

ABSTRACT

CECIL, CHAD ERIC. The Effects of Berberine on Influenza A virus, Influenza A virus-induced Inflammation and the Lipopolysaccharide-induced Synthesis of Prostaglandin E₂. (Under the direction of Dr. Scott Laster).

The innate immune response is essential for the initial recognition and control of invading pathogens. Through the production of cytokines, chemokines and lipid mediators of inflammation, the spread of an infection can be controlled by recruitment and activation of host immune cells. In some cases, the response to certain microorganisms can become too strong and can cause damage to normal healthy tissues. For example, infection with the influenza A virus, or certain bacterial species that cause sepsis, can stimulate a destructive inflammatory response. Current drug therapy is aimed at both controlling the infection and limiting the immunopathology. Our research was focused on goldenseal (*Hydrastis canadensis*), a perennial herb that has a long history of use in traditional and Native American medicine. This herb is commonly used to treat a variety of inflammatory conditions and infections in humans. Many of the effects of goldenseal are attributed to berberine; one of the primary alkaloids present in this plant. The mechanisms of action of goldenseal extracts and berberine have not been well characterized. In these experiments we evaluate the use of goldenseal and berberine to attenuate the growth of the influenza A virus and limit the inflammatory response to the virus. We also characterize the inhibition of lipid mediated responses to lipopolysaccharide (LPS), an important model for bacterial sepsis.

Our first study examined the effects of goldenseal and berberine on the growth of the influenza A virus and the production of inflammatory mediators in

response to viral infection. Results presented in chapter 2 reveal that goldenseal and berberine inhibit the growth of two H1N1 strains of the influenza A virus. Our studies demonstrate that berberine does not prevent the expression of key viral proteins, but may limit the growth of the virus by inducing the formation of viral protein aggregates within the host cell cytoplasm. Additional studies on the anti-inflammatory properties of goldenseal and berberine demonstrate attenuation of the production of proinflammatory cytokines exemplified by tissue necrosis factor α (TNF α) and lipid mediators like prostaglandin E₂ (PGE₂) in response to viral infection.

In chapter 3 we sought to evaluate the effects of berberine on various components of the pathway by which PGE₂ is produced. Berberine has been shown previously to inhibit the production of PGE₂ in response to LPS-induced inflammation. However, a clear mechanism has not been defined. Our experiments sought to systematically evaluate the effects of berberine on the key enzymes that direct the production of PGE₂. Our studies show that berberine does not affect the expression or activity of these enzymes. Our research indicates that berberine is mediating its effect on PGE₂ through an as yet unidentified mechanism.

Taken together, our research indicates that goldenseal, or berberine, may be important inhibitors of the influenza A virus and strong inhibitors of inflammation in response to both viral and bacterial infections. Further studies are ongoing to determine if there is a common mechanism by which berberine mediates these two effects.

The Effects of Berberine on Influenza A virus, Influenza A virus-induced Inflammation
and the Lipopolysaccharide-induced Synthesis of Prostaglandin E₂

by
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DEDICATION

I would like to dedicate this work to my supportive and loving wife Danielle. Thank you for always believing in me. Thank you for always encouraging me to keep working towards my dreams. I could not have done this without you.

I would also like to dedicate this to my beautiful new daughter Ava. I hope I can use some small part of my education to instill in you a sense of wonder and curiosity.

BIOGRAPHY

Chad Cecil was born in High Point, North Carolina to Ronald Jeffrey and Phyllis Darr Cecil. He attended the University of North Carolina at Chapel Hill from 1994-1998 and obtained a Bachelor of Science in Biology and Bachelor of Arts in Anthropology. Following graduation, he worked as a research technician at the Lineberger Comprehensive Cancer Center from 1999-2004. He received his Master of Science in Microbiology and Immunology from the University of North Carolina at Chapel Hill in 2004. He worked in the Research Triangle Park for Bayer and AlphaVax Human Vaccines as a Senior Research Associate Scientist prior to returning to North Carolina State University in 2006 to obtain his Doctor of Philosophy in Microbiology.

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CHAPTER 1
LITERATURE REVIEW

CHAPTER 1

1 Introduction

The human immune system is composed of both innate and adaptive components which are responsible, respectively, for protecting us from both immediate and long term threats to human health. On occasion, these complex systems over-react or react inappropriately resulting in inflammatory and autoimmune disorders. The focus of this dissertation is on the acute inflammatory response that can occur following infection with a number of infectious agents. Viruses such as influenza A virus, Hepatitis C virus, and Dengue virus can trigger this response [1, 2] as can bacteria such as *Neisseria meningitidis*, *Escherichia coli*, and *Staphylococcus aureus* [3-5]. The acute inflammatory response is initiated by the binding of pathogen associated molecular patterns (PAMPS) to host pattern recognition receptors (PRRs) expressed on tissue macrophages, epithelial cells, and other barrier cell types. Signaling, transcription and changes in cell physiology ensue resulting in the secretion of a number of different cytokines, chemokines and inflammatory lipids. These molecules bind receptors throughout the body causing changes in virtually every organ system, and resulting in the painful symptoms that accompany the acute inflammatory response. These pro-inflammatory soluble mediators mediate both localized symptoms such as redness, swelling, and pain; as well as systemic symptoms such as fever, anorexia and low blood pressure.

For the most part, research on these diseases has focused on the development of various classes of drugs that are aimed at controlling the growth of the invading microorganism [6]. However, there is also an emerging effort to limit the acute inflammatory response of the host in order to limit the symptoms and tissue damage that result from the host response to these infections [1]. The focus of my research is the evaluation of a natural product derived small molecule inhibitor of this acute inflammatory response to various microorganisms.

This review describes the cells and molecules responsible for mediating the acute inflammatory response. I also describe the synthetic and natural pharmaceuticals that have been developed in an attempt to combat this response. In particular, we will focus on the isoquinoline alkaloid berberine. This compound, which is found in several plants, including goldenseal (*Hydrastis canadensis*), displays potential for use to combat acute inflammation and is the subject of the experiments presented herein. Emphasis is placed on both the importance of natural products in the drug discovery process and inflammation in response to viral or bacterial stimuli.

2 Acute inflammation

2.1 Cells that participate in acute inflammatory responses

A large number different cell types can participate in the acute inflammatory response. Several immune system cell types such as macrophages, dendritic cells

(DCs), mast cells, neutrophils, eosinophils and natural killer (NK) cells are able to rapidly identify pathogens and initiate the acute inflammatory response. Macrophages, for example, express a variety of receptors for the identification of microbial products; including Toll-like receptors, mannose receptors and scavenger receptors. At the site of infection, various cell types that are not part of the immune system can also participate in initiation of this response. Epithelial cells, keratinocytes, vascular endothelial cells, and fibroblasts can produce many of the same mediators that are produced by the professional immune system cells. A large number of different cell types also participate in the effector portion of the acute inflammatory response. Changes in neuron activity, for example, result in the fever and anorexia [7, 8]. Also, changes in endothelial cell activity cause the precipitous drop in blood pressure that can accompany acute inflammation [9, 10].

2.2 Receptors responsible for initiating the acute inflammatory response

Initial recognition of invading microorganisms occurs through the germline-encoded pathogen recognition receptors (PRRs). These receptors can recognize microbial nucleic acids and conserved structures found on microbial surfaces known as pathogen-associated molecular patterns (PAMPs). These microbial components are often necessary for the survival of the microbe and are therefore less likely to be altered by the microorganism as a means to evade the host immune response. Among this family of receptors, there are several classes of receptors. These include the membrane-bound Toll-like receptors (TLRs) and C-type lectin receptors

(CLRs) as well as the cytoplasmic retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs). Each of these receptors has the unique ability to recognize specific molecular patterns found only in foreign microorganisms or damaged tissues (DAMPs). The PRRs mediate several functions including opsonization, activation of complement and coagulation cascades, phagocytosis, activation of proinflammatory signaling pathways, and induction of apoptosis [11]. Upon receptor binding and activation, several genes mediating these functions are transcriptionally upregulated. These include genes encoding proinflammatory cytokines, type I interferons, chemokines, antimicrobial proteins, enzymes for the production of inflammatory lipids and proteins needed for signaling events that regulate these pathways [12].

The signaling pathways that emanate from the TLRs have been the best characterized to date. Structurally, TLRs consist of a hydrophobic N-terminal leucine-rich repeat region, a transmembrane region and a cytoplasmic Toll/IL-1R (TIR) domain. TLRs are found either on the cell surface or on membranes of cytoplasmic vesicles. Each TLR is capable of sensing specific components of the microorganism (Table 1). TLR signaling begins with the dimerization of the TLR and subsequent recruitment of adaptor proteins containing a TIR (Toll/IL-1 receptor/resistance) domain to the cytoplasmic TIR portion of the receptor. There are four cytoplasmic adaptor proteins; MyD88, TIR-associated protein (TIRAP), TIR-domain-containing adaptor protein-inducing IFN- β (TRIF) and TRIF-related adaptor molecule (TRAM). MyD88 and TRIF are primarily responsible for the propagation of

signals through a downstream signaling cascade from the TLRs through the cytoplasm, and ultimately leading to the activation of NF- κ B which drives the gene expression of the proinflammatory cytokines. The central signaling pathway leading from MyD88 begins with the recruitment of IL-1R-associated kinase 4 (IRAK-4) to MyD88 and the phosphorylation of IRAK-1 by IRAK-4. TRAF-6 is then recruited to the complex and acts as an ubiquitin protein ligase (E3) with IKK- γ /NF- κ B essential modulator (NEMO). TGF- β -activated kinase (TAK1) and the TAK1 binding proteins TAB1, 2 and 3 are also recruited to TRAF6. TAK1 phosphorylates IKK- γ and MAP kinase kinase 6 (MKK6), which leads to the phosphorylation and degradation of IKK- β , and culminates in the nuclear translocation of NF- κ B and subsequent transcriptional activation of various proinflammatory cytokine genes. This cascade is the central pathway for most, but not all, of the human TLRs. For example, TLR3 and TLR4 are to some extent MyD88-independent. For TLR4 signaling, TIRAP is also required to recruit MyD88 [13].

TLR	Associated Proteins	Ligands	Adaptors
TLR1/2	CD14, CD36, Dectin1	Lipoproteins, Glycolipids	MyD88, Tirap
TLR2/6	CD14, Dectin1	Lipoproteins, LTA, Zymosan	MyD88, Tirap
TLR3		polyI:C, dsRNA	TRIF
TLR4	CD14, MD-2	LPS	MyD88, Tirap TRIF, TRAM
TLR5		Flagellin	MyD88
TLR7		ssRNA, imiquimod, loxoribine, other	MyD88
TLR9		CpG, DNA	MyD88
TLR11		Profilin	MyD88
TLR12		?	?
TLR13		?	?

Adapted from Beutler et al., Blood 2009 113: 1399-1407

The signaling pathways emanating from RNA helicases retinoic acid inducible gene-I (RIG-I) protein and the melanoma differentiation associated gene 5 (MDA5) protein have also been partially characterized. These cytoplasmic DEx(D/H) box helicases signal through caspase activation and recruitment domain (CARD) with the mitochondrial IFN β promoter stimulator 1 (IPS-1) protein [14-17]. RIG-I is important for the recognition of negative-stranded RNA viruses, while MDA5 will sense positive-stranded RNA viruses [18]. Following activation of IPS-1, the transcription factors IRF-3 and IRF-7 are activated through TBK-1, IKK ϵ , and NF- κ B

[14, 19]. This in turn leads to the production of IFN α/β production which will activate other interferon-induced genes and induces apoptosis through secondary feedback pathways [14, 20].

2.3 Cytokines and chemokines and their role in acute inflammation

Many of the receptor-mediated pathways involved in the acute inflammatory response lead to the production of cytokines and chemokines. Cytokines are an important class of protein signaling molecules that recruit additional leukocytes to contain the invading microbe and regulate the inflammatory process [21, 22]. Cytokines mediate their effects through interaction with cell surface receptors that signal down intracellular cascades and ultimately activate various transcription factors and genes. This activation can lead to the production of additional cytokines or chemokines, and increase the expression of cell surface receptors. Chemokines such as IL-8, MIP-1 α , MCP-1 and RANTES are chemotactic cytokines that play an important role in the recruitment and activation of immune system cells (neutrophils, basophils, macrophages and T cells) to the site of infection. Chemokines mediate this recruitment through interactions with G-protein-coupled chemokine receptors found on leukocytes. In many cases, the overproduction of these mediators drives much of the pathology associated with acute inflammation. This is especially true for the proinflammatory “cytokine storm” associated with sepsis [23] and infections with the influenza A virus [24]. These infections lead to the production of large amounts

of interleukins (IL-1, IL-6), and tumor necrosis factor alpha (TNF- α). The production of interleukin 1 (IL-1) and TNF- α have been identified as the primary mediator of fever, inflammation and tissue destruction [24-26]. TNF- α functions to increase vascular permeability, and along with IL-1, affects the vascular endothelium to enhance access of effector cells to sites of infection. In addition, IL-1 α works synergistically with TNF- α throughout the body to induce the secretion of other cytokines (IL-6) and acute-phase proteins, activate COX-2, which leads to the production of PGE₂ and induces fever, increase neutrophil recruitment and induce tissue destruction through apoptosis.

2.4 Lipid mediators and their role in inflammation

Eicosanoids are lipid metabolites derived from the polyunsaturated fatty acid arachidonic acid (AA). As with the cytokines and chemokines, eicosanoids are produced by many different cells types, bind to widely-expressed cognate receptors, and produce many changes in cell physiology and gene expression [27]. Among the eicosanoids are a group of lipid mediators of inflammation that are referred to as prostanoids. Included in this group are the prostaglandins PGE₂, PGF_{2 α} , PGD₂, PGI₂ and thromboxane (Tx)A₂. In addition, arachidonic acid can also be converted to other groups of mediators known as leukotrienes (LT), lipoxins (LX) and hydroxyeicosatetraenoic acids (HETEs). Collectively, the products of the eicosanoid biosynthetic pathway play an essential role in mediating many of the hallmark signs

of inflammation, as well as playing an important role in regulating these inflammatory responses.

Among the lipid mediators of inflammation, PGE₂ is the most ubiquitous and is produced under both physiological and pathophysiological conditions. PGE₂ has both pro- and anti-inflammatory properties [28]. Through interaction with specific G-protein-coupled receptors (EP 1-4), expressed on a variety of target cells and tissues, PGE₂ can exert a variety of systemic effects that include vasodilatation, fever generation, hyperalgesia, intestinal motility, uterine contractions, renal function and hormone secretion [27]. PGE₂ interactions with the EP4 receptor have also been shown to enhance inflammation through TH₁ cell differentiation and amplification of interleukin-23-mediated TH₁₇ cell expansion [29, 30]. The production of PGE₂ relies on step-wise modifications of AA by a series of modifying enzymes. Limiting the production of PGE₂ through direct targeting of the inducible COX-2 and mPGES-1 enzymes has shown therapeutic promise to limit lipid-mediated inflammation.

3 Prostaglandin activity and biosynthesis

A number of the experiments in this dissertation focus on disruption of the PGE₂ biosynthetic pathway by berberine and therefore additional information on this segment of the pathway is included below.

3.1 Phospholipase enzymes

The first step in the PGE₂ biosynthetic pathway is the liberation of the 20-carbon unsaturated fatty acid AA from membrane phospholipids [31]. There are several phospholipase A₂ (PLA₂) enzymes in mammalian cells that can liberate this fatty acid including cytosolic PLA₂ (cPLA₂), secretory PLA₂ (sPLA₂), and Ca²⁺-independent PLA₂ (iPLA₂) [32]. The group IV cytoplasmic phospholipase A₂ (cPLA_{2α}) enzyme is believed to be the primary enzyme for liberating the AA necessary for production of eicosanoids and induction of the inflammatory response [33-35]. This enzyme is activated in response to various stimuli, including cytokines, hormones, mitogens, antigens and other physical stress stimuli [31, 36, 37]. This enzyme must translocate from the cytoplasm to the membranes of the perinuclear region upon activation of the cell. This translocation occurs as a result of increased levels of intracellular calcium stimulated by receptor activation within the cell. Calcium is important for the translocation of the enzyme, but does not affect its activation state [38]. The translocated phospholipase is stabilized on the membrane phospholipids through phosphatidylcholine head groups, phosphatidylinositol, 4,5-bisphosphate (PIP₂) and vimentin [32]. MAPKs that are activated through receptor-mediated pathways phosphorylate and activate the cPLA₂ on Ser⁵⁰⁵ which will increase the catalytic activity of the enzyme [39, 40]. Macrophages activated through TLRs can amplify cPLA₂ activation through ERK1/2 phosphorylation cascades [41]. cPLA₂ will preferentially hydrolyze phospholipids in the membrane that contain AA in the *sn*-2 position using the nucleophilic active site at Ser²²⁸ to

cleave the ester bond [42-44]. The translocation and activation of cPLA₂ at the perinuclear membrane spatially localizes the release of AA to a site within the cell that initiates the cascade of eicosanoid biosynthesis. Other important enzymes needed for the production of the eicosanoid end-products are also localized within this area of the cell and are available to transform the free AA into the next intermediate in this cascade.

3.2 Cyclooxygenase enzymes

Following the liberation of AA by the phospholipases, AA is metabolized by the cyclooxygenase enzymes into an intermediate form necessary for the production of the prostaglandins. Cyclooxygenase (COX) enzymes provide the necessary substrate for the production of PGE₂, PGD₂, PGF_{2α}, PGI₂ and TXA₂. COX enzymes localize to the nuclear envelope, in close proximity to cPLA₂, and within the nearby endoplasmic reticulum in response to cell activation [45]. The COX enzymes have received attention as the pharmaceutical industry seeks to develop non-steroidal anti-inflammatory drugs (NSAIDs), which target the COX enzymes, as a means to control the primary symptoms of inflammation. Three isoforms of the COX enzyme have been identified. COX-1 is a constitutively expressed protein that is found in most tissues. It serves the role of maintaining homeostasis through the production of prostaglandins. COX-1 is located in the endoplasmic reticulum (ER) and on the perinuclear envelope. COX-2 is the inducible form of the enzyme that is activated in

response to cytokines, mitogens and endotoxins [45]. The COX-2 promoter contains binding sites for several transcription factors including NFκB. Enhancement of MAPK activity will activate transcription of the *cox-2* gene [46]. The enzyme is located near the perinuclear envelope and within the ER of the cell. COX-3 is a recently discovered protein that is transcribed from the COX-1 gene, but retains intron 1. While the function of the COX-3 enzyme has not been fully elucidated to date, it has some COX activities similar to that of the other isoforms of this enzyme [47]. The COX-2 enzyme is a homodimeric protein and has bifunctional enzyme activity that carries out both the cyclo-oxygenase and peroxidase activity concurrently. This two step process results in the oxidative cyclization of the five central carbons of AA [48, 49]. The first step of this process converts the free AA into the bicyclic peroxide intermediate prostaglandin G₂ (PGG₂) by introducing two molecules of oxygen. The second step reduces the PGG₂ into the unstable cyclic endoperoxide, prostaglandin H₂ (PGH₂) through the peroxidase activity of the second active site of the enzyme [48]. The resulting PGH₂ is the substrate for the downstream terminal prostaglandin and thromboxane synthases.

3.3 Prostaglandin synthase enzymes

Downstream of the COX enzyme, there are several prostaglandin synthases that catalyze the production of the various prostaglandins and thromboxane [50]. These terminal synthases rapidly convert the PGH₂ substrate produced by the COX

enzymes into their respective terminal prostanoids. All of these enzymes are located near the ER and perinuclear membranes so as to be in close proximity to COX and cPLA₂. Conversion of PGH₂ to each prostanoid occurs through specialized synthases. For example, TXA₂ is catalyzed by the thromboxane synthase and is rapidly hydrolyzed to TXB₂. Similarly, the formation of PGI₂ is catalyzed by the PGI₂ synthase. Both of these enzymes are members of the cytochrome P-450 family but their products have opposing actions. PGI₂ (or, prostacyclin) has potent vasodilator functions and can inhibit platelet aggregation, while thromboxane is a vasoconstrictor and platelet aggregator [50-52]. PGD₂ is synthesized by the PGD₂ synthase (PGDS) which occurs in two forms that are encoded by two unrelated genes [50]. One is the lipocalin-type PGDS (L-PGDS), a secreted form that is common in the CNS; the other is the hematopoietic PGDS (H-PGDS). PGD₂ plays a pivotal role in numerous physiological functions, including sleep induction [53], pain perception [54], coagulation [55] and as an allergic inflammatory mediator in mast cells [56]. PGF synthases are cytosolic proteins which belong to the aldo-keto reductase family and lead to the production of PGF_{2α}. PGE synthases (PGES) exist as three isoenzymes, each encoded by a separate gene [57]. The three isoenzymes are known as the cytosolic PGES (cPGES), the membrane-bound PGES-1 (mPGES), and mPGES-2. mPGES-2 is associated with the Golgi and while spatially coupled to the COX enzymes, appears to be constitutively expressed, with little elevation during inflammation [58, 59]. cPGES is also constitutively expressed and is structurally identical to Hsp90. It functions with

COX-1, but not COX-2. It is localized in the cytosol and unaffected by proinflammatory stimuli [60].

Crucial to the development of inflammation, the mPGES-1 enzyme is responsible for the conversion of PGH_2 into PGE_2 [58, 61, 62]. Recent interest has emerged to identify mPGES-1 inhibitors to help overcome some of the negative side effects of general COX inhibitors, while still limiting the potentially devastating effects of pro-inflammatory PGE_2 [57]. mPGES-1 is functionally coupled to COX-2 and both are typically induced and expressed in response to $\text{IL-1}\beta$ and inflammatory stimuli [61, 63]. mPGES-1 is a glutathione (GSH)-requiring protein that is localized to the perinuclear envelope and belongs to the membrane-associated proteins involved in eicosanoid and GSH metabolism (MAPEG) family. mPGES-1 expression is crucial to PGE_2 production under inflammatory conditions. In studies where the mPGES-1 was knocked-out in mice, it was shown to be the major inducible activity leading to PGE_2 production in response to LPS stimulation. mPGES-1 knock-out mice also show reduced pathology in animal models of induced inflammation and inflammation induced cancers [64].

