This is to certify that the
dissertation entitled

EFFECTS OF HERBAL CONSTITUENTS ON
PROINFLAMMATORY CYTOKINES

presented by

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has been accepted towards fulfillment
of the requirements for the

Ph.D. degree in Food Science & Environmental Toxicology

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4-29-03

Date

EFFECTS OF HERBAL CONSTITUENTS ON PROINFLAMMATORY CYTOKINES

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Food Science and Human Nutrition
Institute of Environmental Toxicology

2003

ABSTRACT

EFFECTS OF HERBAL CONSTITUENTS ON PROINFLAMMATORY CYTOKINES

By

Alexa Terese Smolinski

The dietary supplement market and number of supplement consumers has grown significantly since the passage of the Dietary Supplement Health and Education Act of 1994 (DSHEA). Among dietary supplement products, herbals accounted for an estimated total retail value of $3.24 billion in the U.S. in 1996 (Johnston,1997), a considerable portion of total supplement sales which were estimated at $15.7 billion in 2000 (Blendon et al., 2001). Although the industry and number of products has continued to grow, there is still a considerable lack of scientific evidence to clearly support efficacy, and in some cases, safety of these products. Thus, examination of efficacy and safety of specific dietary supplement products is necessary.

The hypothesis of this dissertation is that the anti-inflammatory properties of the herbal constituents apigenin (chamomile), ginsenoside Rb_1 (ginseng), and, particularly, parthenolide (feverfew), are mediated in part by inhibition of proinflammatory cytokines.

The specific objectives of these studies were to 1) evaluate the potential anti-inflammatory properties of the three herbal constituents, apigenin, ginsenoside Rb_1 and parthenolide on lipopolysaccaharide (LPS)-induced proinflammatory cytokine protein production (interleukin [IL]-6 and tumor necrosis factor [TNF]-α), and relate these effects to the intact animal model; 2) determine the effect of route, dose and dose-timing of parthenolide administration on inhibition of LPS-induced serum IL-6 and TNF-α production in vivo; and 3) assess the relationship between serum cytokine protein

production and proinflammatory cytokine gene expression in the spleen and liver of parthenolide co-treated mice.

Apigenin, ginsenoside Rb_1 and parthenolide inhibited LPS-induced IL-6 and/or TNF-α production in cell culture. Although cytokine inhibition was observed in mice sera, the pattern of inhibition differed from cell culture data suggesting the cell culture model employed could only be used to approximate potential in vivo effects, and must be confirmed using appropriately designed animal models. Based on evaluation of route, dose and dose-timing of parthenolide administration on inhibition of LPS-induced IL-6 and TNF-α, intraperitoneal injection of 5 mg/kg parthenolide as a co-treatement with LPS was determined. Protein and mRNA comparison studies revealed that changes in serum IL-6 and TNF-α correlated with mRNA expression in spleen, but not liver. mRNA levels of IL-6 were reduced, TNF-α and COX-2 unchanged, and IL-1β mRNA increased in spleen of parthenolide plus LPS co-treated animals compared to LPS-treated only. No significant effects were observed in liver. The overall expression of each gene was significantly higher in spleen when compared to liver.

Taken together, these studies contribute to the understanding of the anti-inflammatory properties, and potential mechanisms of inhibition, of the herbal constituents apigenin, ginsenoside Rb_1 and, more specifically, parthenolide on inflammatory mediators. The methods and results of these studies can be used to further elucidate parthenolide's, and other herbal constituents' and extracts', molecular mechanism for inhibitory effects on inflammation, and potential as human therapeutics in the treatment of inflammatory conditions.

ACKNOWLEDGMENTS

I would like to thank my dissertation advisor Dr. James J. Pestka for his guidance, support and assistance prior to, and throughout, my Ph.D. study. I also thank him for the opportunity to study and conduct alternative research under a USDA National Needs Fellowship in Chemical Food Safety, and in such a highly regarded laboratory. Your continued support and patience through the ups and downs of this project allowed me to finish this program with a sense of pride and accomplishment.

I would also like to thank the remainder of my committee , Dr. Joseph J. Schroeder, Dr. Venugopal Gangur, Dr. Muralee Nair and Dr. Gale Strasburg, for their valuable advice, suggestions and comments.

Special thanks go to Becky Uzarski and Yongjoo Chung for technical assistance throughout my time in the laboratory. Your help was invaluable to my success in and out of the laboratory. Additional thanks go to our undergraduate students Elana Pottorff, Kate Brackney and Dan Lampen for their assistance with animal studies. I also thank all of the laboratory members in both the Pestka and Linz groups.

Finally, I would like to thank my parents, family and friends for their continued support, encouragement and patience throughout my program. The end is finally here! I eagerly look forward to a new beginning.

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ABBREVIATIONS

TNF-α	Tumor Necrosis Factor-alpha
IL	Interleukin
NF-κB	Nuclear Factor - kappa B
C/EBPβ	CAAT/Enhancer Binding Protein
DSHEA	Dietary Supplement Health & Education Act of 1994
FDA	Food & Drug Administration
NVNM	Non-vitamin, Non-mineral
MAPK	Mitogen Activated Protein Kinase
COX	Cyclooxygenase
PG	Prostaglandin
LT	Leukotriene
LPS	Lipopolysaccharide
i.p.	Intraperitoneal
p.o.	Oral gavage
min	Minute
h	Hour
PMA	Phorbol 12-myristate 13-acetate
NSAID	Non-steroidal Anti-inflammatory Drug
PAF	Platelet-Activating Factor
IFN	Interferon
iNOS	Inducible Nitric Oxide Synthase
MAPKKK	Mitogen - activated Protein Kinase Kinase Kinase
MAPKK	Mitogen - activated Protein Kinase Kinase
JNK	c-Jun Amino-Terminal Kinase
ERK	Extracellular Signal-regulated Protein Kinase
RT-PCR	Reverse Transcription - Polymerase Chain Reaction

AP-1	Activator Protein 1
VCAM	Vascular Cell Adhesion Molecule
ICAM	Intercellular Adhesion Molecule
RHD	Rel-homolgy Domain
GRAS	Generally Recognized as Safe
Ig	Immunoglobulin
IKK	IκB Kinase Complex
STATs	Signal Transducers and Activators of Transcription
DMSO	Dimethylsulfoxide
MTT	Methylthiazolyldiphenyl-tetrazolium bromide

INTRODUCTION

Hundreds of herbal remedies have been used historically for maintenance of health and in the treatment of diseases. Today, such alternatives to over-the-counter medicines and prescriptions are still sought, with 36 percent of the U.S. population using herbal products (McVean et al., 2000; O'Hara et al., 1998). The herbal supplement industry experienced tremendous growth throughout the 1990's with sales doubling every four years (Fleming, 1998) and projected sales expected to reach $6.6 billion by 2003 (Sloan, 2000). Herbal supplements are legally defined as dietary supplements, and thus fall under the regulation of the Dietary Supplement Health and Education Act (DSHEA). The dietary supplement market and number of supplement consumers has grown significantly since the passage of DSHEA in 1994. Among dietary supplement products, herbals accounted for an estimated total retail value of $3.24 billion in the U.S. in 1996 (Johnston,1997), a considerable portion of total dietary supplement sales which were estimated at $15.7 billion in 2000 (Blendon et al., 2001).

Despite continued growth of the industry and number of products (Commission on Dietary Supplement Labels 1997; Sarubin 2000), there is still a considerable lack of scientific evidence to clearly support efficacy, and in some cases, safety of these products. This is due, at least in part, to the lack of pre-market approval required for dietary supplements, which is in strong contrast to the pre-market regulatory requirements of conventional drugs and food additives. As a result, no investigations of the safety and efficacy of dietary supplements, including herbal products, are required by the Food and Drug Administration prior to marketing and sale of the product. Thus, it is often not known if these products are effective or even safe. Therefore, studies verifying

the presumed efficacy, and safety, of specific dietary supplement products should be conducted.

One potential use for herbals is the treatment of inflammatory conditions. Published reports describe putative effects of various herbal extracts and their constituents. Preliminary data suggest the potential anti-inflammatory properties of specific herbal constituents including apigenin (chamomile), ginsenoside Rb_1 (ginseng) and parthenolide (feverfew). Despite the collection of work that has been documented, limited studies have been undertaken to elucidate the specific effects of these constituents on proinflammatory cytokines in vivo.

The specific objectives of this dissertation are divided into three areas. The first objective was to use a cell culture system that could be used to evaluate the potential efficacy of herbal constituents, including apigenin, ginsenoside Rb_1 and parthenolide, or whole herb extracts, with presumed anti-inflammatory properties, and to determine if the results of cell culture studies could accurately predict effects in an animal model. The potential anti-inflammatory properties were evaluated in a murine macrophage cell and mouse model using LPS-induced stimulation of the proinflammatory cytokines interleukin (IL)-6 and tumor necrosis factor (TNF)-α. The second objective was to further examine one constituent, parthenolide, to determine the effect of route, dose and dose-timing of parthenolide administration on inhibition of LPS-induced IL-6 and TNF-α in an animal model system. The results of these studies were used to define conditions to study the potential mechanism by which parthenolide imparts its anti-inflammatory activity. The third objective was to test the hypothesis that parthenolide-induced suppression of LPS-induced IL-6 and TNF-α gene expression correlate with reduced mRNA levels for these genes, and other related proinflammatory genes (IL-1β and

cyclooxygenase [COX]-2), in the spleen and liver. Additionally, we sought to determine the differences in expression levels of each gene in the spleen versus the liver.

Collectively, the studies in this dissertation will contribute to the understanding of how specific herbal constituents, primarily parthenolide, impair inflammation through evaluation of effects on specific mediators of inflammation. In the long term, methods and results of this dissertation could be used to further elucidate parthenolide's, and other herbal constituents' and whole herb extracts', anti-inflammatory effects, and the specific molecular mechanism(s) for inhibition.

CHAPTER 1

Literature Review

INTRODUCTION

Hundreds of herbal remedies have been used historically in maintenance of health and in the treatment of diseases. Today, research is directed towards determining the mechanistic and molecular basis for these therapeutic outcomes. Additional investigations are being performed to ascertain the potential for herbal extracts and/or their constituents to be used in the treatment of conditions not historically treated by these herbs, or to verify the efficacy for their historical uses. The overall objective of the research presented here was to understand how specific herbal constituents affect the inflammatory response of the immune system. The intent of this section is to review recent literature on 1) the herbal supplement market, 2) regulatory status, 3) inflammation and inflammatory mediators, 4) proinflammatory cytokine signal transduction pathways - with emphasis on the role of mitogen activated protein kinases (MAPKs) and transcription factors in inflammation, 5) herbal extracts, constituents and their effects on inflammatory mediators, 6) biological effects of feverfew and/or parthenolide on mediators of inflammation and, 7) the effect of parthenolide on MAPKs and transcription factors.

Herbal supplement market The sale of dietary supplements in the United States has increased dramatically since the passage of the Dietary Supplement Health and Education Act of 1994 (Radimer et al., 2000). When the DSHEA was enacted, an estimated 4000 products made by nearly 600 U.S. dietary supplement manufacturers was

reported (Commission on Dietary Supplement Labels, 1997). Today, the Food and Drug Administration (FDA) estimates more than 29,000 different dietary supplements are available, with an average of 1000 new products being developed annually (Sarubin, 2000). Estimates of total dietary supplement sales have grown to $15.7 billion per year (in 2000) with sales of individual products including echinacea, ginseng, *Ginkgo biloba* and St. John's wort exceeding $200 million per product per year (Blendon et al., 2001).

American adults, at a rate of 48%, report regularly taking some type of vitamin, dietary or mineral supplement (Fontanarosa and Lundberg, 1998). Of this population, many specifically consume non-vitamin, non-mineral (NVNM) dietary supplements like ginseng, echinacea, amino acids or over-the-counter hormones (Blendon et al., 2001). NVNM supplements include herbals, botanicals, protein and amino acids as well as shark cartilage and Brewer's yeast (Radimer et al., 2000).

The majority of users consume dietary supplement products as part of a routine health care regimen (Blendon et al., 2001). Reports of NVNM supplement use among college students indicate primary use for immune benefits, good health, and to prevent disease as well as prevention of depression and anxiety (Newberry et al., 2001). Other studies of NVNM supplement use in university students indicate that the three primary reasons for use were to improve energy, promote weight loss and burn fat (Perkin et al., 2002).

The herbal supplement industry, specifically, has experienced tremendous growth throughout the 1990's with sales doubling every four years (Fleming et al., 1999). Total sales of herbal remedies reached $3.2 billion in 1996, with an increase of 80 percent in mass-market outlets, including grocery stores, pharmacies and retail stores, from 1996 to 1997 (Blumenthal, 1998). The Natural Marketing Institute reported that 67 percent of the

general population shop for natural products, including herbals and healthy foods, at traditional grocery stores (Natural Marketplace Trends Report, 1999). Projected sales of herbs are expected to reach $6.6 billion in 2003 (Sloan, 2000).

Regulation of herbal supplements The overwhelming increase in herbal supplement sales has prompted concern over the need for appropriate regulations. Herbal supplements are legally defined as dietary supplements, and thus fall under the regulation of the Dietary Supplement Health and Education Act of 1994 (DSHEA). Dietary supplements, unlike conventional drugs, do not require approval from the FDA prior to marketing and sale of the product. However, supplements that contain a "new dietary ingredient" require that the manufacturer or distributor file a pre-market notification with the agency at least 75 days prior to marketing the product. The notification must include information supporting evidence that the supplement containing the new ingredient "will reasonably be expected to be safe". However, the DSHEA does not clearly define "reasonably expected to be safe". This approach is very lax in comparison to the rigorous pre-market approval process required for food products containing new food ingredients such as food additives (Table 1.1), which many times takes years to complete. Thus, the DSHEA enables manufacturers to release new herbal products to the general public without considerable restriction, but does not provide the same level of confidence about safety as would the FDA for new food ingredients.

In addition to pre-market notification required by the DSHEA, a manufacturer is also required to notify the FDA no later than 30 days after marketing a product if the product label contains a statement of nutritional support. Statements of nutritional support including structure and function claims, but not disease claims, are allowed on dietary supplement labels. The difference between a structure or function claim in

Table 1.1. Current Status of Foods, Drugs and Dietary Supplements under FDA Regulation

Status	Dietary Supplements	Foods[a]	Food Additives	New Drugs[b]
Premarket approval required	No[c]	No[d]	Yes	Yes
Risk-benefit analysis conducted by FDA prior to marketing	No	No	No	Yes
Postmarket reporting or surveillance by industry required	No	No	Rarely	Yes
Burden of proof for demonstrating safety or lack thereof	FDA	FDA	Manufacturer	Manufacturer

[a]Foods (including conventional foods and dietary supplements), unlike drugs, are considered to be safe (reasonable certainty of no harm), and thus risk-benefit analysis is not applicable.
[b]This description applies to "new" drugs. Many over-the-counter drugs that are not "new drugs" are regulated under FDA's Over-The-Counter Drug Review procedures, which do not provide for postmarketing surveillance.
[c]A 75-day premarket notification, but not premarket approval, is required for dietary supplements containing ingredients not marketed before 1994.
[d]In 2001 FDA proposed a rule requiring marketers of food developed through biotechnology to notify the agency at least 120 days before commercial distribution and to provide information to demonstrate that the product is as safe as its conventional counterpart (FDA, 2001).
Source: Proposed Framework for Evaluating the Safety of Dietary Supplements

comparison to a disease claim is very subtle and is not likely to be understood by the general consumer. Examples of prohibited claims include: "supports the body's antiviral capabilities," or "supports the body's ability to resist infection." An allowable claim would be "supports the immune system." Additionally, the act requires that the manufacturer be able to substantiate that the claim is truthful and not misleading, but does not require that this information be provided to the agency with the post-market notification. Moreover, the product must contain the disclaimer, "This statement has not been evaluated by the FDA. This product is not intended to diagnose, treat, cure or prevent any disease." The subtle difference in the types of claims that are allowable, versus prohibited is not particularly meaningful to the general consumer. Permitting the liberal use of numerous structure and function claims on herbal supplements is vastly different than the limited and strict use of disease claims on food products.

The increase in consumer demand and relaxed regulatory approach of dietary supplements, including herbals, have contributed to the tremendous growth of the market and access to a number of traditional and emerging therapeutics with potential for great benefits. However, with the unrestricted access has come little, if any, guarantee of efficacy of the products, and in some cases, safety. In a recent study evaluating the views of American's on regulation of dietary supplements, a majority of respondents expressed support for increased government regulatory efforts to increase the assurance of dietary supplement safety, purity, dose consistency and truth in advertising claims (Blendon et al., 2001). However, the majority also felt access to these products was important, and a substantial number of respondents were not prepared to be denied access to existing products that have not been tested for safety previously (Blendon et al., 2001).

In light of the overwhelming increase in supplement use and sales, and current questions about safety of specific products, e.g., ephedra containing products and kava kava, the FDA has asked a committee to develop a framework for use by the FDA to evaluate the safety of dietary ingredients. The created report entitled "Proposed Framework for Evaluating the Safety of Dietary Supplements" developed by the Committee on the Framework for Evaluating the Safety of Dietary Supplements, is currently open for comment. The report contains information for a proposed framework for prioritizing and evaluating the safety of dietary ingredients/supplements based on existing information. The committee is currently creating six mock monographs using the process outlined in their report.

Critical to the implementation of the developed framework will be access to adequate research studies which have evaluated the safety, and efficacy, of dietary supplement products including herbals. It has been noted that human and animal studies will be placed at the top of the hierarchy of data types. Thus, it will be essential to conduct more in vivo studies and human clinical trials in order to validate that herbal supplements are both safe and effective for continued wide spread use in America and around the world.

Inflammation One potential use for herbals is the treatment of inflammatory conditions. Inflammation is a complex response to localized injury, trauma or infection, and involves various immune system cells and numerous mediators (Fundamental Immunology, Fourth Edition). Development of an effective inflammatory response is an important part of the body's defense system, but can at times be detrimental. The immune system elicits inflammatory responses that are both acute, characterized by rapid onset and short duration, and chronic, characterized by persistent immune activation. A

chronic inflammatory response may accompany microbial infections, transplants and allergies. Persistence of this inflammation can lead to granuloma formation and tissue injury. Thus, in many cases, therapeutics such as corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs) are employed for reducing inflammation.

Mediators of inflammation A number of inflammatory mediators are released by cells in response to localized injury or trauma (Fundamental Immunology, Fourth Edition). These mediators elicit or enhance particular functions of the inflammatory response. The primary inflammatory mediators are grouped into four main categories: chemokine, plasma enzyme, lipid and cytokine (Figure 1.1). *Chemokines* are small polypeptides which chemotactically attract leukocytes and regulate the expression of integrins in leukocyte membranes. Chemokines, such as interleukin (IL)-8, function in neutrophil extravasation. *Plasma enzyme mediators* include the kinin, clotting, fibrinolytic and complement systems. These four interconnected mediator-producing systems function by increasing vascular permeability, causing vasodilation and neutrophil chemotaxis. *Lipid inflammatory mediators* result from degradation of membrane phospholipids. Two major pathways are involved: lyso-platelet-activating factor (PAF) or arachidonic acid. The production of PAF is responsible for platelet aggregation, eosinophil chemotaxis and neutrophil activation. Metabolism of arachidonic acid by the cyclooxygenase pathway results in production of prostaglandins and thromboxanes important for vasoconstriction, platelet aggregation and increased vascular permeability. Alternatively, the metabolism of arachidonic acid by the lipoxygenase pathway creates leukotrienes responsible for bronchial smooth-muscle contraction and neutrophil chemotaxis. Finally, *cytokines*, including IL-1, IL-6 and

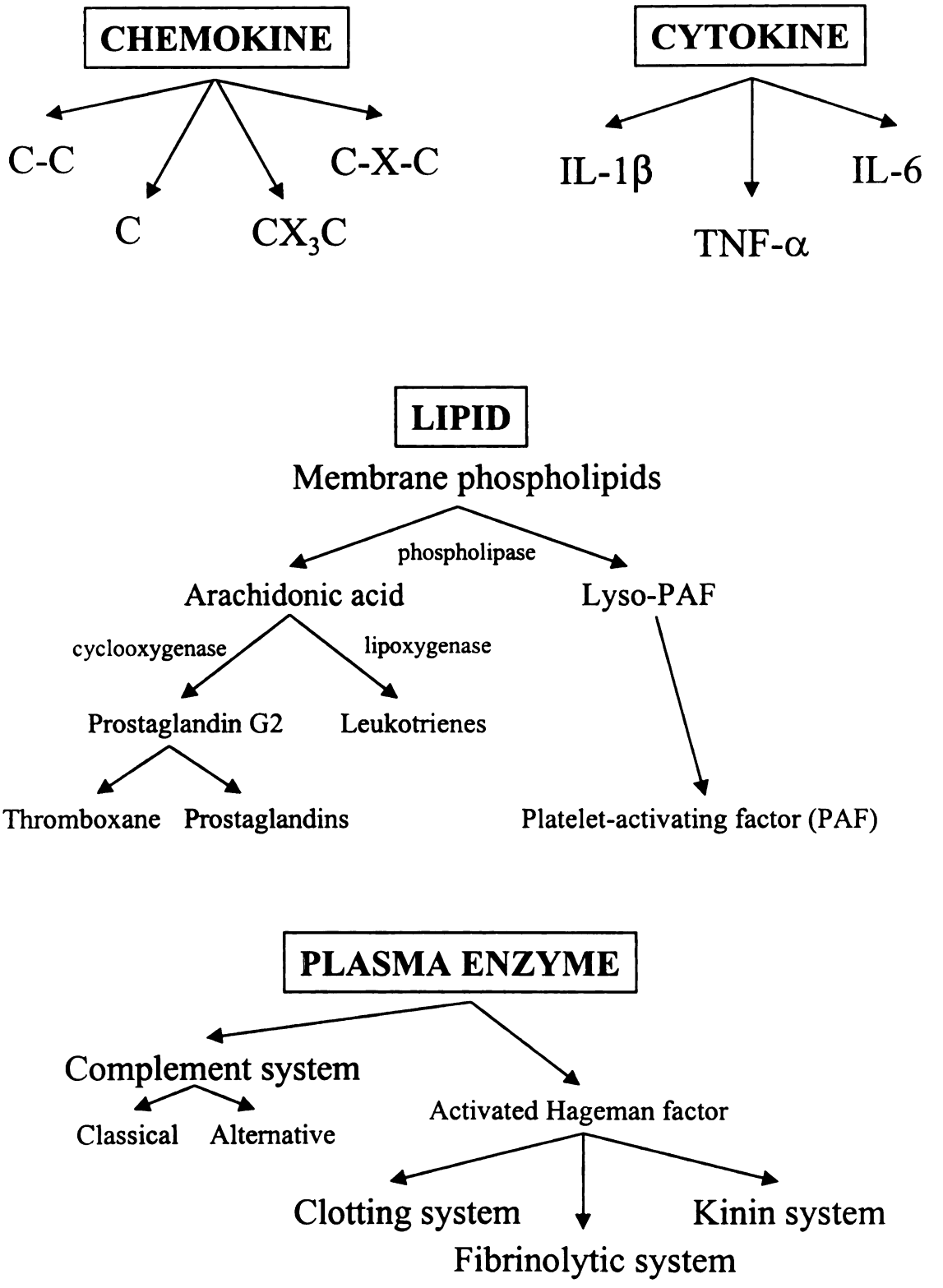

Figure 1.1. Major groups of inflammatory mediators.

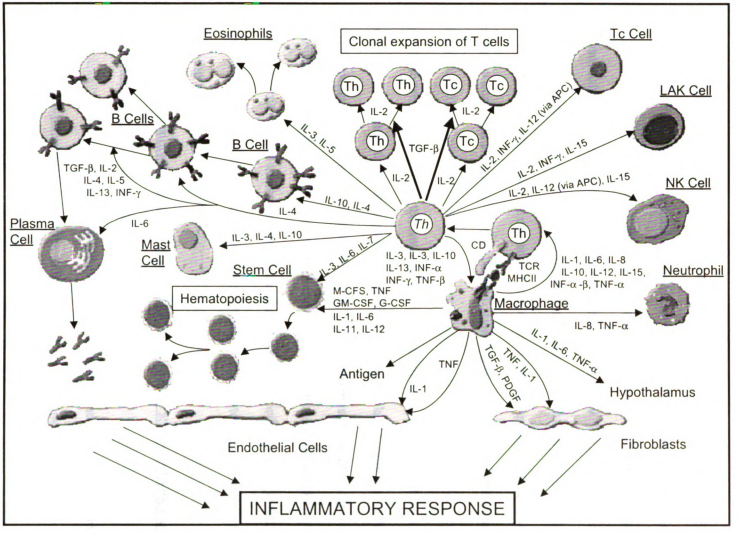

Figure 1.2. Cytokines and inflammation. Adapted from Biocarta.com

tumor necrosis factor (TNF)-α, play an integral role in the inflammatory response (Figure 1.2). They act as mediators of inflammation by inducing fever, acute phase proteins, vascular permeability and by activating B and T cells. The cytokines TNF-α and interferon (IFN)-γ play a central role in the development of chronic inflammation.

