3 kDa.

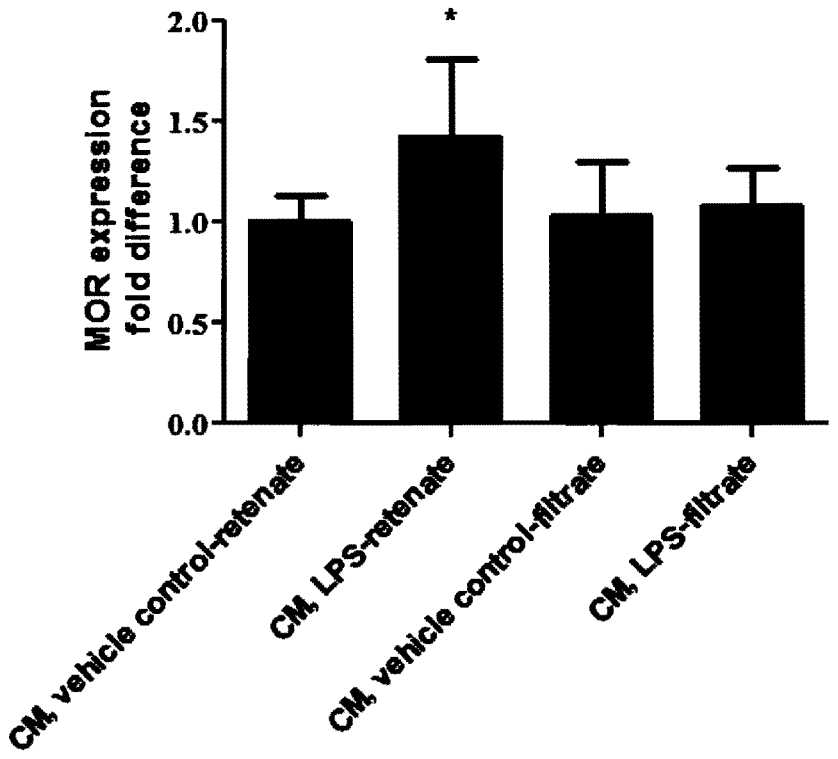

Figure 14. Effect of LPS retentate and filtrate CM on SH-SY5Y MOR expression. Conditioned medium (CM) from TPA-HL-60 cells treated with 0.9% saline + 0.1% ethanol (CM, vehicle control) or 0.500 mg/mL LPS alone (CM, LPS) was separated into retentate and filtrate fractions by centrifugal force ultrafiltration and overlaid onto SH-SY5Y cells. Relative changes in MOR expression (fold difference) were determined using the $2^{-\Delta\Delta Ct}$ equation, with GAPDH serving as the internal standard. Values are the means ± 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 two-way ANOVA followed by Bonferroni's Multiple Comparison Test. $^{*}p < 0.05$ CM, LPS-retenate compared to CM, vehicle control- retenate.

xi. Effects of LPS on TPA-HL-60 cytokine secretion.

Levels of cytokines present in the CM from the TPA-HL-60 cells were 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-1β, IL-2, IL-6, IL-10, IL-12p70, IFN-γ, TNF-α, and GM-CSF secretion, but decreased IL-8 secretion (Table I). Pre-treatment of TPA-HL-60 cells with VE for 3 h significantly attenuated the secretion of both TNF-α and GM-CSF by approximately 30% (Figures 15A, B). A reason for the CM, VE+LPS treatment group TNF-α, and GM-CSF levels not at levels to the controls would suggest other factors aside from ROS modulate their secretion. Although a role in the modulation of MOR expression by IL-8 and IL-12p70 remains unclear, in this experiment CM from the VE alone treatment significantly increased IL-8 and IL-12p70 levels when compared to the vehicle control (Table III).

Table I. Changes inTPA-HL-60 cytokine levels in response to LPS treatment.