4. Acute inflammation and infection by the influenza A virus

Infection by influenza A viruses is accompanied by acute inflammation which is responsible for many of the symptoms and pathology associated with this disease. The cellular response to influenza A virus is one of the two model systems used in

this dissertation to define the mechanism of berberine action. Detailed information on the biology of influenza A virus and the inflammatory response to this virus are presented below.

4.1 The biological characteristics of influenza A virus

Influenza A viruses are classified in the *Orthomyxoviridae* family and are negative-sense, single-stranded, enveloped RNA viruses [65]. There are four genera within the *Orthomyxoviridae* family. These are influenza virus A, B, C and Thogotovirus. Viruses in these genera are distinguished by antigenic differences and the number of genomic segments [66]. While viruses from all of these genera can infect humans; influenza A viruses are responsible for all human pandemics to date [65]. Influenza A viruses can infect humans, wild and domestic birds, swine, horses, seals, whales, canines, and other mammals. These viruses are classified into subtypes based on antigenic variations in the hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins. There are currently 16 known subtypes of HA and 9 subtypes of NA. While each subtype can be isolated from aquatic birds, only H1N1, H2N2, H3N2, H5N1 H7N7 and H9N2 have been isolated from humans [66]. Co-infection of a single host with two different influenza viruses may result in the generation of 'reassortant' progeny viruses having a new combination of RNA segments. This antigenic shift can result in the emergence of antigenically novel pathogens [67].

Influenza A and B virus have an eight segment genome, while influenza C virus has seven. The eight genome segments encode viral RNAs that function as templates for messenger RNA (mRNA) and complementary RNA (cRNA) synthesis [66]. Segment 1 encodes the basic polymerase protein 2 (PB2), a protein which makes up part of the polymerase complex and is important for the “cap-snatching” function of the virus whereby the endonuclease activity of PB2 cleaves host mRNAs to generate 5'-capped primers for viral mRNA synthesis [68, 69]. Segment 2 encodes the basic polymerase protein 1 (PB1), which functions as the RNA polymerase in conjunction with the PB2 and PA subunits [70]. Segment 3 encodes the acidic polymerase protein (PA) which contains the nuclear localization signal needed to move the polymerase complex into the nucleus [71]. Segment 4 encodes the hemagglutinin (HA) protein which binds the virus particle to surface sialic acid residues on the host cell surface to mediate entry [65, 72]. The HA protein is synthesized as a polypeptide precursor (HA₀) that is cleaved post-translationally by extracellular trypsin-like proteases into HA₁ and HA₂. Cleavage of HA is essential for the subsequent conformational changes that occur in the acidic environment of the endosome which mediates membrane fusion in the endosome. In addition, HA₁ is important for receptor-binding specificity [73] and HA₂ contains the “fusion peptide” at the amino terminus necessary for membrane fusion and entry into the host cell cytoplasm [66]. Segment 5 encodes the nucleoprotein (NP). The NP contains a RNA-binding domain that encapsidates the viral RNA and transports it into the nucleus and later to the cytoplasm for packaging [74, 75]. Segment 6

encodes the neuraminidase (NA), a surface glycoprotein which plays an important role in the release of progeny virions through the cleavage of sialic acid residues from cell-surface glycoproteins and gangliosides [76]. Segment 7 encodes the matrix proteins 1 and 2 (M1 and M2). M2 is translated from a spliced mRNA. The M1 protein forms a layer to separate the ribonucleoproteins (RNP) from the viral membrane. It can bind viral RNA, regulate nuclear export and function in the assembly of progeny virions [77-79]. The M2 protein is an integral membrane protein that has ion channel activity needed for the acidification of the virus particle interior. Acidification of the virus particle interior allows for the vRNPs to dissociate from the M1 protein for nuclear import [80, 81]. Segment 8 encodes the nonstructural proteins (NS1 and NS2). NS2 is translated from a spliced mRNA. NS1 accumulates mostly in the nucleus where it can bind RNA [82, 83]. It inhibits splicing, nuclear export of cellular mRNAs and protein kinase (PKR) activation [84, 85]. In addition, NS1 proteins can induce the production of proinflammatory cytokines which is an important component of the pathology of the virus [86]. The viral NS2 protein is believed to be important for vRNP nuclear export [87]; however, the full functions of this protein are unclear.

The structure of the influenza virus particle is pleomorphic, being either spherical or filamentous in shape, and ranges in size from 100-300nm [76]. The virus particle has a lipid envelope derived via the budding process from the host cell membrane. It is studded with the viral proteins HA, NA and M2. The HA spike recognition of host cell N-acetylneuraminic (sialic) acid residues is the first step in

the initiation of the influenza A virus infectious cycle. HA preferentially binds oligosaccharides with specific linkages to the sialic acids to galactose. For example, the carbon-2 of sialic acid binds the carbon-3 or 6 of galactose forming α -2,3 or α -2,6-linkages. Specificity for oligosaccharides with these different linkages explains, to some degree, the tropism of the virus. Human glycoproteins contain primarily the α -2,6 configuration, while the avian cells have α -2,3 linkages. Human cells do have some glycoproteins with the α -2,3 configuration, primarily in the lower respiratory tract, therefore explaining the susceptibility of humans to avian viruses [76]. The host immune response can produce antibodies to HA, so the virus evolves through amino acid changes to avoid this response. These cumulative minor changes render host antibody responses ineffective. This process is termed antigenic drift and is the process which necessitates the development of new vaccines each year. This process is distinct from antigenic shift whereby genetic reassortants between human, avian and porcine strains of influenza lead to the production of entirely novel strains of the virus. When this occurs, it is common to observe widespread morbidity and mortality as the population affected is often immunologically naïve.

Following binding of the influenza A virus HA protein to sialic acid, the virus is endocytosed. Once inside the endosome, the low pH will trigger a conformational change in the HA protein which will expose a fusion peptide that can insert into the endosomal membrane. The M2 protein allows hydrogen ions from the endosome to pass into the virus particle interior, which disrupts the internal protein-protein interactions between M1 and RNP. These two processes mediate the release of the

viral RNP into the host cell cytoplasm [76, 88, 89]. The RNPs traffic to the host nucleus using viral nuclear localization signals located on the NP protein. Once inside the nucleus, the vRNA serve as templates for both cRNA and mRNA synthesis. The PB2, PB1, PA and NP proteins form the polymerase complex for the transcription and replication of the viral genome. The cRNA intermediate is transcribed in order to make additional negative-sense genomic vRNA that will be packaged with the NP protein in progeny virions and exported for packaging with the envelope proteins at the cell surface. The virus mRNA is polyadenylated, not by host poly(A) polymerases, but through an encoded stretch of uracils that upon transcription, provides the poly(A) tail [76, 90]. mRNA capping occurs when the endonuclease activity of the PB2 protein cleaves 5' capped primers from host mRNAs [91]. This process is known as "cap snatching" and allows for the mRNA to be translated using host cell machinery. The HA, NA and M2 proteins are synthesized from these mRNA transcripts and are trafficked through the ER and Golgi apparatus for post-translational modification. Apical sorting signals direct these proteins to the cell membrane for assembly into the budding virion. Release of progeny virions occur as the NA protein cleaves the terminal sialic acid residues from the cell surface glycoproteins and gangliosides. NA is also believed to facilitate virus entry in the host respiratory epithelium by breaking down mucins in respiratory tract secretions [92].

4.2 The innate immune response to influenza A virus

The influenza A virus primarily infects epithelial cells of the upper and lower respiratory tract and alveolar macrophages in humans [93]. Typical symptoms associated with influenza include fever, headaches, fatigue, chills, congestion and body aches. These symptoms are mediated by the host innate inflammatory response, which indirectly leads to much of the pathology of influenza [94]. Indeed, cellular damage associated with influenza is typically not mediated by the virus itself, but rather through the strong induction of inflammatory cytokines (TNF- α , IL-6, IL-1 β) and lipid mediators (PGE₂), which when produced in excess can damage healthy tissues [95]. This “cytokine storm” is initiated by the host innate immune system which recruits other immune system cells and primes the adaptive immune response that will ultimately limit and control the spread of the infection. Infections involving pandemic strains are unique in that they often exhibit enhanced immune dysregulation and altered pro-coagulant activity that leads to further complications and increased risk of secondary bacterial infections [95]. Pandemic strains have higher morbidity and mortality rates due to the lack of specific immunity to novel recombinants [93]. Moreover, they often cause uncontrolled inflammatory responses despite control of the virus load [96]. Understanding the activation of the key pathways leading to the hyper-production of inflammatory cytokines and lipids will provide opportunities to direct therapeutics to dampen the inflammatory

response and thereby potentially limit some of the pathology associated with the disease of influenza.

Upon infection, the epithelial cells of the lung and upper airways secrete large quantities of IFN α/β as well as the chemokines RANTES, MCP-1 and IL-8. In addition, tissue-resident macrophages and dendritic cells (DC) are able to produce MIP-1 α/β , MCP-1 and MCP-3, IL-10, IL-1 β , IL-6, TNF- α and IFN- α/β [97, 98]. IFN α/β plays a key role in activating the host response against influenza A virus. Secreted IFNs can bind to IFN receptors on the cell surface and upregulate JAK/STAT pathways leading to the activation of several IFN stimulated genes such as MxA, 2'-5' oligoadenylate synthetase (OAS) and protein kinase R (PKR). These proteins act to limit the spread of the virus through intervention with cellular translation machinery, degradation of viral and cellular RNAs and interference with transcription and replication of the virus [99-101]. Expression of IFN can lead to the development of an "anti-viral state" whereby these antiviral factors prevent subsequent infections.

Immunostimulatory cytokines and chemokines are essential for the activation of the host cellular response to influenza A virus. There are several transcription factors and associated pathways that must be activated in response to virus interactions with host PRRs. Three general classes of PRRs are cited as having an essential role in the recognition of influenza A virus [14]. These pathways include TLR-3, -7 and -8 [13], the retinoic acid-inducible helicase (RIG-I) [102], and the nucleotide-binding domain and leucine-rich-repeat-containing (NLRs) proteins [103].

Transcriptional activation downstream of these receptors involve nuclear factor kappa B (NF- κ B), interferon regulatory factors (IRFs), activating protein (AP-1), signal transducers and activators of transcription (STATs) and nuclear factor-interleukin 6 (NF-IL6 or C/EBP β) [97, 98]. In addition, mitogen-activated protein (MAP) kinases, such as ERK, p38, and JNK, function to regulate cytokine and inflammatory lipid production in response to influenza A virus infection. Influenza A virus infections, and phosphorylation of the MAPK p38, ERK and JNK, have been shown to lead to increases in COX-2, cPLA₂ phosphorylation, and PGE₂ release [104]. In fact, it has been shown with COX-2 (-/-) mice that lack of COX-2 expression is beneficial to the host during influenza infection and may indicate a role for the inhibition of the lipid mediator pathways as a potential therapeutic target [105].

Influenza A virus has evolved various strategies to subvert and blunt the efficacy of the immune response. Influenza can target RIG-I-dependent expression of IFN genes and block IFN receptor signaling through the NS1 protein. NS1 protein can directly antagonize IFN signaling events, inhibit maturation of cellular mRNAs, and directly inhibit IFN-responsive factors such as PKR [106]. In addition, influenza can influence the overall function of the host cell through the induction of a G₀/G₁ cell cycle arrest which produces favorable conditions for virus protein expression and particle production [107].

4.3 Treatments for influenza A virus

Vaccines for both seasonal and pandemic strains of influenza are the primary strategy for the prevention and control of influenza [108]. Trivalent inactivated vaccines administered intramuscularly and live attenuated vaccines administered intranasally are the two primary forms and routes by which influenza vaccines are administered. These vaccines are generally effective in inducing protective responses in the majority of the population (80-90% efficacy) [109]. Protective responses elicited through natural infection and vaccinations depend on both humoral and cellular responses [110-112]. Current vaccines typically induce virus-neutralizing IgG antibodies as well as IgA antibodies to the major structural proteins HA and NA [113, 114]. These antibody mediated responses are often limited due to antigenic drift and shift and require seasonal vaccination. Effector CD4+ and CD8+ T-cells also play an important role in the control of influenza [115]. Vaccination strategies that elicit a strong CTL response have shown protection against influenza A virus infection [116, 117]. Immunogenicity varies with age and is generally not high among the very young, the very old, or in individuals with compromised immune systems [118]. In these populations, vaccine efficacy can be as low as 50% and may not provide adequate protection [109]. These populations, however, are very susceptible to infection and are therefore representative of the limitations of vaccination strategies to control influenza. Ongoing modifications to influenza vaccines are being made to enhance immunogenicity and identify correlates of protection in these critical populations.

Infections with influenza A virus are also treated with anti-viral pharmaceuticals. Currently, there are two drugs that are approved to treat active influenza infections in humans [119]. M2 inhibitors such as rimantadine and amantadine block the viral ion channel in the virion envelope that mediates the critical pH change needed for release of M1 protein from the viral RNPs. NA inhibitors such as zanamivir and oseltamivir function by inhibiting viral NA activity needed for release of progeny virions. Unfortunately, due to the development of resistance, M2 inhibitors are no longer clinically useful. Recent concerns have also arisen with the development of resistance to NA inhibitors [120]. As a result of this, there is renewed interest in defining new molecular targets to limit the spread of the virus as well as control the inflammation associated with infection [119]. In particular, blockade of the MAPK signaling pathway has been shown to impair the growth of all influenza A virus and influenza B virus strains tested to date [121]. Also, inhibition of the NF- κ B pathway was shown to impair influenza virus replication through a blockade of the critical tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas/FasL [122, 123]. In this case, caspase activation promotes nucleocytoplasmic export of the viral ribonucleoprotein complexes through facilitation of the passive transport process [124]. Recent research has also shown that influenza A virus replication can be inhibited by aspirin [125], an effect that is believed to be due to direct effects on COX-2-mediated NF- κ B activation. This finding opens the possibility of targeting the lipid mediator pathways as a means to control virus pathogenesis as well as inflammation.

Development of amantadine and neuraminidase inhibitor derivatives, application of broad spectrum anti-virals such as ribavirin, development of sialidase fusion inhibitors, interferon inducers and influenza specific siRNAs are just a few of the approaches that are currently being explored to combat the influenza virus infection [126, 127]. Research is also focused on the characterization of phytochemicals with anti-influenza A virus activity. Examples of recently isolated phytochemicals with demonstrated anti-influenza A virus activity generally belong to one of several structural classes of polyphenols [128-130], flavonoids [131-136], and alkaloids [137-141]. For example, a polyphenol rich extract (CYSTUS052) from the Mediterranean plant *Cistus incanus* exerts a potent anti-influenza A virus activity [142, 143]. Also, the flavonoid quercetin, commonly found in a variety of fruits, vegetables and medicinal plants has shown anti-influenza A virus activity and antioxidant protective effects in the lung following infection [144-146]. These studies and many others highlight the importance of natural products in the drug discovery process.

5. Acute inflammation and bacterial infections

Infections of internal body compartments by Gram-negative bacteria can result in acute life-threatening inflammation. Sepsis, meningitis, and peritonitis are examples of disorders where the acute response, triggered by bacterial components, can cause dangerous symptoms and pathology. Our laboratory utilizes treatment with bacterial lipopolysaccharide (LPS) as a model for bacterial sepsis. Background

information on this model, and the molecular mechanisms which control this response, are presented below.

5.1. The symptoms and causes of bacterial sepsis

Sepsis is a multi-factorial clinical syndrome characterized by the release of excessive amounts of inflammatory mediators in response to systemic microbial infections [147]. Sepsis begins as a normal immune response to an invading microbial pathogen, but quickly progresses to the dysregulation of the normal immune response resulting in collateral damage of healthy tissue as the immune response struggles to contain the infection and regulate the production of proinflammatory mediators. Interestingly, at the same time the host is hyper-responsive to the invading microorganism, the patient can also become immunocompromised due to this same dysregulation of the host inflammatory response [148]. This is often referred to as “immunoparalysis” and has been associated with defects in the macrophage whereby there is decreased phagocytic ability, reduced bactericidal activity, and attenuated proinflammatory cytokine production in response to *ex vivo* LPS stimulation [149]. This immunoparalysis can lead to immunosuppression and make the patient more likely to become infected or reduce clearance of the invading pathogen [150]. In cases of sepsis, the balance between proinflammatory responses which control the spread of infection and the anti-inflammatory mechanisms of the host to regulate this response are deregulated leading to the robust and uncontrolled inflammatory response. As these host

inflammatory responses intensify, septic shock occurs as a result of the associated organ dysfunction, disseminated intravascular coagulation (DIC) and effects on the endothelium leading to hypotension which in turn leads to much of the morbidity and mortality associated with the infection [151]. Currently, sepsis is the leading cause of death in critically ill patients with a mortality rate of approximately 30–50% in patients with severe sepsis [152]. There are many microbial (bacterial, viral and fungal) PAMPs associated with sepsis that can activate the initial host immune response in response to infection. Among these are microbial surface molecules such as endotoxin (lipopolysaccharide or LPS), lipoproteins, outer membrane proteins, flagellin, fimbriae, peptidoglycan, peptidoglycan-associated lipoprotein, and lipoteichoic acids. In the case of Gram-negative bacterial infections, which account for approximately 30% of bacterial cases of sepsis, the outer membrane contains endotoxin or LPS [151].

5.2 LPS and its signaling pathways

LPS derived from Gram-negative bacteria is one of the major inducers of sepsis and has been the focus of intensive research. LPS is composed of three main structural elements: a core oligosaccharide, an O-specific chain of repeating sequences of polysaccharides and a proinflammatory lipid A component [153, 154]. In active Gram-negative bacterial infections, LPS is released by the bacteria in several forms; as a free LPS, as an aggregated form associated with serum lipoproteins, or in complexes with membrane proteins [155]. Circulating LPS can

interact with macrophages, endothelial cells and other cells via the Toll-like receptor 4 (TLR4)-MD-2 receptor complex to initiate a proinflammatory cascade that leads to the hyperimmune response associated with sepsis. LPS is initially recognized by the host LPS-binding protein (LBP). This acute-phase protein will bring the LPS to the cell surface CD14 receptor and facilitate the transfer of LPS to the TLR4/MD-2 complex [156]. Following TLR4 engagement with LPS, the TLR4 receptor homodimerizes to enhance LPS binding and recruits the TIR-domain-containing adaptor molecules (TIRAP) to the cytoplasmic domain of the receptor. At this point, there can be either MyD88-dependent or MyD88-independent activation of downstream protein kinases. For the MyD88 dependent pathway, the key adaptors in the signaling cascade include MyD88, TIRAP, TRIF and TRAM which leads to the activation of NF- κ B, MAPK and PI3K/Akt pathways, which in turn drive the expression of proinflammatory cytokines, chemokines and inflammatory lipids [13]. In this process MyD88 interacts with the TLR4 receptor through cytoplasmic TIR domains. This complex will then recruit IRAK1 and 4. TIRAP will also be recruited and is essential for MyD88 signaling. The activation of IRAK1 will facilitate binding to TRAF6. This newly formed complex will bind to TAK1 and TAB1, 2 and 3. TAK1 can then phosphorylate the I κ B kinase (IKK) facilitating the activation of NF- κ B. The MyD88 independent pathway relies upon the activation of IRF3 through TRAF6 and TRIF. Upon activation through both of these pathways, cells will produce a variety of pro-inflammatory mediators. These include the MyD88-dependent production of TNF- α , IL-1, and IL-8 and the MyD88-independent activation of IFN β , IP10, IL-6 and

iNOS. The MAPK and PI3K pathways play a role in this cascade; however, the cross-talk among these pathways likely involves additional unidentified proteins involved in this generalized signaling cascade [13, 153]. For example, PI3K has been shown to be involved in both the positive and negative regulation of NFκB [153]. In addition, the activation of MAPKs can lead to the direct phosphorylation of cPLA₂ which leads to the production of inflammatory lipids such as PGE₂. The exact role TLR4 signaling pathways play beyond the function of MAPK activation of the eicosanoid pathway has not been well characterized and represents an open area of research to be explored.

5.3 Treatments for bacterial sepsis

Current treatments for sepsis typically involve targeting the invading microorganism with broad-spectrum anti-microbials and providing supportive care for shock and organ dysfunction [151]. Therapies targeting the rampant inflammation associated with sepsis have primarily depended on non-specific drug regimes involving high-dose corticosteroids and NSAIDs. For the past 20 years there have been a variety of approaches developed to target LPS itself and or attenuate the signaling pathways triggered by LPS. For example, the development of monoclonal antibodies to LPS [157], synthetic analogues of lipid A [158] and recombinant monoclonal antibody to CD14 [159] have shown promise in animal models of sepsis but have failed to provide sufficient protection in clinical trials in humans. Attempts

to target single inflammatory mediators like TNF- α , IL-1, platelet activating factor, adhesion molecules, arachidonic acid metabolites, oxygen free radicals, bradykinin, phosphodiesterase and C1 esterase, or NO synthase have also failed to show adequate protection against the deregulated inflammatory responses associated with sepsis [151, 160-162]. There is an ongoing need to develop novel therapeutics that can either attenuate the growth of the microorganisms involved in sepsis or attenuate and regulate the induction of the inflammatory response to these microorganisms [163].

To this end, a number of plant-derived phytochemicals show promise for the treatment of sepsis. For example, a major component of green tea, (-)-epigallocatechin-3-gallate (EGCG), has been shown to limit inflammation and rescue mice in lethal models of endotoxemia by targeting the high mobility group box 1 protein (HMGB1) [164]. HMGB1 is secreted by macrophages and functions as a cytokine regulator of inflammation. Also, extracts of *Thuja orientalis* were recently shown to protect mice from endotoxemia through limiting inflammation by targeting NF- κ B and MAPK p38 signaling pathways [165]. There are many examples of other naturally derived compounds which can limit the induction of inflammation and protect mice in lethal endotoxemia models [166, 167]. Of interest to our research is the isoquinoline alkaloid berberine. This alkaloid, commonly found in the medicinal herb goldenseal, has also been identified as a potential inhibitor of inflammatory mediators in response to LPS [168-171].