The proinflammatory cytokines can trigger systemic and local inflammatory processes following development of acute disease (Dinarello, 1997). Local effects of these cytokines result in release of lipid-derived mediators, and at sites of local IL-1 and TNF-α production, emigration of neutrophils, monocytes and lymphocytes takes place. Emigration occurs as a result of chemokine induction and up-regulation of cell adhesion molecules.

The proinflammatory cytokines IL-1 and TNF-α have the ability to induce a wide variety of genes, and are potent inducers of inducible enzymes like phospholipase A_2 and COX-2 resulting in production of prostaglandins and leukotrienes (Dinarello). It is suggested that many biological effects of these cytokines are due to increased PGE_2 production. Initiation of transcription and translation of the inducible form of nitric oxide synthase (iNOS) is observed in a number of cell types. These cytokines are also capable of inducing their own genes.

Proinflammatory cytokine signal transduction pathways - Role of MAPKs and transcription factors in inflammation

MAPKs Herbal constituents, including apigenin and parthenolide, may exhibit their anti-inflammatory activity, at least in part, by inhibiting members of signaling pathways including the mitogen-activated protein kinases (MAPKs) and specific transcription factors. Cellular signaling pathways mediate the response to extracelluar stimuli by converting these signals into intracellular responses. Through a

series of pathways involving cell-surface receptors, MAPKs and transcription factors, cells can respond to inflammatory mediators including TNF-α and IL-6, ultimately resulting in the activation of gene products critical to the inflammatory response.

The MAPK pathways consist of a three-member protein kinase cascade involving successive phosphorylation and subsequent activation of various MAPKs (Garrington and Johnson, 1999). MAPK kinase kinase (MAPKKK or MEKK) phosphorylate and activate the MAPK kinase (MAPKK, MEK or MKK). In turn, the MAPKK catalyze the phosphorylation of MAPKs on tyrosine and threonine residues. Activation of MAPKs controls gene expression, metabolism and other cell regulatory events, and contribute to complex cellular events including differentiation, survival and migration (Garrington and Johnson, 1999).

There are three MAPK subgroups: extracellular signal-regulated kinase (ERK or p42/p44), c-jun amino-terminal kinase (JNK, also referred to as stress-activated protein kinase, SAPK) and p38 (Garrington & Johnson, 1999). The ERK pathway is stimulated primarily by growth factors and mitogens, (Ichijo, 1999) and affects cellular growth and differentiation. The JNK family is stimulated by stress including LPS, UV irradiation and DNA damage, (Tibbles & Woodgett, 1999) and by differentiation and growth factors in addition to the cytokines IL-1 and TNF-α. Activation of JNK affects cellular growth, differentiation, survival and apoptosis (Garrington and Johnson, 1999). The p38 pathway is stimulated by stress, similar to the JNK pathway. Activation of p38 controls cytokine production and apoptosis (Garrington and Johnson, 1999).

MAPKs participate in gene expression through the activation of various transcription factors and/or their family members. The ERKs can activate Elk-1, Sap1a, c-myc, Tal and STATS. The JNKs can activate c-Jun, ATF-2, Elk-1 whereas p38 also

activates ATF-2 and Elk-1 (Garrington and Johnson, 1999). Notably, the transcription factors Elk-1, c-Jun, and ATF-2 are each involved in the expression of genes which control the inflammatory response.

Transcription factors -

Similar to the MAPKs, transcription factors play an integral role in the transduction of extracellular signals. Intracellular signaling cascades result in the activation of specific DNA-binding proteins or transcription factors. These transcription factors, once activated, bind to specific recognition sequences in the promoters of target genes, resulting in the modulation of gene transcription (Adcock et al., 1997). A number of transcription factors participate in the expression of genes involved in the inflammatory response.

AP-1, activating protein 1, refers collectively to the dimeric transcription factors composed of the Jun, Fos or ATF (activating transcription factor) subunits that bind to a common DNA site, the AP-1 binding site (Karin et al., 1997). AP-1 is an important regulator of nuclear gene expression in leukocytes (Foletta et al., 1998) and is activated in response to a diverse range of stimuli including peptide growth hormones, cellular stress, UV irradiation and the cytokines IL-1, TNF-α and TGF-β (Wisdom, 1999; Angel & Karin, 1991). Homodimers (Jun or ATF) or heterodimers (Jun, ATF or Fos) bind to the AP-1 binding site located in the promoter region of target genes including TNF-α, IL-8 (Foletta et al., 1998) and GM-CSF (granulocyte monocyte - colony stimulating factor) (Adcock et al., 1997) and initiate transcription by stabilizing RNA polymerase II at the transcription start site.

CAAT/enhancer binding protein beta (C/EBPβ), formally referred to as NF-IL6, belongs to the C/EBP family of transcription factors (Wedel & Ziegler-Heitbrock,

1995). At least seven family members have been identified, each containing three distinct regions. First, a C-terminal leucine-zipper which is required for dimerization. Second, a basic DNA-binding region responsible for DNA recognition and nuclear translocation. And third, a N-terminal transactivating region. C/EBPβ is strongly up-regulated by inflammatory stimuli including LPS, and the cytokines IL-6, IL-1 and TNF-α (Ledda-Columbano et al., 1998). C/EBPβ binding sites have been identified in the promoters of many cytokine genes including those involved in the inflammatory response i.e. IL-1, IL-6, TNF-α and IL-8 (Ledda-Columbano et al., 1998; Pope et al., 1994) suggesting an involvement in the response to inflammation.

The transcription factor **nuclear factor-kappa B (NF-κB)** consists of five protein family members, RelA (p65), c-Rel, RelB, NF-κB1 (p50) and NF-κB2 (p52) (Mercurio & Manning, 1999). The N-terminal region called the Rel-homology domain (RHD) contains subregions which mediate DNA-binding, dimerization and nuclear localization (Mercurio & Manning, 1999). NF-κB is located in the cytoplasm of cells in an inactive form. Activation of NF-κB by LPS, IL-1 or TNF-α results in phosphorylation, ubiquitination and subsequent degradation of the inhibitory protein IκB (inhibitor of NF-κB). The dimeric NF-κB can now translocate to the nucleus where it activates expression of specific target genes (Baldwin, 1996) including the cell adhesion molecules VCAM-1 and ICAM-1, the cytokines IL-6 and TNF-α and the chemokine IL-8 (Baeuerle & Henkel, 1994).

The role of MAPK and transcription factors in the expression of genes regulating inflammatory responses has been studied extensively. New drugs and potential therapeutics for inflammatory diseases including rheumatoid arthritis are focusing on inhibition of specific members of the intracellular signaling cascades, including various

MAPKs and transcription factors. The investigation of herbal constituents and products affecting these signaling pathways may confirm their role as potential therapeutics in the treatment of inflammatory diseases.

Lipopolysaccharide - a prototypical inflammagen Lipopolysaccaride (LPS), the major biologically active component of endotoxin, is derived from cell walls of Gram-negative bacteria (Roth et al., 1997). LPS is a potent inducer of inflammation (Figure 1.3) and has been used for many years as a tool to induce and study inflammatory responses. In humans and animals, injection of small, non-toxic doses of LPS results in the circulation of soluble inflammatory mediators and increased plasma concentrations of TNF and lipid inflammatory mediators (Roth et al., 1997). The production of these inflammatory mediators by LPS can be monitored and evaluated to study the effect of pharmacological treatment on the inflammatory response.

Herbal extracts, constituents and their effects on inflammatory mediators The overall goal of this research was to assess the potential for specific herbal constituents to possess anti-inflammatory activity, and thus serve as effective treatments for inflammatory conditions. Specifically, the proposed research sought to determine the effect of the herbal constituents apigenin (chamomile), ginsenoside Rb_1 (ginseng) and parthenolide (feverfew) on specific mediators of inflammation.

Chamomile *Matricaria recutita*, also commonly known as German chamomile, has been traditionally used as a popular folk remedy to treat digestive disorders, cramps, various skin conditions and minor infectious ailments (Tyler, 1993). Today, chamomile is most frequently used as a sleep aid and in the promotion of wound healing. Chamomile is most commonly consumed as a tea or applied externally as a compress. The fresh or dried flowering heads of the plant are used when preparing the herb.

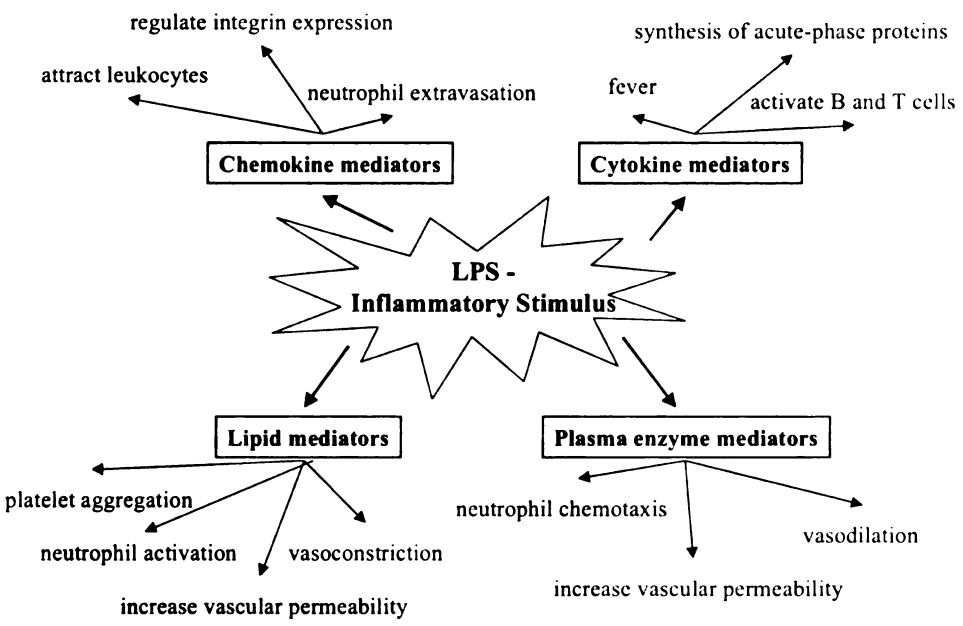

Figure 1.3. Inflammatory mediators and select consequences of their induction by inflammatory stimuli.

Chamomile has a good safety record and is listed as a "generally recognized as safe" (GRAS) substance by the FDA. However, individuals are cautioned against use of the herb in conjunction with other sedatives such as alcohol or benzodiazepines. Additionally, the plant is a possible allergen that may cause contact dermatitis, anaphylaxis or other hypersensitivity reactions in allergic individuals (Tyler, 1993). There are no other reported contraindications, side effects or interactions with other drugs (Blumenthal et al., 1998). Furthermore, no known adverse effects in pregnancy, lactation or childhood are noted (O'Hara et al., 1998).

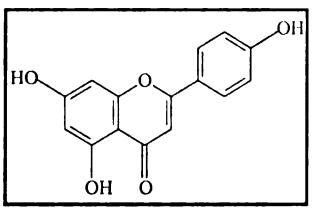

Figure 1.4. Apigenin

The main active components of chamomile are thought to be volatile oils and various flavonoids including apigenin. Apigenin (4, 5, 7 - trihydroxyflavone) (Figure 1.4) is the best studied component of chamomile. The flavone has inhibitory effects on all four major categories of inflammatory mediators; chemokine, plasma enzyme, lipid and cytokine (Figure 1.1).

Apigenin significantly inhibits TNF-α-induced IL-8 (Gerritsten et al., 1995) in human endothelial cells which suggests apigenin may interfere with neutrophil recruitment to sites of inflammation. Gerritsen *et al* (1995) also reported that apigenin was capable of inhibiting intercellular adhesion molecule (ICAM)-1, vascular cell-adhesion molecule (VCAM)-1 and E-selectin protein and mRNA expression in human endothelial cells induced by TNF-α and IFN-γ (Gerritsen et al, 1995). Similar effects were observed by Wolle et al. (1996). The expression of these three cell-adhesion molecules is increased on endothelial cells by a variety of cytokines and inflammatory mediators upon the induction of an inflammatory response. They serve to increase the

strength of functional interactions between cells of the immune system (Kuby, 1997) and thus contribute to a successful inflammatory response.

The complement system, one of the four plasma enzyme mediator systems, forms complement split products that induce localized vasodilation and attract phagocytic cells chemotactically, leading to an inflammatory reaction. At high concentrations (IC_{50}=156 μM), apigenin had an inhibitory effect on the classical, but not alternative, pathway of the complement system in an *in vitro* model system (Pieroni et al., 2000).

Apigenin also inhibits lipid inflammatory mediators. PGE_2 production is significantly decreased by apigenin in LPS stimulated murine macrophage (Liang et al., 1999) and IL-1α-stimulated endothelial cells (Gerritsen et al., 1995). LPS-induced cyclooxygenase (COX)-2 up-regulation of protein and mRNA are dose-dependently inhibited by apigenin in murine macrophage cells (Liang et al., 1999). Prostaglandins resulting from COX metabolism of arachidonic acid are important for increased vascular permeability and dilation suggesting that apigenin's inhibitory effects are likely to decrease this aspect of the inflammatory response.

Additionally, apigenin inhibits IgE-induced TNF-α release in rat basophilic leukemia cells (Mastuda et al., 2002). An inhibitory effect on the proinflammatory cytokine IL-6 was observed in TNF-α-induced human endothelial cells co-treated with apigenin (Gerritsen et al., 1995). This cytokine can induce B- and T-cell activation as well as fever and platelet production in times of inflammation.

Ginseng *Panax ginseng* is one of several ginseng species commonly consumed worldwide. In the Orient, where ginseng is cultivated and utilized extensively, consumption is not generally for curing a disease but used in a supportive role in order to maintain health (Tyler, 1993). In the United States, it is estimated that at least 6 million

Americans consume ginseng (Lawrence Review, 1990) to enhance physical performance, promote vitality and increase resistance to stress and aging (O'Hara et al., 1998). A variety of commercial ginseng products exist including teas, powders, capsules, tablets and extracts. Two studies have shown that many ginseng products contain negligible ginseng, whereas other ginseng products actually contained no ginseng (Tyler, 1993). The medicinal part of the plant is derived from the roots (Fleming, 1999; Tyler, 1993).

Although a good safety record exists from centuries of human use, the safety of ginseng is still questioned. The most commonly documented side effects of ginseng are insomnia, and to a lesser extent, diarrhea and skin irritation (Tyler, 1993). Overdoses can bring about sleeplessness, hypertonia and edema. These symptoms are generally characterized as Ginseng Abuse Syndrome (Fleming, 1999).

Ginseng contains several chemicals which are thought to have biological activity. These include: water soluble polysaccharides, polyynes and saponins including the panaxosides, which are more commonly referred to as ginsenosides. Of these, the ginsenosides are believed to be responsible for most of ginseng's activities (Wakabayashi et al., 1997; Attele et al., 1999) and are used as standardization markers in quality evaluation of ginseng products (Harkey et al., 2001). These compounds are found in the largest quantities in the root (Tyler, 1993). Twelve major ginsenosides have been isolated, along with several dozen minor glycoside derivatives (Lawrence Review, 1990). Ginsenoside Rb_1 (Figure 1.5) is one of the primary ginsenoside components (Bae et al., 2000). The biological activity of ginsenoside Rb_1 is attributed to the hydrolyzed metabolite ginsenoside M1, or compound

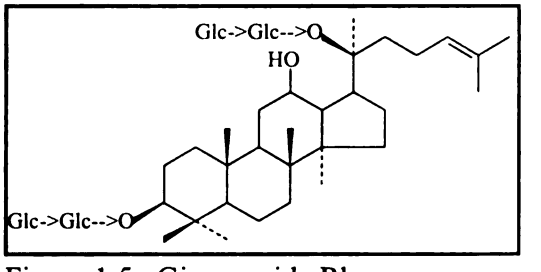

Figure 1.5. Ginsenoside Rb_1

K, which is produced by intestinal cleavage (Wakabayashi et al., 1997; Bae et al., 2000; Hasegawa et al., 1998; Akao et al., 1998). Animal studies show the inhibitory effects of ginseng root saponins, following repeated i.p. injections, on LPS-induced IL-1β and IL-6 mRNA expression in hippocampus of aged rats (Shang-Car & Xiao-Yu, 2000). But little is known about the direct effects of ginsenoside Rb_1, specifically, on inflammatory mediators. However, Cho et al. (2001) reported that ginsenoside Rb_1 inhibited LPS-induced TNF-α production by human and murine macrophage cells.

Feverfew *Tanacetum parthenium* has been used since 78 A.D. for the treatment of fever as well as headache, menstrual irregularities and stomachache (Tyler, 1993). Today, feverfew is widely used as a migraine preventative, and more recently as an aid for those suffering from arthritis. The product is available in a number of forms including capsule, tablet and tea. The medicinal properties of the plant can be found in the fresh or dried leaves.

Safety data on feverfew obtained from clinical trials suggests that consumption of the plant is relatively safe, although the incidence of mouth ulcers was 5-15 % higher in treatment groups compared to placebo groups (O'Hara et al., 1998). This side effect is most commonly associated with consumption of the fresh leaves. Johnson et al. (1985) has noted development of anxiety, poor sleep patterns, and muscle and joint stiffness in patients who stopped taking feverfew after years of consumption - "postfeverfew syndrome"(Johnson et al., 1985). Individuals are cautioned against the use of feverfew during pregnancy since it was historically used to induce menstrual bleeding, and in patients with coagulation problems due to effects on platelet activity (O'Hara etal.,1998). Additionally, it is not recommended for lactating mothers or children under the age of 2 (Awang, 1991).

The volatile oils, flavonoids and polyynes found in feverfew are all thought to impart biological activity, although the majority of the medicinal properties are attributed to the sesquiterpene lactone parthenolide (Figure 1.6).

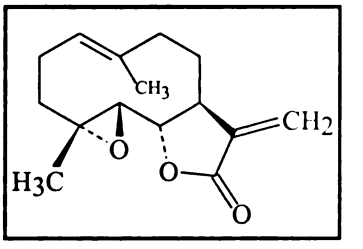

Figure 1.6. Parthenolide

Biological effects of feverfew and/or parthenolide on mediators of inflammation

A number of studies have investigated the effects of parthenolide on inflammatory mediators. Mazor et al. (2000) reported that parthenolide is a potent inhibitor of IL-8 gene expression in cultured human respiratory epithelium (Mazor et al., 2000). TNF-α mediated IL-8 protein and mRNA expression are inhibited in a concentration-dependent manner. One hour pretreatment with parthenolide also inhibits TNF-α-mediated activation of the IL-8 promoter. ICAM-1 expression, also important for cell recruitment at sites of inflammation, is repressed by parthenolide in immortalized human umbilical cord endothelial cells stimulated by IL-1β and LPS (Dietrich et al., 1999).

Parthenolide also has inhibitory effects on lipid inflammatory mediators. One end result of membrane phospholipid breakdown is platelet aggregation. In platelet-rich plasma derived from the blood of healthy volunteers, parthenolide caused a concentration-dependent inhibition of aggregation induced by phorbol ester (PMA) or arachidonic acid (Groenewegen et al., 1990). In LPS-stimulated alveolar macrophages, parthenolide inhibited the expression of COX-2 protein and mRNA (Hwang et al., 1996). The activity of COX-2 is responsible for production of prostaglandins and thromboxanes which can increase vascular permeability, platelet aggregation, neutrophil chemotaxis and vasoconstriction. Parthenolide inhibits prostaglandin synthetase-mediated PGE_2

production from arachidonic acid *in vitro* (Pugh & Sambo, 1988). Parthenolide's inhibition of COX-2 and subsequent prostaglandin production may therefore result in a diminished inflammatory response.

In addition, parthenolide inhibits LPS-induced TNF-α production in alveolar macrophage cells (Hwang et al., 1996). Parthenolide also inhibits steady-state levels of TNF-α and IL-1β mRNA in this model. The production of these proinflammatory cytokines is important for induction of other cytokines, increased expression of adhesion molecules on vascular endothelium and activation of B- and T-cells.

Parthenolide's effect on MAPKs and transcription factors Recent attention has focused on the inhibitory effects of parthenolide on signaling pathways critical to various inflammatory mediators including TNF-α and IL-6 (Garcia-Pineres et al., 2001; Kwok et al., 2001; Uchi et al., 2002. These studies indicate that parthenolide is capable of inhibiting both MAPKs and various transcription factors in cell culture systems, thus providing mechanistic insight into the constituent's anti-inflammatory activity, but no studies of this type have been completed in an animal model system.

Hwang et al (1996) demonstrated the inhibitory effects of parthenolide on MAPKs. Parthenolide pre-treatment caused a dose-dependent inhibition of tyrosine phosphorylation and kinase activity of ERK1 and ERK2 in LPS-stimulated RAW 264.7 macrophage cells. Similar dose-dependent inhibition was noted for JNK1 and p38. The inhibitory effects of parthenolide on tyrosine phosphorylation of MAPKs correlated with reduced expression of COX-2 and IL-1α evaluated in RAW cells.

By contrast, JNK activation and p38 phosphorylation were not inhibited by parthenolide treatment when employing TNF-α-induced HeLa cells (Hehner et al., 1999). In addition, parthenolide treatment in conjunction with the overexpression of the

upstream MEKK1 did not affect JNK activation. In support of these findings, Uchi et al (2002) demonstrated in dendritic cell populations that parthenolide could inhibit LPS- but not TNF-induced p38. These findings suggest that the differences observed in parthenolide's ability to inhibit specific MAPKs may be related to the specific stimuli used to induce MAPK phosphorylation, not the cell type evaluated.

Many studies have focused on the inhibitory effects of parthenolide on transcription factors, particularly NF-κB. Bork et al (1997) used PMA-stimulated HeLa cells pre-incubated with parthenolide to demonstrate the inhibitory effects on activation of NF-κB. Hehner et al (1999) similarly used the HeLa cell model, except with TNF-α-stimulation, to determine the actual target for parthenolide inhibition of NF-κB activation. The findings suggest that parthenolide is targeting the IκB kinase complex (IKK), which is responsible for phosphorylation of the inhibitory protein which prevents nuclear translocation of NF-κB. Mazor et al (2000) used TNF-α induced cultured human respiratory epithelium treated with parthenolide to inhibit nuclear translocation of NF-κB and degradation of the IκBα protein. Alternatively, Garcia-Pineres et al. (2001) report that parthenolide most likely inhibits NF-κB by alkylating p65 at cysteine 38, not through IκB degradation.

Other studies have evaluated the affect of parthenolide treatment on the AP-1 and STAT3 transcription factors. PMA-stimulated HeLa cells treated with parthenolide showed no inhibitory effects on AP-1 DNA-binding activity compared to the PMA-stimulated control cells (Bork et al., 1997). However, parthenolide was effective at blocking STAT3 phosphorylation on tyrosine 705, preventing STAT3 dimerization necessary for nuclear translocation (Sobota et al., 2000).

The inhibitory effects of parthenolide on the activation of MAPK and various transcription factors may explain its inhibitory effects on mediators of inflammation including chemokines, lipid mediators and proinflammatory cytokines. Further studies evaluating the in vitro and in vivo effects of parthenolide on cellular signaling pathways may provide the necessary efficacy information to prove their therapeutic capacity in inflammatory diseases.

Rationale The dietary supplement market and number of supplement consumers has grown significantly since the passage of the Dietary Supplement Health and Education Act of 1994 (DSHEA). Among dietary supplement products, herbals account for an estimated $3.24 billion (Johnston et al., 1997), a considerable portion of total dietary supplement sales estimated at $15.7 billion in 2000 (Blendon et al., 2001). The increased use and sales throughout the 1990's and into the 2000's confirms the continued interest in the dietary supplement market.

Current regulations governing the dietary supplement industry are insufficient to guarantee the same level of safety and efficacy as that of conventional drugs. Although the industry and number of products has continued to grow, there is still a considerable lack of scientific evidence to clearly support efficacy and, in some cases, safety of these products. Thus, studies verifying the presumed efficacy, and safety, of specific dietary supplement products should be undertaken.