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

Table I Legend: 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 μM vitamin E (CM, VE), or 100 μM+ 0.500 mg/mL LPS (CM, VE+LPS) for 24 h was assayed for IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12p70, INFγ, TNF-α, and GM-CSF levels. The concentrations of cytokines measured are expressed in units of pg/mL. Values are the means ± SD of five replicates per treatment from 4 independent experiments. Significant differences between treatment groups were determined using Tukey's post hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to CM, vehicle control, ns, not significant when comparing CM, VE to CM vehicle control.

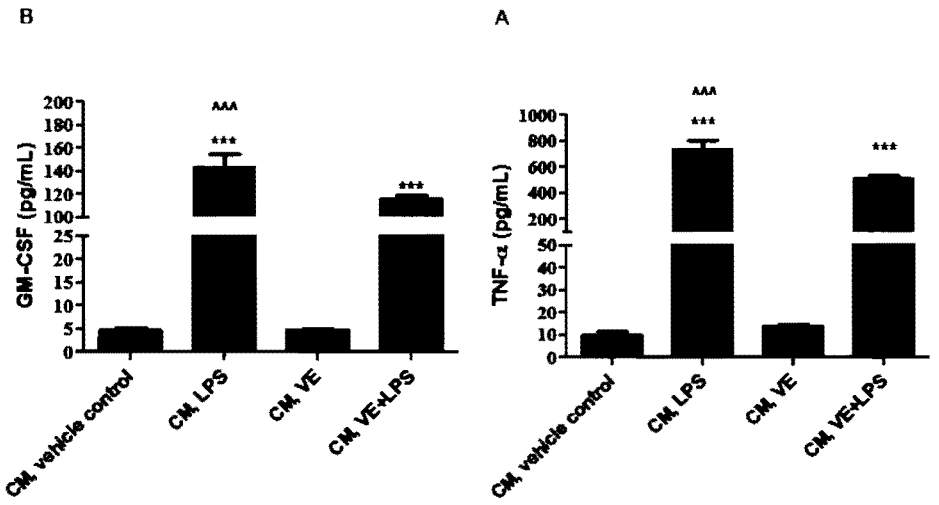

Figure 15. Effects of LPS and VE pre-treatment on TPA-HL-60 cytokine secretion. (A-B) Conditioned medium (CM) from TPA-HL-60 cells treated with 0.9% saline + 0.1% ethanol (CM, vehicle control), 0.500 mg/mL LPS alone (CM, LPS), 100 μM vitamin E alone (CM, VE), or 100 μM+ 0.500 mg/mL LPS (CM, VE+LPS) for 24 h was assayed for TNF-α and GM-CSF levels using a multiplex, electrochemiluminescent sandwich immunoassay. Values are the means ± 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, ^^^ $p <$ 0.001 CM, LPS compared to CM, VE+LPS.

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

TNF-α and GM-CSF are cytokines known to effect MOR expression and therefore neutralization of the cytokines present in TPA-HL-60 CM would presummably have an effect on SH-SY5Y MOR expression. The sandwich immunoassays indicated that both TNF-α and GM-CSF levels were modulated by the antioxidant, VE. This result was expected since LPS is known to modulate cytokine production and at the concentrations of LPS here, VE was effective in blocking ROS accumulation in TPA-HL-60 cells. 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.

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 the vehicle control alone CM (Figure 16A). GM-CSF down-regulates MOR expression through actions mediated on IL-4 (Kraus

et al. 2003) and although at relatively low concentrations in the vehicle control CM, was effective in leading to the observed increase in MOR expression. However, neutralization of TNF-α had no effect on SH-SY5Y MOR expression in the vehicle control+anti-TNF-α group, and partially attenuated SH-SY5Y MOR expression in the LPS+anti-TNF-α group (Figure 16B). Simultaneous neutralization of the GM-CSF and TNF-α present in the TPA-HL-60 CM significantly increased SH-SY5Y MOR expression by 60% and 50% in the vehicle control-anti-GM-CSF+anti-TNF-α and LPS-anti-GM-CSF+anti-TNF-α groups, respectively, when compared to the vehicle control (Figure 16C).