6. Goldenseal and berberine

Natural products and herbal medicines represent a multi-billion dollar industry that has developed from the application of traditional medicines used by individuals for centuries to treat common ailments. Medicinal plants are rich resources of novel pharmaceuticals due to the vast structural diversity of their active components. Pharmaceutical research has historically attempted to identify novel lead compounds from these sources for medicines and has historically led to the successful development of several commonly prescribed medicines [172]. For example, between 1981 and 2002 approximately half of new chemical entities (NCEs) with pharmaceutical potential were derived from natural products [173]. Hundreds of naturally derived products have shown promise at least initially *in vitro* in areas of anti-cancer [174, 175], anti-inflammatory [176-180] and anti-microbial [181-184] product development.

One commonly used herbal medicine that has demonstrated activity in all three of these areas is the goldenseal plant (*Hydrastis canadensis*). Goldenseal is a perennial herb in the family *Ranunculaceae*. This plant has a characteristically yellow rhizome and fibrous rootstock, 5-7 lobed leaves and produces a raspberry like fruit in the summer. Goldenseal grows in shaded areas in the eastern United States and Canada and is native to the North Carolina mountains. The popularity of wild-crafted forms of this plant has threatened it and in 1997, goldenseal was listed on Appendix II of the Convention for International Trade on Endangered Species

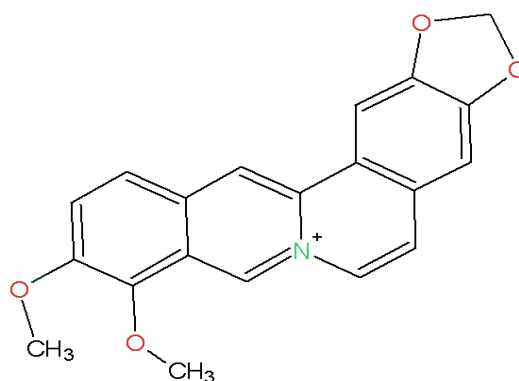
(CITES), an international treaty monitoring trade in threatened and endangered species. Efforts are underway to raise awareness of sustainable growth options and to optimize crop-raised goldenseal to satisfy the demands the natural product consumer.

Traditionally goldenseal was used by Native Americans like the Cherokee and later by early settlers and herbalists for the treatment of generalized skin disorders, digestive problems, cancer, liver conditions, diarrhea, and eye irritations. In addition, extracts of goldenseal are often used for their purported anti-inflammatory, antiseptic, laxative, and muscle stimulant effects. Usage of goldenseal to treat these conditions is often based on traditional use and preparation and has benefited from an ongoing scientific examination and identification of specific mechanisms of action and identification of the key constituents of the extract which are believed to exert these effects. Goldenseal extracts are complex and contain a variety of secondary metabolites in the form of isoquinoline alkaloids. Goldenseal extracts contain several alkaloids; primarily hydrastine, berberine, berberastine, hydrastinine, tetrahydroberberastine, canadine, and canadine [185]. The concentrations of these alkaloids often vary depending on the source of the preparation [186] and the time of year the plant is harvested [187]. The function of these alkaloids within the plant is not well understood, but it is generally accepted that plant alkaloids provide a protective mechanism against herbivore animals and parasites [188]. The alkaloid berberine is the primary alkaloid within the rootstock and is responsible for the bright yellow root color and for the strong bitter taste of the root extract. Many of the

biological effects of goldenseal have been correlated with the levels of the primary alkaloid berberine. Most studies to date utilize a purified and commercially available form of this alkaloid.

6.1 The structure and synthesis of berberine

Berberine is a quaternary ammonium salt derived from the protoberberine group of isoquinoline alkaloids (Figure 1). Berberine is derived from the precursor amino acid tyrosine and is converted through a seven step process of chemical modification by the berberine bridge enzyme and tetrahydroprotoberberine oxidase [188]. In addition to goldenseal, berberine is found in a variety of plants that have been used in traditional, Ayurvedic and Chinese medicine. For example, *Coptis chinensis* (Coptis or goldenthread), *Berberis aquifolium* (Oregon grape), *Berberis vulgaris* (barberry) and *Berberis aristata* (tree turmeric) are all important sources of berberine and have been shown to have similar biological effects as goldenseal [189].



<http://commons.wikimedia.org/wiki/Berberine>

Figure 1. Structure of Berberine

Berberine has been shown to interact with a variety of cellular and microbial targets that may account for many of its effects. For example, berberine can intercalate in DNA, inhibit DNA synthesis, inhibit protein biosynthesis and inhibit reverse transcriptase [190]. It can also bind to the polyadenylic acid tail (polyA tail) of mRNA [191].

6.2 The toxicity and pharmacokinetics of berberine

Toxicity studies with purified berberine administered by intravenous (IV), intraperitoneal (IP) and intragastric (IG) routes demonstrated that the route of administration in mice determines the level of toxicity. In one study, the LD₅₀ by IV was 9.04mg/kg and 57.61mg/kg for IP. The IG group had no quantifiable LD₅₀ and was at least 83.2g/kg [192]. The explanation for this effect is due to the first-pass elimination of berberine through the GI tract which prevents adequate absorption into the blood stream [192, 193]. Interestingly, oral doses of whole root goldenseal supplement gave higher levels of measurable berberine in human serum than did oral administration of purified berberine, indicating that other components of the whole plant may enhance uptake or limit elimination of berberine following oral administration [194]. Pharmacokinetic studies with berberine indicate that it distributes to the heart, kidney, spleen, lung and brain but mainly to the liver where it is metabolized by approximately 33.6% within 1 hour of administration into the four main metabolites M1-M4 (along with others) which pass into circulation and are excreted through the urine [193, 195, 196].

6.3 The anti-microbial effects of goldenseal and berberine

In the early 1950s researchers first established that berberine could have direct anti-microbial properties [197]. Historical use of goldenseal root to alleviate generalized skin disorders, digestive problems, liver conditions, diarrhea, and eye irritations was most likely due to the effects of berberine on the microorganisms responsible for these conditions. Over the next 60 years ongoing research has identified that goldenseal and berberine can limit the growth of a variety of microorganisms (bacterial, fungal, protozoan, and viral) as measured by a reduction in total viable organisms; however, the mechanisms by which this is accomplished are largely poorly understood and vary by organism. To date there has been no central mechanism identified to adequately explain how goldenseal and berberine can target such a wide variety of organisms directly. In the case of infectious causes of diarrhea, the primary culprits are often *Vibrio cholerae*, *Escherichia coli* and *Salmonella Typhi*. Early studies with berberine found direct bacteriocidal activity against *V. cholerae* [198, 199] and direct inhibition of *V. cholerae* and *E. coli* enterotoxins [200]. Berberine can also directly inhibit the growth of *S. Typhi* by up to 80% at 24hrs with a 50 μ M dose [201]. Berberine can also alter the response to the invading pathogen by the host through reduction of smooth muscle contraction, intestinal motility and intestinal fluid secretion [202, 203].

Berberine has been shown to inhibit a variety of other microorganisms [204]. Extracts of goldenseal have been shown to directly inhibit *Helicobacter pylori* in culture with a mean inhibitory concentration (MIC₅₀) of 12.5-50 μ g/ml. In this case,

both berberine and hydrastine were shown to mediate this effect [205]. Berberine and goldenseal extracts have also been shown to inhibit intestinal parasites that can cause diarrhea such as *Giardia lamblia*, *Entamoeba histolytica*, *Trichomonas vaginalis* and *Leishmania donovani* [206, 207]. Berberine can inhibit *Chlamydia trachomatis* infections, an important pathogen of the eye. In this case, berberine performed better in clinical trials than the typically prescribed sulfacetamide eye drops [208, 209]. Berberine can also inhibit the oral pathogens *Streptococcus mutans* and *Fusobacterium nucleatum* [210], and inhibit drug resistant strains of *Mycobacterium tuberculosis* [211]. Berberine has also been shown to inhibit the formation of biofilms of *Staphylococcus epidermidis* at concentrations of 35-40 µg/ml [212, 213].

While berberine has been the focus of many studies involving the anti-microbial effects of goldenseal, there is evidence that in some cases the extract or other alkaloids can perform better than berberine. For example, Scazzocchio et.al; [214] evaluated the bacteriocidal activity of a whole-root goldenseal extract in comparison to individual alkaloids against *Staphylococcus aureus*, *Streptococcus sanguis*, *E. coli* and *Pseudomonas aeruginosa* at varying doses (1.5-10mg/ml) over 30min. In this study, the extract was more effective against *S. aureus* than berberine while canadine (another alkaloid) was more effective against *S. sanguis* and *P.aeruginosa* than either berberine or extract. This study highlights the effectiveness of goldenseal and berberine against a variety of bacteria, but may also indicate the importance of the various other components of goldenseal extracts.

Unfortunately, one of the major limitations of using berberine and other anti-microbials to treat bacterial infections is the presence of multidrug-resistance pumps (MDRs) [215, 216]. Berberine is a strong amphipathic cation and is actively effluxed out of the microbial cell by MDRs [217, 218]. This efflux mechanism limits the efficacy of berberine and other anti-microbial compounds and is often the cause of anti-microbial resistance [219-223]. Recently, the addition of MDR inhibitors have been shown to enhance the effects of various anti-microbials [224]. This strategy has been employed with berberine and has been shown to potentiate the effects of berberine against bacteria [225-227]. Interestingly, whole plant extracts containing berberine have also been shown to contain natural MDR inhibitors that can potentiate the effects of the individual alkaloids [228, 229].

In addition to the anti-bacterial and anti-parasitic effects of berberine, there is also some limited evidence of berberine functioning as an anti-viral compound that can target a variety of viruses. For example, Hayashi et.al., described the ability of berberine to inhibit human cytomegalovirus (HCMV) by plaque assay with an IC_{50} of $0.68\mu M$; similar to that of the commonly prescribed antiviral drug ganciclovir ($IC_{50}=0.91\mu M$) [230]. Berberine extracted from *Coptidis* rhizome was also shown to inhibit herpes simplex virus (HSV) in Vero cells [231]. An abstract published 20 years ago showed that berberine can inhibit the growth of the influenza A virus on chicken allantoic membranes [141]. More recently, we have fully characterized the effects of berberine on the growth of influenza A virus *in vitro* with a number of different cell types [232]. Similarly, Wu, et.al., concurrently reported that berberine

will inhibit the growth of strain A/FM/1/47 (H1N1) influenza in ICR mice in a recent publication examining the anti-influenza effects of berberine *in vivo* [233].

6.4 The anti-inflammatory effects of goldenseal and berberine

In addition to the direct anti-microbial effects of goldenseal and berberine, there is evidence that berberine can independently inhibit proinflammatory cytokine, chemokine and inflammatory lipid responses in a variety of infectious and non-infectious models of inflammation. As mentioned previously, traditional medicine practitioners and herbalists have utilized goldenseal for centuries to treat inflammatory conditions of the eye and skin. Ongoing research has begun to identify the pathways and specific targets of berberine that may mediate these anti-inflammatory effects. Also, there is interest in making chemical analog libraries by making modifications to the structure of berberine that can enhance its anti-inflammatory activities [168]. The results of these findings may have far-reaching implications for the future design of novel therapeutics for infectious disease, chronic inflammatory disease and cancer research.

In many of the studies demonstrating an anti-inflammatory effect of berberine and goldenseal extracts, LPS is used as the ligand to trigger inflammation. Clement-Kruzel et.al. utilized a mouse macrophage cell line (J774A.1), stimulated with 10ng/ml of LPS in the presence or absence of different amounts of a goldenseal extract for 72 hours. Upon analysis of cell supernatants by ELISA, it was determined that goldenseal extracts reduced the expression of TNF- α , IL-6, IL-10

and IL-12 in a dose-dependent manner [234]. This effect can also be observed with mouse models of endotoxemia where berberine was administered daily for five days prior to treatment with LPS. In these animals, mortality rates decreased by 57% and there was a reduction in the production of TNF- α , IFN- γ , and NO [235]. In addition to LPS, berberine has been shown to inhibit inflammation induced by 12-o-tetradecanoylphorbol-13-acetate (TPA), hydrogen peroxide, okadaic acid and ceramide. In these experiments, a 5 μ M concentration of berberine was found to inhibit production of IL-1 β and TNF- α from A549 cells for all the ligands listed above. The mechanism underlying this effect was linked to the inhibition of I κ B- α phosphorylation and degradation [236]. Inhibition of I κ B- α phosphorylation prevents the activation of NF- κ B, an important transcription factor that drives cytokine expression. Berberine has also shown efficacy in other models of inflammation. For example, when THP-1 macrophage-like cells were stimulated with 50 μ g/ml of acetylated low-density lipoproteins (to mimic the proinflammatory activation that leads to atherosclerosis), and treated with 5-10 μ M berberine for 24-48 hours, berberine inhibited production of TNF- α , IL-6 and MCP-1 (both mRNA and protein) [237]. In animal models of colitis, 10-20mg of berberine was administered orally to mice once a day for three days prior to TNBS treatment inhibited expression of iNOS, IL-1 β , IL-6, and TNF- α in colonic tissue. This effect was also linked to the inhibition of NF κ B activation as was the LPS-induced production of cytokines from LPS stimulated peritoneal macrophages [238]. Other models that have been used to demonstrate the anti-inflammatory potential of berberine include experimental

autoimmune encephalomyelitis (EAE) in mice, [239] and expression of IFN- and IL-17 in non-obese diabetic (NOD) mice. In this model, berberine prevented progression to diabetes in 50% of the mice by inhibiting p38 MAPK and JNK activation, which the authors speculate reduces expression of inflammatory cytokines [240]. Berberine was also shown to inhibit production of TNF- α and IL-6 in J774A.1 mouse macrophage-like cells following treatment with HIV protease inhibitors [241]. Similar anti-inflammatory effects were observed in LPS-stimulated cardiac myocytes (decreases in IL-1 β , TNF- α , and NO) [169] and rat mesangial cells stimulated with LPS (decreases in ICAM-1, TGF- β , iNOS) [242].

The effects of berberine on the production of inflammatory lipid mediators have also been examined by a number of labs. For example, Zhang, et.al, demonstrated that berberine can reduce the LPS-induced production of thromboxane B2 and LTB4. These authors linked this effect to a reduction in the expression and phosphorylation of cPLA2 [171]. Studies with LPS-treated human peripheral blood mononuclear cells (PBMCs) have also observed inhibition of COX-2 mRNA and protein and linked this effect to inhibition of ERK1/2 and JNK protein expression. Similarly, berberine has been shown to decrease the expression of COX-2 *in vivo* in rats treated with LPS. Interestingly, in this study the effects of berberine were linked to effects on p38 kinase, not ERK1/2 or JNK. Inhibition of expression of COX-2 was also observed in studies with colon cancer [243] and oral cancer-derived cell lines [244]. In contrast, other studies have failed to reveal an effect of berberine on the expression or activity of COX-2. For example, Kim, et.al,

showed that berberine did not inhibit the expression of COX-2 protein in RAW 264.7 macrophage-like cells treated with LPS [245]. This lack of inhibition was also observed with the use of a 13-alkyl substituted form of berberine [168].

Based on these studies, at present there is not a clear explanation of the ability of berberine to inhibit the production of inflammatory lipids. Studies of the effects of berberine on the expression of COX-2 have revealed contradictory results and only a single study has examined the effects of berberine on the expression and activity of cPLA2. Unfortunately, these studies have utilized a variety of concentrations of berberine, different treatment times and different cell types. Additional studies, comparing the effects of these variables, will be necessary before a consensus emerges on this activity of berberine.

6.5 Additional effects of goldenseal and berberine

In addition to the anti-microbial and anti-inflammatory effects of berberine, several other important activities for this alkaloid have been identified. For example, berberine has been shown to affect adipocyte physiology and gene expression and therefore may be useful for the treatment of a number of metabolic disorders. Berberine has been shown to reduce the expression of adipogenic enzymes such as fatty acid synthase, acetyl-CoA carboxylase and lipoprotein lipase [246]. Berberine has also been shown to reduce expression of proinflammatory genes (IL-1 β , IL-6, iNOS, MCP-1 and COX-2) expression in the adipose tissue of obese mice (db/db) by preventing phosphorylation of p38, ERK, JNK and through activation of

AMP-activated kinase (AMPK) [247, 248]. Goldenseal and berberine can also reduce low density lipoproteins (LDL) in circulation through upregulation of the LDL-receptor [249-251]. Berberine has also been reported to exert effects on the cardiovascular system, including as an antiarrhythmic and vasorelaxant [252, 253]. Berberine has been shown to limit the formation of atherosclerosis and restenosis of vascular stents by inhibiting the regrowth and inflammation of vascular smooth muscle cells in response to mechanical injury [254, 255].

Finally, berberine and goldenseal have been reported to limit the growth and spread of cancer [256]. Berberine can induce a G_1/G_0 growth arrest in many transformed cells [257-259] and exert direct cytotoxic effects towards other cancer cells [260-262]. The pro-apoptotic effects of berberine have been linked to regulation of transcription factors necessary for the induction of apoptosis [263-267]. In conclusion, berberine has been shown to exert many effects on different cellular processes. It is possible that these effects arise from a common effect of berberine on cellular macromolecules. Alternatively, these effects may stem from individual, distinct effects of berberine.

7. References

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CHAPTER 2

**Inhibition of H1N1 Influenza A virus growth and
induction of inflammatory mediators by the
isoquinoline alkaloid berberine and extracts of
goldenseal (*Hydrastis canadensis*)**

CHAPTER 2

1. Abstract

In this study we tested whether the isoquinoline alkaloid berberine can inhibit the growth of influenza A virus. Our experiments showed strong inhibition of the growth of H1N1 influenza A virus strains PR/8/34 or WS/33 in RAW 264.7 macrophage-like cells, A549 human lung epithelial-derived cells and murine bone marrow derived macrophages, but not MDCK canine kidney cells. Studies of the mechanism underlying this effect suggest that berberine acts post-translationally to inhibit virus protein trafficking/maturation which in turn inhibits virus growth. Berberine was also evaluated for its ability to inhibit production of TNF- α and PGE₂ from A/PR/8/34 infected-RAW 264.7 cells. Our studies revealed strong inhibition of production of both mediators and suggest that this effect is distinct from the anti-viral effect. Finally, we asked whether berberine-containing ethanol extracts of goldenseal also inhibit the growth of influenza A virus and production of inflammatory mediators. Here we found strong effectiveness at high concentrations, although upon dilution extracts were somewhat less effective than purified berberine. Taken together, our results suggest that berberine may indeed be useful for the treatment of infections with influenza A virus.

2. Introduction

Influenza A viruses are negative-sense, single stranded RNA viruses which belong to the Orthomyxoviridae family [1, 2]. Each year, seasonal strains of influenza A cause significant morbidity and economic losses worldwide. The Centers for Disease Control estimates that approximately 23,000 people die annually in the United States from flu-related complications [3]. Typically, influenza A infects the tracheal and bronchial epithelial cells as well as alveolar macrophages resulting in localized cell damage and the induction of an acute host inflammatory response [4], which has been characterized as a “cytokine storm”. This response is the cause of the symptoms associated with influenza A infections [5-11] and, in addition, can lead to destruction of healthy tissue [12]. As a result, infected individuals display heightened susceptibility to additional bacterial and viral infections, which are generally the cause of morbidity and mortality associated with this virus [4].

Current treatments for influenza A infection include both pharmacological and non-pharmacological approaches. Two types of influenza A specific anti-viral drugs are currently available; M2 pump inhibitors such as amantadine and rimantidine, and neuraminidase inhibitors including zanamivir and oseltamivir. However, due to side effects and the emergence of drug-resistant strains [13], vaccination has become the dominant approach for control of this disease. Vaccines generally work effectively, but they are contraindicated in neonates and the elderly. Also, vaccine production is a complex process based on the predicted emergence of new seasonal variants.

Goldenseal (*Hydrastis canadensis*) is a plant that has been used for centuries in traditional medicine to treat a variety of conditions, including; skin and eye infections, upper respiratory disorders, diarrhea, and cancer [14]. Many of the effects of goldenseal have been attributed to the isoquinoline alkaloid berberine [15] which is also found in other plants such as barberry (*Berberis vulgaris*), coptis (*Coptis chinensis*), and Oregon grape (*Mahonia aquifolium*). Studies with berberine have revealed effects on a variety of cellular processes. Berberine has been shown to inhibit the growth of certain tumor-derived cell lines [16-20] and to prevent the growth of certain bacteria [21-26]. Berberine can effectively inhibit the growth of several viruses [27-29] including human cytomegalovirus and herpes simplex virus. Berberine has also been shown to inhibit production of cytokines, inflammatory lipids, and nitric oxide from macrophages treated with LPS [3, 30]. Inhibition of cytokine production also occurs *in vivo* in mice treated with LPS, suggesting berberine has potential for the treatment of endotoxemia [30, 31].

In this report we have investigated the effects of berberine on infections with influenza A *in vitro*. An abstract published 20 years ago [32], showed that berberine could inhibit the growth of influenza A on chicken allantoic membranes, but its effects with mammalian cells had not been tested. Therefore, we evaluated berberine for its anti-viral activity against several strains of H1N1 influenza A with a number of different murine and human cell types. We also tested berberine for its ability to suppress production of TNF- α and PGE₂ from infected macrophages. Our experiments show strong inhibition of influenza A growth. We also found strong

inhibition of TNF- α and PGE₂ production from infected macrophages. Similar results were seen with berberine-containing extracts of goldenseal. Taken together, these results suggest the alkaloid berberine and extracts of plants containing berberine may be useful for the treatment of influenza A.

3. Materials and methods

3.1 Cell lines, media and reagents

All cell lines cells were obtained from the American Type Culture Collection (Manassas, VA). RAW 264.7 cells were cultured in Dulbecco's modification of minimal essential medium (DMEM) with 4 mM L-glutamine, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate with 10% fetal calf serum (FCS). A549 cells were cultured in Ham's F-12 nutrient medium with 4mM L-glutamine and 1.5g/L sodium bicarbonate with 10% FCS. Madin-Darby canine kidney (MDCK) cells were cultured in DMEM with 4 mM L-glutamine, 4.5 g/L glucose, and 3.0 g/L sodium bicarbonate and supplemented with 10% FCS, 0.2% BSA and 25mM HEPES buffer. Media, berberine chloride, amantadine, and supplements were obtained from Sigma-Aldrich (St. Louis, MO) and Cellgro (Manassas, VA). FCS was obtained from Atlanta Biologicals (Atlanta, GA) and Gemini Bio-products (West Sacramento, CA). Cells were cultured at 37°C and 5% CO₂.