Published reports describe putative effects of various herbal extracts and their constituents. Research suggests three herbal constituents, apigenin, ginsenoside Rb_1 and parthenolide, posses anti-inflammatory properties in vitro. Despite the collection of work that has documented the anti-inflammatory properties of these constituents in vitro, there is very limited data documenting these inhibitory effects in vivo. Additionally, no

studies have been undertaken to elucidate potential transcriptional mechanisms by which parthenolide affects mediators of inflammation in vivo; and the work in vitro is largely incomplete. Therefore, continued research in this area is warranted.

Overall, these studies will contribute to the understanding of the anti-inflammatory properties, and potential mechanisms of inhibition, of the herbal constituents apigenin, ginsenoside Rb_1 and, more specifically, parthenolide on inflammatory mediators. The methods and results of these studies can be used to further elucidate parthenolide's, and other herbal constituents' and extracts', molecular mechanism for inhibitory effects on inflammation, and potential as human therapeutics in the treatment of inflammatory conditions.

CHAPTER 2

Modulation of Lipopolysaccharide-Induced Proinflammatory Cytokine Production In Vitro and In Vivo by the Herbal Constituents Apigenin (Chamomile), Ginsenoside Rb_1 (Ginseng) and Parthenolide (Feverfew)

INTRODUCTION

Hundreds of herbal remedies have been used historically in maintenance of health and in the treatment of diseases. Today, such alternatives to over-the-counter medicines and prescriptions are still sought, with 36 percent of the U.S. population using herbal products (McVean et al., 2000; O'Hara et al., 1998). The herbal supplement industry experienced tremendous growth throughout the 1990's with sales doubling every four years (Fleming, 1998) and projected sales expected to reach $6.6 billion by 2003 (Sloan, 2000). The overwhelming increase in herbal supplement sales has prompted concern over the need for appropriate regulations. Herbal supplements are legally defined as dietary supplements, and thus fall under the regulation of the Dietary Supplement Health and Education Act of 1994 (DSHEA). Dietary supplements, unlike conventional drugs, do not require approval from the Food and Drug Administration (FDA) prior to marketing and sale of the product. Therefore, no investigations of the safety and efficacy of herbal products are required. Thus, it is often not known if these products are effective or even safe.

One potential use for herbals is the treatment of inflammatory conditions. A number of inflammatory mediators are released by cells in response to localized injury or

trauma. These mediators elicit or enhance particular functions of the inflammatory response and may be monitored to assess an inflammatory response. The primary inflammatory mediators are grouped into four main categories: chemokine, plasma enzyme, lipid and cytokine. Three herbal constituents, apigenin (chamomile), ginsenoside Rb_1 (ginseng) and parthenolide (feverfew), may possess anti-inflammatory properties. A limited amount of information now exists relating these herbal products, and their constituents, to specific anti-inflammatory mechanisms.

German chamomile, derived from *Matricaria recutita*, has been traditionally used as a popular folk remedy to treat digestive disorders, cramps, various skin conditions and minor infectious ailments (Tyler, 1993). Today, chamomile is most frequently used as a sleep aid and in the promotion of wound healing. Apigenin (4, 5, 7 - trihydroxyflavone) is the best studied component of chamomile. This flavonoid has inhibitory effects on adhesion molecule expression (Panes et al., 1996; Wolle et al., 1996), prostaglandin (PG) E_2, (Liang et al., 1999; Panes et al. 1996) cyclooxygenase (COX)-2 (Liang et al., 1999) production and the proinflammatory cytokine interleukin (IL)-6 (Panes et al., 1996) in cell culture models.

The herbal supplement ginseng, derived from the roots of *Panax ginseng* or *Panax quinquefolium*, is estimated to be consumed regularly by more than 6 million Americans (Lawrence Review, 1990). Ginseng extracts are frequently consumed for their ability to enhance physical performance and for their adaptogenic effects. These effects are believed to increase the body's ability to fight stress, increase resistance to disease by strengthening normal body function and as a result, reduce the detrimental effects of the aging process (O'Hara et al., 1998, Tyler, 1998). The ginsenosides are believed to be responsible for most of ginseng's activities (Attele et al., 1999; Fleming,

1998; Tyler, 1993; Wakabayashi et al., 1997). Cell culture studies have shown that ginsenoside Rb_1, one of the primary ginsenoside components (Bae et al., 2000), can inhibit lipopolysaccharide (LPS)-induced expression of the proinflammatory cytokine tumor necrosis factor (TNF)-α (Cho et al., 1998). No in vivo data are available assessing the effect of ginseng on proinflammatory cytokine production.

Feverfew, *Tanacetum parthenium,* has been used for at least two millenia for the treatment of fever as well as headache, menstrual irregularities and stomachache (Tyler, 1993). Today, feverfew is widely used as a migraine preventative, and more recently as an aid for those suffering from arthritis (Jain & Kulkarni, 1999; Schinella et al., 1998; Williams et al., 1999). The majority of the medicinal properties are attributed to the sesquiterpene lactone parthenolide, which inhibits expression of intercellular adhesion molecule (ICAM)-1(Dietrich et al., 1999), COX-2 and TNF-α production (Hwang et al., 1996) in vitro. No studies have been published which evaluated the effects of parthenolide on proinflammatory cytokine production in vivo.

LPS, the major biologically active component of endotoxin, is derived from cell walls of gram-negative bacteria (Roth et al., 1997). LPS is a potent inducer of inflammation and can be used to induce production of inflammatory mediators, including proinflammatory cytokines, in model systems. Induction of the proinflammatory cytokines TNF-α and IL-6 by LPS can be monitored and evaluated to study the effect of pharmacological treatment, including herbals, on the inflammatory response. In this study, we hypothesized that apigenin, ginsenoside Rb_1 and parthenolide inhibit the expression of TNF-α and IL-6 in murine macrophage cell culture and intact animal model systems. Although all three constituents inhibited proinflammatory cytokine production in vitro, these results did not adequately predict effects in vivo.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. For in vitro studies, apigenin, ginsenoside Rb_1 and parthenolide (Aldrich, Milwaukee, WI) were dissolved in tissue culture grade dimethyl sulfoxide (DMSO) and further diluted in Dulbecco's modified Eagle's medium (DMEM, Sigma). The final concentration of DMSO in cell culture (less than 0.01% (v/v)) was not cytotoxic as determined by the colorimetric MTT (tetrazolium) assay (Mosmann, 1983). LPS from *Salmonella typhimurium* [1.5 EU/ng LPS] was diluted in DMEM. For in vivo studies, apigenin, ginsenoside Rb_1 and parthenolide were dissolved in DMSO; LPS was dissolved in endotoxin-free tissue culture grade water.

Experimental design for in vitro studies

The murine macrophage cell line, RAW 264.7, was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in DMEM containing 4 mM L-glutamine, 3.7 g/L sodium bicarbonate and 4.5 g/L glucose. Medium was supplemented with 10% (v/v) fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA), 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate and 1% (v/v) NCTC-135 medium (Gibco BRL, Rockville, MD). Cells were maintained in a 5% CO_2 humidified incubator at 37°C. Cell number and viability were assessed by trypan blue dye exclusion using a hemacytometer (American Optical, Buffalo, NY).

RAW 264.7 cells (5×10^5 cells/ml) were cultured in flat-bottomed 48-well tissue culture plates (0.8 ml/well)(Corning, Corning, NY) with either apigenin, ginsenoside Rb_1

or parthenolide with or without stimulation by LPS. Supernatants were collected after 12, 24 and 48 h incubations (24 and 48 h data not shown) and stored at -80°C until analysis.

Experimental design for in vivo studies

All animal handling was conducted in ordinance with guidelines established by the National Institutes of Health. Experiments were designed to minimize the numbers of animals used. Female B6C3F1 mice (8-10 wks) were obtained from Charles River (Portage, MI). Animals were housed 3-4 per cage with a 12 h light/dark cycle, provided standard rodent chow and water *ad libitum*, and acclimated to their environment as least one week before the start of experiments.

In all cases, treatment with each herbal constituent was via p.o. administration to more closely approximate human exposure through consumption of herbal dietary supplements. Mice were pre-treated for 1 h with apigenin (50 mg/kg, p.o. in 50 µl) based on efficacious results obtained by Panes et al. (1996) and Mascolo et al. (1988) using similar treatment times and doses via i.p. administration. A 2 h pre-treatment with ginsenoside Rb_1 (25 mg/kg, p.o. in 50 µl) was used based on the previously reported kinetics of ginsenoside Rb_1 and its active metabolite, ginsenoside M1 (also known as compound K) (Wakabayashi et al., 1997), and treatment dose reported as efficacious by Jun-tain et al. (1990) and Hasegawa and Uchiyama (1998). Parthenolide (50 mg/kg, p.o. in 50 µl) was administered 1 h prior to LPS based on studies by Jain et al. (1999) and Mittra et al. (2000), and dose based on consideration of studies by Jain et al. (1999) and Schinella et al. (1998). LPS was administration at 1 mg/kg, i.p. in 100 µl. Vehicle-treated mice received 50 µl DMSO, p.o. and/or 100 µl H_2O, i.p. A 90 min time point

sufficient to evaluate both IL-6 and TNF-α from one blood draw was chosen for sample collection based on time course data obtained by Zhou et al. (1999). Blood was obtained by retro-orbital bleeding and allowed to clot overnight at 4°C. Serum was collected and analyzed for IL-6 and TNF-α by ELISA.

IL-6 and TNF-α determination by ELISA

Cytokine analysis for IL-6 (in vitro and in vivo) and TNF-α (in vitro) were performed using purified and biotin-conjugated rat anti-mouse IL-6 and TNF-α antibodies, respectively, from PharMingen (San Diego, CA). Streptavidin-peroxidase (Sigma) and 3,3',5,5'-tetramethylbenzidine (TMB, Fluka, Ronkonkoma, NY) were used for detection. Absorbance was read at 450 nm using a Vmax™ Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA). For TNF-α analysis in vivo, the OptEIA Set: Mouse TNF-α (Mono/Poly) kit was used according to manufacturer's instructions (Sigma).

Statistics

All statistical testing was performed using SigmaStat Statistical Analysis Software (Jandel Scientific, San Rafael, CA). For comparisons of multiple groups using parametric data, one-way analysis of variance (ANOVA) using Bonferroni's method were performed. For comparison of two groups, a Student's *t*-test was used.

RESULTS

In vitro co-treatment with apigenin impairs LPS-induced IL-6, but not TNF-α production

To determine the potential inhibitory effects of herbal constituents on proinflammatory cytokine expression, RAW 264.7 murine macrophage cells were stimulated by LPS and co-treated with various herbal constituents. Apigenin, a flavone found in chamomile extracts and foods including parsley, bell peppers and guava was evaluated in vitro for potential inhibitory effects on LPS-induced IL-6 and TNF-α. RAW cells were co-treated with LPS and apigenin, 0.1 - 10 μg/ml, for 12 h. Apigenin doses evaluated were not cytotoxic as determined by the colorimetric MTT (tetrazolium) assay (results not shown) (Mosmann, 1983). LPS at 100 and 1000 ng/ml significantly induced IL-6 and TNF-α production following incubation (Figure 2.1). No direct induction of proinflammatory cytokines was observed in vehicle or apigenin-only treated cells. Co-treatment with the two highest doses, 1 and 10 μg/ml (3.7 and 37 μM, respectively), of apigenin significantly, and dose-dependently, impaired LPS-induced IL-6. Significant inhibition of LPS-induced TNF-α was not observed.

In vitro co-treatment with ginsenoside Rb_1 dose-dependently inhibits LPS-induced IL-6 and TNF-α production

Ginsenoside Rb_1 is one of 12 major ginsenosides derived from *Panax* species. To test ginsenoside Rb_1's capacity to inhibit LPS-induced IL-6 and TNF-α production, RAW cells were co-treated with LPS and ginsenoside Rb_1, 0.1 to 100 μg/ml. No dose of ginsenoside Rb_1 tested caused cytotoxicity as determined by the MTT assay (results not

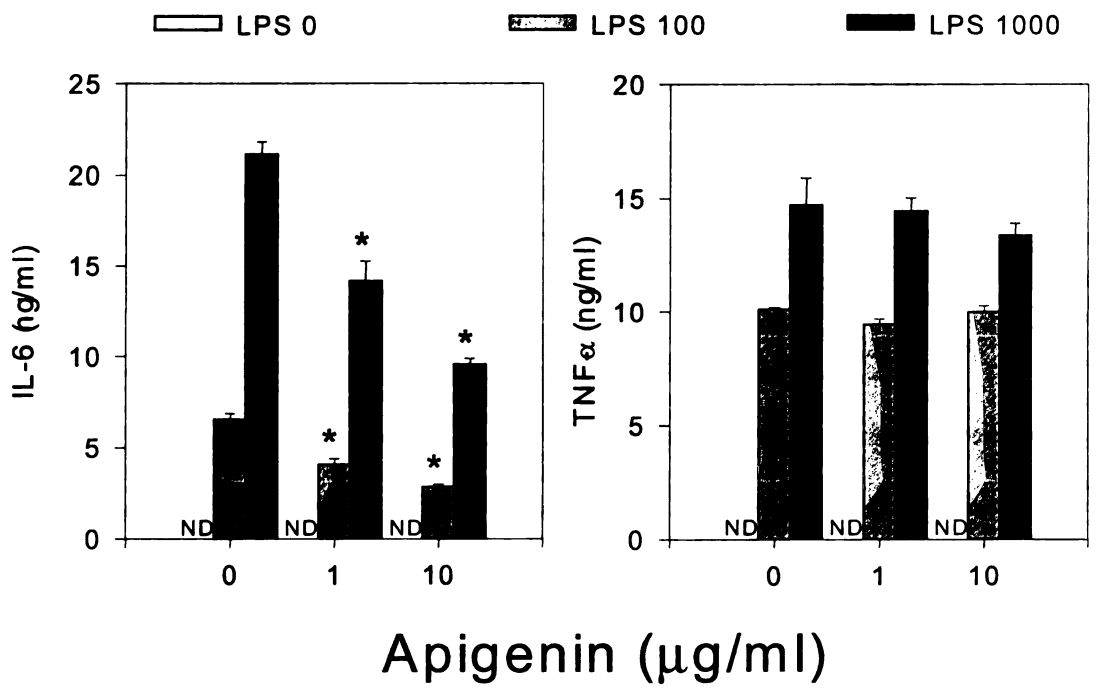

Figure 2.1. Effect of apigenin on LPS-induced interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) production by RAW 264.7 cells. Cells (5 x 10^5 cells/ml) were cultured with LPS (0, 100 or 1000 ng/ml) in the presence or absence of apigenin for 12 h and supernatant analyzed for IL-6 and TNF-α. Data are mean ± SEM (n = 6) of triplicate cultures. Values marked with an asterisk (*) differ significantly from LPS control values ($p< 0.05$). ND indicates nondetectable at less than 3 ng/ml.

shown). Significant production of IL-6 and TNF-α was observed following treatment with 100 and 1000 ng/ml LPS (Figure 2.2). No direct induction of IL-6 or TNF-α was observed in vehicle or ginsenoside Rb_1-only treated cells. Ginsenoside Rb_1 co-treated cells produced significantly, and dose-dependently, less LPS-induced IL-6 following 12 h incubation. Similar dose-dependent impairment of TNF-α was observed, with significant decreases in LPS-induced TNF-α following 10 μg/ml (8.4 μM) co-treatment, and a complete inhibition in cells co-treated with 100 or 1000 ng/ml LPS and 100 μg/ml (84 μM) of ginsenoside Rb_1.

In vitro co-treatment with parthenolide inhibits LPS-induced IL-6 and TNF-α

Parthenolide, used as the constituent for standardization in commercial feverfew products, exhibits a wide variety of anti-inflammatory effects in murine and human cell systems. To determine potential effects on proinflammatory cytokine secretion, RAW cells were co-treated with LPS and 1 to 1000 ng/ml parthenolide. Cytotoxicity, as determined by the MTT assay, was not observed with parthenolide test doses (results not shown). Significant production of IL-6 and TNF-α was observed following 12 h exposure to LPS (Figure 2.3). No direct induction of proinflammatory cytokines was observed in vehicle and parthenolide-only treated cells. Co-treatment with 1000 ng/ml (4 μM) parthenolide significantly impaired both LPS-induced IL-6 and TNF-α.

Apigenin pretreatment in vivo inhibits LPS-induced IL-6 and TNF-α

In order to more closely approximate potential human effects and to compare model systems, an animal model was employed using each herbal constituent and serum accumulation of LPS-induced proinflammatory cytokines as efficacy endpoint. Female

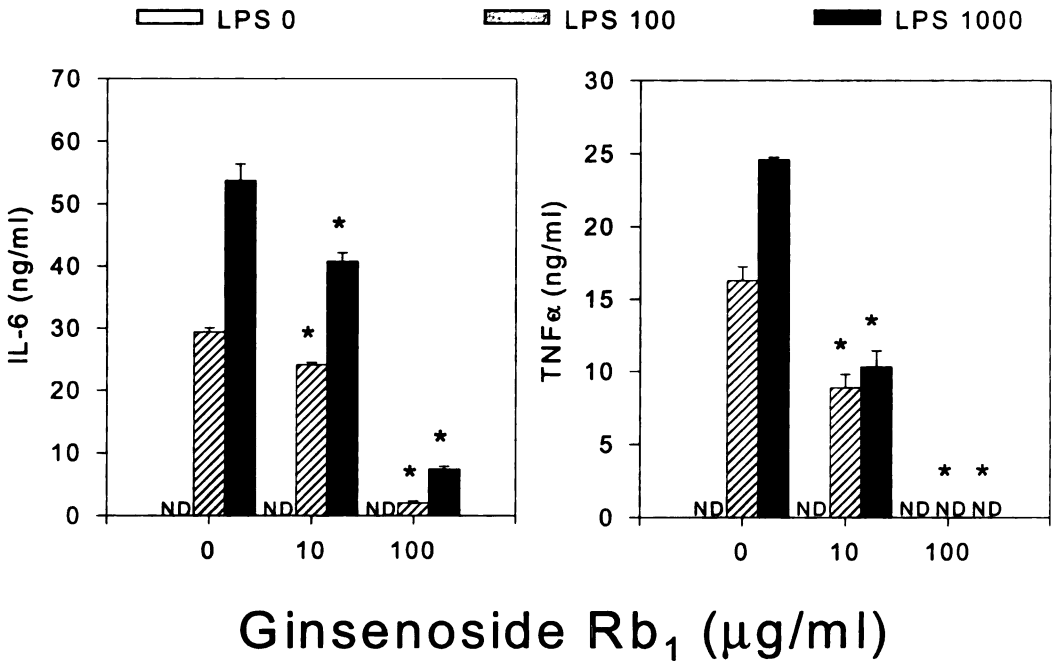

Figure 2.2. Effect of ginsenoside Rb_1 on LPS-induced IL-6 and TNF-α production by RAW 264.7 cells. Cells were cultured as described in Figure 2.1 with the presence or absence of ginsenoside Rb_1 and supernatant analyzed for IL-6 and TNF-α. Data are mean ± SEM (n = 6) of triplicate cultures. Values marked with an asterisk (*) differ significantly from LPS control values ($p< 0.05$). ND indicates nondetectable at less than 3.5 ng/ml.

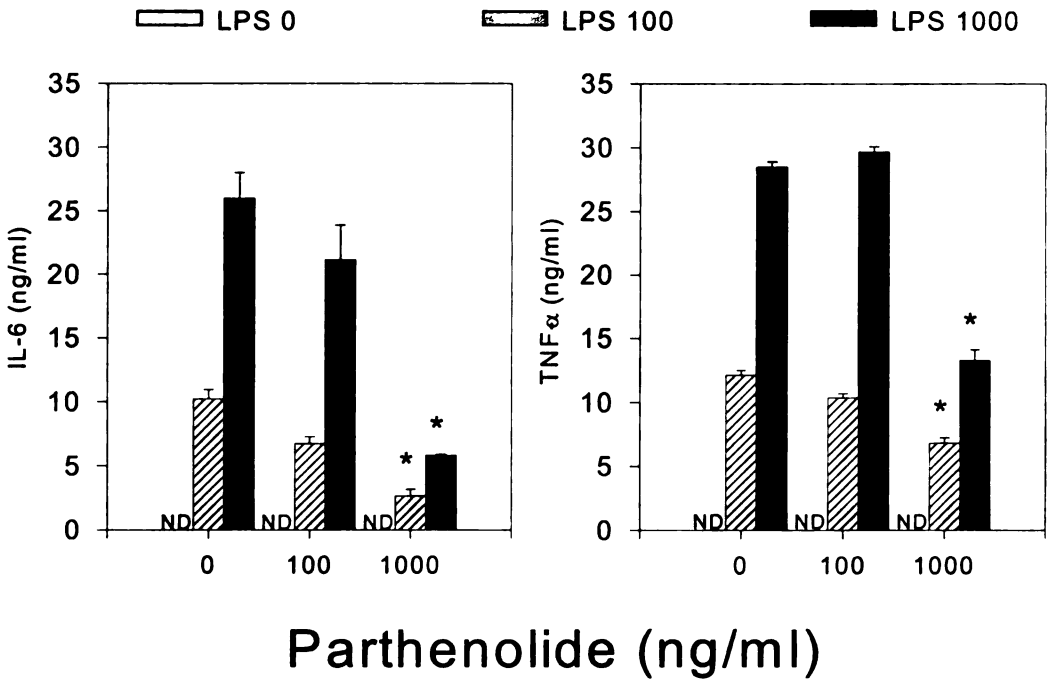

Figure 2.3. Effect of parthenolide on LPS-induced IL-6 and TNF-α production by RAW 264.7 cells. Cells were cultured as described in Figure 2.1 with the presence or absence of parthenolide and supernatant analyzed for IL-6 and TNF-α. Data are mean ± SEM (n = 6) of triplicate cultures. Values marked with an asterisk (*) differ significantly from LPS control values ($p < 0.05$). ND indicates nondetectable at less than 2.25 ng/ml.

mice were pretreated with apigenin (50 mg/kg, p.o.) based on previous in vivo studies by Panes et al. (1996) and Mascolo et al. (1988). After 1 h, LPS (1 mg/kg, i.p.) was administered and blood was collected 90 min later. Analysis of the serum showed significant levels of LPS-induced IL-6 and TNF-α production in controls (Figure 2.4). Serum levels of IL-6 and TNF-α were not detectable in vehicle and apigenin control animals. One h pretreatment with apigenin caused a significant reduction in LPS-induced proinflammatory cytokine production. LPS-induced IL-6 (Figure 2.4A) was significantly reduced (35%), while TNF-α (Figure 2.4B) was significantly reduced (33%) compared to animals treated with vehicle and LPS alone.

Ginsenoside Rb_1 pretreatment in vivo selectively inhibits LPS-induced TNF-α

The in vivo effects of ginsenoside Rb_1 on LPS-induced IL-6 and TNF-α levels in serum were also evaluated. Ginsenoside Rb_1 (25 mg/kg, p.o.) was administered 2 h prior to LPS (1 mg/kg, i.p.) based on the previously reported kinetics of ginsenoside Rb_1 and its active metabolite, ginsenoside M1 or compound K, in the sera of treated mice (Wakabayashi, 1997). Analysis of the serum following 90 min exposure to LPS showed significant induction of IL-6 and TNF-α, whereas these cytokines were unaffected in vehicle and ginsenoside Rb_1 control-treated animals (Figure 2.5). No changes in IL-6 production (Figure 2.5A) were observed with ginsenoside Rb_1 pretreatment. However, TNF-α production was significantly inhibited (Figure 2.5B), (37%), by ginsenoside Rb_1 pretreatment.

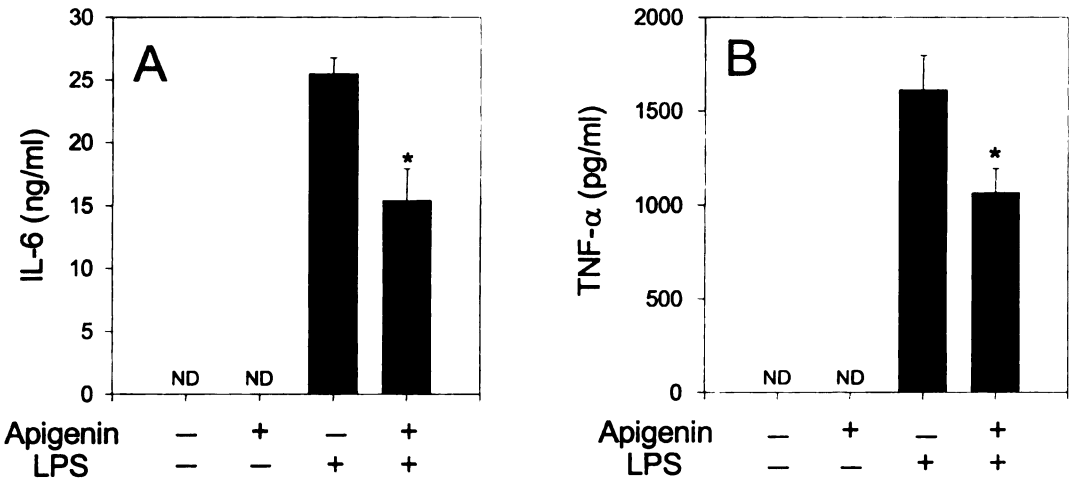

Figure 2.4. Apigenin pretreatment in vivo inhibits LPS-induced (A) IL-6 and (B) TNF-α production. Female B6C3F1 mice were pretreated with apigenin (50 mg/kg, gavage) or vehicle (100 µl DMSO) for 1 hour. LPS (1 mg/kg, i.p.) or vehicle (100 µl sterile water) was administered, and after 90 minutes blood was collected and serum analyzed for IL-6 and TNF-α. Data are mean ± SEM (n=12, controls n=6). Values marked with an asterisk (*) differ significantly from LPS control values ($p< 0.05$). ND indicates nondetectable. Data are a combination of three separate animal experiments.

