The Effects of Plant Derived Antioxidants on LPS-induced Production of Tumor Necrosis Factor Alpha and Nitric Oxide

by

Teri L. Wadsworth

A DISSERTATION

Presented to the Department of Physiology and Pharmacology
and the Oregon Health Sciences University
School of Medicine
in partial fulfillment ofthe requirements for the degree of

Doctor of Philosophy
March 2001

School of Medicine
Oregon Health Sciences University

Certificate of Approval

This is to certify the the Ph.D. thesis of

Teri Wadsworth

Has been approved

[redacted]
Professor in charge of thesis

[redacted]
Member

[redacted]
Member

[redacted]
Member

[redacted]
Member

Table of Contents

List of Figures

List of Abbreviations

AAPH	2,2'-azobis hydrochloride
AP-1	activator protein 1
ARE	AU-rich element
ATF	activating transcription factor
CAT	chloramphenicol acyl transferase
C/EBP	CCAAT/enhancer binding protein (NF-IL6)
COX	cyclooxygenase
CRE	cyclic AMP response element
CREB	cyclic AMP binding protein
CYP	Cytochrome P450
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EGb 761	*Ginkgo biloba* extract
EMSA	electrophoretic mobility shift analysis
ERK	extracellular signal-related kinase
EROD	ethoxyresorufin-O-deethylase
GSH	reduced glutathione
IFN	interferon
IKK	IκB Kinase
IL	interleukin
iNOS	inducible nitric oxide synthase
JNK/SAPK	Jun N terminal kinase/stress activated protein kinase

LBP	lipid binding protein
LDL	low density lipoprotein
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
MEK	MAPK/ERK kinase
MEKK	MAPK/ERK kinase kinase
MZF1	myeloid zinc finger-1
NAC	N-acetyl cysteine
NF-κB	nuclear factor-κB
NO	nitric oxide
PAF	platelet-activating factor
PDGF	platelet derived growth factor
PDTC	pyrrolidinedithiocarbamate
PKC	protein kinase C
PMSF	phenylmethylsulfonyl fluoride
ROIs	reactive oxygen intermediates
ROS	reactive oxygen species
SNP	sodium nitroprusside
TIL	tumor infiltrating lymphocyte
TLR	Toll-like receptor
TNF	tumor necrosis factor
TLR	Toll-like receptor
TPA	12-O-tetradecanoylphorbol-13-acetate

TTP	tristetrapolin
UTR	untranslated region

Acknowledgments

I am indebted to Dr. Dennis Koop for allowing me the opportunity to veer from the land of P450s to explore the chemistry of medicinal herbs. I thank him for his incredible memory and intelligence, patience, insight, guidance and sense of humor.

I thank my family for their incredible support. John Paul, Tawny and Julian were always there to convince me that this was possible and necessary. Thank you John for your love and understanding.

I was fortunate to collaborate with many wonderful residents of Koopville. Thanks to Tasha McDonald for her mischievous smile, Tanya Hudson for her sweet disposition and Julia Parazin for her quiet strength. Thanks also to Jianya Huan, Amy Stuyvesant, Lisa Bleyle and Bethany Klopfenstein, for their help and friendship.

I am obliged to John Brookes for comic relief and to Suz Dziennis and Jason Diegnan for many scientific conversations over coffee and martinis.

Thanks finally to the Department of Physiology and Pharmacology and to my committee members, Dr. Roger Hohimer, Dr. Beth Habecker, Dr. George Olsen, Dr. David Williams and Dr. Mary Stenzel-Poore for their critical thinking, suggestions, time and assistance.

I. Abstract

Polyphenolic antioxidants are thought to be responsible for the beneficial effects of moderate wine consumption and are often the active ingredients in herbal remedies used for the treatment of disorders related to oxidative stress and inflammation. Oxidant stress is an activating stimulus for the mitogen activated protein kinase (MAPK) pathway and the transcription factors nuclear factor (NF)-κB and activator protein (AP)-1, which have binding sites in the promoter regions of genes involved in the inflammatory response such as tumor necrosis factor (TNF)-α and inducible nitric oxide synthase (iNOS). Administration of lipopolysaccharide (LPS) to laboratory animals and cultured macrophages also activates the MAPK pathway, NF-κB and AP-1 and induces the production of TNF-α and iNOS-derived nitric oxide NO.

In these studies, pre-treatment of mice with the antioxidant herbal remedy EGb 761 (*Ginkgo biloba* extract) dose-dependently inhibited the *in vivo* production of TNF-α (measured by ELISA) and NO (measured by the Griess reaction) after challenge with LPS. In order to begin to understand the mechanism of inhibition, I evaluated the *in vitro* effects of EGb 761 and its flavonoid component quercetin on LPS-induced signaling cascades in RAW 264.7 macrophages. Pre-treatment with EGb 761 or quercetin dose dependently inhibited TNF-α and NO release. Both substances exhibited unique effects on the MAPK pathway, NF-κB and AP-1. EGb 761 and quercetin suppressed NO release by scavenging NO generated from sodium nitroprusside. Both inhibited iNOS transcription (measured by northern blot analysis) by inhibiting activation of p38 MAPK (measured by western blot analysis and an *in vitro* kinase assay). Both inhibited phosphorylation of extracellular signal-related kinase (ERK1/2), which is important in

post-transcriptional regulation of TNF-α mRNA. Although EGb 761 and quercetin inhibited TNF-α release, quercetin was unique in its ability to inhibit TNF-α transcription (measured by northern blot analysis), possibly by inhibiting activation of Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK, measured by western blot analysis) and its downstream substrate AP-1 (assessed by electrophoretic mobility shift analysis, EMSA). EGb 761 uniquely suppressed LPS-induced NF-κB-dependent transcriptional activity in a luciferase reporter construct and decreased nuclear translocation of the p50 subunit of NF-κB (measured by western blot analysis). Effects on NF-κB may contribute to inhibition of iNOS transcription. My results suggest that quercetin contributes to the inhibition of TNF-α and NO, but it is not the only active component in EGb 761.

This thesis also addresses the *in vitro* effects of the polyphenolic resveratrol on LPS-induced NO and TNF-α formation in RAW 264.7 macrophages. Results from northern blot analysis, the Griess reaction and EMSA illustrated that resveratrol had no effect on LPS-induced NF-κB or iNOS mRNA and therefore decreased LPS-induced NO release at a post-transcriptional level. In contrast to its effects on NO, resveratrol increased basal levels of TNF-α mRNA and protein and enhanced LPS-induced TNF-α mRNA and cytokine release. Resveratrol-mediated release of TNF-α may contribute to the antitumorigenic properties of this wine component.

In summary, quercetin, resveratrol and EGb 761 exhibited unique and selective effects on LPS-induced signaling. Therefore, their mechanisms of action cannot be merely explained by their ability to act as antioxidants.

II. Introduction

A. Antioxidant Phytochemicals

Herbal medicine, the therapeutic use of plants or plant-derived preparations (also referred to as botanicals or phytomedicinals) has in the last several years emerged from the fringes of the health care system in this country. It is estimated that approximately one third of the US population and about three quarters of the world's population use herbs or other forms of alternative medicine to treat their ailments and in many cases, phytomedicinal treatment is used in conjunction with traditional therapy. Sales of botanicals reached approximately $4 billion in 1998.

Herbal medicines are legally classified as dietary supplements and must be labeled with clear statements indicating that they have not been evaluated by the Food and Drug Administration (Angell and Kassirer, 1998; Fontanarosa and Lundberg, 1998). Anecdotally, herbs are often considered safe and nontoxic. However as with all drugs, there is the potential for adverse effects and drug interactions. Cardiac arrhythmias, anticholinergic poisoning and liver, neurological, gastrointestinal and renal toxicity have been reported following ingestion of herbal medicines (Chan *et al.*, 1993; Perharic *et al.*, 1995; Tsiodras *et al.*, 1999; Takei *et al.*, 1997). St John's wort, a common herbal remedy for depression contains potent inducers of several cytochrome P450 (CYP) activities and has been shown to decrease cyclosporin concentrations in kidney transplantation patients (Obach, 2000; Mai *et al.*, 2000). Passionflower, juniper and verbena contain significant concentrations of vitamin K and can lessen the effect of oral anticoagulants while Ginkgo contains an antagonist of platelet activating factor (PAF) and therefore potentiates the action of warfarin (Argento *et al.*, 2000). The importance and relevance of studies

focusing on the toxicity, efficacy, mechanism of action and potential interactions between herbal medicines and traditional medicines is therefore warranted.

A growing body of literature suggests that polyphenolic antioxidants are the active ingredients in phytomedicinals used for the treatment of disorders related to oxidative stress and inflammation such as cancer, cardiovascular and liver disease (Joyeux *et al.*, 1990; Dehmlow *et al.*, 1996; Chen *et al.*, 2000; Mantle *et al.*, 2000; Shao *et al.*, 1999; Craig, 1999). The overall objective of my research has been an investigation of the effects of *Ginkgo biloba* extract and the antioxidants quercetin and resveratrol on the initial phase of the inflammatory process.

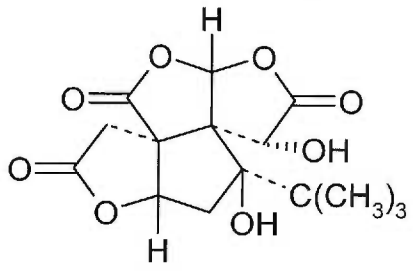
1) Ginkgo biloba extract (EGb 761)

Gingko biloba is the last remaining species of the Gingko tree, which once flourished during Jurassic times. *Ginkgo biloba* originated in eastern China, survived the last ice age, but is now nearly extinct in its native habitat and has been placed on the list of "species in urgent need of conservation" (Masood, 1997). The name *Ginkgo* is derived from the Chinese word for apricots, which resemble the Ginkgo fruit; "biloba" refers to the 2-lobed leaf. The medicinal uses of Ginkgo have been traced to the Chinese materia medica (2800 BC). In the United States, use of *Ginkgo biloba* is increasing at 26% a year, one of the highest for any medicinal plant. The standardized Extract of *Ginkgo biloba* (EGb 761) is the most commonly prescribed herbal remedy in France and Germany. It has been prescribed for the treatment of peripheral vascular, cardiovascular and cerebrovascular disease, reperfusion injury and acute mountain sickness, all of which have components associated with oxidative stress (Itil and Martorano, 1995; Bradbury *et al.*, 1993; Hickman *et al.*, 1994; Roncin *et al.*, 1996; Ramassamy *et al.*, 1999; Pitchumoni

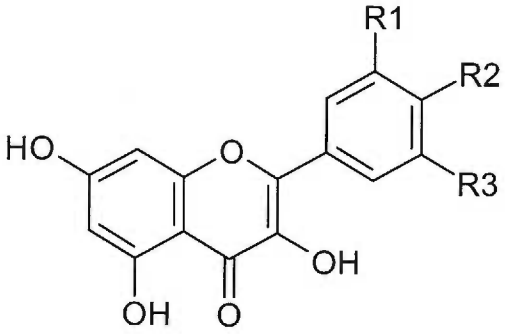
and Doraiswamy, 1998).

EGb 761 is prepared by organic extraction of dried *Ginkgo biloba* leaves, followed by concentration by evaporation. Although an extremely complex mixture, the components have been verified by two-dimensional thin layer chromatography, high-pressure liquid chromatography, mass spectrometry and nuclear magnetic resonance analysis. It is standardized at 24% flavonoid glycosides, the aglycones of which are quercetin, kaempferol and isorhamnetine (Fig 1, Introduction). Rutin, the glycoside of quercetin, accounts for 11.1% of EGb 761. In addition to flavonoids, EGb 761 contains unique diterpenes, ginkgolides A, B and C (which comprise 3% of the extract), and 3% is the sesquiterpene, bilobalide. The terpenes impart bitterness to the extract. Although the flavonoids and terpenes are individually insoluble in water, the extract itself is water soluble (Drieu, 1986; Kleijnen and Knipschild, 1992).

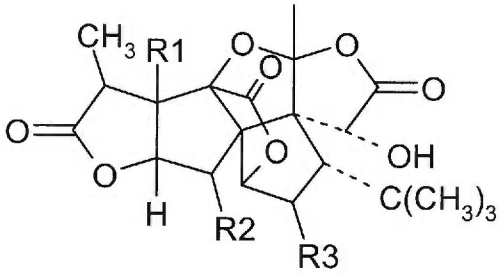
Pharmacokinetic studies using gas chromatography/mass spectrometry indicate that the terpenoid constituents of EGb 761 (ginkgolides and bilobalide) are bioavailable after intravenous and oral ingestion, the elimination half lives in man ranging from 3 to 11 hours (Fourtillan et al., 1995). There is controversy regarding the oral bioavailability of flavonoid glycosides. Animal studies suggest flavonoid glycosides are not absorbed; rather intestinal microflora hydrolyze β-glycosidic bonds and the aglycone is subsequently absorbed (Di Carlo et al., 1999). Absorption studies with ileostomists, however report absorptions of 52% for quercetin glucosides from onions, 17% for quercetin rutinosides and 24% for quercetin aglycone (Hollman *et al.*, 1997). Reverse phase liquid chromatography and electrospray mass spectrometry (ES-MS) analysis of urine, feces and blood from rats administered EGb 761 by gastric probe suggest that



Bilobalide



	R1	R2	R3
Kaempferol	H	OH	H
Quercetin	OH	OH	H
Isorhamnetin	OCH_3	OH	H



	R1	R2	R3
Ginkgolide A	OH	H	H
Ginkgolide B	OH	OH	H
Ginkgolide C	OH	OH	OH

Fig 1. Components of EGb 761

ginkgo flavonoids are extensively degraded to phenylalkyl acids within 24 h of administration (Pietta *et al.*, 1995). The pharmacokinetics of the components of natural products such as EGb 761 is an extremely important yet relatively unexplored area that requires extensive research.

Numerous *in vitro* studies have established the antioxidant properties of EGb 761. It scavenges various reactive oxygen species (ROS) generated *in vitro*, including superoxide, hydrogen peroxide, hydroxyl, oxoferryl and peroxyl radicals (Maitra *et al.*, 1995; Pincemail *et al.*, 1989; Marcocci *et al.*, 1994; Scholtyssek *et al.*, 1997; Noda *et al.*, 1997). The extract prevents oxidative modification of human low densitiy lipoproteins exposed to the free radical initiator 2,2'-azobis (2-amidinopropane) hydrochloride (AAPH) and prevents oxidation of α-tocopherol in this system (Maitra *et al.*, 1995). In addition, EGb 761 slows O_2 consumption (the respiratory burst) of human neutrophils stimulated with phorbol ester by inhibiting NADPH-oxidase (Pincemail *et al.*, 1987). It inhibits NO production in LPS/IFNγ-activated macrophages by concomitantly scavenging NO and inhibiting iNOS mRNA and enzyme activity (Kobuchi *et al.*, 1997; Marcocci *et al.*, 1994). Hydroxyl radical-induced apoptosis and lipid peroxidation is attenuated in rat cerebellar granule cells treated with EGb 761 (Xin *et al.*, 2000). The extract dose-dependently protects primary hippocampal neurons against apoptosis and reactive oxygen intermediate (ROI) accumulation induced by beta-amyloid-derived peptides and hydrogen peroxide, suggesting that the neuroprotective effects of the extract are partly associated with its antioxidant properties (Bastianetto *et al.*, 2000). Ginkgolide B is an antagonist of platelet-activating factor (PAF), a potent mediator of inflammatory reactions (Braquet *et al.*, 1989). The synthetic ginkgolide B analog BN 50739 inhibits

LPS-induced mobilization of NF-κB and expression of TNF-α in J774 macrophages (Im *et al.*, 1997).

Support for the protective effects of EGb 761 in models of oxidative stress have also been provided by animal studies. *In vivo* administration of a terpene-free fraction of EGb 761 was more efficient than *in vitro* administration of the terpene-free fraction in reducing free radical formation (as assessed by electron spin resonance) in isolated ischemic reperfused rat hearts. EGb 761 itself was more effective than the terpene-free fraction in reducing free radicals. Taken together, this indicates that flavonoid metabolites and terpenoid components of EGb 761 act in a complementary manner to protect against ischemia-reperfusion injury (Liebgott *et al.*, 2000). Improved myocardial functional recovery following ischemia-reperfusion, as assessed by hemodynamic measurements, has been reported for rats pre-treated with oral EGb 761 (Pietri *et al.*, 1997a). Oral treatment of rats with EGb 761 protected against cerebral free radical formation and lipid peroxidation associated with the rodenticide bromethalin and protected against electrolyte shifts induced by ischemia reperfusion in diabetic rats (Dorman *et al.*, 1992; Szabo *et al.*, 1995). Intravenous EGb 761 significantly decreased levels of malonaldehyde formation in a rat model of ischemia-induced lipid peroxidation associated with spinal cord injury (Koc *et al.*, 1995).

A limited number of controlled clinical trials document the efficacy of EGb 761 in treating a variety of impairments. The extract shows promise in treating some of the sequelae associated with Alzheimer's disease such as ischemia, stroke, edema, tinnitus and macular degeneration (Diamond *et al.*, 2000). Efficacy in treatment of Alzheimer's disease has been measured as the delay in symptom progression rate or improvement in

cognitive, walking or social performance (Wettstein, 2000; Blume *et al.*, 1996; Le Bars *et al.*, 1997; Oken *et al.*, 1998). EGb 761 inhibited release of thiobarbituric acid reactive species and decreased plasma free radical levels (as measured by electron spin resonance) in a clinical study of coronary bypass patients (Pietri *et al.*, 1997b). In a double blind, placebo controlled clinical trial, the extract significantly decreased circulating levels of endothelial cells (an alteration associated with blood stasis and ischemia) in patients with chronic venous insufficiency (Janssens *et al.*, 1999). Topical EGb 761 reduced allergic contact dermatitis from various allergens in the European standard series (Castelli *et al.*, 1998). Oral EGb 761 decreased symptoms such as headache, insomnia, dizziness, nausea and ataxia associated with acute mountain sickness in a Himalayan expedition (Roncin, Schwartz, and D'Arbigny, 1996). It decreased vertigo and dizziness in a controlled study evaluating patients with vascular vestibular disorders (Cesarani *et al.*, 1998). In general, there are few adverse side effects or drug interactions reported for EGb 761 therapy, however caution is advised for patients taking anticoagulants. Proposed mechanisms of action of EGb 761 include its antioxidant and anti-PAF properties.

2) Quercetin

Quercetin (Fig 1, Introduction) is one of the most abundant flavonoids in the human diet. In addition to EGb 761, it is present in wine, tea, and a wide variety of fruits, vegetables and herbal remedies. Quercetin is produced by plants in response to exposure to ultraviolet light. Grapes exposed to full sun, for instance, have substantially higher levels of quercetin than shaded berries (Price *et al.*, 1995). In plants, flavonoids such as quercetin naturally occur as glycosides such as rutin, with a sugar moiety bound to the C-3 position (Fig 1, Introduction). At present, over 130 preparations containing quercetin

or rutin are registered as drugs worldwide (Erlund *et al.*, 2000).

In vitro studies have established that quercetin is an antioxidant, inhibiting LDL oxidation, lipid peroxidation, and α-tocopherol oxidation in free radical based assays (Csokay *et al.*, 1997; Bors *et al.*, 1990) Quercetin has also been reported to act as a non-specific inhibitor of various tyrosine and serine/threonine kinases, including mitogen activated protein kinases (MAPKs) (Bird *et al.*, 1992; Nishioka *et al.*, 1989; Ferriola *et al.*, 1989; Hagiwara *et al.*, 1988). In human myeloid U-937 cells, quercetin suppresses tumor necrosis factor (TNF-α)-induced activation of the transcription factor NF-κB (Natarajan *et al.*, 1998). In addition, it inhibits purified recombinant IκB kinases, IKK1 and IKK2 *in vitro* by serving as a mixed type inhibitor versus ATP (Peet and Li, 1999). Quercetin is also a potent and selective inhibitor of 5-lipoxygenase, inhibiting calcium ionophore-stimulated generation of leukotrienes in rat peritoneal leukocytes (Moroney *et al.*, 1988).

Literature suggests that repeated dietary intake of quercetin can lead to accumulation in plasma and contribute to plasma antioxidant capacity (Hollman *et al.*, 1997; Hollman *et al.*, 1996). Absorption kinetics and bioavailability of quercetin varies based on the type of glycoside and source. For example, absorption from tea and apples is less than 50% of that absorbed from onions (Hollman *et al.*, 1997; de Vries *et al.*, 1998). Flavonoid glycosides are hydrolyzed by intestinal microfloral enzymes in the small intestine and colon and absorbed as the aglycone (Terao and Piskula, 1999; Walgren *et al.*, 1998; Morand *et al.*, 1998). Clinical trials show that the absorption of quercetin aglycone is more predictable than that of rutin, absorption of the latter varying with gender and use of oral contraceptives (Erlund *et al.*, 2000). The elimination half life

of dietary quercetin in humans is 17-24 h (Hollman, *et al.*, 1997). HPLC analysis indicates that in humans and rats, quercetin is metabolized into methylated, glucurono-sulfated metabolites and is also found in small amounts in the plasma as the unconjugated aglycone. Conjugated derivatives from human plasma retain approximately 50% of the antioxidant properties of free quercetin as established by decreased rates of Cu^{+2}- induced oxidation of human LDL (Manach *et al.*, 1998; Erlund *et al.*, 2000).

Literature suggests that the daily consumption of flavonols in the human diet is 3-80 mg, more than 50% of which is quercetin (de Vries *et al.*, 1997). Commercially available quercetin preparations recommend dosages of 400-1200 mg as a daily dietary supplement. Ingestion of 64 mg of quercetin results in plasma concentrations of 650 nM quercetin in hydrolyzed plasma (Hollman *et al.,* 1996). Assuming first order kinetics, a 1200 mg dose of quercetin could therefore lead to plasma concentrations up to 12 μM, with higher levels occurring if metabolic enzymes become saturated. In addition, the circulating levels of quercetin glycosides may not necessarily reflect therapeutic concentrations. It is conceivable that as with many drugs, quercetin and its metabolites may exhibit tissue binding and therefore tissue concentrations much greater than found in the plasma. Digitalis glycosides, for instance, concentrate in cardiac tissue, with concentrations reaching 15-30 times that of plasma (Hardman *et al.*, 1996). The antimalarial drug chloroquine exhibits lysosomal sequestration, thus concentrating in parasitized red cells (Rang *et al.*, 1995). Studies illustrating the *in vitro* effects of quercetin on cultured cells have used quercetin concentrations ranging between 1-200 μM (Nicholson *et al.*, 1996; Kawada *et al.*, 1998; Kuo *et al.*, 1997; Kobuchi *et al.*, 1999; Musonda *et al.*, 1997; Wadsworth and Koop, 1999). It is therefore possible that dietary

consumption of quercetin from foods or supplements could exert physiological effects.

3) Resveratrol

Resveratrol (Fig 1, manuscript 1) is a polyphenolic compound found in relatively few plants, with the richest sources being grapes, peanuts, and the Asian medicinal herb *Polygonum cuspidatum*. It is synthesized by the plant in response to fungal infection or injury and is therefore included in a class of plant antibiotics known as phytoalexins (Soleas *et al.*, 1997). Because it concentrates in grape skins, it is found in significant concentrations in Pinot noir, a red variety grown in cool climates where fungal infections are prevalent.

In vitro, resveratrol is an inhibitor of cyclooxygenase (COX-1) (Jang *et al.*, 1997). It has been shown to reduce inflammation in carageenan-induced rat pedals and block thrombin-induced platelet aggregation and eicosanoid synthesis in human platelet rich plasma (Jang *et al.*, 1997; Pace-Asciak *et al.*, 1995). As an antioxidant, resveratrol inhibits oxidation of porcine low density lipoproteins in the presence of metal ions and AAPH (Belguendouz *et al.*, 1997). Epidemiological studies suggest that wine antioxidants such as quercetin and resveratrol may contribute to the overall decrease in mortality from all causes, coronary heart disease and cancer amongst moderate wine consumers (Gronbaek *et al.*, 2000; Gronbaek *et al.*, 1995; Rimm *et al.*, 1996; Camargo, Jr. *et al.*, 1997; Burr, 1995).

Antioxidant polyphenolics are therefore implicated as active ingredients responsible for the preventative and beneficial effects of herbal medicines, red wine and a diet rich in fruits and vegetables on disorders related to oxidative stress and inflammation. This thesis specifically evaluates the mechanism by which EGb 761,

quercetin and resveratrol affect the inflammatory process by analyzing the ability of these substances to inhibit bacterial lipopolysaccharide (LPS)-induced production of two representative inflammatory mediators, tumor necrosis factor alpha (TNF-α) and inducible nitric oxide synthase (iNOS) in the murine macrophage cell line, RAW 264.7. Based on preliminary results indicating that EGb 761 inhibited LPS-induced release of TNF-α and NO *in vivo* and *in vitro*, I hypothesized that inhibition was a result of the antioxidant and tyrosine/serine/threonine kinase inhibitory properties of the EGb 761 component, quercetin.

B. LPS-induced signal transduction cascades

It is estimated that 20,000 people in the United States die each year from septic shock as a result of gram-negative bacterial infection (Pinner *et al.*, 1996). The lethal effect is linked in part to the biological effects of LPS, the major structural component of gram negative bacterial outer membranes. Recent observations suggest that the cellular response to LPS is transduced from the cell surface to the interior in a molecular framework analogous to the IL-1 signaling cascade. LPS is transferred from lipid binding protein (LBP, a plasma lipid transfer protein) to CD14 (a glycosylphospatidylinositol linked protein on the surface of leukocytes) and via one or more intermediate steps, the Toll-like receptor 4 (TLR4) is activated (Zhang *et al.*, 1999; Yang *et al.*, 1998; Chow *et al.*, 1999; Wright, 1999; Ulevitch, 1999; Du *et al.*, 1999). The extracellular region of Toll contains multiple leucine-rich and carboxy-terminal cysteine-rich domains, while the intracellular portion contains domains that share homology with the mammalian IL-1 receptor (Means *et al.*, 2000a). The gene encoding TLR4 was first identified as the gene responsible for the failure of several mouse strains (C3H/HeJ and C57/10ScCr) to

respond to LPS (Poltorak *et al.*, 1998; Qureshi *et al.*, 1999). Recent studies suggest that heat shock protein 60 (hsp60) is the endogenous ligand for the TLR4 complex. The innate immune response exhibits similarity in its response to LPS and hsp60, as evidenced by studies showing that macrophages containing functional TLR4s up-regulate inflammatory mediators such as IL-6, NO and TNF-α when treated with recombinant human hsp60 (Chen *et al.*, 1999b). Macrophages from LPS-resistant C3H/HeJ mice are non responsive to hsp60 (Ohashi *et al.*, 2000). TLR4 activation results in a downstream signaling pathway involving phosphorylation and activation of IRAK (IL-1 receptor associated kinase) which interacts with the activated TLR4 receptor complex via the adapter protein MyD88. Phosphorylated IRAK dissociates from the receptor complex and interacts with TRAF6 (TNF receptor associated factor 6) (Wesche *et al.*, 1997; Adachi *et al.*, 1998; Cao *et al.*, 1996a; Cao *et al.*, 1996b; Robinson *et al.*, 1997; Swantek *et al.*, 2000). This sequence of events is supported by several lines of evidence, including immunoprecipitation studies and transient transfections with dominant negative mutants of MyD88, IRAK and TRAF6 (Zhang *et al.*, 1999; Swantek *et al.*, 2000; Means *et al.*, 2000a; Aderem and Ulevitch, 2000). Following activation of the TLR4 signaling cascade, additional signal transduction pathways are activated, including tyrosine kinases, the ERK, p38 and JNK mitogen activated protein kinase (MAPK) pathways, and transcription factors such as NF-κB/Rel and the activator protein 1 (AP-1) complex (Swantek *et al.*, 1997; Han *et al.*, 1993; Hambleton *et al.*, 1996; Weinstein *et al.*, 1992; Siebenlist *et al.*, 1994; Baeuerle and Baltimore, 1996; Hwang *et al.*, 1997). Various members of the MAPK cascade have been shown to directly or indirectly activate the transcription factors NF-κB and AP-1 and promoters for several

inflammatory cytokines contain consensus sequences which bind these transcription factors. These processes are therefore potential sites to evaluate the effects of natural product antioxidants on the LPS-induced inflammatory process. A schematic diagram of LPS-induced TLR signaling is illustrated in Fig 2.

1) MAP kinase cascades

Three mammalian MAPK cascades, p44/42 (Erk1 and Erk2), p38 and JNK/SAPK play critical roles in the regulation of cell growth and differentiation and the control of cellular responses to cytokines and stress. MAPK cascades are usually organized in a three kinase architecture consisting of a MAPK/extracellular-signal related kinase (ERK) kinase kinase (MEKK) which activates a MAPK/ERK kinase (MEK) which in turn activates a MAPK/ERK. Transmission of signals is achieved by sequential phosphorylation and activation of components specific to a respective cascade. The MEKs are dual specificity kinases, phosphorylating MAPKs on threonine and tyrosine on specific TXY sequences. Phosphorylation of a MAPK induces dimerization and translocation from the cytoplasm to the nucleus. MAPKs are proline-directed serine/threonine kinases. X-ray crystallographic studies demonstrate that individual MAPKs exhibit substrate specificity due to a combination of intrinsic specificity of the catalytic region for serine or threonine with a proline at the +1 or +2 position and domains that determine stable substrate binding. MAPK scaffolds and adaptors are thought to tether components of specific MAPK cascades into oligomeric complexes which increase local concentrations of enzyme and substrate and exclude related components that operate in parallel cascades, thus allowing independent signaling (Khokhlatchev *et al.*, 1998; Schaeffer and Weber, 1999). A schematic overview of

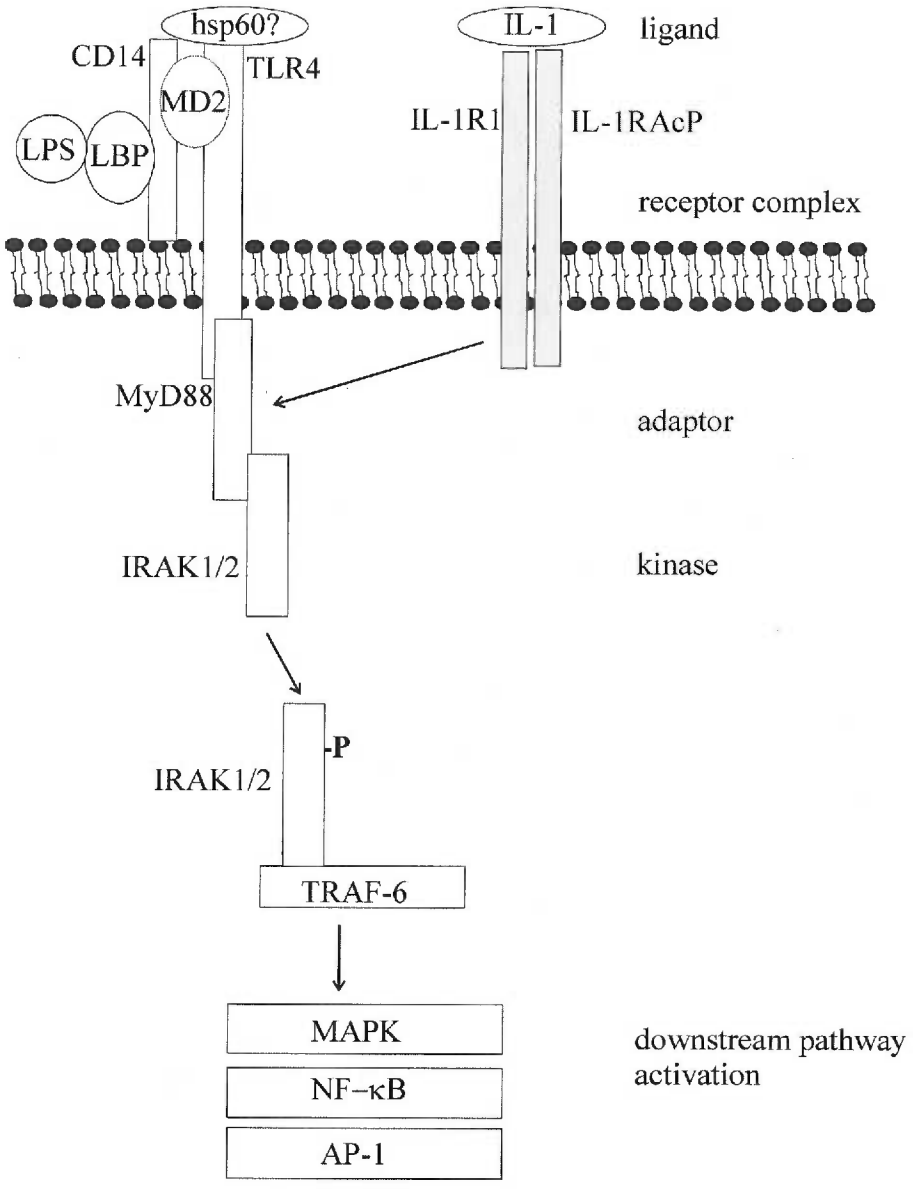


Fig 2. Signaling in the IL-1/TLR superfamily. IL-1 binds to and activates IL-1R1. LPS is opsonized by LBP and the complex is recognized by CD14, a receptor which associates with the cell surface by a glycolipid linkage. TLR4 and CD14 each contain leucine rich repeats which facilitate protein-protein interactions. MD2 is a secreted protein that binds to the extracellular domain of TLR4. Hsp60 is a putative endogenous TLR4 ligand. IL-1R1 and TLR4 contain intracellular domains with high homology. Both signal through the adaptor protein MyD88 and the serine kinase IRAK to subsequently activate the MAPK cascade.

MAPK modules, scaffold and adaptor molecules is illustrated in Fig 3. Specific protein phosphatases regulate the magnitude and duration of MAPK signaling (Keyse, 1998).

Studies utilizing specific MAPK inhibitors in conjunction with 12-O-tetradecanoylphorbol-13-acetate (TPA) in RAW 264.7 macrophages suggest that ERK1/2 is downstream of protein kinase C (PKC), Ras, Raf and MEK1/2 (Cobb and Goldsmith, 1995; Schaeffer and Weber, 1999; Chen and Wang, 1999). ERK 1/2 activation can be inhibited by the noncompetetive MEK1/2 inhibitors PD98059 and UO126 (Dudley *et al.*, 1995; Favata *et al.*, 1998; Alessi *et al.*, 1995). Co-tranfection assays illustrate that the upstream members of p38 MAP kinase family are MEKK-1 and MEK3/6 (Derijard *et al.*, 1995; Raingeaud *et al.*, 1995). In the family of p38 MAP kinases, the α and β isoforms are both activated by proinflammatory cytokines but differ in their preference of activating MKK and substrate (Jiang *et al.*, 1997; Wang *et al.*, 1998). Activation of p38 can be selectively inhibited by SB203580, which binds to its ATP-binding site (Lee *et al.*, 1994b; Cuenda *et al.*, 1995). The JNK/SAPK pathway consists of MEKK-1, and MKK 4/7. c-Jun, a component of the transcription factor AP-1 is a major downstream target of the JNK/SAPK signaling pathway (Derijard *et al.*, 1994; Kyriakis *et al.*, 1994). To the best of my knowledge, no specific inhibitor of JNK/SAPK has been identified.

Although MAPKs exhibit substrate specificity, they do not all operate in a linear fashion and may converge on the same kinase or transcription factor. For instance, MAPKAP kinase 3 can be phosphorylated by ERK, JNK and p38 MAPK. The transcription factor ATF-2 is regulated by JNK and p38 MAPK while Elk-1 can be activated by ERK1/2, JNK and p38. How specificity of signals is achieved in the face of such convergence is currently unknown. MAPK modules may be linked to downstream

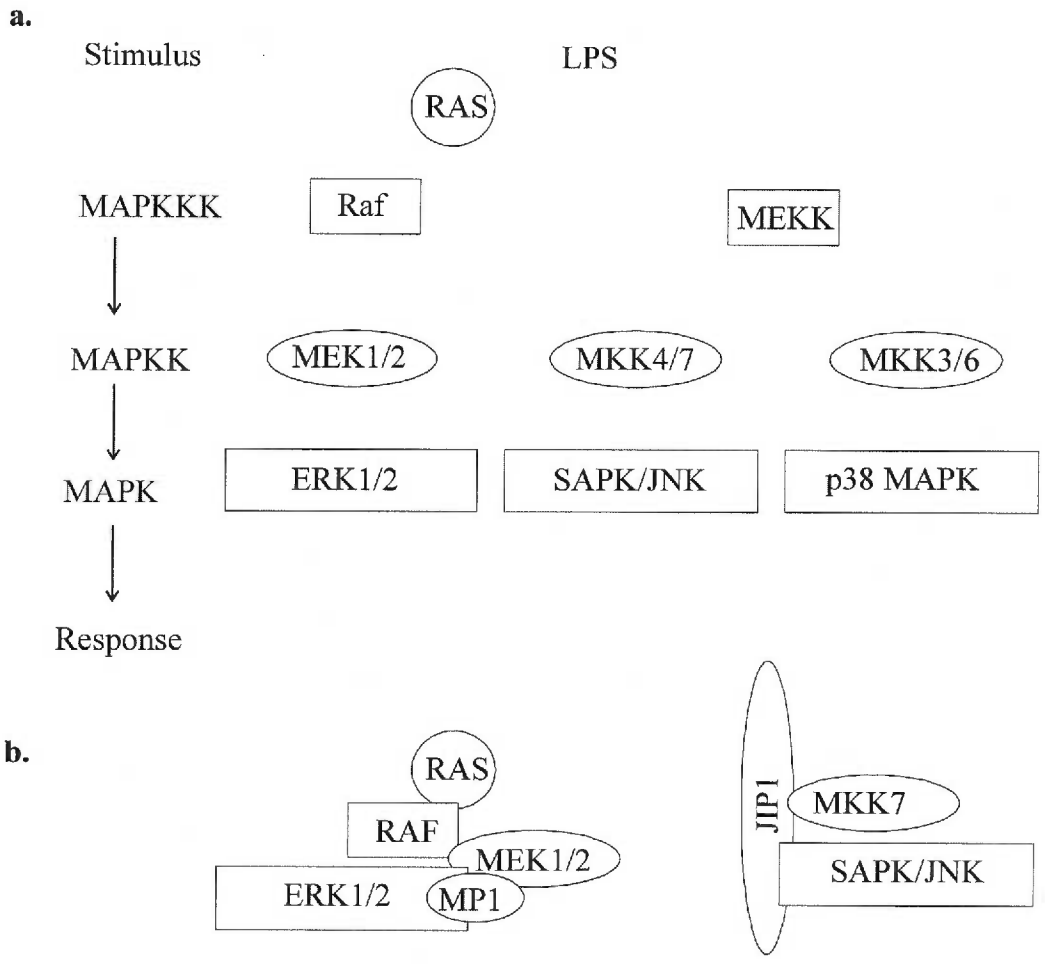


Fig 3. a. Schematic overview of MAPK modules. b. Scaffold and adaptor molecules such as MP1 and JIP1 are thought to tether components of specific MAPK cascades into oligomeric complexes, thus increasing local concentrations of enzyme and substrate and excluding components from other signaling cascades. MP1 = Mek partner 1, JIP1 = JNK interacting protein 1.

effectors in a cell-type specific manner, or common downstream targets may cooperate with unique input signals to a achieve a unique biological response. The activation of signaling cascades in various combinations has been compared to the different sounds an orchestra creates by varying harmony, rhythm and instrumentation (Schaeffer and Weber, 1999). The challenge is to understand how the coordination and regulation of these pathways results in a specific biological response.

2) Transcription factors

a. NF-κB/Rel

NF-κB/Rel proteins are dimeric, sequence-specific transcription factors involved in the activation of numerous genes in response to inflammation, viral and bacterial infection. The precise mechanism by which LPS stimulation induces NF-κB has yet to be elucidated. The most abundant form of the transcription factor is a heterodimer of p50 and p65 subunits, in which the p65 subunit contains the transcriptional activation domain. In unstimulated cells, NF-κB is sequestered in the cytoplasm by the binding of a family of inhibitors (IκBα, IκBβ, IκBγ). Activation by LPS involves rapid sequential phosphorylation of IκB by an IκB-kinase (IKK1 or IKK2), ubiquitination and degradation of IκB by the proteasome. Free NF-κB translocates to the nucleus and activates transcription of its target genes (Rothwarf, 2000).

Several studies have advanced our understanding of the role of protein kinases in LPS-mediated NF-κB activation. Tyrosine kinase inhibitors prevent the activation of NF-κB and subsequent release of pro-inflammatory cytokines (Hwang et al., 1997; Weinstein *et al.,*1992; Stefanova *et al.*, 1993; Chanmugam *et al.*, 1995; Akarasereenont *et al.*, 1994b). IKK1 and IKK2 appear to be differentially regulated. In LPS-stimulated

monocytes, prolonged NF-κB activity correlates with IKK2 dependent IκB-β phosphorylation and degradation. MEKK-1, the upstream activator of SAPK/JNK and p38 MAP kinase pathways, preferentially stimulates IKK2 activity (O'Connell *et al.*, 1998; Nakano *et al.*, 1998). Following IκB degradation, phosphorylation of the p65 subunit by an associated catalytic subunit of PKA is essential for efficient transcriptional activation by NF-κB (Zhong *et al.*, 1998; Zhong *et al.*, 1997). p38 MAP kinase regulates NF-κB-dependent gene expression, in part, by phosphorylating the transcription factor TFIID (TBP), which is important for transcriptional activation of NF-κB (Xu *et al.*, 1993; Blair *et al.*, 1994; Schmitz *et al.*, 1995; Lee and Young, 1996; Carter *et al.*, 1999b; Carter *et al.*, 1999a; Kerr *et al.*, 1993). Inhibition of p38 MAP kinase does not affect NF-κB DNA binding (Beyaert *et al.*, 1996).

The role ROIs in the regulation of NF-κB is controversial. Indirect evidence for the role of ROIs in NF-κB activation is based on the inhibition of NF-κB and κB-responsive cytokines by overexpression of antioxidant enzymes and antioxidants such as N-acetylcysteine (NAC), pyrrolidinedithiocarbamate (PDTC) and the natural products curcumin and epigallocatechin gallate (Manna *et al.*, 1998; Kretz-Remy *et al.*, 1996; Baeuerle and Henkel, 1994; Katiyar *et al.*, 1994; Sen and Packer, 1996; Chan *et al.*, 1995). Hydrogen peroxide-induced NF-κB activation is highly cell-type dependent and may be dependent on intracellular levels of reduced glutathione (GSH). To date, data suggests that IκB phosphorylation and degradation may be the steps that are sensitive to oxidative stress as these processes can be inhibited by antioxidants or overexpression of peroxidases (Li and Karin, 1999).

b. AP-1

The transcription factor AP-1 was first defined as a DNA binding activity specific for positive regulatory elements in the SV40 early promoter. It is activated in response to a wide array of stimuli, including UV and ionizing irradiation, peptide growth factors, cytokines and LPS. AP-1 is not a single transcription factor, but a series of related dimeric complexes of Fos and Jun family proteins that bind to the DNA sequence 5' TGAGTCA 3' (Wisdom, 1999). Supershift analysis indicates that LPS-activated AP-1 complexes in murine macrophages consist of c-Fos, Jun-B, Jun-D and c-Jun (Medvedev *et al.*, 1999). In addition to forming Jun-Jun and Jun-Fos dimers, Jun proteins can also form heterodimeric Jun-ATF-2 complexes which recognize the CRE (cAMP response element) binding sequence. Unlike the human TNF-α promoter, which contains perfectly matched AP-1 sites, murine AP-1 sites are imperfectly matched (Chu *et al.*, 1998). The promoter region of the murine iNOS gene contains motifs corresponding to elements involved in the binding of AP-1 (Xie and Nathan, 1994; Lowenstein *et al.*, 1993).

Activation of ERK and/or JNK/SAPK results in AP-1 activation. Both c-Jun and c-Fos are activated by JNK/SAPK, c-Jun by direct phosphorylation and c-Fos by activation of Elk-1, a transcriptional element on the c-Fos promoter (Whitmarsh *et al.*, 1995). AP-1 and NF-κB may form synergistic complexes that enhance transcription (Stein *et al.*, 1993).

C. Inflammatory mediators

1) TNF-α

TNF-α is a polypeptide cytokine released predominantly by macrophages and monocytes in response to bacterial infection and inflammation. Although beneficial

when released in small amounts in a localized fashion, massive upregulation of TNF-α has been implicated as a mediator of endotoxic shock and several chronic inflammatory diseases.

The murine TNF-α gene, which was cloned in 1985, encodes a 26 kDa membrane bound polypeptide that is activated by proteolytic cleavage into a 17 kDa subunit. Three of these subunits subsequently trimerize to form the bioactive form of TNF-α (Tracey and Cerami, 1994; Camussi *et al.*, 1991; Fransen *et al.*, 1985). The induction of TNF-α production upon stimulation by LPS results from both an enhancement of gene transcription and translational derepression. Following LPS stimulation, TNF-α mRNA levels rise by a factor of 50-100, while secretion of mature cytokine rises by a factor of 10,000 (Han *et al.*, 1990; Camussi *et al.*, 1991).

The TNF-α promoter and 3'-untranslated region (UTR) each contain sequence elements that cooperatively mediate responses to LPS (Han *et al.*, 1991). The promoter contains potential binding sites for several transcription factors, including NF-κB, CRE, AP-1, and C/EBPβ (NF-IL6) (Rhoades *et al.*, 1992; Zagariya *et al.*, 1998). Studies utilizing 5' deletion mutants linked to the CAT reporter in primary macrophages suggest that transcriptional control of TNF-α is mediated primarily by NF-κB (Shakhov *et al.*, 1990a). EMSA of nuclear extracts from LPS-induced murine macrophages suggest that NF-κB dependent transcriptional activation by LPS is mediated by c-Rel and p65 (Kuprash *et al.*, 1995). The TNF-α CRE is critical for autoregulation, as shown by studies in which mutation of this promoter element prevented TNF-α receptor-mediated stimulation of TNF-α expression (Brinkman *et al.*, 1999).