3.2 Virus propagation and focus forming assays

A/PR/8/34 and A/WS/33 viruses were originally obtained from the American Type Culture Collection (Manassas, VA). Virus stocks were prepared by infecting MDCK cells at a multiplicity of infection (moi) of 0.001 (1 infectious particle per thousand cells). The virus was added to cells for 30 min in a small volume of serum free virus growth media (DMEM with 4 mM L-glutamine, 4.5 g/L glucose, and 3.0 g/L sodium bicarbonate and supplemented with 0.2% BSA, 2 μ g/ml Trypsin-TPCK and 25mM HEPES buffer) followed by the addition of fresh virus growth media and incubated for 36-48 h or until cells displayed 90+% cytopathic effect (CPE). Cell supernatants were collected, cell debris was removed by centrifugation (1,000 rpm for 10 min.), aliquots prepared, and stored at -80°C. For production of experimental supernatants, viruses were added to cells at either low (0.002) or high (5) moi following the same protocol. Berberine and/or extracts were added with the virus and supernatants were collected at the indicated time points.

Virus titers were determined using a focus forming assay (FFA). MDCK cells were seeded in 24 or 48 well plates and incubated overnight at 37°C and 5% CO₂. Virus containing cell supernatants were added for 30 min followed by the addition of an overlay containing 1.2% tragacanth. Plates were incubated for 24 h; cell monolayers were washed with 1X PBS (Sigma, St. Louis, MO) and fixed with 1:1 acetone/methanol at -20°C overnight. The acetone/methanol was removed and plates were allowed to fully dry before blocking in 1% normal horse serum/PBS for approximately 1 h. Primary mouse anti-HA monoclonal antibody (Fitzgerald, Acton,

MA) was diluted 1:3000 in blocking buffer and incubated for 1 h (2.3 µg/ml final Ab concentration). Cell monolayers were washed three times with 1X PBS and incubated with 1:1000 diluted goat anti-mouse IgG – HRP conjugated secondary Ab (Sigma St. Louis, MO) for 30-45 minutes (0.8 µg/ml final Ab concentration). Foci were visualized with Vector VIP peroxidase substrate kit (Vector Burlingame, CA). Foci were enumerated using GelDoc XR (BioRad Hercules, CA) imaging software to determine focus forming units/ml. Titers derived from FFAs which are expressed as focus forming units (FFU) are equivalent to plaque forming units (PFU).

3.3 Preparation of bone marrow derived macrophages

C57BL/6 murine bone marrow-derived macrophages (BMDM) were obtained from the laboratory of Dr. Frank Scholle, PhD, Department of Microbiology, North Carolina State University. Cells were cultured in DMEM with 10% FCS, 30% L929 conditioned media, 1X L-glutamine and 1X non-essential amino acids. Virus inoculations to generate experimental supernatants were performed as in Section 3.2 using serum free medium supplemented with 2µg/ml Trypsin-TPCK.

3.4 Immunoblot analysis

RAW 264.7 cells were plated at a cell density of 5×10^5 cells/60mm tissue culture dish (Corning, Corning, NY) for 24 h, then infected at either low (0.002) or high (5) moi with the A/PR/8/34 virus in the absence or presence of 25 µM berberine as described above. At indicated times, cell monolayers were washed twice with

cold phosphate buffered saline (PBS) (Gibco, Carlsbad, California), lysis buffer added (50 mM HEPES, pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM leupeptin, 0.5% SDS), lysates collected by scraping, and centrifuged for 30 min at 14,000 rpm. The protein concentration for each sample lysate was determined using the Pierce BCA system (Pierce, Rockford, IL). Equal protein samples (10 µg) were loaded on 4-12% Bis-Tris gels (Invitrogen, Carlsbad, CA) and subjected to electrophoresis using the Novex Mini-Cell System (Invitrogen). Following transfer to PVDF membranes, (Millipore, Billerica, MA) membranes were blocked for 24 h with 5% powdered milk in TBS/0.1% Tween-20 and probed with a primary goat anti-H1N1 polyclonal antibody (Fitzgerald, Acton, MA) for 1 h and secondary rabbit anti-goat HRP for 45 minutes (Southern Biotech, Birmingham, Alabama) diluted in 1% powdered milk in TBS/0.1% Tween-20. Bands were visualized using ECL Plus Western Blotting Detection System (GE Healthcare, Piscataway, NJ).

3.5 Immunofluorescence

RAW 264.7 cells were plated in 8-well chamber slides (Lab-Tek II, Chamber Slide System, NUNC, Rochester, NY), incubated for 24 h, and then infected and/or treated with berberine as indicated above. Infections were allowed to proceed for indicated times then processed for immunofluorescence detection of viral hemagglutinin (HA) protein. Media was removed and monolayers were rinsed with PBS 1X and then fixed with 10% formaldehyde for 20 minutes at room temperature.

Fixative was removed and monolayers were rinsed with 1X with PBS. Cells were then permeabilized with 0.5% Triton X 100 (Sigma, St. Louis, MO) in PBS for 10 min at room temperature. Blocking buffer was added (2% BSA, 5% NHS, 10mM glycine) for 1 h at room temperature followed by a 1 h incubation with a viral HA specific monoclonal antibody (mAb) (Fitzgerald Acton, MA). The primary mAb was removed, 2 additional PBS washes were performed, followed by addition of the secondary rabbit anti-mouse Tetramethyl rhodamine iso-thiocyanate (TRITC) conjugated IgG secondary antibody. Staining was visualized using a Zeiss Axioscop 2 plus (Carl-Zeiss Oberkochen, Germany) microscope equipped with a SPOT camera (Diagnostic Imaging, Sterling Heights, MI). Images were captured using SPOT software and analyzed with Photoshop (Adobe, San Jose, CA) software.

3.6 ELISA

TNF- α and PGE₂ ELISA kits were purchased from eBioscience (San Diego, CA). Assays were performed according to manufacturer's recommendations. In each case, sample values were interpolated from standard curves. Optical density was determined using a PolarStar microplate reader (BMG Labtechnologies, Durham, NC).

3.7 Extract preparation and analysis of berberine content

Goldenseal (*Hydrastis canadensis* L. Ranunculaceae) roots were cultivated in a hardwood forest in western North Carolina, as described elsewhere [26], and a

voucher was deposited at the Herbarium of the University of North Carolina (NCU583414). Three individual extracts were used in these studies; one prepared from a pooled sample of goldenseal roots, one from a pooled sample of goldenseal leaves, and one prepared from a single root sample. Extracts were prepared in a solvent of 50:50 ethanol: nanopure water at a ratio of 1 mL solvent: 5 g plant material. The extracts were analyzed for alkaloid content using LC-MS, as described in detail previously [26], and diluted to contain the indicated concentrations of berberine.

3.8 Cell viability assays

RAW 264.7 cells (1.5×10^5 /well) were plated in 24-well tissue culture dishes in 1 ml DMEM and incubated for 24 h. Berberine (25 μ M) or a goldenseal extract containing an equivalent berberine concentration were added and incubated for an additional 12 or 24 hours. Cells were harvested with 0.25% Trypsin-EDTA (Sigma, St. Louis, MO) and counted by hemocytometer with a 0.04% solution of trypan blue. Cell viability was determined by calculation of the ratio of trypan blue stained cells to the number of total cells.

For DNA cleavage experiments, RAW 264.7 cells (5×10^6 /dish) were plated in 100mm tissue culture dishes and incubated for 24 h. Cells were treated with berberine or goldenseal extract as above and, in addition, a treatment with cycloheximide (100 μ g/ml, Sigma, St. Louis, MO) was used as a positive control for the induction of apoptosis. Following 24 h incubation, cells were harvested in lysis

buffer (50 mM HEPES, pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM leupeptin, 0.5% SDS) and digested with proteinase K overnight at 56°C. Lysates were then extracted using phenol and chloroform and DNA precipitated with isopropyl alcohol. Resulting DNA was resuspended in TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA) and treated with 1 µl of RNase A (Sigma, St. Louis, MO) for 30 minutes at room temperature. DNA (5µg) was separated by electrophoreses on a 2% agarose gel containing ethidium bromide and visualized using BioRad XR Gel documentation system (Bio-Rad Hercules, CA).

4. Results

4.1 Berberine inhibits the growth of H1N1 influenza A in RAW 264.7 cells and bone marrow derived macrophages

The effect of berberine on the growth of influenza A was first tested with the murine macrophage-like cell line RAW 264.7 and the influenza A strain PR/8/34, a mouse-adapted strain of influenza A that has been used extensively in studies of influenza A pathogenesis and vaccine production. Typically, the replication time for A/PR/8/34 is 6-8 hr depending on cell type [33, 34]. Fig. 1A shows the results of a typical time course experiment for the growth of this virus in RAW 264.7 cells where infections were performed at a low moi (0.002) to approximate conditions of infection *in vivo*. A total of 10^3 FFU of virus was added to 5×10^5 cells in a 1 ml culture. As

shown in Fig. 1A, six h after the infection was initiated, levels of infectious virus in the culture supernatant had not increased; in fact only small amounts of virus were detectable likely representing residual inoculating virus. However, by 12 h we found that infectious virus reappeared in the culture supernatant and by 24 h levels of infectious virus had increased by 2-3 log units. Our experiments revealed that a concentration of 25 μM berberine strongly inhibited virus growth under these conditions (Fig. 1A). The increase in virus titer noted at 12 h was blocked completely and at 24 h the level of inhibition was 90%. Based on these experiments, a series of dose-response curves were performed at the 24 h time point. As shown in Fig. 1B, near complete inhibition of the growth of A/PR/8/34 in RAW 264.7 cells by berberine occurred at concentrations above 1 μM , and the IC_{50} was 0.01 μM . For this virus, berberine was more effective than amantadine, a known anti-influenza compound that targets the M2 protein of influenza. Amantadine displayed an IC_{50} of 27 μM in these experiments, which is comparable to the IC_{50} reported previously (33 μM) [35]. The inhibitory effect of berberine on the growth of influenza A was also seen with a second H1N1 virus (WS/33) (IC_{50} = 0.44 μM) (Fig. 1B).

The effect of berberine on the growth of influenza A was also tested with cultures of primary macrophages. Bone marrow derived murine macrophages (BMDM) were infected with strains A/PR/8/34 or A/WS/33 in the absence or presence of 25 μM berberine. Supernatants were harvested after 24-48 h and titers determined using the FFA. As shown in Fig. 1C, berberine inhibited the growth of

both viruses, but that the effect was variable. With strain A/PR/8/34, which produced only 10^2 FFU after 48 h of growth (after subtracting inoculating dose); we measured 30% inhibition by berberine (not statistically significant). On the other hand, we found that strain A/WS/33 grew faster, and to higher titers in BMDM. This growth was inhibited 53% by berberine at 24 h, which was statistically significant ($p=0.0001$)

4.2 Berberine inhibits the growth of influenza in A549 human lung epithelial cells but not MDCK cells

The effects of berberine were also tested with A549 human lung epithelial cells. These experiments were of interest because epithelial cells are a key host target cell for influenza A *in vivo*. As shown in Fig. 2A, berberine completely blocked the growth of strain A/PR8/34 with these cells (98% inhibition at 48 h), although we did note that this mouse-adapted viral strain grew poorly in this human cell type. Finally, we tested whether berberine could inhibit the growth of influenza A in the MDCK canine kidney cell line. This cell line is noted for its highly efficient replication of influenza A viruses (note the scale on the y-axis) [36-38] and is used routinely in plaque and focus forming assays. As shown in Fig. 2B, although we did measure 39 and 32 % growth inhibition with A/WS/33 and A/PR/8/34 strains, respectively, these values were not statistically significant.

4.3 Studies on the mechanism of viral growth inhibition by berberine

To gain insight into the mechanism of growth inhibition by berberine, we investigated its effects on the expression of several influenza A proteins using a polyclonal goat anti-H1N1 antiserum. Four proteins are typically recognized by Abs during influenza A infections, including HA (75 kDa), NA (58 kDa), NP (60 kDa), and M (25 kDa)[1]. As shown in Fig. 3A, in proteins prepared from cells infected at a low moi (0.002), this antiserum revealed three bands at the 12 and 24 h time points. Based on predicted molecular weights, the bands running at approximately 70 and 25 kDa are HA and M proteins, respectively, while the identity of the protein(s) running at approximately 60 kDa is not as clear. This band may represent either NA or NP proteins since under these SDS-PAGE conditions, their mass differences cannot be resolved. Fig. 3A also shows that treatment of infected cells with berberine strongly inhibited production of these proteins.

One possible interpretation of these data is that berberine is blocking virus protein production by inhibiting an early step in the virus replication pathway (i.e., entry, uncoating, transcription). Alternatively, it may be directly inhibiting the translation of viral proteins. However, since low multiplicity infections require several rounds of virus replication, release, and re-infection to achieve detectable levels of viral protein (or infectious virus) in the culture, it is also possible that berberine is inhibiting a later step in the replication pathway such as protein translocation or virus release. Inhibition of these processes would reduce the spread of the virus in culture and produce the same result as shown in Fig. 3A.

To address this question we also examined protein production in high multiplicity infections (moi=5). Under these conditions, each cell is infected at the start of the experiment (super-infections do not occur with H1N1 influenza A [39]) and proceeds through the early stages of the viral life cycle relatively synchronously with viral RNAs and proteins produced simultaneously by all cells in the culture. Release of progeny virus to fully infect the cells in culture is not required. As shown in Fig. 3B, under these conditions, berberine did not exert a strong effect on the production of any of proteins detected. This result indicates that the effects of berberine on viral protein production observed under low moi conditions (Fig. 3A) arose from inhibition of spread of the virus through the culture not from the inhibition of protein production by individual infected cells. The results shown in Fig. 3B also suggest that berberine is interfering with the growth of influenza A at a post-translational stage in the virus life cycle.

We addressed this hypothesis by examining intracellular trafficking of the influenza A HA protein, an important aspect of influenza A virus replication that occurs post-translationally. The influenza A HA protein follows a well defined translocation process through the endoplasmic reticulum (ER) and Golgi *en route* to the plasma membrane for the formation of virus particles [40]. To determine whether berberine interferes with this process, immunofluorescence experiments were performed using a mAb against the viral HA protein, in both low and high multiplicity infections. As shown in Fig. 4A, 20 h after low moi infections were initiated in the absence of berberine, HA displayed a pattern of cell surface

expression with weak intracellular staining. As shown in Fig. 4B, we found that berberine treatment of similarly infected cells produced a change in the pattern of HA staining. Intracellular HA staining was more pronounced while surface staining was reduced. Berberine also caused enhanced intracellular HA staining in cells infected under high moi conditions (compare Figs. 4C and D). These results suggest that the effect of berberine on the growth of influenza A may stem from a post-translational effect on the intracellular movement and/or maturation of viral proteins.

Finally, we considered the hypothesis that treatment with berberine is causing cell death and thereby reducing production of infectious virus or viral proteins. Previous studies with A549 cells [41] and THP-1 [42] macrophage-like cells cell lines have failed to find any cytotoxic effects with berberine. In agreement, as shown in Fig. 5A, we did not find any decrease in cell viability when RAW 264.7 cells were treated with 25 μ M berberine or a goldenseal extract with an equivalent berberine concentration. In addition, we did not find any evidence of apoptosis, when assayed by DNA fragmentation, in RAW 264.7 cells treated by berberine or goldenseal extract with equivalent berberine concentration (Fig. 5B).

4.4 Berberine inhibits influenza-induced production of TNF- α and PGE₂

In these experiments, we evaluated the effects of berberine on the ability of influenza A to induce inflammatory mediators from infected macrophages. We focused on TNF- α , one of the major pro-inflammatory cytokines associated with influenza pathogenesis; [43-46] and PGE₂, which recent reports suggest is also

responsible for many of the symptoms associated with infections by influenza A [47, 48]. RAW 264.7 cells were infected with strain A/PR/8/34 and levels of TNF- α and PGE₂ in culture supernatants were determined by ELISA. In preliminary experiments with low moi infections, we found that levels of inflammatory mediator production were low and inconsistent. Therefore, in these experiments, infections were performed at an moi of 5. As shown in Figs. 6A and B, we found that cells infected in this manner produced consistent high levels of TNF- α and PGE₂, respectively. Both mediators were readily detected at both 12 and 24 h time points. In contrast, in the presence of 25 μ M berberine, we found strong, significant inhibition of both TNF- α and PGE₂ (Figs. 6A and B) ($p < 0.05$) at both 12 and 24 h time points.

4.5 Goldenseal extracts can inhibit influenza growth and block the production of TNF- α and PGE₂

Previous results from our laboratories have shown that the activity of botanical compounds may differ when tested in purified form or as components of crude extracts [49]. Therefore, we tested whether berberine-containing extracts of goldenseal also display anti-viral and anti-inflammatory activity. Samples of goldenseal were collected, extracts produced, and levels of berberine measured by LC-MS [26]. As shown in Fig. 7A, with extract dilutions containing greater than a 2.5 μ M concentration of berberine, we found complete suppression of virus growth, equivalent to the effect of purified berberine. However, when extracts were diluted

to contain a concentration of 0.25 μM berberine, we found that the extracts were not as effective as purified berberine. As a result, IC_{50} values for extracts were greater than for purified berberine. The *H. canadensis* root extract displayed an IC_{50} value of 0.22 μM , while an extract produced from *H. canadensis* leaves displayed an IC_{50} of 0.40 μM . Finally, a dilution of a root extract with a concentration of 25 μM berberine was tested for its ability to suppress production of inflammatory mediators. As shown in Figs. 7B and C, the root extract strongly inhibited production of $\text{TNF-}\alpha$ and PGE_2 , at levels similar to those seen with purified berberine (see Fig. 6).

5. Discussion

Our investigations have revealed that berberine can inhibit the growth of influenza A *in vitro* with two different H1N1 strains of influenza A. This effect was observed with two murine macrophage type cells (RAW 264.7 macrophage-like cells and normal bone marrow derived macrophages) and the A549 human lung epithelial-derived cell line. Dose response curves with the A/PR/8/34 virus growing on RAW 264.7 cells revealed several orders of magnitude more inhibition by berberine than with amantadine, a well characterized M2 inhibitor. The A/PR/8/34 virus is known to be relatively resistant to amantadine [50] and its susceptibility to berberine may indicate that berberine is working through a distinct mechanism. In addition, we showed that berberine can inhibit the influenza A-induced production of $\text{TNF-}\alpha$ and PGE_2 from RAW 264.7 macrophages.

Berberine has been shown to exert a large number of effects on cellular machinery; including the reduction of F-actin polymerization [51], moderation of lipid and glucose metabolism [52], direct binding to polyadenylic acid [53], and modulation of MAP family kinase activity [54]. To gain insight into the effect of berberine on the growth of influenza A, we focused our studies on the production of viral proteins. We reasoned that finding inhibitory effects on protein production in high multiplicity infections would allow us to concentrate future studies on early events in the viral replication cycle, such as entry or transcription of viral genes. Conversely, a lack of effect of berberine on protein production would allow us to shift our focus to later events in the replication cycle. Our experiments with high moi infections did not reveal an effect of berberine on the expression of several different viral proteins, suggesting that berberine is not affecting the entry and uncoating of the virus or transcription and translation of viral mRNAs. Berberine also likely does not interfere with production of viral genomic RNAs (vRNA, cRNA), since these molecules are produced prior to mRNA synthesis. On the other hand, we did detect a change in the intracellular position of HA in infected cells that were treated with berberine. The HA protein did not display its normal pattern of cell surface staining. Instead, a more punctuate intracellular pattern was observed. It is possible, therefore, that berberine is acting to block the intracellular translocation as HA as it progresses toward the cell surface. Alternatively, berberine may be causing HA to misfold, and the punctate intracellular staining we observed arises from

accumulations of HA targeted for degradation. Finally, we did not observe any evidence of berberine-induced cell death.

Interestingly, we did not find a significant inhibitory effect of berberine on growth of influenza A with the MDCK cell line. This cell line is used extensively *in vitro* for the growth of influenza A, and produces many orders of magnitude more virus than any of the other cells we have tested (Fig 2B). It is possible, therefore, that the changes in this cell that allow for highly efficient production of influenza A also enable it to be resistant to the effects of berberine. MDCK cells are, for example, more efficient in the folding, maturation, and subsequent transport of HA and NA proteins, and it is these changes that may endow them with resistance to berberine [55]. The inability of berberine to inhibit influenza A growth in MDCK cells may also support a role for the host interferon response in the effect of berberine. Seitz et.al, have shown that the canine interferon response is ineffective against influenza A [56]. Finally, it is also possible that these cells are resistant to all effects of berberine because they have elevated levels of an MDR protein responsible for berberine efflux. This type of berberine resistance is common among tumor derived cells lines [57, 58].

In addition to the effects of berberine on the growth of the virus, we found that berberine could strongly inhibit production of TNF- α and PGE₂, two mediators linked to the symptoms and pathology associated with infections by influenza A. Activation of the host innate response to influenza A has been studied extensively [59, 60]. These studies have shown that recognition of influenza A v- and cRNA by host

(TLR)-3, -7, -8, RIG-I/MDA5, and NLRs is the key to initiating this response [61-64]. Collectively, activation of these TLR pathways leads to activation of a number of different transcription factors including NF- κ B, IRF-3, IRF-7, AP-1 and IKK ϵ [65-69]. The inhibitory effects of berberine on mediator production could arise at any number of points in these pathways. Production of a viral RNA ligand could be blocked, although this is unlikely since our experiments were conducted under high moi conditions, where all cells will contain high levels of viral RNAs and proteins (which we confirmed). It is also possible that berberine is interfering with ligand recognition by one of the TLRs. As noted above, berberine has been shown to bind polyadenylic acid [53, 70, 71], and it may be that berberine-bound influenza A RNAs do not effectively trigger signaling through one or more TLR pathways. It is also possible that berberine is acting downstream in the TLR pathways, preventing activation of key transcription factors. Berberine has been shown to block the activity of a number of kinases [54, 72-74], several of which are known to regulate the activity of pro-inflammatory transcription factors.