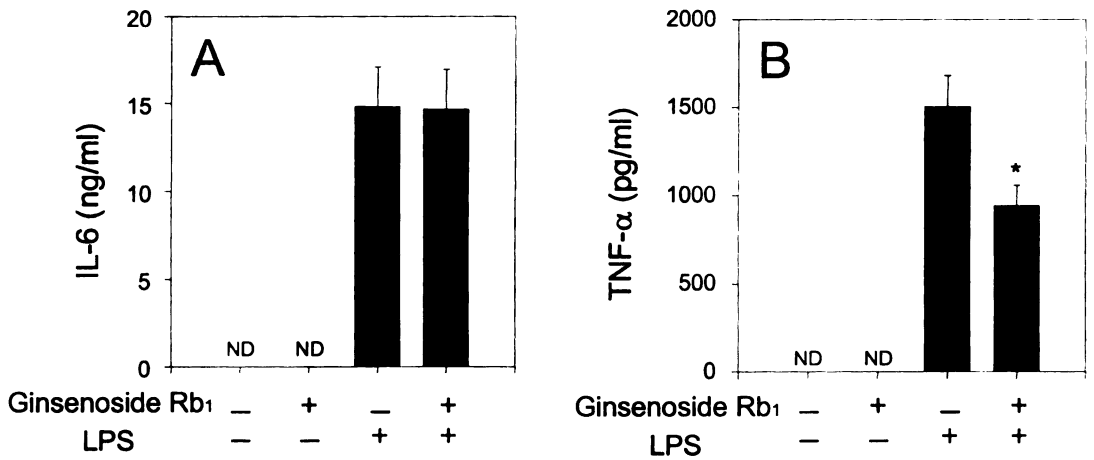

Figure 2.5. Ginsenoside Rb_1 pretreatment in vivo inhibits LPS-induced (B) TNF-α production, but not (A) IL-6. Female B6C3F1 mice were pretreated with ginsenoside Rb_1 (25 mg/kg, gavage) or vehicle (100 µl DMSO) for 2 hours. LPS (1 mg/kg, i.p.) or vehicle (100 µl sterile water) was administered, and after 90 minutes blood was collected and serum analyzed for IL-6 and TNF-α. Data are mean ± SEM (n=12, controls n=6). Values marked with an asterisk (*) differ significantly from LPS control values ($p< 0.05$). ND indicates nondetectable. Data are a combination of three separate animal experiments.

Parthenolide pretreatment in vivo shows no inhibitory effects on LPS-induced IL-6 and TNF-α

Parthenolide's ability to impair LPS-induced IL-6 and TNF-α was also evaluated in vivo. Mice were pretreated with parthenolide (50 mg/kg, p.o.) for 1 h prior to LPS (1 mg/kg, i.p.) administration. Serum was analyzed for IL-6 and TNF-α following a 90 min exposure to LPS. Serum levels of IL-6 and TNF-α were below detection in vehicle and parthenolide control-treated animals (Figure 2.6). Although there was significant production of IL-6 and TNF-α by LPS, parthenolide pretreatment caused no significant changes in cytokine production for either IL-6 (Figure 2.6A) or TNF-α (Figure 2.6B).

DISCUSSION

Consumer use of dietary supplements has grown significantly since the passage of the DSHEA in 1994 and herbals account for a considerable portion of total supplement sales (Blendon et al., 2001). Despite this growth, (Commission on Dietary Supplement Labels, 1998; Sarubin, 2000) there is still a considerable lack of scientific evidence to clearly support efficacy and, in some cases, safety of these products. The objective of this study was to establish a cell culture system that could be used to evaluate the potential efficacy of herbal constituents with presumed anti-inflammatory properties, and to determine if the results of cell culture studies could accurately predict effects in an animal model. The potential anti-inflammatory properties of apigenin, ginsenoside Rb_1 and parthenolide were evaluated in RAW 264.7 cells because this clonal macrophage model produces high concentrations of IL-6 and TNF-α in culture upon activation with LPS, and reduced the need for additional animals required to obtain and use primary

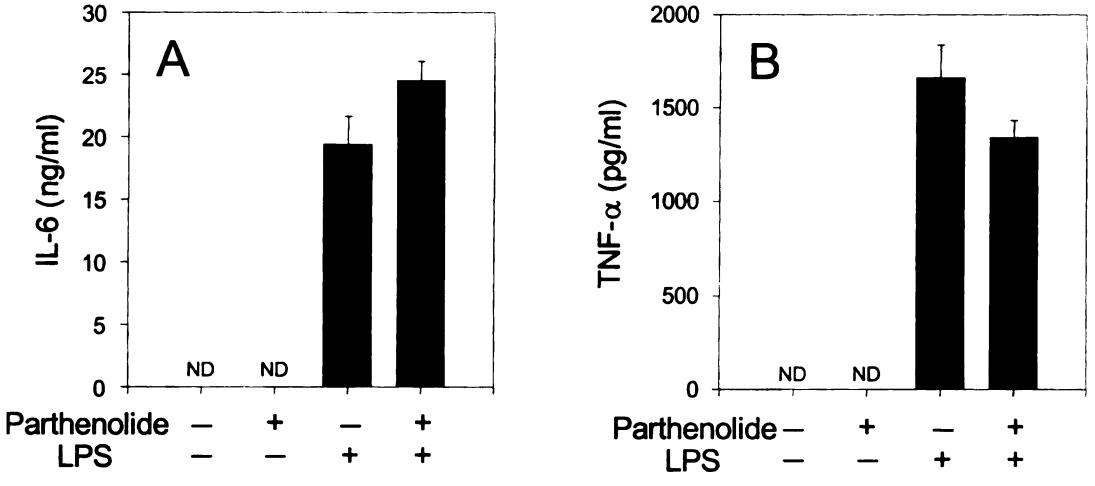

Figure 2.6. Parthenolide pretreatment in vivo shows no inhibitory effect on LPS-induced (A) IL-6 and (B) TNF-α production. Female B6C3F1 mice were pretreated with parthenolide (50 mg/kg, gavage) or vehicle (100 µl DMSO) for 1 hour. LPS (1 mg/kg, i.p.) or vehicle (100 µl sterile water) was administered, and after 90 minutes blood was collected and serum analyzed for IL-6 and TNF-α. Data are mean ± SEM (n=12, controls n=6). ND indicates nondetectable. Data are a combination of three animal separate experiments.

cultures. Cytokine levels were also evaluated in mice using LPS as a stimulant. The cytokines IL-6 and TNF-α were evaluated because these proinflammatory cytokines play a key role in the inflammatory response and can be easily quantified in supernatant and serum samples.

Studies of apigenin's effect on proinflammatory cytokines are limited. The results of this in vitro study showed that apigenin significantly inhibited LPS-induced IL-6, but not TNF-α. Gerritsen et al. (1995) stimulated human endothelial cells with TNF-α and found that apigenin co-treatment inhibited IL-6 production as was observed herein for LPS treatment. However, in another study evaluating the inhibitory effects of apigenin on TNF-α production, Mastuda et al. (2002) showed that apigenin, at an IC_{50} = 5.3 μM, inhibited antigen-IgE-mediated TNF-α secretion in RBL-2H3 mast cells. A potential reason for the differences in results may be the differences in stimuli and cell types used.

Although significant reductions in LPS-induced IL-6 by apigenin were observed here in cell culture, both IL-6 and TNF-α accumulation were inhibited in the animal model. No previous studies have evaluated the effect of apigenin on proinflammatory cytokine levels in vivo. Engelmann et al. (2002) assessed apigenin's in vitro and in vivo effects on cytostatic and anti-angiogenic action. The results showed effects in vitro, but lack of efficacy in vivo. However, animal studies evaluating the effect of apigenin on TNF-induced ICAM-1 expression (Panes et al., 1996) and carrageenan-induced PGE_2 and leukotriene $(LT)B_4$ (Mascolo et al., 1988) revealed inhibitory effects of apigenin similar to those observed in this study. The Panes study employed a 100 mg/kg dose and administered the apigenin i.p. 1 h prior to and 2 h following TNF-stimulation, while the Mascolo study employed a single 24 mg/kg, i.p. dose administered 15 min prior to the

stimulus. The doses differed from the single 50 mg/kg, p.o. pretreatment employed in the present study, which was chosen as a result of observed anti-inflammatory effects in the above named studies. It is not clear how differences in mode of administration impact the results based on the specific doses evaluated in each of these studies since no evaluation of the pharmacokinetics of apigenin in mice are available. Additionally, because no reported levels of apigenin in chamomile products are available, it is unclear how the 50 mg/kg dose used in this study compares to reasonable human consumption through chamomile extracts.

Although ginsenoside compounds, including Rb_1, have been studied frequently relative to their metabolism (Akao et al., 1998; Attele et al., 1999; Chen et al., 1980; Hasegawa & Uchiyama, 1998; Wakabayashi et al., 1997), relatively few studies have evaluated the effect of ginsenoside Rb_1 on proinflammatory cytokine production in vitro and in vivo. The results of those latter studies are generally consistent with the findings herein. For example, Cho et al. (2001) showed that ginsenoside Rb_1 at an IC_{50} of 56.5 μM significantly impaired LPS-induced TNF-α in mouse and human macrophage cells. In another study that investigated the immunostimulatory activity of *Panax quinquefolius* (American ginseng) extracts containing ginsenoside compounds but no polysaccharides, significant TNF-α stimulating activity was observed (Assinewe et al., 2002). This effect, however, could not be induced using pure ginsenoside Rb_1. This finding agrees with our results showing that ginsenoside Rb_1 alone did not affect TNF-α levels in culture supernatants and suggests that the immunostimulatory properties of the ginseng extract were not due to the presence of ginsenoside Rb_1, generally the ginsenoside in highest abundance in *P. quinquefolius* extracts (Harkey et al., 2001).

Although our in vitro data showed significant inhibitory effects of ginsenoside Rb_1 on LPS-induced IL-6 and TNF-α, our in vivo data did not follow the same pattern. Rather, oral gavage with ginsenoside Rb_1 inhibited LPS-induced TNF-α production but not IL-6 levels. Other studies evaluating ginsenosides in vivo support the anti-inflammatory claims attributed to ginsenoside Rb_1. A model of chronic inflammation in aged rats induced with multiple daily injections of LPS was used by Yu & Li (2000) to study the effect of ginseng root saponins (ginsenosides) on IL-1β and IL-6 mRNA expression. Daily treatment with 10, 20 or 40 mg/kg, i.p. ginseng root saponins markedly decreased the mRNA expression levels of both proinflammatory cytokines in the hippocampus compared to LPS-stimulated animals which did not receive ginseng root saponin treatment. These findings of IL-6-related effects contrast with our model, possibly because of the different route of exposure, target tissue and endpoint.

Compared to apigenin and ginsenoside Rb_1, many more studies have been undertaken to elucidate the anti-inflammatory properties of feverfew extract and parthenolide. For example, Hwang et al. (1996) showed LPS-stimulated TNF-α synthesis is inhibited by parthenolide in alveolar macrophage cells. Similarly, Uchi et al. (2002) showed that parthenolide impairs LPS-induced TNF-α production in human monocyte-derived dendritic cells. Additionally, parthenolide pre-incubation of HeLa cells prevents the induction of transcription from the IL-6 promoter (Bork et al., 1997). The results of previous in vitro studies are similar to our findings that parthenolide significantly impaired LPS-induced IL-6 and TNF-α production.

Although in vitro results showed here that parthenolide inhibited LPS-induced IL-6 and TNF-α, the in vivo findings were not in agreement. Only a limited amount of in

vivo data are available for parthenolide, and specifically, no studies have been found in the literature which have evaluated its effects on proinflammatory cytokine responses in animals. However, other in vivo anti-inflammatory endpoints have been evaluated with respect to feverfew extracts and parthenolide in mice and rats (Jain & Kulkarni, 1999). When feverfew extracts from *Tanacetum parthenium* were administered by oral gavage, or pure parthenolide was injected i.p., both anti-inflammatory and antinociceptive effects were observed. Similarly, Tournier et al. (1999) showed that feverfew extracts and parthenolide from *Tanacetum vulgare*, administered by oral gavage, significantly reduced gastric ulcer index induced by ethanol in rats. Although these findings support the anti-inflammatory properties of parthenolide in animals, the anti-inflammatory effects could be the result of inhibition of multiple aspects of the inflammatory response. Other proinflammatory mediators including chemokine (MIP-2), plasma enzyme mediators (complement, kinin and clotting systems) and lipid mediators (COX, PG, platelet activating factor), may be affected rather than proinflammatory cytokines. This may explain the lack of effect observed here on the reduction of LPS-induced proinflammatory cytokine levels in the serum of parthenolide pretreated animals when compared to other anti-inflammatory studies using parthenolide.

In these studies, specific herbal constituents that are believed to be responsible for a majority of the extract's activity were chosen for evaluation. This was done to minimize variability that is frequently encountered in whole herb extract products (Harkey et al., 2001; Nelson et al., 2002). The intention was to use the results as a potential predictor of effects of whole herbal extracts. Groenewegen & Heptinstall (1990), in a comparison of parthenolide and whole feverfew extracts on human platelet activity, showed that there are great similarities of feverfew extract and parthenolide on

platelet aggregation and concluded, in platelets, that parthenolide from feverfew was responsible for the biological effects of the extract. In vivo studies by Tournier et al. (1999) showed similar effectiveness of parthenolide in comparison to whole extracts of feverfew in the cytoprotection of gastric mucosa against alcohol injury. However, Mittra et al. (2000) showed that parthenolide alone inhibited 5-hydroxytryptamine, but that it was evident the feverfew plant extract, which contains many other compounds, was more potent than parthenolide alone. These studies showed that focusing on specific individual constituents derived from herbal extracts may be used to predict the potential effect of a whole herb extract, but that additional, confirmatory studies using whole extracts need to be completed. These approaches may be used as a potential means of standardization of activities for parthenolide in feverfew and ginsenosides in ginseng.

Overall, the results of this study showed the compounds tested could inhibit proinflammatory cytokine responses. However, cell culture data did not always accurately predict the results in an animal model. This was supported by the findings that none of the three compounds evaluated displayed similar inhibitory activity in the cell and animal model systems for both IL-6 and TNF-α production levels. Discrepancies between cell culture data and animal data are not entirely surprising considering only one cell type exists in the culture studies, whereas multiple cell types capable of producing proinflammatory cytokines are present in a whole animal system. Additionally, other inflammatory mediators may influence the cytokine response. Finally, other key factors are metabolism and distribution of the herbal constituents. Future perspectives should include understanding the molecular basis for inhibitory effects of these specific herbal constituents on proinflammatory cytokine gene expression and specifically the role of altered signal transduction.

CHAPTER 3

Effect of Route, Dose and Dose-Timing of Parthenolide Administration on Inhibition of LPS-Induced IL-6 and TNF-α In Vivo

INTRODUCTION

The dietary supplement market and number of supplement consumers has grown significantly since the passage of the Dietary Supplement Health and Education Act of 1994 (DSHEA). Among dietary supplement products, herbals account for an estimated $3.2 billion (Johnston, 1997), a considerable portion of total supplement sales (Blendon et al., 2001; U.S. Consumer Supplement Use Summary, 2000). Although the industry and number of products has continued to grow (Commission on Dietary Supplement Labels, 1997; Sarubin, 2000), there is still a considerable lack of scientific evidence to clearly support efficacy, and in some cases, safety of these products. This is due, at least in part, to the lack of pre-market approval required of dietary supplements, which is in strong contrast to the pre-market regulatory requirements of conventional drugs and food additives. Thus, studies verifying the presumed efficacy, and safety, of specific dietary supplement products are warranted.

Feverfew (*Tanacetum parthenium*) extracts have been used since 78 A.D. (Tyler, 1993). Historically, feverfew has been used to treat headaches as well as stomachache and fever. Recently, research has focused on feverfew's principal active constituent - parthenolide, a sesquiterpene lactone. Specifically, studies have investigated their ability to function as anti-inflammatory agents.

Feverfew and parthenolide's anti-inflammatory properties were studied relative to inhibition of platelet aggregation in platelet-rich plasma obtained from healthy volunteers (Groenewegen & Heptinstall, 1990). Parthenolide caused a concentration-dependent inhibition of aggregation induced by phorbol ester (PMA) and lipopolysaccahride (LPS). Additional studies in cell culture models have supported parthenolide's effects on inflammation through inhibition of cyclooxygenase (COX)-2 (Hwang et al., 1996), the chemokine interleukin (IL)-8 (Mazor et al., 2000), the proinflammatory cytokine tumor necrosis factor (TNF)-α (Hwang et al., 1996) and the transcription factor nuclear factor (NF)-κB (Bork et al., 1997; Mazor et al., 2000; Kwok et al., 2001; Hehner et al., 1998 & 1999).

Studies conducted in our laboratory evaluating the effect of herbal constituents, including parthenolide, using LPS to induce proinflammatory cytokines, support the anti-inflammatory properties of parthenolide demonstrated in in vitro studies (Smolinski & Pestka, accepted 2003). LPS, the major biologically active component of endotoxin, is derived from the cell walls of Gram-negative bacteria (Roth et al., 1997). LPS is a potent inducer of inflammation and can be used to study inflammatory responses. Parthenolide and LPS co-treatment significantly impaired LPS-induced production of IL-6 and TNF-α in murine macrophage cells.

Although clear support of parthenolide's anti-inflammatory properties have been demonstrated in vitro, only a few studies have been performed in animal model systems. Cumulative evaluation of parthenolide's effect on LPS-induced proinflammatory cytokines in vivo via gavage administration showed no significant inhibitory effects on serum cytokine production (Smolinski & Pestka, 2003). Alternatively, evaluation of parthenolide's anti-inflammatory properties in vivo via i.p. administration showed

promising inhibitory properties against carrageenan-induced paw edema in rats (Jain & Kulkarni, 1999).

The objective of this study was to determine the effect of route, dose and dose-timing of parthenolide administration on inhibition of LPS-induced IL-6 and TNF-α using an animal model system. The results of these studies provide a basis for further investigation of mechanisms by which parthenolide imparts its anti-inflammatory activity.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Parthenolide (Aldrich, Milwaukee, WI; or Calbiochem, San Diego, CA) was dissolved in tissue culture grade dimethyl sulfoxide (DMSO). LPS from *Salmonella typhimurium* [1.5 EU/ng LPS; Stimulation index (SI) 3.6 @15.6 μg/ml LPS] was dissolved in endotoxin-free tissue culture grade water.

Experimental design for in vivo studies

All animal handling was conducted in ordinance with guidelines established by the National Institutes of Health. Experiments were designed to minimize the numbers of animals used. Male C57BL/6 or female B6C3F1 mice were obtained from Charles River (Portage, MI). Animals were housed 3-4 per cage with a 12 h light/dark cycle, provided standard rodent chow and water *ad libitum*, and acclimated to their environment at least one week before the start of experiments.

Chow and water were removed from cages one hour prior to the start of each experiment unless otherwise indicated. Mice were treated with various doses of parthenolide (0.05 - 50 mg/kg, p.o. or i.p. in 100 or 50 µl DMSO) 7 days, 1 h, 30 min or 0 h prior to LPS administration (1 or 5 mg/kg, i.p. in 100 µl water). Vehicle-treated mice received 50 or 100 µl DMSO, p.o. or i.p. and 100 µl water, i.p. Parthenolide control animals received respective parthenolide dose and 100 µl water, i.p. After 90 min, blood was obtained by retro-orbital bleeding under metofane anesthesia and allowed to clot overnight at 4°C. Serum was collected and analyzed for IL-6 and TNF-α by ELISA.

IL-6 and TNF-α determination by ELISA

Cytokine analysis for IL-6 was performed using purified and biotin-conjugated rat anti-mouse IL-6 antibodies from PharMingen (San Diego, CA). Streptavidin-peroxidase (Sigma) and 3,3',5,5'-tetramethylbenzidine (TMB, Fluka, Ronkonkoma, NY) were used for detection. Absorbance was read at 450 nm using a Vmax™ Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA). For TNF-α analysis the OptEIA Set: Mouse TNF-α (Mono/Poly) kit was used according to manufacturer's instructions (PharMingen).

Statistics

All statistical testing was performed using SigmaStat Statistical Analysis Software (Jandel Scientific, San Rafael, CA). For comparison of two groups, a Student's *t*-test was used. For comparisons of multiple groups using parametric data, one-way analysis of variance (ANOVA) using Bonferroni's method were performed.

RESULTS

Evaluation of the anti-inflammatory properties of parthenolide on the LPS-induced proinflammatory cytokines IL-6 and TNF-α indicated inhibitory activity in a murine macrophage cell line. The observed inhibitory effects in vitro suggested that similar effects may be observed in vivo. Preliminary studies of parthenolide's effect on LPS-induced serum production of IL-6 and TNF-α in C57BL/6 male mice suggested promising inhibitory capacity with parthenolide pre-treatment and LPS 1 mg/kg, i.p. (see Figure 3.1). As a result of these small scale studies, a series of experiments was planned to determine an optimal route of exposure, dose and dose-timing relative to LPS administration of parthenolide on inhibition of LPS-induced IL-6 and TNF-α production using an animal model. Additional experiments were executed to replicate preliminary results, but inconsistencies were evident. This led to additional studies which evaluated the specific effects of routes of exposure, sources of parthenolide, feed withdrawal and cumulative dosing. The following is a summary of the work that was completed.

Pre-treatment via Intraperitoneal.(i.p.) Administration

EXPERIMENT 1 In the first (preliminary) in vivo study male C57BL/6 mice were pre-treated with parthenolide, 50 mg/kg, i.p., 1 h prior to either 1 or 5 mg/kg, i.p. LPS. These two LPS doses were chosen based on previous work completed in our laboratory which evaluated the effects of LPS administration on serum cytokine production (Zhou et al., 1999). Blood was collected 90 min following LPS administration. This time period was adequate to evaluate the levels of both IL-6 and TNF-α from one blood collection based on time course data obtained by Zhou et al.

(1999). The results indicated a significant, and almost complete, impairment of the 1 mg/kg LPS-induced IL-6 and TNF-α by pre-treatment (1 h) with 50 mg/kg parthenolide, i.p. (Figure 3.1). Parthenolide pre-treatment of the 5 mg/kg LPS-stimulated animals significantly increased IL-6 but had no effect on TNF-α. No IL-6 or TNF-α were detected in vehicle control animals. Interestingly, LPS-induced TNF-α was lower following 5 mg/kg treatment when compared to 1 mg/kg.

EXPERIMENT 2 This experiment was repeated using the 50 mg/kg parthenolide pre-treatment and 1 mg/kg LPS treatment. However, a different stain and sex mouse was used - female B6C3F1. The results were in partial agreement with the initial experiment. IL-6 was significantly decreased but TNF-α was not affected (Figure 3.2). No detectable IL-6 or TNF-α were observed in vehicle control animals, however, parthenolide control animals included in this study exhibited IL-6 levels similar to those of LPS- treated only animals, whereas TNF-α levels were not significantly induced.

EXPERIMENT 3 A third experiment was performed which evaluated conditions identical to those in second experiment. The results showed that both IL-6 and TNF-α were inhibited by parthenolide treatment but only the TNF-α decrease was significant (Table 3.1, Expt. 3).

Pre-treatment via Oral Gavage (p.o.) Administration

EXPERIMENT 4 The apparent suppression of IL-6 and TNF-α resulting from parthenolide pre-treatment via i.p. injection prompted evaluation of other routes of exposure. Oral gavage administration was chosen because this route more closely approximates human exposure to parthenolide through consumption of whole herb extracts in supplement or tea forms, making this a more relevant model. Gavage and i.p.