Post-transcriptional regulation (mRNA instability and translational repression) is

governed by an AU-rich element (ARE) in the 3'-UTR of TNF-α mRNA. Using constructs in which the CAT coding sequence was followed by varying segments of the TNF 3'-UTR, Han *et al.* (1990a) demonstrated that this region mediates LPS-induced enhancement of translational efficiency. This response is referred to as derepression and is conferred by the translationally repressive TTATTTAT element, acting in concert with essential flanking sequences. The proteins involved in translational control are yet to be fully elucidated. Two protein complexes from macrophage cytosolic extracts that bind to TNF-α mRNA are implicated. Protein complex 1, which forms in both stimulated and unstimulated macrophages, contains an RNA-binding protein and binds to clustered AUUUA pentamers independently of LPS. Complex 1 is thought to be responsible for translational repression (Gueydan *et al.*, 1999). Complex 2, which is only detected after LPS treatment, is composed of a 55-kDa protein. This complex binds to UUAUUUAUU sequences following LPS-stimulation and may mediate translational de-repression (Lewis *et al.*, 1998). Destabilization of TNF-α mRNA appears to be mediated by binding of the zinc finger protein tristetrapolin (TTP) to the ARE. TTP is a cytosolic protein whose synthesis is induced by TNF-α and LPS. Immunoprecipitation studies revealed that constructs containing human TTP bind to probes containing the 3' UTR of murine TNF-α mRNA and northern blots illustrate that TTP decreases the half-life of ARE-containing mRNA. TTP knockout mice display symptoms of chronic excess TNF-α such as inflammatory arthritis, dermatitis and cachexia (Carballo *et al.*, 1998).

Several studies have illustrated the role of MAPK in the post-transcriptional regulation of TNF-α biosynthesis. The protein kinase inhibitor radicolol inhibits the expression of TNF-α in LPS-stimulated macrophages in part due to accelerated decay of

mRNA (Feng *et al.*, 1997). Upstream components in the ERK pathway, Ras and Raf are required for TNF-α production at the level of both transcription and translation in RAW 264.7 macrophages (Geppert *et al.*, 1994). Direct evidence supporting a role for ERK1/2 in TNF-α production has not been reported. The use of specific p38 inhibitors and kinase defective mutants of JNK/SAPK established that these kinases are necessary for LPS-induced translation of TNF-α mRNA (Lee *et al.*, 1994b;Swantek *et al.*, 1997). MAPKAP kinase 2, a substrate of p38 MAPK, is essential for LPS-induced TNF-α translation (Khokhlatchev *et al.*, 1998). Both SB203580 and PD98059 block TNF-α accumulation in RAW macrophages stimulated with LPS plus interferon-γ (Ajizian *et al.*, 1999). Taken together, these studies implicate the involvement of p38 and p44/42 MAPKs in TNF-α regulation.

2) iNOS

NO, a short-lived gas (with a biological half life in the range of seconds) and highly reactive free radical, is synthesized by monocytes and macrophages upon exposure to stimuli such as LPS, IFN-γ or TNF-α. In macrophages NO is synthesized by an inducible calcium indpendent isoform of nitric oxide synthase, iNOS, which converts L-arginine to L-citrulline and NO. Although NO is an important mediator of host cell defense, overproduction of NO has been linked to diverse pathophysiological conditions associated with vascular, inflammatory and neuro-degenerative disorders (Gross and Wolin, 1995). In oxygenated biologic media, NO rapidly reacts with superoxide radical to form peroxynitrite ($OONO^-$). The toxicity of NO is thought to be mediated by direct reactions between peroxynitrite and thiol or iron-sulfur containing proteins (Rubbo *et al.*, 1996).

Control of iNOS expression is complex, but appears to occur primarily at the level of transcription. Computer analysis of the iNOS promoter reveals numerous regulatory regions incorporating binding sites for NF-κB, C/EBP, tumor necrosis factor response element, interferon-related transcription factors and AP-1 (Xie *et al.*, 1993; Xie and Nathan, 1994; Chu *et al.*, 1998; Lowenstein *et al.*, 1993). Reporter constructs containing truncated promoter regions from murine iNOS transfected into murine macrophages reveal that NF-κB mediates the enhanced expression of the iNOS gene in macrophages exposed to LPS (Xie *et al.*, 1994; Lowenstein *et al.*, 1993). Mobility shift analysis indicates that in RAW macrophages the NF-κB sites bind to p50/65 and p50/c-rel heterodimers (Xie and Nathan, 1994; Xie *et al.*, 1994). Activation of NF-κB is necessary but not sufficient for iNOS expression, as iNOS is also regulated at a posttranscriptional level. Mobility shift analysis reveals a cycloheximide-sensitive NF-κB binding complex in nuclear extracts from LPS-induced macrophages which may represent additional transcription factors known to interact with NF-κB such as Fos/Jun (Xie *et al.*, 1994; Xie and Nathan, 1994). Cycloheximide is also known to inhibit the induction iNOS mRNA by LPS in murine macrophages. These studies suggest the importance of newly synthesized proteins for LPS-inducibility (Nathan and Xie, 1994; Xie *et al.*, 1994; Xie and Nathan, 1994; Chu *et al.*, 1998; Weisz *et al.*, 1994).

The importance of tyrosine phosphorylation in mediating the induction of iNOS by LPS is illustrated by the dose dependent inhibition of nitrite accumulation by the tyrosine kinase inhibitors erbstatin and genistein in J774.2 macrophages (Akarasereenont, *et al.,* 1994). As with TNF-α, comparison of studies evaluating the specific role of MAPKs on iNOS regulation are complicated due to differences in cell type and inducing

agent. Using specific inhibitors of p38 and p44/42 MAPK, Chen and Wang (1999) reported that only p38 is involved in the LPS-induced stimulation of iNOS in RAW 264.7 macrophages. In contrast, Paul *et al.* (1999) reported that neither p38 or p42/44 MAP kinases are involved in the induction of iNOS in RAW 264.7 macrophages stimulated with LPS. In primary glial cells and RAW 264.7 macrophages, the induction of iNOS by IFN-γ plus LPS is dependent on both p38 and p42/44 (Ajizian, *et al.*, 1999; Bhat *et al.*, 1998). TNFα-induced iNOS requires activation of JNK/SAPK in primary mouse macrophages (Chan *et al.*, 1999; Chan and Riches, 1998). Additional studies are necessary to determine the exact involvement of MAPK family members in LPS-induced expression of iNOS in RAW 264.7 macrophages.

A scheme illustrating LPS-induced signaling cascades is shown in Fig 4. Relationships are incorporated from data in the literature.

D. Specific Aims Addressed in this Thesis

1) To test whether plant derived antioxidants suppress LPS-induced NO and TNF-α in vitro

The murine macrophage cell line, RAW 264.7, was utilized to determine whether EGb 761 and the polyphenolic antioxidants resveratrol and quercetin inhibit LPS-induced release of the representative inflammatory mediators, NO and TNF-α *in vitro*. Cells were pretreated with either resveratrol, quercetin or EGb 761, treated with LPS, and culture supernatants analyzed for TNF-α (by ELISA or bioassay) or nitrite (using the Griess reaction). Manuscript 1 discusses the effects of quercetin and resveratrol on LPS-induced NO release and TNF-α secretion. Manuscript 2 discusses the effects of EGb 761 on

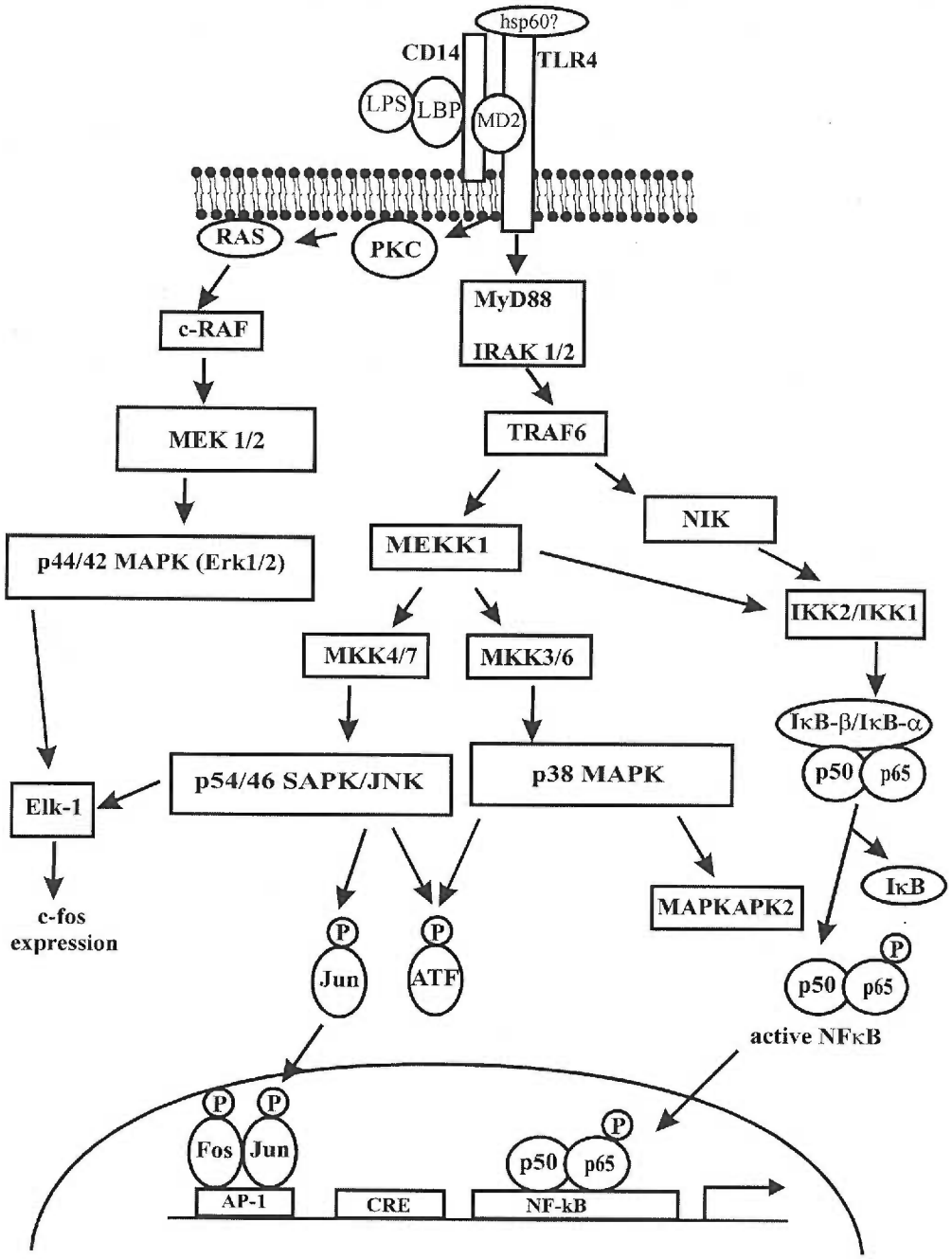


Fig 4. LPS-induced signaling pathways. Relationships are supported by data in the literature.

LPS-induced TNF-α and manuscript 3 the effects of EGb 761 on LPS-induced NO.

2) To evaluate whether EGb 761 inhibits serum levels of NO and TNF-α in C57Bl/6 mice

The literature reports *in vivo* protective effects of EGb 761 on reperfusion injury, age-related neuronal membrane fluidity, 4-O-methylpyridoxine-induced convulsions, free radical formation and lipid peroxidation (Szabo *et al.*, 1995; Tosaki *et al.*, 1996; Pietri, *et al.*, 1997a; Sasaki *et al.*, 1997; Marcilhac *et al.*, 1998; Stoll *et al.*, 1996). With dose and duration of treatment based on these *in vivo* studies, I hypothesized that EGb 761 would inhibit LPS-induced iNOS and TNF-α in C57Bl/6 mice. C57Bl/6 mice were chosen for these studies because of the availability of iNOS and TNF-α knockouts for this strain (Jackson Labs). Manuscripts 2 and 3 discuss the effects of intraperitoneal EGb 761 pretreatment on LPS-induced serum levels of TNF-α (measured by ELISA) and NO (measured as nitrite by the Griess reaction).

3) To establish whether EGb 761 and quercetin scavenge nitrite

Antioxidant mechanisms have been proposed to underlie the beneficial pharmacological effects of EGb 761 and moderate red wine consumption. As antioxidants, quercetin, resveratrol and EGb 761 exhibit protection against biological free radicals in oxidative injury models by inhibiting lipid peroxidation and/or oxidative damage to human low density lipoprotein (LDL) (Ratty and Das, 1988; Belguendouz *et al.*, 1997; Chanvitayapongs *et al.*, 1997; Maitra *et al.*, 1995). Manuscript 3 addresses whether suppression of NO release by polyphenolics can be partially attributed to NO scavenging by examining the effects of EGb 761 and quercetin on NO generated from the decomposition of sodium nitroprusside (SNP).

4) To determine whether inhibition of NO release by EGb 761 & quercetin is due

suppression of LPS-induced levels of iNOS protein

LPS-induced production of NO in macrophages is catalyzed by the enzyme iNOS. Manuscript 3 describes western blot analysis evaluating whether EGb 761, quercetin and specific MAPK inhibitors suppress levels of LPS-induced iNOS protein. This analysis does not address whether protein suppression is due to decreased protein stability or inhibition of protein synthesis.

5) To determine the effects of polyphenolics on LPS-induced transcription of iNOS and TNF-α

Suppression of LPS-induced iNOS and TNF-α protein could result from an inhibition of gene transcription. Manuscript 1 tests the effects of resveratrol, quercetin and the antioxidants NAC and PDTC on steady state levels of LPS-induced iNOS and TNF-α mRNA. Manuscript 2 describes the effects of quercetin, EGb 761 and specific MAPK inhibitors on steady state levels of LPS-induced TNF-α mRNA and the effect of quercetin on TNF-α mRNA half life. The effects of EGb 761, quercetin and specific MAPK inhibitors on the steady state levels of and the half life of iNOS mRNA is described in manuscript 3.

6) To determine effect EGb 761, quercetin, resveratrol, N-acetylcysteine and pyrollidine dithiocarbamate on LPS-induced NF-κB

As described in section B2a, many cell types respond to LPS and oxidative stress by up-regulating the transcription factor NF-κB, which plays a central role in TNF-α and iNOS transcription. Although the role of reactive oxygen intermediates in NF-κB regulation is controversial, NF-κB is inhibited by a variety of structurally diverse antioxidants, including polyphenolic phytochemicals. Manuscript 1 utilizes

electrophoreticmobility shift analysis (EMSA) to compare the effects of quercetin, resveratrol and the antioxidants NAC and PDTC on LPS and hydrogen peroxide-induced NF-κB/DNA binding. Manuscript 2 compares the effects of quercetin and EGb 761 on LPS-induced NF-κB/DNA binding and analyzes the NF-κB signaling cascade in more detail by utilizing western blot analysis to describe the effects of EGb 761 and quercetin on LPS-induced IκB degradation and nuclear localization of the p50 and p65 subunits of NF-κB. A commercially available inhibitor of I-κB kinase was not available for use as a positive control to illustrate the effects of inhibition of IκB phosphorylation on these processes. Instead MG132, which prevents I-κB degradation and subsequent NF-κB activation, was utilized to illustrate the effects of a proteasome inhibitor on NF-κB activation.

The effect of EGb 761 and quercetin on LPS-induced, NF-κB-mediated transcriptional activity is described in manuscript 2. RAW 264.7 macrophages were transiently transfected with the pNFκB-luc plasmid, containing the luciferase reporter gene driven by a basic promoter element plus five repeats of the NF-κB/Rel binding site. Treatments were analyzed for luciferase activity.

7) To determine the effect of EGb 761 and quercetin on the MAPK signaling cascade

As described in section B1, the MAPK pathway is important in the regulation of iNOS and TNF-α, and MAP kinases activate Fos and Jun, components of the transcription factor, AP-1. Manuscript 2 investigates the *in vitro* effects of EGb 761 and quercetin on LPS-induced stimulated activation of the MAPK cascade in RAW 264.7 macrophages. Western blot analysis was utilized to examine the LPS-induced phosphorylation of p38 MAPK, p44/42 MAPK, and JNK/SAPK and its substrates c-Jun

and ATF. p38 MAPK activity was examined by immunoprecipitation kinase assays. Specific inhibitors of p38 MAPK and p44/42 MAPK were utilized as positive controls.

8) To determine the effects of EGb 761 and quercetin on AP-1

The promoter region of murine iNOS and TNF-α genes contains motifs corresponding to elements involved in the binding the AP-1 transcription factor, which is activated in response to a variety of stimuli, including LPS. In manuscript 2, the effects of EGb 761 and quercetin on LPS-induced AP-1/DNA binding were examined by EMSA. The effects of these polyphenolics on LPS-induced AP-1-dependent transcriptional activity is illustrated in Appendix I. RAW 264.7 macrophages were transiently transfected with an AP-1 plasmid, containing the luciferase reporter gene driven by a basic promoter element plus seven repeats of the AP-1 binding site. Treatments were analyzed for luciferase activity.

9) To determine the effect of EGb 761 and quercetin on CRE

As described in section B2b, activated Jun proteins can form heterodimeric Jun-ATF-2 dimers which recognize the CRE binding sequence. The TNF-α promoter contains potential binding sites for CRE, which is thought to be critical for autoregulation. Manuscript 2 explores the effect of EGb 761 and quercetin on LPS-induced CRE-dependent transcriptional activity in RAW 264.7 macrophages transiently transfected with the pCRE-luc reporter plasmid, which contains the luciferase reporter gene driven by a basic promoter element plus four repeats of the CRE binding site. Forskolin, which activates CRE via activation of adenylate cyclase and CRE binding protein (CREB) was used as a positive control. A representative EMSA, illustrating the effects of LPS and quercetin on CRE/DNA binding, is shown in Appendix B.

10) To determine the effect of EGb 761 and quercetin on primary macrophages

The complex cellular interactions that occur in the upregulation of inflammatory cytokines during acute toxemia are difficult to assess in whole animal studies. Kupffer cells, the resident macrophages of the liver, are thought to be the major cells responsible for the clearance of circulating LPS and are also the source of cytokines, ROIs and NO (Decker, 1990; Milosevic *et al.*, 1999). Macrophages display phenotypic heterogeneity and regional specialization in different tissues. For example, the functional properties of macrophages present in the mouse peritoneal cavity (resident macrophages) differ markedly from blood monocyte-derived macrophages recruited to the peritoneal cavity by inflammatory stimuli (elicited macrophages) (Gordon, 1995). There is evidence to suggest that LPS-inducible cytokine genes and transcription factors are differentially regulated in macrophages derived from different tissues. PD98059 blocks LPS-induced activation of TNF-α gene expression in a murine cell line derived from alveolar macrophages but not in a non-pulmonary macrophage cell line (Means *et al.*, 2000b). Resident and elicited peritoneal macrophages from mice lacking the c-Rel gene exhibit differences in cytokine release and express different NF-κB complexes in response to LPS stimulation (Grigoriadis *et al.*, 1996). The utilization of an immortalized cell line to assess the mechanism by which EGb 761 inhibits LPS-induced cytokine release *in vivo* may therefore not always reflect what is occurring in other macrophage populations.

Results from my studies clearly establish the importance of the MAPK cascade, AP-1 and NF-κB in the induction of NO and TNF-α in LPS-induced RAW 264.7 macrophages. To begin to understand if similar pathways are involved in a primary cell population, preliminary studies were undertaken to determine the effects of EGb 761,

quercetin and MAPK inhibitors on TNF-α and NO release from thioglycollate-elicited peritoneal macrophages from C57BL/6 mice. Because LPS alone is a poor inducer of TNF-α in these primary macrophages, cells were stimulated with LPS plus murine IFN-γ. RTPCR was utilized to analyze iNOS and TNF-α RNA. Results from these studies are shown in Appendix A.

III. Results

A. Manuscript 1

Effects of the Wine Polyphenolics Quercetin and Resveratrol on Pro-Inflammatory Cytokine Expression in RAW 264.7 Macrophages

Teri L. Wadsworth and Dennis R. Koop

Department of Physiology and Pharmacology

Oregon Health Sciences University

Biochemical Pharmacology, Vol 57, pp 941-949, 1999

ABSTRACT

The beneficial effects of moderate red wine consumption have been attributed in part to the presence of antioxidant components. Oxidant stress is an activating stimulus for the NF (nuclear factor)-κB/Rel family of transcription factors, which have binding sites in the promoter region of many genes involved in inflammatory and immune responses. The effect of lipopolysaccharide (LPS)-stimulated activation of NF-κB and the subsequent production of tissue necrosis factor (TNF-α) and nitric oxide was determined in the macrophage cell line, RAW 264.7. Unexpectedly, the wine polyphenolics quercetin and resveratrol and the antioxidant N-acetyl cysteine (NAC) did not inhibit LPS-induced activation of the NF-κB complex, p50/65, as determined by mobility shift. Quercetin inhibited LPS-induced p50/50. Northern blot analysis indicated that quercetin (0.1 and 0.2 mM) inhibited LPS-dependent production of inducible nitric oxide synthase (iNOS) mRNA and decreased NO release, as measured by the Griess reaction. This flavonoid had no effect on LPS-induced TNF-α mRNA, but decreased LPS-stimulated TNF-α release, as measured by ELISA. Resveratrol (0.05 and 0.1 mM) postranscriptionally decreased LPS-induced nitrite release. It increased basal levels of TNF-α mRNA and protein, and enhanced LPS-induced TNF-α mRNA and cytokine release. Our results do not support the view that wine antioxidants inhibit LPS-induced NF-κB activation but instead have a more selective action on genes activated by LPS.

INTRODUCTION

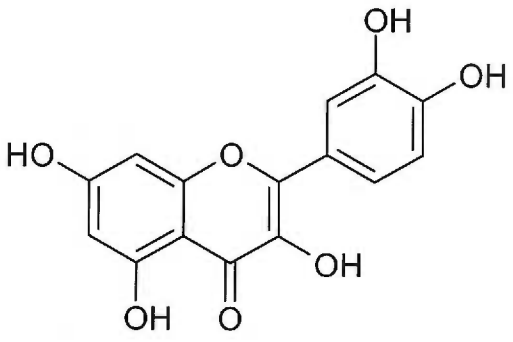
A growing body of literature indicates that polyphenolics are the active ingredients in dietary plants and traditional medicines used for treatment of disorders related to oxidative stress and inflammation (Joyeux *et al.*,1990; Kobuchi *et al.*, 1997; Dehmlow et al., 1996). Inflammation involves a complex web of intracellular and intercellular cytokine signals. Activated monocytes and/or macrophages release a variety of inflammatory mediators such as TNF-α, IL-1, IL-6, ROIs, and NO (Wang *et al.*, 1994). Curcumin, a polyphenolic derived from turmeric and used for centuries in Asia as an anti-inflammatory remedy, suppresses the activation of the transcription factor NF-κB thereby reducing the production of TNF-α and IL-1 in human macrophages (Chan, 1995). Green tea, attributed with numerous biological activities including antioxidant (Katihar *et al*, 1994) and anti carcinogenic properties (Yang and Wang, 1993; Valcic *et al.*, 1996), contains the flavonoid epigallocatechin gallate, which inhibits LPS-induced nitrite production in mouse peritoneal macrophages (Chan, *et al.*, 1995).

Phenolics present in red wine have been shown to exhibit cancer preventative properties (Jang *et al.*,1997; Agullo *et al.*, 1994; Kuo *et al.*, 1997), stimulate endothelial vasorelaxation (Fitzpatrick *et al.*, 1993), inhibit oxidation of human low density lipoproteins (Frankel *et al.*, 1993; Kondo *et al.*, 1994), platelet aggregation and the synthesis of pro-atherogenic eicosanoids (Pace-Asciak *et al.*, 1995). As antioxidants, these phytochemicals exhibit protective effects against biological free radicals in oxidative injury models by inhibiting lipid peroxidation (Inoue *et al.*, 1996; Joyeux *et al.*,1990; Ratty and Das, 1988; Belguendouz *et al.*, 1997; Chanvitayapongs *et al.*, 1997).

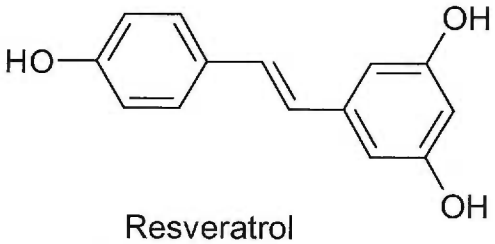
Epidemiological studies linking moderate wine consumption to a reduced risk of coronary heart disease (Gronbaek *et al.*, 1995; Rimm et al., 1996; Camargo et al.,1997; Burr, 1995) partially attribute reduced mortality rates to the antioxidant properties of wine phenolics, but the mechanism by which these phytochemicals exert such effects are not fully elucidated.

To obtain insight into the biological effects of quercetin and resveratrol (Fig 1) on the inflammatory response of macrophages, the influence of these compounds on the activity of the transcription factor NF-κB and the NF-κB responsive genes TNF-α and iNOS was determined. Although NF-κB is induced by a wide variety of agents, in RAW 264.7 macrophages, LPS is the most widely studied stimulant. Macrophage sensitivity to LPS is mediated by three major classes of transcription factors: the C/EBP, Fos-Jun and Rel homology families (Bethea *et al.*, 1997).

NF-κB, a member of the Rel family, is a common regulatory element in the promoter region of many pro-inflammatory cytokines. In activated macrophages, NF-κB in synergy with other transcriptional activators plays a central role in coordinating the expression of genes encoding TNF-α , IL-1, IL-6 and iNOS (Baeuerle and Henkel, 1994). The Rel family of transcription factors are dimeric proteins that reside in the cytoplasm bound to the inhibitory subunit, I-κB . In response to a variety of stimuli, including TNF-α , IL-1, oxygen radicals, viruses, LPS, or UV light, I-κB is phosphorylated and degraded by the cytosolic proteosome. Active Rel dimers translocate to the nucleus and activate genes containing κ-B regulatory elements (Thanos and Maniatis, 1995; Baeuerle and Baltimore, 1996). The diverse signal transduction pathways that converge on I-κB phosphorylation and the specificities of the multiple DNA binding and inhibitory



Quercetin



Resveratrol

Fig 1. Chemical structures of two wine polyphenolics, quercetin and resveratrol.

subunits of the Rel family are yet to be fully elucidated.

Since a variety of structurally diverse antioxidants inhibit NF-κB activation (Sen and Packer, 1996), we hypothesized that quercetin and resveratrol would inhibit the LPS-induced nuclear localization of this transcription factor in RAW 264.7 macrophages. Here we report the effects of quercetin, resveratrol and the antioxidanats NAC and PDTC on NF-κB activation and the expression of TNF-α and iNOS, which have NF-κB responsive elements in their promoters.

MATERIALS AND METHODS

Reagents

LPS, quercetin, resveratrol, penicillin/streptomycin, Denhardt's reagent, Griess reagent, deionized formamide, E-Toxate®, sodium nitroprusside and DMSO were from Sigma Chemical Company (St. Louis, MO). Antibodies to p50, p65 and c-Rel were from Santa Cruz Biotechnology (Santa Cruz, CA). NF-κB consensus oligonucleotide (5'-AGT TGA GGG GAC TTT CCC AGG C-3') and single base pair mutant (5'-AGT TGA GGC GAC TTT CCC AGG C-3') were from Promega (Madison, WI). Dulbecco's minimal essential medium (DMEM) was from Gibco BRL (Gaithersburg, MD). Fetal bovine serum was from Research Sera (Fort Collins, CO). Tissue culture plates were from Nalge Nunc International .^{32}P- ATP and Gene Screen Plus were from DuPont NEN (Boston, MA). Bradford protein dye reagent was from Bio-Rad (Hercules, CA). TNF-α ELISA kit was from Endogen (Boston, MA). High purity neutral red was from Molecular Probes (Eugene, OR). Riboquant® Multiprobe Rnase Protection Assay System was from PharMingen (San Diego, CA). Bioxytech® GSH-400 was from Oxis International, Inc (Portland, OR). TRI® Reagent was from Molecular Research Center (Cincinnati, OH).

The mouse macrophage-like cell line, RAW 264.7 and plasmid clone of mouse TNF-α were from ATCC (Rockville, MD). The cDNA for iNOS was a generous gift from Yoichi Osawa, University of Michigan.

Cell culture

Cells were cultured in phenol red free DMEM containing 50 units/ml penicillin, 50 μg/ml streptomycin, 44 mM sodium bicarbonate and 10% fetal bovine serum at 37°C in humidified air containing 5% CO_2. For preparation of RNA or nuclear extracts, cells were plated in 2.5 ml of media in 5.5 cm dishes and cultured for 2 days until cells reached 80% confluency (approximately 2 x 10^7 cells/dish) then treated as described in the text. When nitrite and TNF-α were determined, cells were plated in 500 μl of media in 24 well plates, cultured for 2 days (approximately 2 x 10^6 cells/well) then treated . Cells were washed and fresh complete media without phenol red was added 2 hour before the indicated stimuli. Stock solutions of quercetin and resveratrol were prepared in DMSO and added to the media. Doses of quercetin and resveratrol were similar to those used in studies reporting antioxidant and antitumor effects (Chanvitayapongs *et al.*, 1997; Jang *et al.*, 1997; Csokay *et al.*, 1997; Joyeux *et al.*, 1990). The final concentration of DMSO did not exceed 0.2%.

Northern blot analysis and ribonuclease protection assay

After 6 h of treatment, total RNA was isolated with TRI® Reagent as specified by the manufacturer. For Northern blot analysis, total RNA (20 μg) was electrophoresed in 1% agarose/15% formaldehyde gels, transferred overnight to GeneScreen Plus® membranes and cross-linked to the membrane by UV irradiation. For iNOS mRNA detection , the membrane was prehybridized 4 h at 37 °C in hybridization buffer (50%

deionized formaldehyde, 5x Denhardt's reagent, 5x sodium chloride/sodium citrate buffer (SSC), 0.1% SDS for and 100 μg/ml denatured salmon sperm DNA for iNOS; 50% deionized formaldehyde, 5x Denhardt's reagent, 5x SSPE; 0.5% SDS and 100 μg/ml denatured salmon sperm DNA. For TNF-α, the membrane was hybridized overnight in the same buffer containing 1-2 x 10^6 cpm/ml denatured cDNA probe. For iNOS, following hybridization, the filters were washed twice for 30 min in 2x SSC/0.1% SDS at room temperature, twice for 30 min in 0.5x SSC/0.1% SDS at 60 °C. The cDNA probe for iNOS was a 781 base pair BamHI fragment excised from full length murine iNOS cDNA. For TNF-α , the membrane was washed once with 2x sodium chloride/sodium phosphate/EDTA buffer (SSPE), 0.1% SDS for 10 min at room temperature; twice in 1x SSPE, 0.1%SDS for 15 min at 60 °C. A portion (10 μg) of total RNA was analyzed for the mRNA of TNF-α using the RiboQuant® Multi-Probe RNase Protection Assay System as described by the manufacturer. Signals for Northern blot analysis and RNase protection were detected with a GS-363 BioRad Molecular Imager with a BI imaging screen and signal intensity quantified with BioRad Molecular Analyst software.

Nitrite assay

After 24 h of treatment, 100 μl of medium from the 24 well plates was incubated with an equal volume of Griess reagent for 15 min at room temperature. The absorbance at 550 nm was measured using an Anthos htIII microplate reader. Nitrite concentration was determined using dilutions of sodium nitrite in phenol red free DMEM as a standard. Additions of quercetin and resveratrol to standard solutions of sodium nitrite confirmed that the wine components did not interfere with the nitrite assay.

TNF-α

Cells were plated and grown to confluency in 24-well tissue culture dishes. Following 24 h of treatment in 0.5 ml of medium, supernatants were collected and centrifuged for 30 sec at 3,500 x g. Conditioned medium (50 µl) was analyzed for TNF-α by ELISA as per manufacturer's instructions. For samples containing LPS, the supernatant was diluted 50% with culture medium prior to analysis.

Preparation of Nuclear Extracts

Nuclear extracts were prepared by a modified method of Dignam *et al.* (Dignam *et al.*, 1983). Treated cells were washed then scraped into 1.5 ml of ice cold Tris-buffered saline (pH 7.9) and pelleted at 12,000 x g for 30 sec. The pellet was resuspended in 10 mM HEPES, pH 7.9, with10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10 µg/ml leupeptin, aprotinin and peptstatin, incubated on ice for 15 min, then vortexed for 10 sec with 0.6% Nonidet P-40. Nuclei were separated from cytosol by centrifugation at 12,000 x g for 60 sec. The supernatant was removed and the pellet suspended in 50-100 µl of 20 mM HEPES, pH 7.9, with 25% glycerol, 0.4 M NaCl, 1 mM EDTA , 1 mM EGTA, 1 mM DTT , 0.5 mM PMSF, 10 µg/ml leupeptin, aprotinin and peptstatin. The samples were incubated with rocking at 4 °C for 15 min, then centrifuged for 5 minutes at 12,000 x g. Protein concentration of the supernatant was determined by the method of Bradford.

Electrophoretic Mobility Shift Assay (EMSA)

The activation of NFκB was assayed by gel mobility shift assays using nuclear extracts from control and treated cells. Mixtures containing 10 µg of nuclear protein extract were incubated for 15 min at 4 °C in 20 µl of total reaction volume containing 10

mM HEPES (pH 7.9), 50 mM KCl, 0.2 mM EDTA, 2.5 mM DTT, 10% glycerol and 1 μg of sonicated salmon sperm DNA. Radiolabeled NFκB concensus oligonucleotide (about 150,000 cpm of ^{32}P) was added, and the complete mixture incubated an additional 20 min at room temperature. Complexes were separated on a 7% native polyacrylamide gel containing 0.5x TBE with a 5 mM Tris/38 mM glycine running buffer. The gel was dried and complexes detected by autoradiography. The identity of the complexes was established with excess cold NF-κB oligonucleotide and antibody supershifts using rabbit polyclonal antibodies to p50 (nuclear localization signal), and goat polyclonal antibodies to p65 (C-20) and c-Rel (N-terminus).

Cell Viability Assay

Uptake of the dye neutral red was used as a measure of cell viability (Gardner and Johnson, 1996). Cells were plated in 24-well plates and samples (N=3) treated for various times in 0.5 ml of medium. Following treatment, the media was removed and replaced with 500 μl of media containing 50 μg/ml neutral red for 90 min at 37 °C. Following neutral red treatment, the media was removed and the wells washed 3 times with 500 μl of 37 °C phosphate-buffered saline (PBS). The neutral red was extracted with 500 μl of 50% ethanol, 50 mM sodium citrate, pH 4.2. Duplicate 200 μl samples from each well were transferred to a 96 well plate and the absorbance at 510 nm measured with an Anthos htIII microplate reader.

Endotoxin Assay

The E-Toxate® (Limulus Amebocyte Lysate) test was used, following the manufacturer's instructions, to test all solutions for the presence of gram-negative bacterial endotoxin.

Statistical Analysis

The student's unpaired *t*-test was used to assess the statistical significance of differences.

RESULTS

Effect of Antioxidants on NF-kB Binding Activities & Characterization of Binding Complexes

Induction of pro-inflammatory cytokines is modulated, in part, through the activation of NF-κB. As a result, we sought to determine what effects the antioxidants in wine had on basal NF-κB levels and whether they could prevent LPS-induced nuclear localization of this transcription factor. Following 1 h pretreatment with quercetin (0.1 or 0.2 mM) , resveratrol (0.05 or 0.1 mM), NAC (30 mM) or PDTC (0.1 mM), RAW 264.7 cells were treated with 100 ng/ml LPS or 0.5 mM hydrogen peroxide for 2 h. Neutral red uptake assays and the E-Toxate® test verified that cellular responses to the various treatments were not due to general cellular toxicity or bacterial endotoxin contamination (results not shown).

LPS-induced NF-κB binding activities exhibited time dependence and sequence motif specificity (Fig 2A). Distinct NF-κB binding complexes were detected and identified using antibodies to three members of the Rel homology family, p50, p65, and c-Rel, and excess cold NF-κB consensus oligonucleotide. Antibodies to p50 completely shifted all complexes, whereas antibodies to p65 shifted the larger complex (labeled C3). This suggests that C2 consists of the p50/50 homodimer, and the uppermost complex (labeled C3), consists of classic NF-κB (p50/p65). Antibodies to c-Rel shifted the slow

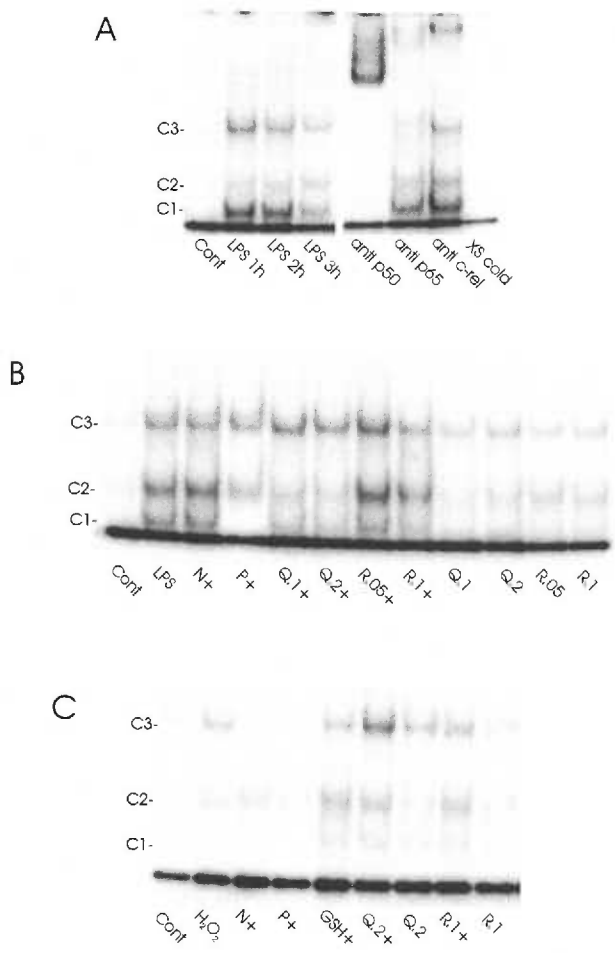


Figure 2. Effect of antioxidants and wine polyphenolics on NF-κB binding activities in EMSA. Binding activity was assayed using ^{32}P-labelled NF-κB consensus oligonucleotide in the presence or absence of indicated antibodies and assessed by EMSA. Dried gels were analyzed by autoradiography. The results shown are from a single experiment and are representative of what was observed in two or more additional experiments. A. Time course for LPS activation and characterization of binding complexes using anti-Rel family antibodies. RAW 264.7 cells were either untreated (Cont), or treated with LPS (100 ng/ml) for 1, 2 or 3 h prior to the preparation of nuclear extracts. Antibody analysis was performed on nuclear extracts obtained after 2 h LPS treatment. Anti c-rel: antibody to N-terminus; XS cold: 100-fold excess cold NF-κB oligonucleotide. B. Effect of antioxidants on LPS-induced NF-κB binding activities. Cells were either untreated (Cont), treated with LPS (100 ng/ml) for 2h, treated with quercetin (Q.1, 0.1 mM ; Q.2, 0.2 mM) or resveratrol (R.05, 0.05 mM; R.1, 0.1 mM) for 3 h, or pretreated 1 h with antioxidants then treated with LPS 2 h prior to the preparation of nuclear extracts (N+, 30 mM NAC; P+, 0.1 mM PDTC; Q.1+, 0.1 mM quercetin; Q.2+, 0.2 mM quercetin; R.05+, 0.05 mM resveratrol; R.1+, 0.1 mM resveratrol). C. Effect of antioxidants on hydrogen peroxide-induced NF-κB binding activities. Cells were either untreated (Cont), treated with H_2O_2 (0.5 mM) for 2 h, treated with resveratrol (R.1, 0.1 mM), quercetin (Q.2, 0.2 mM) for 3 h or pretreated for 1 h with antioxidants, then H_2O_2 (0.5 mM) 2 h prior to the preparation of nuclear extracts (N+, 30 mM NAC; P+, 0.1 mM PDTC; GSH+, 30 mM glutathione; Q.2+, 0.2 mM quercetin R.1+, 0.1 mM resveratrol).

migrating complex above C3, suggesting that this consists of p50 and c-Rel. Complex identification is in agreement with results previously reported for this cell line (Ohmori *et al.*, 1994; Brown and Taffet, 1995). C1 may be a complex that results from proteolysis of p50/50. This complex was observed in LPS-activated murine macrophages (Kuprash *et al.*, 1995). Our analysis focused on the well characterized Rel dimers p50/50 (C2) and p50/65 (C3).

Compared to untreated cells, all complexes were enhanced in cells stimulated with LPS for 2h (Fig 2B). Pretreatment with the antioxidant NAC (30 mM) did not protect against LPS-induced complex formation. PDTC pretreatment (0.1 mM) and quercetin (0.1 and 0.2 mM) decreased LPS-stimulated binding of p50/50 (C2). Resveratrol, like NAC, did not protect against LPS-induced nuclear localization of NF-κB complexes. Quercetin (0.2 mM) and resveratrol (0.05 and 0.1 mM) alone appeared to slightly enhance the signals associated with p50/50 (C2) and p50/65 (C3).

Hydrogen peroxide is known to activate NF-κB in a redox-sensitive manner (Baeuerle and Henkel, 1994; Sen and Packer, 1996). We therefore investigated the effect of antioxidants on H_2O_2-stimulated NF-κB activation (Fig 2C). Relatively high doses (0.5 mM) of hydrogen peroxide were required to observe NF-κB activation in this cell line. Unlike results with LPS, pretreatment with NAC decreased H_2O_2-stimulated binding of p50/65 (C3), while PDTC inhibited H_2O_2-induced binding of all complexes. Glutathione (30 mM), quercetin (0.2 mM), and resveratrol (0.1 mM) did not inhibit and may have enhanced hydrogen peroxide-induced formation of all complexes.

NF-κB analysis by mobility shift assay is difficult to quantify. We therefore

examined the regulation of the NF-κB inducible genes, iNOS and TNF-α, which may reflect the activation or inhibition of NF-κB complexes by antioxidants.

Effect of antioxidants on expression of iNOS and TNF-α mRNA

RAW 264.7 cells were pretreated with quercetin or resveratrol for 1 h, then LPS was added and the cells incubated for an additional 5 h before mRNA for TNF-α and iNOS was analyzed (Fig 3). The intensity of mRNA signals was normalized to 18S rRNA and compared to either LPS treatment (iNOS) or untreated cells (TNF-α). Northern blot analysis using a cDNA probe for iNOS revealed a 4.4 kb iNOS mRNA transcript in cells treated with LPS. The mRNA was not detectable in untreated cells or those treated with quercetin (0.1 and 0.2 mM) or resveratrol (0.05 and 0.1 mM) alone. Quercetin (0.2 mM) inhibited LPS-induced expression of iNOS mRNA. Resveratrol (0.1 mM), NAC (30 mM)and PDTC (0.1 mM) caused a small increase in the levels of LPS-induced iNOS mRNA.

Quercetin (0.1 and 0.2 mM) inhibited constitutive expression of TNF-α mRNA but had no effect on LPS-dependent increases in TNF-α mRNA. Resveratrol (0.05 and 0.1 mM) increased basal levels of TNF-α mRNA by 3.5 and 6.9 fold, respectively. There was a 2.9 fold enhancement of LPS-induced TNF-α mRNA by resveratrol (0.1 mM). NAC and PDTC enhanced LPS-induced TNF-α mRNA by about 6-fold. These findings were confirmed by RNase protection assays (results not shown).

Effects of antioxidants on cytokine expression and enzyme activity

Consistent with an enhanced expression of TNF-α mRNA in the presence of resveratrol, 0.05 and 0.1 mM doses of this phytoalexin increased basal levels of TNF-α

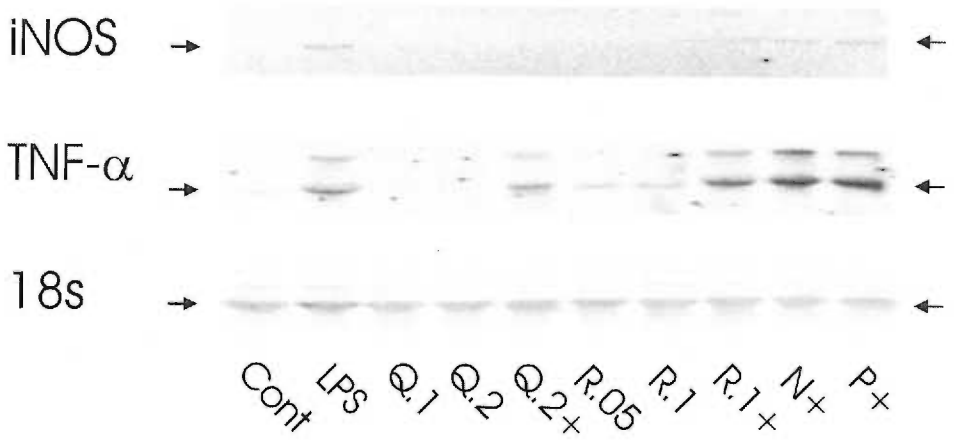


Figure 3. Northern blot analysis of iNOS and TNF-α mRNA expression in RAW 264.7 macrophages. Cells were cultured for 6 hours with media only (Cont), quercetin (Q.1, 0.1 mM; Q.2, 0.2 mM) or resveratrol (R.05, 0.05 mM; R.1, 0.1 mM) or 5 h with LPS (100 ng/ml), or pretreated with NAC (N+, 30 mM), PDTC (P+, 0.1mM), quercetin (Q.2+, 0.2 mM), or resveratrol (R.1+, 0.1 mM) for 1h, then LPS for 5 h prior to the isolation of total RNA. Following cell lysis and RNA purification, i-NOS and TNF-α mRNA and 18S rRNA were assessed in 20 μg of total cellular RNA by Northern blot analysis as described in Materials and Methods.

protein by 2 and 8-fold, respectively (Fig 4A). Resveratrol also caused a 1.6-fold enhancement of LPS-induced levels of TNF-α. Quercetin (0.2 mM), while having no effect on mRNA, caused an 85% decrease in LPS-stimulated release of TNF-α . A lower dose of quercetin (0.1 mM) caused a small but significant increase in LPS-stimulated TNF-α secretion. NAC (30 mM) had no effect on LPS-induced TNF-α release while PDTC (0.1 mM) enhanced LPS-induced TNF-α secretion by 3.6 fold.

Treatment of cells with resveratrol, quercetin, NAC, or PDTC had no effect on basal NO formation, as measured by the Griess reaction (Fig 4B). However, resveratrol (0.05 and 0.1 mM), which enhanced iNOS mRNA, decreased LPS-induced NO release 16% and 34%. Quercetin, which inhibited LPS-induced iNOS mRNA, decreased LPS-induced NO release in a dose dependent fashion. Nitrite release was inhibited 24% and 74% by 0.1 and 0.2 mM quercetin. NAC (30 mM) and PDTC (0.1 mM) inhibited the formation of nitrite 83% and 99%, respectively. PDTC is a known inhibitor of iNOS mRNA translation (Eberhardt *et al.*, 1994; Sherman *et al.*, 1993).