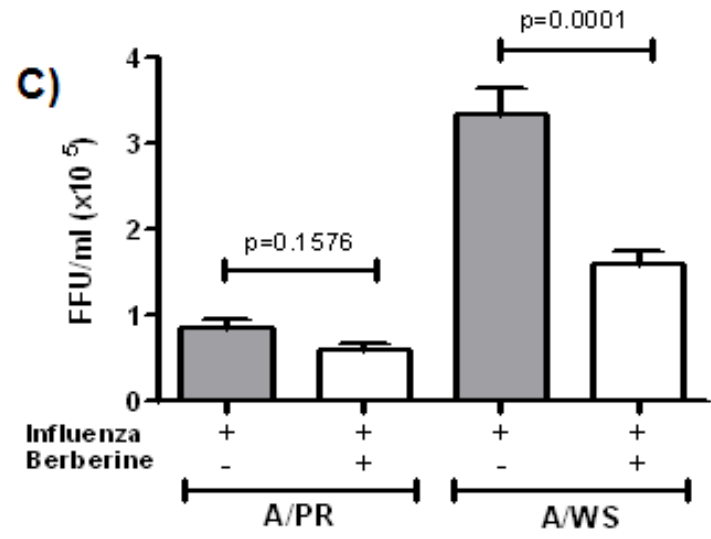
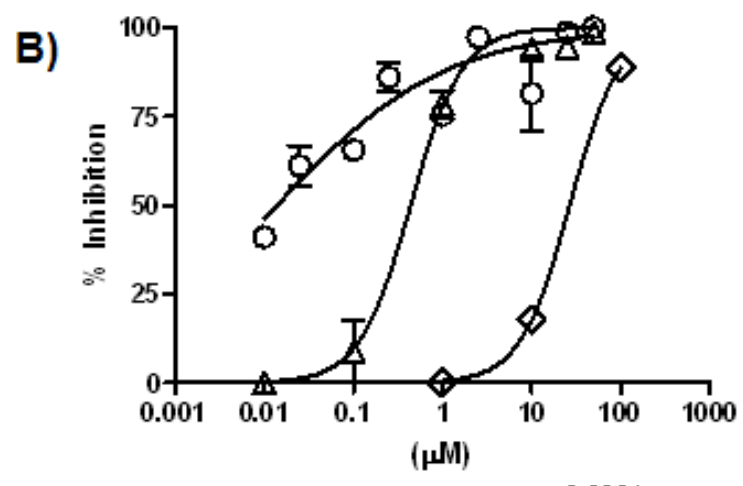
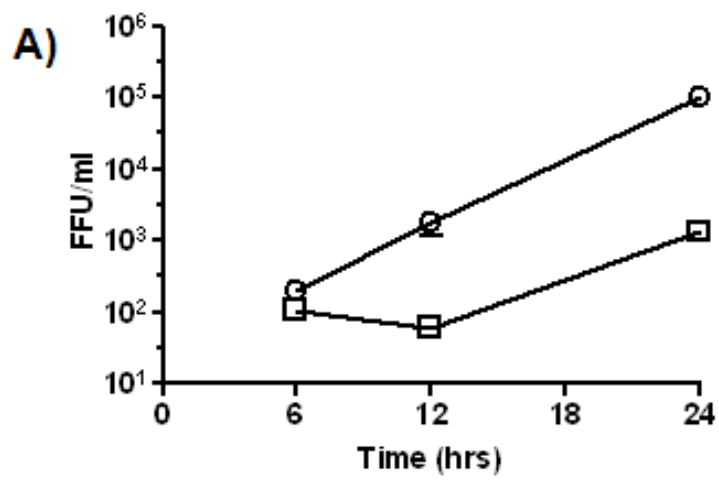
The results of our experiments suggest that berberine may be useful for the treatment of influenza A. Since berberine is derived from a natural product, many individuals may prefer to use a berberine-containing plant extract rather than the purified compound. Therefore, we sought to determine whether goldenseal extracts containing berberine also exert the anti-viral and anti-inflammatory effects. The results of our investigation revealed that at high concentrations, the extracts inhibited the growth of influenza A at levels that would be predicted from their

berberine concentration. However, in more dilute samples; the extracts were less effective than would have been predicted from their berberine concentration. The molecular basis for this finding is not clear. It is possible that the extracts contain other compounds that reduce the available concentration of berberine effectively causing a shift in the dose response curve. Alternatively, the extracts may contain compounds that counteract the effects of berberine on the infected cell.

In summary, we have shown that berberine can effectively inhibit the growth of two H1N1 strains of influenza A with a number of different cell types. We have also shown that berberine can inhibit the production of TNF- α and PGE₂ from infected RAW 264.7 cells. Experiments using animal models will be necessary to determine whether these effects are also seen *in vivo*. However, given that berberine has proven effective for suppressing acute inflammation in mice treated with LPS [31], there is no a priori reason why it would not be effective for the treatment of influenza A infections *in vivo*.

Figure 1. Effects of Berberine on the growth of influenza A in macrophage-type cells.

A) RAW264.7 cells were infected with A/PR/8/34 (moi=0.002, 1000 FFU) for varying times in the absence (○) or presence (□) of 25 μM berberine. Supernatants were collected and virus titers determined using a focus forming assay with MDCK cells. Values shown are means +/- S.E.M. of triplicate measurements of virus titers from a single representative experiment. B) RAW 264.7 cells were infected with A/PR/8/34 (○) or A/WS/33 (Δ) at moi=0.002 for 24 h in the presence or absence of varying doses of berberine. Titers were determined as described above with % inhibition calculated by comparison with controls. Values shown are from three independent experiments with virus titers determined in duplicate in each experiment. Growth of strain A/PR/8/34 in the presence of amantadine (◇) is also shown. C) Bone marrow derived macrophages infected with either A/PR/8/34 or A/WS/33 virus (moi=0.002, 1000 FFU) in the absence or presence of 25 μM berberine for 48 h or 24 h, respectively. Values shown are means +/- S.E.M. from three independent experiments with virus titers determined in duplicate in each experiment.



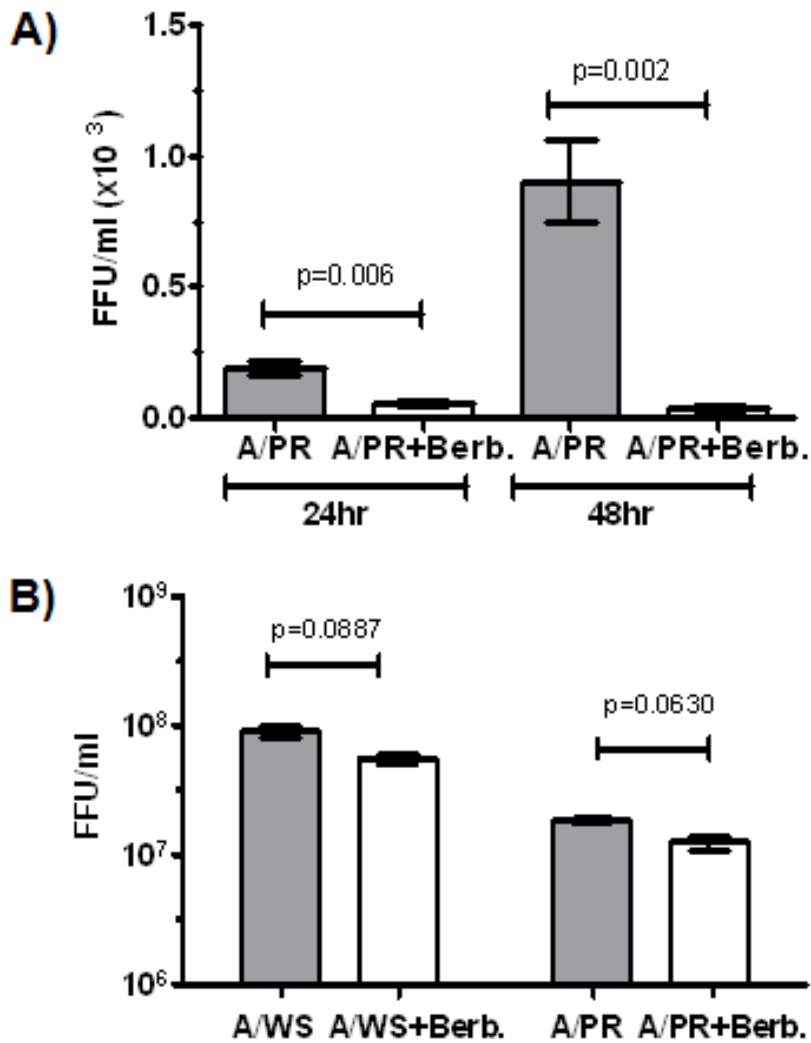
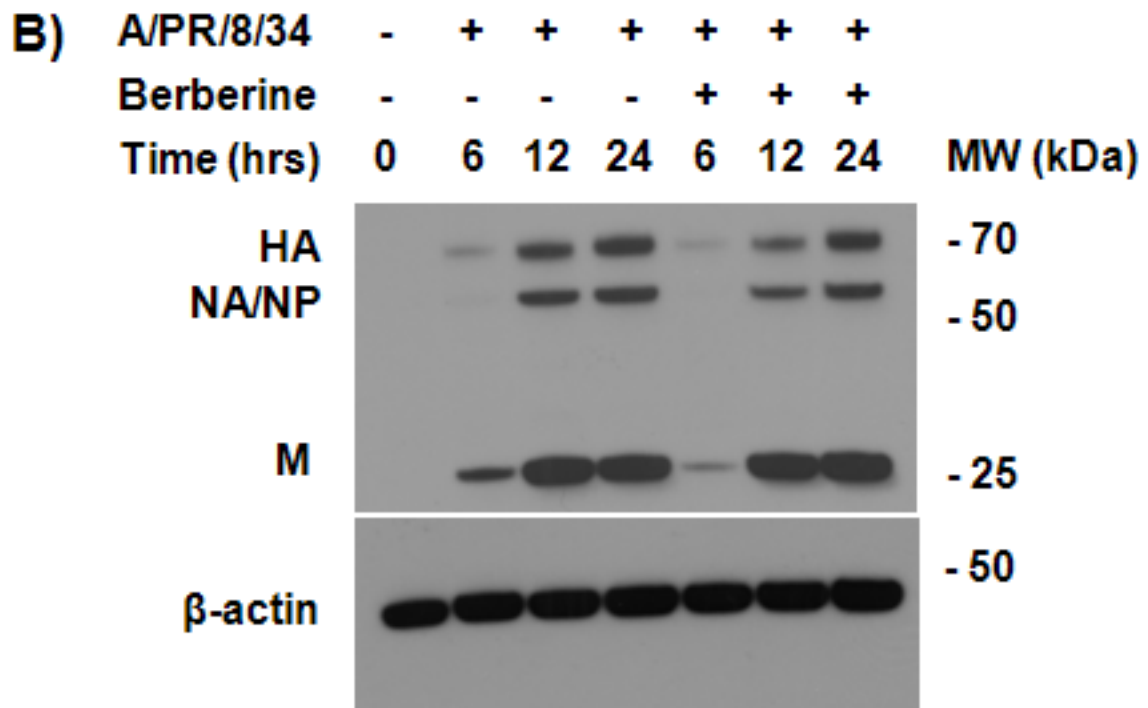
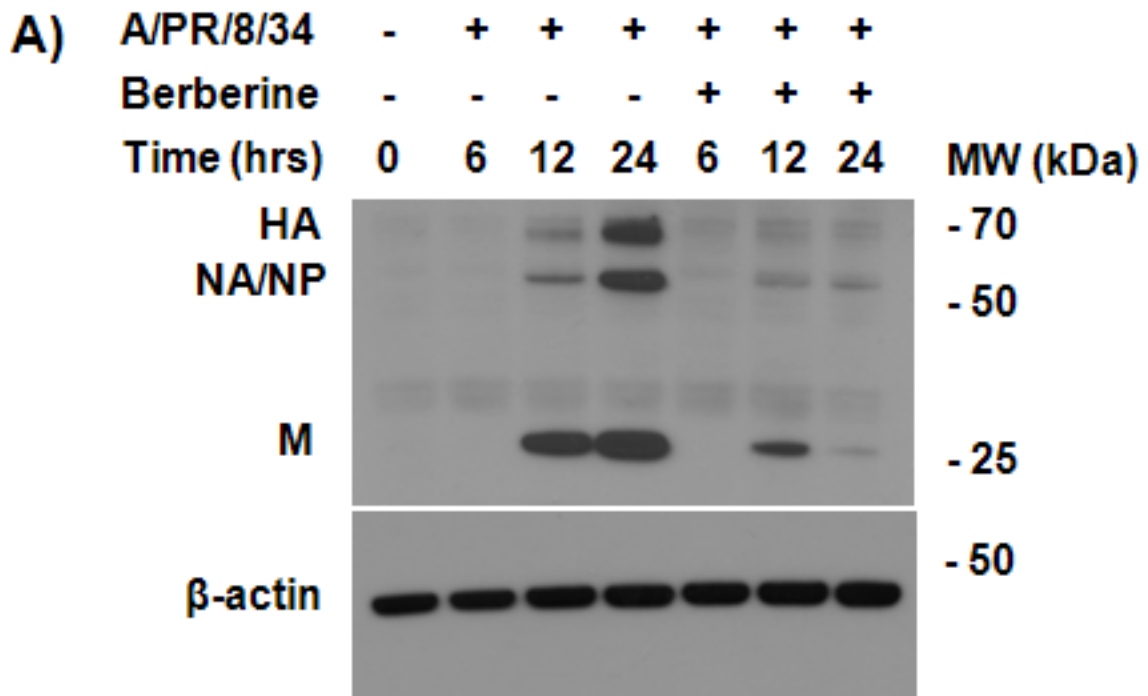


Figure 2. Effects of berberine on the growth of influenza A in additional cell types.

A) A549 human epithelial cells were infected with A/PR/8/34 (moi=0.002, 300 FFU) for 24 or 48 h in the absence or presence of 25 μ M berberine. Supernatants were collected and viral titers determined using a focus forming assay with MDCK cells. B) MDCK cells were infected with influenza strains WS/33 or PR/8/34 (moi=0.002, 400 FFU) for 24 h in the presence or absence of 25 μ M berberine. Values shown are means \pm S.E.M. from three independent experiments with virus titers determined in duplicate in each experiment.

Figure 3. Effects of berberine on expression of influenza A proteins.

RAW 264.7 cells were infected with A/PR/8/34 at (A) low moi (0.002) or (B) high moi (5) for indicated times in the absence or presence of 25 μ M berberine. Expression of influenza A proteins were detected by immunoblot assay using a polyclonal goat anti-H1N1 primary antibody and anti-goat-HRP secondary antibody. Identification of viral proteins based on predicted molecular weights. Blots were reprobed with mAb to β -actin to ensure equal loading of samples. Blots shown are representative of two experiments at each multiplicity of infection.



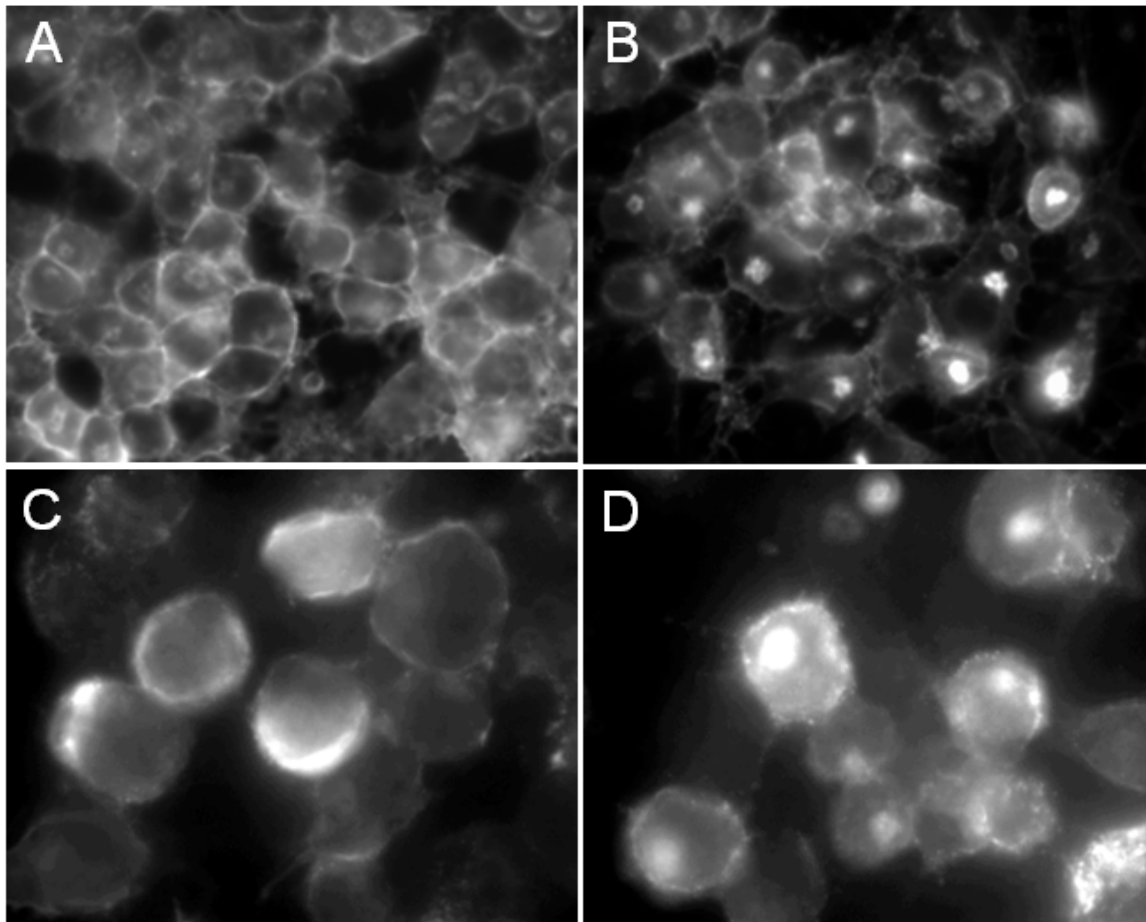


Figure 4. Effects of berberine on HA protein localization.

RAW 264.7 cells were grown on 8-well chamber slides and infected with strain A/PR/8/34 at low moi (0.002) for 20h in the absence (A) or presence of 25 μ M berberine (B). Cells were also infected with A/PR/8/34 at high moi (5) for 12 h in the absence (C) or presence of 25 μ M berberine (D). Cells were fixed, permeabilized and HA protein detected using an anti-HA mAb followed by a TRITC-labeled goat anti-mouse secondary Ab. Staining was visualized using a Zeiss Axioskop 2 plus fluorescence microscope. Magnifications are 40X (A and B) and 100X (C and D). Images are representative of at least five experiments performed at each multiplicity of infection.

Figure 5. Effect of berberine and goldenseal extract on cell viability.

RAW 264.7 cells were treated with media, 25 μ M berberine, or the goldenseal root extract containing an equivalent berberine concentration. A) Following 12 and 24 h incubation periods cells were harvested and viable cells counted by staining with trypan blue. B) Identically treated cultures were harvested; DNA isolated as indicated in the Materials and Methods, and separated on 2% agarose gels. A treatment with 100 μ g/ml cycloheximide was also included as a positive control for induction of apoptosis. Results shown are representative of four independent experiments.

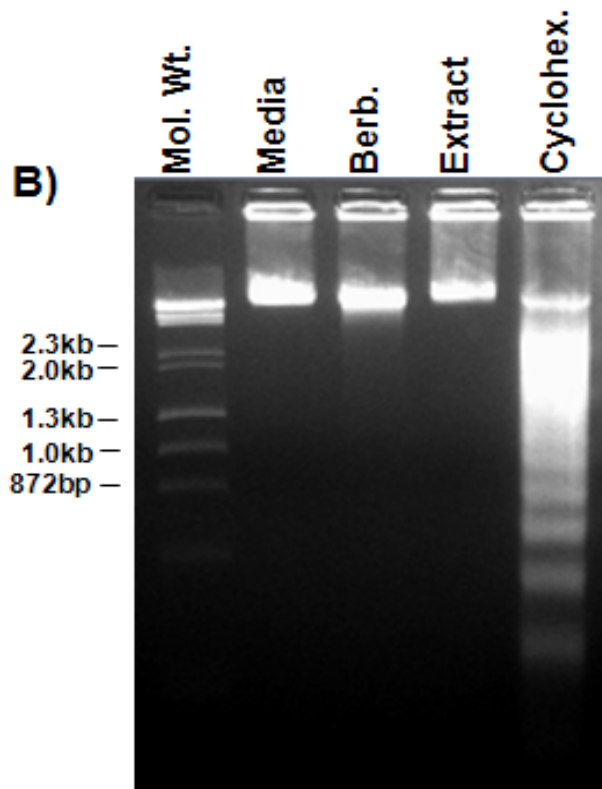
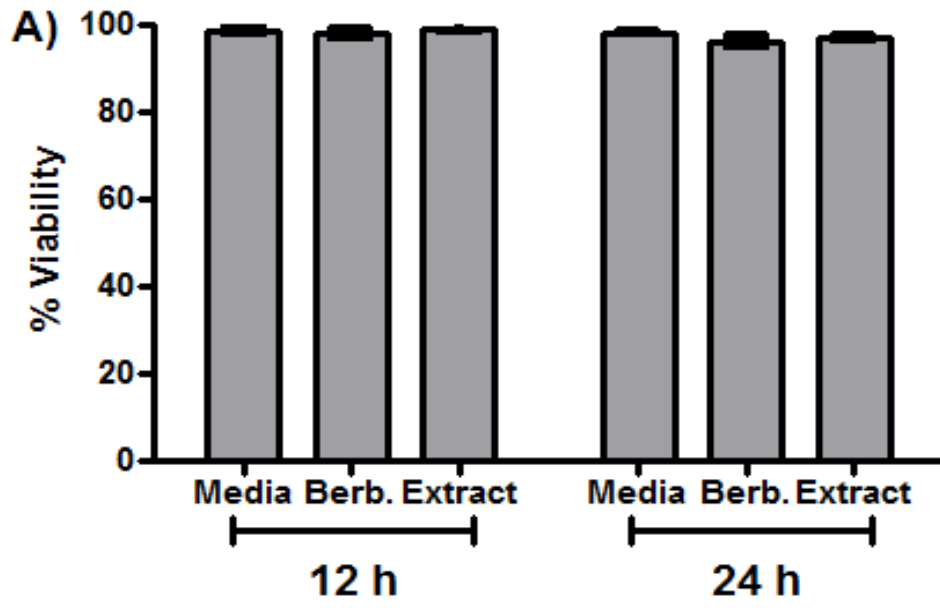


Figure 6. Effect of berberine on the production of TNF- α and PGE₂ from RAW 264.7 macrophage-like cells.