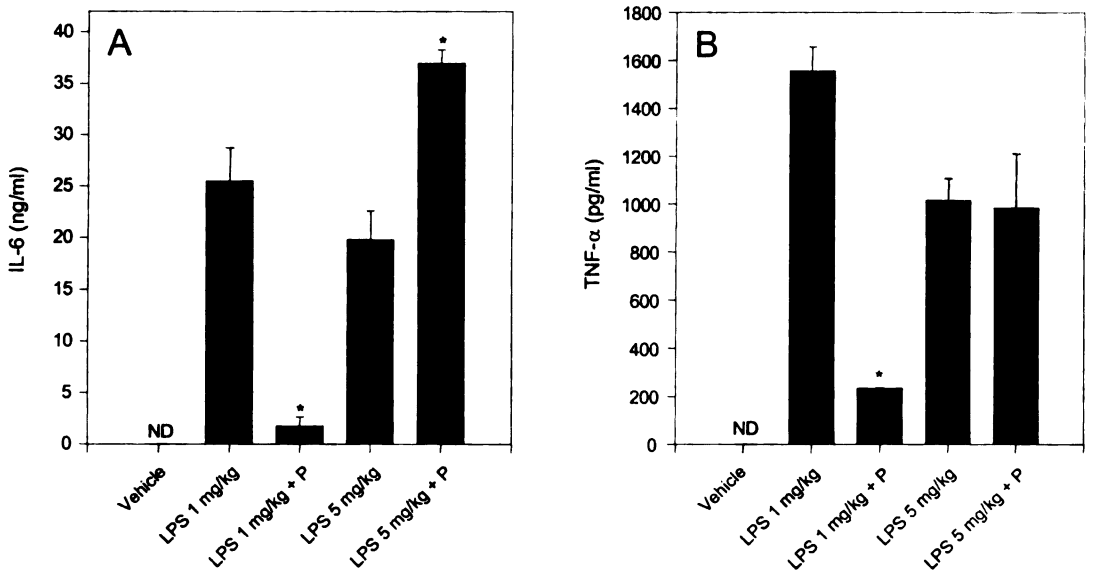

Figure 3.1. Effect of parthenolide 1 hour pre-treatment on LPS-induced (A) IL-6 and (B) TNF-α production in male C57BL/6. Mice were treated with parthenolide (50 mg/kg, i.p.) or vehicle (100 µl DMSO) one hour prior to LPS (1 mg/kg, i.p.) or vehicle (200 µl sterile water). After 90 minutes, blood was collected and serum analyzed for IL-6 and TNF-α. Data are mean ± SEM (control groups n=2, treatment groups n=4). Values marked with an asterisk (*) differ significantly from LPS control values ($p< 0.05$). ND indicates nondetectable.

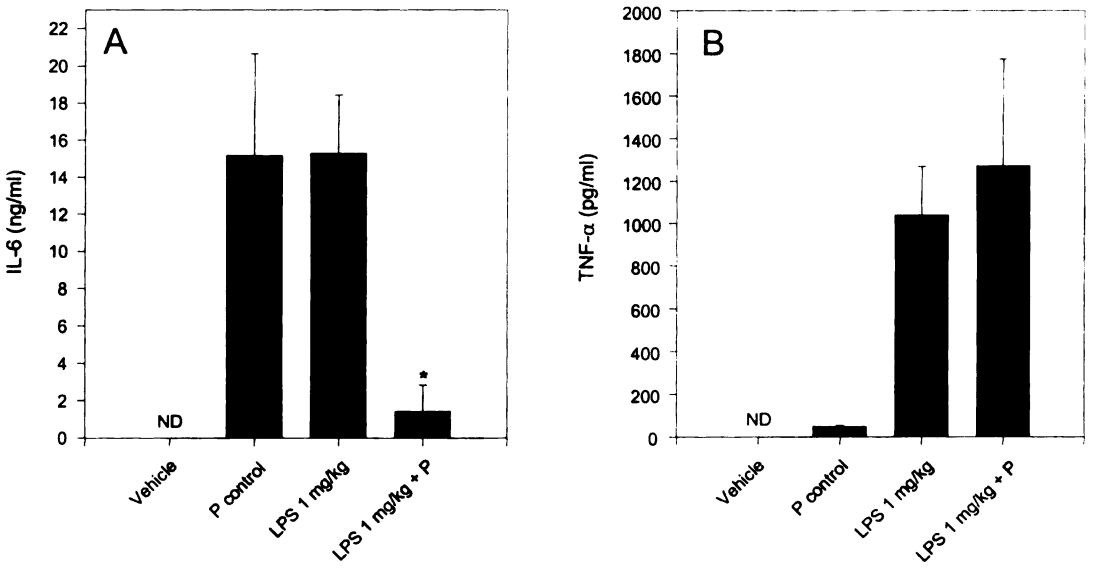

Figure 3.2. Effect of parthenolide 1 hour pre-treatment on LPS-induced (A) IL-6 and (B) TNF-α production in female B6C3F1. Mice were treated with parthenolide (50 mg/kg, i.p.) or vehicle (50 µl DMSO) one hour prior to LPS (1 mg/kg, i.p.) or vehicle (100 µl sterile water). After 90 minutes, blood was collected and serum analyzed for IL-6 and TNF-α. Data are mean ± SEM (control groups n=2, treatment groups n=4). Values marked with an asterisk (*) differ significantly from LPS control values ($p< 0.05$). ND indicates nondetectable.

Table 3.1. Summary of studies evaluating parthenolide 1 h pre-treatment via intraperitoneal (i.p.) administration on LPS-induced serum IL-6 and TNF-α in vivo

Parthenolide dose	Route of exposure	Treatment time	LPS dose (i.p.)	IL-6 (ng/ml)	TNF (pg/ml)	Expt. number	Animals #
None	NA	NA	1 mg/kg	25.5 ± 4.3	1555.0 ± 101.5	1	n=2
50 mg/kg	i.p.	1h prior	1 mg/kg	1.7 ± 0.9*	235.3 ± 1.8*		n=2
None	NA	NA	5 mg/kg	19.8 ± 2.8	1014.1 ± 92.0		n=2
50 mg/kg	i.p.	1h prior	5 mg/kg	37.0 ± 1.3*	982.45 ± 227.6		n=2
None	NA	NA	1 mg/kg	15.3 ± 3.2	1037.0 ± 232.2	2	n=4
50 mg/kg	i.p.	1h prior	1 mg/kg	1.4 ± 1.4*	1269.8 ± 503.2		n=4
None	NA	NA	1 mg/kg	48.3 ± 5.9	3265.3 ± 651.2	3	n=4
50 mg/kg	i.p.	1h prior	1 mg/kg	28.0 ± 8.5	1097.5 ± 314.6*		n=4

Values marked with an asterisk (*) differ significantly from LPS control values ($p< 0.05$)

pre-treatment using parthenolide 1 h prior to LPS exposure yielded remarkable results, similar to those observed in our first study (Figure 3.1). IL-6 and TNF-α were significantly decreased with parthenolide administration, regardless of route of exposure (Figure 3.3). In fact, both treatment routes reduced cytokine production to less than 25% that of LPS-only animals, and the gavage route appeared more effective at reducing cytokine levels when compared to i.p. administration, with near complete elimination of LPS-induced IL-6 and TNF-α. No induction of cytokines was noted in vehicle or parthenolide control animal groups.

EXPERIMENT 5 The gavage experiment was repeated using a larger number of animals (n=4 per treatment group). Only two animals were initially used in the gavage group. Pathenolide was administered at 10 or 50 mg/kg through oral gavage 1 h prior to LPS 1 mg/kg i.p. This experiment did not validate the initial gavage experiment. There was no effect on TNF-α, and only a very small decrease in IL-6 using the 50 mg/kg dose, whereas the 10 mg/kg dose had no effect on IL-6 and a small stimulatory effect on TNF-α (Table 3.2, Expt. 5), although no significant differences existed between treatment groups. Overall levels of LPS-induced IL-6 and TNF-α were similar to all other previous experiments suggesting that there was not a procedural problem.

EXPERIMENT 6 Because the results of the second gavage experiment were contrary to the first attempt, a third experiment was designed to clarify the results. This experiment was identical to the second except a 25 mg/kg dose was used in place of the 10 mg/kg dose, along with 50 mg/kg. The results were comparable to those found in the second, and larger, gavage experiment and, therefore, were again contrary to the initial gavage experiment. Parthenolide 1 h pre-treatment using a 50 mg/kg dose did not affect LPS-induced TNF-α. There was, however, a statistically insignificant decrease in IL-6 at

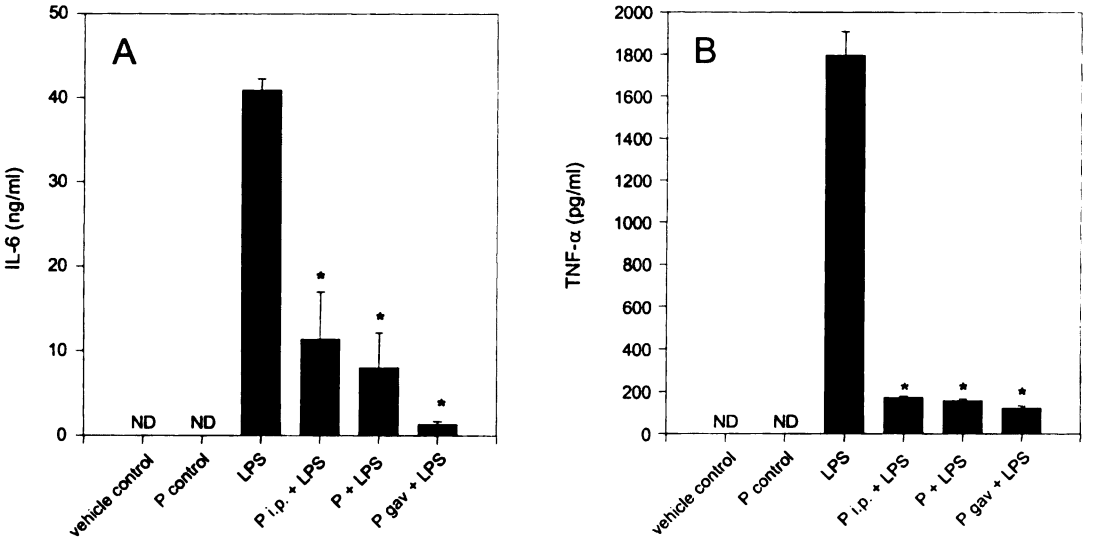

Figure 3.3. Effect of parthenolide (PTL) 1 hour pre-treatment on LPS-induced (A) IL-6 and (B) TNF-α production. Female B6C3F1 mice were treated with parthenolide (50 mg/kg, i.p. or p.o.) or vehicle (50 µl DMSO) one hour prior to LPS (1 mg/kg, i.p.) or vehicle (100 µl sterile water). After 90 minutes blood was collected and serum analyzed for IL-6 and TNF-α. Data are mean ± SEM (control groups and PTL p.o. + LPS n=2, treatment groups n=4). Values marked with an asterisk (*) differ significantly from LPS control values ($p< 0.05$). ND indicates nondetectable.

Table 3.2. Summary of studies evaluating parthenolide 1 h pre-treatment via oral gavage (p.o.) administration on LPS-induced serum IL-6 and TNF-α in vivo

Parthenolide dose	Route of exposure	Treatment time	LPS dose (i.p.)	IL-6 (ng/ml)	TNF (pg/ml)	Expt. number	Animal #
None	NA	NA	1 mg/kg	40.9 ± 1.4	1793.7 ± 113.4	4	n=4
50 mg/kg	i.p.	1h prior	1 mg/kg	11.4 ± 5.6*	170.8 ± 9.7*		n=4
	gavage	1h prior	1 mg/kg	1.3 ± 0.4*	122.3 ± 9.1*		n=2
None	NA	NA	1 mg/kg	13.5±5.4	731.7±330.5	5	n=4
50 mg/kg	gavage	1h prior	1 mg/kg	10.3 ± 3.8	832.1 ± 382.0		n=3
10 mg/kg	gavage	1h prior	1 mg/kg	12.4 ± 8.0	1054.3 ± 829.7		n=2
None	NA	NA	1 mg/kg	20.2 ± 1.1	2120.5 ± 150.3	6	n=4
50 mg/kg	gavage	1h prior	1 mg/kg	20.8 ± 3.0	1642.4 ± 208.9		n=4
25 mg/kg	gavage	1h prior	1 mg/kg	10.3 ± 0.2	977.6 ± 16.7		n=1
None	NA	NA	1 mg/kg	16.3 ± 6.8	940.5 ± 232.7	7	n=4
50 mg/kg	gavage	1h prior	1 mg/kg	26.3 ± 0.9	1109.3 ± 79.3		n=5

Values marked with an asterisk (*) differ significantly from LPS control values ($p< 0.05$)

this dose. Using the 25 mg/kg dose there was a decrease in both TNF-α and IL-6, but neither were significant and at this dose, only one animal was evaluated (Table 3.2, Expt. 6).

EXPERIMENT 7 The gavage route of exposure was again tested using the 50 mg/kg dose of parthenolide 1 h prior to LPS treatment. Again, there was no change in TNF-α and a small, insignificant increase in IL-6 was noted (Table 3.2, Expt. 7). No detectable levels of cytokines were observed in control groups.

Re-evaluation of i.p. and Gavage Pre-treatments

EXPERIMENT 8 The results from all three larger scale experiments focusing on gavage administration of parthenolide did not agree with the results from the initial experiment. Therefore, an additional experiment was performed to test the effect of parthenolide pre-treatment using either gavage or i.p. administration on LPS-induced IL-6 and TNF-α. Additionally, this experiment was performed to confirm that the i.p. route of exposure was effective at suppressing LPS-induced IL-6 and TNF-α. Gavage administration of parthenolide produced results similar to the most previous experiment. There was no change in TNF-α levels but there was a small, insignificant increase in IL-6. On the other hand, i.p. administration resulted in decreases of both IL-6 and TNF-α (Figure 3.4). Although these decreases were obvious, they were not significant, nor were they as pronounced as in the initial experiments depicted in Figure 3.1.

EXPERIMENT 9 Because the data from the most recent experiment were not consistent with those of the initial experiments, another attempt was made to clarify the effect. Parthenolide, 50 mg/kg, i.p. was administered 1 h prior to LPS. There was no

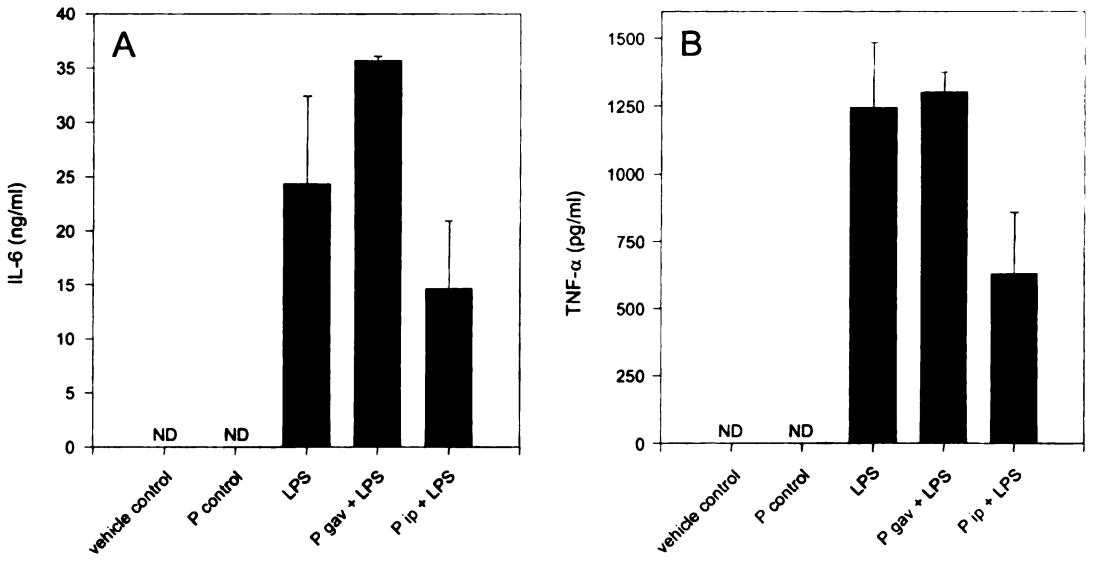

Figure 3.4. Effect of parthenolide 1 hour pre-treatment via i.p. or oral gavage administration on LPS-induced (A) IL-6 and (B) TNF-α production. Female B6C3F1 mice were pre-treated with parthenolide (50 mg/kg, i.p. or p.o.) or vehicle (100 µl DMSO) one hour prior to LPS (1 mg/kg, i.p.) or vehicle (100 µl sterile water). After 90 minutes, blood was collected and serum analyzed for IL-6 and TNF-α. Data are mean ± SEM (control groups and PTL p.o. + LPS n=2, treatment groups n=4). Values marked with an asterisk (*) differ significantly from LPS control values ($p< 0.05$). ND indicates nondetectable.

change in LPS-induced IL-6 and a small but non-statistically significant decrease in TNF-α (Table 3.3, Expt. 9).

Weight/Dose Testing

EXPERIMENT 10 After these numerous, unsuccessful attempts to reproduce the results observed in the initial i.p. and gavage experiments, possible differences that may have existed between experiments were considered. It was determined that the initial experiments assumed an animal weight of 25g. However, after weighing many animals of similar age for use in subsequent experiments, it appeared that the average weight had been overestimated. The average animal weight near 21g. Based on this finding another experiment was performed using a larger dose of parthenolide. This would translate to a 63.8 mg/kg dose, not 50 mg/kg if a 25g weight was assumed as it was in the initial experiments. Parthenolide was administered by oral gavage or i.p. injection 1 h prior to LPS. The results were inconclusive because no animals received only LPS. However, if the average LPS value from all previous experiments was used, a small increase in IL-6 and no change in TNF-α would be concluded for the gavage administration of parthenolide (Figure 3.5). For i.p. administration of parthenolide, there was no change in IL-6 and a decrease in TNF-α, although significance could not be determined. Although there was no direct comparison, it did not appear that the dose/weight difference effected the results.

Effect of Cumulative Exposure

EXPERIMENT 11 It was possible that repeated exposure to parthenolide may increase its potential to act as an anti-inflammatory agent in vivo. Therefore, an

Table 3.3. Re-evaluation experiments of parthenolide 1 h pre-treatment via oral gavage (p.o.) and intraperitoneal (i.p.) administration on LPS-induced serum IL-6 and TNF-α in vivo

Parthenolide dose	**Route of exposure**	**Treatment time**	**LPS dose (i.p.)**	**IL-6 (ng/ml)**	**TNF (pg/ml)**	**Expt. number**	**Animal #**
None	NA	NA	1 mg/kg	24.3 ± 8.0	1242.6 ± 240.8	8	n=4
50 mg/kg	gavage	1h prior	1 mg/kg	35.6 ± 0.4	1300.5 ± 74.5		n=2
	i.p.	1h prior	1 mg/kg	14.6 ± 6.3	628.5 ± 228.9		n=3
None	NA	NA	1 mg/kg	20.0 ± 3.0	948.6 ± 178.1	9	n=4
50 mg/kg	i.p.	1h prior	1 mg/kg	16.4 ± 6.3	640.7 ± 196.3		n=4

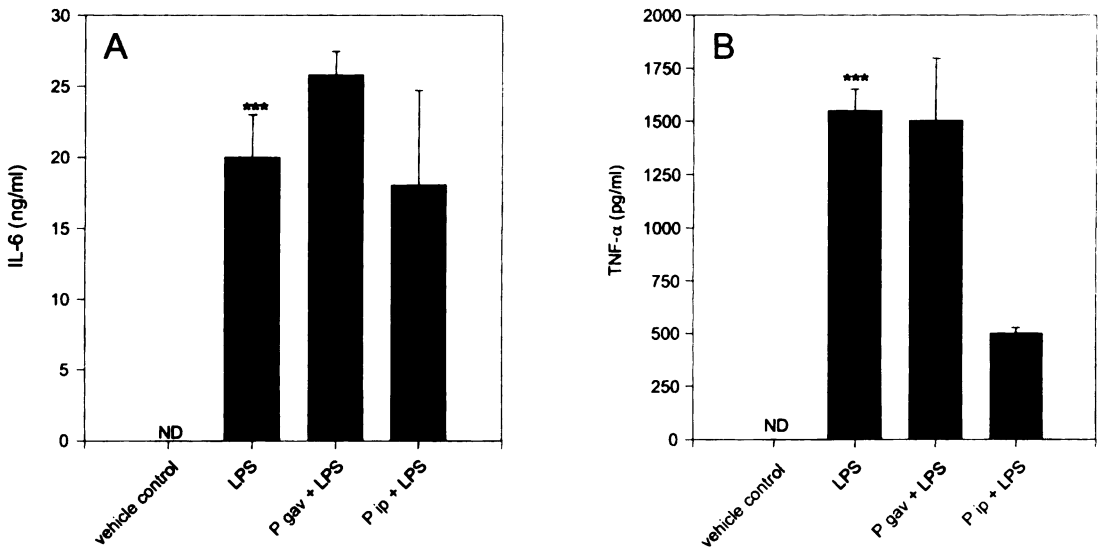

Figure 3.5. Effect of parthenolide 1 hour pre-treatment on LPS-induced (A) IL-6 and (B) TNF-α, comparison of i.p. and oral gavage administration with dose adjustment for animal weight. Female B6C3F1 mice were pre-treated with parthenolide (64 mg/kg, i.p. or p.o.) or vehicle (50 µl DMSO) one hour prior to LPS (1 mg/kg, i.p.) or vehicle (100 µl sterile water). After 90 minutes, blood was collected and serum analyzed for IL-6 and TNF-α. Data are mean ± SEM (n=3). Values marked with three asterisks (***) represent LPS values determined based on an average of all animal studies to date. ND indicates nondetectable.

experiment was performed using repeated gavage administration of parthenolide. Animals were orally gavaged with 50 mg/kg of parthenolide for 7 consecutive days. On day 7 the last dose was administered 1 h prior to LPS administration. Serum was collected after 90 mins as in all previous experiments. Using an average LPS induction value, there did not appear to be any changes in the level of IL-6 or TNF-α (Figure 3.6); thus, a cumulative effect of repeated parthenolide administration on LPS-induced proinflammatory cytokine levels seemed unlikely via oral gavage administration.

Parthenolide Stock/Quality

EXPERIMENT 12 After determining that the animal weight differences and cumulative dosing did not affect LPS-induced serum IL-6 or TNF-α, we considered the quality of the parthenolide stock. Degradation of parthenolide stock has been noted (Heptinstall et al., 1992), and has been associated with reduced biological activity (Mittra et al., 2000), indicating that older stocks, or new batches or lots used may not be exact product matches to those used in initial experiments. All previous experiments used parthenolide from Aldrich Chemical Company. To test the source of parthenolide we obtained the constituent from Calbiochem and tested its effect in vivo. Parthenolide at 50 mg/kg was administered by oral gavage 1 h prior to LPS. There was no change in IL-6 but a significant decrease in TNF-α was observed (Figure 3.7). The new source of parthenolide did not appear to make a remarkable difference in cytokine production levels, but did show more promising results compared to other prior experiments. The remaining parthenolide experiments used only the Calbiochem product.

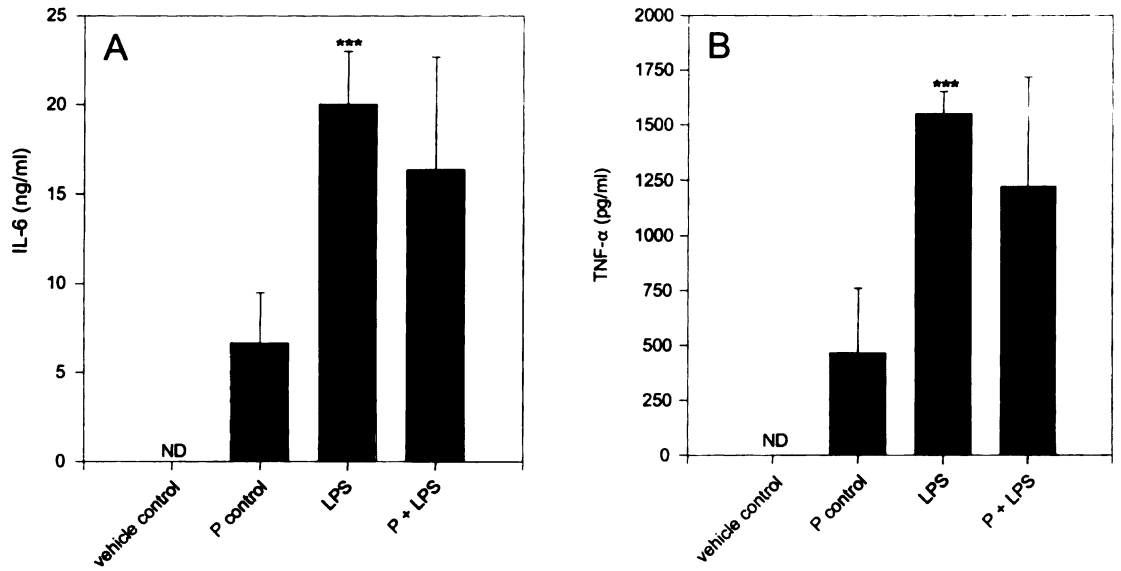

Figure 3.6. Effect of 7-day parthenolide pre-treatment on LPS-induced (A) IL-6 and (B) TNF-α. Female B6C3F1 mice were treated with parthenolide (50 mg/kg, p.o.) or vehicle (50 µl DMSO) 6 days and 1 hour prior to LPS (1 mg/kg, i.p.) or vehicle (100 µl sterile water) on day 7. After 90 minutes, blood was collected and serum analyzed for IL-6 and TNF-α. Data are mean ± SEM (controls n=2; PTL + LPS n=3). Values marked with three asterisks (***) represent LPS values determined based on an average of all animal studies to date. ND indicates nondetectable.