In order to confirm that inhibition of nitrite formation was not due to an effect on the ability of the Griess reaction to detect nitrite, the reaction was repeated with the artificial NO donor, SNP (Fig 5). Only PDTC (0.1 mM) inhibited NO formation from SNP while resveratrol (0.05 and 0.1 mM), quercetin (0.1 and 0.2 mM), and NAC (30 mM) all increased the amount detected. Enhancement of nitrite production from donor molecules such as SNP in the presence of NAC was previously reported (Foresti *et al.*, 1997).

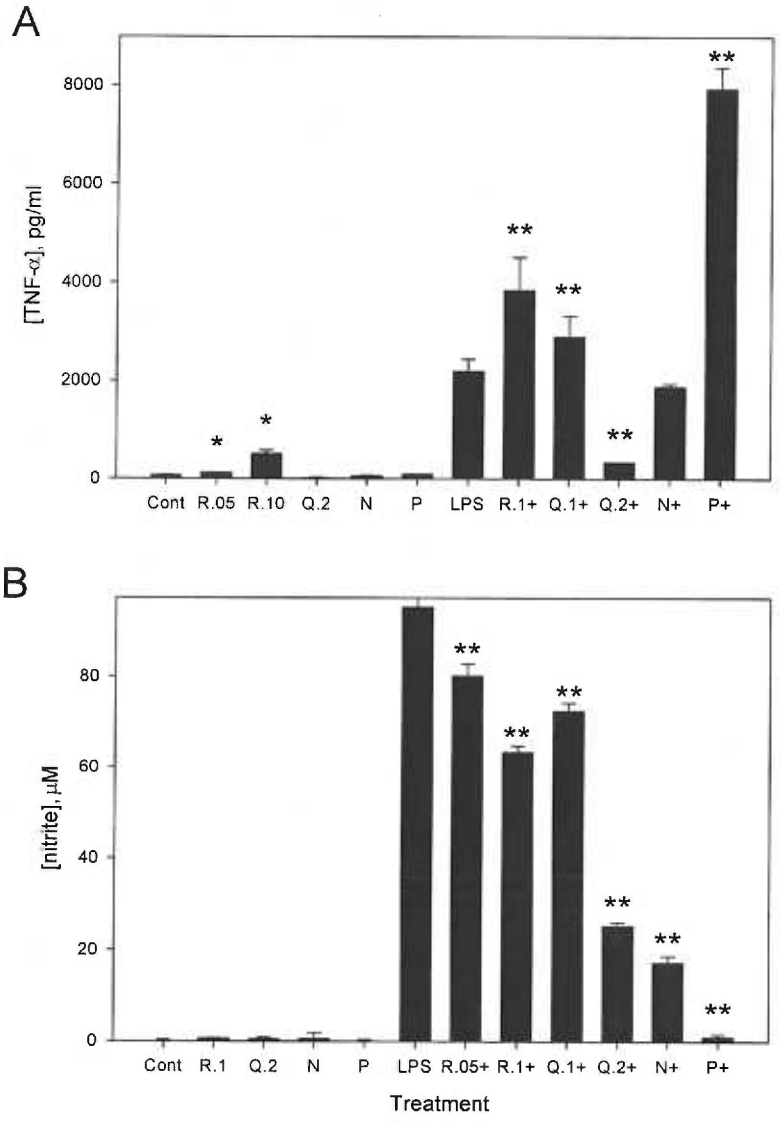


Fig 4. Effect of wine phenolics on TNF-α and NO production. RAW 264.7 macrophages were cultured in the presence of resveratrol (R.05, 0.05 mM; R.1, 0.1 mM), quercetin (Q.2; 0.2 mM), NAC (N, 30 mM), or PDTC (P, 0.1 mM), LPS (100 ng/ml) or antioxidants plus LPS (R.05+, 0.05 mM resveratrol; R.1+, 0.1 mM resveratrol; Q.1+, 0.1 mM quercetin; Q.2+, 0.2 mM quercetin; N+, 30 mM NAC; P+, 0.1 mM PDTC). After 24 hours, supernatants were collected and the concentrations of TNF-α and nitrite determined. Values are means ± standard deviation, N=3. A. TNF-α analysis by ELISA. Similar trends were observed in 4 separate experiments. (*) $P < .01$ compared to control; (**) $P < .05$ compared with LPS stimulation. B: NO analysis by the Griess reaction. Similar results were obtained in a second independent experiment. (**) $P < .01$ compared to LPS stimulation.

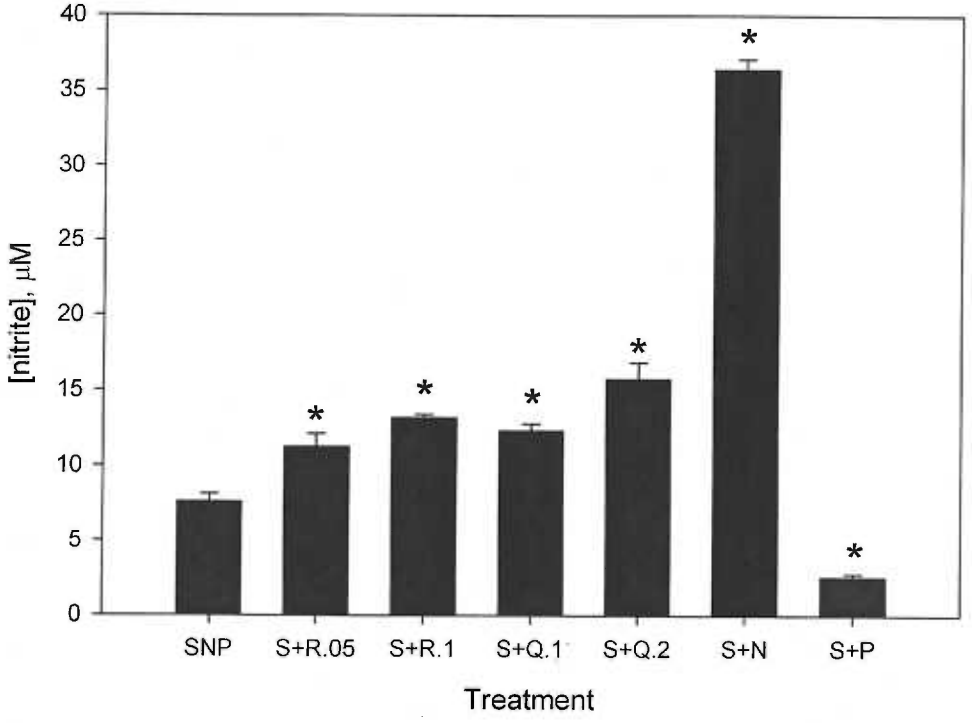


Fig 5. Effect of antioxidants and wine polyphenolics on nitrite production by SNP. Nitrite levels were measured by the Griess reaction in culture media incubated with SNP (1 mM), SNP + 0.05 mM resveratrol (S+R.05), SNP +0.1 mM resveratrol (S+R.1), SNP + 0.1 mM quercetin (S+Q.1), SNP + 0.2 mM quercetin (S+Q.2), SNP + 30 mM NAC (S+N), or SNP + 0.1 mM PDTC (S+P), for 5 h at 37 °C. All values represent the mean ± standard deviation of six independent experiments. (*) $P < .01$ compared to SNP treatment.

DISCUSSION

Effects on NF-κB activation

Activity of the transcription factor NF-κB is induced by a wide variety of agents, including ROIs, TNF-α , LPS, viral infection, UV irradiation, phorbol esters and nitric oxide. Though no common second messenger has been identified, most NF-κB activating signals can be inhibited by antioxidants (Baeuerle and Henkel, 1994; Sen and Packer, 1996). LPS-induced NF-κB activation in other cell lines appears to be mediated through its ability to stimulate the production of superoxide, hydrogen peroxide, and other ROIs (Schreck *et al.*, 1992). Our results suggest that LPS and H_2O_2 exhibit differential effects on Rel transcription factors in RAW 264.7 cells. Activation of p50/65 by LPS is resistant to PDTC, NAC , resveratrol and quercetin. Hydrogen peroxide-induced p50/65 activation, on the other hand, is inhibited by NAC and PDTC but not by quercetin or resveratrol. LPS-induced p50/50 activation is attenuated by PDTC and quercetin, with NAC and resveratrol having little effect. Only PDTC protects against H_2O_2-induced p50/50 activation. LPS therefore appears to induce p50/65 and p50/50 through unique mechanisms and via signaling pathways which differ from the activation triggered by hydrogen peroxide.

Quercetin, while enhancing constitutive levels of p50/65, inhibited LPS-induced p50/50. This inhibition may be attributed to quercetin's free radical scavenging activity. This seems unlikely, given that it did not diminish H_2O_2-induced p50/50 activation. The inhibition of NF-κB by PDTC does not necessarily implicate the involvement of ROIs. Although this metal chelator is often utilized to block NF-κB, its mechanism does not

appear to involve its antioxidant properties but rather an inhibition of NF-κB-DNA binding (Brennan and O'Neill, 1996). The inability of NAC to attenuate LPS-induced p50/50 or p50/65 activation suggests that glutathione depletion is not the mechanism by which LPS induces NF-κB. Similarly, glutathione and NAC had no effect on H_2O_2-induced p50/50 binding. It should be noted that NAC inhibits p50/50 and 50/65 binding in RAW 264.7 cells activated at 100-fold higher concentration of LPS than that used in our studies (Chen *et al.*, 1995).

Effect of Quercetin on iNOS and TNF-α

In the present study, quercetin inhibited iNOS mRNA, the release of NO, and production of TNF-α. These results are consistent with studies reporting that quercetin inhibits LPS-induced release of TNF-α from mouse peritoneal macrophages (Hu and Qian, 1993) and NO and TNF-α release from LPS-stimulated rat Kupffer cells (Kawada, *et al.*, 1998). Quercetin is a major component in *Ginkgo biloba* extract, which was shown to inhibit iNOS mRNA and NO production in LPS/IFN-γ activated macrophages while having no effect on NF-κB activation (Kobuchi *et al.*, 1997).

It seems likely that quercetin inhibits NO production by several mechanisms. Quercetin inhibits various tyrosine and serine/threonine kinases, including PKC and MAP kinase (Bird *et al.*, 1992; Nishioka *et al.*, 1989; Serriola *et al.*, 1989; Hagiwara *et al.*, 1988; Csokay *et al.*, 1997; Kawaka *et al.*, 1998). Tyrosine kinases are known to attenuate LPS-induced increases in nitrite in murine macrophages and TNF-α and NO in LPS-treated rats (Ruetten and Thiemermann, 1997). PKC is directly involved in the induction of NOS in rat hepatocytes (Hortelano *et al.*, 1992). Quercetin may also inhibit LPS-

induced iNOS mRNA expression through inhibition of transcription factors other than NF-κB. In addition to NF-κB, the promoter region for the iNOS gene is known to contain consensus sequences for binding IFN-γ and IL-6 regulatory factors (Martin *et al.*, 1994; Kamijo *et al.*, 1994; Xie *et al.*, 1993). The antioxidant PDTC, while inhibiting p50/50, did not suppress iNOS mRNA, ruling out inhibition of this heterodimer as a likely explanation. Suppression of NO release by quercetin may be attributed to direct free radical and NO scavenging activity, however it did not decrease levels of nitrite when added to solutions of the NO generator, SNP.

Transfection experiments with reporter constructs indicate that p50 exhibits negative effects on LPS-induced TNF-α transcription in murine macrophages (Kuprash, *et al.*, 1995). The resistance of LPS-induced TNF-α mRNA to quercetin may therefore be attributed to removal of the transcriptional inhibition imparted by p50/50. Our results are similar to findings by Kawada *et al.* (1998) who reported that quercetin inhibits NO and TNF-α production in LPS-stimulated rat Kupffer cells at a posttranscriptional level. Further studies are necessary to determine the exact mechanism by which quercetin inhibits TNF-α translation.

Effects of Resveratrol on iNOS and TNF-α

Resveratrol, though slightly enhancing iNOS mRNA, dose dependently decreased NO production in activated RAW 264.7 macrophages. It did not inhibit nitrite generated by SNP, abrogating the explanation that this phenolic lowers LPS-induced nitrite production by scavenging NO. Resveratrol also inhibits NO production in LPS-stimulated rat Kupffer cells at a posttranscriptional level (Kawaka *et al.*, 1998).

A dramatic and unique effect in our study was a 3.5 to 6.9 fold increase in basal TNF-α mRNA expression with a concomitant increase in TNF-α secretion from undetectable levels to 100 and 500 pg/ml with 0.05 and 0.1 mM resveratrol, respectively. Addition of resveratrol to LPS treated cells resulted in a 1.5- fold increase in TNF-α secretion. The biological effects of resveratrol were recently reviewed by Soleas *et al.* (Soleas *et al.*, 1997). By acting as a cyclooxygenase-1 inhibitor (Jang *et al.*, 1997), resveratrol may interfere with the negative feedback loop of prostaglandins, which downregulate TNF-α production at the mRNA level (Jongeneel, 1994). The induction of TNF-α by activated macrophages can lead to cytotostatic and cytotoxic activities on malignant cells (Camussi *et al.*, 1991). Resveratrol-mediated release of TNF-α may therefore contribute to the antitumorigenic properties of this wine component.

In summary, while quercetin and resveratrol can act as antioxidants in some assays, our results do not support the view that their mechanism of action is via inhibition of LPS-induced NF-κB activation in this cell line. Instead, these compounds have more selective actions on genes activated by LPS.

This work was supported by grants from the Oregon Wine Advisory Board and the National Institute on Alcohol Abuse and Alcoholism (AA08608).

B. Manuscript 2

Effects of *Ginkgo biloba* extract (EGb 761) and Quercetin on Lipopolysaccharide-induced Signaling Pathways Involved in the Release of Tumor Necrosis Factor Alpha

Teri L. Wadsworth, Tasha L. McDonald and Dennis R. Koop[1]

Department of Physiology and Pharmacology

Oregon Health Sciences University

Biochemical Pharmacology, in press

ABSTRACT

Administration of bacterial lipopolysaccharide (LPS) to laboratory animals and cultured macrophages induces the pro-inflammatory cytokine, tumor necrosis factor-α (TNF-α). Pre- treatment with *Ginkgo biloba* extract (EGb 761) inhibited the *in vivo* production of TNF-α (measured by ELISA) after challenge with LPS. In order to begin to understand the mechanism of this inhibition, we evaluated *in vitro* effects of EGb 761 and its flavonoid component, quercetin, on LPS-treated RAW 264.7 macrophages. Pre-treatment with EGb 761 or quercetin dose-dependently inhibited TNF-α release, as measured by the L929 fibroblast assay. Northern blotting demonstrated that quercetin inhibited LPS-induced TNF-α mRNA, but did not alter its half life. Activation of mitogen activated protein kinases (MAPKs) and the redox-sensitive transcription factors, nuclear factor-κB (NF-κB) and activator protein 1 (AP-1) are key events in the signal transduction pathways mediating TNF-α induction. Phosphorylation of the MAPK family members extracellular signal-related kinases 1 and 2 (ERK 1/2), p38 MAPK and Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK) was analyzed by western blotting. Our results suggest that quercetin was unique in its ability to inhibit TNF-α transcription by inhibiting phosphorylation and activation of JNK/SAPK and therefore suppressing AP-1-DNA binding (assessed by mobility shift analysis (EMSA)). Results from western analysis, EMSA and transient transfections suggest that EGb 761 diminished LPS-induced NF-κB but had no effect on LPS-induced TNF-α transcription. Both EGb 761 and quercetin inhibited ERK1/2 phosphorylation and p38 MAPK activity, which are important in post-transcriptional regulation of TNF-α mRNA.

INTRODUCTION

The standardized extract of Ginkgo, EGb 761, is the most commonly prescribed herbal remedy in France and Germany. It has been used in the treatment of peripheral vascular, cardiovascular and cerebrovascular disease and acute mountain sickness, all of which have components associated with oxidative stress (Roncin *et al.*, 1992). The antioxidant properties of EGb 761 have been proposed to underlie its beneficial effects. *In vitro*, it is a potent free radical scavenger and inhibitor of NADPH-oxidase, which significantly decreases superoxide radical, hydrogen peroxide and hydroxyl radical production in human neutrophils stimulated with phorbol ester (Maitra *et al.*, 1995; Gueydan *et al.*, 1999). EGb 761 is composed of 24% flavonoid glycosides; rutin, the glycoside of quercetin, accounts for 11.1% . In addition to flavonoids, EGb 761 consists of unique terpenes (3% bilobalide and 3% ginkgolides A, B and C). (Drieu, 1986). It has yet to be determined whether the effects of EGb 761 are caused by a single active ingredients or the combined action of the many components.

Exposure of laboratory animals or cells in culture to bacterial lipopolysachharide (LPS) triggers gene induction and generation of reactive oxygen intermediates (ROIs) by monocytes and macrophages. The inducible genes encode pro-inflammatory cytokines and enzymes such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, cyclooxygenase (COX-2) and inducible nitric oxide synthase (iNOS), which upregulate the host defense systems but unfortunately also contribute to pathological conditions such as bacterial sepsis, ischemia/reperfusion injury, chronic inflammatory disease and downregulation of hepatic drug metabolizing enzymes. Recent observations suggest that

the murine Toll-like receptor 4 (TLR4), in concert with CD14 and LBP (lipid binding protein), transduces the cellular responses to LPS in a molecular framework analogous to the IL-1 signaling cascade (Ulevitch, 1999). In turn, many signal transduction pathways are activated, including mitogen activated protein kinase (MAPK) pathways, and transcription factors such as nuclear factor-κB (NF-κB/Rel) and the activator protein 1 (AP-1) complex (Sweet and Hume, 1996).

The induction of TNF-α production upon stimulation by LPS results from both an enhancement of gene transcription and translational derepression (Han *et al.*, 1990a). The TNF-α promoter contains potential binding sites for several transcription factors, including NF-κB, AP-1, cyclic AMP response element (CRE) and CCAAT/enhancer binding protein β (C/EBPβ, also called NF-IL6) (Zagariya *et al.*, 1998). Transcriptional control appears to be mediated primarily by NF-κB, whereas the TNF-α CRE is critical for autoregulation (Brinkman *et al.*, 1999; Shakhov *et al.*, 1990a). Post-transcriptional regulation (mRNA instability and translational repression) is governed by an AU-rich element (ARE) in the 3'-untranslated region (-UTR) of TNF-α mRNA. Three well defined MAPK cascades, extracellular signal-related kinase (ERK), p38 MAPK and Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK) are activated upon stimulation with LPS. Several reports have described the importance of these pathways in LPS-induced TNF-α secretion in murine macrophages (Swantek *et al.*, 1997; Geppert, *et al.*, 1994).

Our recent studies addressed the hypothesis that the flavonoid quercetin would inhibit LPS-induced production of TNF-α and nitrite in the macrophage cell line, RAW

264.7 (Wadsworth and Koop, 1999). In addition to EGb 761, quercetin is present in wine and a wide variety of fruits, vegetables and herbs. *In vitro*, it is a potent antioxidant and non specific inhibitor of various tyrosine and serine/threonine kinases, including MAPKs (Kawada *et al.*, 1998). Quercetin inhibits purified recombinant IκB kinases, IKK1 and IKK2, by serving as a mixed type inhibitor versus ATP (Peet and Li, 1999). Unexpectedly, in our previous studies, quercetin inhibited LPS-induced TNF-α release at both transcriptional and posttranscriptional levels, but did not inhibit NF-κB-DNA binding (Wadsworth and Koop, 1999). Here we examine the mechanism by with EGb 761 and its aglycone component, quercetin inhibit TNF-α by examining the effects of these natural product antioxidants on LPS-induced stimulation of MAPK, NF-κB, AP-1 and CRE. Our results show that EGb 761 and quercetin have selective effects on TNF-α and the MAPK cascade.

MATERIALS AND METHODS

Reagents

Escherichia coli LPS, serotype 0127:B8, quercetin, penicillin/streptomycin, DMSO, Denhardt's reagent, sodium orthovanadate and E-toxate® reagent were from Sigma Chemical Co. (St. Louis, MO). Antibodies to p50, p65, c-Rel, c-Jun/AP-1, ATF-2 and IκB-α were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to phospho-MAPK (Erk1/2), phospho-p38 MAPK, and phospho-SAPK/JNK, and the p38 MAP kinase assay kit were from New England Biolabs (Beverly, MA). The antiphosphotyrosine antibody, 4G10 was a generous contribution from Dr. Brian Druker (Oregon Health Sciences University). The protease inhibitors aprotinin, leupeptin,

Pefabloc and E-64 and the selective MAPK inhibitors, SB203580 and UO126 were from Calbiochem (La Jolla, CA). Phenylmethyl-sulfonyl fluoride (PMSF) was from Boehringer Mannheim Corp. (Indianapolis, IN). NF-κB (5'-AGT TGA GGG GAC TTT CCC AGG C-3'), AP-1 (c-jun) (5'-CGC TTG ATG AGT CAG CCG GAA-3') and CRE (5'-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3') consensus oligonucleotides, Passive Lysis Buffer, and luciferin were from Promega (Madison, WI). Recombinant mouse TNF-α was from R&D Systems (Minneapolis, MN). Dulbecco's minimal essential medium (DMEM), RPMI , TRIzol® reagent and salmon sperm DNA were from Gibco BRL (Gaithersburg, MD). The pAP1-Luc, pNF-κB-Luc, pCRE-Luc and negative control pCIS-CK cis-reporter plasmids were from Stratagene (La Jolla, CA). SuperFect Transfection Reagent and EndoFree Plasmid Maxi Kit were from Qiagen (Valencia, CA). DNA Etox columns were from Sterogene (Carlsbad, CA). Fetal bovine serum (FBS) was from Research Sera (Fort Collins, CO). Tissue culture plates were from Fisher Scientific (Pittsburgh, PA). ^{32}P-ATP and GeneScreen Plus® membranes were from DuPont NEN (Boston, MA). Bradford and D_c protein assay reagents were from Bio-Rad (Hercules, CA). TNF-α ELISA kit and recombinant mouse IFN-γ were from Endogen (Boston, MA). PVDF membrane was from Millipore (Burlington, MA). Nitrocellulose membrane was from Schleicher & Schuell (Keene, NH). ECL™ Western blotting detection reagents were from Amersham Life Science (Arlington Heights, IL). SuperSignal® Ultra chemiluminescent substrate was from Pierce (Rockford, IL). The mouse macrophage RAW 264.7 and L-929 fibroblast cell lines were obtained from the American Type Culture Collection (Rockville, MD). C57BL/6 mice were from Jackson Laboratories

(Bar Harbor, ME). EGb 761 was a generous gift from Dr. Willmar Schwabe GmbH (Karlsruhe, Germany).

Animals

Male C57BL/6 mice, 8 weeks old, were allowed free access to food and water at all times and were allowed to acclimatize in the facilities for 7 days before use. Immediately prior to treatment, EGb 761 was dissolved in sterile saline, adjusted to pH 7.4 and administered intraperitoneally to the mice at a dose of 0, 5, 20, 50 or 100 mg/kg for 7 days. On day 7, LPS, (dissolved in sterile saline) was intraperitoneally injected at a dose of 1.3 mg/kg body weight. At 1 h after LPS injection, animals were sacrificed by CO_2 asphyxiation. All procedures were approved by the Institutional Animal Care and Use Committee of Oregon Health Sciences University.

Serum

Blood samples were collected by cardiac puncture. Blood was allowed to clot for 2 h at room temperature or overnight at 4°C before centrifuging for 20 minutes at 2000 x g. Serum was removed and stored at -70°C.

Cell culture

RAW 264.7 cells were cultured in phenol red free DMEM and L929 fibroblasts in RPMI containing 50 units/ml penicillin, 50 μg/ml streptomycin, 44 mM sodium bicarbonate and 10% fetal bovine serum at 37°C in humidified air containing 5% CO_2. For preparation of RNA, nuclear extracts, or cell lysates for western blot analysis, RAW 264.7 cells were plated in 2.5 ml of media in 5.5 cm dishes and cultured for 2 days until cells reached 80% confluency (approximately 2 x 10^7 cells/dish) then treated as described

in the text. For western blot analysis of p44/42 MAPK, cells were cultured for 2 days in 0.5% FBS to reduce basal levels of MAPK phosphorylation. For determination of TNF-α, RAW 264.7 cells were plated in 1 ml of media in 24 well plates, cultured for 2 days to 80% confluency (approximately 1 x 10^6 cells/well) then treated. For the p38 MAPK immunoprecipitation-kinase assay, cells were plated in 10 cm tissue culture dishes at a density of 6 x 10^6 cells/dish and treated 48 h later. In all cases, cells were washed and fresh complete media added before the indicated treatments. Stock solutions of EGb 761, quercetin and MAPK inhibitors were prepared in DMSO, the final concentration of DMSO did not exceed 0.5%. Crystal violet uptake assays confirmed that results were not due to general cellular toxicity and the E-toxate® test verified that culture media was not contaminated with bacterial endotoxin.

Measurement of TNF-α

Following 24 h of LPS treatment, supernatants from RAW 264.7 macrophages were collected and centrifuged for 30 sec at 3,500 x g. Mouse serum was collected 1 h following LPS treatment. Medium (50 µl) and serum (50 µl) were analyzed for TNF-α by ELISA (as described by the manufacturer) or with TNF-sensitive L929 fibroblasts as previously described (Gardner and Johnson, 1996). Cell viability was determined by the crystal violet cytotoxicity assay (Gillies *et al.*, 1986).

Preparation of Cytosolic and Nuclear Extracts

Nuclear extracts were prepared by a modified method of Dignam *et al.*(Dignam *et al.*, 1983). Treated cells were washed then scraped into 1.5 ml of ice cold Tris-buffered saline (pH 7.9) and pelleted at 12,000 x g for 30 sec. The pellet was suspended in 10 mM

HEPES, pH 7.9, with 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10 μg/ml leupeptin, aprotinin and pepstatin, incubated on ice for 15 min, then vortexed for 10 sec with 0.6% Nonidet P-40. Nuclei were separated from cytosol by centrifugation at 12,000 x g for 60 sec. The supernatant (cytosolic fraction) was removed and the pellet suspended in 20-50 μl of 20 mM HEPES, pH 7.9, with 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10 μg/ml leupeptin, aprotinin and pepstatin. The samples were incubated with rocking at 4°C for 15 min, then centrifuged for 5 min at 12,000 x g. Protein concentrations were determined by the Bradford method.

Electrophoretic Mobility Shift Assay (EMSA)

To assay DNA-NF-κB binding, mixtures containing 5 μg of nuclear protein extract were incubated for 15 min at 4°C in 20 μl of total reaction volume containing 10 mM HEPES (pH 7.9), 50 mM KCl, 0.2 mM EDTA, 2.5 mM DTT, 10% glycerol and 1 μg of sonicated salmon sperm DNA. Radiolabeled NF-κB consensus oligonucleotide (100,000-150,000 cpm of ^{32}P) was added, and the complete mixture incubated an additional 20 min at room temperature. Complexes were resolved on 7% native polyacrylamide gels containing 0.5x TBE (0.045 M Tris-borate/ 0.001 M EDTA) with 5 mM Tris/38 mM glycine running buffer. Gels were loaded while running at 50 V and run at 400 V until the bromophenol blue dye front reached the bottom. The gel was dried and complexes detected by autoradiography. The identity of the complexes was established with excess cold oligonucleotide and antibody supershifts using rabbit polyclonal antibodies to p50 (nuclear localization signal), and goat antibodies to p65 (C-20) and c-

Rel (N-terminus). For DNA binding to AP-1 and CRE, mixtures containing 5 μg of nuclear protein extract, 0.01 mg/ml poly(dI-dC)-poly(dI-dC), 5 mM DTT, 4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 50 mM NaCl and 10 mM Tris-HCl, pH 7.5, were incubated at room temperature for 10 minutes. Radiolabeled oligonucleotide (100,000-150,000 cpm) was added and the complete mixture incubated at room temperature for 20 min. The mixtures were loaded onto 6% nondenaturing acrylamide gels while running at 50 V in 0.5X TBE running buffer. The voltage was increased to 350 V and the gels run until the bromophenol blue dye front reached the bottom. Gels were dried and complexes detected by autoradiography. The identity of the AP1 and CRE complexes were established with excess cold AP1 or CRE nucleotide and antibody supershift using rabbit polyclonal antibodies to c-Jun/AP1and/or ATF-2. For all supershift analyses, 2 μg of antibody was added and the reaction mixtures incubated for 1 hour at 4°C before the addition of ^{32}P.

Western blot analyses

Phosphorylation of MAPK (Erk1/2), p38 MAPK and SAPK/JNK were analyzed with a commercially available phospho-MAPK antibody kit according to the manufacturer's instructions. Briefly, after indicated treatments, cells were lysed in SDS sample buffer, and proteins separated by 8% SDS-PAGE. Separated proteins were electrophoretically transferred to nitrocellulose membranes, blotted with specific MAPK antibodies and analyzed by enhanced chemiluminescence (ECL). To determine p65 and p50 levels, nuclear (20 μg protein) and cytoplasmic (40 μg protein) extracts were resolved on 8% SDS PAGE, electrophoretically transferred to nitrocellulose membranes,

probed with rabbit polyclonal antibodies against p50 or p65 (1:4000 dilution of TransCruz Gel Supershift reagents) and detected by ECL. For IκB analysis, treated cells were lysed in RIPA (150 mM NaCl, 1% w/v deoxycholate, 0.1% w/v SDS in 50 mM Tris, pH 8.0) containing 10 μg/ml PMSF, 10 μg/ml aprotinin, 100 μg/ml leupeptin, 100 μg/ml E-64 and 1 mM sodium orthovanadate. Proteins (20 μg) were resolved on 10% SDS-PAGE, transferred to PVDF membranes, probed with rabbit polyclonal antibodies to IκBα or IκBβ (1:1000 dilution) and detected by ECL. A time course to determine optimal LPS-induced phosphorylation was performed for each MAPK (data not shown). When indicated, signal intensity was quantified with Bio-Rad Molecular Analyst Software.

p38 MAPK Immunoprecipitation-Kinase Assay

The activity of p38 MAPK was analyzed with a commercially available kit according to the manufacturer's instructions. Briefly, RAW 264.7 macrophages were untreated (control) or treated with LPS for 15 min. Cells were harvested, lysed, and p38 MAPK immunoprecipitated with a monoclonal phospho-specific antibody to p38 MAPK. The immunoprecipitate was incubated with ATF-2 fusion protein (a substrate of p38 MAPK), ATP and either EGb 761, quercetin, SB203580 or DMSO. Samples were separated by 10% SDS-PAGE, transferred to nitrocellose, immunoblotted with anti phospho-ATF-2 and analyzed by ECL.

Northern Blot Analysis

After treatment, total RNA was isolated with TRIzol® Reagent as specified by the manufacturer. Total RNA (20 μg) was electrophoresed in 1% agarose/15% formaldehyde

gels, transferred overnight to GeneScreen Plus® membranes, and cross-linked to the membrane by UV irradiation. The membrane was prehybridized for 4 h at 37 °C in 50% deionized formaldehyde, 5X Denhardt's reagent, 5X saline-sodium phosphate-EDTA buffer (SSPE), 0.5% SDS and 100 μg/ml denatured salmon sperm DNA and hybridized overnight in the same buffer containing 1-2 x 10^6 cpm/ml of denatured ^{32}P-labeled cDNA probe. Following hybridization, the membrane was washed once with 2X SSPE, 0.1% SDS for 10 min at room temperature, and twice in 1X SSPE, 0.1% SDS at 15 min at 60^0C. Signals were detected with a GS-363 Bio-Rad Molecular Imager with a BI imaging screen, and signal intensity quantified with Bio-Rad Molecular Analyst software.

Transient Transfections

Endotoxin free plasmid DNA was prepared with the EndoFree Plasmid Maxi Kit (Qiagen) followed by purification with DNA Etox resin (Sterogene) according to the manufacturers' instructions. For each transfection, 1 x 10^6 RAW cells were distributed in wells of 6-well plates and incubated for 16-24 h. Transfections were performed according to manufacturer's guidelines using 5 μl of SuperFect reagent and 2 μg of plasmid DNA per well. Reagent/DNA was incubated with the cells for 2 h at 37^0C, then washed 3 times in 2 ml of serum free DMEM. Wells were replenished with 2 ml of complete medium and incubated at 37^0C for 24 h from the start of transfection. Transfected cells were stimulated for 4 h (for pNF-κB-luc and pAP-1-luc) and 4.5 h (for pCRE-luc) with 1 μg/ml LPS then solubilized in Passive Lysis Buffer (Promega) and assayed for luciferase activity. Stimulation with an LPS concentration of 1μg/ml was based on studies by Thompson *et al.* (1999) describing optimal methods for transient

transfection of RAW 264.7 macrophages. We found no changes in LPS-induced TNF-α levels at 100 ng/ml LPS compared to 1 μg/ml LPS in RAW 264.7 cells (data not shown). After normalization to protein concentration with the Biorad D_c protein assay reagent, data were expressed as fold activation.

Statistical Analysis

The statistical significance of the results were analyzed by the Student's t test for unpaired observations.

RESULTS

Effect of EGb 761 on LPS-induced TNF-α in vivo

Animal studies suggested that EGb 761 protects against free radical formation and lipid peroxidation *in vivo* (Tosaki *et al.,* 1996; Stoll *et al.,* 1996). With dose and duration of treatment based on these studies, we hypothesized that EGb 761 would inhibit the release of LPS-induced TNF-α in C57BL/6 mice. Consistent with our hypothesis, intraperitoneal injection of LPS caused increased serum levels of TNF-α and pre-treatment with EGb 761 protected against LPS-induced increases of this cytokine (Fig. 1).

Effects of EGb 761, quercetin, and MAPK inhibitors on LPS-induced TNF-α in vitro

In vitro studies with the murine macrophage cell line, RAW 264.7 were utilized to probe the mechanism by which EGb 761 and quercetin inhibit TNF-α. As shown in Fig 2, EGb 761 (400 μg/ml) and quercetin (200 μM) alone had no effect on culture supernatant levels of TNF-α as measured by the L929 fibroblast assay. LPS increased TNF-α levels. Pre-treatment with EGb 761 (400 μg/ml), quercetin (200 μM) , UO126

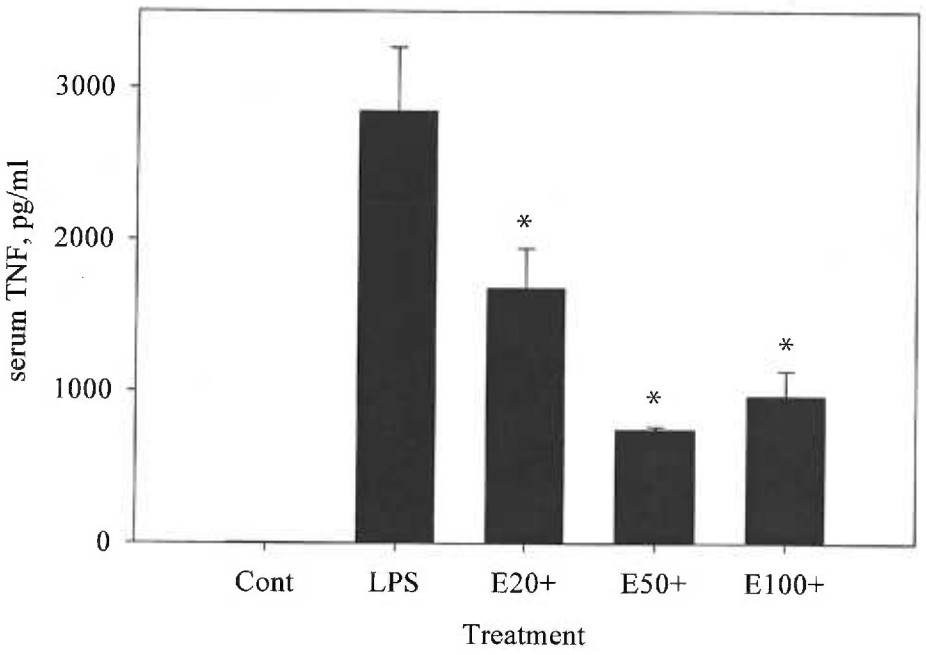


Fig 1. Effects of EGb 761 on LPS-induced TNF-α *in vivo*. C56BL/6 mice were pre-treated with EGb 761 or saline for 7 days. On day 7, immediately following EGb 761 treatment, mice were injected with LPS (1.3 mg/kg) or saline and sacrificed 1 h later for TNF-α analysis by ELISA. Cont, saline injections only (n=2); LPS, pre-treatment with saline, injection with LPS (n=5); E20+ (n=5), E50+ (n=4), E100+ (n=4), pre-treatment with 20, 50 or 100 mg/kg EGb 761 and LPS-injected. The data presented represents mean ± SE. *, significantly different from mice treated with LPS only, $p<0.05$.

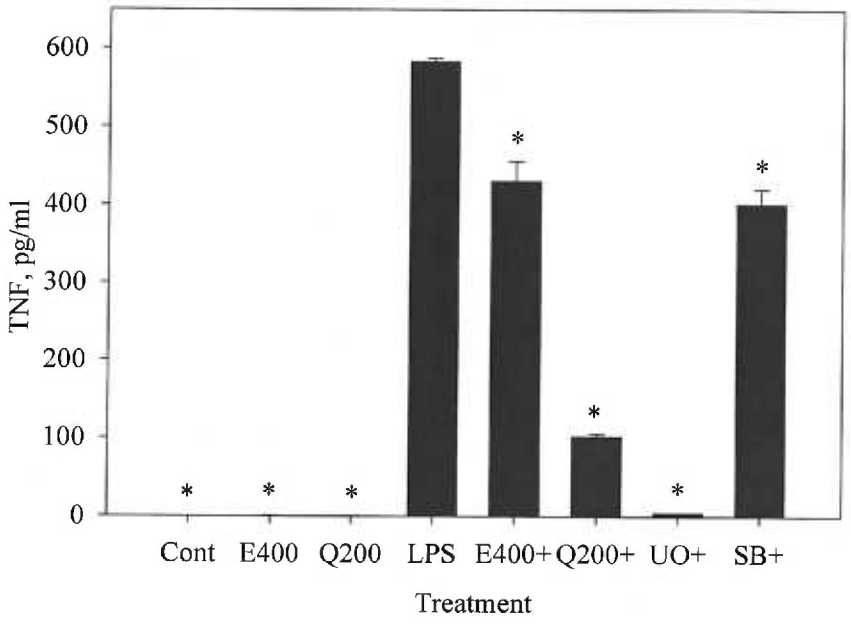


Fig 2. Effects of EGb 761, quercetin and MAPK inhibitors on LPS-induced TNF-α *in vitro*. RAW 264.7 macrophages were pre-treated with vehicle only (Cont), EGb 761 only (E400, 400 μg/ml),or quercetin only (Q200, 200 μM) or pre-treated with DMSO (LPS), EGb 761 (E400+, 400 μg/ml), quercetin (Q200+, 200 μM), UO126 (UO+, 20 μM) or SB203580 (SB+, 30 μM) for 1h, followed by treatment with LPS (100 ng/ml, 18h). Supernatants were collected and analyzed for TNF-α using the L929 fibroblast assay. The data represents mean ± SE, n=3. Similar results were observed in three independent experiments. *, significantly different from treatment with LPS only, $p < 0.05$.

(20 μM) or SB203580 (30 μM) inhibited LPS-induced TNF-α. Similar results were observed in independent experiments using ELISA to measure TNF-α in culture supernatants (data not shown). Initial dose response studies using either ELISA or the L929 fibroblast assay established that 400 μg/ml EGb 761 inhibited 30-50% of LPS-induced TNF-α (data not shown). This dose is approximately equivalent to 73 μM quercetin glycoside and 160 μM total flavonoid glycoside.

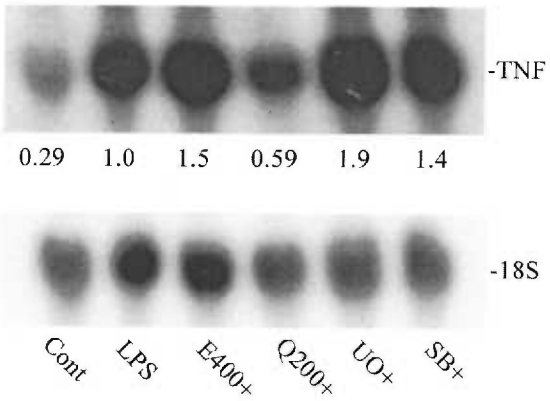
Effect of Querctin on LPS-induced TNF-α transcription

The suppression of LPS-induced TNF-α protein by EGb 761, quercetin and MAPK inhibitors could result from an inhibition of gene transcription. To test this possibility, TNF-α mRNA was assessed by northern blot analysis (Fig 3a). EGb 761, UO126 and SB203580 did not inhibit, while quercetin decreased LPS-induced up-regulation of TNF-α mRNA. Since quercetin had no effect on the half life of TNF-α mRNA (Fig 3b), inhibition of TNF-α secretion by quercetin most likely occurs at the transcriptional level.

Effects of EGb 761 and quercetin on LPS-induced IκB degredation and of EGb 761 on NF-κB-DNA binding

Because NF-κB is important in mediating transcriptional control of TNF-α, we analyzed the effects of EGb 761 and quercetin on the LPS-induced NF-κB signaling cascade. In unstimulated cells, NF-κB is retained in the cytoplasm by binding to a member of the inhibitory protein family, I-κB. Activation by LPS requires sequential phosphorylation of I-κB, ubiquitination and degradation by the proteasome, followed by translocation of NF-κB to the nucleus. Western blot analysis indicates that while the

a.



b.

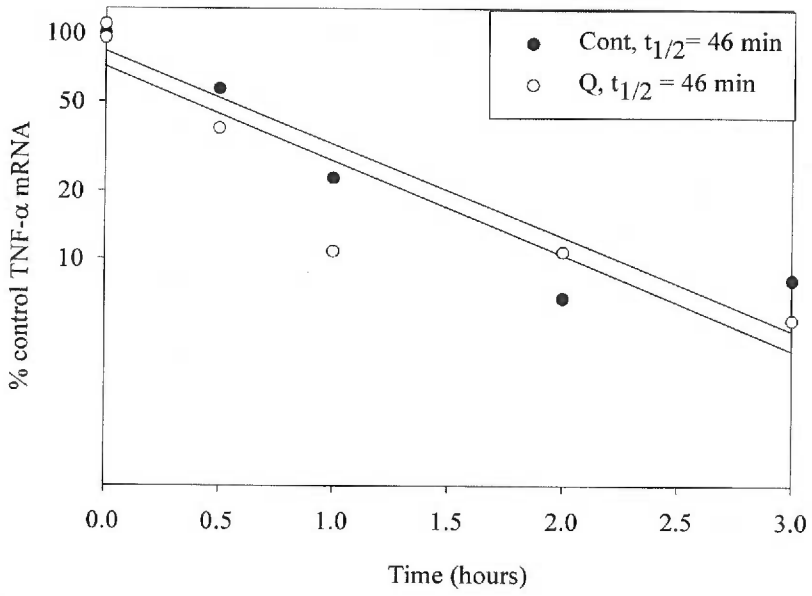


Fig 3. Effects of Quercetin on LPS-induced TNF-α transcription. a. RAW 264.7 cells were treated with vehicle only (Cont) or pre-treated for 1 h with DMSO (LPS), EGb 761 (E400+, 400 μg/ml), quercetin (Q200+, 200 μM) UO 126 (UO+, 20 μM) or SB203580 (SB+, 30 μM) prior to treatment with LPS (100 ng/ml, 6 h). Total cellular RNA was isolated and TNF-α and 18S RNA assessed by northern analysis. The relative intensity of the signal after normalization to 18S RNA is shown below each lane. b. RAW 264.7 macrophages were stimulated with LPS (100 ng/ml) for 6 h, then treated with quercetin (200 μM) and actinomycin D (2.5 μg/ml) (Q), or actinomycin alone (Cont). Total cellular RNA was isolated at the indicated times, assessed for TNF-α and 18S RNA by northern blot analysis and the intensity of the TNF-α signal normalized to the18S signal. Results include data from two independent experiments. Linear regression analysis, r^2: Cont = 0.884, Q = 0.843.

proteasome inhibitor MG132 protected IκB-α and IκB-β from LPS-induced degradation, neither quercetin or EGb 761 prevented this phenomenon (Fig 4a).

Mobility shift analysis was utilized to determine the effects of EGb 761 on LPS-induced NF-κB-DNA binding. Our previous studies (Wadsworth and Koop, 1999) demonstrated that quercetin (0.1 and 0.2 mM) decreased LPS-stimulated binding of p50/50, but had no effect on p50/65. These studies established that maximal LPS-induced NF-κB-DNA binding occured at 1-2 h. Fig 4b illustrates that pre-treatment with EGb 761 diminished binding of LPS-induced p50/50 and p50/65. The NF-κB-DNA binding complexes, p50/65 and p50/50 were identified with supershift analysis, using antibodies to Rel homology family members, p50 and p65 and correspond to complexes identified in our previous studies (Wadsworth and Koop, 1999).