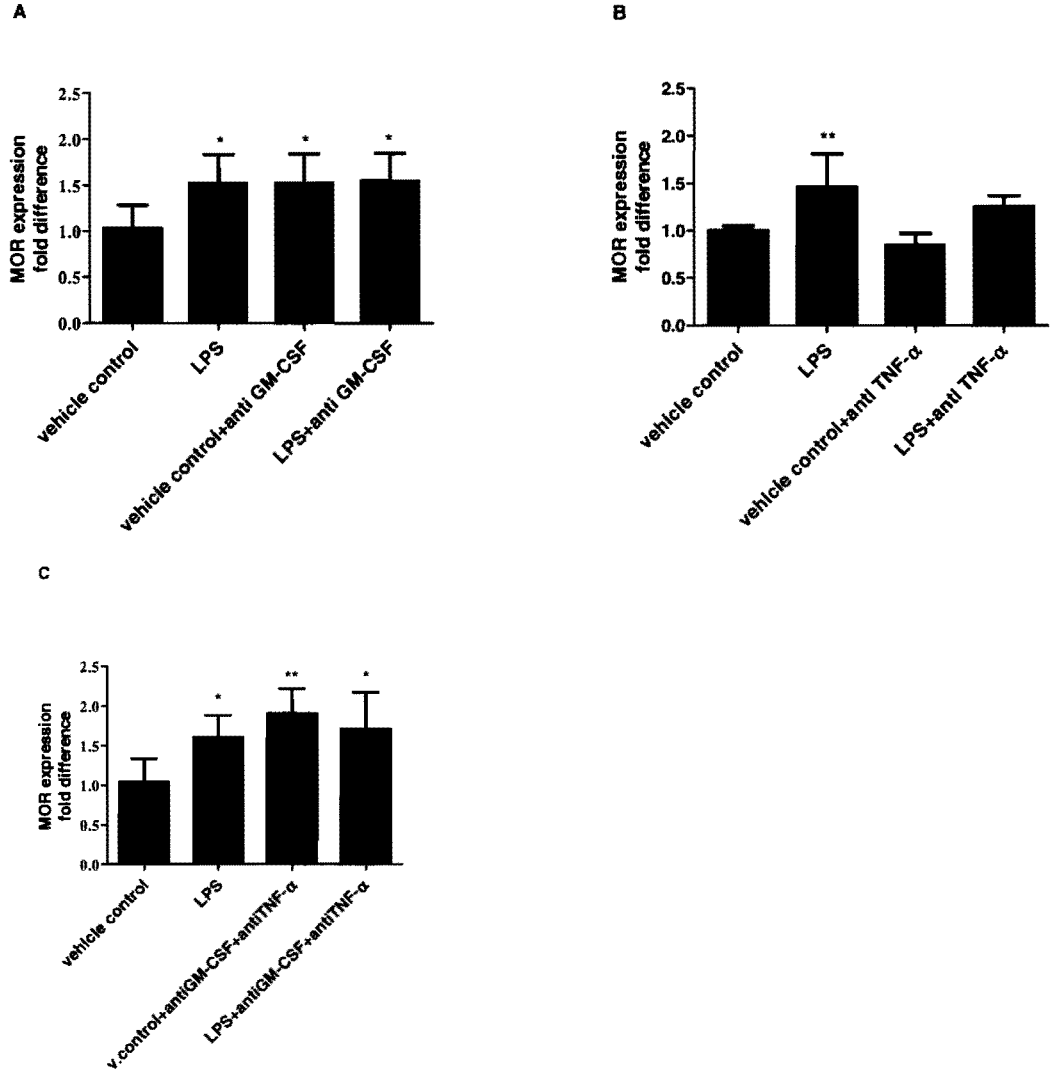

Figure 16. Neutralization of TNF-α and GM-CSF present in TPA-HL-60 CM. TPA-HL-60 cells were treated with 0.9% saline (vehicle control) or 0.500 mg/mL LPS (LPS) for 24 h. Neutralizing antibodies to (A, C) GM-CSF (2 μg/mL), and/or (B, C) TNF-α (10 μg/mL) were added first to TPA-HL-60 CM from vehicle control 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 $2^{-\Delta\Delta Ct}$ equation, with GAPDH serving as the internal standard. Values are the means ± SD of six replicates per treatment from two independent experiments. Significant differences between treatment groups were determined using a Tukey's post hoc test. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to vehicle control. All neutralization experiments were performed 2 times.