RAW 264.7 cells were infected with strain A/PR/8/34 (moi=5) 12 or 24 h in the presence or absence of 25 μ M berberine. Cell supernatants were collected and levels TNF- α (A) or PGE₂ (B) determined using commercial ELISA kits. Values shown are means \pm S.E.M. from two independent experiments with mediator determinations performed in duplicate in each ELISA assay.

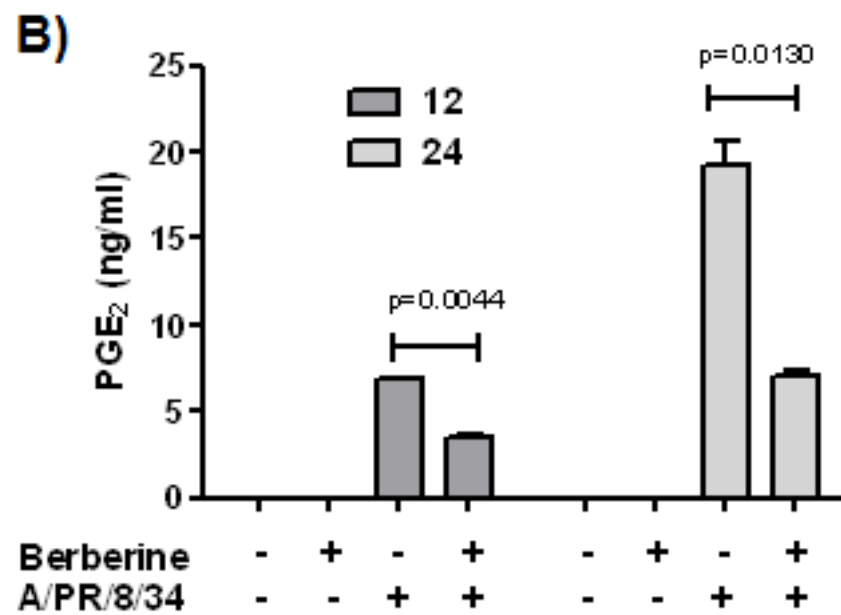
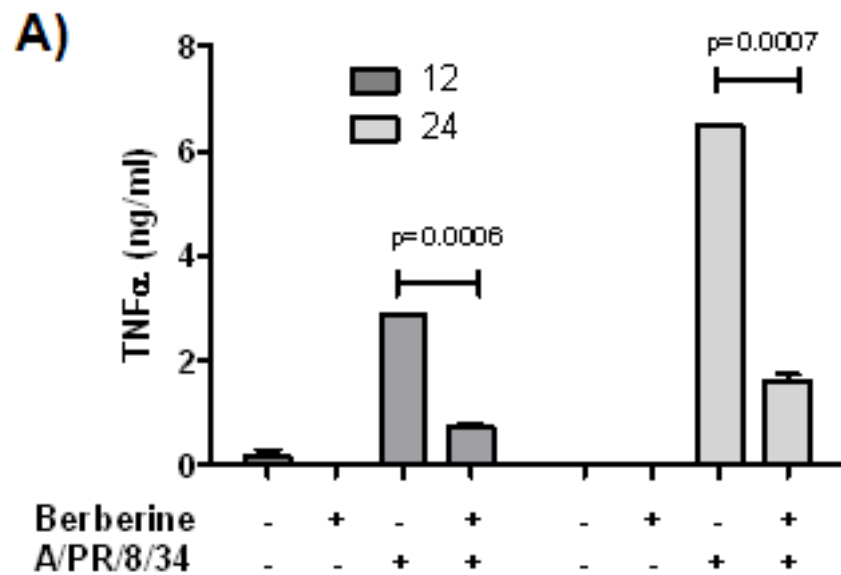
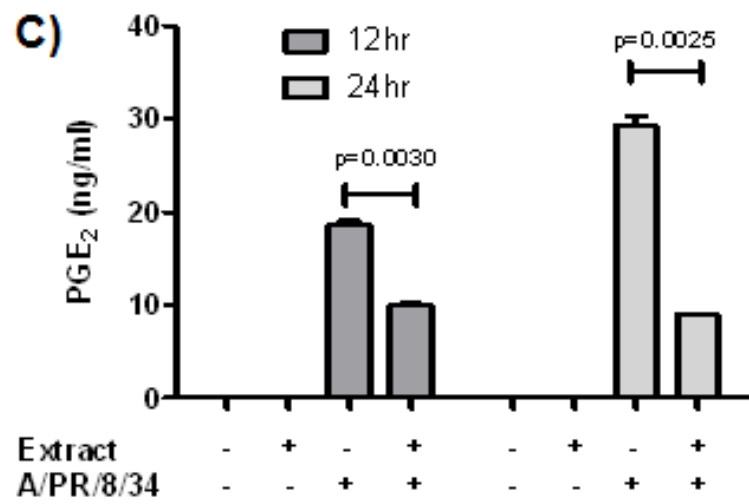
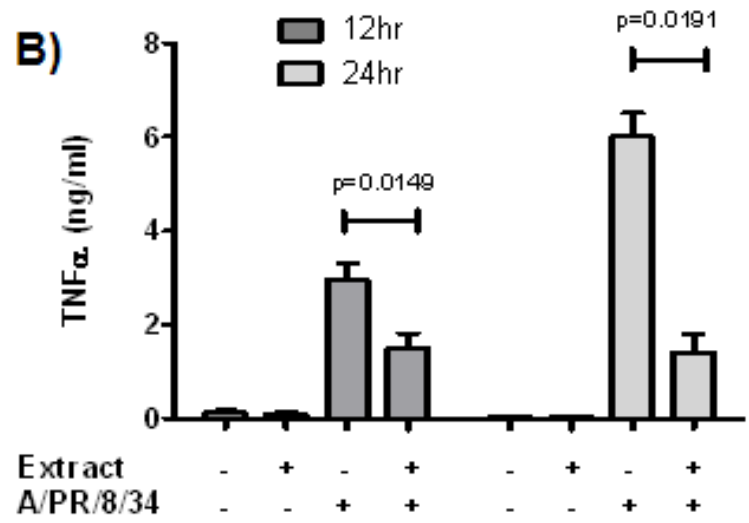
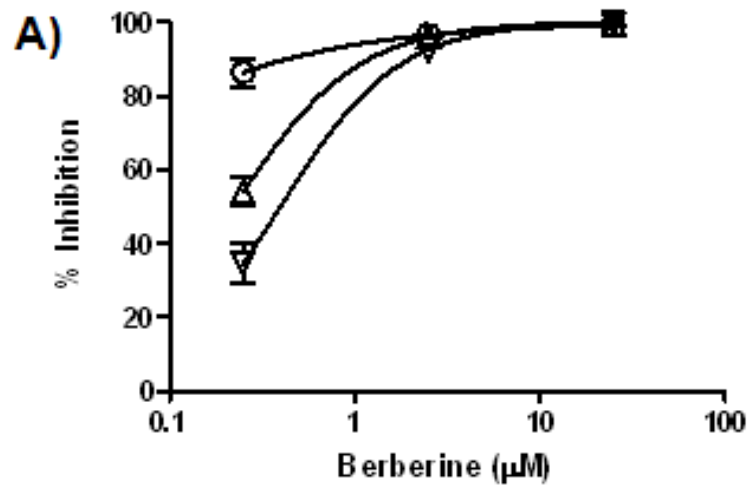


Figure 7. Effect of goldenseal extracts on the growth of influenza A and the influenza A-induced production of TNF- α and PGE₂.

A) RAW 264.7 cells were infected with strain A/PR/8/34 (moi=0.002) for 24 h in the absence or presence of 25 μ M berberine (\circ) or a dilution of pooled root (Δ) or leaf (∇) extracts containing equivalent berberine concentrations. Culture supernatants were collected and virus titers determined using a focus forming assay with MDCK cells. Percent inhibition of viral growth was calculated as in Fig. 1. Values shown are means \pm S.E.M. from three independent experiments with virus titers determined in duplicate in each experiment. *B and C*) RAW 264.7 cells were infected with strain A/PR/8/34 (moi=5) for 24 h in the absence or presence of a dilution of a single root goldenseal extract containing a 25 μ M concentration of berberine. Levels of TNF- α (*B*) or PGE₂ (*C*) were determined by ELISA. Values shown are means \pm S.E.M. from 5 (12 h) or 2 (24 h) independent experiments with mediator determinations performed in duplicate in each ELISA assay.



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CHAPTER 3

Characterization of the effects of berberine on LPS- induced prostaglandin E₂ (PGE₂) production

CHAPTER 3

1. Abstract

In this study we evaluated the effects of the isoquinoline alkaloid berberine (BBR) on the production of PGE₂ in response to LPS stimulation. Our studies examined the effects of berberine on the key enzymes in the PGE₂ biosynthetic pathway. Our experiments demonstrated a minor effect on the cPLA₂-mediated release of arachidonic acid. Our studies indicate that berberine does not affect the expression or activity of the COX-2 enzyme. Also, we demonstrate that berberine does not inhibit the expression or activity of the mPGES-1 enzyme. Taken together, our results suggest that berberine may inhibit the production of PGE₂ through a novel and as yet unidentified mechanism.

2. Introduction

Eicosanoids are a family of arachidonic acid (AA)-derived lipid molecules that mediate many of the hallmark signs of inflammation (fever, pain, swelling and redness) [1, 2]. Eicosanoids consist of the prostanoids (prostaglandins, thromboxanes, and prostacyclins) and leukotrienes. Prostaglandin E₂ (PGE₂) is the most abundant prostanoid [3] and an important mediator of inflammation [4-6]. PGE₂ has been shown to be one of the causes of the chronic inflammation and pain associated with rheumatoid arthritis and osteoarthritis [7, 8]. Levels of PGE₂ are

also elevated in acute responses to microbially-induced inflammation as in cases of bacterial endotoxemia [9] and influenza infection [10].

The production of PGE₂ depends upon a series of enzymatic modifications of AA. In the first reaction, the release of AA from the cell membrane in response to inflammatory stimuli depends upon the cytosolic phospholipases A₂ (cPLA₂) [11, 12]. cPLA₂ liberates AA by cleaving the fatty acid from cell membrane phospholipids at the *sn*-2 position [2]. This calcium dependent enzyme is activated through phosphorylation by mitogen-activated protein kinases (MAPK) [13, 14]. In the second reaction, the inducible membrane-bound glycoprotein, cyclooxygenase 2 (COX-2) cyclizes the AA into PGG₂ and reduces the hydroperoxy group of PGG₂ to form PGH₂ [15, 16]. PGH₂ is the substrate for the various prostanoid synthases. The microsomal prostaglandin E synthase-1 (mPGES-1) enzyme is co-regulated with COX-2, is localized to the perinuclear region with COX-2 and is primarily responsible for the production of PGE₂ in response to inflammatory stimuli [17, 18].

The COX-2 and mPGES-1 enzymes are attractive targets for drug development to limit inflammation through inhibition of PGE₂ production. The use of NSAIDs to reduce PGE₂ production has been limited due to indiscriminate targeting of COX enzymes which with long term use may result in gastrointestinal complications. The development of COX-2-specific inhibitors has also been limited due to the renal and cardiovascular side effects [19]. Recently, the development of novel mPGES-1 inhibitors has shown promise to inhibit the production of PGE₂ [20]. However, this approach has also been criticized due to the possible redirection of

PGH₂ towards other prostaglandin synthases with potential unknown consequences [21]. Based on these potential complications of current therapies, it is critical that novel compounds with unique mechanisms to limit production of PGE₂ be investigated.

The isoquinoline alkaloid berberine is found in a variety of medicinal plants such as goldenseal (*Hydrastis canadensis*), barberry (*Berberis vulgaris*), coptis (*Coptis chinensis*), and Oregon grape (*Mahonia aquifolium*). Our group and others have demonstrated that berberine can attenuate PGE₂ production in a variety of cells in response to various proinflammatory ligands [22-24]. Several mechanistic studies suggest that berberine decreases expression of the COX-2 enzyme [23, 25-27], reduces expression and phosphorylation of cPLA₂ [28], and reduces expression and phosphorylation of upstream MAPKs [25, 26]. However, the results from a number of other studies do not agree with these findings and indicate that the effects of berberine on PGE₂ production are not fully understood. For example, Kim et.al., [29] did not see inhibition of PGE₂ or COX-2 in 100ng/ml LPS treated RAW 264.7 cells with up to 40μM berberine. In other studies using a 13-alkyl-substituted berberine with 10ng/ml LPS treated RAW 264.7 cells, berberine reduced PGE₂, but had no effect on COX-2 expression [24].

In this report, we examine the effects of berberine on the expression and enzyme activity of the key proteins involved in the production of PGE₂ in response to LPS stimulation. Our studies confirm that berberine can strongly inhibit PGE₂ production in response to LPS stimulation. However, we did not observe substantial

effects on the expression or activity of any of the individual enzymes in the PGE₂ biosynthetic pathway. These results indicate that berberine may inhibit the production of PGE₂ by another mechanism not directly related to the activity or expression of the key enzymes of the PGE₂ biosynthesis pathway.

3. Materials and methods

3.1 Cell lines, media, reagents

Cell lines were obtained from the American Type Culture Collection (Manassas, VA). RAW 264.7 cells were cultured in Dulbecco's modification of minimal essential medium (DMEM) with 4 mM L-glutamine, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate with 10% fetal calf serum (FCS). Media, berberine chloride and supplements were obtained from Sigma-Aldrich (St. Louis, MO) and Cellgro (Manassas, VA). FCS was obtained from Atlanta Biologicals (Atlanta, GA) and Gemini Bio-products (West Sacramento, CA). Cells were cultured at 37°C and 5% CO₂.

3.2 PGE₂ ELISA

PGE₂ ELISA kits were purchased from Enzo Life Sciences (Farmingdale, NY). Assays were performed according to manufacturer's recommendations. In each case, sample values were interpolated from standard curves. Optical density was

determined using a PolarStar microplate reader (BMG Lab technologies, Durham, NC).

3.3 Arachidonic acid release assay

A total of 2.5×10^4 cells was plated into 24-well flat-bottom tissue culture plates (Fisher Scientific) and labeled overnight with 0.1 $\mu\text{Ci/ml}$ [^3H] AA. The following morning, the cells were washed twice with HBSS, allowed to recover for an additional 2 h, and washed again before treatment. At indicated time points, 275- μl aliquots of medium were removed from the wells and centrifuged to remove debris. A total of 200 μl of the supernatant was removed for scintillation counting (Beckman Coulter model LS 5801), and total [^3H]AA release was calculated by multiplying by a factor of 2. Each point was performed in triplicate, and maximum radiolabel incorporation was determined by lysing untreated controls with 0.01% SDS and counting the total volume.

3.4 Immunoblot analysis

RAW 264.7 cells were plated at a cell density of 5×10^5 cells/60mm tissue culture dish (Corning, Corning, NY) for 24 h, then treated with 1 $\mu\text{g/ml}$ of LPS. At indicated times, cell monolayers were washed twice with cold phosphate buffered saline (PBS) (Gibco, Carlsbad, California), lysis buffer added (50 mM HEPES, pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM leupeptin, 0.5% SDS), lysates collected by

scraping, and centrifuged for 30 min at 14,000 rpm. The protein concentration for each sample lysate was determined using the Pierce BCA system (Pierce, Rockford, IL). Equal protein samples (10 µg) were loaded on 4-12% Bis-Tris gels (Invitrogen, Carlsbad, CA) and subjected to electrophoresis using the Novex Mini-Cell System (Invitrogen). Following transfer to PVDF membranes, (Millipore, Billerica, MA) membranes were blocked for 24 h with 5% powdered milk in TBS/0.1% Tween-20 and probed with a primary rabbit anti-COX-2 (Cell Signaling Danvers, MA) for 1 h and secondary goat anti-rabbit HRP for 45 minutes (Sigma St. Louis, MO) diluted in 1% powdered milk in TBS/0.1% Tween-20. Bands were visualized using ECL Plus Western Blotting Detection System (GE Healthcare, Piscataway, NJ).

3.5 qRT-PCR

Total RNA was extracted using the RNAeasy kit (Qiagen, Valencia, CA) according to the manufacturer's specifications. Amount and purity of RNA was determined using a Nanodrop 1000 spectrophotometer (ThermoFisher Scientific, Waltham, MA). RNA (1 µg) was denatured and reverse transcription was performed with the Improm II reverse transcription kit (Promega, Madison, WI) in a reaction mix containing oligo dT as primers (50 ng/µl) for 60 min at 42°C. The iQTM SYBR Green supermix kit (BioRad, Hercules, CA), was used for Real-time PCR analysis. cDNA was amplified using primers specific for murine GAPDH, COX-2 and mPGES-1 genes. Primer combinations are GAPDH [antisense: 5' ATG TCA GAT CCA CAA CGG ATA GAT 3'; sense: 5' ACT CCC TCA AGA TTG TCA GCA AT 3']; COX-2

[antisense: 5' AAC TGCV AGG TTC TCA GGG ATG TGA 3'; sense: 5' ACT GGG CCA TGG AGT GGA CTT AAA 3']; mPGES-1 [antisense: 5' ATG GGT CTG GAG AAA TGG CTC AGT 3'; sense: 5' TGC CCA TGG AGA CCA GAA GAA GTT 3']. All primer pairs were purchased from Integrated DNA Technologies (Coralville, IA). PCR was performed in 96 well plates (Eppendorf AG, Hamburg, Germany). Samples were amplified for a total of 50 cycles, followed by a meltcurve analysis to ensure the specificity of reactions. Experimental samples were normalized to GAPDH and fold induction was calculated by the $\Delta\Delta C_T$ method.

3.6 COX-2 enzyme assay

The activity of the COX-2 enzyme was measured using the protocol by Gierse and Koboldt [30]. Briefly, purified COX-2 enzyme (38.04 μ g/rxn) (Cayman Chemical Ann Arbor, Michigan) was added to 1 cm glass cuvettes containing a 3ml final volume of peroxidase assay buffer containing 100mM Tris-HCl, pH 8.1, and 1 μ M Bovine Hemin in dH₂O in the presence or absence of the known inhibitor NS-398 (10 μ M) or indicated doses of berberine (Sigma St. Louis, MO). Samples were equilibrated to 25°C in a temperature regulated spectrophotometer for 30 seconds (Shimadzu UV-2401 PC UV-Vis Spectrophotometer Kyoto, Japan). The reaction was initiated with the addition of 200 μ M N,N,N',N'-Tetramethyl-p-phenylenediamine (TMPD) and 100 μ M arachidonic acid. The TMPD serves as a cosubstrate electron donor that turns blue upon reduction by the intrinsic peroxidase activity of the COX-2 enzyme. This color change is read at 611nm using the kinetic read function of the

spectrophotometer software (Shimadzu UV Probe Software v2.10 Kyoto, Japan) for 2 minutes at 25°C. The rate of the reaction is calculated as the change in OD units per minute using the following equation: $(\text{OD units/min}) \times (0.17\text{mM TMPD} / 13.5 \text{ OD units}) \times (1 \text{ mol arachidonic acid} / 2 \text{ mol TMPD}) \times (2\text{mol O}_2 / 1\text{mol AA}) \times (0.003\text{liter} / 0.03804\text{mg enzyme}) = \text{mmol O}_2 / \text{min} \bullet \text{mg}$.

3.7 mPGES-1 enzyme assay

The activity of the mPGES-1 enzyme was measured by the conversion of PGH₂ to PGE₂ in an *in vitro* cell free assay. In a 96 well plate, 5µg of purified mPGES-1 enzyme (Cayman Chemical) was added to reaction buffer (100mM NaPO₄ pH 7.0, 5mM Triton X-100, 1mM EDTA, 2.5mM reduced glutathione) in the presence or absence of varying doses of the known mPGES-1 inhibitor MK886 (Cayman Chemical) or indicated doses of berberine for 20 minutes at 25°C. The reaction was initiated with 4µM PGH₂ and incubated for 1 minute at 25°C. Final volume for each reaction was 125µl. To quench the reaction, 10µl of each reaction was transferred to 490µl of 25mM FeCl₂ (1:50) then diluted to 1:5000 final in PGE₂ ELISA assay diluent provided in the PGE₂ ELISA kit (Enzo Life Sciences Farmingdale, NY). Production of PGE₂ was measured according to manufacturer's instructions.

4. Results

4.1 Berberine inhibits LPS-induced production of PGE₂

Several, but not all, research studies have demonstrated that berberine can inhibit the production of PGE₂ in response to LPS stimulation. As shown in Fig.1A, we found strong inhibition of PGE₂ by berberine. Fig. 1A shows the results of a typical dose response curve in response to 1µg/ml LPS in the presence of berberine ranging from 3.25-25µM. Berberine demonstrated strong dose-dependent inhibition of PGE₂ in response to LPS stimulation at 24 hrs (IC₅₀=5.2µM). Fig. 1B shows the results of a typical time course experiment in response to 1µg/ml LPS in the presence or absence of a concentration of 25µM berberine. Typically, we found that in the absence of berberine, RAW 264.7 cells began producing PGE₂ appx. 6 hrs after LPS stimulation and production continued linearly during the remainder of the experimental period. In the presence of berberine, this effect was strongly attenuated. Berberine inhibited the production of PGE₂ by 66% at 12 hrs and 85% at 24 hrs.

4.2 Effect of berberine on arachidonic acid release

In order to define the mechanism behind the inhibition of PGE₂ production, we sought to determine what effects berberine had on the various enzymes in the PGE₂ biosynthetic pathway. Production of PGE₂ begins with the release of arachidonic acid from cell membranes by PLA₂-type enzymes. cPLA₂ is known to be important for the release of arachidonic acid during inflammatory reactions although there are

several additional enzymes that can display this activity. To confirm the role of cPLA₂ in this response, we tested a known cPLA₂ inhibitor as well as an inhibitor of another PLA₂, the calcium-independent phospholipase 2 known as iPLA₂, in an arachidonic release assay. Following the labeling of RAW 264.7 cells for 24 hrs with ³H-arachidonic acid, cells were treated with 1µg/ml LPS in the presence or absence of either cPLA₂ inhibitor or the iPLA₂ inhibitor bromoenol lactone. As shown in Fig. 2A, we found that the cPLA₂ inhibitor, but not the iPLA₂ inhibitor, strongly inhibited the release of ³H-AA. Levels of ³H-AA release in the presence of the cPLA₂ were comparable to background levels of release. These data suggest that cPLA₂ is the enzyme responsible for release of arachidonic acid in RAW 264.7 cells treated with LPS.