Effects of EGb 761 and quercetin on LPS-induced nuclear localization of the p50 and p65 subunits of NF-κB

EMSA revealed a decrease in p50/50 and p50/65-DNA binding by EGb 761 but no substantial differences in the levels of LPS-induced p50/65-DNA binding with quercetin treatment. Cytoplasmic and nuclear pools of the p50 (Figs 5a & 5b) and p65 (Figs 5c &5d) subunits were therefore examined by western blot analysis to determine if nuclear translocation of these subunits was altered by EGb 761 or quercetin. Treatment of cells with LPS induced the reduction of p50 and p65 from the cytoplasm (Figs 5a & 5c) and their appearance in the nucleus (Figs 5b & 5d). Pretreatment of cells with EGb 761 diminished LPS-induced nuclear levels of p50 by 25% when signal intensities were quantified as described in Materials and Methods. Quercetin had no effect on nuclear

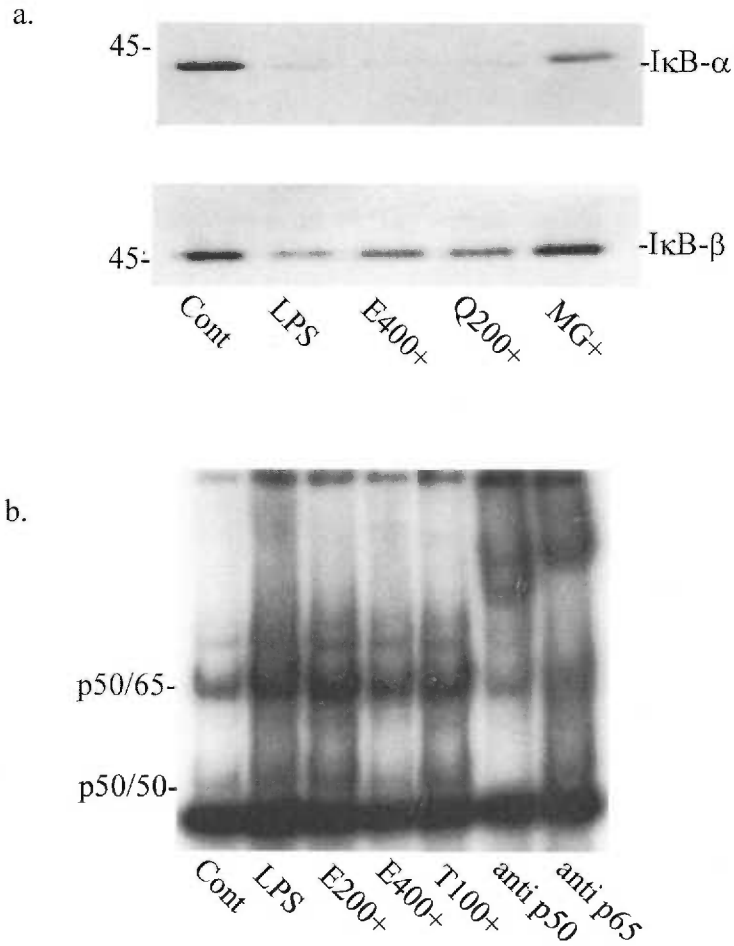


Fig 4. Effects of EGb 761 and quercetin on LPS-induced IκB degradation and of EGb 761 on NF-κB-DNA binding. a. RAW 264.7 cells were pretreated with DMSO only (Cont) or pretreated for one hour with DMSO (LPS), EGb 761 (E400+, 400 μg/ml), quercetin (Q200+, 200 μM) or MG132 (MG+, 25 μM) prior to treatment with LPS (100 ng/ml, 20 min). Cell lysates were immunoblotted with specific antibodies to IκB-α or IκB-β and analyzed by western blot. b. Mixtures containing 5 μg of nuclear protein were assayed for NF-κB-DNA binding by EMSA using ^{32}P-labeled NF-κB consensus oligonucleotides. Cells were treated with DMSO only (Cont), pre-treated for one hour with DMSO (LPS) ,EGb 761 (E200+, 200 μg/ml; E400+, 400 μg/ml) or the non-specific tyrosine kinase inhibitor tyrophostin (T100+, 100 μM) then stimulated with LPS (100 ng/ml for 60 min). Supershift analysis was performed on nuclear extract from cells treated with LPS that were preincubated for 30 min with the indicated antibodies against members of the NF-κB/Rel family. Complex identity is in agreement with results from Manuscript 1.

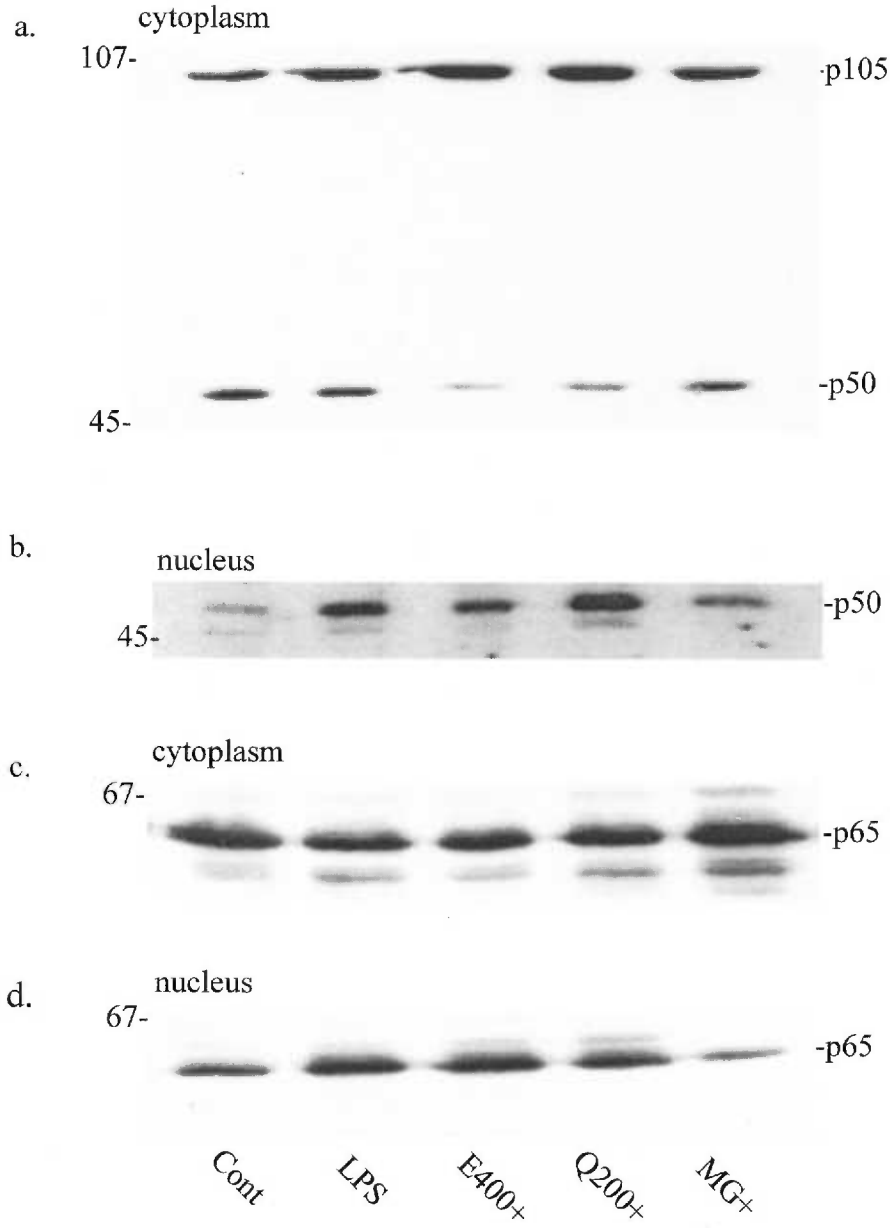


Fig 5. Effect of EGb 761 on LPS-induced nuclear localization of the p50 subunit of NF-κB. Cell extracts from RAW 264.7 cells were treated with DMSO only (Cont) or pretreated for one hour with DMSO (LPS), EGb 761 (E400+, 400 μg/ml), quercetin (Q200+, 200 μM) or MG132 (MG+, 25 μM) prior to treatment with LPS (100 ng/ml, 15 min). p50 (a and b) and p65 (c and d) were analyzed by western blot. a and c represent cytoplasmic extracts, b and d nuclear extracts. Two independent experiments gave similar results.

translocation of this subunit. EGb 761 and quercetin had no effect on cytoplasmic or nuclear levels of p65. The proteasome inhibitor, MG132 blocked nuclear translocation of both p50 and p65 subunits.

Effects of EGb 761 and quercetin on NF-κB-dependent transcriptional activity

Because quercetin and EGb 761 diminished LPS-induced NF-κB-DNA-binding, we examined the effects of these compounds on NF-κB-dependent transcriptional activation. RAW 264.7 macrophages were transiently transfected with the pNFκB-luc plasmid, containing the luciferase reporter gene driven by a basic promoter element (TATA box) plus five repeats of the NF-κB/Rel binding site. LPS treatment caused a 4.5-fold increase in luciferase activity compared to control (Fig 6). EGb 761 diminished LPS-induced luciferase activity by approximately 25%, while quercetin had no effect. Cells transfected with the negative control pCIS-CK plasmid (-Cont+, which does not contain *cis*-acting elements) did not respond to LPS. Treatment with EGb 761 or quercetin in the absence of LPS had only a small effect on cells transfected with the pNFκB-luc plasmid.

Selective effects of EGb 761 and quercetin on p44/42 MAPK and SAPK/JNK

The MAPK pathway is important in the post-transcriptional regulation of TNF-α. Transcription factors that bind to AP-1 and CRE are activated by MAPKs. We therefore investigated the effects of EGb 761 and quercetin on LPS-stimulated activation of the MAPK cascade. As shown in Fig 7a, LPS induced phosphorylation of p44/42 MAPK. EGb 761 and quercetin partially inhibited and the specific MEK 1/2 inhibitor, UO126, completely inhibited LPS-induced phosphorylation. In contrast, the specific inhibitor of

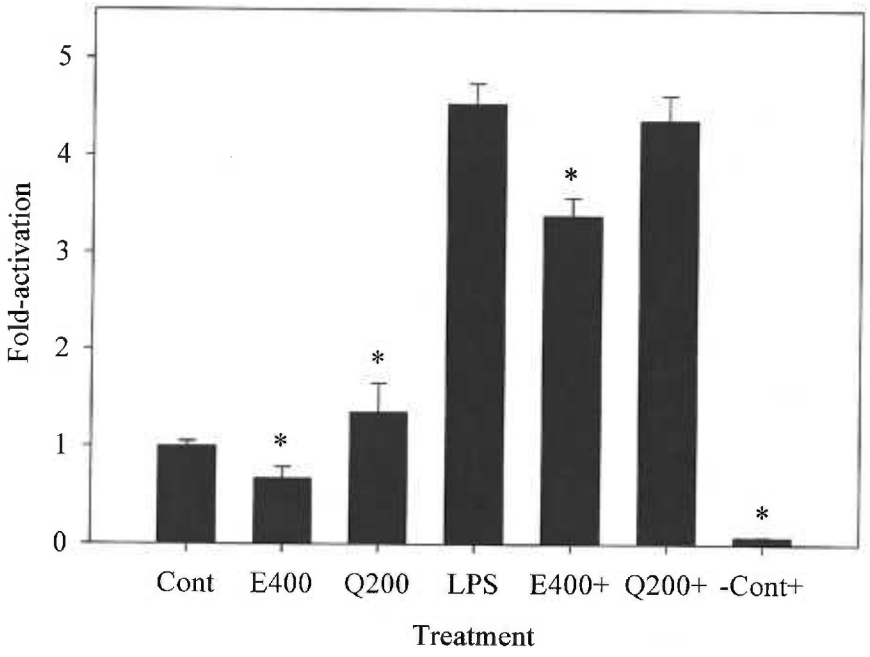


Fig 6. Effects of quercetin and EGb 761 on NF-κB-dependent transcriptional activity. Twenty four hours after transient transfection with the pNFκB-luc reporter plasmid, RAW 264.7 cells were treated with vehicle only (Cont), EGb 761 only (E400, 400 μg/ml) or quercetin only (Q200, 200 μM) or with vehicle (LPS), EGb 761 (E400+, 400 μg/ml) or quercetin (Q200+, 200 μM) for one hour followed by a four hour treatment with LPS (1000 ng/ml). -Cont+ represents cells transiently transfected with the negative control plasmid, pCIS-CK and treated with vehicle for one hour followed by treatment with LPS (1000 ng/ml for 4 h). Cells were harvested for luciferase activity and protein determination. Data, representative of two separate experiments, n = 3 for each experiment, is expressed relative to control luciferase activity, normalized to protein, and plotted as mean ± S.E, n=6. *, significantly different than control treatment, $p < 0.05$.

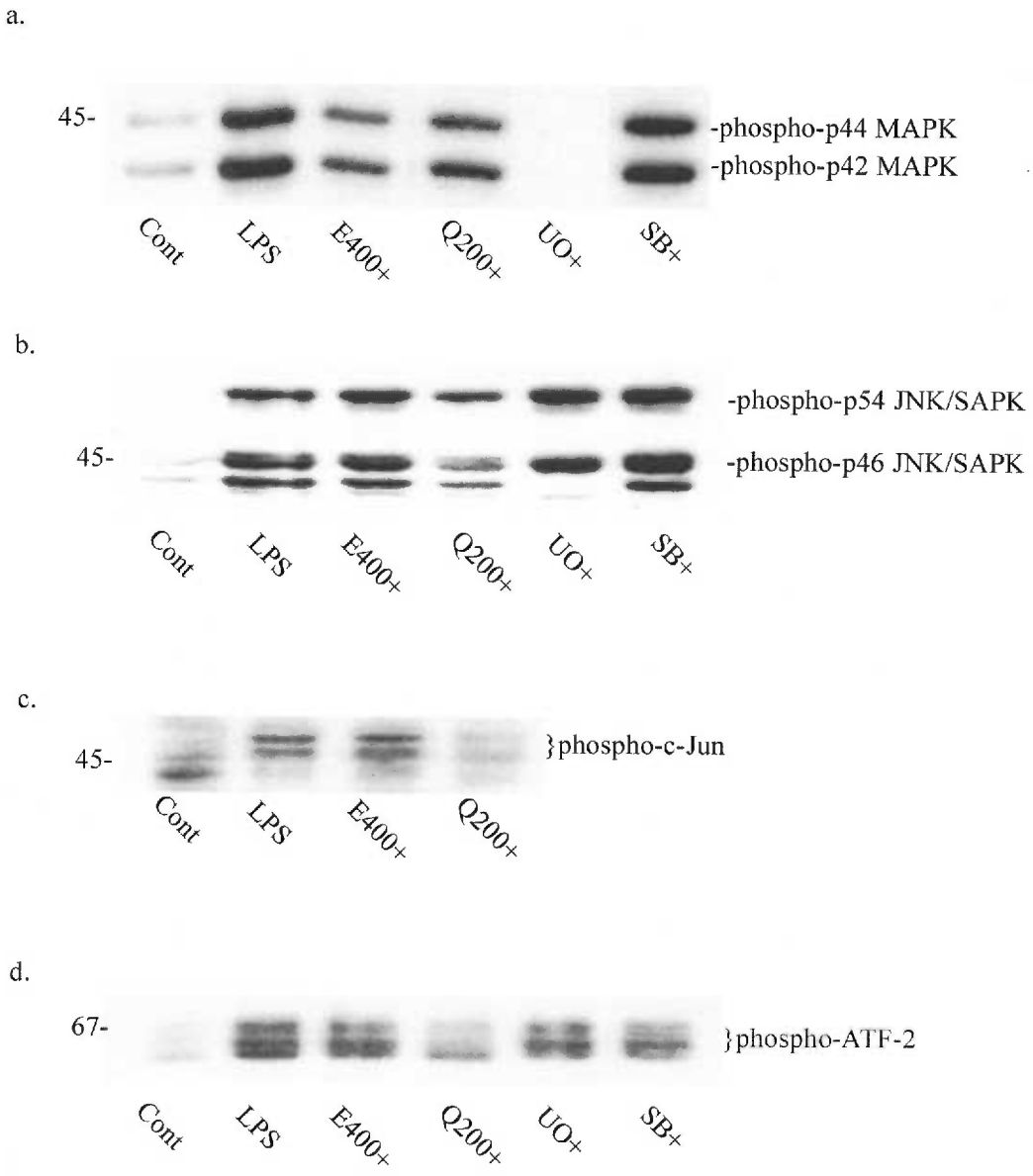


Fig 7. Effects of EGb 761 and quercetin on LPS-induced phosphorylation of p44/42 MAPK (ERK 1/2), SAPK/JNK, c-Jun and ATF-2. RAW 264.7 cells were unstimulated (Cont) or stimulated with LPS (100 ng/ml) for 15 min. Prior to LPS treatment, cells were pre-treated for 1 h with EGb 761 (E400+, 400 μg/ml), quercetin (Q200+, 200 μM), UO126 (UO+, 20 μM) or SB203580 (SB+, 30 μM) Cell lysates were immunoblotted with a specific antibody to a. phosho-p44/42 MAPK, b. phospho- SAPK/JNK, c. phospho-c-Jun or d. phospho-ATF-2. In each case, at least two independently performed western analyses gave similar results.

p38 MAPK, SB203580, was without effect. EGb 761 had no effect on and quercetin inhibited LPS-induced phosphorylation of SAPK/JNK (Fig 7b) and its substrates, c-Jun (Fig 7c) and ATF (Fig 7d). UO126 did not effect and SB 203580 showed slight inhibition of LPS-induced ATF-2 phosphorylation (Fig 7d). Since ATF-2 is a substrate for both p38 MAPK and SAPK/JNK, the *in situ* inhibition of phosphorylation of this protein by quercetin but not EGb 761 (Fig 7c) stresses the importance of SAPK/JNK in the activation of this transcription factor. The MEK/ERK inhibitor, PD98059, at a concentration of 50 μM, caused inhibition of LPS-induced phosphorylation of JNK/SAPK and c-Jun (data not shown). Compared to control, treatment with EGb 761 or quercetin in the absence of LPS had no effect on ERK1/2, SAPK/JNK or p38 MAPK (data not shown). Preliminary time course studies using the general antiphosphotyrosine antibody 4G10 established that maximal MAPK phosphorylation occurs15-30 min following LPS stimulation (data not shown).

Inhibitory effects of EGb 761 and quercetin on p38 MAPK activity

Immunoblot analysis of p38 MAPK revealed that only SB203580 diminished LPS-induced phosphorylation of p38 MAPK (Fig 8a). p38 MAPK activity, analyzed by an *in vitro* kinase assay after specific immunoprecipitation with anti-p38 MAPK antibody demonstrated that LPS induced p38 MAPK activity as shown by the increased phosphorylation of the p38 MAPK substrate, ATF-2 (Fig 8b). EGb 761 and quercetin dose dependently inhibited ATF-2 phosphorylation. The p38 MAPK inhibitor, SB203580, at a dose of 30 μM, inhibited *in vitro* p38 MAPK activity. Similar doses of SB203580 have been used to inhibit p38 MAPK activity from RAW 264.7 macrophages

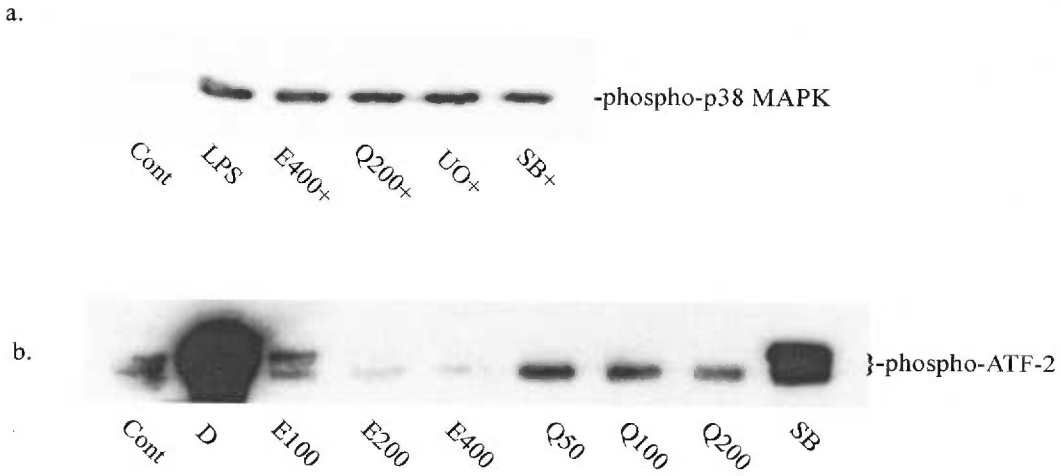


Fig 8. Effects of EGb 761, quercetin and SB 203580 on p38 MAPK activity. a. RAW 264.7 cells were unstimulated (Cont) or stimulated with LPS (100 ng/ml) for 15 min. Prior to LPS treatment, cells were pre-treated for 1 h with EGb 761 (E400+, 400 μg/ml), quercetin (Q200+, 200 μM), UO126 (UO+, 20 μM) or SB203580 (SB+, 30 μM). Cell lysates were immunoblotted with a specific antibody to phosho-p38 MAPK. Three identical experiments independently performed gave similar results. b. *in vitro* p38 MAPK activity was analyzed by an immunoprecipitation kinase assay. The kinase assay solution contained immunoprecipitated p38 MAPK from untreated cells (Cont) or LPS treated cells and DMSO (D), EGb 761 (E100, 100 μg/ml; E200, 200 μg/ml; E400, 400 μg/ml), quercetin (Q50, 50 μM; Q100, 100 μM; Q200, 200 μM) or SB203580 (SB, 30 μM). A second, independent experiment gave similar results.

(Paul *et al.*, 1999).

Inhibitory effects of EGb 761 and quercetin on LPS-induced AP-1-DNA binding

Because activation of JNK/SAPK and or Erk1/2 results in AP-1 activation, we examined the effects of EGb 761 and quercetin on LPS-induced AP-1-DNA binding by EMSA (Fig 9). Consistent with our results from western analysis, which demonstrated that quercetin inhibited activation of JNK/SAPK and both EGb 761 and quercetin diminished p44/42 MAPK phosphorylation, quercetin dramatically decreased and EGb 761 diminished binding of nuclear extract proteins to the AP-1 complex. The identity of the AP-1 complex was confirmed using antibodies to c-Jun/AP-1, which did not result in a supershift, but did decrease AP-1-DNA binding. Excess unlabeled AP-1 oligonucleotide inhibited AP-1-DNA binding.

Effects of LPS on ATF/CRE binding and transcriptional activity in RAW macrophages

The TNF-α promoter contains the CRE sequence, which binds Jun-ATF-2 heterodimers. Since quercetin inhibits LPS-induced c-Jun and ATF phosphorylation, we examined whether transcriptional inhibition of TNF-α by quercetin may be due to inhibition of binding to the CRE. Transient transfection of RAW 264.7 macrophages with the pCRE-luc reporter plasmid (Fig 10) revealed that LPS did not stimulate transcriptional activity. In contrast, forskolin treatment, which activates CRE via activation of adenylate cyclase and CRE binding protein (CREB), resulted in a 9-fold enhancement of luciferase activity. EMSA did not reveal a dramatic difference in nucleoprotein binding between control and LPS-treated macrophages and quercetin had no effect on c-Jun/ATF heterodimer levels (data not shown).

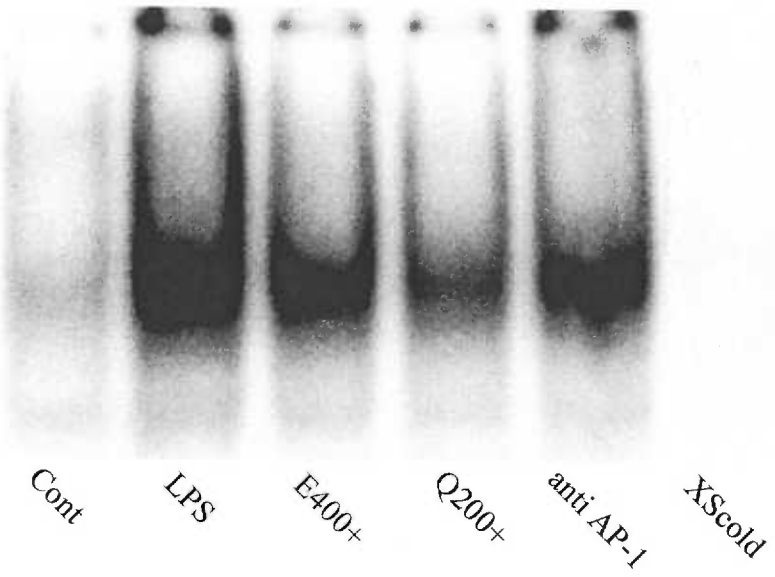


Figure 9. Effects of EGb 761 and quercetin on LPS-induced AP-1 binding. Nuclear extracts were prepared and assayed for AP-1 induction by EMSA as described in materials and methods. RAW 264.7 cells were treated with DMSO (Cont), or pre-treated for 1 h with DMSO (LPS), EGb 761 (E200+, 200 μg/ml), or quercetin (Q200+, 200 μM) prior to stimulation with LPS (100 ng/ml for 60 min). Antibody and competition analysis was performed on nuclear extracts from cells treated with LPS. Anti AP-1: antibody to c-Jun/AP-1; XS cold: 100 fold excess cold AP-1 consensus oligonucleotide. Similar results were obtained in three independent experiments.

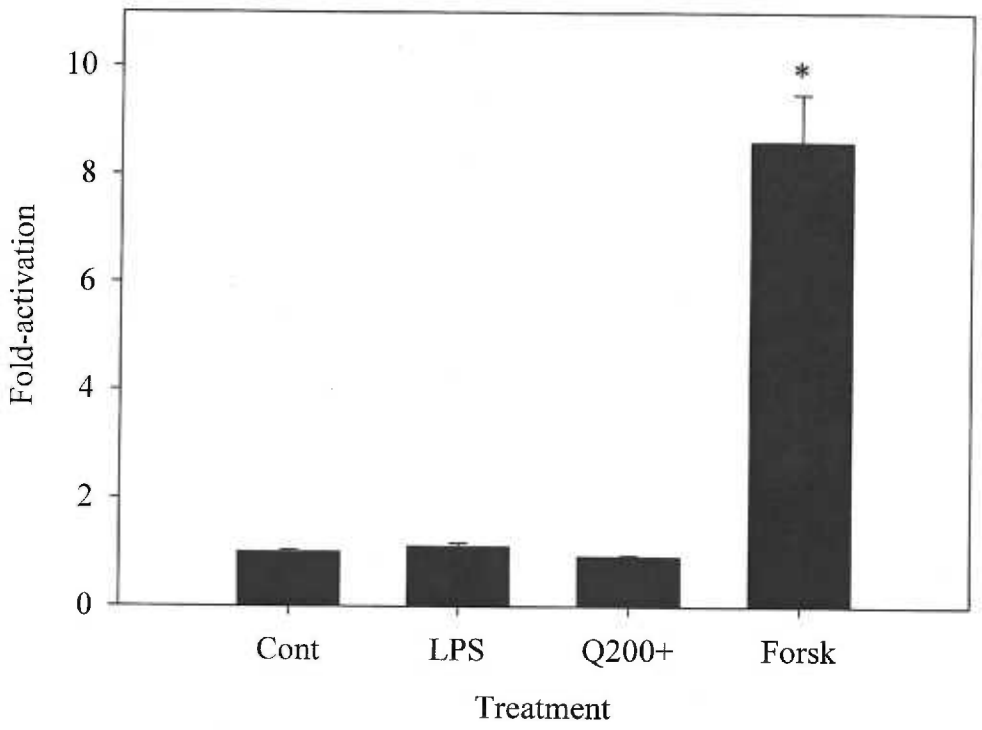


Fig 10. Effect of LPS on CRE-dependent transcriptional activity in RAW 264.7 macrophages. Twenty four hours after transient transfection with pCRE-luc, cells were treated with vehicle only (Cont, n=6), pre-treated for one hour with DMSO (LPS, n=6) or quercetin (Q200+, 200 μM, n=3) followed by treatment with LPS (1000 ng/ml for 4.5 h) or pre-treated with vehicle for one hour, followed by treatment with forskolin (Forsk, 10 μM, n=3) for 4.5 h. Cells were harvested for luciferase activity and protein determination. Data, representative of two separate experiments, is expressed relative to control luciferase activity normalized to protein and plotted as mean ± S.E, n=6. *, significantly different than control, $p < 0.05$.

DISCUSSION

Many cell types respond to oxidative stress by up-regulating the Rel/NF-κB family of transcription factors which play a central role in coordinating the expression of genes involved in the inflammatory process. NF-κB is inhibited by a variety of structurally diverse antioxidants and overexpression of antioxidant enzymes (Li and Karin, 1999). Polyphenolic phytochemicals with antioxidant properties such as curcumin and epigallocatechin gallate have been shown to inhibit LPS-induced activation of NF-κB and κB-responsive cytokines (Chan, 1995; Lin and Lin, 1997). Here, we investigated whether the natural product antioxidants, EGb 761 and quercetin inhibit LPS-induced release of TNF-α by blocking activation of NF-κB.

The precise mechanism by which LPS stimulation induces NF-κB has yet to be elucidated. The most abundant form of the transcription factor is a heterodimer of p50 and p65 subunits, in which the p65 subunit contains the transcriptional activation domain. In unstimulated cells, NF-κB is retained in the cytoplasm by the binding of a family of inhibitors (IκBα, IκBβ, IκBγ). Activation by LPS requires sequential phosphorylation of IκB by an IκB-kinase (IKK1 or IKK2), ubiquitination and degradation by the proteasome, followed by translocation of NF-κB/Rel proteins to the nucleus (Rothwarf, 2000). Several studies have advanced our understanding of the role of protein kinases in LPS-mediated NF-κB activation. Tyrosine kinase inhibitors inhibit the activation of NF-κB and subsequent release of pro-inflammatory cytokines (Hwang *et al.*, 1997). IKK1 and IKK2 appear to be differentially regulated. In LPS-stimulated monocytes, prolonged NF-κB activity correlates with IKK2 dependent IκB-β phosphorylation and degradation.

MEKK-1, the upstream activator of SAPK/JNK and p38 MAP kinase pathways, preferentially stimulates IKK2 activity (O'Connell *et al.*, 1998). p38 MAP kinase regulates NF-κB-dependent gene expression, in part, by phosphorylating the transcription factor TFIID (TBP), which is important for transcriptional activation of NF-κB (Carter *et al.*, 1999a). The role of reactive oxygen intermediates (ROIs) in the regulation of NF-κB is controversial. To date, data suggests that IκB phosphorylation and degradation may be the steps that are sensitive to oxidative stress and antioxidants (Li and Karin, 1999).

Based on our results from western analysis of I-κB degradation and nuclear translocation of the p50 and p65 subunits of NF-κB, mobility shift analysis of NF-κB-DNA binding and transient transfections with the pNF-κB-luc reporter, we conclude that although quercetin inhibits LPS-induced TNF-α mRNA, it has no effect on LPS-induced binding of the p50/65 heterodimer or NF-κB-dependent transcriptional activity. As described in the introduction, the 5' flanking region of the TNF-α promoter contains binding sites for numerous transcription factors, including NF-κB, AP-1, CRE and C/EBPβ. A reporter plasmid, containing the promoter region of murine TNF-α may help illuminate the mechanism by which quercetin inhibits LPS-induced TNF-α transcription. Most studies examining the *in vivo* effects of quercetin on cultured cells use quercetin concentrations ranging between 1-100 μM (Shih *et al.*, 2000; Kawada *et al.*, 1998; Kuo, *et al.*, 1999; Musonda *et al.*, 1997). Although we used a higher concentration of quercetin (200 μM) to evaluate the inhibition of LPS-induced TNF-α, crystal violet uptake assays confirmed that this concentration does not exhibit cytotoxicity.

EGb 761 decreased LPS-induced p50/50 binding, nuclear translocation of the p50

subunit and NF-κB-transcriptional activity in the pNFκB-luc reporter construct, but this does not appear to be important in TNF-α transcriptional inhibition since EGb 761 does not diminish LPS-induced TNF-α mRNA. Components other than quercetin are most likely responsible for the effects of EGb 761 on NF-κB.

Since quercetin and EGb 761 can act as radical scavengers, the mechanism by which LPS induces NF-κB in RAW 264.7 macrophages may not involve ROS . TNF-α inhibition by EGb 761 and quercetin cannot be explained merely by their antioxidant properties.

Literature suggests that components of the MAPK pathway are important in regulation of TNF-α (Swantek *et al.*, 1997; Carter *et al.*, 1999b). The p44/42 (Erk1/2), p38 and JNK/SAPK play a critical role in the regulation of cell growth and differentiation and the control of cellular responses to cytokines and stress. MAPK cascades are usually organized in a three kinase architecture consisting of a MAPK/extracellular-signal related kinase (Erk) kinase kinase (MEKK) which activates a MAPK/ERK kinase (MEK) which in turn activates a MAPK/ERK. The MEKs are dual specificity kinases, phosphorylating MAPKs on threonine and tyrosine on specific TXY sequences. Phosphorylation induces dimerization and translocation of the MAPK from the cytoplasm to the nucleus. In RAW 264.7 macrophages, p44/42 MAPK appears to be downstream of PKC, Raf and MEK1/2 (Cobb and Goldsmith, 1995). Erk 1/2 activation can be inhibited by the noncompetetive MEK1/2 inhibitor UO126 (Favata, *et al.*, 1998). The upstream members of p38 MAP kinase family are MEKK-1 and MEK3/6. p38 MAPK can be selectively inhibited by SB203580, which binds to its ATP-binding site

(Lee *et al.*, 1994b). The JNK/SAPK pathway consists of MEKK-1, and MKK 4/7. c-Jun, a component of the transcription factor AP-1 is a major downstream target of the SAPK/JNK signaling pathway (Kyriakis *et al.*, 1994). To the best of our knowledge, no specific inhibitor of SAPK/JNK has been identified. Signals downstream of the three distinct MAPK cascades do not operate in a purely linear fashion, and may ultimately converge. For instance, the transcription factor, Elk-1 can be activated by Erk1/2, SAPK/JNK and p38, while ATF-2 is regulated by SAPK/JNK and p38. The challenge is to understand how the coordination and regulation of these pathways results in a specific biological response.

Our results from western blot analysis indicate that quercetin inhibits LPS-induced phosphorylation of SAPK/JNK and its downstream substrates, c-Jun and ATF-2. Both quercetin and EGb 761 diminish phosphorylation of ERK1/2 and p38 MAPK activity. Inhibition occurs downstream of TRAF6 and MEKK1, as degradation of IκB is not affected by EGb 761 or quercetin. Erk1/2 and p38 MAPK are not required for TNF-α transcription as evidenced by northern blots illustrating that the specific inhibitors UO126 and SB203580 have no effect on LPS-induced TNF-α mRNA. Quercetin's unique effect on TNF-α transcription is therefore not due to its effects on Erk1/2 or p38 MAPK.

We propose that inhibition of TNF-α transcription by quercetin is due to inhibition of SAPK/JNK via effects on the transcription factor, AP-1. AP-1 was first defined as a DNA binding activity specific for positive regulatory elements in the SV40 early promoter. It is activated in response to a wide array of stimuli, including LPS, UV and ionizing irradiation, peptide growth factors, and cytokines. AP-1 is not a single

transcription factor, but a series of related dimeric complexes of Fos and Jun family proteins that bind to the DNA sequence 5' TGAGTCA 3' (Wisdom, 1999). In addition to forming Jun-Jun and Jun-Fos dimers, Jun proteins can form heterodimeric Jun-ATF-2 complexes which recognize the CRE binding sequence. Activation of Erk and/or SAPK/JNK results in AP-1 activation. Both c-Jun and c-Fos are activated by SAPK/JNK, c-Jun by direct phosphorylation and c-Fos by activation of Elk-1, a transcriptional element on the c-Fos promoter (Whitmarsh *et al.*, 1995). AP-1 and NF-κB may form synergistic complexes that enhance transcription (Stein *et al.*, 1993). Consistent with our hypothesis, EMSA results demonstrate that quercetin dramatically decreases AP-1/DNA binding.

Transient transfections with the pCRE-luc reporter and EMSAs show that LPS does not stimulate transcriptional activity or ATF/CRE binding in RAW 264.7 macrophages. A lack of a statistically significant difference between control and LPS-stimulated RAW 264.7 nuclear extracts in EMSAs has been previously reported (Proffitt *et al.*, 1995). These results suggest that ATF/CRE is not involved in the LPS-induced transcription of TNF-α. The post-transcriptional effects on TNF-α by EGb 761 and quercetin may be explained by inhibition of Erk1/2 phosphorylation and p38 MAPK activity. In our system, both UO126 and SB203580 block LPS-induced release of TNF-α, yet have no effect on steady state levels of TNF-α mRNA. Several studies have illustrated the role of MAPKs in the post-transcriptional regulation of TNF-α biosynthesis. Upstream components in the Erk pathway, Ras and Raf are required for TNF-α production at the level of both transcription and translation in RAW 264.7

macrophages (Geppert *et al.*, 1994). The use of specific p38 inhibitors and kinase defective mutants of SAPK/JNK established that these kinases are necessary for LPS-induced translation of TNF-α mRNA (Lee *et al.*, 1994b; Swantek *et al.*, 1997). MAPKAP kinase 2, a substrate of p38 MAPK, is essential for LPS-induced TNF-α translation (Khokhlatchev *et al.*, 1998). We were not able to monitor the phosphorylation of MAPKAP kinase 2 directly. The inhibition of p38 MAPK *in vitro* with ATF as a substrate suggests that this is a likely site of EGb 761 and quercetin action. Quercetin may exhibit more profound effects on TNF-α translation than EGb 761 by concomitantly inhibiting SAPK/JNK. The proteins involved in translational control are yet to be fully elucidated, but two protein complexes that bind to TNF-α mRNA are implicated. Protein complex 1, containing an RNA-binding protein, binds to clustered AUUUA pentamers independently of LPS, and may be responsible for translational repression (Gueydan *et al.*, 1999). Complex 2, composed of a 55-kDa protein, binds to UUAUUUAUU sequences following LPS-stimulation and may mediate translational de-repression (Lewis *et al.*, 1998). It will be necessary to evaluate the effect of EGb 761 and quercetin on the 3'UTR of TNF-α in order to understand the effects of these natural products on post-transcriptional regulation of TNF-α.

In conclusion, EGb 761 and its aglycone component, quercetin have selective effects on TNF-α and the MAPK cascade. Though both EGb 761 and quercetin inhibit TNF-α secretion in LPS-stimulated RAW 264.7 macrophages, our results suggest that quercetin is unique in its ability to inhibit TNF-α transcription by inhibiting phosphorylation and activation of SAPK/JNK, and therefore suppressing activation of the

transcription factor AP-1. EGb 761 diminishes LPS-induced NF-κB transcriptional activity slightly but has no effect on TNF-α transcription. Both EGb 761 and quercetin also can inhibit TNF-α production at a post transcriptional level. Erk1/2 and p38 MAPK activities, which are important in the post-transcriptional regulation of TNF-α mRNA are inhibited by EGb 761 and quercetin.

Acknowledgements

The authors thank Dr. T. Schmeller (Dr. Willmar Schwabe GmbH, Karlsruhe, Germany) for the kind gift of EGb 761 and Ms. Tanya Hudson for technical assistance.

This work was supported by grants from the Oregon Wine Advisory Board and the National Institute on Alcohol Abuse and Alcoholism (AA08608).

C. Manuscript 3

Effects of *Ginkgo biloba* extract (EGb 761) and Quercetin on Lipopolysaccharide-induced Release of Nitric Oxide

Teri L. Wadsworth and Dennis R. Koop

Department of Physiology and Pharmacology

Oregon Health Sciences University

Chemico-Biological Interactions, in press

ABSTRACT

Administration of bacterial lipopolysaccharide (LPS) to laboratory animals and cultured macrophages is known to induce the production of nitric oxide (NO) from inducible nitric oxide synthase (iNOS). Here we show that pre-treatment with *Ginkgo biloba* extract (EGb 761) suppresses the *in vivo* production of NO (measured by the Griess reaction) after challenge with LPS. In order to begin to understand the mechanism of this inhibition, we evaluated *in vitro* effects of EGb 761 and its flavonoid component, quercetin, on LPS-treated RAW 264.7 macrophages. Pre-treatment with EGb 761 or quercetin dose-dependently inhibited NO release. Both substances scavenged NO generated from the decomposition of sodium nitroprusside. Western analysis showed that EGb 761 and quercetin inhibited LPS-induced levels of iNOS protein. Northern blotting demonstrated that EGb 761 and quercetin decreased LPS-induced iNOS mRNA levels without altering the half-life. Activation of mitogen activated protein kinases (MAPKs) and the redox-sensitive transcription factors, nuclear factor-κB (NF-κB) and activator protein 1 (AP-1) are key events in the signal transduction pathways mediating iNOS induction. In our studies, both EGb 761 and quercetin inhibited p38 MAPK activity, which is necessary for iNOS expression in LPS-stimulated RAW 264.7 macrophages. However, differences in the response of NF-κB, AP-1, and Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK) and its downstream substrates to EGb 761 and quercetin suggest that quercetin is not the sole component responsible for the *in vivo* inhibition of LPS-induced iNOS activation by EGb 761.

INTRODUCTION

Extracts from *Ginkgo biloba* have been used in Chinese medicine for centuries. Today, the standardized extract of Ginkgo, EGb 761, is prescribed for treatment of disorders such as Alzheimer's disease and neuronal hypoxia, both of which have etiologies associated with oxidative stress (Kleijnen and Knipschild, 1992; Itil and Martorano, 1995; Oken *et al.,* 1998; Pitchumoni and Doraiswamy, 1998). *In vitro*, EGb 761 is a potent free radical scavenger and inhibitor of NADPH-oxidase (Maitra *et al.,* 1995; Pincemail *et al.,* 1987). The extract inhibits nitric oxide (NO) production in lipopolysaccharide/gamma interferon (LPS/IFNγ)-activated macrophages by concomitantly scavenging NO and inhibiting inducible nitric oxide synthase (iNOS) mRNA and enzyme levels (Kobuchi and Packer, 1997; Marcocci *et al.,* 1994a). EGb 761 is a complex mixture of natural products, including 24% flavonoid glycosides (11% is the glycoside of quercetin) and 6% terpenes unique to *Ginkgo biloba* (Drieu, 1986). In addition to EGb 761, quercetin is present in wine and a wide variety of fruits, vegetables and herbs. Quercetin is a powerful antioxidant and *in vitro* can inhibit various tyrosine and serine/threonine kinases, including mitogen-activated protein kinases (MAPKs) (Csokay *et al.,* 1997; Kawada *et al.,* 1998). Whether the therapeutic effects of EGb 761 are caused by single active ingredients or the combined action of its many components has yet to be determined.

NO, a short lived gas and highly reactive free radical, is produced by monocytes and macrophages upon exposure to LPS. Physiological production of NO plays an important role in host defense. Overproduction of NO and its metabolites, however, have been implicated in the pathogenesis of conditions such as bacterial sepsis and chronic

inflammation (Laskin *et al.,* 1995; Evans *et al.*, 1993). When activated by stimuli such as LPS, IFN-γ, IL (interleukin)-1 or TNF-α, macrophages generate NO from L-arginine via the inducible form of nitric oxide synthase (iNOS) (Leone *et al.*, 1991). The regulation of iNOS expression is complex, but appears to occur primarily at the level of transcription. The murine iNOS promoter contains regulatory regions incorporating binding sites for nuclear factor-κB (NF-κB), activator protein 1 (AP-1), CCAAT/enhancer binding protein (C/EBP), tumor necrosis factor response element, and interferon-related transcription factors (Xie *et al.,*1993; Chu *et al.,* 1998; Lowenstein *et al.,* 1993). Of these, activation of the redox-sensitive transcription factor, NF-κB has been shown to mediate the enhanced expression of the iNOS gene in macrophages exposed to LPS (Xie, *et al.,* 1994; Sherman *et al.*, 1993). The inhibition of iNOS mRNA expression by cycloheximide suggests the importance of newly synthesized proteins for LPS-inducibility (Nathan and Xie, 1994; Xie and Nathan, 1994; Weisz *et al.,*1994).

In murine macrophages, the cellular response to LPS is transmitted from the plasma membrane to the cytoplasm through the Toll-like receptor 4 (TLR4) in concert with CD14 and lipid binding protein (LBP) (Ulevitch, 1999). In turn, a myriad of signal transduction pathways and transcription factors such as NF-κB and AP-1 are activated. Signaling pathways involving tyrosine phosphorylation appear to be important in mediating the induction of iNOS by LPS (Akarasereenont *et al.*, 1994). In macrophages, LPS stimulates the phosphorylation and activation of three, well defined mitogen active protein kinases (MAPKs), extracellular signal related kinase (Erk), p38 MAPK and Jun N-terminal kinase/stress activated protein kinases (JNK/SAPK) (Weinstein *et al.*, 1992). Studies evaluating the involvement of specific members of the MAPKs in macrophage

iNOS induction are not in complete agreement (Chen and Wang, 1999; Paul *et al.,* 1999; Carter *et al.,* 1999b; Ajizian *et al.,* 1999).

Previous studies in our lab demonstrated that quercetin inhibited LPS-induced transcription of iNOS, but not via effects on NF-κB-DNA binding (Wadsworth and Koop, 1999). Our more recent studies evaluating the effects of EGb 761 and quercetin on LPS-induced activation of the transcription factors NF-κB and AP-1 and the MAPK cascade illustrate that while EGb 761 and quercetin have selective effects on each of these processes, both inhibit p38 MAPK activity (Wadsworth *et al.*, in press). Here, we evaluate the mechanism by which EGb 761 and quercetin inhibit LPS-induced NO by examining the *in vitro* effects of these natural product antioxidants on NO scavenging, LPS-induced iNOS protein and mRNA. Our results show that EGb 761, quercetin, and the p38 MAPK inhibitor, SB203580, inhibit LPS-induced upregulation of iNOS mRNA. We demonstrate that p38 MAPK activity is necessary for iNOS expression in LPS-stimulated RAW 264.7 macrophages. Direct NO scavenging may also contribute to the suppression of NO release by EGb 761 and quercetin.

MATERIALS AND METHODS

Reagents

Escherichia coli LPS, serotype 0127:B8, quercetin, penicillin/streptomycin, Griess reagent, DMSO, nitrate reductase, lactate dehydrogenase, pyruvic acid, NADPH, FAD, sodium nitroprusside, Denhardt's reagent and E-toxate® reagent were from Sigma Chemical Co. (St. Louis, MO). Polyclonal iNOS antibodies were from Transduction Laboratories (Lexington, KY). The protease inhibitors aprotinin, leupeptin, Pefabloc, E-64 and the selective MAPK inhibitors, SB203580 and UO126 were from Calbiochem (La

Jolla, CA). Dulbecco's minimal essential medium (DMEM), RPMI , and TRIzol® reagent were from Gibco BRL (Gaithersburg, MD). Fetal bovine serum was from Research Sera (Fort Collins, CO). Tissue culture plates were from Fisher Scientific (Pittsburgh, PA). ^{32}P-CTP and GeneScreen Plus® membranes were from DuPont NEN (Boston, MA). Ready To Go ™ DNA labeling beads (-dCTP) were from Amersham Pharmacia Biotech Inc (Piscataway, NJ). Bradford and D_c protein assay reagents were from Bio-Rad (Hercules, CA). Nitrocellulose membrane was from Schleicher & Schuell (Keene, NH). ECL™ Western blotting detection reagents were from Amersham Life Science (Arlington Heights, IL). SuperSignal® Ultra chemiluminescent substrate was from Pierce (Rockford, IL). The mouse macrophage RAW 264.7 cell line was obtained from the American Type Culture Collection (Rockville, MD). C57BL/6 mice were from Jackson Laboratories (Bar Harbor, ME). EGb 761 was a generous gift from Dr. Willmar Schwabe GmbH (Karlsruhe, Germany).

Animals

Male C57BL/6 mice, 8 weeks old, were allowed free access to food and water at all times and were allowed to acclimatize in the facilities for 7 days before use. Immediately prior to treatment, EGb 761 was dissolved in sterile saline, adjusted to pH 7.4 and administered intraperitoneally to the mice at a dose of 0, 5, 20, 50 or 100 mg/kg for 7 days. On day 7, LPS, (dissolved in sterile saline) was intraperitoneally injected at a dose of 1.3 mg/kg body weight. At 6 h after LPS injection, animals were sacrificed by CO_2 asphyxiation. All procedures were approved by the Institutional Animal Care and Use Committee of Oregon Health Sciences University.