IV.

Discussion

In the first part of this project, treatment of SH-SY5Y cells, a dopaminergic neuronal cell model, with METH, a psychostimulant, increased the intracellular accumulation of ROS. The concentrations of METH and H_2O_2 used here are similar to those reported in other *in vitro* studies where the investigators examined a correlation between cell viability and changes in dopmaine receptor and heme oxygenase-1 expression (Larouche et al., 2008; Huang et al., 2009). METH treatment also produced a significant increase in the expression of the MOR that coincided with the increased level of intracellular ROS. In addition, vitamin E, an antioxidant, reduced the accumulation of ROS and attenuated METH-induced MOR expression. These findings indicate a role for ROS signaling in METH regulation of MOR expression in SH-SY5Y cells.

METH time-course experiments demonstrated that the increase in ROS preceded the increase in MOR expression, suggesting a potential role for ROS in modulating MOR expression. Additional evidence that ROS can affect MOR expression was observed when SH-SY5Y cells were treated with H_2O_2 (Figure 6A). However, the data indicated that the effect of ROS on MOR expression was not concentration-dependent (Figure 6B). However, while METH-induced

ROS accumulation was dose-dependent (Figure 3D), METH-increased MOR expression was found not to be dose-dependent (Figure 5A). Also, 0.85 mM METH induced a significant increase in ROS at 6 h post treatment, but increased MOR expression was not observed until 48 h. This suggested that a critical level of ROS is needed to influence the expression of the MOR. This was clearly apparent since shifting the redox status of the cytoplasm to a more oxidized state, by way of increased ROS accumulation, failed to increase MOR expression. Furthermore, the lack of a dose-dependent effect on H_2O_2-induced MOR expression supports our hypothesis that a critical intracellular concentration of ROS is necessary to produce a change in MOR expression. Taken together, these data indicate that the modulation of MOR expression by METH involves some degree of ROS signaling and may involve other factors known to modulate MOR expression e.g. cytokines (Kraus, 2009).

More importantly, these results highlight the existence of the possible coupling of the dopaminergic and opioidergic pathways in neuronal cells by ROS potentially signaling via transcription factors known to regulate MOR expression, e.g. NFκB. Preliminary evidence from this study indicated the up-regulation of SH-SY5Y MOR expression subsequent to METH treatment at concentrations that increased intracellular ROS accumulation in SH-SY5Y lead to an increase in the p50/p65 mRNA levels (data not shown). Convergence of the two pathways has been documented with several addictive substances

including nicotine, alcohol, and cocaine (Zhu et al., 2006). The dopaminergic pathway encompasses the motivational reward circuitry in the brain and utilizes dopamine as a key signaling molecule in the pathway's activation; whereas, the activation of the opioidergic pathway is often associated with euphoria and pleasure. These two pathways have previously been linked, and studies have shown that MOR antagonists limit dopamine's activation of the dopaminergic pathway (Wu et al., 2007b).

Studies have shown that cross-sensitization of METH to morphine in rats correlates with alterations in MOR binding (Lett, 1989; Chiu et al., 2005; Chiu et al., 2006). In this study, the effects of METH on MOR expression (a key component of the opioidergic pathway) in a dopaminergic neuronal cell line suggests that the drug seeking motivational behaviors of METH users could potentially be enhanced by exposure to opioid drugs and that alteration of intracellular ROS levels may be one of the molecular mechanisms underlying the coupling between the dopaminergic and opioidergic pathways. This seems reasonable to suggest since SH-SY5Y cells treated with METH exhibited increased levels of MOR expression correlating to increased ROS accumulation. This METH increased MOR expression could by attenuated with the pre-treatment of vitamin E and concomitant blocking of ROS accumulation.