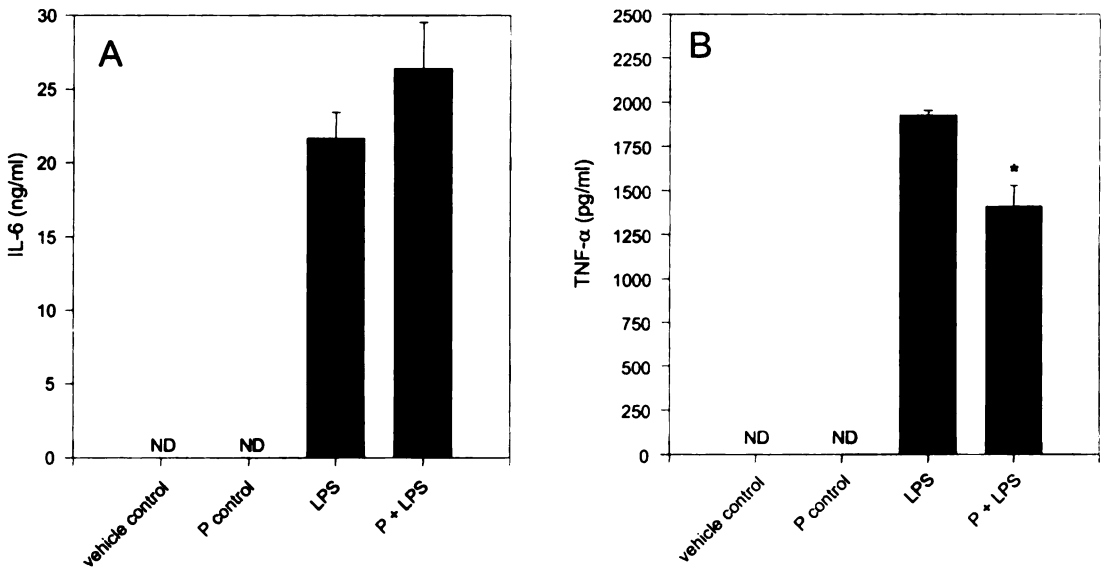

Figure 3.7. Effect of parthenolide 1 hour pre-treatment via oral gavage, using a new source, on LPS-induced (A) IL-6 and (B) TNF-α. Female B6C3F1 mice were pre-treated with parthenolide (50 mg/kg, p.o.) or vehicle (50 µl DMSO) one hour prior to LPS (1 mg/kg, i.p.) or vehicle (100 µl sterile water). After 90 minutes, blood was collected and serum analyzed for IL-6 and TNF-α. Data are mean ± SEM (control groups n=2, treatment groups n=4 or 5). Values marked with an asterisk (*) differ significantly from LPS control values ($p< 0.05$). ND indicates nondetectable.

Effect of Feed Withdrawal Duration

EXPERIMENT 13 According to the National Academy of Science it is suggested that feed be withdrawn from mice 4-6 hours prior to oral gavage administration of any compound (Arnold, 1990). Because all previous experiments, both i.p. and p.o., were evaluated using a 1 h feed withdrawal period, we designed an experiment to investigate the possibility that experimental variability may be due, at least in part, to the feed withdrawal period. In this experiment parthenolide was administered 1 h prior to LPS by oral gavage at 50 or 100 mg/kg. However, feed was withdrawn from cages six hours prior to parthenolide dosing. The results showed no overall changes in IL-6 levels although a decreasing trend was noted at both doses tested (Figure 3.8). TNF-α levels were not changed by the 50 mg/kg treatment, though there was an increasing trend with the 100 mg/kg dose. Overall, the change in parthenolide source and length of feed withdrawal did not effect the levels of LPS-induced IL-6 and TNF-α produced in the serum of parthenolide pre-treated animals.

Time Course

EXPERIMENT 14 Using the new source of parthenolide and i.p. administration, significant reductions in LPS-induced IL-6 and TNF-α were once again observed in pre-treated animals (Figure 3.9). EXPERIMENT 15 This experiment was repeated but the pre-treatment period was modified to include multiple time points. Pre-treatment times included 1 h (as done in all previous experiments), 30 mins and 0 mins (co-treatment). LPS-induction of TNF-α was significantly inhibited with parthenolide, regardless of dose-timing (Figure 3.10). The co-treated animals, those that received parthenolide and LPS at the same time, appeared to have the greatest reductions in serum TNF-α. IL-6

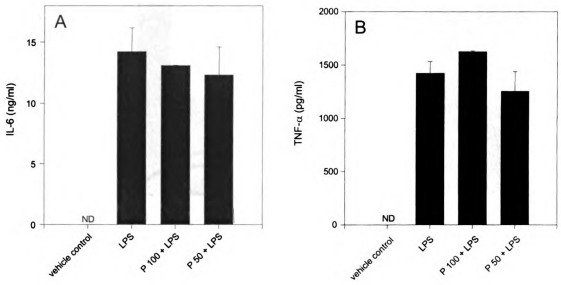

Figure 3.8. Effect of feed withdrawal duration and parthenolide 1 hour pre-treatment on LPS-induced (A) IL-6 and (B) TNF-α. Feed was withdrawn from cages 6 hours prior to the start of experiments. Female B6C3F1 mice were pretreated with parthenolide (100 or 50 mg/kg, p.o.) or vehicle (100 µl DMSO) one hour prior to LPS (1 mg/kg, i.p.). After 90 minutes, blood was collected and serum analyzed for IL-6 and TNF-α. Data are mean ± SEM (control groups n=2, treatment groups n=4 or 5).

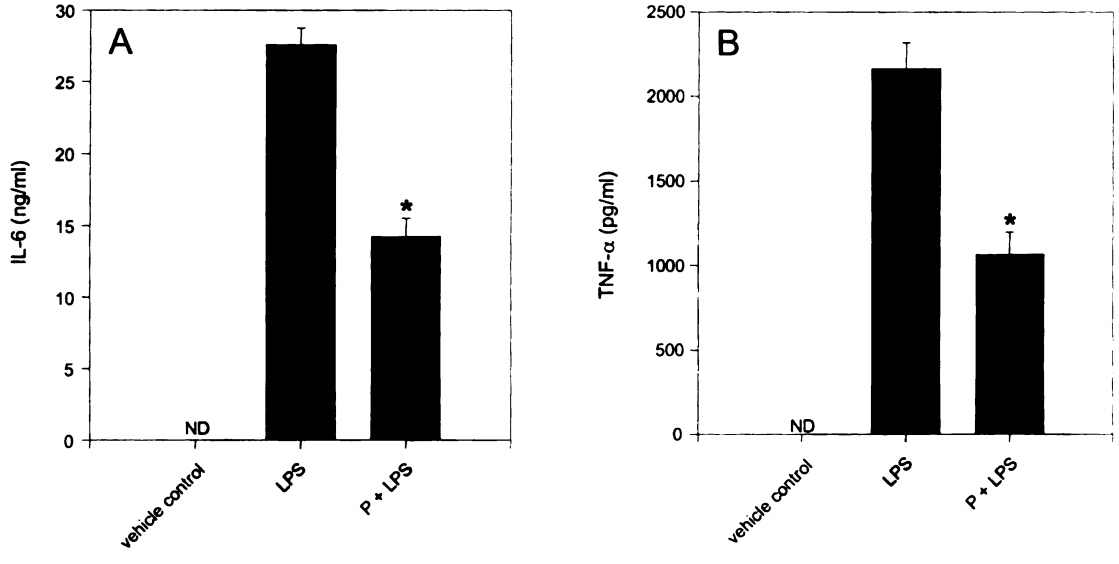

Figure 3.9. Effect of parthenolide 1 hour pre-treatment on LPS-induced (A) IL-6 and (B) TNF-α production. Female B6C3F1 mice were treated with parthenolide (50 mg/kg, i.p.) or vehicle (50 µl DMSO) one hour prior to LPS (1 mg/kg, i.p.) or vehicle (100 µl sterile water). After 90 minutes, blood was collected and serum analyzed for IL-6 and TNF-α. Data are mean ± SEM (control groups n=2, treatment groups n=4). Values marked with an asterisk (*) differ significantly from LPS control values ($p< 0.05$). ND indicates nondetectable.

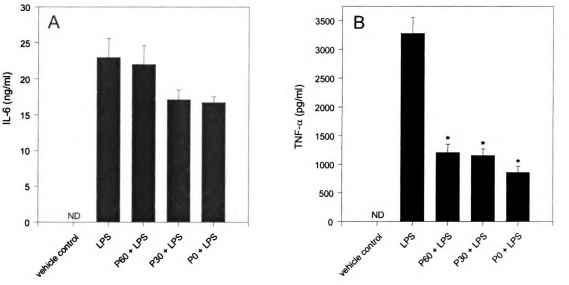

Figure 3.10. Effect of parthenolide pre-treatment time on LPS-induced (A) IL-6 and (B) TNF-α. Female B6C3F1 mice were pretreated with parthenolide (50 mg/kg, i.p.) or vehicle (50 µl DMSO) 60, 30 or 0 minutes prior to LPS (1 mg/kg, i.p.) or vehicle (100 µl sterile water). After 90 minutes, blood was collected and serum analyzed for IL-6 and TNF-α. Data are mean ± SEM (control groups n=2, treatment groups n=3). Values marked with an asterisk (*) differ significantly from LPS control values ($p < 0.05$). ND indicates nondetectable.

induction, however, was inhibited in only the 30 and 0 min pretreated animals, but neither of these reductions were significant. Co-treated animals also exhibited the greatest reduction in serum IL-6.

EXPERIMENT 16 The time course for parthenolide administration was repeated. The conditions used in this experiment were identical to the prior experiment. A 50 mg/kg, i.p. dose of parthenolide was administered 60, 30 or 0 mins prior to LPS 1 mg/kg, i.p. The results of LPS and parthenolide administration on serum TNF-α levels was virtually identical to those of the previous experiment. Significant reductions in LPS-induced serum TNF-α levels were observed following treatment with parthenolide, regardless of dose-timing. The results of serum IL-6 were not as consistent. Unlike the previous experiment, there was a small decrease with 60 min pretreatment, but no changes with 30 or 0 min (Table 3.4, Expt. 16).

Dose Response

EXPERIMENT 17 Following completion of the two time course experiments, a dose-response study was undertaken to determine the minimum dose at which parthenolide administration could inhibit LPS-induced IL-6 and TNF-α in treated mice. The typical dose of parthenolide used in previous experiments was 50 mg/kg by oral gavage or i.p. injection. The later studies focused on i.p. administration. In the first dose response study animals were administered parthenolide, i.p., at 25, 10 or 5 mg/kg. It was determined, based on the findings of the two time course studies, that a co-treatment would be used. In this study, co-treated animals receiving the 25 mg/kg dose of parthenolide and LPS 1 mg/kg, i.p. showed decreases in both serum IL-6 and TNF-α, but no significance was noted (Figure 3.11). At the 10 mg/kg dose there was a decrease in

Table 3.4. Summary of time course studies of parthenolide 50 mg/kg via intraperitoneal (i.p.) administration on LPS-induced serum IL-6 and TNF-α in vivo

Parthenolide dose	Route of exposure	Treatment time	LPS dose (i.p.)	IL-6 (ng/ml)	TNF (pg/ml)	Expt. number	Animal #
None	NA	NA	1 mg/kg	23.0 ± 2.7	3273.3 ± 282.5	15	n=3
50 mg/kg	i.p.	1h prior	1 mg/kg	22.0 ± 2.6	1202.5 ± 151.1*		n=3
		30 min prior	1 mg/kg	17.1 ± 1.4	1155.0 ± 116.5*		n=3
		cotreat	1 mg/kg	16.7 ± 0.8	857.1 ± 109.2*		n=3
None	NA	NA	1 mg/kg	18.6 ± 1.5	1869.0 ± 57.9	16	n=3
50 mg/kg	i.p.	1h prior	1 mg/kg	13.9 ± 6.9	650.6 ± 316.5*		n=3
		30 min prior	1 mg/kg	20.4 ± 4.7	507.5 ± 195.1*		n=3
		cotreat	1 mg/kg	21.6 ± 1.8	662.0 ± 52.7*		n=3

Values marked with an asterisk (*) differ significantly from LPS control values ($p< 0.05$)

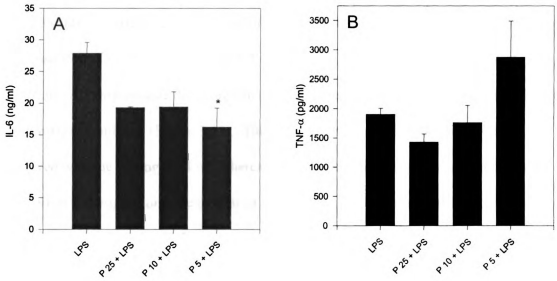

Figure 3.11. Effect of parthenolide dose on LPS-induced (A) IL-6 and (B) TNF-α. Female B6C3F1 mice were co-treated with parthenolide (25, 10 or 5 mg/kg, i.p.) or vehicle (50 µl DMSO) and LPS (1 mg/kg, i.p.) or vehicle (100 µl sterile water). After 90 minutes, blood was collected and serum analyzed for IL-6 and TNF-α. Data are mean ± SEM (treatment groups n=3). Values marked with an asterisk (*) differ significantly from LPS control values ($p < 0.05$). ND indicates nondetectable.

IL-6 but not TNF-α. The 5 mg/kg resulted in significant decreases in IL-6, but a possible increase in TNF-α. This insignificant increase may not be biologically relevant, as there was a small number of animals (n=3 per group) and there was also large animal to animal variation.

EXPERIMENT 18 Based on the effects observed using the 5 mg/kg dose a second study investigating lower doses of parthenolide was designed. In this second dose response study four doses, 10, 5, 1 or 0.5 mg/kg, of parthenolide were tested via i.p. injection. All parthenolide doses significantly impaired LPS-induction of IL-6 and TNF-α in co-treated animals (Figure 3.12). The most suppressive effects on IL-6 were observed with the 0.5 mg/kg dose, whereas the 5 mg/kg dose appeared most suppressive for TNF-α. These findings are similar to the previous dose response study, with the exception of the 5 mg/kg TNF-α response.

EXPERIMENT 19 The observation that lower doses of parthenolide could impair LPS-induced proinflammatory cytokines lead to the attempt at another dose response study using 5, 0.5 or 0.05 mg/kg, i.p. of parthenolide co-treated with LPS 1 mg/kg, i.p. All parthenolide doses evaluated resulted in insignificant decreases in LPS-induced TNF-α, with the largest reduction observed with the 5 mg/kg dose (Table 3.5, Expt. 19). IL-6 was decreased in co-treated animals with 5 and 0.05 mg/kg treatments, but not 0.5 mg/kg. None of the changes were statistically significant.

EXPERIMENT 20 Based on the findings of the three dose response studies the 5 mg/kg, i.p. dose of parthenolide was evaluated using a larger number of animals. The conditions were identical to the previous studies. Serum cytokine levels were evaluated 90 min after receiving injections of parthenolide 5 mg/kg, i.p. and LPS 1 mg/kg, i.p. LPS-induced IL-6 and TNF-α were both significantly impaired in co-treated animals, but

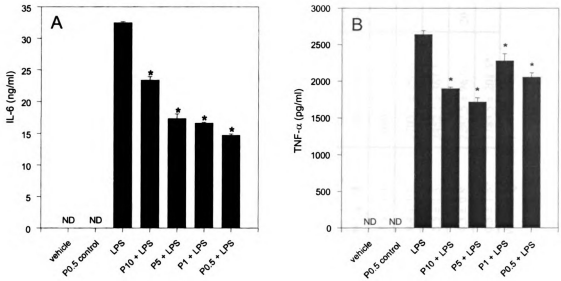

Figure 3.12. Effect of parthenolide dose on LPS-induced (A) IL-6 and (B) TNF-α. Female B6C3F1 mice were co-treated with parthenolide (10, 5, 1 or 0.5 mg/kg, i.p.) or vehicle (50 µl DMSO) and LPS (1 mg/kg, i.p.) or vehicle (100 µl sterile water). After 90 minutes, blood was collected and serum analyzed for IL-6 and TNF-α. Data are mean ± SEM (controls n=2; treatment groups n=3). Values marked with an asterisk (*) differ significantly from LPS control values ($p< 0.05$). ND indicates nondetectable.

Table 3.5. Summary of dose response studies of co-treatment with parthenolide via intraperitoneal (i.p.) administration on LPS-induced serum IL-6 and TNF-α in vivo

Parthenolide dose	Route of exposure	Treatment time	LPS dose (i.p.)	IL-6 (ng/ml)	TNF (pg/ml)	Expt. number	Animal #
None	NA	NA	1 mg/kg	27.9 ± 1.7	1900.8 ± 104.8	17	n=3
25 mg/kg	i.p.	cotreat	1 mg/kg	19.3 ± 0.2	1427.4 ± 140.1		n=3
10 mg/kg	i.p.	cotreat	1 mg/kg	19.4 ± 2.4	1758.7 ± 298.6		n=3
5 mg/kg	i.p.	cotreat	1 mg/kg	16.2 ± 3.0*	2868.8 ± 619.3		n=3
None	NA	NA	1 mg/kg	32.5 ± 0.2	2635.8 ± 55.8	18	n=3
10 mg/kg	i.p.	cotreat	1 mg/kg	23.3 ± 0.6*	1897.6 ± 20.2*		n=3
5 mg/kg	i.p.	cotreat	1 mg/kg	17.3 ± 0.8*	1716.7 ± 57.5*		n=3
1 mg/kg	i.p.	cotreat	1 mg/kg	16.6 ± 0.2*	2275.8 ± 99.6*		n=3
0.5 mg/kg	i.p.	cotreat	1 mg/kg	14.7 ± 0.2*	2052.4 ± 60.9*		n=3
None	NA	NA	1 mg/kg	20.1 ± 1.9	3168.8 ± 214.5	19	n=3
5 mg/kg	i.p.	cotreat	1 mg/kg	13.9 ± 1.1	2090.8 ± 125.8		n=3
0.5 mg/kg	i.p.	cotreat	1 mg/kg	19.4 ± 1.5	2703.7 ± 150.8		n=3
0.05 mg/kg	i.p.	cotreat	1 mg/kg	16.5 ± 1.3	2452.1 ± 367.1		n=3
None	NA	NA	1 mg/kg	24.3 ± 0.9	2287.5 ± 96.9	20	n=7
5 mg/kg	i.p.	cotreat	1 mg/kg	20.5 ± 1.5*	1783.5 ± 201.5*		n=7

Values marked with an asterisk (*) differ significantly from LPS control values ($p< 0.05$)

the overall level of reduction was low - 0.16 - 0.22-fold compared to LPS alone for IL-6 and TNF-α, respectively (Table 3.5, Expt. 20). Although there was a significant reduction in the overall level of LPS-induced IL-6 and TNF-α in parthenolide co-treated animals, the reduction was small, and likely not biologically relevant.

LPS Dose

EXPERIMENT 21 There was some speculation that the LPS treatment used may be overwhelming the model, and that the effect of the parthenolide treatment was minimal because the response to LPS was simply too high. Therefore, an additional experiment was performed to evaluate the effect of parthenolide in combination with lower doses of LPS. The 5 mg/kg dose of parthenolide was administered via both i.p. and gavage along with 0.1 mg/kg, i.p. of LPS as a co-treatment. This dose of LPS still produced appreciable levels of serum IL-6 and TNF-α (Figure 3.13). In both gavage and i.p. treated animals, the effect of parthenolide on LPS-induced IL-6 and TNF-α was minimal. There were no effects on IL-6 levels, and a small decrease in TNF-α following gavage, but there appeared to be small increases with i.p. administration. The results of this study suggested that lower doses of LPS did not necessarily lead to a greater effectiveness of the parthenolide treatment.

Finally, another lower dose of LPS was tested using the same conditions. LPS 0.01 mg/kg, i.p. was administered with parthenolide 5 mg/kg, i.p. or gavage. The gavage treatment had no effect on IL-6 or TNF-α induction, whereas the i.p. treatment slightly increased IL-6 and decreased TNF-α (Figure 3.13). Again, it appeared that parthenolide's effect is not dependent on the level of LPS the animals received.

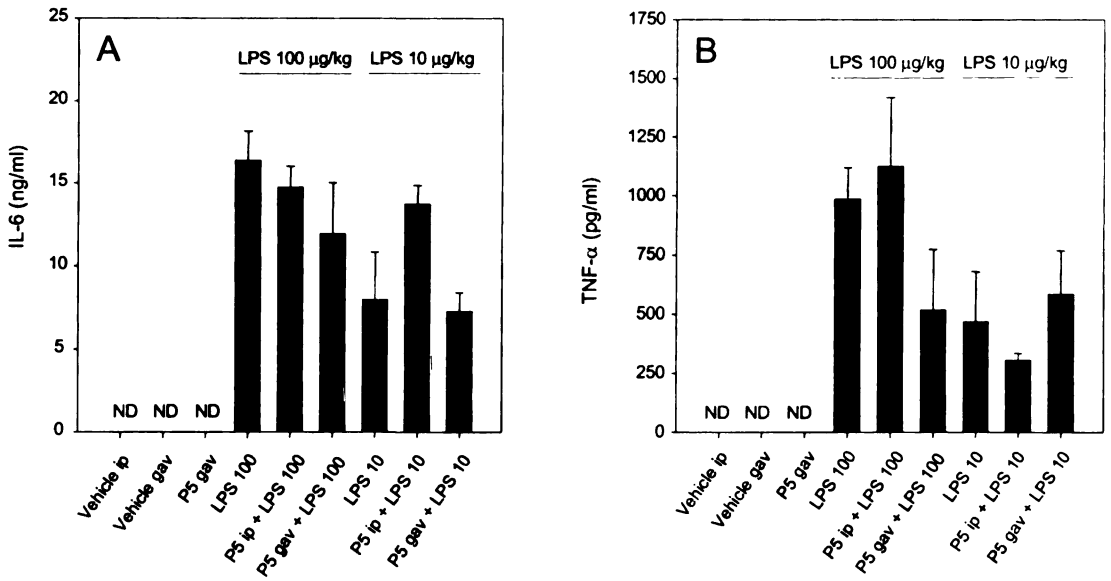

Figure 3.13. Effect of parthenolide on reduced levels of LPS-induced (A) IL-6 and (B) TNF-α. Female B6C3F1 mice were co-treated with parthenolide (5 mg/kg, i.p. or p.o.) or vehicle (50 µl DMSO) and LPS (100 or 10 µg/kg, i.p.) or vehicle (100 µl sterile water). After 90 minutes, blood was collected and serum analyzed for IL-6 and TNF-α. Data are mean ± SEM (controls n=2; treatment groups n=3 or 4). Values marked with an asterisk (*) differ significantly from LPS control values ($p< 0.05$). ND indicates nondetectable.

Combined Data

A summary of all in vivo studies of parthenolide are included in Table 3.6. These data represent a combination of experiments using identical parthenolide doses and treatment times on LPS-induced serum IL-6 and TNF-α production. No significant effects were observed following 1 h pre-treatment of 50 mg/kg parthenolide via oral gavage, whereas there were significant reductions in both LPS-induced IL-6 (0.47-fold) and TNF-α (0.58-fold) following i.p. administration of parthenolide. Co-treatment with 5 mg/kg parthenolide via i.p. administration significantly impaired LPS-induced serum IL-6 (0.35-fold), but had no significant effect on serum TNF-α production.