Serum

Blood samples were collected by cardiac puncture. Blood was allowed to clot for 2 h at room temperature or overnight at 4°C before centrifuging for 20 minutes at 2000 x g. Serum was removed and stored at -70°C.

Cell culture

RAW 264.7 cells were cultured in phenol red free DMEM containing 50 units/ml penicillin, 50 μg/ml streptomycin, 44 mM sodium bicarbonate and 10% fetal bovine serum at 37°C in humidified air containing 5% CO_2. For preparation of RNA, nuclear extracts, or cell lysates, RAW 264.7 cells were plated in 2.5 ml of media in 5.5 cm dishes and cultured for 2 days until cells reached 80% confluency (approximately 2 x 10^7 cells/dish) then treated as described in the text. For determination of nitrite, RAW 264.7 cells were plated in 1 ml of media in 24 well plates, cultured for 2 days to 80% confluency (approximately 1 x 10^6 cells/well) then treated. In all cases, cells were washed and fresh complete media added before the indicated treatments. Stock solutions of EGb 761, quercetin and MAPK inhibitors were prepared in DMSO, the final concentration of DMSO did not exceed 0.5%. Crystal violet uptake assays confirmed that results were not due to general cellular toxicity and the E-toxate® test verified that culture media was not contaminated with bacterial endotoxin.

Measurement of Nitrite

Six hours after LPS-treatment, the stable end products of L-arginine-dependent NO synthesis, nitrate and nitrite were measured in serum using the Griess reaction as previously described (Sewer *et al.*, 1998). Aliquots of serum (50 μl) were deproteinized by addition of 2 μl of 35% sulfosalicylic acid. Deproteinized samples were vortexed

every 5 min for 30 min and centrifuged at 10,000 x g at 4°C for 15 min. To convert nitrate to nitrite, 40 µl aliquots of supernatant were mixed with 40 µl of 0.31 M phosphate buffer, pH 7.5, 20 µl of 0.1 mM FAD, 20 µl of 1 mM NADPH, 20 µl of nitrate reductase (10 U/ml) and 60 µl of water and the reaction allowed to proceed in the dark for 1 h. NADPH was removed by adding 2 µl of lactate dehydrogenase (1500 U/ml) and 20 µl of 100 mM pyruvic acid and incubating at 37 °C for 15 min. Duplicate 100 µl samples were added to an equivalent volume of Griess reagent in a 96 well plate and incubated for 10 min at room temperature. Nitrite levels were determined colorimetrically at 550 nm with a PowerWave$_x$ 340 microplate reader (Bio-Tek Instruments, Inc., Winooski, VT) and a sodium nitrite standard curve. For *in vitro* studies, after 24 h of LPS treatment, 100 µl of medium from 24 well plates containing RAW 264.7 macrophages was incubated with an equal volume of Griess reagent for 10 minutes in 96 well plates at room temperature. The reaction was analyzed colorimetrically as described for serum. Additions of quercetin and EGb 761 to standard solutions of sodium nitrite confirmed that these additions did not interfere with the nitrite assay.

NO Production from Sodium Nitroprusside

Immediately before the experiment, 100 mM stock solutions of sodium nitroprusside (SNP) were prepared in PBS that had been bubbled with argon. SNP solution (50 µl) was added to 950 µl PBS or culture medium containing DMSO, EGb 761 or quercetin. Solutions were incubated at 25°C for 2.5 hours. Aliquots (100 µl) were added to an equal volume of Griess reagent and analyzed for nitrite as described above.

Western blot analyses

For analysis of iNOS protein, RAW 264.7 cells were scraped and pelleted in PBS.

Pellets were resuspended in lysis buffer (50 mM Tris·HCl, pH 7.4, 10 mM EDTA, 1% Triton X, 1 mM PMSF , 1 μg/ml aprotinin, 10 μg/ml leupeptin, 200 μg/ml Pefabloc and 10 μg/ml E-64). Proteins (40 μg per lane) were electrophoresed on 8% SDS-polyacrylamide gels (PAGE) and transferred to nitrocelluose. Blots were blocked 1 h in blocking buffer (5% non-fat dry milk (NFDM) dissolved in 10 mM Tris·HCl, pH 7.4, 200 mM sodium chloride and 0.1% Tween-20 (TBS-T)). Blots were incubated overnight with 1:10,000 anti-iNOS in blocking buffer, washed in TBS-T and incubated 30 min with 1:25,000 secondary antibody. After washing, the blots were analyzed by enhanced chemiluminescence (ECL).

Northern Blot Analysis

After treatment, total RNA was isolated with TRIzol® reagent as specified by the manufacturer. Total RNA (20 μg) was electrophoresed in 1% agarose/15% formaldehyde gels, transferred overnight to GeneScreen Plus® membranes, and cross-linked to the membrane by UV irradiation. The membrane was prehybridized for 3 h at 37°C in hybridization buffer (50% deinoized formaldehyde, 5X Denhardt's reagent, 5X SSC, 0.1% SDS and 100 μg/ml denatured salmon sperm DNA) then hybridized overnight in the same buffer containing 1-2 x 10^6 cpm/ml of denatured cDNA probe. Following hybridization, the filters were washed twice for 30 min in 2X SSC (0.03 M sodium citrate/0.3 M sodium chloride), 0.1% SDS at room temperature and twice for 30 min in 0.5 X SSC, 0.1% SDS at room temperature. The probe was a 781 *Bam*HI fragment excised from full-length murine iNOS. Signals were detected with a GS-363 Bio-Rad Molecular Imager with a BI imaging screen, and signal intensity quantified with Bio-Rad Molecular Analyst software.

Statistical Analysis

The statistical significance of the results were analyzed by the Student's t-test for unpaired observations.

RESULTS

EGb 761 suppresses LPS-induced nitrate/nitrite in vivo

Animal studies have examined the protective effects of EGb 761 on reperfusion injury, age-related neuronal membrane fluidity and 4-O-methylpyridoxine-induced convulsions (Szabo *et al.,* 1995; Tosaki *et al.,* 1996; Pietri *et al.,* 1997a; Sasaki *et al.,* 1997). With dose and duration of treatment based on these studies, we hypothesized that EGb 761 would inhibit iNOS in C57BL/6 mice. Consistent with our hypothesis, intraperitoneal injection of LPS increased serum levels of nitrate/nitrite and pre-treatment with EGb 761 dose-dependently protected against LPS-induced increases (Fig. 1).

EGb 761, quercetin and SB203580 inhibit LPS-induced NO in vitro

In vitro studies with RAW 264.7 macrophages were utilized to evaluate the mechanism by which EGb 761 inhibits LPS-induced NO *in vivo*. EGb 761 has been shown to inhibit LPS/IFN-γ-induced nitrite release in RAW 264.7 macrophages (Kobuchi *et al.*, 1997). As shown in Fig 2, LPS increased culture supernatant levels of NO, as measured by the Griess reaction. Pre-treatment with EGb 761 or quercetin inhibited NO release in a dose-dependent fashion. The p38 MAPK inhibitor, SB203580 decreased and the MEK1/2 inhibitor UO126 enhanced LPS-induced NO. EGb 761, quercetin, UO126 and SB203580 did not interfere with the Griess reaction when added to standard solutions of potassium nitrite (data not shown).

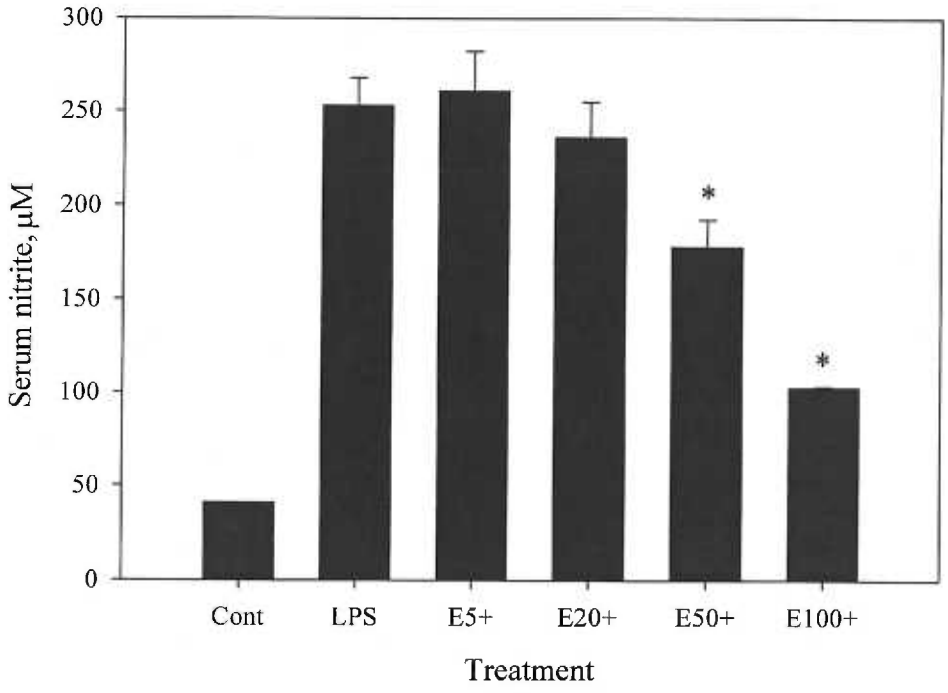


Fig 1. EGb 761 suppresses LPS-induced NO *in vivo*. C56BL/6 mice were pre-treated with EGb 761 or saline for 7 days. On day 7, immediately following EGb 761 treatment, mice were injected with LPS (1.3 mg/kg) or saline and sacrificed 1 h later. Serum was collected and analyzed for concentrations of nitrate and nitrite by the Griess reaction. Cont, saline injections only (n=2); LPS, pre-treatment with saline, injection with LPS (n=9); E5 (n=5), E20 (n=5), E50 (n=8), E100 (n=5), pre-treatment with 5, 20, 50 or 100 mg/kg EGb 761 and LPS-injected. The data presented represents mean ± SE. *, Significantly different from mice treated with LPS only, $p<0.05$.

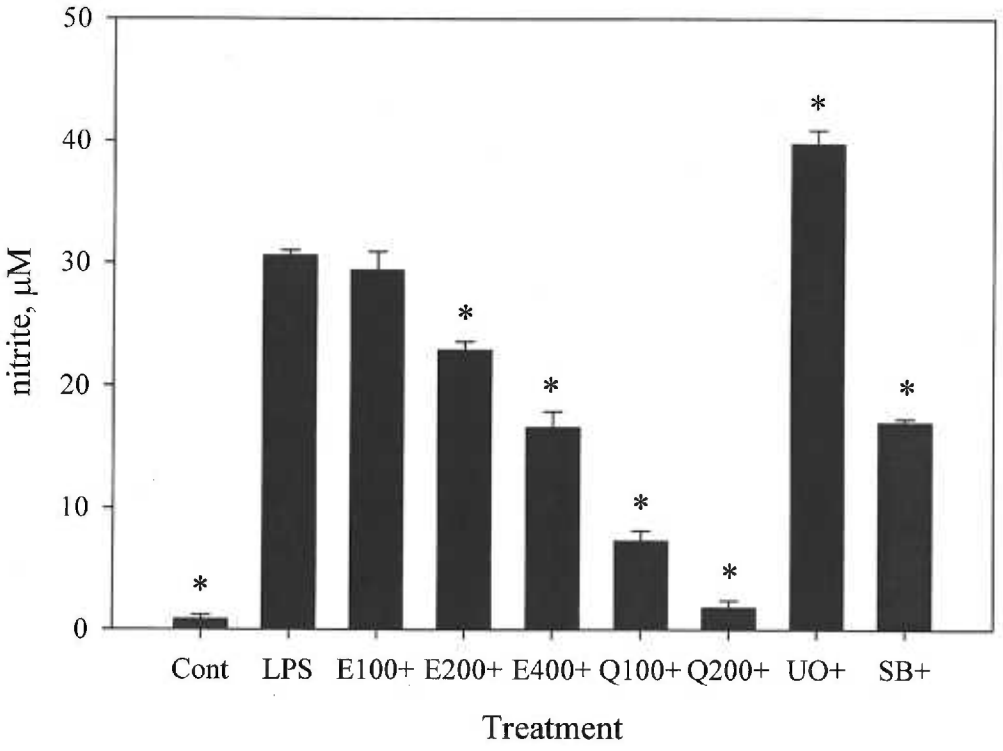


Figure 2. EGb 761, quercetin and the p38 MAPK inhibitor, SB203580 decrease LPS-induced NO *in vitro*. RAW 264.7 macrophages were pre-treated with vehicle only (Cont), or pre-treated with vehicle (LPS), EGb 761 (E100+, 100 μg/ml; E200+, 200 μg/ml; E400+, 400 μg/ml), quercetin (Q100+,100 μM; Q200+, 200 μM), UO126 (UO+, 20 μM) or SB203580 (SB, 30 μM) for 1h, followed by treatment with LPS (100 ng/ml, 18h). Supernatants were collected and analyzed for nitrite using the Griess reagent. The data represents mean ± SE, n=3. Similar results were observed in two independent experiments. *, Significantly different from treatment with LPS only.

Inhibition of NO release by EGb 761, quercetin and SB203580 is due to inhibition of LPS-induced production of iNOS protein

Because LPS-induced production of NO is catalyzed by iNOS, we studied the induction of iNOS in RAW macrophages by western blot analysis. Pre-treatment with EGb 761 or quercetin inhibited LPS-induced iNOS protein synthesis (Fig 3a). Consistent with results from *in vitro* studies measuring LPS-induced NO release, the p38 MAPK inhibitor, SB203580, inhibited and the Erk1/2 inhibitor, UO126, had no effect on iNOS protein levels (Fig 3b).

EGb 761 and quercetin scavenge nitric oxide

Studies have shown that EGb 761 scavenges NO generated from the decomposition of sodium nitroprusside (SNP) in PBS (Marcocci *et al.*, 1994). We evaluated the effects of EGb 761 and quercetin on SNP-derived nitrite in both PBS and tissue culture media (Fig 4). Both EGb 761 and quercetin caused a small, dose-dependent inhibition of nitrite accumulation in culture media. In contrast, SNP dissolved in PBS generated more nitrite than SNP dissolved in culture media and inhibition by EGb 761 and quercetin was more profound in PBS.

EGb 761 and quercetin inhibit LPS-induced iNOS transcription, SB 203580 decreases the half life of iNOS mRNA

The suppression of LPS-induced iNOS protein by EGb 761, quercetin and SB203580 could result from an inhibition of gene expression. To test this possibility, iNOS mRNA was assessed by northern analysis (Fig 5a). EGb 761 and SB203580 decreased and quercetin inhibited LPS-induced up-regulation of iNOS mRNA. Quercetin and EGb 761 did not have a significant effect on the half life of iNOS mRNA (Fig 5b).

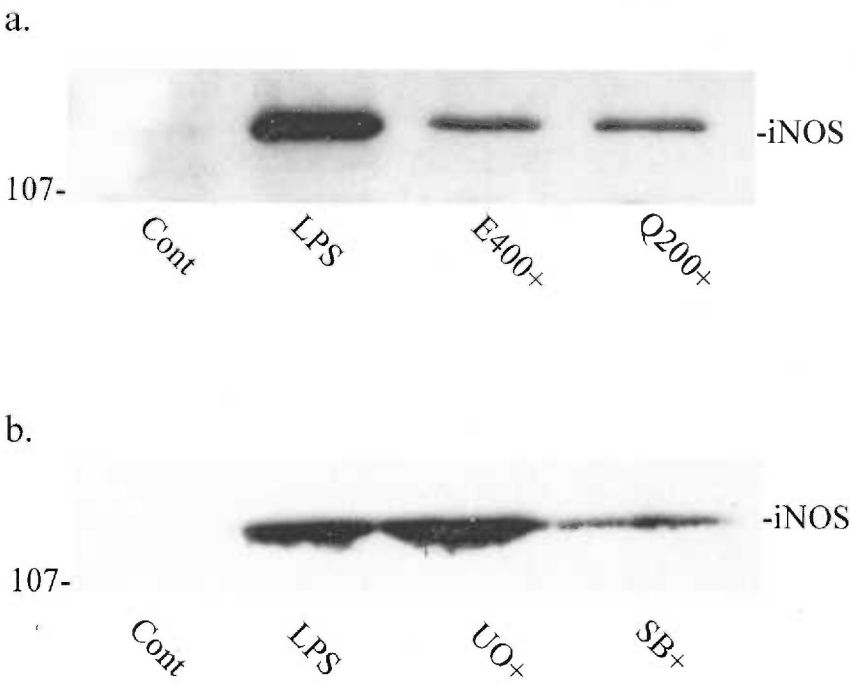


Fig 3. EGb 761, quercetin and SB203580 inhibit LPS-induced iNOS protein. Cell extracts from RAW 264.7 cells treated with vehicle only (Cont) or pre-treated for one hour with vehicle (LPS), EGb 761 (E400+, 400 μg/ml), quercetin (Q200+, 200 μM), UO126 (UO+, 20 μM) or SB203580 (SB+, 30 μM) prior to treatment with LPS (100 ng/ml, 18 h). Cell lysates were examined for iNOS protein levels by western blot analysis as described in materials and methods. Similar results were observed in three independent experiments.

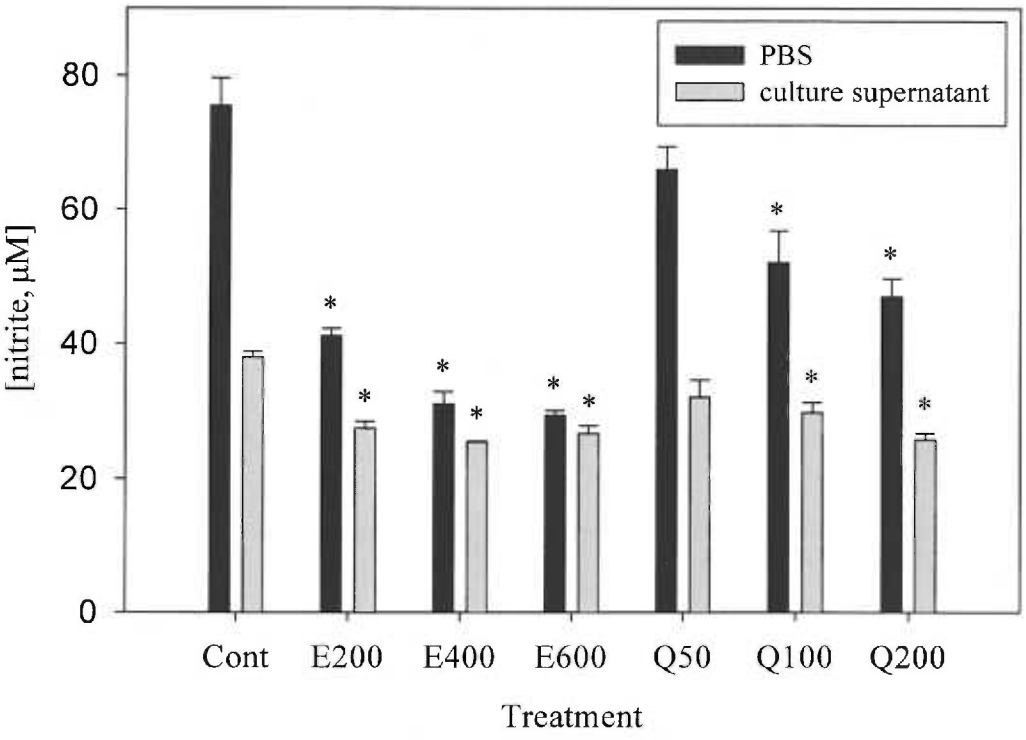


Fig 4. EGb 761 and quercetin scavenge nitric oxide. A solution of 5 mM SNP in PBS (dark fills) or tissue culture medium (light fills) was incubated for 2.5 h at 25°C in the presence of vehicle only (Cont), EGb 761 (E200, 200 μg/ml; E400, 400 μg/ml; E600, 600 μg/ml) or quercetin (Q50, 50 μM; Q100, 100 μM; Q200, 200 μM). Nitrite was measured by the Griess reaction. Each bar represents the mean ± SE of three individual assays. * Significantly different than corresponding control. Similar results were obtained in a second independent experiment with three assays.

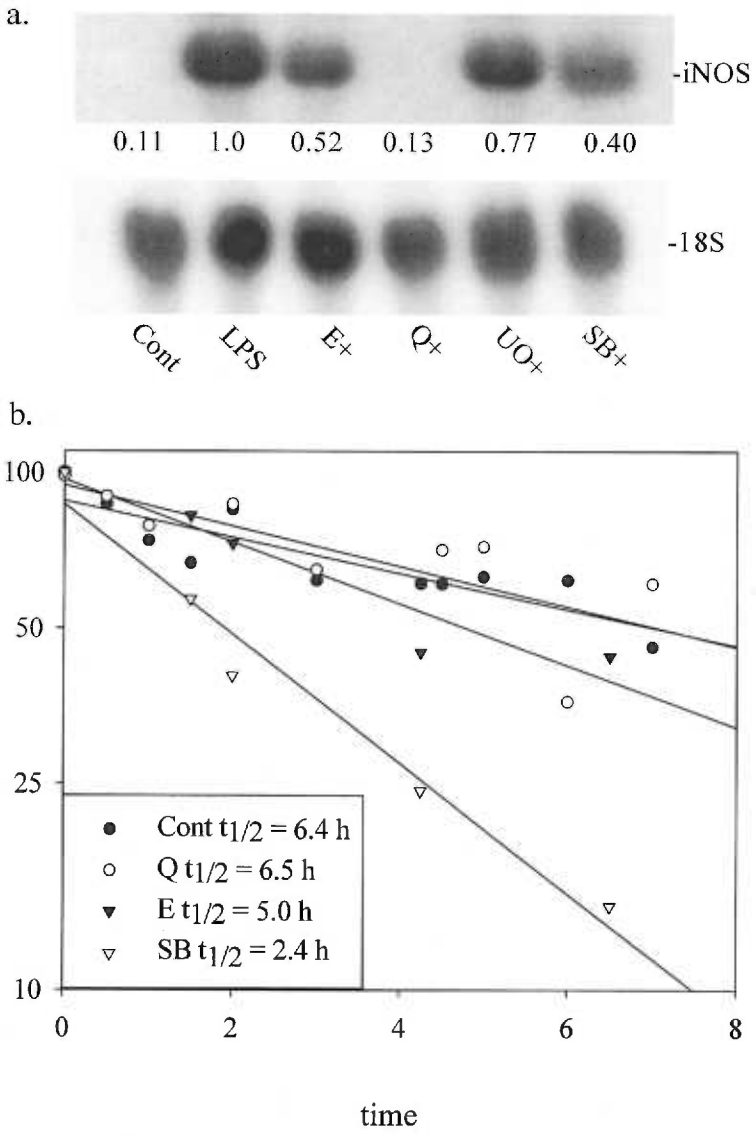


Fig 5. EGb 761 and quercetin inhibit iNOS transcription, SB203580 decreases the half life of iNOS mRNA. a. RAW 264.7 cells were treated with vehicle only (Cont.) or pre-treated for 1 h with vehicle (LPS), EGb 761 (E400+, 400 μg/ml), quercetin (Q200+, 200 μM), UO 126 (UO+, 20 μM) or SB203580 (SB+, 30 μM) prior to treatment with LPS (100 ng/ml, 6 h). Total cellular RNA was isolated and iNOS mRNA and 18S rRNA assessed by northern analysis. The relative intensity of the signal after normalization to 18S rRNA is shown below each lane. b. RAW 264.7 macrophages were stimulated with LPS (100 ng/ml) for 6 h, then treated with actinomycin D (2.5 μg/ml) and quercetin (Q, 200 μM), EGb 761 (E, 400 μg/ml) or SB 203580 (SB, 30 μM). Total cellular RNA was isolated at the indicated times and assessed for iNOS and 18S RNA by northern blot analysis. Data, representative of three, independent experiments, is presented as relative amounts of iNOS mRNA remaining after normalization to 18S RNA. Curve fit by linear regression analysis, r^2 Cont = 0.824, Q = 0.735, E = 0.910, SB = 0.971.

In contrast, SB203580 decreased the half life of iNOS mRNA by 62%.

DISCUSSION

The inhibition of SNP-generated nitrite by EGb 761 and quercetin in culture media suggest that direct NO scavenging activity is one of the mechanisms by which these natural products decrease NO levels. However, NO scavenging cannot account for all the observed effects since EGb 761 and quercetin also inhibit LPS-induced iNOS mRNA and protein. It is not clear why SNP generates more nitrite in PBS when compared to complete tissue culture medium. This difference may reflect a reaction of NO with protein present in the medium thus reducing the overall nitrite produced.

The transcription factor, NF-κB has been shown to be essential but not sufficient for enhanced iNOS gene expression in RAW macrophages (Xie *et al.*, 1994; Lowenstein *et al.,* 1993; Xie and Nathan, 1994). NF-κB can be activated by oxidative stress and inhibited by a variety of structurally diverse antioxidants (Sherman *et al.* 1993; Li and Karin, 1999; Sen and Packer, 1996). Natural product polyphenolic antioxidants such as curcumin, (-)-epigallocatechin-3-gallate, and oroxylin A (an active component in the Chinese medicine, Huang Qin) inhibit both activation of NF-κB and iNOS (Takeuchi *et al.*, 1999; Lin and Lin, 1997; Chen *et al.,* 2000). In recent studies (Wadsworth *et al.* in press), we investigated whether the antioxidant phytochemicals, EGb 761 and quercetin, inhibit LPS-induced TNF-α production by blocking activation of NF-κB. Based on results from western blot analysis of I-κB degradation and nuclear translocation of the p50 and p65 subunits of NF-κB, EMSA of NF-κB/DNA binding and transient transfections with a pNFκB-luciferase reporter plasmid, which contains the luciferase reporter driven by a basic promoter containing five repeats of the NF-κB/Rel binding site

(pNFκB-luc), we concluded that quercetin had no effect on LPS-induced activation of NF-κB. EGb 761, on the other hand, diminished LPS-induced binding of the NF-κB homodimer, p50/50, and heterodimer p50/65 (as determined by EMSA), nuclear levels of p50 protein (as determined by western blot analysis) and NF-κB-dependent transcriptional activity (as determined by transient transfections with pNFκB-luc). A partial inhibition of LPS-induced NF-κB activity may contribute to the inhibition of iNOS transcription by EGb 761. Components other than quercetin in EGb 761 are most likely responsible for these effects.

Literature suggests that components of the MAPK pathway are important in regulation of iNOS in murine macrophages (Chen and Wang, 1999; Ajizian *et al.*, 1999; Chen *et al.*, 1999a). In our recent studies (Wadsworth *et al.*, in press), immunoprecipitation-kinase assays indicated that both quercetin and EGb 761 dose-dependently inhibited p38 MAPK activity. p38 MAPK is required for LPS-induced iNOS transcription and the synthesis of iNOS, as evidenced by northern blots (Fig 5a) and western blots (Fig 3b) where the p38 MAPK inhibitor, SB203580, inhibited LPS-induced iNOS mRNA and protein. SB203580 also inhibited LPS-induced NO release *in vitro* (Fig 2). While Paul *et al.* (1999) reported no significant changes in LPS-induced iNOS protein levels with SB203580 treatment, interpretation of their data reveals a trend towards inhibition. This trend is consistent with our data as well as reports from others who suggest the importance of p38 MAPK in transcriptional regulation of LPS-induced iNOS in murine macrophages (Chen *et al.*, 1999a; Chen and Wang, 1999; Jeon *et al.*, 2000; Lee *et al.*, 2000).

In order to evaluate the mechanism by which EGb 761, quercetin and the p38

MAPK inhibitor SB203580 decreased steady state levels of LPS-induced iNOS mRNA, we evaluated the effects of these compounds on iNOS mRNA half life. SB203580 decreased iNOS mRNA half life by approximately 62%, suggesting that p38 MAPK is important for iNOS message stability. To our knowledge, this is the first time that the destabilizing effect of of SB203580 on iNOS mRNA has been reported. Because quercetin and EGb 761 did not alter the half life of iNOS mRNA, inhibition of iNOS mRNA by this flavonoid most likely occurs at the transcriptional level. Since EGb 761 and quercetin can inhibit p38 MAPK-dependent phosphorylation of the p38 MAPK substrate, ATF-2 activity *in vitro* (Wadsworth *et al.*, in press), it is not clear why these compounds did not exhibit the same effect as SB203580 on iNOS mRNA half life. SB203580 may inhibit activation of a protein involved in iNOS mRNA stability that is not affected by EGb 761 or quercetin.

Although EGb 761 and quercetin suppress LPS-induced Erk1/2 phosphorylation (Wadsworth *et al.*, in press), this suppression most likely does not contribute to the mechanism of iNOS inhibition by EGb 761 and quercetin because the MEK1/2 inhibitor UO126 had no effect on LPS-induced NO release or steady state levels of LPS-induced iNOS mRNA. A comparison of our data with studies evaluating the specific role of Erk1/2 on iNOS regulation are complicated due to differences in cell type and inducing agent. Our results are in agreement with Chen and Wang (1999) who reported that p44/42 MAPK is not involved in LPS-induced expression of iNOS and NO release in RAW 264.7 macrophages. Ajizian *et al.*, (1999) suggest that the Erk1/2 pathway is involved in the up-regulation of iNOS in RAW 264.7 macrophages; however these studies utilized the MEK/Erk inhibitor, PD98059, and cells were stimulated with LPS (at

a 100-fold lower concentration) plus IFN-γ. The authors noted that in their experience, tyrosine kinase inhibitors are less effective at high concentrations of LPS. Utilizing PD98059 as a MEK inhibitor, Bhat *et al.* (1998) used microglia and astrocytes to demonstrate the importance of Erk1/2 in LPS/IFN-γ-induced iNOS. In our studies, PD98059, at a concentration of 50 μM, did not inhibit LPS-induced NO release but did inhibit phosphorylation of JNK/SAPK and c-Jun (Wadsworth *et al.*, in press).

Quercetin inhibits phosphorylation of SAPK/JNK and its downstream substrates, c-Jun and ATF-2 and dramatically decreases binding of the transcription factor, AP-1 to DNA in LPS-induced RAW 264.7 macrophages (Wadsworth *et al.*, in press). The dramatic inhibition of LPS-induced iNOS mRNA by quercetin (Fig 3a) may be due to effects on p38 MAPK coupled with inhibition the AP-1 via effects on JNK/SAPK and c-Jun. The murine promoter for iNOS contains binding sites for AP-1, and this transcription factor is activated by LPS (Lowenstein *et al.*, 1993). On the other hand, our findings showing that PD98059 inhibits JNK/SAPK but not NO release suggests that this MAPK pathway may not be necessary for LPS-induced iNOS activation in RAW 264.7 macrophages. A selective inhibitor for the SAPK/JNK pathway would confirm the importance of JNK/SAPK in iNOS transcription.

Dietary flavonoid glycosides are hydrolyzed in the intestine, absorbed as aglycones and metabolized to methylated, glucurono-sulfated derivatives (Teraod *et al.*, 1999; Walgren *et al.*, 1998; Morand *et al.*, 1998; Manach *et al.*, 1998; Sharma *et al.*, 1998). The conjugated derivatives of quercetin retain approximately 50% of the antioxidant properties of the aglycone (Manach *et al.*, 1998; Eklund *et al.*, 2000). Literature suggests that repeated dietary intake of quercetin can lead to accumulation in

plasma and contribute to plasma antioxidant capacity. The elimination half life of quercetin in humans is 17-24 h and ingestion of 64 mg of quercetin results in concentrations of 650 nM in hydrolyzed plasma (Hollman *et al.*, 1996; Hollman *et al.*, 1997; Erlund *et al.*, 2000). The daily consumption of flavonoids in the human diet is 3-80 mg, more than 50% of which is quercetin (de Vries *et al.*, 1997). Commercially available preparations of quercetin recommend dosages of 400-1200 mg daily as a dietary supplement. Assuming first order kinetics, a 1200 mg dose of quercetin could lead to plasma concentrations up to12 μM, Studies evaluating the effects of quercetin on cultured cells have used quercetin concentrations ranging between 1-200 μM (Nicholson *et al.*, 1997; Kobuchi, *et al.*, 1999; Musonda *et al.*, 1997; Wadsworth and Koop, 1999). It is possible that dietary consumption of quercetin from foods and nutritional supplements could exert physiological effects, especially if quercetin, like many drugs, exhibits tissue binding. Although concentrations of quercetin used to treat RAW 264.7 macrophages in this study are relatively high, crystal violet uptake assays confirmed that this concentration does not exhibit cytotoxicity in RAW 264.7 cells.

A dose of 400 μg/ml EGb 761 contains approximately 73 μM quercetin glycoside and 160 μM total flavonoid glycoside. Commercial purveyors of *Ginkgo biloba* tablets recommend a daily dose of 120-240 mg. Thus, a single dose of commercial *Ginkgo biloba* would not reach the levels of the extract used in the current studies. However, the pharmacokinetics of the components of *Ginkgo biloba* have not been well studied. It is possible that accumulation of some of the components may occur with prolonged treatment and may also concentrate in different tissues.

In conclusion, our studies uniquely demonstrate that EGb 761 inhibits the *in vivo*

production of LPS-induced nitric oxide. In agreement with previous reports, EGb 761 and quercetin inhibited LPS-induced transcription of iNOS in murine macrophages (Kobuchi and Packer, 1997; Wadsworth and Koop, 1999). Our recent results (Wadsworth *et al.*, 2000) suggest that EGb 761 and quercetin exhibit selective effects on the MAPK pathway and transcription factors AP-1 and NF-κB . Therefore, the mechanism by which EGb 761 and quercetin inhibit iNOS appears more complex than mere inhibition of a single transcription factor or kinase cascade. Suppression of NO release may be partially attributed to direct NO scavenging, as both EGb 761 and quercetin decreased the amount nitrite generated from the decomposition of sodium nitroprusside *in vitro*. Both EGb 761 and quercetin inhibited p38 MAPK activity, which is required for LPS-induced iNOS transcription. Quercetin is unique in its ability to inhibit JNK/SAPK and its substrate c-Jun and may inhibit iNOS transcription, in part, by suppressing activation of the transcription factor AP-1. While quercetin had no effect on LPS-induced NF-κB activation, EGb 761 suppressed LPS-induced p50/50 and p50/65 DNA binding, nuclear translocation of p50 protein and NF-κB-dependent transcriptional activity in a luciferase reporter. Inhibition of NF-κB may contribute to EGb 761-mediated inhibition of iNOS transcription. Although EGb 761 and quercetin also inhibit p44/42 MAPK phosphorylation, the use of the specific MEK1/2 inhibitor, UO126, demonstrates that p44/42 is not required for iNOS induction in RAW 264.7 macrophages. Collectively, our findings suggest that although EGb 761 and quercetin exhibit unique effects on components of LPS-induced signaling cascades, both EGb 761 and quercetin scavenge NO, inhibit LPS-induced iNOS mRNA and protein and inhibit p38 MAPK, which is important in iNOS transcription. Quercetin may therefore contribute to the

inhibition of iNOS by EGb 761.

Acknowledgements

The authors thank Dr. T Schmeller (Dr. Willmar Schwabe GmbH, Karlsruhe, Germany) for the kind gift of EGb 761 and Ms. Tanya Hudson and Ms.Tasha McDonald for technical assistance.

This work was supported by grants from the Oregon Wine Advisory Board and the National Institute on Alcohol Abuse and Alcoholism (AA08608).

IV. Discussion and Conclusions

The studies presented in this thesis address the effects of EGb 761 and the polyphenolic antioxidants resveratrol and quercetin on LPS-induced signaling cascades. *In vitro* studies with the immortalized peritoneal macrophage cell line RAW 264.7 have helped elucidate mechanisms by which quercetin and EGb 761 inhibit LPS-induced release of TNF-α, synthesis of iNOS, and production of NO. My studies suggest that quercetin may contribute to the action of EGb 761, yet it is not the sole active component. EGb 761 and quercetin exhibited selective effects on the MAPK pathway and the transcription factors NF-κB and AP-1. Both compounds suppressed NO release by scavenging NO and decreased LPS-induced iNOS transcription by inhibiting activation of p38 MAPK. Quercetin, on the other hand, was unique in its ability to inhibit TNF-α transcription, possibly by inhibiting activation of JNK/SAPK and therefore AP-1. Inhibition of AP-1 by quercetin may also contribute to the inhibition of iNOS transcription. EGb 761 exhibited a unique ability to suppress LPS-induced nuclear translocation of p50 and NF-κB-dependent transcriptional activity. Inhibition of NF-κB by EGb 761 may contribute to the inhibition of iNOS transcription, but has no effect on TNF-α transcription. The selective actions of EGb 761 and quercetin on the LPS-induced MAPK cascade and transcription factors NF-κB and AP-1 are summarized in Fig 1, a simplified scheme which incorporates data from the literature and my studies.

My findings have raised interesting new questions that may be addressed in future studies. A synopsis of several speculations and key issues that have emerged in the course of this project are presented below.

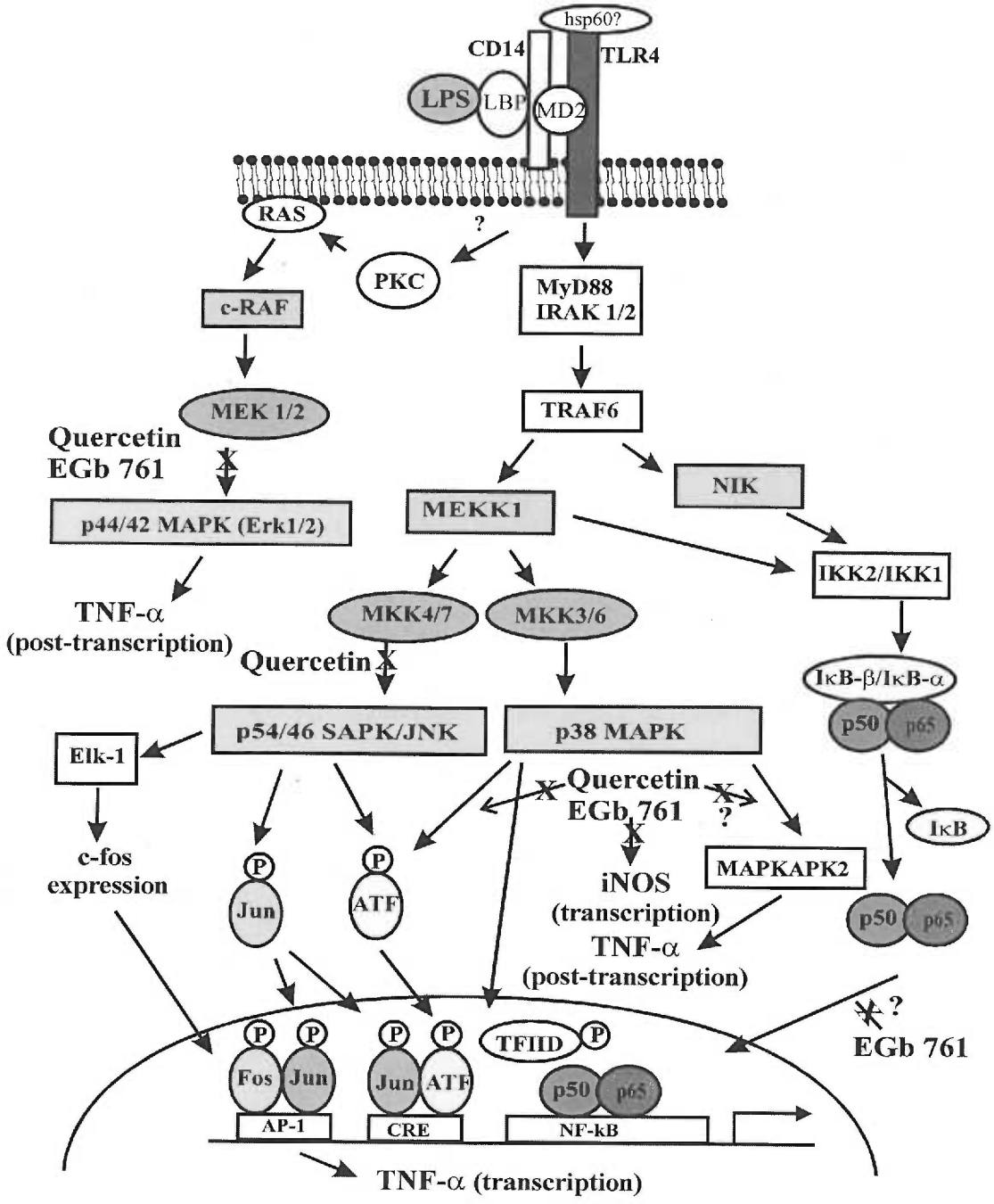


Fig 1. The selective actions of EGb 761 and quercetin on the LPS-induced MAPK cascade, NF-κB and AP-1 incorporating data from the literature and my studies.

A. Can the transcriptional effects of EGb 761 and quercetin on iNOS and TNF-α be more clearly defined?

Analysis of the effect of EGb 761, quercetin and MAPK inhibitors on LPS-induced AP-1 and NF-κB-dependent transcriptional activity was performed on RAW 264.7 cells transiently transfected with the luciferase gene driven by synthetic promoters containing basic TATA elements plus direct repeats of either AP-1 or NF-κB. Further examination of *cis*-elements that mediate iNOS and TNF-α transcription would help clarify the mechanism by which EGb 761 and quercetin inhibit transcription of these proteins. In addition, nuclear runoff assays would confirm that inhibition of LPS-induced iNOS or TNF-α mRNA is due to an inhibition of transcription rather than an effect on mRNA stability.

Literature describes the use of luciferase reporter plasmids containing the full length promoter region of murine macrophage-inducible iNOS (mac-NOS-luc) as well as deletion mutants of the mac-NOS 5' flanking region (Lowenstein *et al.*, 1993). As described in the Introduction, the 5' flanking region of the mac-NOS promoter contains binding sites for numerous transcription factors, including NF-κB, NF-IL6 and AP-1. RAW 264.7 cells, transiently transfected with macNOS-luc or its deletion mutants would illustrate the importance of the full length iNOS promoter and potential interactions between cis-elements. Transient transfection with a deletion mutant for the region which putatively binds AP-1, for instance, could be utilized to verify whether this transcription factor is involved in quercetin-mediated inhibition of LPS-induced iNOS. Transient transfection with a mutant containing a deleted or mutated NF-κB binding region would illustrate the importance of EGb 761-mediated inhibition of NF-κB in LPS-induced iNOS

transcription. Similar studies could be performed on the 5' flanking region of the murine TNF-α reporter. RAW 264.7 cells would be utilized for transient transfections in order to correlate results with my previous studies and because LPS-induced signaling mechanisms are well defined for this cell line.

My results from northern analysis show that quercetin inhibits steady state levels of LPS-induced TNF-α but does not decrease mRNA half-life, suggesting that quercetin inhibits TNF-α at a transcriptional level. Similarly, both EGb 761 and quercetin appear to inhibit iNOS transcription, as these substances inhibited steady state levels of LPS-induced iNOS mRNA without altering mRNA half life. Nuclear runoff would be an alternative and more sensitive procedure for confirming that quercetin inhibits the rate of TNF-α transcription and that EGb 761 and quercetin inhibit the rates of iNOS transcription. In addition, nuclear runoff would help confirm the importance of p38 MAPK on iNOS transcription. This was difficult to assess by northern analysis because SB203580 also decreased the half life of iNOS mRNA. Nuclei from treated cells would be isolated, incubated with ^{32}P-labeled UTP to label nascent RNA transcripts and hybridizied with cDNA probes to evaluate levels of LPS-induced iNOS or TNF-α mRNA in the presence or absence of EGb 761 or quercetin.

Quercetin inhibited LPS-induced transcription of TNF-α and iNOS and also blocked LPS-induced JNK/SAPK activity. If JNK/SAPK inhibition is the mechanism by which this flavonoid inhibits LPS-induced TNF-α and iNOS transcription, overexpression of SAPKβ would overcome quercetin-induced transcriptional inhibition. Swantek *et al.* (1997) utilized this technique to demonstrate that RAW 264.7 cells

cotransfected with a TNF-α translational reporter and wild type SAPKβ overcome dexamethasone-induced suppression of TNF-α translation. Cotransfection of wild type SAPKβ with a transcriptional reporter, or nuclear runoff analysis of RAW 264.7 cells transiently transfected with SAPKβ could be similarly utilized to address whether quercetin suppresses LPS-induced iNOS and TNF-α transcription by virtue of its effects on JNK/SAPK. Alternatively, a dominant inhibitor of SAPKβ would mimic the inhibitory effects of quercetin on LPS-induced iNOS and TNF-α mRNA if JNK/SAPK inhibition is important in iNOS and TNF-α transcriptional regulation. Swantek *et al.* (1997) confirmed the importance of JNK/SAPK in TNF-α translation by illustrating that transient transfection of RAW 264.7 macrophages with a kinase-defective mutant of JNK/SAPK blocked LPS-induced derepression of the translational blockade normally imposed by the TNF-α 3-UTR.

There is evidence to suggest that NF-κB and AP-1 work in concert to regulate gene transcription. However, very little is known about interactions between these transcripiton factors in cell-type-specific regulation of iNOS and TNF-α (Stein *et al.*, 1993). Cotransfection assays in Jurkat T cells (which do not possess endogenous C/EBPβ) with C/EBPβ, wild-type c-Jun and a TNF-α promoter-reporter construct demonstrated that C/EBPβ and c-Jun acted synergistically to activate the expression of TNF-α in phorbol myristate acetate (PMA)-treated Jurkat cells. In addition, U937 cells (that possess endogenous C/EBPβ) stably transfected with wild-type c-Jun secreted more TNF-α than control lines in response to PMA. EMSA using oligonucleotides derived from the TNF-α promoter suggested that C/EBPβ and c-Jun interact *in vitro* (Zagariya *et*

al., 1998). RAW 264.7 macrophages transfected with murine TNF-α or iNOS promoter-reporter constructs could be used to establish whether cells co-transfected with wild-type c-Jun secrete more TNF-α or iNOS than control. Oligonucleotides derived from the TNF-α and iNOS promoters could also be used in EMSAs to detect NF-κB/AP-1/DNA interactions.