Vitamin E is an antioxidant capable of chelating free radicals. To further examine if METH-induced increase in MOR expression is related exclusively to

ROS signaling, I pre-treated SH-SY5Y cells with vitamin E and found that vitamin E was able to reduce ROS levels and attenuate MOR expression to control levels. The findings that vitamin E and ROS accumulation are directly related are supported by the studies of Wu et.al. (2007), which demonstrated that vitamin E is capable of attenuating METH-induced neurotoxicity and increased ROS levels. Taken together, the evidence clearly points to a correlation between METH-induced ROS accumulation and MOR expression in neuronal dopaminergic cells.

In these experiments, it was observed that an increase in accumulation of ROS coincides with increased MOR expression and decreased cell viability in SH-SY5Y cells. However, it is not clear as to the extent that ROS contributes to neurotoxicty or whether changes occurring within the cells during the onset of toxicity contribute to the observed increase in MOR expression. It is known that METH-induced neurotoxicity is associated with other variables independent of ROS (Yamamoto et al., 2010). However, these data show that concentrations of H_2O_2 that did not have an effect on cell viability, e.g., 10 and 20 μM, significantly increased MOR expression. The H_2O_2 experiments shown here further underscore a role for ROS signaling in modulation of SH-SY5Y MOR expression.

Additional studies will need to be performed to assess the effects of ROS accumulation on the functionality of the METH-induced MOR in SH-SY5Y cells.

Raut et al. (2006) have shown that oxidative stress induced by 3-nitroproprionic acid (3-NPA) decreases MOR function in SK-N-SH cells, the parent population of the SH-SY5Y cell line. Impairment of MOR function seems to be a consequence of a reduction in membrane MOR protein levels following 3-NPA-induced oxidative stress (Raut et al., 2007). However, as the authors of those reports point out, decreased MOR function could well be a consequence of inhibited mitochondrial succinate dehydrogenase activity (Raut et al., 2007). Whereas highly oxidative cellular environments impact MOR function, increased MOR expression has been shown to coincide with increased binding of MOR agonist and function in SH-SY5Y cells (Borner et al., 2004; Borner et al., 2007). Therefore, it seems reasonable to speculate that the increased expression of MOR seen with sub-lethal levels of METH and H_2O_2 in this study would be indicative of increased MOR function.

The data presented here show that METH treatment of a dopaminergic cell model can affect components of the opioidergic pathway. Delineating how METH modulates MOR expression and function could also provide new clues as to how METH abuse impacts both innate and adaptive immune system function (Yu et al., 2002; In et al., 2005). Research has shown MOR activation suppresses immune function through down regulation of macrophage pro-inflammatory cytokine secretion (Bidlack, 2000). It seems reasonable to speculate that, since METH increases constitutive expression of the MOR, the

detrimental consequences of METH abuse might involve opioidergic signaling if the increase in MOR expression results in a greater number of MOR receptors being activated by either endogenous or exogenous opiates (Roy et al., 1998). In addition, oxidative stress-related events are often associated with pro-inflammatory cytokine activity. Tumor necrosis factor-alpha (TNF-α) has been shown to increase MOR expression (Kraus et al., 2003) and could be a signaling pathway involved in METH-induced ROS accumulation and MOR expression. The transcription factor, NFκB, has been shown to be activated by TNF-α and ROS, and could potentially play a role in regulating METH-induced MOR expression (Li et al., 2001). Supporting this idea is the fact that NFκB binding sites have been located in the MOR promoter region (Min et al., 1994; Kraus, 2009).

In the second part of this project, LPS stimulated the intracellular accumulation of ROS and the expression of the MOR in TPA-HL-60 cells (Figures 10 and 11), a macrophage cell model, and CM from those cultures significantly increased MOR expression in SH-SY5Y cells, a neuronal cell model (Figure 12). However, CM TPA-HL-60 cells pre-treated vitamin E prior to LPS treatment did not increase MOR expression in SH-SY5Y (Figure 12). These findings suggest that an indirect ROS signaling mechanism could be responsible, at least in part, for the modulation of MOR expression in SH-SY5Y cells overlaid with CM from LPS-stimulated TPA-HL-60 cells.