To ask whether berberine's effect on the production of PGE₂ could be mediated through effects on cPLA₂ we evaluated the effect of BBR in this assay. As shown in Fig. 2B, berberine only partially inhibited the release of AA (i.e., 27% at 24 hrs). Based on the 60-80% inhibition seen in PGE₂ production it is therefore unlikely that the minor inhibition of cPLA₂ by berberine could completely account for the reduction in PGE₂. This observation led us to consider the effects of berberine on enzymes further downstream in this pathway.

4.3 Berberine does not affect COX-2 expression

In order to evaluate the effects of berberine on the expression of the COX-2 enzyme, we examined both the mRNA and protein levels of COX-2 in response to

LPS stimulation in RAW 264.7 cells. For the measurement of mRNA, cells were stimulated with 1µg/ml LPS in the presence or absence of 25µM berberine for 6, 12 or 24 hours. Following treatment, total RNA was isolated from each sample and cDNA was prepared for use in qRT-PCR reactions. As shown in Fig. 3A, berberine did not exert a significant effect on the expression of the COX-2 gene. We next evaluated the effects of berberine on the expression of COX-2 protein in response to LPS stimulation. RAW 264.7 cells were treated for 3, 6, 12 or 24 hours with 1µg/ml of LPS in the presence or absence of 1, 10 or 25µM berberine. At each time point, cells were lysed and 10µg of protein used for Western blot analysis with antibodies specific for COX-2. Figure 3B demonstrates that the amount of COX-2 protein increases over time and reaches a maximum by 12 hours. In all time points evaluated, there was no inhibition of COX-2 protein expression with any dose of berberine tested. These results coupled with the data from Fig. 3A indicate that berberine does not affect the expression of COX-2 *in vitro*.

4.4 Berberine does not inhibit COX-2 enzyme activity

In addition to evaluating the effects of berberine on the expression of COX-2 we also considered effects on enzyme activity. The activity of recombinant COX-2 was evaluated *in vitro* using the standard TMPD assay. TMPD reacts with the oxygen liberated by COX-2 during the conversion of PGG₂ to PGH₂ causing a color change which can be monitored spectrophotometrically. For this assay, NS-398, a well characterized specific inhibitor of the COX-2 enzyme was used as a positive

control. Berberine and NS-398 were added to the enzyme just prior to the initiation of the enzymatic reaction. As shown in Fig. 4A, while NS-398 significantly inhibited the COX-2 enzyme, berberine did not. Taken together, these data indicate that the reduction in PGE₂ by berberine is not mediated through inhibition of COX-2 expression or activity.

4.5 Berberine does not affect mPGES-1 mRNA production or enzyme activity

The final enzyme in the pathway leading to the production of PGE₂ is mPGES-1. This enzyme is believed to be co-regulated with COX-2 and is induced under inflammatory conditions [31]. mPGES-1 will convert the PGH₂ produced by the COX-2 into PGE₂. In order to evaluate whether berberine is mediating its anti-PGE₂ effects by inhibiting the mPGES-1 enzyme, we first examined the effects of berberine on the expression of the enzyme. Expression studies were limited to mRNA expression due to the lack of a reliable antibody to mPGES-1. To generate expression data, RAW 264.7 cells were treated with 1µg/ml LPS for 6, 12, or 24 hours in the presence or absence of 25µM berberine. Total RNA from these samples was used to prepare cDNA and then samples were analyzed by RT-PCR using mPGES-1 specific primers. Values represent fold induction relative to GAPDH expression. Fig. 5A shows that at 6 and 12 hours there was a significant induction of mPGES-1 mRNA expression by LPS over media alone controls. However, berberine did not inhibit expression of mPGES-1 at these time points. At 24 hours, berberine treatment itself did increase expression of mPGEs mRNA to levels

comparable with LPS treatment. Again, however, we found that when BBR was combined with LPS it did not inhibit expression of mPGES-1 mRNA. In fact, a small increase, although not statistically significant, was noted. In summary, these data indicate that berberine does not inhibit the expression of mPGES-1 mRNA.

Next we tested the ability of berberine to inhibit the activity of recombinant mPGES-1 in an *in vitro* enzyme assay. Purified PGH₂ was supplied as the substrate and after the reaction was terminated, PGE₂ production was quantified by ELISA. In these experiments we also included MK-886, a well characterized specific inhibitor of mPGES-1. As shown in Fig. 5B, while MK-886 strongly inhibited the production of PGE₂, BBR did not. Based on these data, it does not appear that berberine can exert its anti-PGE₂ activity through inhibition of mPGES-1 enzyme expression or activity.

5. Discussion

A number of studies have previously tested berberine for its effects on the production of PGE₂ [23, 24, 27]. The majority of these studies show strong inhibition of PGE₂; however, in at least one study this effect was not observed [29]. The studies demonstrating strong inhibition of PGE₂ production were conducted either in carcinoma cells or with modified forms of berberine. The study that failed to observe an effect of BBR on PGE₂ utilized RAW 264.7 macrophage-like cells. Previous research by our group demonstrated strong anti-PGE₂ activity by berberine in the RAW 264.7 macrophage in response to influenza A virus infection [22]. This

inhibitory effect was readily observed in our investigations presented here with LPS in the RAW 264.7 macrophage-like cell model. The effect was strong; 60-80% inhibition, and highly reproducible. We focused our studies on the mechanism of inhibition. This area of study has been controversial and primarily focused on the COX-2 enzyme. Our lab has considerable expertise in this area, so we felt we could help to resolve the controversy. The first step in the PGE₂ biosynthetic pathway we examined was the enzyme cPLA₂. Initially, using specific inhibitors, we confirmed that cPLA₂ was indeed the phospholipase A2-type enzyme responsible for the release of AA in RAW 264.7 cells treated with LPS. In contrast, berberine treatment did not strongly inhibit the release of AA. Typically, we found 30% inhibition of AA when cells were treated with LPS and 25µM berberine (Fig.2). Berberine has previously been reported to inhibit the activity of the MAP kinases [25, 26]. It is possible, therefore, that the reduction in AA-release we observed may be a result from the effects of BBR on the MAP kinases since these molecules are known activators of cPLA₂ [14, 32]. Alternatively, it is possible that the effects of BBR on the release of arachidonic acid stem from effects on other molecules that regulate the activity of cPLA₂ such as Ca⁺⁺ [13, 33, 34]. However, since the effect of BBR on the release of AA was relatively small, we decided not to investigate this area of the pathway. Instead we focused our attention downstream in the pathway from cPLA₂ where a more major effect was observed.

The first enzyme we examined was COX-2, the enzyme responsible for converting AA to PGG₂ and subsequently to PGH₂. Examination of the expression

of COX-2 mRNA or protein did not reveal a significant inhibitory effect of BBR (Fig. 3A). In addition, BBR did not inhibit the LPS-induced expression of the COX-2 protein (Fig. 3B). Our data supports similar findings by Kim et.al. [29] and Lee et.al. [24], who found that BBR does not affect expression of COX-2 in RAW 264.7 macrophage-like cells. On the other hand, several groups have reported that BBR does inhibit the expression of COX-2 [25, 26]. For example, Jeong et.al., showed inhibition of LPS-induced COX-2 mRNA expression in RAW 264.7 cells following treatment with BBR. In addition, berberine has also been shown to inhibit constitutive COX-2 expression in certain cancer cell lines [27, 35]. At present the reasons for these discrepancies are not clear. It is possible that the effects of BBR could depend upon the differences in dosage, timing and pre-treatment in these studies. We also considered the hypothesis that BBR could be inhibiting the activity of the COX-2 enzyme itself. However, the results of our experiments did not reveal any direct inhibitory activity of BBR towards COX-2. Similar results have been reported by Seaver et. al., [36] who also used a recombinant *in vitro* enzyme assay. Collectively, these data would tend to rule out a direct effect of BBR on the activity of COX-2. On the other hand, these experiments have not ruled out an indirect effect of BBR on the activity of COX-2. It is possible, that BBR could be inducing an inhibitor of COX-2 that could only be detected if cell lysates, not recombinant protein, were used as the source of COX-2 enzyme. Typically, assays which rely on purified cellular enzyme as the source of COX-2 activity are performed using seminal vesicle tissues, which expresses extraordinarily high levels of this protein. It will likely be

more difficult to perform these experiments with inflammatory cell types since these cells express much lower levels of COX-2.

Finally, we examined the effects of BBR on the expression and activity of the final enzyme in the PGE₂ biosynthetic pathway, mPGES-1. Here, our expression studies were limited to measurements of mPGES-1 mRNA since an effective Ab was not available to us. To our knowledge, our work was the first attempt to address the effect of berberine on this enzyme. Our experiments failed to reveal an effect of berberine on the expression of mPGES-1 mRNA at the 6 and 12 hr time points. In fact, berberine slightly enhanced the expression at these time points. We did note that BBR acutely induced expression of mPGES-1 at the 24 h time point. BBR also slightly enhanced the LPS-induced expression of mPGES-1 mRNA at this time point, although this effect was not statistically significant. While there is no clear explanation for the increase at 24 h, berberine has been shown to induce the transcriptional activity of some genes. For example, this effect has been observed with the upregulation of the LDL-receptor gene by berberine [37]. Finally, we examined the effects of BBR on the activity of mPGES-1. We evaluated the enzymatic conversion of PGH₂ to PGE₂ in a cell-free system using with recombinant enzyme and purified PGH₂. Our experiments did not reveal any direct effect of BBR on the activity of mPGES-1 under the conditions tested suggesting that BBR is not acting as an inhibitor of this enzyme. Again, however, our studies do not rule out BBR acting indirectly on the activity of this enzyme. BBR could be inducing the expression of an inhibitory protein. Alternatively, BBR could be preventing mPGES-

1 from positioning itself properly on the ER/perinuclear membrane so that it can efficiently receive PGH_2 from the COX-2 enzyme. These and other hypotheses will be investigated in future studies.

In summary, our findings show that BBR is a potent inhibitor of PGE_2 production from RAW 264.7 cells treated with bacterial LPS. Similar inhibitory effects were noted with RAW 264.7 cells infected with influenza A virus suggesting that this is a general effect of BBR and may occur with all ligands. Support for this hypothesis also comes our finding that the majority of the inhibitory effect targets the effector phase of the prostanoid biosynthetic pathway. However, our examination of this phase of the pathway failed to reveal a direct effect of BBR on either expression or activity of the COX-2 or mPGEs-1 enzymes. There were, however, several limitations to our experiments. Most prominent was our use of recombinant enzymes in the in vitro activity assays we performed. While these studies did attempt to address direct effects of BBR on enzyme activity, we did not address indirect effects. We could not rule out the induction of inhibitory proteins by BBR nor could we rule out effects of BBR on the assembly of the COX-2 and mPGEs-1 proteins into a complex for efficient product synthesis. Future studies utilizing confocal microscopy to identify the position of these proteins in the cell in the presence of berberine should address some of these important questions. In addition, analysis of the intermediate PGH_2 by HPLC-MS could potentially indicate the functional activity of the COX enzyme in the context of the cell.

Figure 1. Effects of berberine on PGE₂ production.

A) RAW 264.7 cells were stimulated with 1µg/ml of LPS for 24 hours in the presence (▼) or absence (O) of 3-25µM berberine. B) RAW 264.7 cells were treated cell growth media (□), 25µM berberine (▲) or with 1µg/ml LPS in the presence (▼) or absence (O) of 25µM berberine. Supernatants were collected from both assays and PGE₂ levels were determined using commercial ELISA kits. Values shown are means +/- S.E.M. from two independent experiments with mediator determinations performed in duplicate in each ELISA assay.

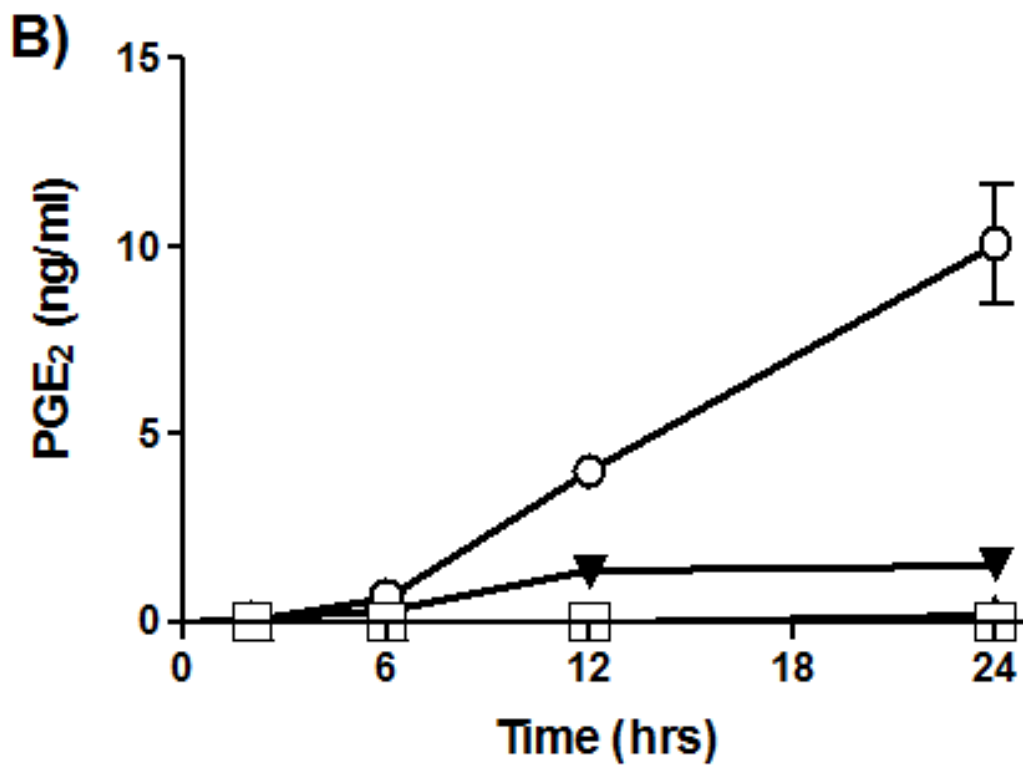
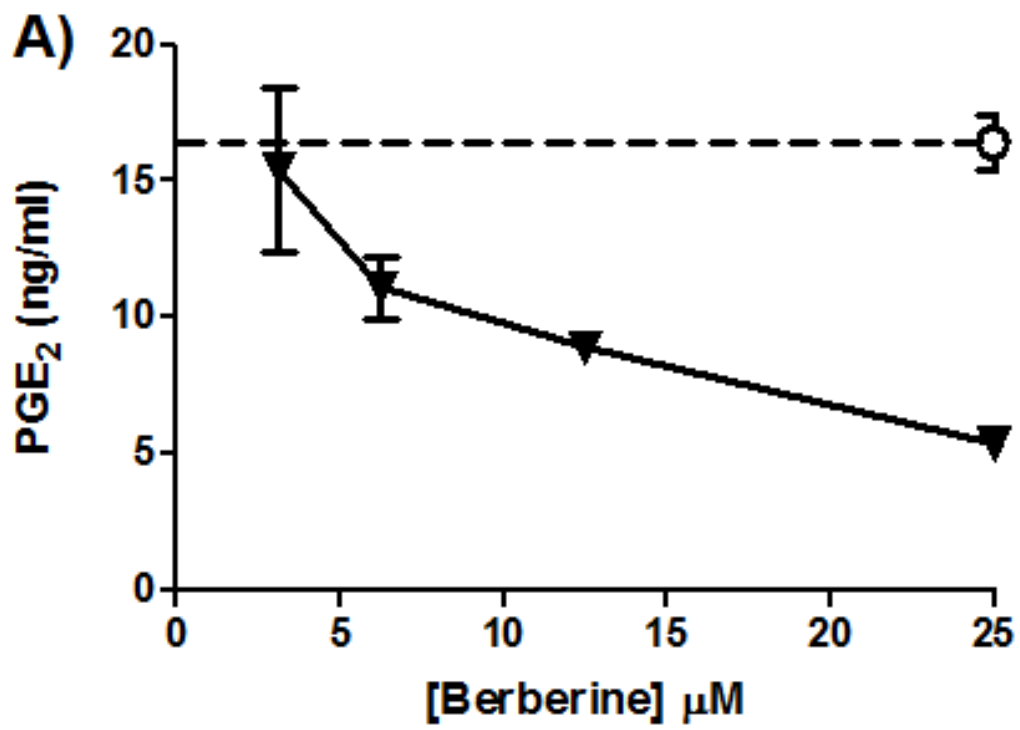


Figure 2. Effects of berberine on the release of arachidonic acid and cPLA₂ activity.

A) RAW 264.7 cells were seeded in 24 well tissue culture plates and incubated overnight with .1 $\mu\text{Ci/ml}$ [^3H] AA. Cells were then treated with cell growth media, the i-PLA₂ inhibitor bromoenol lactone (**BEL**) or the cPLA₂ inhibitor (**cPLA₂ inh.**) in the presence or absence of LPS for 24 hours. Cell supernatants were collected and analyzed by scintillation counter and reported as disintegrations per minute (DPM).

B) Cells were labeled with ^3H -AA as above and treated with media (\square), 25 μM berberine (\blacktriangle), LPS (O) or LPS with 25 μM berberine (\blacktriangledown) for the indicated times. At each timepoint, cell supernatants were collected and analyzed by scintillation counting reported as disintegrations per minute (DPM). Values shown are means \pm S.E.M. from three independent experiments. Significance ($p < 0.05$) determined by unpaired t-test.