B. Are repressor proteins involved in the inhibition of iNOS transcription?

Support for the possibility that repressor proteins may be involved in the inhibition of iNOS transcription by EGb 761 and quercetin is derived from studies by Chan and Riches (1998). These investigators describe the inhibition of iNOS transcription by IL-4 in macrophages stimulated with TNF-α and IFN-γ. IL-4, which activates the transcription factor STAT 6, inhibits iNOS transcription with only modest inhibition of JNK/SAPK and Erk1/2. By competing for binding to a region in the E-selectin gene promoter, which contains overlapping STAT6 and NF-κB binding sites, STAT 6 acts as an antagonist of NF-κB binding and transcriptional activation (Bennett *et al.*, 1997). My EMSA analyses, in which NF-κB/DNA binding was assessed using the NF-κB consensus oligonucleotide would not reveal NF-κB binding inhibition from overlapping transcription factor binding sites on the iNOS promoter.

A search for established consensus sequences for transcription factor binding sites on the murine iNOS promoter reveals that STAT-6 sites do not overlap with either of the NF-κB binding sites. The search did reveal, however, the presence of overlapping NF-κB and MZF1 (myeloid zinc finger-1) sites. MZF-1 contains several serine and threonine residues, potential phosphorylation sites for MAPK. Synthesis of an extended

oligonucleotide containing both the NF-κB sites and the MZF1 sites and examination of the effects of EGb 761 and quercetin on binding of LPS-induced nuclear proteins to this sequence would test whether MZF1 is involved in the inhibition of iNOS release by polyphenolics. Supershift analysis, using MZF1 antibodies would confirm whether MZF1 was a component of the DNA binding complex.

C. Do EGb 761 and quercetin post-transcriptionally inhibit LPS-induced TNF-α by impeding translational derepression?

As described in the Introduction, TNF-α mRNA translation is tightly controlled. TNF-α mRNA is translationally repressed in unstimulated cells, however LPS treatment results in repression of translational inhibition. MAPKAP kinase 2, a substrate of p38 MAPK is essential for TNF-α translation (Khokhlatchev *et al.,* 1998). We were not able to monitor phosphorylation of this kinase directly, however inhibition of p38 MAPK *in vitro* with ATF as a substrate suggest that this is a potential site for EGb 761 and quercetin action. The key element in translational regulation is an AU-rich region in the 3'-UTR of TNF-α mRNA. The literature describes translational reporter constructs containing either the CAT or luciferase coding sequences followed by the TNF-α 3'UTR (Han *et al.*, 1990b; Stein *et al.,* 1993). Reporter plasmids such as these, when transiently transfected into RAW 264.7 cells, could be utilized to examine the effects of EGb 761 and quercetin on TNF-α translation. If these compounds inhibit LPS-induced derepression of TNF-α translation, LPS-induced reporter activity would be inhibited. Section A of the Discussion describes the utilization of TNF-α translational reporting systems in concert with SAPKβ constructs to establish that glucocorticoids inhibit TNF-

α translation by blocking JNK/SAPK (Swantek *et al.,* 1997).

If future studies with translational reporter constructs suggest that EGb 761 and/or quercetin inhibit LPS-induced derepression of TNF-α, further studies could address the mechanism. Two proteins present in macrophage cytosolic extracts are known to form complexes in EMSA with the AU-rich sequence of TNF-α mRNA. Complex 1, which contains the RNA-binding protein TIAR, forms in both unstimulated and LPS-stimulated macrophages and is thought to be involved in translational repression (Gueydan *et al.,* 1999). Complex 2, containing a 55-kDA protein, is detected only after LPS treatment and is putatively involved in translational derepression (Lewis *et al.,* 1998). The two protein complexes differ in their electrophoretic mobilities and recognition motifs within the ARE (Gueydan *et al.,* 1999). It is possible that inhibition of p38 MAPK by quercetin or EGb 761 interferes with phosphorylation events that result in the binding of complex 2 to TNF-α mRNA in activated macrophages. EMSAs utilizing 3' UTR riboprobes and cytoplasmic extracts from RAW 264.7 macrophges could examine whether EGb 761 or quercetin alters formation of either complex 1 or 2 in the presence of LPS. Antibodies to TIAR would confirm the identity of complex 1 via supershift analysis.

D. Inflammatory cytokines are involved in the LPS-induced downregulation of hepatic drug metabolizing enzymes. What effect does EGb 761 have on this process?

Cytochrome P450 (CYP) enzymes constitute a family of proteins involved in the oxidative metabolism of many endogenous compounds, environmental chemicals, herbal components and drugs. It is well established that the ability of the liver to carry out cytochrome P450 dependent drug biotransformation is compromised during inflammation

or bacterial infection (Gorodischer *et al.*, 1976, Iber *et al.*, 1999). Administration of LPS to laboratory animals and cultured hepatocytes impairs catalytic activity and downregulates the expression of several CYP families (Morgan, 1997; Gorodischer *et al.*, 1976). Both *in vitro* and *in vivo* evidence indicates that TNF-α is involved in this phenomenon. Steady state levels of CYP1A2, 2C, 2E1 and 3A mRNA levels as well as ethoxyresorufin-O-deethylase (EROD) and nifedipine oxidation (which are indicative of CYP1A1/1A2 and CYP3A activity) are depressed in human hepatocytes following TNF-α treatment (Abdel-Razzak *et al.*, 1993). Expression of CYP 2C11 mRNA is depressed in rat hepatocytes treated with TNF-α (Sewer and Morgan, 1997). Pentoxifylline, which inhibits LPS-induced TNF-α production, selectively prevents LPS-induced downregulation of CYP1A2 and 2B in rats (Monshouwer *et al.*, 1996).

The involvement of nitric oxide in mediating decreased activity and expression of several P450 isozymes is controversial. Inhibition of catalytic activity is attributed to the interaction of NO with the heme-moiety and the nitrosylation of cytochrome P450 (Quaroni *et al.*, 1996). LPS-induced inhibition of CYP1A, 2B, 2C and 3A protein and activity in rat liver is prevented by NOS inhibitors (Khatsenko and Kikkawa, 1997). Studies by Sewer *et al.* (Sewer *et al.*, 1998) with iNOS knockout mice, however, suggest that LPS-induced downregulation of CYP2C, 3A and 2E1 occur independently of NO production from iNOS.

Bacterial endotoxin is cleared from the portal circulation by phagocytosis from Kupffer cells, which in turn become activated to produce ROS, NO, cytokines and eicosanoids (Knolle *et al.*, 1995; Decker, 1990). Kupffer cell activation is dependent on a

functional TLR4 as evidenced by studies illustrating that Kupffer cells from C3H/HeJ mice to not respond to LPS in the presence of LBP (Su *et al.*, 2000). Studies utilizing coculture of primary hepatocytes with Kupffer cells suggest that soluble factors released from Kupffer cells may be involved in LPS-induced downregulation of hepatic enzymes. The simultaneous treatment of coculture with phenobarbital and LPS, for instance, results in a strong down-regulation of phenobarbital-induced CYP2B1 in hepatocytes after 24 h of LPS treatment. This down regulation is mediated exclusively by TNF-α, as antibodies to TNF-α inhibit down-regulation, while treatment with the iNOS inhibitor 1-N(6)-(1-iminoethyl) lysine is without effect. Treatment of hepatocyte cultures with LPS in the absence of Kupffer cells does not result in TNF-α release (Milosevic *et al.*, 1999).

Because EGb 761 inhibits LPS-induced iNOS and TNF-α *in vivo* and because inhibitors of TNF-α and possibly iNOS protect against microsomal downregulation of CYP protein and activity in animals treated with LPS, EGb 761 may also protect against LPS-induced downregulation of hepatic drug metabolizing enzymes. I propose future studies evaluating the effects of EGb 761 pre-treatment on LPS-induced inhibition of CYP expression and activity. Hepatocytes and Kupffer cells can be isolated and maintained in culture for approximately 7 days. Methodology for coculture of Kupffer cells and hepatocytes and time-response relationships of TNF-α and NO release and CYP down regulation to LPS-treatment is available in the literature. The effects of EGb 761 on LPS-induced CYP down-regulation would be determined from enzyme activity, western analysis of S9 extracts, and northern analysis. If EGb 761 prevents LPS-induced CYP *in vitro*, the *in vivo* effects of EGb 761 pre-treatment on LPS-induced inhibition of

CYP expression and activity could then be determined. Because a variety of flavonoids inhibit cytochrome P450 activity (Doostdar *et al.*, 2000; Lee *et al.*, 1994a; Doostdar *et al.*, 2000), control studies would first be performed to evaluate the *in vivo* effect of EGb 761 alone on CYP expression and activity.

E. Are there other active ingredients in EGb 761?

As noted in the Introduction, EGb 761 is a well-defined but complex product consisting of flavonoid glycosides and unique terpenes. It is not known whether the effects of EGb 761 can be attributed to a single active ingredient or the combined action of the many components found in the extract. The differential effects of EGb 761 and quercetin on the MAPK cascade (described in manuscripts 2 and 3) suggest that quercetin is not the sole component responsible for the *in vivo* inhibition of LPS-induced activation of TNF-α and iNOS.

It is possible that the effects of EGb 761 can, in part, be attributed to antagonism of PAF by ginkgolide B (Braquet *et al.*, 1989). PAF is a potent autocoid involved in pathological conditions such as asthma, ischemia, septic shock and inflammatory bowel disease (Braquet *et al.*, 1987; Braquet and Hosford, 1991; Beutler *et al.*, 1985; Hsueh *et al.*, 1990). PAF and TNF-α stimulate each other's release via positive feedback mechanisms *in vivo* and *in vitro* (Im *et al.*, 1996; Poubelle *et al.*, 1991). Studies investigating the kinetics of PAF and TNF-α release in LPS-injected C57BL/6 mice indicated that PAF is released immediately after LPS injection and suggested that PAF induced TNF-α gene expression and protein synthesis via activation of NF-κB. Pre-treatment with BN 50739, a ginkgolide-derived synthetic PAF analogue, inhibited LPS-

induced NF-κB and TNF-α gene expression in vivo and in J774A.1 macrophages (Ling *et al.*, 1998). Pre-treatment with ginkgolide B significantly reduced neuronal damage in the hippocampus of rats subjected to forebrain ischemia (Oberpichler *et al.*, 1990).

The effects of EGb 761, ginkgolides A and B and bilobalide on post-ischemic myocardial free radical generation, *in vitro* free radical scavenging and/or antioxidant activity have been compared. Post-ischemic coronary effluent free radicals were decreased by both *in vivo* oral pre-treatment and *in vitro* perfusion with EGb 761 and individual terpenes. Ginkgolide A was the most effective. For each constituent, cardioprotective effects appeared to involve inhibition of free radical formation rather than direct free radical scavenging. Interestingly, functional recovery (as assessed by coronary flow and ventricular pressures) was greater for hearts reperfused with individual terpenes than with EGb 761. This phenomenon was attributed to the putative binding to and inactivation of antioxidant enzymes by phenolic polymer constituents of EGb 761 (Pietri *et al.*, 1997a).

Ginkgolides A, B and C, bilobalide , kaempferol, isorhamnetin and the glycoside of quercetin (rutin) are now available. Initial *in vitro* studies with RAW 264.7 cells, utilizing each component at a concentration that corresponds to their concentration in 200 μg/ml and 400 μg/ml EGb 761 would establish the involvement of each component in the inhibition of LPS-induced TNF-α and NO by EGb 761. If components other than quercetin exhibit inhibitory effects, then research could be expanded to evaluate the effects of these compounds on the MAPK cascade, iNOS and TNF-α transcription, translation, and mRNA stability.

F. Do EGb 761 and quercetin have selective effects on MAPK isoforms?

In the past ten years, cDNA cloning and expression studies have resulted in a literal explosion of information about MAPK family members. To date, at least ten different isoforms of JNK, four isoforms of p38 MAPK and three isoforms of Erk have been identified in mammalian tissues (Moriguchi *et al.*, 1997; Bost *et al.*, 1999; Wang *et al.*, 1998; Dowd *et al.*, 1998; Enslen *et al.*, 1998; Li *et al.*, 1996). Multiple isoforms may differ in tissue distribution, physiological function, be coupled to different upstream signalling pathways and/or differ in substrate specificities.

Within the p38 MAPK signal transduction pathway, for instance, p38 α and β isoforms are ubiquitously expressed, while p38γ is most prominent in muscle (Jiang *et al.*, 1997). The upstream MAPK kinases, MKK3 and MKK6 are coupled to distinct, but overlapping p38 MAPKS. For instance, MKK6 activates p38α, p38β and p38γ, while MKK3 activates p38α and p38γ (Enslen *et al.*, 1998). P38α and β have different substrate specificities, p38β being 20 times more effective in activating ATF2 than p38α. The α and β isoforms of p38 also have opposing effects on cardiac myosites, the β isoform contributing to hypertrophy and the α isoform to apoptosis (Wang *et al.*, 1998).

As the tools to monitor specific members of the MAPK family become available, it will be important to return to this model to analyze the effects of EGb 761 and quercetin on different MAPK isoforms. The p38 MAPK antibody used for immunoprecipitation kinase assays, for instance, did not differentiate between isoforms and it is possible that EGb 761 and quercetin exhibit differential inhibitory activity.

There are also multiple isoforms Jun. Activated AP-1 complexes can contain

different subunit compositions and the trans activating potential of AP-1 is determined by its subunit composition (Karin, 1995). LPS-induced AP-1 complexes from murine peritoneal macrophages, for instance, consist of c-Fos, Jun-B, Jun-D and c-Jun, while ceramide induced macrophages contain only Jun-D and c-Jun. (Medvedev *et al.,* 1999). In EMSA analysis, EGb and quercetin inhibit LPS-induced AP-1- DNA binding, but only c-Jun was analyzed by supershift analysis. Supershift analysis utilizing antibodies to other Jun family members would test whether quercetin affects other Jun family members. Supershift analysis could also be performed with antibodies to p50, p65 and c-Rel to determine whether interactions between AP-1 and NF-κB are occurring in LPS-induced macrophage nuclear extract.

G. Is the mechanism by which EGb 761 and quercetin inhibit TNF-α similar to the mechanism of endotoxin tolerance?

Strict regulation of TNF-α is necessary to safeguard against the potentially disastrous effects of rampant TNF-α secretion. When humans or experimental animals are exposed to prolonged or repeated exposure to LPS, increased resistance to the pyrogenic and toxic effects are observed. This phenomenon, termed endotoxin tolerance, is associated with marked decrease in the synthesis of proinflammatory cytokines such as TNF-α by macrophages and monocytes (Blackwell *et al.*, 1997; Sanchez-Cantu *et al.*, 1989; Virca *et al.*, 1989).

Four NF-κB sites in the murine TNF-α promoter (labeled κB1-4) are thought to contribute to transcriptional activation by LPS. An essential component in the downregulation of TNF-α in the LPS tolerant state is overexpression of the p50 subunit

of NF-κB and preferential binding of p50 homodimers to κB3 elements of the murine TNF-α promoter (Bohuslav *et al.*, 1998; Liu *et al.*, 2000). Extended treatment with LPS results in transcriptional activation of the p50 precursor, p105 but not other κB family members (Ziegler-Heitbrock *et al.*, 1994). Upregulation of p50 results in the formation of p50 homodimers, which do not bind IκB, but directly translocate to the nucleus, bind to κB3 and contribute to the inability of endotoxin tolerant murine macrophages to respond to LPS (Urban *et al.*, 1991; Shakhov *et al.*, 1990b; Collart *et al.*, 1990). C-rel can compete with p50 homodimers for the κB3 site (Bohuslav *et al.,* 1998). The binding characteristics of individual κB sites may therefore represent fine tuning mechanisms for pro-inflammatory mediators.

Manuscripts 1 and 2 address the *in vitro* and *in vivo* effects of natural product antioxidants on acute, not chronic LPS exposure. In keeping with this paradigm, endotoxin-tolerance does not appear to explain the inhibition of LPS-induced TNF-α by EGb 761 or quercetin. In EMSAs, the levels of p50/50 homodimer in nuclear extracts of LPS-treated macrophages did not increase, and actually appeared to decrease with EGb 761 or quercetin pre-treatment. Because EMSA in these studies utilized a generic NF-κB consensus oligonucleotide, to specifically rule out involvement of p50/50 homodimer in the downregultaion of TNF-α transcription by quercetin, a specific oligonucleotide for κB3 could be utilzed. Western blot analysis of cytoplasmic and nuclear levels of p50 protein in manuscript 3 indicated that pre-treatment with EGb 761 decreased and quercetin had no effect on nuclear translocation of p50 subunits and support the hypothesis that the effects of EGb 761 and quercetin on acute LPS-induced TNF-α are

not due to overexpression of p50/50 homodimers.

H. Do EGb 761 and quercetin alter non-LPS dependent activation of the MAPK cascade?

Mammalian MAPK modules are intertwined in a complex network of signaling cascades. Cross talk between MAPKs and other pathways such as those downstream of cAMP allows coordination, integration and amplification of biological responses to a given stimulus. In addition to LPS, members of the MAPK cascade can be activated by a host of stimuli, including heat shock, high osmolarity, H_2O_2, TNF-α, IL-6, IL-1, ceramide, arachidonate and the peptide urocortin (Han *et al.*, 1994; Raingeaud *et al.*, 1995; Rouse *et al.*, 1994; Freshney *et al.*, 1994, Schaeffer and Weber, 1999).

Activation of the MAPK cascade can lead to contrasting physiological responses. While LPS-induced activation of the MAPK in macrophages is implicated in the pathophysiology of ischemia reperfusion injury, urocortin protects against ischemia reperfusion injury in primary cardiac myocytes and isolated rat hearts *ex vivo* and it is proposed that this protective effect is mediated by the MAPK cascade. Binding of urocortin to a corticotrophin releasing factor receptor results in phosphorylation of Erk1/2. The protective effects of urocortin are abrogated by the Erk1/2 inhibitor, PD98059 (Brar *et al.*, 2000). Arachidonic acid stimulates the phosphorylation and activation of p38 MAPK in HL60 cells, HeLa cells, neutrophils and metastatic human mammary carcinoma cells and plays a role in adhesion of neutrophils to fibrinogen and carcinoma cells to type IV collagen. p38 MAPK activation by arachidonate may play a role in metastasis through the modulation of cell adhesion (Paine *et al.*, 2000; Hii *et al.*,

1998; Detmers *et al.*, 1998).

Although the physiological responses associated with a MAPK module are most likely specific for a given cell type and agonist, it is interesting to speculate on the ramifications of MAPK inhibition by dietary components or herbal supplements. What are the effects of EGb 761 and quercetin on urocortin induced phosphorylation of Erk or arachidonate-induced p38 MAPK activation? Does increased quercetin in the diet decrease susceptibility to tumor metastasis by inhibiting adhesion of metastatic cells? On the other hand, would quercetin abrogate the protective effects of urocortin on cardiovascular cells exposed to ischemia? Self administration and dietary supplementation of biologically active natural products such as EGb 761 and quercetin may lead to far reaching and unpredictable consequences which will only be discerned though wide reaching epidemiological studies that include careful dietary records.

I. Does induction of TNF-α contribute to the chemopreventative properties of resveratrol?

The wine polyphenolic resveratrol has been shown to exhibit cancer chemopreventative activity (Schneider *et al.*, 2000; Gautam *et al.*, 2000; Garcia-Garcia *et al.*, 1999; Nielsen *et al.*, 2000; Holmes-McNary and Baldwin, Jr., 2000). Cancer inhibitory properties are associated with induction of phase II drug-metabolizing enzymes and antioxidant ability (anti-initiation activity), anti-inflammatory effects from inhibition of COX and hydroperoxidase (anti-promotion) and induction of leukemia cell differentiation (anti-progression activity) (Jang *et al.*, 1997).

Manuscript 1 describes the effects of resveratrol on RAW 264.7 macrophages.

Resveratrol treatment resulted in an 8-18 fold increase in basal TNF-α mRNA expression with a concomitant increase in TNF-α secretion from undetectable levels to 100-500 pg/ml. Addition of resveratrol to LPS-treated macrophages resulted in a 1.5-fold increase in TNF-α secretion.

The mechanism of TNF-α enhancement by resveratrol was not pursued in my thesis however this observation leads to several interesting future studies. Transcriptional activation of the TNF-α promoter is down regulated by compounds such as prostaglandins, which increase intracellular levels of cAMP (Jongeneel, 1994). The inhibition of COX by resveratrol may therefore interfere with this negative feedback loop and this hypothesis could be tested by monitoring the effects of other COX inhibitors on LPS-induced TNF-α mRNA in RAW 264.7 cells.

An exciting possibility that emerges from this phenomenon is another potential explanation for the cancer chemopreventative properties of resveratrol. The induction of TNF-α by activated macrophages can lead to cytostatic and cytotoxic activities on malignant cells (Camussi *et al.,* 1991). Since there are well established differences in cellular responses to LPS (discussion section J), it would be of interest to determine whether resveratrol induces TNF-α in other TNF-α secreting cells such as tumor infiltrating lymphocytes (TILs), a subpopulation of CD4+ (helper) T cells that invade growing cancers. TILs possess autologous tumor-killing activity and participate in the anti tumor response by secreting cytokines such as IL-2, IFN-γ and TNF-α, which activate killer T-cells at the tumor site (Noguchi *et al.*, 1997; Vetto *et al.*, 1997).

Pretreatment of tumor cells with cytokines such as IFN-γ and TNF-α enhance

susceptibility to TIL-mediated lysis (Stotter *et al.*, 1989). TILs have been shown to mediate regression of metastatic tumors in human and murine models of adoptive immunotheraphy (Rosenberg *et al.*, 1986; Burger *et al.*, 1995). Studies investigating whether *in vitro* resveratrol treatment enhances TNF-α release and tumor cell lysis by murine TILs would clarify whether resveratrol-mediated cytokine release contributes to the antitumorigenic properties of this wine component.

J. Can an in vitro model from one representative cell type be used to address the in vivo mechanism of action?

Although cell culture is a relatively easy and rapid method for investigating molecular processes *in vitro*, the utilization of an immortalized cell line to assess the mechanism by which natural products inhibit LPS-induced cytokine release may not necessarily reflect what is occurring *in vivo* or in other primary macrophage populations. In addition, assessment of p38 MAPK inhibition by EGb 761 and quercetin was based in part on an in *vitro kinase* assay and there are limitations in attempting to generalize mechanisms of action based on such *in vitro* observations. *In vivo* compartmentalization or enzyme localization, for instance would not be observed.

The importance of exercising caution when attributing the actions of pharmacological agents in intact cells to effects observed *in vitro* are illustrated in recent studies evaluating the mechanism by which sodium salicylate inhibits NF-κB activation. Literature suggests that salicylate inhibits NF-κB activation by preventing the phosphorylation and subsequent degradation of IκBα (Kopp and Ghosh, 1994). However, the mechanism of inhibition of IκB kinase activity *in vitro* does not reflect the

inhibitory mechanism in intact cells. When intact COS-1 cells are treated with inflammaotry cytokines in the presence of salicylate and IKK is subsequently immunoprecipitated from these activated cells, salicylate selectively inhibits IκB kinase activity induced by TNF-α but not IL-1. Inhibition of TNF-α-induced IKK activity in whole cells is prevented by the p38 MAPK inhibitor, SB203580. In contrast, if IKK is first immunoprecipitated, then treated with salicylate, inhibition is neither selective for TNF-α or affected by SB203580 (Alpert and Vilcek, 2000).

Numerous reports suggest that the mechanisms of cytokine induction are also cell-type specific. Alveolar macrophages, for instance, respond to stimuli in a manner that is distinct from peritoneal macrophages, implying that alternative signaling pathways exists in these cells (Ryan and Vermeulen, 1995; Monick *et al.*, 1999; Monick *et al.*, 1998). For example, transcriptional mechanisms of COX-2 induction are agonist, context and cell-type specific. Serum and platelet derived growth factor (PDGF) mediate fibroblast COX-2 induction via JNK and Erk signaling pathways while IL-1β-induction of COX-2 in NIH3T3 and primary rat messangial cells involves JNK and p38 MAPK (Reddy *et al.*, 2000).

In studies directed at determining the requirement for the MEK/Erk pathway in inflammatory cytokine expression in distinct macrophage populations, Means *et al.* (2000b) found that the effects of the Erk inhibitor PD98059 were cytokine and cell-type specific but not strain specific. PD98059 inhibited TNF-α transcription and secretion in primary alveolar macrophages from C57BL/6 mice, BALB/c mice and C-8 cells (derived from alveolar macrophages from C57BL/6 mice), but did not inhibit transcription or

secretion in resident peritoneal macrophages from either C57BL/6 mice, BALB/c mice or RAW 264.7 macrophages (derived from peritoneal macrophages from BALB/c mice). LPS-induced IL-1β mRNA and protein were inhibited by PD98059 in all macrophage populations. Differences in MAPK-regulated transcription were not mediated by NF-κB, CREB or ATF2/c-Jun, suggesting a novel, yet-unidentified MAPK-dependent factor in activation of TNF-α in a cell-type-specific manner .

In recent preliminary studies, I evaluated the effects of EGb 761, quercetin and MAPK inhibitors on LPS/IFN-γ-induced peritoneal macrophages from C57BL/6 mice. Results, which are shown in Fig 1, Appendix A, illustrate that RAW 264.7 macrophages and peritoneal macrophages exhibit different responses to EGb 761 and MAPK inhibitors. While EGb 761 inhibited LPS-induced TNF-α in RAW 264.7 macrophages, it enhanced LPS/IFN-γ-induced TNF-α in primary peritoneal macrophages. UO126, which had no effect on LPS-induced NO release in LPS-induced RAW 264.7 macrophages, tended to decrease LPS/IFN-γ-induced NO in primary peritoneal macrophages. Figure 2, Appendix A, illustrates the effects of EGb 761 and quercetin on LPS-induced mRNA in peritoneal macrophages. EGb 761, quercetin and MAPK inhibitors have similar effects on LPS-induced iNOS and TNF-α mRNA when compared to RAW 264.7 macrophages.

Future studies should address whether the differential effects of EGb761 and UO126 on TNF-α and iNOS are due to strain specificities (RAW 264.7 macrophages are from BALB/c mice) or differences in signaling mechanisms between LPS and LPS/IFN-γ. For instance, induction of the murine iNOS promoter by LPS/IFNγ involves binding

of transcription factors to IFN-stimulated response elements (ISREs) and γ-IFN activated site (GAS), which binds Stat1α. A study evaluating the effects of EGb 761 and UO126 on LPS/IFNγ-induced TNF-α and iNOS in RAW 264.7 macrophages would determine whether the observed differences between the two models are due to IFNγ. *In vitro* studies evaluating the effect of EGb 761 on LPS-induced TNF-α and iNOS on BALB/c mice and comparison of these results from C57BL/6 mice would address questions of strain specificity.

Although all macrophages are ultimately derived from the bone marrow, individual types of macrophages exhibit functional specialization. Literature suggests that the release of reactive oxygen species and inflammatory cytokines from activated Kupffer cells, the resident macrophages of the liver, is instrumental in initiating the pathophysiology of conditions such as sepsis, endotoxemia, ischemia-reperfusion and alcoholic liver disease (Lands, 1995; Decker, 1990). In order to project my observations from RAW 264.7 macrophages to an explanation of the *in vivo* effects of EGb 761 on LPS-induced TNF-α and iNOS, it is therefore important to determine whether EGb 761, quercetin and MAPK inhibitors have the same effects on other macrophage populations such as Kupffer cells, peritoneal macrophages or glial cells in the brain.

V. Appendices

A. Unpublished Data

MATERIALS AND METHODS

Isolation of peritoneal macrophages

C57BL/6 mice were injected intraperitoneally with 3 ml 3% thioglycollate (Sigma Chemical Co., St. Louis, MO). Four days after thioglycollate injection, elicited peritoneal macrophages were harvested by peritoneal lavage, using 6 ml of sterile DMEM. Lavage fluids were pooled and centrifuged at 300g for 10 min at 4 °C. The cell pellet was washed in sterile 10 ml ice cold DMEM supplemented with 10% fetal bovine serum. Yield was approximately 6 x 10^6 cells/mouse. Cells were seeded in DMEM containing 50 units/ml penicillin, 50 μg/ml streptomycin and 10% fetal bovine serum at a density of 1 x 10^6 cells/well in a total volume of 1 ml in a 24 well tissue culture plate. After a 2 h incubation at 37 °C in a 5% CO_2 atmosphere, nonadherant cells were removed by washing with DMEM and fresh complete media added before the indicated treatments.

Measurement of LPS/γIFN-induced levels of TNF-α and NO

TNF-α was measured by the L929 fibroblast bioassay and NO by the Griess reaction, as described for LPS-induced in TNF-α and NO in manuscripts 2 and 3.

Reverse Transcription and Polymerase Chain Reaction (RT-PCR)

Total cellular RNA from macrophages was isolated by the Rneasy Mini Kit and Rnase-Free Dnase Set (Qiagen) following manufacturers instructions. cDNA was synthesized in a series of standard 20 μl RT reactions each containing 1.0 μg of total RNA, 2.5 μM random primers (Promega), 5x 1st Strand Buffer (Gibco-BRL), 1 mM

dNTP, 10 mM DTT, 2 U/μl Rnasin (Promega) and 10 U/μl M-MLV Reverse Transcriptase (Gibco-BRL). The reaction was incubated at 37^{0}C for 1 hour and 95^{0}C for 5 minutes. DNA amplification was performed in a standard 50 μl PCR which contained 2.5 μl of the RT reaction, 25 μl of HotStarTaq Master Mix (Qiagen) and 1 μl of each forward and reverse PCR primer (15 μM).

For TNF-α, the primers were:

Forward: 5'-AGCCCACGTCGTAGCAAACCACCAA-3'

Reverse: 5'-ACACCCATTCCCTTCACAGAGCAAT-3'

For iNOS, the primers were:

Forward: 5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3'

Reverse: 5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3'

The positive control primers for β-actin were:

Forward: 5'-GAGCTATGAGCTGCCTGACG-3'

Reverse: 5'-CACTTGCGGTGCACGATG-3'

Amplification reactions were carried out in a MJ PTC-200 Peltier Thermal Cycler with 40 sequential cycles at 94^{0}C for 1 min, 60^{0}C for 1 min and 72^{0}C for 1 min, followed by 7 minutes at 72^{0}C for final extension. An initial cycle of 15 minutes at 95^{0}C was required for activation of the HotStarTaq DNA Polymerase.

Transient transfections

The pAP-1- luc plasmid was transiently transfected into RAW 264.7 macrophages as described in Materials and Methods, Manuscript 2.

Results

Effects of EGb 761, quercetin, UO126 and SB203580 on LPS/γ-IFN induced TNF-α and NO production

In studies directed at comparing the effects of EGb 761, quercetin and MAPK inhibitors on a primary cell line, elicited peritoneal macrophages were isolated from C57BL/6 mice as described above. LPS alone is a poor inducer of TNF-α in peritoneal macrophages (Grigoriadis *et al.*, 1996), therefore cells were stimulated with LPS (1000 ng/ml) and recombinant murine γ-IFN (10^3 U/ml, Endogen, Woburn MA) for 24 h (for TNF-α) or 48 h (for nitrite). As shown in Fig 1a, EGb 761 (400 μg/ml) enhanced LPS/γ-IFN-induced release of TNF-α as measured by the L929 fibroblast assay. Quercetin (200 μM), UO 126 (20 μM) and SB203580(30 μM) inhbited LPS/γ-IFN-induced TNF-α release. Figure 1b illustrates that EGb 761 (400 μg/ml), quercetin (200 μM) and SB203580 (30 μM) decreased NO release. UO126 significantly decreased NO levels.

Effects of EGb 761, quercetin, UO126 and SB203580 on LPS/γIFN -induced TNF-α and iNOS mRNA

RT-PCR was used to analyze RNA from LPS/γIFN-induced peritoneal macrophages. As shown in Fig 2, quercetin (200 μM) decreased LPS/γIFN-induced TNF-α mRNA. Quercetin completely inhibited and EGb 761, UO126 and SB203580 decreased levels of LPS/γIFN-induced iNOS mRNA.

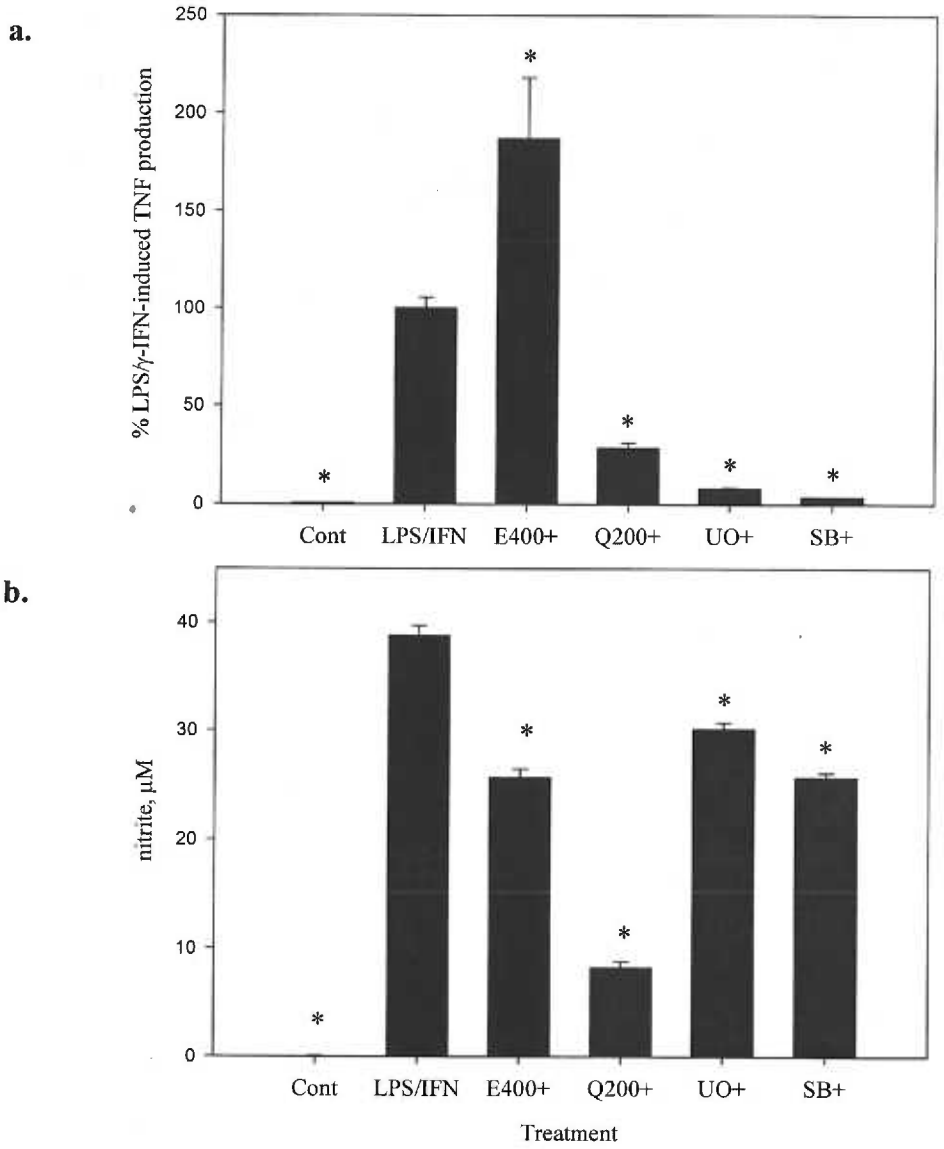


Fig 1. Effects of EGb 761, quercetin and MAPK inhibitors on LPS-γIFN induced release of TNF-α and NO from peritoneal macrophages. A. Elicited peritoneal macrophages were pretreated with DMSO only (Cont) or pretreated with DMSO (LPS/IFN), EGb 761 (E400+, 400 μg/ml), quercetin (Q200+, 200 μM), UO 126 (UO+, 20 μM) or SB203580 (SB+, 30 μM) for 1 h followed by treatment with LPS (1000 ng/ml) and murine γIFN (10^3 U/ml) for 24 h. Supernatants were collected and analyzed for TNF-α using the L929 fibroblast assay. B. Elicited peritoneal macrophages were pretreated as described above, then treated with LPS- γIFN for 48 h. Supernatants were collected and analyzed for nitrite using the Griess reagent. The data represents mean ± SE, n = 3. *, significantly different from treatment with LPS-γIFN.

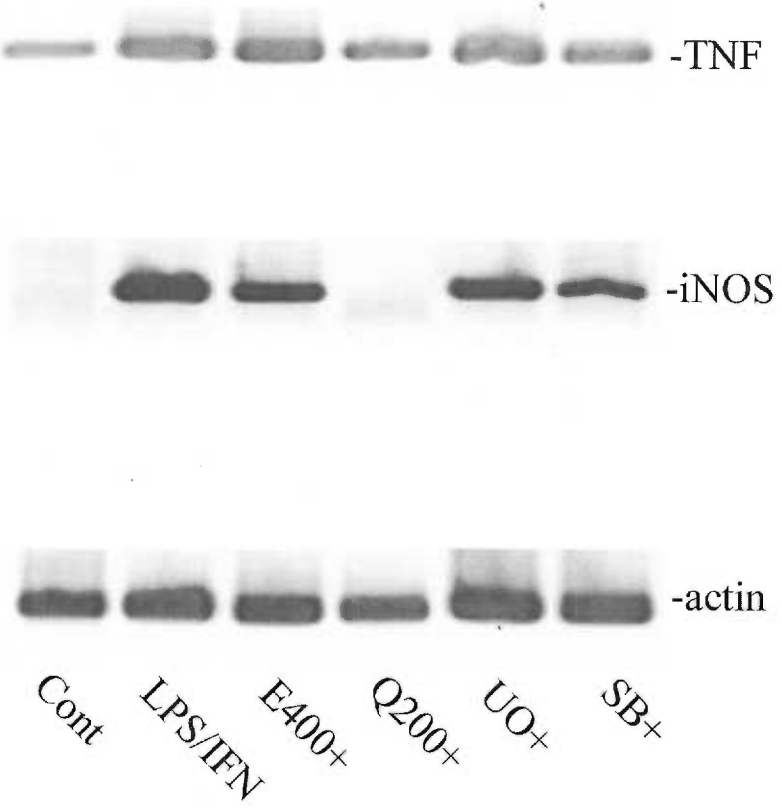


Fig 2. Effects of EGb 761, quercetin, UO 126 and SB203580 on LPS-γIFN induced iNOS and TNF-α mRNA from peritoneal macrophages. Elicited peritoneal macrophages were pretreated with DMSO only (Cont) or pretreated with DMSO (LPS/IFN), EGb 761 (E400+, 400 μg/ml), quercetin (Q200+, 200 μM), UO 126 (UO+, 20 μM) or SB203580 (SB+, 30 μM) for 1h followed by treatment with with LPS (1000 ng/ml) and murine γ-IFN (10^3 U/ml) for 6 h. Total cellular RNA was isolated and TNF-α, iNOS and actin RNA assessed by RTPCR as described in Materials and Methods.

Effects of EGb 761 and quercetin on AP-1 dependent transcriptional activity in RAW 264.7 macrophages

The TNF-α promoter contains the AP-1 sequence, which binds Fos-Jun heterodimers. Since quercetin inhibited LPS-induced c-Jun phosphorylation and AP-1-DNA binding, I examined whether transcriptional inhibition of TNF-α by quercetin may be due to inhibition of AP-1-dependent transcriptional activity. Transient transfection of RAW 264.7 macrophages with the pAP-1-luc reporter plasmid, containing the luciferase reporter gene driven by a basic promoter element (TATA box) plus seven repeats of the AP-1 binding site. LPS caused a 6-fold increase in luciferase activity compared to control (Fig 3). EGb 761 had no effect on LPS-induced luciferase activity. Surprisingly, quercetin increased LPS-induced luciferase activity in a dose-dependent fashion. Quercetin has been reported to activate luciferase activity in a HepG2-derived cell line stably integrated with a region of the human CYP1A2 5'-flanking gene containing two AP-1 binding sites linked to a luciferase reporter gene (Shih *et al.*, 2000). Future studies could address whether this phenomenon occurs in RAW 264.7 cells transiently transfected with a full length TNF-α promoter-reporter construct.

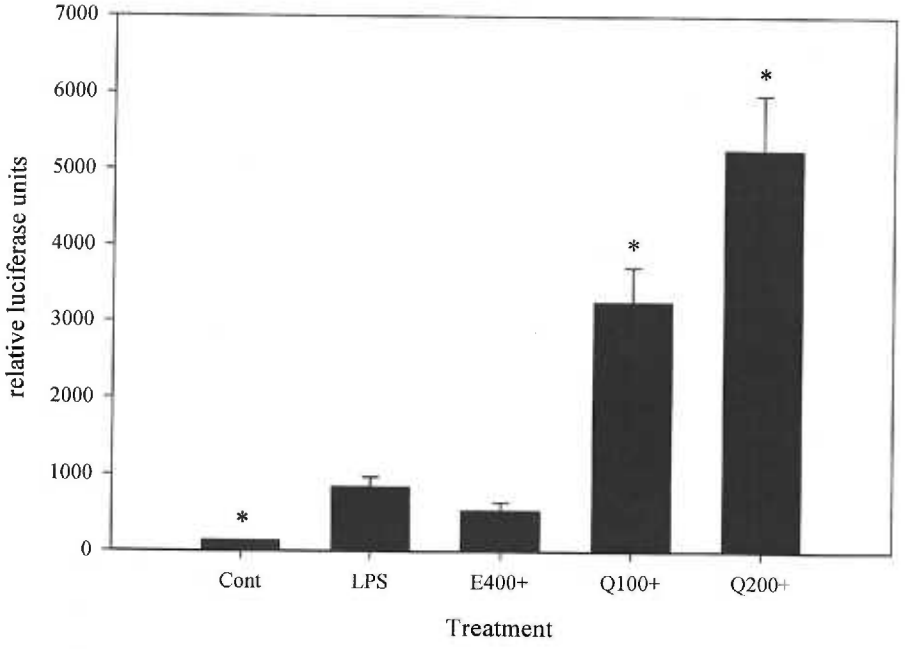
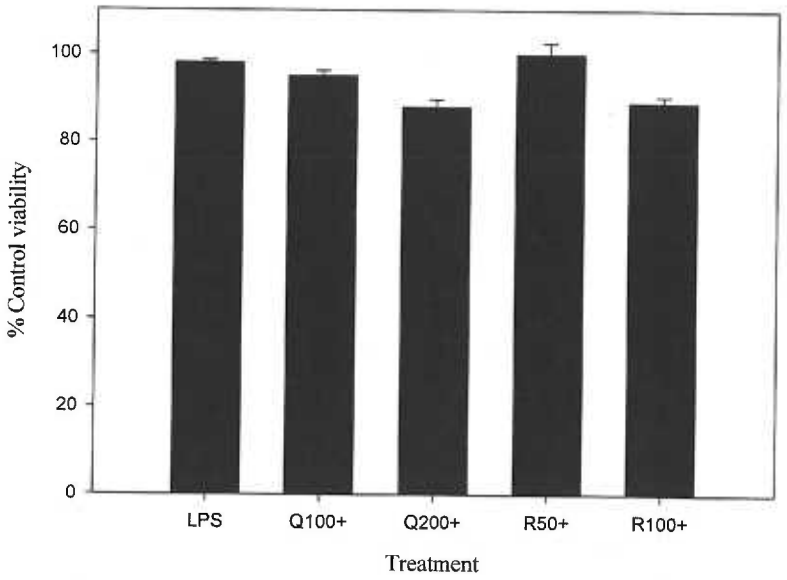


Fig 3. Effect of LPS, EGb 761 and quercetin on LPS-induced AP-1-dependent transcriptional activity in RAW 264.7 macrophages. Twenty four hours after transient transfection with pAP-1-luc, cells were treated with vehicle only (Cont) or pre-treated for one hour with DMSO (LPS, n=3), EGB 761 (E400+, 400 μg/ml), or quercetin (Q100+, 100 μM; Q200+, 200 μM) followed by a four hour treatment with LPS (1000 ng/ml). Cells were harvested for luciferase activity and protein determination. Data is expressed as relative luciferase activity normalized to protein and plotted as mean ± SE, n = 3. *, significantly different than LPS treatment, $p < 0.05$.

B. Data Not Shown

a.



b.

Sample	gelation
MilliQUFplus™ water	-
complete medium	-
Q200	-
R100	-
0.5% DMSO	-
negative control	-
positive control	+

Fig. 1. Cellular responses to LPS, quercetin and resveratrol are not due to general cellular toxicity or LPS contamination. a. RAW 264.7 cells were pre-treated for 1 h with DMSO (LPS), quercetin (Q100+, 100 μM; Q200+, 200 μM) or resveratrol (R50+, 50 μM; R100+, 100 μM), then treated with LPS (100 ng/ml) for 18 h. Uptake of the dye neutral red was measured as described in Materials and Methods, Manuscript 1. Viability was compared to control cells treated with DMSO only. The data represents mean ± SE, n = 3. *, significantly different from control cells, $P < 0.05$. b. Treatments other than LPS were not contaminated with bacterial endotoxin. Filtered water (MilliQUFplus™ water), complete medium, 200 μM quercetin (Q200), 100 μM resveratrol (R100) and 0.5% DMSO in complete medium, endotoxin free water (negative control) and 0.4 EU/ml endotoxin standard (positive control) were tested for the presence of gram-negative bacterial endotoxin by the E-Toxate™ (Limulus Amebocyte Lysate) test as described in Materials and Methods, Manuscript 1. A positive test is the formation of a hard gel.