DISCUSSION

Our initial work evaluating the potential anti-inflammatory effects of parthenolide on LPS-induced proinflammatory cytokine production was accomplished using a murine cell culture model. The results indicated that parthenolide co-treatment could significantly impair LPS-induced IL-6 and TNF-α production by RAW 264.7 macrophage cells. Following repeated in vitro studies with consistent results, a similar model of parthenolide's effect on LPS-induced cytokine production was evaluated in vivo using mice.

At the time animal studies were first undertaken, little data in the literature were available for parthenolide use in vivo. Schinella et al. (1998) evaluated the inhibitory effect of parthenolide (100 mg/kg, p.o.) on carrageenan-induced paw edema in mice demonstrating a 25% reduction in paw volume. One additional study evaluated parthenolide (1 or 2 mg/kg, i.p.) and feverfew extracts (40 or 60 mg/kg, p.o) in vivo, also

Table 3.6. Summary of serum IL-6 and TNF-α production following treatment with parthenolide and LPS 1 mg/kg, i.p. 90 minutes after LPS administration

	Serum IL-6	Serum TNF-α
Parthenolide 50 mg/kg, p.o. 1h pretreatment (n=14)	↑ 0.26-fold	↓ 0.19-fold
Parthenolide 50 mg/kg, i.p. 1h pretreatment (n=31)	↓* 0.47-fold	↓* 0.58-fold
Parthenolide 5 mg/kg, i.p. 0h pretreatment (n=15)	↓* 0.35-fold	↓ 0.15-fold

Values marked with an asterisk (*) differ significantly from LPS control values ($p< 0.05$)

on carrageenan-induced paw edema using rats (Jain & Kulkarni, 1999). Feverfew extract and parthenolide both significantly decreased paw volume following carrageenan-induced edema.

Based on consideration of the two aforementioned studies, a dose of 50 mg/kg, by i.p. injection, was chosen for parthenolide administration. This dose was on the high side, but our initial interest was to determine if an effect was evident, and to adjust the dose in later experiments depending on the response observed in the preliminary studies. Additionally, this higher dose allowed us to consider potential safety concerns that may be associated with administration of higher doses of parthenolide. No detrimental observational effects were evident following parthenolide exposure. However, in an initial experiment parthenolide control animals had significantly elevated serum IL-6. It is not clear why this effect was observed, as no additional studies under similar conditions resulted in measurable cytokine levels in parthenolide control animals. Of potential safety concern is the trend of elevated serum cytokine production in parthenolide control animals receiving parthenolide for 7-consecutive days. Additional studies would be required to determine if this was a reproducible effect.

Results of the dose response study showed that parthenolide, as low as 0.5 mg/kg, i.p., could significantly reduce LPS-induced serum IL-6 and TNF-α concentrations. Although these findings were significant, they were not reproducible. It is not clear if this was a dose-specific effect, or if it was caused by the same unknown factor responsible for variability observed in other in vivo studies. As a result, the lowest dose which caused reductions in cytokines, and was reproducible, was chosen (5 mg/kg, i.,p.) for future studies.

It is not clear how this specific dose compares with typical human exposure. Feverfew extracts typically contain a minimum 0.2% parthenolide, but frequently contain 0.8%. Based on suggested daily doses of feverfew extracts, ranging from 50 mg - 1.2 g, a typical parthenolide dose could be expected between 0.1 - 9.6 mg per day. Assuming an average human weight of 70 kg, an expected dose on a mg/kg basis would range from 0.006 - 0.14. Thus, our experimental dose was much higher than would be a typical human dose. However, supplement consumption in humans is not typically limited to a single dose. It is not clear how cumulative doses of parthenolide, at a lower level via an alternate route, would effect the LPS-induced cytokine response.

In our animal studies, parthenolide was administered 1 h prior to LPS. This pre-treatment period was determined based on a review of in vitro and in vivo studies (Hwang et al., 1996; Tournier et al., 1999; Jain et al., 1999). In general, these studies used a similar pre-treatment period. As a component of the optimization studies, the dose-timing was evaluated. Cumulative effects were assessed through a 7-day oral administration study, but no beneficial effects were noted. As a result, additional time course studies evaluated 60, 30 and 0 min pre-treatment periods. Overall, no overwhelming differences were noted between time periods, but the co-treatment conditions did appear to be as effective, if not more so, than 60 and 30 min pre-treatments. Ross et al. (1999) demonstrated similar beneficial effects of parthenolide co-treatment in comparison to pre-treatment using an in vitro model system. In this study, the co-treatment conditions were therefore pursued based on the observed inhibitory effects, and simplicity added to the experimental design of studies.

An additional component of this research was to determine potential reasons for observed inconsistences in parthenolide's inhibitory capacity on LPS-induced IL-6 and

TNF-α from experiment to experiment. As a result, specific experimental parameters were adjusted to determine their impact on parthenolide's anti-inflammatory properties in vivo.

The impact of feed withdrawal period was evaluated. Feed withdrawal allows an animal's stomach to empty, thereby preventing altered absorption and subsequent pharmacokinetic characteristics of test compounds, and should help minimize animal to animal variation. The effect of feed withdrawal period was evaluated in this study to determine if this contributed to the observed experiment to experiment variability, since all previous experiments used a 1 h withdrawal period. No altered effect of parthenolide treatment on LPS-induced IL-6 and TNF-α concentrations were noted, suggesting that the amount of feed in an animals stomach likely did not contribute to the observed experimental variability.

Degradation of parthenolide stocks and amounts in feverfew extracts has been observed frequently (Heptinstall et al., 1992; Mittra et al., 2000), and is associated with reduced biological activity (Sumner et al., 1992; Barsby et al., 1992; Mittra et al., 2000). Additional studies evaluating parthenolide content in feverfew products demonstrate the variability of supplement products available to American consumers (Abourashed and Khan, 2000; Nelson et al., 2002). Heptinstall et al. (1992) confirmed the instability of parthenolide over time, and showed parthenolide levels in powdered leave products fell during storage. The lower than expected levels of parthenolide observed in feverfew extracts (<0.02%) are likely a result of degradation.

As a component of this study, we specifically evaluated the effect of a highly purified compound (>97% pure) opposed to a whole herb extract. This was done to minimize the variability that is frequently encountered in whole herb extract products

(Harkey et al., 2001; Nelson et al., 2002). However, as parthenolide's inhibitory capacity fluctuated, an additional source was tested to determine if degradation or product to product variability was responsible for observed differences. The results suggested that the specific parthenolide source was not the sole cause for observed differences of the inhibitory capacity of parthenolide.

Neither feed withdrawal period or parthenolide source appeared to contribute to the observed variability. Therefore, we tested the potential effect of reduced levels of LPS used in the co-treatment conditions. Different responses are observed in animals exposed to large versus small doses of LPS (Roth et al., 1997). The doses of LPS tested in this study likely do not represent large doses, but results from the preliminary study do show differences in the observed inhibitory capacity of parthenolide pre-treatment in response to elevated LPS administration. Therefore, the effect of parthenolide on lower doses of LPS was evaluated. Reduction in LPS dose did not appear to influence the effect of parthenolide on inhibition of cytokine production. It is not clear, therefore, why no inhibitory effects were observed at the higher LPS dose (5 mg/kg). However, it may simply be another example of experimental variability, and therefore may not be reproducible.

Although the results of specific treatments, when grouped together, show that parthenolide can reduce LPS-induced serum IL-6 and TNF-α (see Table 3.6), great variability continued to exist from experiment to experiment. No specific reason could be determined to explain the potential variability, but it is important to note that the response in LPS-only treated animals was consistent from experiment to experiment.

Additional factors that were not specifically evaluated were strain and gender differences. It is not clear if the change in strain contributed to the observed differences

of parthenolide's effect on LPS-induced IL-6 and TNF-α. In our first in vivo study (expt. 1), C57BL/6 mice were used. In all remaining experiments B6C3F1 mice were used. This strain was chosen for its hardiness and common use in research studies, including those of the National Toxicology Program. Numerous studies evaluating various endpoints indicate differences are evident with changes in strains of test animals (Goldsworthy & Fransson-Steen, 2002; He et al., 1994; Butler et al., 1988). Many additional experiments would be required to specifically determine if mouse strain was a true variable in our in vivo studies. This is based on observations, in B6C3F1 mice, of similar inhibitory effects of parthenolide pre-treatment on LPS-induced IL-6 and TNF-α using identical treatment conditions as those of the C57BL/6. Our results showed that similar inhibition was observed in B6C3F1 mice, but this inhibition was not consistent from experiment to experiment. It is not clear if a continued trend of variability would similarly exist if C57BL/6, or other mouse strains, were continually tested as was the B6C3F1 mice.

A similar scenario exists with respect to potential gender differences. Gender differences are commonly observed in animal studies (Harbuz et al., 1999; Miyamoto et al., 1999; Spitzer & Spitzer, 2000). Kim et al. (1999) observed gender differences on corticosterone and IL-6 and TNF-α production following immune challenges with LPS and IL-1β. Both cytokines were significantly higher in female animals when compared to males. However, the levels of IL-6 and TNF-α observed in the sera of male mice were within the same range observed for females in our studies, and were not considerably lower than the female response.

In our study, the C57BL/6 animals used in the preliminary experiment were male mice. All additional experiments were performed using female mice. Females were

chosen because they are generally more docile and tend not to fight with one another while housed 3-4 per cage, making their use in experiments more preferable than males. Although the affect of gender was also not specifically tested, many additional experiments would be required to determine if gender was a true variable, as for the determination of strain effects. This is based on the same rationale described for strain. Parthenolide pre-treatment impaired LPS-induced cytokine production in female mice, similar to inhibition observed in male mice. However, this pattern of inhibition could not be repeated continually. To make a clear determination of the effect of gender, numerous additional studies would be required.

An additional variable related to gender differences pertains to the estrous cycle of the female mouse. The average length of individual cycles typically lasts 4-5 days, but is highly variable and can range from 2-28 days (Biology of the Laboratory Mouse; Chapter 11 Reproduction). During this period, many changes in cell biology occur in the uterus. The stage of the cycle is best determined by changes in cellular patterns obtained through evaluation of vaginal swabs. Also accompanying these changes are fluctuations in hormone levels of estrogen and progestin.

In vivo studies suggest that sex steroids are capable of modulating the inflammatory response to LPS (Trentzsch et al., 2003; Zuckerman et al., 1996). Administration of estrogen agonists in LPS-treated mice resulted in elevated serum TNF-α and IL-6. Additional in vitro studies highlight the role of 17 β-estradiol in the outcome of the immune response during inflammation (Deshpande et al., 1997). Fluctuations in estrogen levels during the estrous cycle may effect the inhibitory properties of parthenolide on LPS-induced serum IL-6 and TNF-α, and may explain, at least in part, the observed experimental variability. Studies which specifically evaluate the effect of

parthenolide on LPS-induced serum IL-6 and TNF-α in ovariectomized female mice, treated with or without 17 β-estradiol, are warranted.

Additionally, other key factors are metabolism and distribution of the constituent. To our knowledge, no studies have evaluated the pharmacokinetics of parthenolide in animals or humans. It is possible that the efficiency of metabolism of parthenolide differs between individual animals, and groups, contributing to observed variability that existed from experiment to experiment. Addition considerations include the specific animal age at the time of each experiment and the cage environment for each animal. Until appropriate metabolism and distribution studies are undertaken, and sex hormone effects are determined, the specific cause of the experimental variability observed in these series of experiments will continue to be unknown.

CHAPTER 4

Comparative effects of parthenolide on IL-6, TNF-α, IL-1β and COX-2 mRNA expression in spleens and livers of lipopolysaccharide-treated mice

INTRODUCTION

Parthenolide, the major sesquiterpene lactone derived from the feverfew extract (*Tanacetum parthenium)*, has been studied for its inhibitory effects on inflammation in cell culture and, to a limited extent, in intact animals. This constituent has been shown to attenuate a variety of inflammatory endpoints. Recent attention has turned to the determination of the molecular mechanisms by which parthenolide imparts its effects on inflammatory responses.

Investigations of the anti-inflammatory properties of parthenolide, and feverfew, focused on primary inflammatory endpoints such as platelet aggregation (Groenewegen and Heptinstall, 1990) and carrageenan-induced mouse (Schinella et al, 1998) and rat (Jain et al, 1999) paw edema. Additional studies have evaluated parthenolide's inhibitory effect on inflammatory mediators including cyclooxygenase (COX) (Sumner et al, 1992; Hwang et al, 1996), prostaglandins (O'Neill et al, 1987; Pugh and Sambo, 1988), leukotrienes (LT) (Sumner et al, 1992) proinflammatory cytokines (Hwang et al, 1996; Uchi et al, 2002) and most recently, the transcription factor nuclear factor (NF)-κB (Henher et al, 1998 & 1999; Bork et al, 1997; Rungeler et al, 1999).

Previous research in our laboratory focused on the inhibitory effects of parthenolide on lipopolysaccharide (LPS)-induced proinflammatory cytokine production

in the supernatant of murine cell culture and sera of animals (Smolinski & Pestka, 2003). The data showed that parthenolide could impair LPS-induced tumor necrosis factor (TNF)-α and interleukin (IL)-6 upregulation in culture and in sera of animals when parthenolide was administered via intraperitoneal injection.

Although protein levels of LPS-induced proinflammatory cytokines are reportedly reduced by parthenolide treatment, there are limited data evaluating the effect of parthenolide on these cytokines at the transcriptional level. Hwang et al (1996) showed that parthenolide suppresses LPS-induced steady state levels of TNF-α and IL-1β mRNA in cell culture. Parthenolide had no inhibitory effect on IL-6 mRNA levels in LPS-stimulated macrophages, but did attenuate IL-12 p40 and p35 mRNA expression (Kang et al, 2001) as well as the chemokine IL-8 in cultured human respiratory epithelium (Mazor et al, 2000).

Although parthenolide's effect on specific cytokine gene expression has been documented in vitro, to our knowledge, similar data are not available for mRNA expression of cytokines or other inflammatory genes such as COX-2 in vivo. The objective of this study was to test the hypothesis that parthenolide-induced suppression of serum LPS-induced IL-6 and TNF-α correlate with reduced mRNA levels for these genes, and other related proinflammatory genes, in the spleen and liver. Additionally, we sought to determine whether differences in expression levels of each gene existed between the spleen and liver.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Parthenolide (Calbiochem, San Diego, CA) was dissolved in tissue culture grade dimethyl sulfoxide (DMSO). Lipopolysaccarhide (LPS) from *Salmonella typhimurium* [1.5 EU/ng LPS; Stimulation index (SI) 3.6 @15.6 μg/ml LPS] was dissolved in endotoxin-free tissue culture grade water.

Experimental design for in vivo studies

All animal handling was conducted in accordance with guidelines established by the National Institutes of Health. Experiments were designed to minimize the numbers of animals used. Female B6C3F1 mice (8-10 weeks) were obtained from Charles River (Portage, MI). Animals were housed 3 or 4 per cage with a 12 h light/dark cycle, provided standard rodent chow and water *ad libitum*, and acclimated to their environment at least one week before the start of experiments.

Chow and water were removed from cages one hour prior to the start of each experiment. Mice were co-treated with 5 mg/kg, i.p. (in 50 μl DMSO) parthenolide based on dose-response data obtained in our laboratory (data not shown) and 1 mg/kg, i.p. LPS (in 100 μl water). Vehicle-treated mice received 50 μl DMSO, i.p. and 100 μl water, i.p. Parthenolide control animals received parthenolide 5 mg/kg, i.p. and 100 μl water, i.p. After 90 minutes, blood was collected by retro-orbital bleeding under metofane (methoxyflurane) anesthesia. Animals were then immediately euthanized by cervical dislocation and spleen and liver were collected.

Serum IL-6, TNF-α, and IL-1β determination by ELISA

Collected blood was allowed to clot overnight at 4°C. Serum was collected and analyzed for IL-6, TNF-α and IL-1β by ELISA. IL-6 analysis was performed using purified and biotin-conjugated rat anti-mouse IL-6 antibodies from PharMingen (San Diego, CA). Streptavidin-peroxidase (Sigma) and 3,3',5,5'-tetramethylbenzidine (TMB, Fluka, Ronkonkoma, NY) were used for detection. Absorbance was read at 450 nm using a Vmax™ Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA). For TNF-α analysis the OptEIA Set: Mouse TNF-α (Mono/Poly) kit was employed (PharMingen). For IL-1β analysis the DuoSet® ELISA Development System was used (R&D Systems, Minneapolis, MN).

Total RNA extraction from spleen and liver

Collected spleens and livers were cut into small pieces and placed into TRIzol® Reagent (Invitrogen Life Technologies, Carlsburg, CA). Samples were homogenized for 30 seconds at setting 8 using a Polytron® Homogenizer (Brinkmann, Westbury, NY) and RNA extractions were completed according to manufacturer's instructions. Total RNA was quantified specrophotometricly at 260 nm using a GeneQuant RNA/DNA Calculator (Pharmacia Biotech, Cambridge, England).

mRNA quantification from spleen and liver

Relative IL-6, TNF-α, IL-1β and COX-2 mRNA levels were determined according to manufacturer's instructions using TaqMan® real-time reverse transcription (RT)- polymerase chain reaction (PCR), ABI Prism® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) and Applied Biosystems reagents unless indicated

otherwise. The RT-PCR reaction was carried out in a total reaction volume of 25 μl containing: 1) RNase-free water (Sigma) to 25 μl; 2) 12.5 μl TaqMan® One-Step RT-PCR Master Mix Reagent; 3) 1.25 μl either IL-6, TNF-α or IL-1β Pre-Developed Assay Reagent (primer and probe sets); 4) 1.25 μl 18S rRNA Pre-Developed Assay Reagent; 5) 50 ng total RNA in RNase-free water and 6) 0.63 μl MultiScribe and RNase Inhibitor Mix. COX-2 mRNA was similarly analyzed using forward 5'-CAGAAC CGCATT GCCTCTG-3' and reverse 3'-AGCTGTACTCCTGGTCTTCAATGTT-5' primers (900 nM each) (Michigan State University Genomics Faciltiy, East Lansing, MI) and probe 6FAM-CAACACACTCTATCACTGGCACCCCCTG-TAMRA (250 nM) designed using Primer Express™ software (Applera Corporation, Norwalk, CT). All samples were multiplexed with 18S rRNA which served as an endogenous reference for cytokine mRNA normalization. All samples were assayed in duplicate and serial dilutions of standard (total RNA from LPS-treated mouse spleen) in triplicate. No template control and no RT negative control reactions were also performed. Reaction conditions were: 48°C for 30 min; 95°C for 10 min; and 40 cycles of 95°C for 10 seconds and 60°C for 1 min.

Statistics

All statistical analyses were performed using SigmaStat Statistical Analysis Software (Jandel Scientific, San Rafael, CA). For comparison of two groups, a Student's *t*-test was used. For comparisons of multiple groups using parametric data, one-way analysis of variance (ANOVA) using Student-Newman-Keuls Method for all pairwise multiple comparisons was performed.

RESULTS

Parthenolide co-treatment in vivo inhibits LPS-induced IL-6 protein production in serum

In order to determine the effect of parthenolide co-treatment on LPS-induced IL-6 production, female mice were treated with parthenolide (5 mg/kg, i.p.) and LPS (1 mg/kg, i.p.) for 90 minutes. Blood was collected and serum analyzed for IL-6. Animals treated with LPS alone produced significant levels of IL-6 (Figure 4.1). Serum concentrations of IL-6 were not detectable in vehicle and parthenolide control animals. Co-treatment with parthenolide caused a significant reduction ($p<0.05$) in LPS-induced IL-6 production compared to animals treated with LPS alone.

Parthenolide co-treatment in vivo does not inhibit LPS-induced TNF-α protein production in serum

The effect of parthenolide and LPS co-treatment on TNF-α serum production was also evaluated. LPS treated animals exhibited significantly increased TNF-α concentration in sera compared to both vehicle and parthenolide control animals (Figure 4.2). The concentrations of TNF-α in both control groups were below detection. TNF-α concentrations in animals co-treated with parthenolide plus LPS were not significantly different from LPS-only treated animals.

Parthenolide co-treatment in vivo impairs LPS-induced IL-6 mRNA expression in spleen but not liver

Relative IL-6 mRNA expression in the spleen and liver of co-treated animals was also determined by real-time RT-PCR. IL-6 mRNA expression was significantly induced

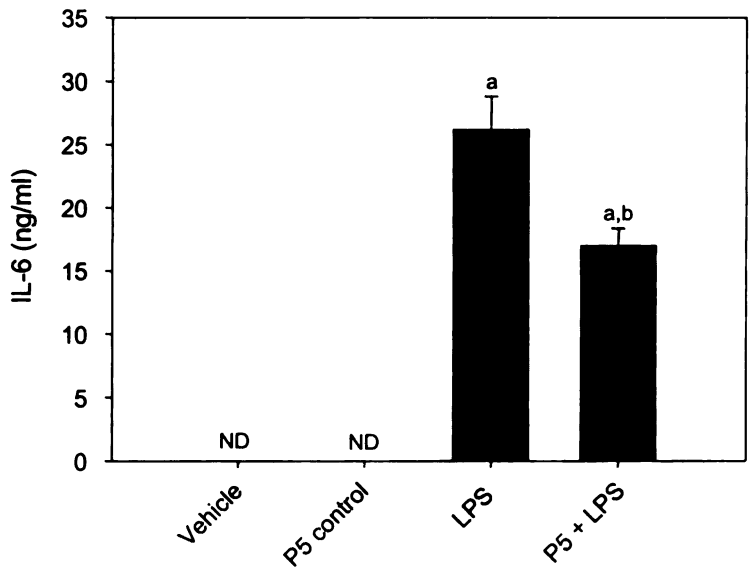

Figure 4.1. IL-6 protein production in sera following parthenolide and LPS co-treatment. Female B6C3F1 mice were co-treated with parthenolide (5 mg/kg, i.p.) or 50 µl DMSO and LPS (1 mg/kg, i.p.) or 100 µl water. After 90 minutes, blood was collected and serum analyzed for IL-6 by ELISA. The letter (a) indicates a significant difference compared to vehicle and parthenolide controls; (b) indicates a significant difference compared to LPS. Data are mean ± SEM (n=16, controls n=4), and is a combination of 4 separate experiments.

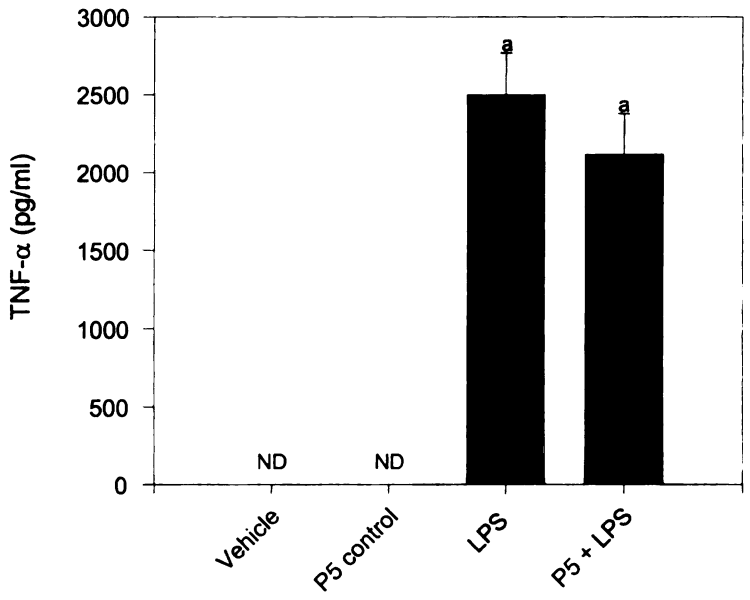

Figure 4.2. TNF-α protein production in sera following parthenolide and LPS co-treatment. Female B6C3F1 mice were co-treated with parthenolide (5 mg/kg, i.p.) or 50 µl DMSO and LPS (1 mg/kg, i.p.) or 100 µl water. After 90 minutes, blood was collected and serum analyzed for TNF-α by ELISA. The letter (a) indicates a significant difference compared to vehicle and parthenolide controls. Data are mean ± SEM (n=16, controls n=4), and is a combination of 4 separate experiments.

in the spleen (239-fold over control) and liver (117-fold over control) of LPS-treated animals (Figure 4.3). IL-6 expression in vehicle and parthenolide control animals was insignificant in both spleen and liver samples. The level of IL-6 mRNA in parthenolide and LPS co-treated animals was significantly reduced ($p<0.05$) compared to LPS-only treated animals in spleen samples, but no difference was observed in liver samples. Overall, IL-6 mRNA expression in spleen was 2.8-fold higher than the liver in LPS-only treated animals, and 1.4-fold higher in the spleen of animals receiving LPS plus parthenolide co-treatment compared to that of the liver.