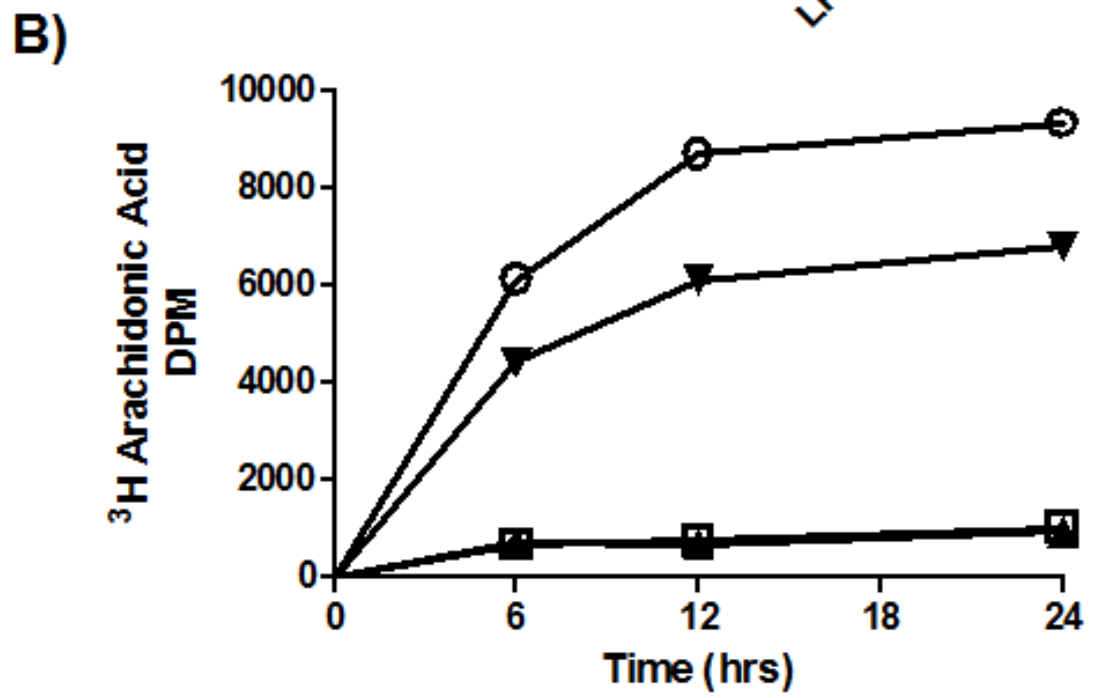
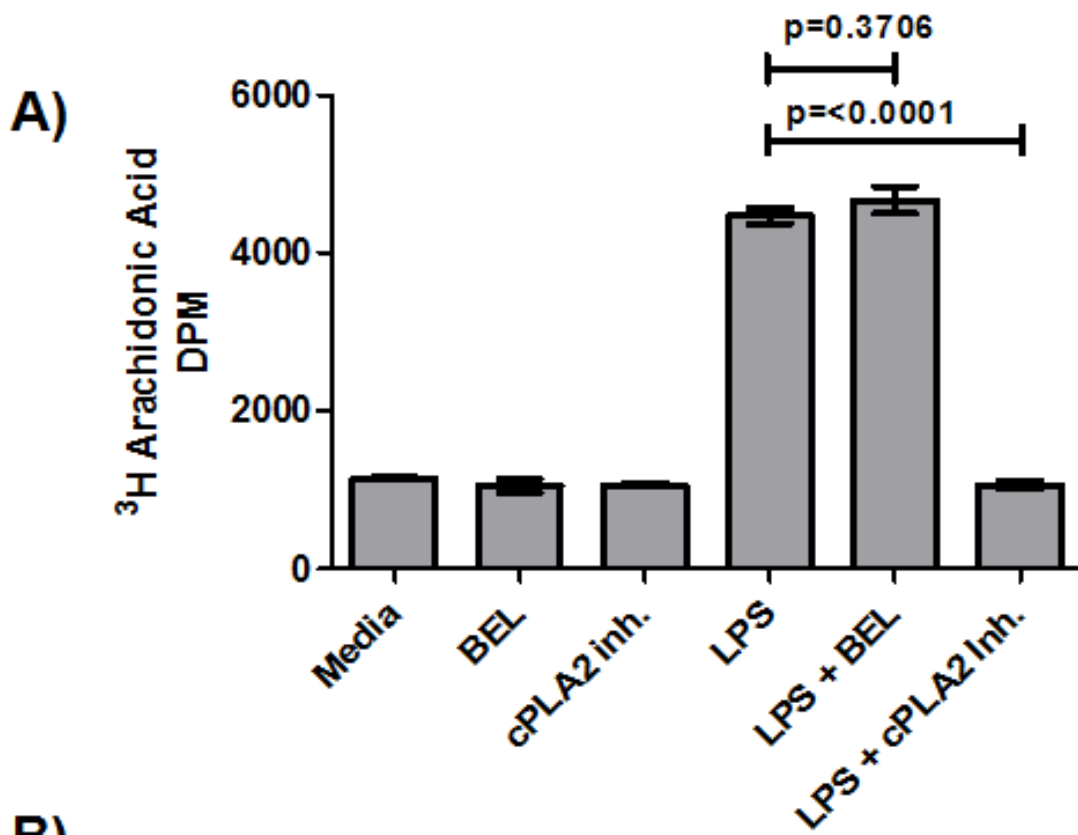
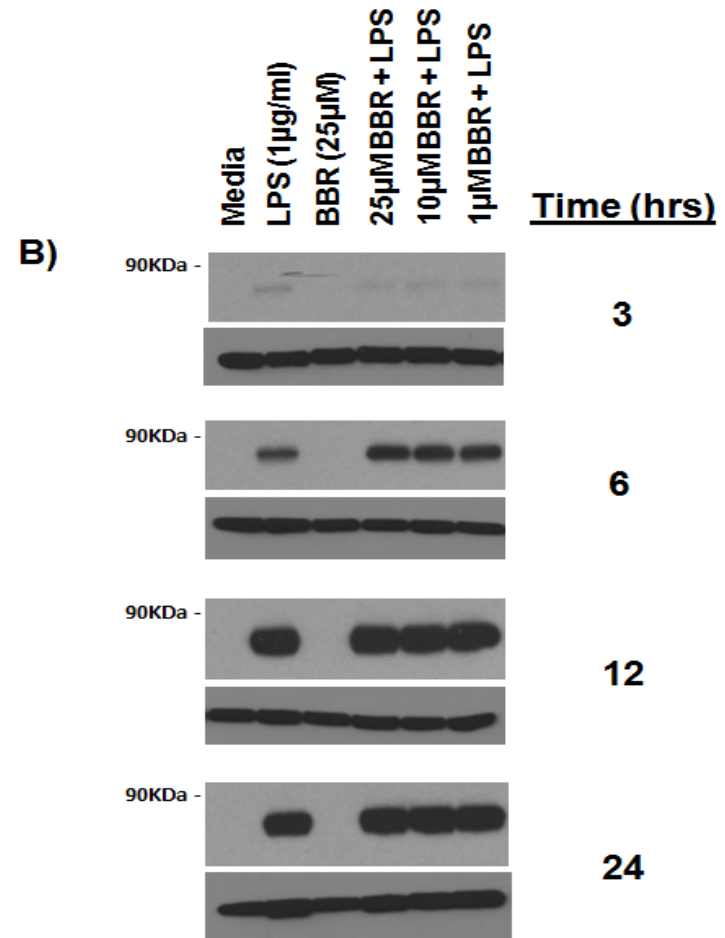
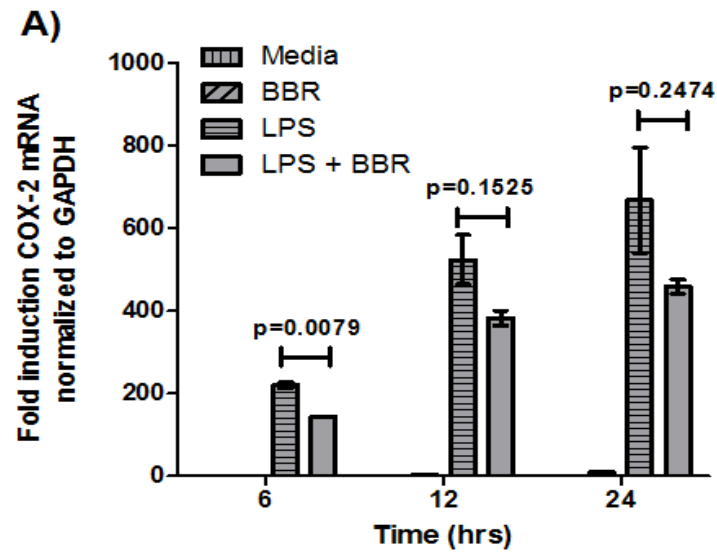


Figure 3. Effects of berberine on COX-2 expression.

A) RAW 264.7 cells were treated for 6, 12 or 24 hours with LPS (1 $\mu\text{g/ml}$) alone or in combination with berberine (25 μM) and copy number of COX-2 mRNA determined by qRT-PCR as described in the Materials and Methods. Values shown are means \pm S.E.M. from three independent experiments. Significance ($p < 0.05$) determined by unpaired t-test. B) RAW 264.7 cells were treated with LPS (1 $\mu\text{g/ml}$) and/or berberine (25, 10, 1 μM) for 3, 6, 12 or 24 hours. Whole cell lysates were made and the expression of COX-2 protein was examined by Western blot. β -Actin used to ensure equal loading. Images are representative of typical results from multiple experiments.



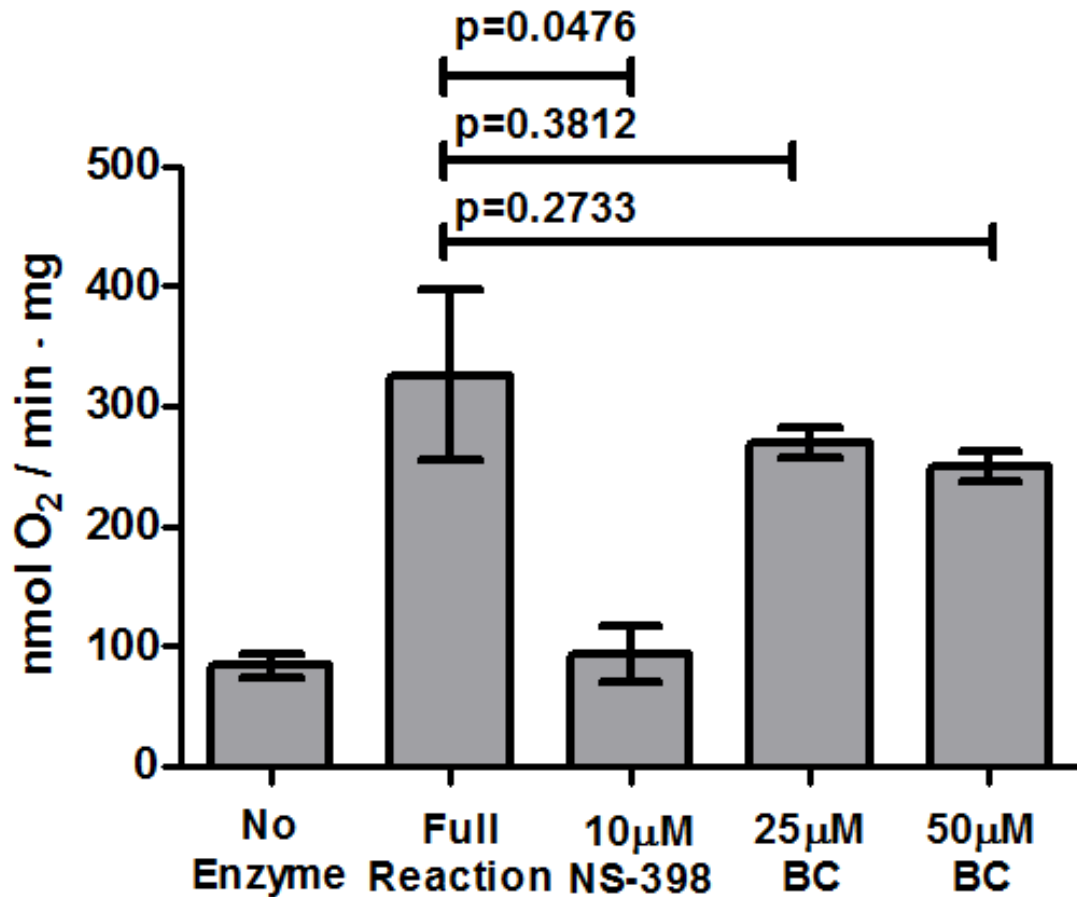
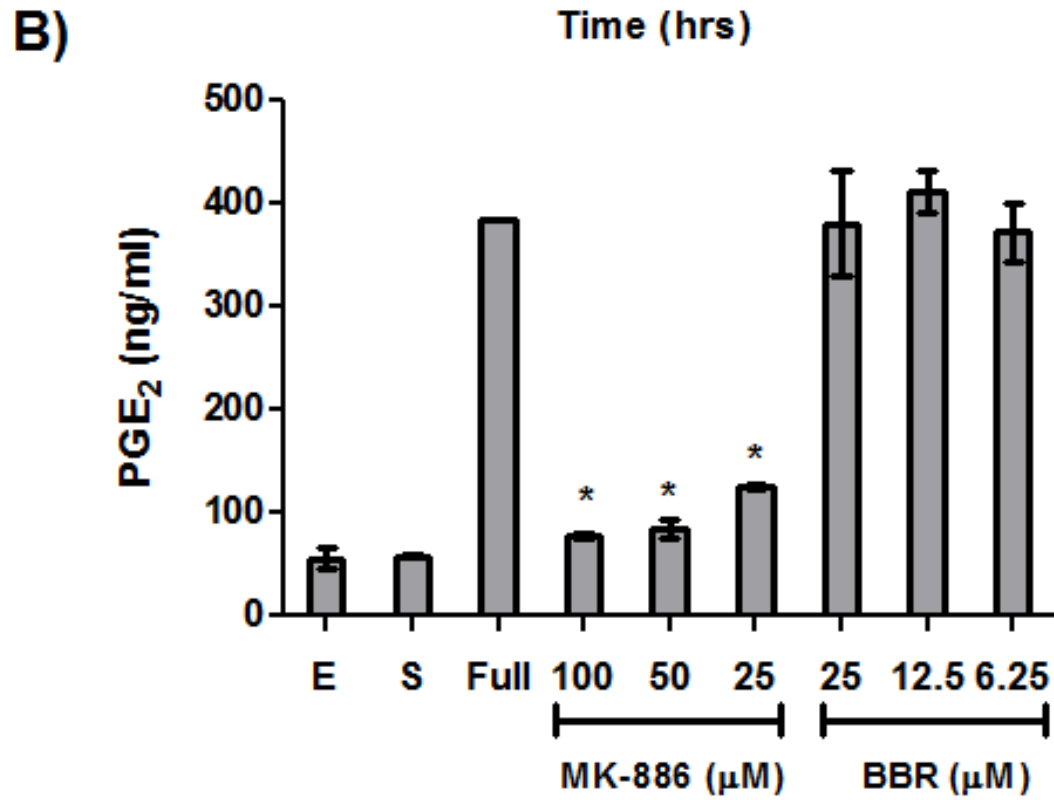
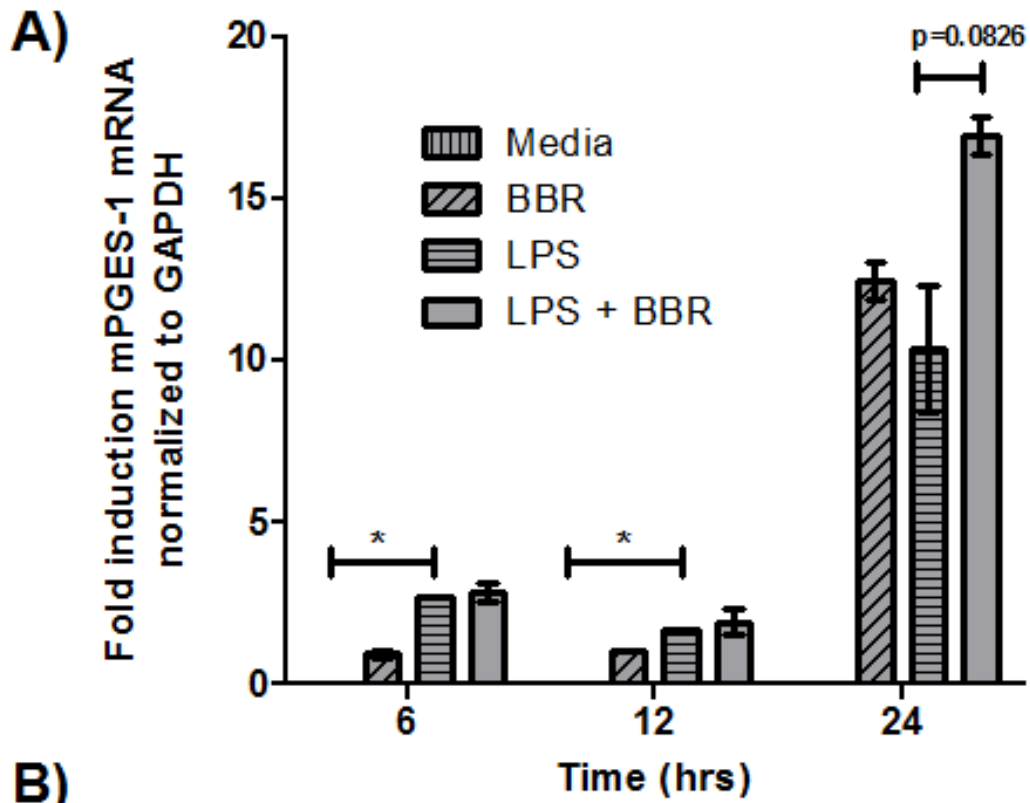


Figure 4. Effects of berberine on COX-2 enzyme activity.

A) Reaction of purified arachidonic acid with purified ovine COX-2 enzyme in the presence of the known inhibitor NS-398 (10µM) or berberine (25 or 50µM) as described in Materials and Methods. Values shown are means +/- S.E.M. from two independent experiments. Significance ($p < 0.05$) determined by unpaired t-test.

Figure 5. Effects of berberine on mPGES-1 expression and activity.

A) RAW 264.7 cells were treated for 6, 12 or 24 hours with LPS (1 $\mu\text{g/ml}$) alone or in combination with berberine (25 μM) and copy number of mPGES-1 mRNA determined by qRT-PCR as described in the Materials and Methods. B) Reaction of purified PGH_2 and mPGES-1 enzyme in the presence of known inhibitor MK-886 (25,50 or 100 μM) or berberine (6.25, 12.5 or 25 μM) was performed as described in Materials and Methods. Following the reaction, total PGE_2 was determined by commercially available ELISA. Control reaction with enzyme alone and no substrate PGH_2 (E) and substrate without enzyme (S) were used as negative controls for the full reaction (Full) with no inhibitors present. Values shown are means \pm S.E.M. from three independent experiments. Significance ($p < 0.05$) determined by unpaired t-test.



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CHAPTER 4

Summary

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1. Summary

The work described in this dissertation centers around the isoquinoline alkaloid berberine. This alkaloid, commonly found in a variety of medicinal herbs like goldenseal, is responsible for many of the activities often attributed to whole plant extracts. Several studies indicate that berberine can limit the production of inflammatory mediators and limit the growth of certain microbes. Our work sought to extend this knowledge by examining the effects of berberine on the growth of the influenza A virus and to determine the effects on virus induced inflammation. In addition, we characterized the effect of berberine on the production of a lipid mediator of inflammation (PGE₂) in response to LPS stimulation. Our studies sought to clarify previously published studies by systematically examining the effect of berberine on the critical enzymes (cPLA₂, COX-2 and mPGES-1) of the PGE₂ biosynthetic pathway.

In chapter 2, we evaluated the effects of berberine and goldenseal extracts on the influenza A virus. Our observations demonstrated for the first time that berberine can inhibit the reproduction of two strains of influenza A virus in several cell lines. However, the overall levels of total virus growth and inhibition varied with each cell type. The reasons for this cell to cell variation are not clear at this time. In the case of A549 cells, this may be attributed to the use of a mouse-adapted virus in a human cell line. Future studies utilizing a human isolate of the virus may be useful for

characterizing the effect of berberine in human cell lines. In MDCK cells, the virus replicated to high levels and berberine had no effect on the virus. We attributed this to the documented ability of this cell line to efficiently package and transport viral proteins through the cell. Mechanistic studies presented here revealed that berberine does not directly inhibit the production of key viral proteins, but impedes the movement of the HA protein in RAW 264.7 macrophage-like cells. We demonstrated that berberine treatment lead to the aggregation of the HA protein within the cell. We hypothesize that berberine may cause a block within the ER/Golgi complex leading to the reduction in viral progeny. Additional studies with MDCK cells, which may overcome this berberine-induced block due to the efficient processing of the viral protein, may help elucidate the anti-viral mechanism of berberine. Future studies addressing the localization of the HA and other key viral proteins with key cellular structures in both RAW 264.7 and MDCK cells should provide insight into the mechanism by which berberine limits virus growth. Based on these findings, it is likely that berberine can inhibit the influenza A virus through an entirely novel mechanism. This finding has implications for the development of a new class of anti-influenza A therapeutics.

In addition to the anti-viral effects observed in chapter 2, we also tested the ability of berberine to attenuate the production of the inflammatory mediators, TNF- α and PGE₂, in response to influenza A virus infection. Our data demonstrate for the first time that berberine can significantly inhibit the production of these important mediators of inflammation. In these studies, we utilized a high MOI model for

infection to elicit a consistent and equal response that is not influenced by the anti-viral effect of berberine. Based on the work of Bola Oyegunwa in our lab (data not shown), we know that in the presence of berberine the viral RNAs are not affected. Therefore sufficient ligand is present for the stimulation of the various TLR and RIG-I pathways leading to the production of these inflammatory mediators. We hypothesize that berberine may be mediating an additional effect on one or several of the signaling pathways emanating from these receptors. Further studies are underway to identify the role of berberine in the phosphorylation of the key kinases involved in the activation of these pathways.

In this chapter, we also evaluated the ability of whole root and whole plant extract to mediate the effects observed with berberine alone. Our studies indicate that treatment with goldenseal extracts, normalized to contain equal amounts of berberine, can mediate the inhibition of the influenza A virus and the inhibition of inflammation associated with infection. We show that the whole root and plant extracts do not inhibit the production of the virus to the same extent as berberine alone. This finding potentially indicates that the complex mixtures of an extract may have other compounds which are interfering with the effects of the berberine. Future studies which carefully examine the alkaloid (and other chemical) profiles of these extracts from a variety of plants may provide insight into the identification and cultivation of goldenseal with optimal alkaloid ratios thereby increasing their efficacy and market value.

Taken together, the findings from chapter 2 provide evidence that berberine may be useful to limit the spread of the influenza A virus as well as to control inflammation associated with infection. Future studies that address the mechanism may provide insight into the development of berberine-based treatments for influenza and thereby limit mortality and morbidity associated with the infection. The question remains whether the anti-viral and anti-inflammatory effects of berberine have a common denominator and can be attributed to single or multiple effects upon the cell.

In chapter 3, we chose to focus our attention on the inhibition of PGE₂ production by berberine in response to LPS stimulation. Previously, the data concerning the effects of berberine on PGE₂ production have not yielded a definitive mechanism. Several reports point to inhibition of the COX-2 enzyme as a mechanism to mediate the inhibition of PGE₂. However, in these studies, the effect on COX-2 varied. In addition, no one has conclusively addressed the effect of berberine on the other key enzymes in the biosynthetic pathway. Therefore, we chose to examine the key enzymes responsible for the ultimate production of PGE₂ using the RAW 264.7 mouse macrophage-like cell line stimulated with LPS.

Our studies confirmed that berberine strongly inhibited the production of PGE₂ in a dose-dependent manner over time. We next examined the activity of the cPLA₂ enzyme by measuring the ability of the cell to release radioactively labeled AA in response to LPS stimulation. We demonstrated that the cPLA₂ enzyme is responsible for the production of PGE₂ using PLA-specific inhibitors. We showed

that berberine can attenuate this effect by approximately 20-30%. This level of inhibition may reflect an effect of berberine on the upstream kinases responsible for the phosphorylation of cPLA₂. Alternatively, berberine could exert a direct effect on the PLA enzymes that cumulatively affects the release of AA. It is also possible that berberine could affect the movement of the cPLA₂ enzyme to the nuclear membrane through direct effects on the cell. For example, berberine may modulate the intracellular calcium and/or ATP levels needed to facilitate the movement of this enzyme. Future studies directed at examining the movement and activation of this enzyme may provide insight into cellular effects of berberine that could not only account for this observation, but also for the anti-viral effects described in chapter 2.

Despite the 20-30% inhibition of AA release observed here, we felt that the strong inhibition of PGE₂ production (60-80%) must indicate an additional effect downstream of cPLA₂. We therefore evaluated the effect of berberine on the expression and activity of the COX-2 enzyme. In our studies, we did not observe inhibition of either mRNA or protein expression of COX-2. Likewise, we did not observe any effect on COX-2 activity in an *in vitro* enzyme assay. We next examined the effect of berberine on the mPGES-1 enzyme. Again, no effect was observed on the expression of the mRNA or activity of this enzyme. These findings reveal that the effect of berberine on PGE₂ production must occur through a mechanism that does not alter the expression or activity of these key enzymes. Perhaps berberine alters the movement and localization of these enzymes within the cell. Other hypotheses that have been considered include the possibility of

berberine directly binding the intermediates of the biosynthetic pathway, thereby inhibiting downstream production of PGE₂ through substrate limitation. Also, we have considered the possibility of berberine inducing an as yet unidentified negative regulator within the cell that could alter the cell function and affect the eicosanoid pathway. These hypotheses remain to be tested in order to fully understand the mechanisms mediating the anti-PGE₂ effects observed in our studies.

In summary, our work provides a foundation for future studies on both the anti-viral and anti-inflammatory effects of berberine and goldenseal extracts. Our findings on the anti-influenza A virus effects, as well as the effects on virus-induced inflammation revealed for the first time that berberine could potentially be used to treat deadly influenza virus infections. In addition, our characterization of the effects on the PGE₂ biosynthetic pathway provides insight into the mechanism by which berberine controls the production of lipid mediators of inflammation. It is our hope that future studies will lead to the development of new classes of berberine-based drugs to treat influenza A virus infections and to limit immune-mediated pathologies.