Cytokines have been shown to regulate MOR expression (Chang et al., 1998; Wei and Loh, 2002; Kraus et al., 2003; Borner et al., 2004; Kraus, 2009). Our lab previously demonstrated that co-treatment of microvascular endothelial cells with IL-1α and IL-1β 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, the data indicated that cytokines present in the CM from LPS-treated TPA-HL-60 cells are involved in the control of MOR expression in the SH-SY5Y cells. This suggests that ROS, produced by LPS-stimulated TPA-HL-60 cells, indirectly influences the up-regulation of SH-SY5Y MOR expression. But more importantly points to a molecule, ROS, that can couple components of the neuro (SH-SY5Y)- immune (TPA-HL-60) axis.

To test the hypothesis that ROS plays a role in the LPS-stimulated cytokine secretion in TPA-HL-60 cells, I 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 (Figure 16B). TNF-α stimulates transcription of the MOR gene through activation of NFκB (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). These data and data presented in this project, suggest that ROS can increase SH-SY5Y MOR expression by inducing the secretion of TNF-α by TPA-HL-60 in response to LPS treatment, again highlighting the coupling of the neuroimmune axis by signaling molecules such as ROS.

GM-CSF down-regulates MOR expression in dendritic cells, which may involve inhibitory actions by IL-4 (Kraus et al., 2003). There was an observed 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 III).

Results from these studies indicated that neutralization of TNF-α in the LPS treatment group 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 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.

Our lab has previously 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 endotoxic 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 et al., 1998; Wang et al., 2002).

These findings suggest that the LPS-induced ROS signaling that occurs in immune cells may indirectly regulate the opioidergic pathway by modulating MOR expression in neurons. Data presented here 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 bacterial infection promotes molecular communication within the neuroimmune axis.

In summary, the data presented in this project show that METH increases the expression of the MOR in SH-SY5Y cells through a ROS signaling mechanism. There was a temporal sequence of events that demonstrated that a significant accumulation of ROS precedes increased MOR expression. Taken together, these findings suggest that the dopaminergic and opioidergic signaling pathways can converge at the level of ROS signaling in neuronal cells. Additional findings suggest that the LPS-induced ROS signaling that occurs in immune cells may indirectly regulate the opioidergic pathway by modulating MOR expression in neurons.

Future experiments should examine if METH-induced ROS in an animal model increases the response to morphine. If an increased response to morphine is observed it would be interesting to see if removal of ROS could reduce the response. If so, a result of this kind would seem to suggest that cross-sensitization of METH to morphine could be minimized by managing redox homeostasis; therefore, highlighting the possibility that antioxidant treatment or targeting sources of ROS may have a therapeutic potential is reducing cross-sensitization of these two drugs of abuse. However, in order to determine the importance of METH-induced ROS on an increased response to

morphine, experiments should include treatment with ROS alone, absent of METH. In addition, addressing METH-induced ROS levels may provide relief for METH addicted individuals by modulating the motivational behaviors linked to activation opioidergic pathway through MOR.

Also, it would be interesting to see if an LPS treated animal's accelerated progression from sepsis to septic shock, resulting from morphine exposure, could be attenuated with ROS clearance. If possible, this would seem to suggest that attenuating ROS production in immune cells or clearance of ROS could prevent the immunosuppressive effects associated with MOR activation in the CNS.

Finally, additional experiments should examine the specific ROS involved in the modulation of MOR. Moreover, the data from these studies indicate ROS, a molecule that participates in a multitude of signaling pathways, leads to an increase in MOR expression, a receptor that has an important function in pain and immune responses, as well as addiction. And until now, a role for ROS in the regulation of the opioidergic pathway via MOR has not been identified. Insight into the precise ROS could point to a specific biosynthetic route, responsible for a particular ROS production, which could be exploited for therapeutic gain for the diseases noted above.

V.

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