Parthenolide co-treatment in vivo does not affect LPS-induced TNF-α mRNA in spleen and liver

TNF-α mRNA was also monitored in the spleen and liver of parthenolide plus LPS co-treated animals. In both the spleen and liver there were no differences in TNF-α mRNA expression between LPS-treated and LPS plus parthenolide-treated mice (Figure 4.4). In fact, there was no statistical difference between any of the groups evaluated in this study. As found for IL-6, TNF-α mRNA levels in spleen were considerably higher than those in the liver. TNF-α expression was 3.3-fold higher in the spleen of LPS-only treated animals, and 4.1-fold higher in LPS plus parthenolide co-treated animals when compared to expression levels for this cytokine in the liver.

Parthenolide co-treatment in vivo elevates LPS-induced IL-1β mRNA in spleen but not liver

IL-1β mRNA expression was significantly elevated in spleen and liver of LPS-only treated animals in comparison to vehicle and parthenolide control animals

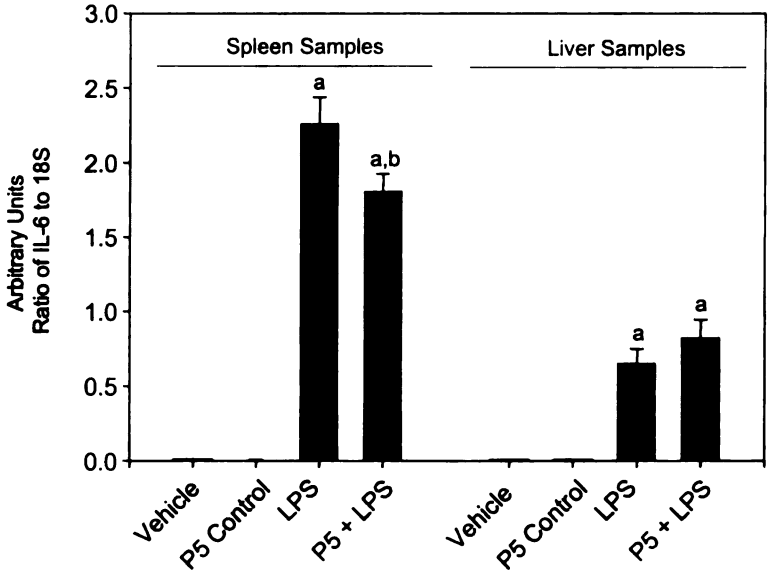

Figure 4.3. IL-6 mRNA expression levels in spleen and liver following parthenolide and LPS co-treatment. Female B6C3F1 mice were co-treated with parthenolide (5 mg/kg, i.p.) or 50 µl DMSO and LPS (1 mg/kg, i.p.) or 100 µl water. Spleen and liver were collected after 90 minutes and total RNA was extracted and subjected to real-time, one-step RT-PCR using TaqMan primers and probes. IL-6 levels were normalized using 18S rRNA. (a) indicates a significant difference compared to vehicle and parthenolide controls; (b) indicates a significant difference compared to LPS. Data are mean ± SEM (n=16, controls n=4), and is a combination of four separate experiments.

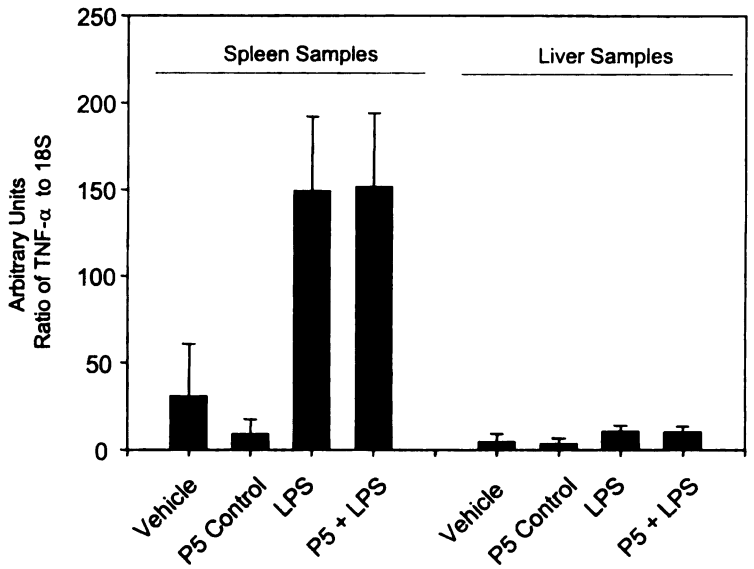

Figure 4.4. TNF-α mRNA expression levels in spleen and liver following parthenolide and LPS co-treatment. Female B6C3F1 mice were co-treated with parthenolide (5 mg/kg, i.p.) or 50 µl DMSO and LPS (1 mg/kg, i.p.) or 100 µl water. Spleen and liver were collected after 90 minutes and total RNA was extracted and subjected to real-time, one-step RT-PCR using TaqMan primers and probes. TNF-α levels were normalized using 18S rRNA. Data are mean ± SEM (n=16, controls n=4), and is a combination of 4 separate experiments.

(Figure 4.5). The level in spleen and liver of vehicle and parthenolide control animals was negligible. In the spleen of co-treated animals, there was a significant ($p<0.05$) increase in IL-1β mRNA compared to LPS-only treated animals. IL-1β mRNA expression levels were 3.2-fold higher in spleen of LPS-only treated animals, and 4.5-fold higher in LPS plus parthenolide co-treated animals, when compared to expression levels of the liver.

Parthenolide co-treatment in vivo does not affect LPS-induced COX-2 mRNA in spleen and liver

Relative COX-2 mRNA expression was assessed in the spleen and liver of parthenolide plus LPS co-treated animals. COX-2 mRNA expression was significantly induced in the spleen of LPS treated animals (44-fold over control) (Figure 4.6). No significant effects were observed in the liver. In both spleen and liver samples there were no significant differences in COX-2 mRNA expression between LPS-treated and LPS plus parthenolide-treated mice. Overall, COX-2 mRNA expression levels were 15.6- and 14.2-fold higher in spleen of LPS-only and parthenolide plus LPS, respectively, when compared to expression levels observed in liver.

DISCUSSION

The results of this study are important because it is the first, to our knowledge, to evaluate parthenolide's effect on proinflammatory cytokine gene expression in two primary sites of the LPS response - the spleen and the liver. Overall, the results presented here show that protein concentrations in serum followed a similar trend to

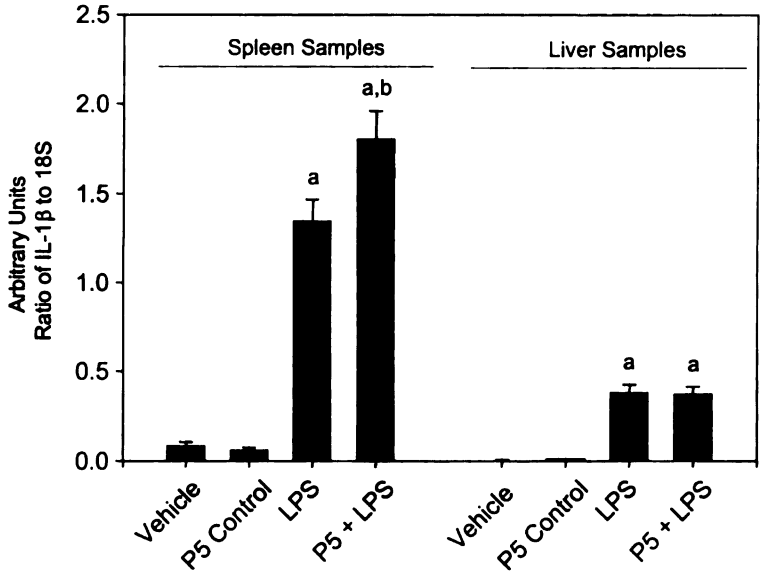

Figure 4.5. IL-1β mRNA expression levels in spleen and liver following parthenolide and LPS co-treatment. Female B6C3F1 mice were co-treated with parthenolide (5 mg/kg, i.p.) or 50 µl DMSO and LPS (1 mg/kg, i.p.) or 100 µl water. Spleen and liver were collected after 90 minutes and total RNA was extracted and subjected to real-time, one-step RT-PCR using TaqMan primers and probes. IL-1β levels were normalized using 18S rRNA. The letter (a) indicates a significant difference compared to vehicle and parthenolide controls; (b) indicates a significant difference compared to LPS. Data are mean ± SEM (n=16, controls n=4), and is a combination of 4 separate experiments.

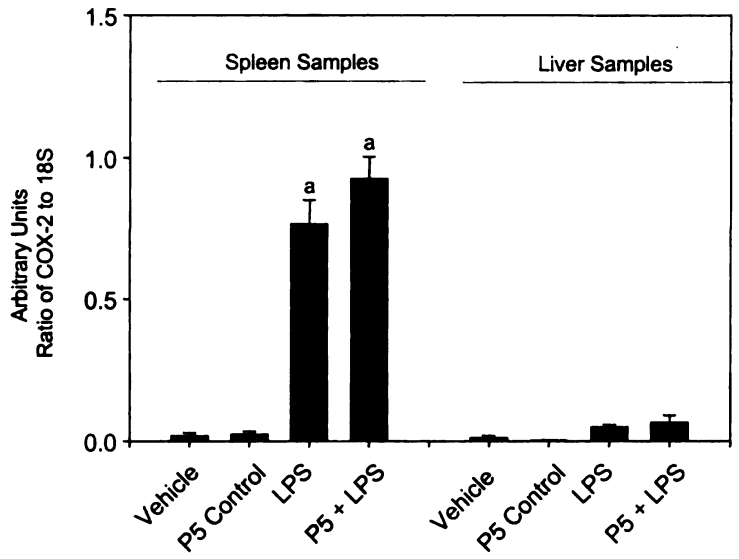

Figure 4.6. COX-2 mRNA expression levels in spleen and liver following parthenolide and LPS co-treatment. Female B6C3F1 mice were co-treated with parthenolide (5 mg/kg, i.p.) or 50 µl DMSO and LPS (1 mg/kg, i.p.) or 100 µl water. Spleen and liver were collected after 90 minutes and total RNA was extracted and subjected to real-time, one-step RT-PCR using TaqMan primers and probes. COX-2 levels were normalized using 18S rRNA. The letter (a) indicates a significant difference compared to vehicle and parthenolide controls. Data are mean ± SEM (n=16, controls n=4), and is a combination of 4 separate experiments.

splenic mRNA accumulation for both IL-6 and TNF-α in LPS and parthenolide co-treated mice. Although protein levels correlated well with splenic mRNA levels, the results were not consistent in liver samples. The mRNA levels of each cytokine in the liver was not changed, irrespective of parthenolide co-treatment, when compared to LPS alone.

Parthenolide's anti-inflammatory effects have been well documented to date. Inhibitory effects on numerous mediators of inflammation including cytokines (TNF-α , IL-1β and IL-6) (Smolinski & Pestka, 2003; Hwang et al., 1996), chemokine (IL-8) (Mazor et al., 2000) and lipid mediators (prostaglandins (Pugh et al., 1988; O'Neill et al., 1987), COX (Hwang et al., 1996; Sumner et al., 1992) and leukotrienes (Sumner et al., 1992)) have been studied. Recently, the research focus has shifted to the determination of the molecular basis for these observed inhibitory effects through evaluation of the transcription factor NF-κB (Uchi et al, 2002; Rungeler et al, 1999; Hehner et al, 1998; Bork et al, 1997).

Transcriptional regulation of cytokine genes including TNF-α (Mercurio and Manning, 1999), IL-6 and IL-8 (Baldwin, 1996) has been strongly linked to NF-κB activation. Interestingly, parthenolide has been shown to inhibit expression of each of these cytokines (Smolinski & Pestka, 2003; Hwang et al., 1996), as well as activation of NF-κB (Uchi et al, 2002; Rungeler et al, 1999; Hehner et al, 1998; Bork et al, 1997) in cell culture studies. Parthenolide appears to inhibit NF-κB by targeting the IκB (inhibitor of NF-κB) kinase complex (Hehner et al, 1999). Parthenolide may, in part, inhibit proinflammatory cytokine and chemokine gene expression through down-regulation of transcriptional activation by NF-κB.

Serum IL-6, but not TNF-α, was significantly reduced following co-treatment with parthenolide (5 mg/kg, i.p.) and LPS (1 mg/kg, i.p.) compared to animals receiving LPS alone. Similarly, IL-6 mRNA concentration in the spleens of co-treated animals was significantly reduced, whereas splenic TNF-α mRNA, and COX-2, were not changed as compared to LPS treated animals. However, the level of IL-1β mRNA in the spleen was significantly elevated in co-treated mice but no effects were observed in the liver. Serum IL-1β were also evaluated, but levels were below the limit of detection in all treatment groups (data not shown). This may be a result of low induction levels in combination with a time point too early to detect IL-1β in sufficient quantities. Therefore, no comparison can be made between protein production and mRNA expression for IL-1β in this study.

To our knowledge, the pharmacokinetics of parthenolide in animal or human tissues has not been studied. It is possible that the differences in metabolism and/or distribution of parthenolide may explain observed differences in cytokine expression levels between the spleen and liver of mice co-treated with LPS and parthenolide. The observed mRNA expression levels of the proinflammatory cytokines and COX-2 might be higher in spleen than liver based on the inherent phagocytic capacities of the macrophage cell populations within each organ. In the spleen, macrophages play key roles in phagocytosis, especially of nonopsonized particles, whereas the macrophage of the liver, Kupffer cells, play a major role in the removal of opsonized particles (Haschek and Rousseaux, 1998). LPS, injected intraperitoneally, does not become opsonized and therefore may be preferentially processed in the spleen rather than liver. This may account, at least in part, for the increased cytokine and COX-2 gene expression observed in the spleen when compared to the liver.

mRNA concentrations in this study were determined using real-time RT-PCR. This sensitive method allowed quantification of very low transcript levels and facilitated comparison of mRNA quantities of each cytokine in the spleen versus that of the liver. Interestingly, the overall expression level of each cytokine in the spleen was at least twice as high as expression levels in the liver. Bhandari et al (2002) also compared cytokine mRNA levels in mouse liver and spleen and found that fumonisin B_1-induced TNF-α, IL-1β and IL-6 mRNA concentrations were comparable in the liver and spleen, although basal-level expression of both TNF-α and IL-1β appeared to be higher in spleen when compared to liver. In that study, mRNA levels were determined by densitometry, a less sensitive method of quantification, and did so following stimulation with fumonisin B_1, not LPS. These experimental differences may contribute to the observed discrepancies of relative spleen and liver mRNA expression of cytokines between the two studies.

Specific cell populations of spleen and liver are likely to play roles in observed cytokine expression. The liver contains a large number of hepatocytes, the parenchymal cells of the liver, which account for 60% of total liver cells and 80% of the liver's volume (Haschek and Rousseaux, 1998). The primary function of these cells is exocrine and metabolic functions of the tissue, and although they are capable of functioning as antigen-presenting cells in certain situations, they are not primary mediators of immune regulation in the liver (Lau and Thomson, 2003). The nonparenchymal cells include Kupffer cells, the resident macrophage of the liver, and interstitial dendritic cells as well as additional cell population types. Both Kupffer and dendritic cells are capable of producing proinflammatory cytokines. When compared to the spleen, the liver's population of macrophage is greater than three times higher, approximately 3% versus

10% (Zhang et al., 2002). However, the opposite is observed with respect to dendritic cell populations. In the spleen, the dendritic cell population is approximately ten times higher than that of the liver. It is possible that dendritic cells, which are constitutively activated and respond more readily to antigen exposure, cause a more rapid activation of macrophage cell populations, and as a result, increase their production of proinflammatory cytokines (Banchereau and Steinman, 1998). This may explain, in part, the observed differences in spleen and liver mRNA expression.

Recent attention to parthenolide's anti-inflammatory effects have been related to the determination of a molecular basis for these observed effects. The inhibitory effect of parthenolide on the transcritpion factor NF-κB has been a major focus of research efforts in this area. NF-κB plays an important role in regulation of both innate and adaptive immune responses (Li and Verma, 2002), and is one of the key regulators of proinflammatory gene expression, and induces transcription of proinflammatory cytokines as well as chemokines, adhesion molecules and COX-2 (Tak and Firestein, 2001; Baeuerle and Baichwal, 1997). Regulation of IL-6 in other models of inflammation demonstrate a dominant role of NF-κB relative to other transcritption factors like CCAAT/enhancer binding protein (C/EBP)β, in IL-6 expression (Georganas et al., 2000). The reduction of IL-6 protein and mRNA accumulation in the spleen observed here might be explained by an inhibition of NF-κB resulting in down-regulation of transcription of IL-6.

In contrast to the observed effects of LPS and parthenolide co-treatment on IL-6 production and gene expression, no inhibitory effect was observed for TNF-α. Although NF-κB has been implicated in the transcriptional regulation of TNF-α, the functional concert of NF-κB with other transcription factors such as activator protein (AP)-1 (Tak

and Firestein, 2001) and C/EBPβ (reviewed by Poli, 1998) may override the importance of NF-κB in LPS-induced TNF-α expression. Notably, the dual pathway of NF-κB and AP-1 has been shown to enhance production of some proinflammatory cytokines, notably TNF-α (Yokoo and Kitamura, 1996). Parthenolide has been shown to inhibit NF-κB, but has no effect on AP-1 (Bork et al., 1997). Therefore, the expression of TNF-α may be compensated for by transcriptional activation by AP-1.

Another transcription factor that acts in concert with NF-κB is C/EBPβ (reviewed by Poli, 1998). C/EBPβ also plays a role in transcriptional activation of TNF-α, IL-1β and IL-6 (Akira et al., 1992). Through the use of C/EBP mutant mice, C/EBPβ appears to be more critical to the regulation of the TNF-α gene in comparison to IL-6 (Poli, 1998). Although no studies have evaluated the effect of parthenolide on C/EBPβ, it is possible that no inhibitory effect would be observed, much as for AP-1. This would provide a possible explanation for the lack of inhibition by LPS and parthenolide co-treatment on TNF-α levels, whereas inhibitory effects were still evident for IL-6.

Similar to the effects observed for TNF-α mRNA expression, there were no significant changes in COX-2 mRNA expression of LPS versus LPS plus parthenolide co-treated animals. Hwang et al. (1996) demonstrated the inhibitory effects of parthenolide on LPS-induced COX-2 protein and mRNA, but using alveolar macrophage cells, not an in vivo model. No other studies have directly evaluated the affect of parthenolide on COX-2 mRNA. Like the other genes evaluated, transcriptional regulation of the COX-2 gene may play a key role in explaining the observed effects.

The COX-2 gene is regulated by a number of transcription factors including NF-κB, C/EBPβ and AP-1 as well as cAMP response element-binding protein (CREB) and others. Site-directed mutagenesis studies of basal COX-2 expression in murine lung

tumor derived cell lines highlight the role of C/EBPβ and CREB as major transcriptional regulators of COX-2 (Wardlaw et al., 2002). Whereas NF-κB appeared to have no role in COX-2 transcriptional regulation using this model. Because the inhibitory effects of parthenolide on transcription factors has been limited to NF-κB, the lack of inhibitory effect on COX-2 mRNA expression may be explained, in part, by the limited role of NF-κB in COX-2 transcriptional regulation.

IL-1β mRNA expression followed a different pattern than the other two cytokines. Although IL-6 was decreased and TNF-α was unchanged, IL-1β levels were increased following co-treatment with LPS and parthenolide. Although IL-1β is also transcriptionally regulated by NF-κB (Tak and Firestein, 2001; Mercurio and Manning, 1999) and C/EBPβ (reviewed by Wedel and Ziegler-Heitbrock, 1995), similar to IL-6 and TNF-α, it may be differentially regulated in response to LPS. In support of this hypothesis, in vivo studies by Zhou et al. (accepted 2003) show that mRNA levels of IL-1β in the spleen are not effected under conditions of LPS tolerance. In that study, it was found that although mRNA levels of both TNF-α and IL-6 are reduced following two injections of LPS, IL-1β levels continued to rise. This suggests that IL-1β is regulated differentially.

CONCLUSIONS

In summary, parthenolide could selectively inhibit proinflammatory cytokine gene expression and subsequent protein production in vivo. These findings also highlight the role of the spleen on cytokine gene expression in response to LPS administration. Further clarification of the specific role of parthenolide on NF-κB, and other

transcription factors including AP-1 and C/EBPβ, in relation to cytokine gene expression using an animal model system will be critical to our understanding of the molecular mechanism for parthenolide's anti-inflammatory effects, as well as it's potential benefit to humans who may consume the compound through supplementation with feverfew products.

CHAPTER 5

SUMMARY

Dietary supplement products, including herbals, are estimated to be consumed by 48% of the American population (Blendon et al., 2000). The relaxed regulatory status of dietary supplements under the DSHEA brings into question the true efficacy and safety of supplement products currently available to consumers, and those that may be released in the future.

Evaluation of the potential anti-inflammatory properties of three herbal constituents, apigenin (chamomile), ginsenoside Rb_1 (ginseng) and parthenolide (feverfew) on lipopolysaccaharide (LPS)-induced proinflammatory cytokine (IL-6 and TNF-α) production were performed in murine macrophage cell culture and intact animal model systems. Although all three constituents inhibited proinflammatory cytokine production in vitro, these results did not adequately predict effects in vivo. These findings suggest the cell culture model employed could only be used to approximate potential in vivo effects, and must be confirmed using appropriately designed animal models.

The results of these specific in vivo studies can only be used as potential predictors of effects of whole herbal extracts. Future studies are necessary to confirm the inhibitory effects of whole herbal extracts of chamomile, ginseng and feverfew on the inhibition of LPS-induced proinflammatory cytokine production in both cell culture and animal model systems. These results may clarify the specific contribution of specific constituents in whole herbal extract preparations. Additional studies which evaluate the

cumulative effects of repeated low level exposure to each herbal constituent, and the whole herbal extract, at doses typical of human use, will contribute to our knowledge of the efficacy of specific herbal products as therapeutics for inflammatory conditions. Additionally, because our studies only evaluated specific mediators (proinflammatory cytokines) of inflammation as an endpoint, and not the inflammatory response as a whole, more studies which evaluate additional endpoints such as PG, LT and chemokine production are warranted, specifically in the animal model system.

Parthenolide, the major sesquiterpene lactone in feverfew extracts, possess clear anti-inflammatory properties in vitro, but a limited number of studies have demonstrated these effects in animal model systems. The studies presented here determined the effect of route, dose and dose-timing of parthenolide administration on inhibition of LPS-induced IL-6 and TNF-α using a mouse model system. Although a determination of intraperitoneal administration of 5 mg/kg parthenolide co-treated with LPS was chosen, it is not clear why our studies demonstrated large experiment to experiment variability in parthenolide plus LPS treatment groups.

The metabolism and distribution of parthenolide may play a role. Studies which evaluate the specific pharmacokinetics of parthenolide in animals, and humans, will contribute to our understanding of parthenolide's anti-inflammatory properties. Additional factors include stain and gender effects. Studies which specifically evaluate the effect of parthenolide on LPS-induced serum IL-6 and TNF-α in ovariectomized female mice, treated with or without 17 β-estradiol, are warranted, and may help explain the observed experimental variability in our studies.

Numerous in vitro studies have demonstrated the inhibitory effect of parthenolide on the transcription factor nuclear factor (NF)-κB (Henher et al, 1998 & 1999; Bork et al,

1997; Rungeler et al, 1999). These studies suggest parthenolide's effect on protein production of IL-6 and TNF-α is related to mRNA expression in the spleen, which may be further regulated by transcription factors including NF-κB. These findings also highlight the role of the spleen on cytokine gene expression in response to LPS administration.

Further clarification of the specific role of parthenolide on NF-κB, and other transcription factors including AP-1 and C/EBPβ, in relation to cytokine gene expression using an animal model system will be critical to our understanding of the molecular mechanism for parthenolide's anti-inflammatory effects, as well as it's potential benefit to humans who may consume the compound through supplementation with feverfew products.

Taken together, these studies contribute to the understanding of the anti-inflammatory properties, and potential mechanisms of inhibition, of the herbal constituents apigenin, ginsenoside Rb_1 and, more specifically, parthenolide on inflammatory mediators. Consideration of the results presented in this dissertation, in concert with previously published studies, likely provide evidence sufficient to claim that these whole herbal extracts "impair the immune system" and as a result may be useful in treating inflammatory conditions. The methods and results of these studies can be used to further elucidate parthenolide's, and other herbal constituents' and extracts', molecular mechanism for inhibitory effects on inflammation, and potential as human therapeutics in the treatment of inflammatory conditions.

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