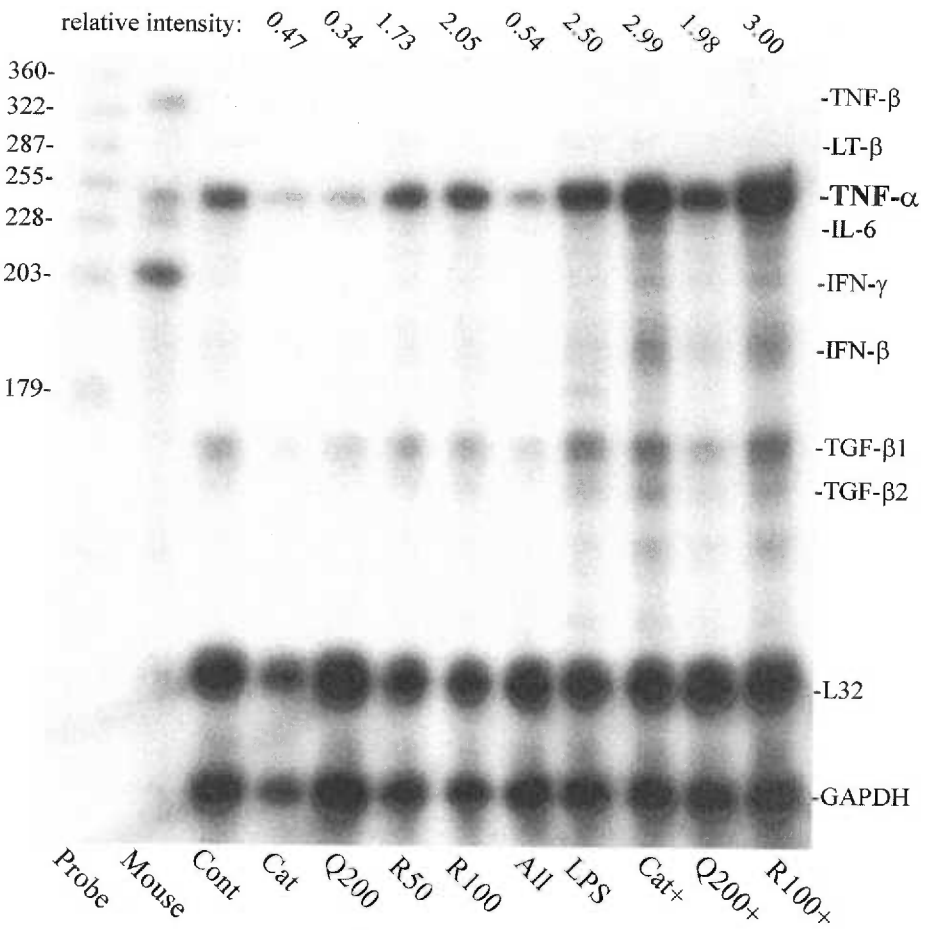


Fig 2. Analysis of cytokine mRNA expression in RAW 264.7 macrophages by RPA. Cells were cultured for 6 h with DMSO only (Cont), catechin (Cat, 600 μM), quercetin (Q200, 200 μM), resveratrol (R50, 50 μM; R100, 100 μM), 600 μM, catechin, 200 μM quercetin and 100 μM resveratrol (all), or pre-treated for 1 h with DMSO (LPS), Catechin (Cat+, 600 μM), quercetin (Q200+, 200 μM) or resveratrol (R100+, 100 μM), followed by 6h treatment with LPS (100 ng/ml). Total RNA was isolated and 10 μg subjected to RPA with ^{32}P-labeled antisense probes complimentary to the indicated mRNA species as described in Materials and Methods in Manuscript 1. Two housekeeping gene transcripts, L32 and GAPDH are included for normalization. Lane 1 represents a diluted probe set, which serves as a size marker. Lane 2 represent PharMingen positive RNA integrity control (total RNA from mouse thymoma cells cultured 4 h with phorbol ester and ionomycin). Similar results were obtained in a second, independent experiment. The intensity of each mRNA signal was quantified with a BioRad Molecular Imager using Bio-Rad Molecular Analyst software. The relative intensity of the TNF-α signal was obtained by normalizing each signal to both GAPDH and L32, then averaging these results. Abbreviations: LT, leukotriene; IL, interleukin; TGF, tissue growth factor.

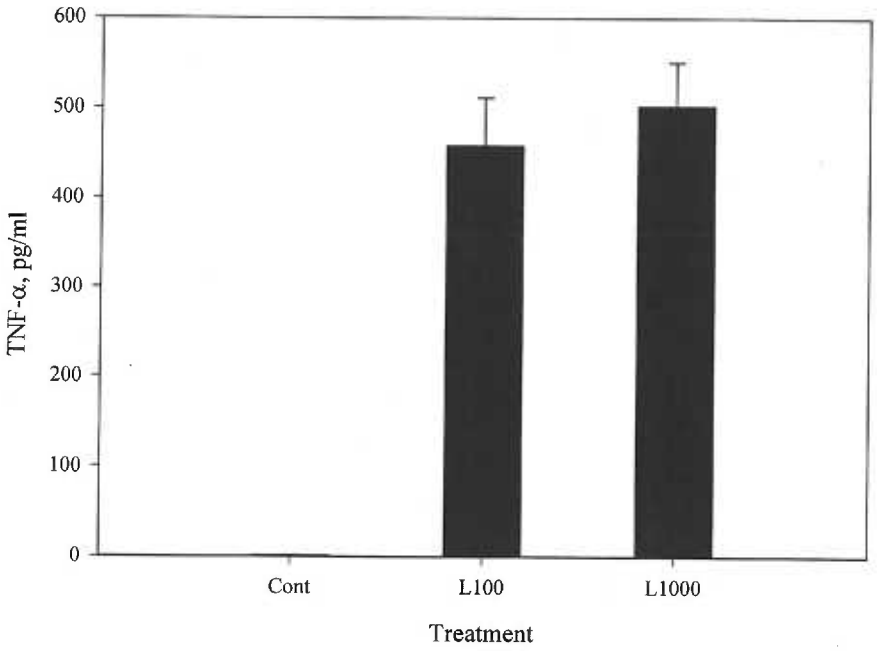
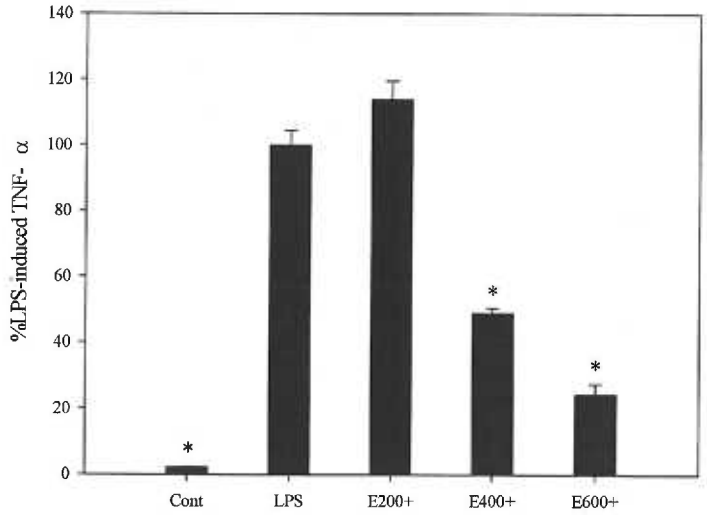


Fig 3. Levels of TNF-α are the same when RAW 264.7 cells are induced with 100 ng/ml or 1000 ng/ml LPS. RAW 264.7 cells were untreated (Cont) or treated with LPS (LPS 100, 100 ng/ml; L1000, 1000 ng/ml) for 18 h. Supernatants were collected and analyzed by the L929 fibroblast assay as described in Materials and Methods in Manuscript 2.

a.



b.

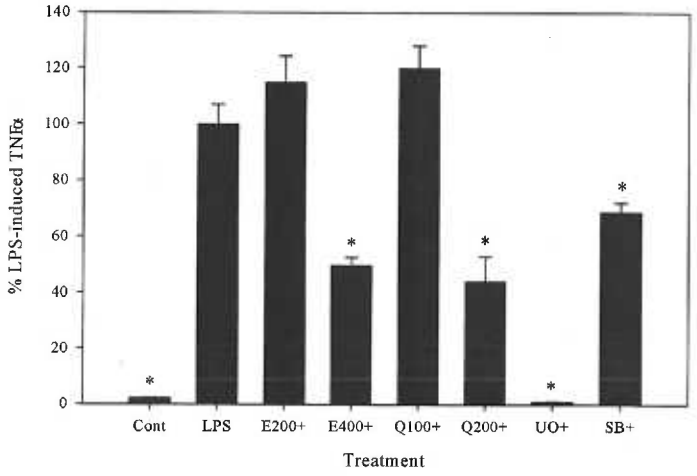


Fig 4. Dose response for the effects of EGb 761 on LPS-induced TNF-α and effects of EGb 761, quercetin, UO126 and SB203580 on LPS-induced TNF-α as measured by ELISA. a. RAW 264.7 macrophages were pre-treated with DMSO only (Cont) or with EGb 761 (E200+, 200 μg/ml; E400+, 400 μg/ml; E600+, 600 μg/ml) for 1 h followed by treatment with LPS (100 ng/ml, 18h). Supernatants were collected and analyzed by ELISA as described in Manuscripts 1 and 2. b. RAW 264.7 macrophages were pretreated with DMSO only (Cont) or with EGb 761 (E200+, 200 μg/ml; E400+, 400 μg/ml), quercetin (Q100+, 100 μM; Q200+, 200 μM), UO 126 (UO+, 20 μM) or SB203580 (SB+, 30 μM) for 1 h followed by treatment with LPS (100 ng/ml, 18 h). Supernatants were analyzed by ELISA. In each graph, data represents mean ± SE, n = 3. Similar results were observed in two independent experiments. *, significantly different from treatment with LPS only, $p<0.05$.

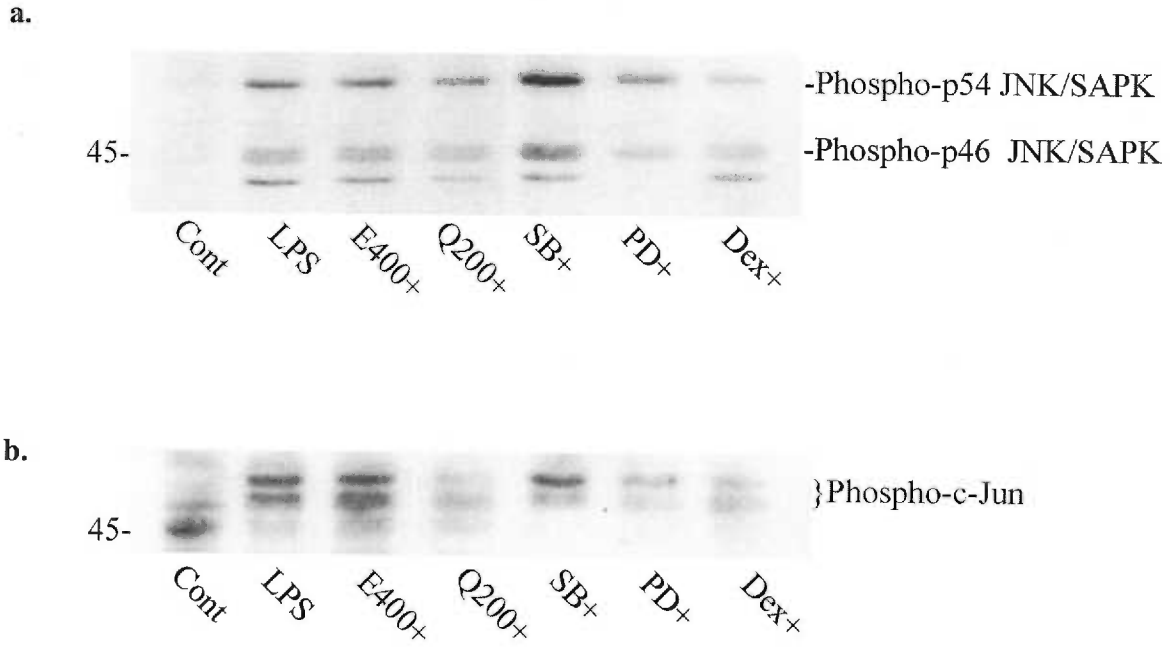


Fig 5. The MEK/Erk inhibitor, PD98059, at a concentration of 50 μM caused inhibition of LPS-induced phosphorylation of p46-JNK/SAPK and c-Jun. RAW 264.7 macrophages were unstimulated (Cont) or stimulated with LPS (100 ng/ml) for 15 min. Prior to LPS treatment, cells were pre-treated for 1 h with EGB 761 (E400+, 400 μg/ml), quercetin (Q200+, 200 μM), SB203580 (SB+, 30 μM), PD98059 (PD+, 50 μM) or dexamethasone (Dex+, 10 μM). Cell lysates were immunoblotted with a specific antibody to a. phospho-JNK/SAPK or b. phospho-c-Jun. In each case, two independently performed western analyses gave similar results. Dexamethasone, which has been shown to inhibit LPS-induced phosphorylation of JNK/SAPK and c-Jun in RAW 264.7 cells, was included as a positive control.

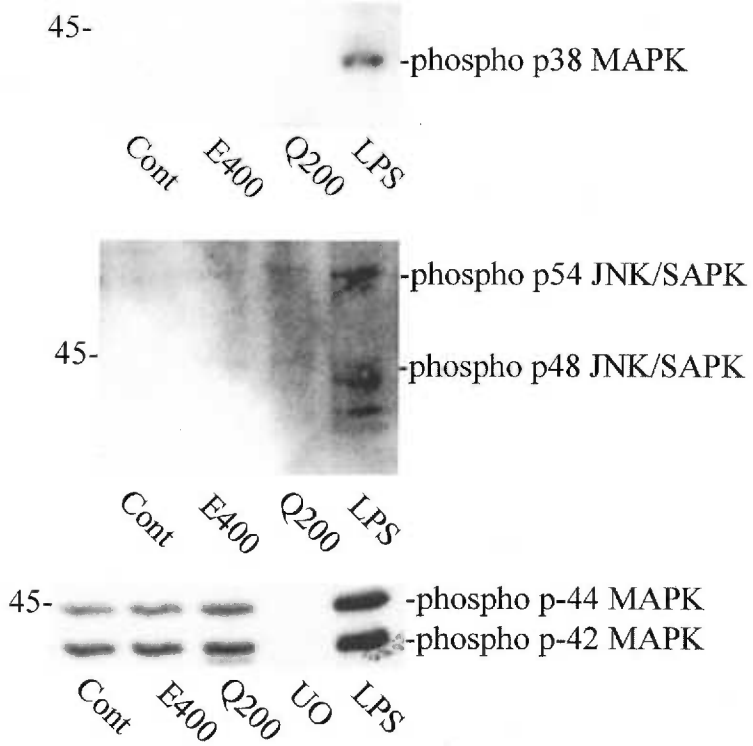


Fig 6. Compared to control, treatment with EGb 761 or quercetin in the absence of LPS had no effect on p38 MAPK, JNK/SAPK or p44/42 MAPK. RAW 264.7 macrophages were treated with DMSO only (Cont) or treated with 400 μg/ml EGb 761 (E400) 200 μM quercetin (Q200) or UO 126 (UO, 20 μM) for 1 h or with vehicle for 1 h then with 100 ng/ml LPS for 15 minutes (LPS). Cell lysates were immunoblotted with a specific antibody to a. Phospho p38 MAPK, b. Phospho JNK/SAPK or c. Phospho p44/42 MAPK.

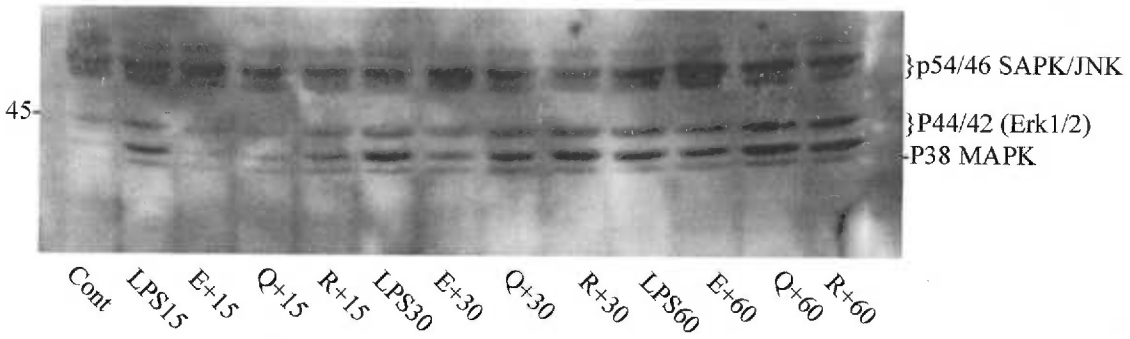


Fig 7. Maximal MAPK phosphorylation occurs 15-30 minutes following LPS stimulation. RAW 264.7 macrophages were unstimulated (Cont) or pre-treated with DMSO only, 400 μg/ml EGb 761 (E+), 200 μM quercetin (Q+) or 100 μM resveratrol (R+) for 1 h followed by treatment with LPS (100 ng/ml) for 15, 30 or 60 minutes. Cell lysates were immunoblotted with the general antiphosphotyrosine antibody 4G10 as described in Materials and Methods in Manuscript 2. Similar results were obtained in a second independent experiment.

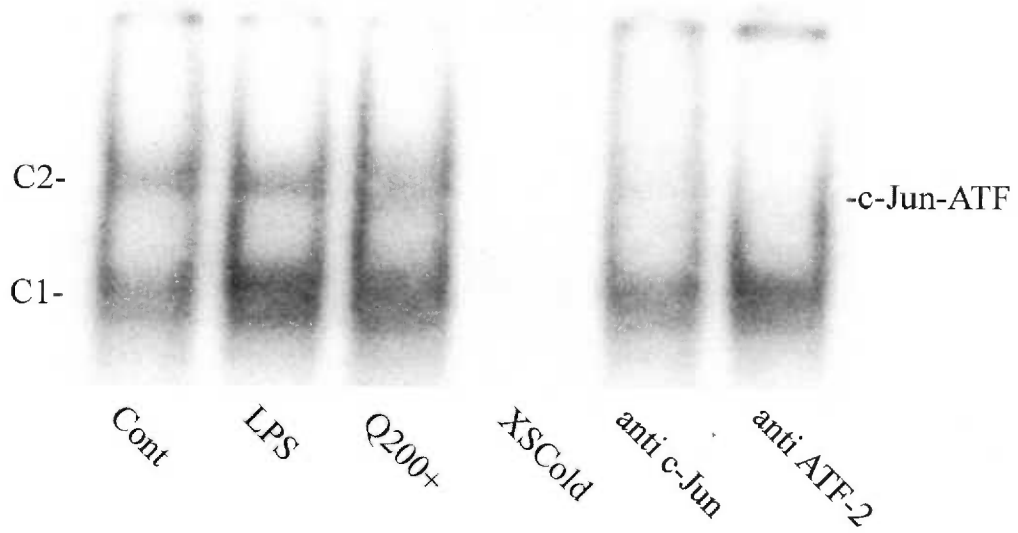


Fig. 8. EMSA did not reveal a dramatic difference in nucleoprotein binding between control and LPS-treated macrophages; quercetin had no effect on C-Jun/ATF heterodimer levels. Nuclear extracts were prepared and assayed for CRE induction by EMSA as described in Materials and Methods in Manuscript 2. RAW 264.7 cells were treated with DMSO only for 2 h (Cont) or pretreated with DMSO (LPS) or quercetin (Q200+, 200 μM) for 1 h followed by LPS (100 ng/ml, 1 h) . Competition and antibody analysis was performed on nuclear extracts from cells treated with LPS. XS cold: 100 fold excess unlabeled CRE consensus oligonucleotide. anti c-Jun: antibody to c-Jun/AP-1; anti ATF-2: antibody to ATF-2 (N-96). Similar results were obtained in two independent experiments.

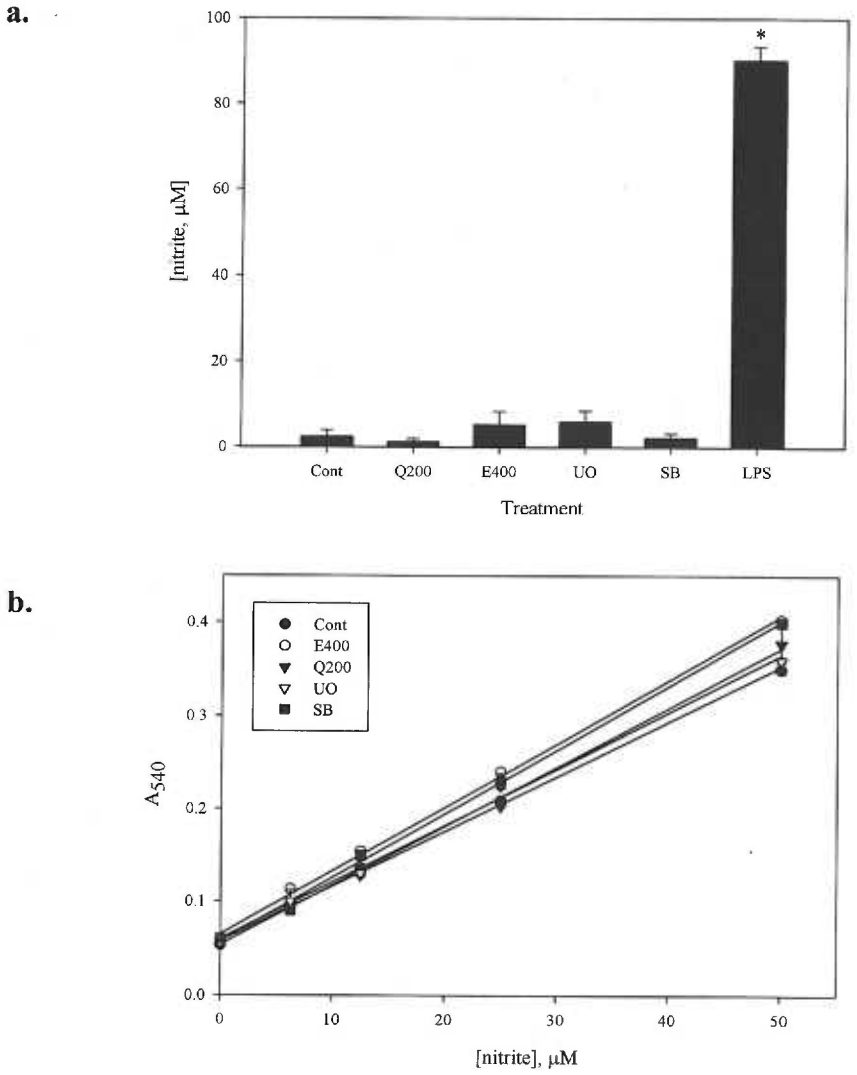


Fig 9. EGb 761, quercetin, UO126 and SB203580 alone had no effect on culture supernatant levels of NO and did not interfere with the Griess reaction when added to standard solutions of potassium nitrite. a. RAW 264.7 macrophages were pre-treated with vehicle only (Cont), quercetin (Q200, 200 μM) EGb 761 (E400, 400 μg/ml), UO 126 (UO, 20 μM), SB203580 (SB, 30 μM) or LPS (LPS, 100 ng/ml) for 18 h. Supernatants were collected and analyzed for nitrite using the Griess reagent. The data represents mean ± SE, n = 3. *, significantly different from control treatment, $P<0.05$. b. Standard solutions of potassium nitrite containing DMSO (Cont), EGb 761 (E400, 400 μg/ml), quercetin (Q200, 200 μM), UO 126 (UO, 20 μM) or SB203580 (SB, 30 μM) were prepared in tissue culture medium and added to an equal volume of Griess reagent and the absorbance at 540 nM measured as described in Materials and Methods, Manuscript 3.

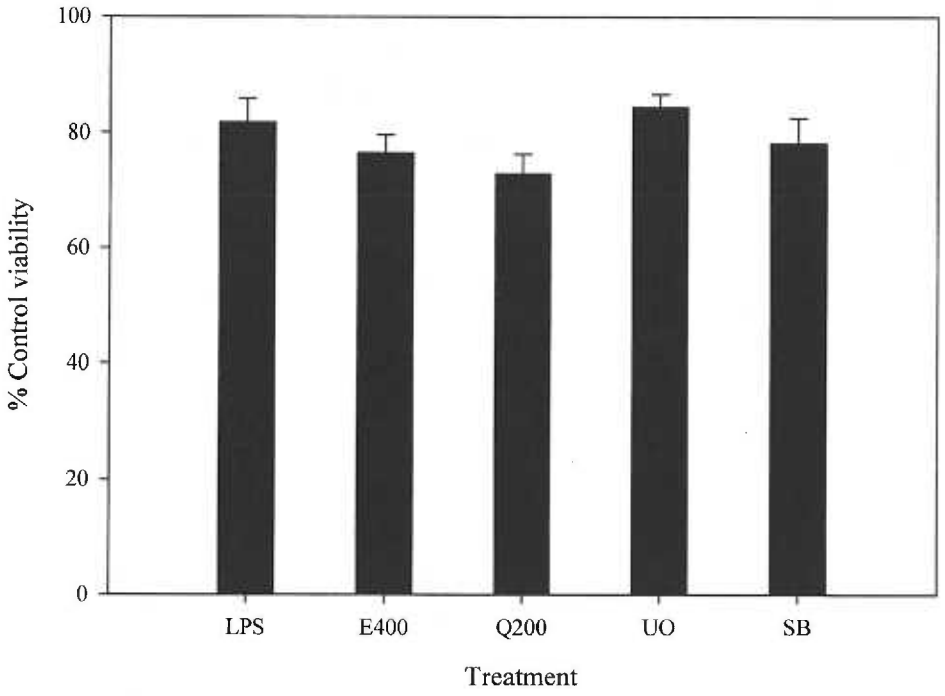


Fig 10. Crystal violet uptake assays confirmed that results were not due to general cellular toxicity. RAW 264.7 cells were treated with DMSO only (Cont), or pretreated for 1 h with DMSO (LPS), EGb 761 (E400, 400 μg/ml), quercetin (Q200, 200 μM, UO126 (UO, 20 μM) or SB203580 (SB, 30 μM), then treated with LPS (100 ng/ml) for18 h. Uptake of crystal violet was measured described in Materials and Methods, manuscripts 2 and 3. Uptake of crystal violet was compared to cells treated with DMSO only. The data represents mean ± SE.

VI. References

Abdel-Razzak Z, Loyer P, Fautrel A, Gautier JC, Corcos L, Turlin B, Beaune P, and Guillouzo A (1993) Cytokines down-regulate expression of major cytochrome P-450 enzymes in adult human hepatocytes in primary culture. *Mol.Pharmacol.* **44**:707-715.

Adachi O, Kawai T, Takeda K, Matsumoto M, Tsutsui H, Sakagami M, Nakanishi K, and Akira S (1998) Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity* **9**:143-150.

Aderem A and Ulevitch RJ (2000) Toll-like receptors in the induction of the innate immune response. *Nature* **406**:782-787.

Agullo G, Gamet L, Besson C, Demigne C, and Remesy C (1994) Quercetin exerts a preferential cytotoxic effect on active dividing colon carcinoma HT29 and Caco-2 cells. *Cancer Lett.* **87**:55-63.

Ajizian SJ, English BK, and Meals EA (1999) Specific inhibitors of p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways block inducible nitric oxide synthase and tumor necrosis factor accumulation in murine macrophages stimulated with lipopolysaccharide and interferon-gamma. *J.Infect.Dis.* **179**:939-944.

Akarasereenont P, Mitchell JA, Appleton I, Thiemermann C, and Vane JR (1994) Involvement of tyrosine kinase in the induction of cyclo-oxygenase and nitric oxide synthase by endotoxin in cultured cells. *Br.J.Pharmacol.* **113**:1522-1528.

Alessi DR, Cuenda A, Cohen P, Dudley DT, and Saltiel AR (1995) PD 098059 is a specific inhibitor of the activation of mitogen- activated protein kinase kinase in vitro and in vivo. *J.Biol.Chem.* **270**:27489-27494.

Alpert D and Vilcek J (2000) Inhibition of IkappaB kinase activity by sodium salicylate in vitro does not reflect its inhibitory mechanism in intact cells. *J.Biol.Chem.* **275**:10925-10929.

Angell M and Kassirer JP (1998) Alternative medicine-the risks of untested and unregulated remedies. *N.Engl.J.Med.* **339**:839-841.

Argento A, Tiraferri E, and Marzaloni M (2000) Oral anticoagulants and medicinal plants. An emerging interaction. *Ann.Ital.Med.Int.* **15**:139-143.

Baeuerle PA and Baltimore D (1996) NF-kappa B: ten years after. *Cell* **87**:13-20.

Baeuerle PA and Henkel T (1994) Function and activation of NF-kappa B in the immune system. *Ann.Rev.Immunol.* **12**:141-179.

Bastianetto S, Ramassamy C, Dore S, Christen Y, Poirier J, and Quirion R (2000) The

Ginkgo biloba extract (EGb 761) protects hippocampal neurons against cell death induced by beta-amyloid. *Eur.J.Neurosci.* **12**:1882-1890.

Belguendouz L, Fremont L, and Linard A (1997) Resveratrol inhibits metal ion-dependent and independent peroxidation of porcine low-density lipoproteins. *Biochem.Pharmacol.* **53**:1347-1355.

Bennett BL, Cruz R, Lacson RG, and Manning AM (1997) Interleukin-4 suppression of tumor necrosis factor alpha-stimulated E- selectin gene transcription is mediated by STAT6 antagonism of NF- kappaB. *J.Biol.Chem.* **272**:10212-10219.

Bethea JR, Ohmori Y, and Hamilton TA (1997) A Tandem GC Box Motif Is Necessary for Lipopolysaccharide-Induced Transcription of the Type II TNF Receptor Gene. *J.Immunol.* **158**:5815-5823.

Beutler B, Milsark IW, and Cerami AC (1985) Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. *Science* **229**:869-871.

Beyaert R, Cuenda A, Vanden Berghe W, Plaisance S, Lee JC, Haegeman G, Cohen P, and Fiers W (1996) The p38/RK mitogen-activated protein kinase pathway regulates interleukin-6 synthesis response to tumor necrosis factor. *EMBO J.* **15**:1914-1923.

Bhat NR, Zhang P, Lee JC, and Hogan EL (1998) Extracellular signal-regulated kinase and p38 subgroups of mitogen- activated protein kinases regulate inducible nitric oxide synthase and tumor necrosis factor-alpha gene expression in endotoxin-stimulated primary glial cultures. *J.Neurosci.* **18**:1633-1641.

Bird TA, Schule HD, Delaney PB, Sims JE, Thoma B, and Dower SK (1992) Evidence that MAP kinase activation may be necessary but not sufficient signal for a restricted subset of responses in Il-1-treated epidermoid cells. *Cytokine* **4**:429-440.

Blackwell TS, Blackwell TR, and Christman JW (1997) Induction of endotoxin tolerance depletes nuclear factor-kappaB and suppresses its activation in rat alveolar macrophages. *J.Leukoc.Biol.* **62**:885-891.

Blair WS, Bogerd HP, Madore SJ, and Cullen BR (1994) Mutational analysis of the transcription activation domain of RelA: identification of a highly synergistic minimal acidic activation module. *Mol.Cell Biol.* **14**:7226-7234.

Blume J, Kieser M, and Holscher U (1996) Placebo-controlled double-blind study of the effectiveness of Ginkgo biloba special extract EGb 761 in trained patients with intermittent claudication. *Vasa* **25**:265-274.

Bohuslav J, Kravchenko VV, Parry GC, Erlich JH, Gerondakis S, Mackman N, and Ulevitch RJ (1998) Regulation of an essential innate immune response by the p50 subunit

of NF-kappaB. *J.Clin.Invest* **102**:1645-1652.

Bors W, Heller W, Michel C, and Saran M (1990) Flavonoids as antioxidants: determination of radical-scavenging efficiencies. *Methods Enzymol.* **186**:343-355.

Bost F, McKay R, Bost M, Potapova O, Dean NM, and Mercola D (1999) The Jun kinase 2 isoform is preferentially required for epidermal growth factor-induced transformation of human A549 lung carcinoma cells. *Mol.Cell Biol.* **19**:1938-1949.

Bradbury AW, Murie JA, and Ruckley CV (1993) Role of the leucocyte in the pathogenesis of vascular disease. *Br.J.Surg.* **80**:1503-1512.

Braquet P, Drieu K, and Etienne A (1989) Le Ginkgolide B (BN52021): un puissant inhibiteur du PAF-acether isole du Ginkgo biloba. *Actual Chim Ther (Paris)* **13**:237-254.

Braquet P and Hosford D (1991) Ethnopharmacology and the development of natural PAF antagonists as therapeutic agents. *J.Ethnopharmacol.* **32**:135-139.

Braquet P, Touqui L, Shen TY, and Vargaftig BB (1987) Perspectives in platelet-activating factor research. *Pharmacol.Rev.* **39**:97-145.

Brar BK, Jonassen AK, Stephanou A, Santilli G, Railson J, Knight RA, Yellon DM, and Latchman DS (2000) Urocortin protects against ischemic and reperfusion injury via a MAPK- dependent pathway. *J.Biol.Chem.* **275**:8508-8514.

Brennan P and O'Neill LA (1996) 2-mercaptoethanol restores the ability of nuclear factor kappa B (NF kappa B) to bind DNA in nuclear extracts from interleukin 1-treated cells incubated with pyrollidine dithiocarbamate (PDTC). Evidence for oxidation of glutathione in the mechanism of inhibition of NF kappa B by PDTC. *Biochem.J.* **320**:975-981.

Brinkman BM, Telliez JB, Schievella AR, Lin LL, and Goldfeld AE (1999) Engagement of tumor necrosis factor (TNF) receptor 1 leads to ATF-2- and p38 mitogen-activated protein kinase-dependent TNF-alpha gene expression. *J.Biol.Chem.* **274**:30882-30886.

Brown MC and Taffet SM (1995) Lipoarabinomannans derived from different strains of Mycobacterium tuberculosis differentially stimulate the activation of NF-kappa B and KBF1 in murine macrophages. *Infect.Immun.* **63**:1960-1968.

Burger UL, Chang MP, Goedegebuure PS, Eberlein TJ, and Adams-Hodgins S (1995) Recruitment of host CD8+ T cells by tumor-infiltrating lymphocytes and recombinant interleukin-2 during adoptive immunotherapy of cancer. *Surgery* **117**:325-333.

Burr ML (1995) Explaining the French paradox. *J.Royal.Soc.Health* **115**:217-219.

Camargo CA, Jr., Stampfer MJ, Glynn RJ, Grodstein F, Gaziano JM, Manson JE, Buring

JE, and Hennekens CH (1997) Moderate alcohol consumption and risk for angina pectoris or myocardial infarction in U.S. male physicians. *Ann.Int.Med.* **126**:372-375.

Camussi G, Albano E, Tetta C, and Bussolino F (1991) The molecular action of tumor necrosis factor-alpha. *Eur.J.Biochem* **202**:3-14.

Cao Z, Henzel WJ, and Gao X (1996a) IRAK: a kinase associated with the interleukin-1 receptor. *Science* **271**:1128-1131.

Cao Z, Xiong J, Takeuchi M, Kurama T, and Goeddel DV (1996b) TRAF6 is a signal transducer for interleukin-1. *Nature* **383**:443-446.

Carballo E, Lai WS, and Blackshear PJ (1998) Feedback inhibition of macrophage tumor necrosis factor-alpha production by tristetraprolin. *Science* **281**:1001-1005.

Carter AB, Knudtson KL, Monick MM, and Hunninghake GW (1999a) The p38 mitogen-activated protein kinase is required for NF-kappaB- dependent gene expression. The role of TATA-binding protein (TBP). *J.Biol.Chem.* **274**:30858-30863.

Carter AB, Monick MM, and Hunninghake GW (1999b) Both Erk and p38 kinases are necessary for cytokine gene transcription. *Am.J.Respir.Cell Mol.Biol.* **20**:751-758.

Castelli D, Colin L, Camel E, and Ries G (1998) Pretreatment of skin with a Ginkgo biloba extract/sodium carboxymethyl- beta-1,3-glucan formulation appears to inhibit the elicitation of allergic contact dermatitis in man. *Contact Dermatitis* **38** :123-126.

Cesarani A, Meloni F, Alpini D, Barozzi S, Verderio L, and Boscani PF (1998) Ginkgo biloba (EGb 761) in the treatment of equilibrium disorders. *Adv.Ther* **15**:291-304.

Chan ED and Riches DW (1998) Potential role of the JNK/SAPK signal transduction pathway in the induction of iNOS by TNF-alpha. *Biochem.Biophys.Res.Commun.* **253**:790-796.

Chan ED, Winston BW, Uh ST, Wynes MW, Rose DM, and Riches DW (1999) Evaluation of the role of mitogen-activated protein kinases in the expression of inducible nitric oxide synthase by IFN-gamma and TNF- alpha in mouse macrophages. *J.Immunol.* **162**:415-422.

Chan MM (1995) Inhibition of tumor necrosis factor by curcumin, a phytochemical. *Biochem.Pharmacol.* **49**:1551-1556.

Chan MM, Ho C, and Huang H (1995) Effects of three dietary phytochemicalas from tea, rosemary and turmeric on inflammation-induced NO production. *Cancer Lett.* **96**:23-29.

Chan TY, Chan JC, Tomlinson B, and Critchley JA (1993) Chinese herbal medicines revisited: a Hong Kong perspective. *Lancet* **342**:1532-1534.

Chanmugam P, Feng L, Liou S, Jang BC, Boudreau M, Yu G, Lee JH, Kwon HJ, Beppu T, and Yoshida M (1995) Radicicol, a protein tyrosine kinase inhibitor, suppresses the expression of mitogen-inducible cyclooxygenase in macrophages stimulated with lipopolysaccharide and in experimental glomerulonephritis. *J.Biol.Chem.* **270**:5418-5426.

Chanvitayapongs S, Draczynska-Lusiak B, and Sun AY (1997) Amelioration of oxidative stress by antioxidants and resveratrol in PC12 cells. *NeuroReport* **8**:1499-1502.

Chen C, Chen YH, and Lin WW (1999a) Involvement of p38 mitogen-activated protein kinase in lipopolysaccharide-induced iNOS and COX-2 expression in J774 macrophages. *Immunology* **97** :124-129.

Chen CC and Wang JK (1999) p38 but not p44/42 mitogen-activated protein kinase is required for nitric oxide synthase induction mediated by lipopolysaccharide in RAW 264.7 macrophages. *Mol.Pharmacol.* **55**:481-488.

Chen F, Kuhn DC, Sun SC, Gaydos LJ, and Demers LM (1995) Dependence and Reversal of Nitric Oxide Production on NF-kB in Silica and Lipopolysaccharide-Induced Macrophages. *Biochem. Biophys.Res.Commun.* **214**:839-846.

Chen W, Syldath U, Bellmann K, Burkart V, and Kolb H (1999b) Human 60-kDa heat-shock protein: a danger signal to the innate immune system. *J.Immunol.* **162**:3212-3219.

Chen Y, Yang L, and Lee TJ (2000) Oroxylin A inhibition of lipopolysaccharide-induced iNOS and COX-2 gene expression via suppression of nuclear factor-kappaB activation. *Biochem.Pharmacol.* **59**:1445-1457.

Chow JC, Young DW, Golenbock DT, Christ WJ, and Gusovsky F (1999) Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J.Biol.Chem.* **274**:10689-10692.

Chu SC, Marks-Konczalik J, Wu HP, Banks TC, and Moss J (1998) Analysis of the cytokine-stimulated human inducible nitric oxide synthase (iNOS) gene: characterization of differences between human and mouse iNOS promoters. *Biochem.Biophys.Res.Commun.* **248**:871-878.

Cobb MH and Goldsmith EJ (1995) How MAP kinases are regulated. *J.Biol.Chem.* **270**:14843-14846.

Collart MA, Baeuerle P, and Vassalli P (1990) Regulation of tumor necrosis factor alpha transcription in macrophages: involvement of four kappa B-like motifs and of constitutive and inducible forms of NF-kappa B. *Mol.Cell Biol.* **10**:1498-1506.

Craig WJ (1999) Health-promoting properties of common herbs. *Am.J.Clin.Nutr.* **70**:491S-499S.

Csokay B, Prajda N, Weber G, and Olah E (1997) Molecular mechanisms in the antiproliferative action of quercetin. *Life Sci.* **60**:2157-2163.

Cuenda A, Rouse J, Doza YN, Meier R, Cohen P, Gallagher TF, Young PR, and Lee JC (1995) SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.* **364**:229-233.

de Vries JH, Hollman PC, Meyboom S, Buysman MN, Zock PL, Van Staveren WA, and Katan MB (1998) Plasma concentrations and urinary excretion of the antioxidant flavonols quercetin and kaempferol as biomarkers for dietary intake. *Am.J.Clin.Nutr.* **68**:60-65.

de Vries JH, Janssen PL, Hollman PC, Van Staveren WA, and Katan MB (1997) Consumption of quercetin and kaempferol in free-living subjects eating a variety of diets. *Cancer Lett.* **114**:141-144.

Decker K (1990) Biologically active products of stimulated liver macrophages (Kupffer cells). *Eur.J.Biochem.* **192**:245-261.

Dehmlow C, Erhard J, and de Groot H (1996) Inhibition of Kupffer cell functions as an explanation for the hepatoprotective properties of silibinin. *Hepatology* **23**:749-754.

Derijard B, Hibi M, Wu IH, Barrett T, Su B, Deng T, Karin M, and Davis RJ (1994) JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* **76**:1025-1037.

Derijard B, Raingeaud J, Barrett T, Wu IH, Han J, Ulevitch RJ, and Davis RJ (1995) Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. *Science* **267**:682-685.

Detmers PA, Zhou D, Polizzi E, Thieringer R, Hanlon WA, Vaidya S, and Bansal V (1998) Role of stress-activated mitogen-activated protein kinase (p38) in beta 2-integrin-dependent neutrophil adhesion and the adhesion-dependent oxidative burst. *J.Immunol.* **161**:1921-1929.

Di Carlo G, Mascolo N, Izzo AA, and Capasso F (1999) Flavonoids: old and new aspects of a class of natural therapeutic drugs. *Life Sci.* **65**:337-353.

Diamond BJ, Shiflett SC, Feiwel N, Matheis RJ, Noskin O, Richards JA, and Schoenberger NE (2000) Ginkgo biloba extract: mechanisms and clinical indications. *Arch.Phys.Med.Rehabil.* **81**:668-678.

Dignam JD, Lebovitz RM, and Roeder RG (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11**:1475-1489.

Doostdar H, Burke MD, and Mayer RT (2000) Bioflavonoids: selective substrates and inhibitors for cytochrome P450 CYP1A and CYP1B1. *Toxicology* **144**:31-38.

Dorman DC, Cote LM, and Buck WB (1992) Effects of an extract of Gingko biloba on bromethalin-induced cerebral lipid peroxidation and edema in rats. *Am.J.Vet.Res.* **53**:138-142.

Dowd S, Sneddon AA, and Keyse SM (1998) Isolation of the human genes encoding the pyst1 and Pyst2 phosphatases: characterisation of Pyst2 as a cytosolic dual-specificity MAP kinase phosphatase and its catalytic activation by both MAP and SAP kinases. *J.Cell Sci.* **111**:3389-3399.

Drieu K (1986) Preparation and definition of Ginkgo biloba extract. *Presse Med.* **15**:1455-1457.

Du X, Poltorak A, Silva M, and Beutler B (1999) Analysis of Tlr4-mediated LPS signal transduction in macrophages by mutational modification of the receptor. *Blood Cells Mol.Dis.* **25**:328-338.

Dudley DT, Pang L, Decker SJ, Bridges AJ, and Saltiel AR (1995) A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc.Natl.Acad.Sci.U.S.A* **92**:7686-7689.

Eberhardt W, Kunz D, and Pfeilschifter J (1994) Pyrrolidine dithiocarbamate differentially affects interleukin 1 beta- and cAMP-induced nitric oxide synthase expression in rat renal mesangial cells. *Biochem. Biophys.Res.Commun.* **200**:163-170.

Enslen H, Raingeaud J, and Davis RJ (1998) Selective activation of p38 mitogen-activated protein (MAP) kinase isoforms by the MAP kinase kinases MKK3 and MKK6. *J.Biol.Chem.* **273**:1741-1748.

Erlund I, Kosonen T, Alfthan G, Maenpaa J, Perttunen K, Kenradi J, Parantainen, J, Aro A (2000) Pharmacokinetics of quercetin from quercetin aglycone and rutin in healthy volunteers. *Eur.J.Clin.Pharmacol* **56**:545-553.

Evans T, Carpenter A, Kinderman H, and Cohen J (1993) Evidence of increased nitric oxide production in patients with the sepsis syndrome. *Circ.Shock* **41**:77-81.

Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, and Trzaskos JM (1998) Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J.Biol.Chem.* **273**:18623-18632.

Feng L, Jang BC, and Hwang D (1997) Inhibitor of protein tyrosine kinase, radicicol, suppresses the expression of cyclooxygenase and pro-inflammatory cytokines in LPS-stimulated rat alveolar macrophage in part by accelerating degradation of mRNA. *Adv.Exp.Med.Biol.* **407**:281-288.

Ferriola PC, Cody V, and Middleton E, Jr. (1989) Protein kinase C inhibition by plant flavonoids. Kinetic mechanisms and structure-activity relationships. *Biochem.Pharmacol.* **38**:1617-1624.

Fitzpatrick DF, Hirschfield SL, and Coffey RG (1993) Endothelium-dependent vasorelaxing activity of wine and other grape products. *Am.J.Physiol.* **265**:H774-8.

Fontanarosa PB and Lundberg GD (1998) Alternative medicine meets science. *JAMA* **280**:1618-1619.

Foresti R, Clark JE, Green CJ, and Molterlini R (1997) Thiol Compounds Interact with Nitric Oxide in Regulating Heme Oxygenase-1 Induction in Endothelial Cells. *J.Biol.Chem.* **272**:118411-18417.

Fourtillan JB, Brisson AM, Girault J, Ingrand I, Decourt JP, Drieu K, Jouenne P, and Biber A (1995) Pharmacokinetic properties of Bilobalide and Ginkgolides A and B in healthy subjects after intravenous and oral administration of Ginkgo biloba extract (EGb 761). *Therapie* **50**:137-144.

Frankel EN, Kanner J, German JB, Parks E, and Kinsella JE (1993) Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. *Lancet* **341**:454-457.

Fransen L, Muller R, Marmenout A, Tavernier J, Van der HJ, Kawashima E, Chollet A, Tizard R, Van Heuverswyn H, and Van Vliet A (1985) Molecular cloning of mouse tumour necrosis factor cDNA and its eukaryotic expression. *Nucleic Acids Res.* **13**:4417-4429.

Freshney NW, Rawlinson L, Guesdon F, Jones E, Cowley S, Hsuan J, and Saklatvala J (1994) Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of Hsp27. *Cell* **78**:1039-1049.

Garcia-Garcia J, Micol V, de Godos A, and Gomez-Fernandez JC (1999) The cancer chemopreventive agent resveratrol is incorporated into model membranes and inhibits protein kinase C alpha activity. *Arch.Biochem.Biophys.* **372**:382-388.

Gardner AM and Johnson GL (1996) Fibroblast Growth Factor-2 Suppression of Tumor Necrosis Factor a-Mediated Apoptosis Requires RAS and the Activation of Mitogen-activated Protein Kinase. *J.Biol.Chem.* **271**:14560-14566.

Gautam SC, Xu YX, Dumaguin M, Janakiraman N, and Chapman RA (2000) Resveratrol selectively inhibits leukemia cells: a prospective agent for ex vivo bone marrow purging. *Bone Marrow Transplant.* **25**:639-645.

Geppert TD, Whitehurst CE, Thompson P, and Beutler B (1994) Lipopolysaccharide signals activation of tumor necrosis factor biosynthesis through the ras/raf-

1/MEK/MAPK pathway. *Mol.Med.* **1**:93-103.

Gillies RJ, Didier N, and Denton M (1986) Determination of cell number in monolayer cultures. *Anal.Biochem.* **159**:109-113.

Gordon S (1995) The macrophage. *Bioessays* **17**:977-986.

Gorodischer F, Krasner J, McDevitt JJ, Nolan JP, and Yaffe SJ (1976) Hepatic microsomal drug metabolism after administration of endotoxin in rats. *Biochem.Pharmacol.* **25**:351-353.

Grigoriadis G, Zhan Y, Grumont RJ, Metcalf D, Handman E, Cheers C, and Gerondakis S (1996) The Rel subunit of NF-kappaB-like transcription factors is a positive and negative regulator of macrophage gene expression: distinct roles for Rel in different macrophage populations. *EMBO J.* **15**:7099-7107.

Gronbaek M, Becker U, Johansen D, Gottschau A, Schnohr P, Hein HO, Jensen G, and Sorensen TI (2000) Type of alcohol consumed and mortality from all causes, coronary heart disease, and cancer. *Ann.Intern.Med.* **133**:411-419.

Gronbaek M, Deis A, Sorensen TI, Becker U, Schnohr P, and Jensen G (1995) Mortality associated with moderate intakes of wine, beer, or spirits. *BMJ.* **310**:1165-1169.

Gross SS and Wolin MS (1995) Nitric oxide: pathophysiological mechanisms. *Annu.Rev.Physiol.* **57**:737-769.

Gueydan C, Droogmans L, Chalon P, Huez G, Caput D, and Kruys V (1999) Identification of TIAR as a protein binding to the translational regulatory AU-rich element of tumor necrosis factor alpha mRNA. *J.Biol.Chem.* **274**:2322-2326.

Hagiwara M, Inoue S, Tanaka T, Nunoki K, Ito M, and Hidaka H (1988) Differential effects of flavonoids as inhibitors of tyrosine protein kinases and serine/threonine protein kinases. *Biochem.Pharmacol.* **37**:2987-2992.

Hambleton J, Weinstein SL, Lem L, and DeFranco AL (1996) Activation of c-Jun N-terminal kinase in bacterial lipopolysaccharide- stimulated macrophages. *Proc.Natl.Acad.Sci.U.S.A.* **93**:2774-2778.

Han J, Brown T, and Beutler B (1990a) Endotoxin-responsive sequences control cachectin/tumor necrosis factor biosynthesis at the translational level *J.Exp.Med.* **171**:465-475.

Han J, Huez G, and Beutler B (1991) Interactive effects of the tumor necrosis factor promoter and 3'- untranslated regions. *J.Immunol.* **146**:1843-1848.

Han J, Lee JD, Bibbs L, and Ulevitch RJ (1994) A MAP kinase targeted by endotoxin

and hyperosmolarity in mammalian cells. *Science* **265**:808-811.

Han J, Lee JD, Tobias PS, and Ulevitch RJ (1993) Endotoxin induces rapid protein tyrosine phosphorylation in 70Z/3 cells expressing CD14. *J.Biol.Chem.* **268**:25009-25014.

Han J, Thompson P, and Beutler B (1990b) Dexamethasone and pentoxifylline inhibit endotoxin-induced cachectin/tumor necrosis factor synthesis at separate points in the signaling pathway. *J.Exp.Med.* **172**:391-394.

Hardman JG, Molinoff PB, Ruddon RW, and Gilman AG (1996) *The Pharmacological Basis of Therapeutics*. McGraw-Hill.

Hickman P, McCollum PT, and Belch JJ (1994) Neutrophils may contribute to the morbidity and mortality of claudicants. *Br.J.Surg.* **81**:790-798.

Hii CS, Huang ZH, Bilney A, Costabile M, Murray AW, Rathjen DA, Der CJ, and Ferrante A (1998) Stimulation of p38 phosphorylation and activity by arachidonic acid in HeLa cells, HL60 promyelocytic leukemic cells, and human neutrophils. Evidence for cell type-specific activation of mitogen-activated protein kinases. *J.Biol.Chem.* **273**:19277-19282.

Hollman PC, Gaag M, Mengelers MJ, van Trijp JM, de Vries JH, and Katan MB (1996) Absorption and disposition kinetics of the dietary antioxidant quercetin in man. *Free Radic.Biol.Med.* **21**:703-707.

Hollman PC, van Trijp JM, Mengelers MJ, de Vries JH, and Katan MB (1997) Bioavailability of the dietary antioxidant flavonol quercetin in man. *Cancer Lett.* **114**:139-140.

Holmes-McNary M and Baldwin AS, Jr. (2000) Chemopreventive properties of trans-resveratrol are associated with inhibition of activation of the IkappaB kinase. *Cancer Res.* **60**:3477-3483.

Hortelano S, Genaro AM, and Bosca L (1992) Phorbol esters induce nitric oxide synthase activity in rat heaptocytes. Antagonism with the induction elicited by lipopolysaccharide. *J.Biol.Chem.* **267**:24937-24940.

Hsueh W, Sun X, Rioja LN, and Gonzalez-Crussi F (1990) The role of the complement system in shock and tissue injury induced by tumour necrosis factor and endotoxin. *Immunology* **70** :309-314.

Hu ZL and Qian DH (1993) Effects of protein kinase C activator and inhibitor on release of tumor necrosis factor from mouse peritoneal macrophages. *Acta Pharm.Sin.* **14**:183-186.

Hwang D, Jang BC, Yu G, and Boudreau M (1997) Expression of mitogen-inducible cyclooxygenase induced by lipopolysaccharide: mediation through both mitogen-activated protein kinase and NF-kappaB signaling pathways in macrophages. *Biochem.Pharmacol.* **54**:87-96.

Iber H, Sewer MB, Barclay TB, Mitchell SR, Li T, and Morgan ET (1999) Modulation of drug metabolism in infectious and inflammatory diseases. *Drug Metab.Rev.* **31**:29-41.

Im SY, Han SJ, Ko HM, Choi JH, Chun SB, Lee DG, Ha TY, and Lee HK (1997) Involvement of nuclear factor-kappa B in platelet-activating factor- mediated tumor necrosis factor-alpha expression. *Eur.J.Immunol.* **27**:2800-2804.

Im SY, Ko HM, Kim JW, Lee HK, Ha TY, Lee HB, Oh SJ, Bai S, Chung KC, Lee YB, Kang HS, and Chun SB (1996) Augmentation of tumor metastasis by platelet-activating factor. *Cancer Res.* **56**:2662-2665.

Inoue Y, Trevanich S, Tsujimoto Y, Miki T, Miyabe S, Sugiyama K, Izawa S, and Kimura A (1996) Evaluation of catechin and its derivatives as antioxidant: Recovery of growth arrest of *Escherichia coli* under oxidative conditions. *J.Sci.Food Agric.* **71**:297-300.

Itil T and Martorano D (1995) Natural substances in psychiatry (Ginkgo biloba in dementia). *Psychopharmacol.Bull.* **31**:147-158.

Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW, Fong HH, Farnsworth NR, Kinghorn AD, Mehta RG, Moon RC, and Pezzuto JM (1997) Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* **275**:218-220.

Janssens D, Michiels C, Guillaume G, Cuisinier B, Louagie Y, and Remacle J (1999) Increase in circulating endothelial cells in patients with primary chronic venous insufficiency: protective effect of Ginkor Fort in a randomized double-blind, placebo-controlled clinical trial. *J.Cardiovasc.Pharmacol.* **33**:7-11.

Jeon YJ, Kim YK, Lee M, Park SM, Han SB, and Kim HM (2000) Radicicol suppresses expression of inducible nitric-oxide synthase by blocking p38 kinase and nuclear factor-kappaB/Rel in lipopolysaccharide- stimulated macrophages. *J.Pharmacol.Exp.Ther.* **294**:548-554.

Jiang Y, Gram H, Zhao M, New L, Gu J, Feng L, Di Padova F, Ulevitch RJ, and Han J (1997) Characterization of the structure and function of the fourth member of p38 group mitogen-activated protein kinases, p38delta. *J.Biol.Chem.* **272**:30122-30128.

Jongeneel CV (1994) Regulation of the TNF-alpha gene. *Prog.Clin.Biolog.Res.* **388**:367-381.

Joyeux M, Rolland A, Fleurentin J, Mortier F, and Dorfman P (1990) Tert-Butyl Hydroperoxide-Induced Injury in Isolated Rate Hepatocytes: A Model for Studying Anti-Hepatotoxic Crude Drugs. *Planta Medica* **56**:171-173.

Kamijo R, Harada H, Matsuyama T, Bosland M, Gerecitano J, Shapiro D, Le J, Koh SI, Kimura R, Green SJ, Mak TW, Taniguchi T, and Vilcek J (1994) Requirement for transcription factor IRF-1 in NO synthase induction in macrophages. *Science* **263**:1612-1615.

Karin M (1995) The regulation of AP-1 activity by mitogen-activated protein kinases. *J.Biol.Chem.* **270**:16483-16486.

Katiyar SK, Agarwal R, and Mukhtar H (1994) Inhibition of spontaneous and photo-enhanced lipid peroxidation in mouse epidermal microsomes by epicatechin derivatives from green tea. *Cancer Lett.* **79**:61-66.

Kawada N, Seki S, Inoue M, and Kuroki T (1998) Effect of antioxidants, resveratrol, quercetin, and N-acetylcysteine, on the functions of cultured rat hepatic stellate cells and Kupffer cells. *Hepatology* **27**:1265-1274.

Kerr LD, Ransone LJ, Wamsley P, Schmitt MJ, Boyer TG, Zhou Q, Berk AJ, and Verma IM (1993) Association between proto-oncoprotein Rel and TATA-binding protein mediates transcriptional activation by NF-kappa B. *Nature* **365**:412-419.

Keyse SM (1998) Protein phosphatases and the regulation of MAP kinase activity. *Semin.Cell Dev.Biol.* **9**:143-152.

Khatsenko O and Kikkawa Y (1997) Nitric oxide differentially affects constitutive cytochrome P450 isoforms in rat liver. *J.Pharmacol.Exp.Ther.* **280**:1463-1470.

Khokhlatchev AV, Canagarajah B, Wilsbacher J, Robinson M, Atkinson M, Goldsmith E, and Cobb MH (1998) Phosphorylation of the MAP kinase ERK2 promotes its homodimerization and nuclear translocation. *Cell* **93**:605-615.

Kleijnen J and Knipschild P (1992) Ginkgo biloba [see comments]. *Lancet* **340**:1136-1139.

Knolle P, Schlaak J, Uhrig A, Kempf P, Meyer zum Buschenfelde KH, and Gerken G (1995) Human Kupffer cells secrete IL-10 in response to lipopolysaccharide (LPS) challenge. *J.Hepatol.* **22**:226-229.

Kobuchi H, Droy-Lefaix M, Christen Y, and Packer L (1997) Ginkgo biloba Extract (EGb 761): Inhibitory Effect on Nitric Oxide Production in the Macrophage Cell Line RAW 264.7. *Biochem.Pharmacol.* **53**:897-903.

Kobuchi H and Packer L (1997) Bio-normalizer modulates interferon-gamma-induced

nitric oxide production in the mouse macrophage cell line RAW 264.7. *Biochem.Mol.Biol.Int.* **43**:141-152.

Kobuchi H, Roy S, Sen CK, Nguyen HG, and Packer L (1999) Quercetin inhibits inducible ICAM-1 expression in human endothelial cells through the JNK pathway. *Am.J.Physiol.* **277**:C403-C411.

Koc RK, Akdemir H, Kurtsoy A, Pasaoglu H, Kavuncu I, Pasaoglu A, and Karakucuk I (1995) Lipid peroxidation in experimental spinal cord injury. Comparison of treatment with Ginkgo biloba, TRH and methylprednisolone. *Res.Exp.Med.(Berl)* **195**:117-123.

Kondo K, Matsumoto A, Kurata H, Tanahashi H, Koda H, Amachi T, and Itakura H (1994) Inhibition of oxidation of low-density lipoprotein with red wine. *Lancet* **344**:1152.

Kopp E and Ghosh S (1994) Inhibition of NF-kappa B by sodium salicylate and aspirin. *Science* **265**:956-959.

Kretz-Remy C, Mehlen P, Mirault ME, and Arrigo AP (1996) Inhibition of I kappa B-alpha phosphorylation and degradation and subsequent NF-kappa B activation by glutathione peroxidase overexpression. *J.Cell Biol.* **133**:1083-1093.

Kuo SM, Morehouse HF, Jr., and Lin CP (1997) Effect of antiproliferative flavonoids on ascorbic acid accumulation in human colon adenocarcinoma cells. *Cancer Lett.* **116**:131-137.

Kuprash DV, Udalova IA, Turetskaya RL, Rice NR, and Nedospasov SA (1995) Conserved kB element located downstream of the tumor necrosis factor a gene: distinct NF-kB binding patternn and enhancer activity in LPS activated murine macrophages. *Oncogene* **11**:97-106.

Kyriakis JM, Banerjee P, Nikolakaki E, Dai T, Rubie EA, Ahmad MF, Avruch J, and Woodgett JR (1994) The stress-activated protein kinase subfamily of c-Jun kinases. *Nature* **369**:156-160.

Lands WE (1995) Cellular signals in alcohol-induced liver injury: a review. *Alcohol.Clin.Exp.Res.* **19**:928-938.

Laskin DL and Pendino KJ (1995) Macrophages and inflammatory mediators in tissue injury. *Annu.Rev.Pharmacol.Toxicol.* **35**:655-677.

Le Bars PL, Katz MM, Berman N, Itil TM, Freedman AM, and Schatzberg AF (1997) A placebo-controlled, double-blind, randomized trial of an extract of Ginkgo biloba for dementia. North American EGb Study Group. *JAMA* **278**:1327-1332.

Lee H, Wang HW, Su HY, and Hao NJ (1994a) The structure-activity relationships of

flavonoids as inhibitors of cytochrome P-450 enzymes in rat liver microsomes and the mutagenicity of 2-amino-3-methyl-imidazo[4,5-f]quinoline. *Mutagenesis* **9**:101-106.

Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, McNulty D, Blumenthal MJ, Heys JR, and Landvatter SW (1994b) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature* **372**:739-746.

Lee JC and Young PR (1996) Role of CSB/p38/RK stress response kinase in LPS and cytokine signaling mechanisms. *J.Leukoc.Biol.* **59**:152-157.

Lee YW, Han SH, Lee M, Yang KH, Kim HM, and Jeon YJ (2000) 2-Amino-3-methylimidazo[4,5-f]quinoline inhibits nitric oxide production in lipopolysaccharide-stimulated RAW 264.7 cells by blocking p38 kinase activation. *Cancer Lett.* **156**:133-139.

Leone AM, Palmer RM, Knowles RG, Francis PL, Ashton DS, and Moncada S (1991) Constitutive and inducible nitric oxide synthases incorporate molecular oxygen into both nitric oxide and citrulline. *J.Biol.Chem.* **266**:23790-23795.

Lewis T, Gueydan C, Huez G, Toulme JJ, and Kruys V (1998) Mapping of a minimal AU-rich sequence required for lipopolysaccharide- induced binding of a 55-kDa protein on tumor necrosis factor-alpha mRNA. *J.Biol.Chem.* **273**:13781-13786.

Li N and Karin M (1999) Is NF-kappaB the sensor of oxidative stress? *FASEB J.* **13**:1137-1143.

Li Z, Jiang Y, Ulevitch RJ, and Han J (1996) The primary structure of p38 gamma: a new member of p38 group of MAP kinases. *Biochem.Biophys.Res.Commun.* **228**:334-340.

Liebgott T, Miollan M, Berchadsky Y, Drieu K, Culcasi M, and Pietri S (2000) Complementary cardioprotective effects of flavonoid metabolites and terpenoid constituents of Ginkgo biloba extract (EGb 761) during ischemia and reperfusion. *Basic Res.Cardiol.* **95**:368-377.

Lin YL and Lin JK (1997) (-)-Epigallocatechin-3-gallate blocks the induction of nitric oxide synthase by down-regulating lipopolysaccharide-induced activity of transcription factor nuclear factor-kappaB. *Mol.Pharmacol.* **52**:465-472.

Ling L, Cao Z, and Goeddel DV (1998) NF-kappaB-inducing kinase activates IKK-alpha by phosphorylation of Ser- 176. *Proc.Natl.Acad.Sci.U.S.A* **95**:3792-3797.

Liu H, Sidiropoulos P, Song G, Pagliari LJ, Birrer MJ, Stein B, Anrather J, and Pope RM (2000) TNF-alpha gene expression in macrophages: regulation by NF-kappa B is independent of c-Jun or C/EBP beta. *J.Immunol.* **164**:4277-4285.

Lowenstein CJ, Alley EW, Raval P, Snowman AM, Snyder SH, Russell SW, and Murphy

WJ (1993) Macrophage nitric oxide synthase gene: two upstream regions mediate induction by interferon gamma and lipopolysaccharide. *Proc.Natl.Acad.Sci.U.S.A.* **90**:9730-9734.

Mai I, Kruger H, Budde K, Johne A, Brockmoller J, Neumayer HH, and Roots I (2000) Hazardous pharmacokinetic interaction of Saint John's wort (Hypericum perforatum) with the immunosuppressant cyclosporin. *Int.J.Clin.Pharmacol.Ther.* **38**:500-502.

Maitra I, Marcocci L, Droy-Lefaix MT, and Packer L (1995) Peroxyl radical scavenging activity of Ginkgo biloba extract EGb 761. *Biochem.Pharmacol.* **49**:1649-1655.

Manach C, Morand C, Crespy V, Demigne C, Texier O, Regerat F, and Remesy C (1998) Quercetin is recovered in human plasma as conjugated derivatives which retain antioxidant properties. *FEBS Lett.* **426**:331-336.

Manna SK, Zhang HJ, Yan T, Oberley LW, and Aggarwal BB (1998) Overexpression of manganese superoxide dismutase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-kappaB and activated protein-1. *J.Biol.Chem.* **273**:13245-13254.

Mantle D, Eddeb F, and Pickering AT (2000) Comparison of relative antioxidant activities of British medicinal plant species in vitro. *J.Ethnopharmacol.* **72**:47-51.

Marcilhac A, Dakine N, Bourhim N, Guillaume V, Grino M, Drieu K, and Oliver C (1998) Effect of chronic administration of Ginkgo biloba extract or Ginkgolide on the hypothalamic-pituitary-adrenal axis in the rat. *Life Sci.* **62**:2329-2340.

Marcocci L, Maguire JJ, Droy-Lefaix MT, and Packer L (1994) The nitric oxide-scavenging properties of Ginkgo biloba extract EGb 761. *Biochem.Biophys.Res.Commun.* **201**:748-755.

Marcocci L, Packer L, Droy-Lefaix MT, Sekaki A, and Gardes-Albert M (1994) Antioxidant action of Ginkgo biloba extract EGb 761. *Methods Enzymol.* **234**:462-475.

Martin E, Nathan C, and Xie Q (1994) Role of interferon regulatory factor 1 in induction of nitric oxide synthase. *J.Exp.Med.* **180**:977-984.

Masood E (1997) Medicinal plants threatened by over-use. *Nature* **385**:570.

Means TK, Golenbock DT, and Fenton MJ (2000a) The biology of Toll-like receptors. *Cytokine Growth Factor Rev.* **11**:219-232.

Means TK, Pavlovich RP, Roca D, Vermeulen MW, and Fenton MJ (2000b) Activation of TNF-alpha transcription utilizes distinct MAP kinase pathways in different macrophage populations. *J.Leukoc.Biol.* **67**:885-893.

Medvedev AE, Blanco JC, Qureshi N, and Vogel SN (1999) Limited role of ceramide in lipopolysaccharide-mediated mitogen- activated protein kinase activation, transcription factor induction, and cytokine release. *J.Biol.Chem.* **274**:9342-9350.

Milosevic N, Schawalder H, and Maier P (1999) Kupffer cell-mediated differential down-regulation of cytochrome P450 metabolism in rat hepatocytes. *Eur.J.Pharmacol.* **368**:75-87.

Monick MM, Carter AB, Gudmundsson G, Geist LJ, and Hunninghake GW (1998) Changes in PKC isoforms in human alveolar macrophages compared with blood monocytes. *Am.J.Physiol* **275**:L389-L397.

Monick MM, Carter AB, and Hunninghake GW (1999) Human alveolar macrophages are markedly deficient in REF-1 and AP-1 DNA binding activity. *J.Biol.Chem.* **274**:18075-18080.

Monshouwer M, McLellan RA, Delaporte E, Witkamp RF, van Miert AS, and Renton KW (1996) Differential effect of pentoxifylline on lipopolysaccharide-induced downregulation of cytochrome P450. *Biochem.Pharmacol.* **52**:1195-1200.

Morand C, Crespy V, Manach C, Besson C, Demigne C, and Remesy C (1998) Plasma metabolites of quercetin and their antioxidant properties. *Am.J.Physiol* **275**:R212-R219.

Morgan ET (1997) Regulation of cytochromes P450 during inflammation and infection. *Drug Metab.Rev.* **29**:1129-1188.

Moriguchi T, Toyoshima F, Masuyama N, Hanafusa H, Gotoh Y, and Nishida E (1997) A novel SAPK/JNK kinase, MKK7, stimulated by TNFalpha and cellular stresses. *EMBO J.* **16**:7045-7053.

Moroney MA, Alcaraz MJ, Forder RA, Carey F, and Hoult JR (1988) Selectivity of neutrophil 5-lipoxygenase and cyclo-oxygenase inhibition by an anti-inflammatory flavonoid glycoside and related aglycone flavonoids. *J.Pharm.Pharmacol.* **40**:787-792.

Musonda CA, Helsby N, and Chipman JK (1997) Effects of quercetin on drug metabolizing enzymes and oxidation of 2',7- dichlorofluorescin in HepG2 cells. *Hum.Exp.Toxicol.* **16**:700-708.

Nakano H, Shindo M, Sakon S, Nishinaka S, Mihara M, Yagita H, and Okumura K (1998) Differential regulation of IkappaB kinase alpha and beta by two upstream kinases, NF-kappaB-inducing kinase and mitogen-activated protein kinase/ERK kinase kinase-1. *Proc.Natl.Acad.Sci.U.S.A.* **95**:3537-3542.

Natarajan K, Manna SK, Chaturvedi MM, and Aggarwal BB (1998) Protein tyrosine kinase inhibitors block tumor necrosis factor-induced activation of nuclear factor-kappaB, degradation of IkappaBalpha, nuclear translocation of p65, and subsequent gene

expression. *Arch.Biochem.Biophys.* **352**:59-70.

Nathan C and Xie Q (1994) Regulation of Biosynthesis of Nitric Oxide. *J.Biol.Chem.* **269**:13725-13728.

Nicholson WJ, Slight J, and Donaldson K (1996) Inhibition of the transcription factors NF-kappa B and AP-1 underlies loss of cytokine gene expression in rat alveolar macrophages treated with a diffusible product from the spores of Aspergillus fumigatus. *Am.J.Respir.Cell Mol.Biol.* **15**:88-96.

Nielsen M, Ruch RJ, and Vang O (2000) Resveratrol reverses tumor-promoter-induced inhibition of gap- junctional intercellular communication. *Biochem.Biophys.Res.Commun.* **275**:804-809.

Nishioka H, Imoto M, Sawa T, Hamada M, Naganawa H, Takeuchi T, and Umezawa K (1989) Screening of phosphatidylinositol kinase inhibitors from Streptomyces. *J.Antibiot.* **42**:823-825.

Noda Y, Anzai K, Mori A, Kohno M, Shinmei M, and Packer L (1997) Hydroxyl and superoxide anion radical scavenging activities of natural source antioxidants using the computerized JES-FR30 ESR spectrometer system. *Biochem.Mol.Biol.Int.* **42**:35-44.

Noguchi K, Tanimura H, Yamaue H, Iwahashi M, Tsunoda T, Tani M, Tamai M, Hotta T, Mizobata S, and Arii K (1997) Enhancement of tumor cell susceptibility to tumor-infiltrating lymphocytes by cisplatin. *J.Cancer Res.Clin.Oncol.* **123**:345-351.

O'Connell MA, Bennett BL, Mercurio F, Manning AM, and Mackman N (1998) Role of IKK1 and IKK2 in lipopolysaccharide signaling in human monocytic cells. *J.Biol.Chem.* **273**:30410-30414.

Obach RS (2000) Inhibition of human cytochrome P450 enzymes by constituents of St. John's Wort, an herbal preparation used in the treatment of depression. *J.Pharmacol.Exp.Ther.* **294**:88-95.

Oberpichler H, Sauer D, Rossberg C, Mennel HD, and Krieglstein J (1990) PAF antagonist ginkgolide B reduces postischemic neuronal damage in rat brain hippocampus. *J.Cereb.Blood Flow Metab.* **10**:133-135.

Ohashi K, Burkart V, Flohe S, and Kolb H (2000) Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. *J.Immunol.* **164**:558-561.

Ohmori Y, Tebo J, Nedospasov S, and Hamilton TA (1994) Kappa B binding activity in a murine macrophage-like cell line. Sequence-specific differences in kappa B binding and transcriptional activation functions. *J.Biol.Chem.* **269**:17684-17690.

Oken BS, Storzbach DM, and Kaye JA (1998) The efficacy of Ginkgo biloba on cognitive function in Alzheimer disease. *Arch.Neurol.* **55**:1409-1415.

Pace-Asciak CR, Hahn S, Diamandis EP, Soleas G, and Goldberg DM (1995) The red wine phenolics trans-resveratrol and quercetin block human platelet aggregation and eicosanoid synthesis: implications for protection against coronary heart disease. *Clin.Chim.Acta* **235**:207-219.

Paine E, Palmantier R, Akiyama SK, Olden K, and Roberts JD (2000) Arachidonic acid activates mitogen-activated protein (MAP) kinase- activated protein kinase 2 and mediates adhesion of a human breast carcinoma cell line to collagen type IV through a p38 MAP kinase- dependent pathway. *J.Biol.Chem.* **275**:11284-11290.

Paul A, Cuenda A, Bryant CE, Murray J, Chilvers ER, Cohen P, Gould GW, and Plevin R (1999) Involvement of mitogen-activated protein kinase homologues in the regulation of lipopolysaccharide-mediated induction of cyclo-oxygenase- 2 but not nitric oxide synthase in RAW 264.7 macrophages. *Cell Signal.* **11**:491-497.

Peet GW and Li J (1999) IkappaB Kinases alpha and beta Show a Random Sequential Kinetic Mechanism and Are Inhibited by Staurosporine and Quercetin. *J.Biol.Chem.* **274**:32655-32661.

Perharic L, Shaw D, Leon C, De Smet PA, and Murray VS (1995) Possible association of liver damage with the use of Chinese herbal medicine for skin disease. *Vet.Hum.Toxicol.* **37**:562-566.

Pietri S, Maurelli E, Drieu K, and Culcasi M (1997a) Cardioprotective and anti-oxidant effects of the terpenoid constituents of Ginkgo biloba extract (EGb 761). *J.Mol.Cell Cardiol.* **29**:733-742.

Pietri S, Seguin JR, D'Arbigny P, Drieu K, and Culcasi M (1997b) Ginkgo biloba extract (EGb 761) pretreatment limits free radical- induced oxidative stress in patients undergoing coronary bypass surgery. *Cardiovasc.Drugs Ther.* **11**:121-131.

Pietta PG, Gardana C, Mauri PL, Maffei-Facino R, and Carini M (1995) Identification of flavonoid metabolites after oral administration to rats of a Ginkgo biloba extract. *J.Chromatogr.B.Biomed.Appl.* **673**:75-80.

Pincemail J, Dupuis M, Nasr C, Hans P, Haag-Berrurier M, Anton R, and Deby C (1989) Superoxide anion scavenging effect and superoxide dismutase activity of Ginkgo biloba extract. *Experientia* **45**:708-712.

Pincemail J, Thirion A, Dupuis M, Braquet P, Drieu K, and Deby C (1987) Ginkgo biloba extract inhibits oxygen species production generated by phorbol myristate acetate stimulated human leukocytes. *Experientia* **43**:181-184.

Pinner RW, Teutsch SM, Simonsen L, Klug LA, Graber JM, Clarke MJ, and Berkelman RL (1996) Trends in infectious diseases mortality in the United States. *JAMA* **275**:189-193.

Pitchumoni SS and Doraiswamy PM (1998) Current status of antioxidant therapy for Alzheimer's Disease. *J.Am.Geriatr.Soc.* **46**:1566-1572.

Poltorak A, He X, Smirnova I, Liu MY, Huffel CV, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, and Beutler B (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* **282**:2085-2088.

Poubelle PE, Gingras D, Demers C, Dubois C, Harbour D, Grassi J, and Rola-Pleszczynski M (1991) Platelet-activating factor (PAF) enhances the concomitant production of tumour necrosis factor-alpha and interleukin-1 by subsets of human monocytes. *Immunology* **72**:181-187.

Price SF, Breen PJ, Valladao M, and Watson BT (1995) Cluster Sun Exposure and Quercetin in Pinot noir Grapes and Wine. *Am.J.Enol.Vitic.* **46**:187-194.

Proffitt J, Crabtree G, Grove M, Daubersies P, Bailleul B, Wright E, and Plumb M (1995) An ATF/CREB-binding site is essential for cell-specific and inducible transcription of the murine MIP-1 beta cytokine gene. *Gene* **152**:173-179.

Quaroni L, Reglinski J, Wolf R, and Smith WE (1996) Interaction of nitrogen monoxide with cytochrome P-450 monitored by surface-enhanced resonance Raman scattering. *Biochim.Biophys.Acta* **1296**:5-8.

Qureshi ST, Lariviere L, Leveque G, Clermont S, Moore KJ, Gros P, and Malo D (1999) Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). *J.Exp.Med.* **189:** 615-625.

Raingeaud J, Gupta S, Rogers JS, Dickens M, Han J, Ulevitch RJ, and Davis RJ (1995) Pro-inflammatory cytokines and environmental stress cause p38 mitogen- activated protein kinase activation by dual phosphorylation on tyrosine and threonine. *J.Biol.Chem.* **270**:7420-7426.

Ramassamy C, Averill D, Beffert U, Bastianetto S, Theroux L, Lussier-Cacan S, Cohn JS, Christen Y, Davignon J, Quirion R, and Poirier J (1999) Oxidative damage and protection by antioxidants in the frontal cortex of Alzheimer's disease is related to the apolipoprotein E genotype. *Free Rad.Biol.Med.* **27**:544-553.

Rang HP, Dale MM, Ritter JM, and Gardner P (1995) *Pharmacology*. Churchill Livingstone.

Ratty AK and Das NP (1988) Effects of flavonoids on nonenzymatic lipid peroxidation:

structure-activity relationship. *Biochem.Med.Metab.Biol.* **39**:69-79.

Reddy ST, Wadleigh DJ, and Herschman HR (2000) Transcriptional regulation of the cyclooxygenase-2 gene in activated mast cells. *J.Biol.Chem.* **275**:3107-3113.

Rhoades KL, Golub SH, and Economou JS (1992) The regulation of the human tumor necrosis factor alpha promoter region in macrophage, T cell, and B cell lines. *J.Biol.Chem.* **267**:22102-22107.

Rimm EB, Klatsky A, Grobbee D, and Stampfer MJ (1996) Review of moderate alcohol consumption and reduced risk of coronary heart disease: is the effect due to beer, wine, or spirits. *Br.Med.J.* **312**:731-736.

Robinson D, Shibuya K, Mui A, Zonin F, Murphy E, Sana T, Hartley SB, Menon S, Kastelein R, Bazan F, and O'Garra A (1997) IGIF does not drive Th1 development but synergizes with IL-12 for interferon-gamma production and activates IRAK and NFkappaB. *Immunity.* **7**:571-581.

Roncin JP, Schwartz F, and D'Arbigny P (1996) EGb 761 in control of acute mountain sickness and vascular reactivity to cold exposure. *Aviat.Space.Environ.Med.* **67**:445-452.

Rosenberg SA, Spiess P, and Lafreniere R (1986) A new approach to the adoptive immunotherapy of cancer with tumor- infiltrating lymphocytes. *Science* **233**:1318-1321.

Rothwarf, D. M Karin M. The NF-kB Activation Pathway: a Paradigm in Information Transfer from Membrane to Nucleus. Science's STKE www.stke.org/cgi/content/full/OC_sigtrans;1999/5/rel. 2000.

Rouse J, Cohen P, Trigon S, Morange M, Alonso-Llamazares A, Zamanillo D, Hunt T, and Nebreda AR (1994) A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell* **78**:1027-1037.

Rubbo H, Darley-Usmar V, and Freeman BA (1996) Nitric oxide regulation of tissue free radical injury. *Chem.Res.Toxicol.* **9**:809-820.

Ruetten H and Thiemermann C (1997) Organ dysfunction caused by endotoxin in the rat: a possible role for protein tyrosine kinase. *Br.J.Pharmacol.* **122**:59-70.

Ryan LK and Vermeulen MW (1995) Alveolar macrophages from C3H/HeJ mice show sensitivity to endotoxin. *Am.J.Respir.Cell Mol.Biol.* **12**:540-546.

Sanchez-Cantu L, Rode HN, and Christou NV (1989) Endotoxin tolerance is associated with reduced secretion of tumor necrosis factor. *Arch.Surg.* **124**:1432-1435.

Sasaki K, Wada K, Hatta S, Ohshika H, and Haga M (1997) Bilobalide, a constituent of Ginkgo biloba L., potentiates drug- metabolizing enzyme activities in mice: possible mechanism for anticonvulsant activity against 4-O-methylpyridoxine-induced convulsions. *Res.Commun.Mol.Pathol.Pharmacol.* **96**:45-56.

Schaeffer HJ and Weber MJ (1999) Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol.Cell Biol.* **19**:2435-2444.

Schmitz ML, Stelzer G, Altmann H, Meisterernst M, and Baeuerle PA (1995) Interaction of the COOH-terminal transactivation domain of p65 NF-kappa B with TATA-binding protein, transcription factor IIB, and coactivators. *J.Biol.Chem.* **270**:7219-7226.

Schneider Y, Vincent F, Duranton B, Badolo L, Gosse F, Bergmann C, Seiler N, and Raul F (2000) Anti-proliferative effect of resveratrol, a natural component of grapes and wine, on human colonic cancer cells. *Cancer Lett.* **158**:85-91.

Scholtyssek H, Damerau W, Wessel R, and Schimke I (1997) Antioxidative activity of ginkgolides against superoxide in an aprotic environment. *Chem.Biol.Interact.* **106**:183-190.

Schreck R, Meier DN, Mannel W, Droge W, and Baeuerle PA (1992) Dithiocarbamates as potent inhibitors of nuclear factor kB activation in intact cells. *J.Exp.Med.* **175**:1185-1194.

Sen CK and Packer L (1996) Antioxidant and redox regulation of gene transcription. *FASEB* **10**:709-720.

Sewer MB, Barclay TB, and Morgan ET (1998) Down-regulation of cytochrome P450 mRNAs and proteins in mice lacking a functional NOS2 gene. *Mol.Pharmacol.* **54**:273-279.

Sewer MB and Morgan ET (1997) Nitric oxide-independent suppression of P450 2C11 expression by interleukin-1beta and endotoxin in primary rat hepatocytes. *Biochem.Pharmacol.* **54**:729-737.

Shakhov AN, Collart MA, Vassalli P, Nedospasov SA, and Jongeneel CV (1990) Kappa B-type enhancers are involved in lipopolysaccharide-mediated transcriptional activation of the tumor necrosis factor alpha gene in primary macrophages. *J.Exp.Med.* **171**:35-47.

Shao ZH, Li CQ, Vanden Hoek TL, Becker LB, Schumacker PT, Wu JA, Attele AS, and Yuan CS (1999) Extract from Scutellaria baicalensis Georgi attenuates oxidant stress in cardiomyocytes. *J.Mol.Cell Cardiol.* **31**:1885-1895.

Sharma SV, Agatsuma T, and Nakano H (1998) Targeting of the protein chaperone, HSP90, by the transformation suppressing agent, radicicol. *Oncogene* **16**:2639-2645.

Sherman MP, Aeberhard EE, Wong VZ, Griscavage JM, and Ignarro LJ (1993) Pyrrolidine dithiocarbamate inhibits induction of nitric oxide synthase activity in rat alveolar macrophages. *Biochem.Biophys.Res.Commun.* **191**:1301-1308.

Shih H, Pickwell GV, and Quattrochi LC (2000) Differential effects of flavonoid compounds on tumor promoter-induced activation of the human CYP1A2 enhancer. *Arch.Biochem.Biophys.* **373**:287-294.

Siebenlist U, Franzoso G, and Brown K (1994) Structure, regulation and function of NF-kappa B. *Annu.Rev.Cell Biol.* **10**:405-455.

Soleas GJ, Diamandis EP, and Goldberg DM (1997) Resveratrol: A molecule whose time has come? And gone? *Clin.Biochem.* **30**:91-113.

Stefanova I, Corcoran ML, Horak EM, Wahl LM, Bolen JB, and Horak ID (1993) Lipopolysaccharide induces activation of CD14-associated protein tyrosine kinase p53/56lyn. *J.Biol.Chem.* **268**:20725-20728.

Stein B, Baldwin AS, Jr., Ballard DW, Greene WC, Angel P, and Herrlich P (1993) Cross-coupling of the NF-kappa B p65 and Fos/Jun transcription factors produces potentiated biological function. *EMBO J.* **12**:3879-3891.

Stoll S, Scheuer K, Pohl O, and Muller WE (1996) Ginkgo biloba extract (EGb 761) independently improves changes in passive avoidance learning and brain membrane fluidity in the aging mouse. *Pharmacopsychiatry* **29**:144-149.

Stotter H, Wiebke EA, Tomita S, Belldegrun A, Topalian S, Rosenberg SA, and Lotze MT (1989) Cytokines alter target cell susceptibility to lysis. *J.Immunol.* **142**:1767-1773.

Su GL, Klein RD, Aminlari A, Zhang HY, Steinstraesser L, Alarcon WH, Remick DG, and Wang SC (2000) Kupffer cell activation by lipopolysaccharide in rats: role for lipopolysaccharide binding protein and toll-like receptor 4. *Hepatology* **31**:932-936.

Swantek JL, Cobb MH, and Geppert TD (1997) Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is required for lipopolysaccharide stimulation of tumor necrosis factor alpha (TNF-alpha) translation: glucocorticoids inhibit TNF-alpha translation by blocking JNK/SAPK. *Mol.Cell Biol.* **17**:6274-6282.

Swantek JL, Tsen MF, Cobb MH, and Thomas JA (2000) IL-1 receptor-associated kinase modulates host responsiveness to endotoxin. *J.Immunol.* **164**:4301-4306.

Sweet MJ and Hume DA (1996) Endotoxin signal transduction in macrophages. *J.Leukoc.Biol.* **60**:8-26.

Szabo ME, Droy-Lefaix MT, and Doly M (1995) EGb 761 and the recovery of ion imbalance in ischemic reperfused diabetic rat retina. *Ophthalmic Res.* **27**:102-109.

Takei A, Nagashima G, Suzuki R, Hokaku H, Takahashi M, Miyo T, Asai J, Sanada Y, and Fujimoto T (1997) Meningoencephalocele associated with Tripterygium wilfordii treatment. *Pediatr.Neurosurg.* **27**:45-48.

Takeuchi O, Kawai T, Sanjo H, Copeland NG, Gilbert DJ, Jenkins NA, Takeda K, and Akira S (1999) TLR6: A novel member of an expanding toll-like receptor family. *Gene* **231**:59-65.

Terao J and Piskula MK (1999) Flavonoids and membrane lipid peroxidation inhibition *Nutrition* **15**:790-791.

Thanos D and Maniatis T (1995) NF-kappa B: a lesson in family values. *Cell* **80**:529-532.

Thompson CD, Frazier-Jessen MR, Rawat R, Nordan RP, and Brown RT (1999) Evaluation of methods for transient transfection of a murine macrophage cell line, RAW 264.7. *Biotechniques* **27**:824-30.

Tosaki A, Pali T, and Droy-Lefaix MT (1996) Effects of Ginkgo biloba extract and preconditioning on the diabetic rat myocardium. *Diabetologia* **39**:1255-1262.

Tracey KJ and Cerami A (1994) Tumor necrosis factor: a pleiotropic cytokine and therapeutic target. *Annu.Rev.Med.* **45**:491-503.

Tsiodras S, Shin RK, Christian M, Shaw LM, and Sass DA (1999) Anticholinergic toxicity associated with lupine seeds as a home remedy for diabetes mellitus. *Ann.Emerg.Med.* **33**:715-717.

Ulevitch RJ (1999) Endotoxin opens the Tollgates to innate immunity. *Nat.Med.* **5**:144-145.

Urban MB, Schreck R, and Baeuerle PA (1991) NF-kappa B contacts DNA by a heterodimer of the p50 and p65 subunit. *EMBO J.* **10**:1817-1825.

Valcic S, Timmerman B, Alberts DS, Wachter GA, Krutzsch M, Wymer J, and Guillen JM (1996) Inhibitory effect of six green tea catechins and caffeine on the growth of four selected human tumor cell lines. *Anti-Cancer Drugs* **7**:461-468.

Vetto JT, Lum S, Morris A, Sicotte M, Davis J, Lemon M, and Weinberg A (1997) Presence of the T-cell activation marker OX-40 on tumor infiltrating lymphocytes and draining lymph node cells from patients with melanoma and head and neck cancers. *Am.J.Surg.* **174** :258-265.

Virca GD, Kim SY, Glaser KB, and Ulevitch RJ (1989) Lipopolysaccharide induces hyporesponsiveness to its own action in RAW 264.7 cells. *J.Biol.Chem.* **264**:21951-21956.

Wadsworth TL and Koop DR (1999) Effects of the wine polyphenolics quercetin and resveratrol on pro- inflammatory cytokine expression in RAW 264.7 macrophages. *Biochem.Pharmacol.* **57**:941-949.

Wadsworth, TL, McDonald, TL, and Koop, DR (in press) Effects of Ginkgo biloba extract (EGb 761) and quercetin on lipopolysaccharide-induced signaling pathways involved in the release of tumor necrosis factor alpha. *Biochem.Pharmacol.*

Walgren RA, Walle UK, and Walle T (1998) Transport of quercetin and its glucosides across human intestinal epithelial Caco-2 cells. *Biochem.Pharmacol.* **55**:1721-1727.

Wang SC, Rossignol DP, Christ WJ, Geller DA, Freeswick PD, Thai NL, Su GL, and Simmons RL (1994) Suppression of lipopolysaccharide-induced macrophage nitric oxide and cytokine production in vitro by a novel lipopolysaccharide antagonist. *Surgery* **116**:339-46; discussion 446-7.

Wang Y, Huang S, Sah VP, Ross J, Jr., Brown JH, Han J, and Chien KR (1998) Cardiac muscle cell hypertrophy and apoptosis induced by distinct members of the p38 mitogen-activated protein kinase family. *J.Biol.Chem.* **273**:2161-2168.

Weinstein SL, Sanghera JS, Lemke K, DeFranco AL, and Pelech SL (1992) Bacterial lipopolysaccharide induces tyrosine phosphorylation and activation of mitogen-activated protein kinases in macrophages. *J.Biol.Chem.* **267**:14955-14962.

Weisz A, Oguchi S, Cicatiello L, and Esumi H (1994) Dual mechanism for the control of inducible-type NO synthase gene expression in macrophages during activation by interferon-gamma and bacterial lipopolysaccharide. Transcriptional and post-transcriptional regulation. *J.Biol.Chem.* **269**:8324-8333.

Wesche H, Henzel WJ, Shillinglaw W, Li S, and Cao Z (1997) MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. *Immunity* **7**:837-847.

Wettstein A (2000) Cholinesterase inhibitors and Gingko extracts--are they comparable in the treatment of dementia? Comparison of published placebo-controlled efficacy studies of at least six months' duration. *Phytomedicine.* **6**:393-401.

Whitmarsh AJ, Shore P, Sharrocks AD, and Davis RJ (1995) Integration of MAP kinase signal transduction pathways at the serum response element. *Science* **269**:403-407.

Wisdom R (1999) AP-1: one switch for many signals. *Exp.Cell Res.* **253**:180-185.

Wright SD (1999) Toll, a new piece in the puzzle of innate immunity. *J.Exp.Med.* **189**:605-609.

Xie Q, Kashiwabara Y, and Nathan C (1994) Role of transcription factor NF-kB/Rel in induction of nitric oxide synthase. *J.Biol.Chem.* **269**:4705-4708.

Xie Q and Nathan C (1994) The high-output nitric oxide pathway: role and regulation. *J.Leukoc.Biol.* **56**:576-582.

Xie Q, Whisnant R, and Nathan C (1993) Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon gamma and bacterial lipopolysaccharide. *J.Exp.Med.* **177**:1779-1984.

Xin W, Wei T, Chen C, Ni Y, Zhao B, and Hou J (2000) Mechanisms of apoptosis in rat cerebellar granule cells induced by hydroxyl radicals and the effects of EGb761 and its constituents. *Toxicology* **148**:103-110.

Xu X, Prorock C, Ishikawa H, Maldonado E, Ito Y, and Gelinas C (1993) Functional interaction of the v-Rel and c-Rel oncoproteins with the TATA-binding protein and association with transcription factor IIB. *Mol.Cell Biol.* **13**:6733-6741.

Yang CS and Wang ZY (1993) Tea and cancer. *J.Nat.Cancer Inst.* **85**:1038-1049.

Yang RB, Mark MR, Gray A, Huang A, Xie MH, Zhang M, Goddard A, Wood WI, Gurney AL, and Godowski PJ (1998) Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signaling. *Nature* **395**:284-288.

Zagariya A, Mungre S, Lovis R, Birrer M, Ness S, Thimmapaya B, and Pope R (1998) Tumor necrosis factor alpha gene regulation: enhancement of C/EBPbeta- induced activation by c-Jun. *Mol.Cell Biol.* **18**:2815-2824.

Zhang FX, Kirschning CJ, Mancinelli R, Xu XP, Jin Y, Faure E, Mantovani A, Rothe M, Muzio M, and Arditi M (1999) Bacterial lipopolysaccharide activates nuclear factor-kappaB through interleukin-1 signaling mediators in cultured human dermal endothelial cells and mononuclear phagocytes. *J.Biol.Chem.* **274**:7611-7614.

Zhong H, SuYang H, Erdjument-Bromage H, Tempst P, and Ghosh S (1997) The transcriptional activity of NF-kappaB is regulated by the IkappaB- associated PKAc subunit through a cyclic AMP-independent mechanism. *Cell* **89**:413-424.

Zhong H, Voll RE, and Ghosh S (1998) Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. *Mol.Cell* **1**:661-671.

Ziegler-Heitbrock HW, Wedel A, Schraut W, Strobel M, Wendelgass P, Sternsdorf T, Bauerle PA, Haas JG, and Riethmuller G (1994) Tolerance to lipopolysaccharide involves mobilization of nuclear factor kappa B with predominance of p50 homodimers. *J.Biol.Chem.* **269**:17001-17004.