THE EFFECTS OF ACUTE INFECTION AND INFLAMMATION ON PHASE II DETOXIFYING ENZYMES

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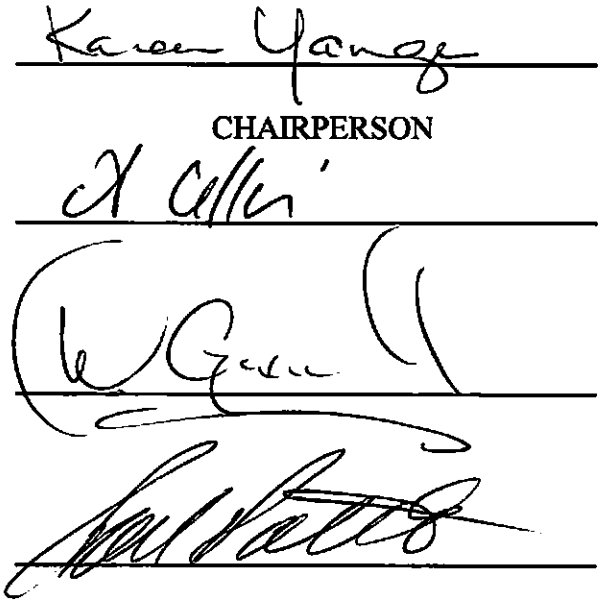

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Abstract

Infection and inflammation may alter liver metabolism causing significant changes in drug efficacy and toxicity. The expression and activity of the Cytochromes P450 in human liver are significantly down-regulated during acute infection and inflammation but little is known about Phase II metabolizing enzymes. We treated the human liver cell line HepG2 with TNF-α and IL-1β to at 0.1 – 1000 U/mL for 0-48 hours as a model of acute infection and inflammation. Cells were harvested at each time point and assessed for cell death (MTT assay) and levels of reactive oxygen species (ROS) and for the expression and activity of the major Phase II enzymes UDP-glucuronosyl transferase (UGT), Glutathione-S-transferase (GST) and Sulfotransferase (SULT). Since all of these enzymes have hepatic nuclear factor (HNF) recognition sequences in their genes, we assessed the effects of the cytokines on HNF1 and HNF4 expression and nuclear translocation with immunofluorescence. UGT enzymes are not significantly affected by pro-inflammatory cytokines and SULT enzymes showed no change in activity while GST enzymes showed a significant increase in activity but returned to normal levels by 48 hours. Higher GST activity may confer lower efficacy of drugs during acute infection and inflammation.

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Abbreviations

αMSH	alpha melanocyte stimulating hormone
4MU	4-methylumbelliferone
AIDS	acquired immunodeficiency syndrome
ANOVA	analysis of variance
CF	cystic fibrosis
cGST	cytosolic gluthathione *S*-transferase
CYP450	cytochrome P450
DCF	dichlorofluorescein
DCFH-DA	dichlorofluorescein diacetate
DDT	dichloro-diphenyl-trichloroethane
DHEA	dehydroepiandrosterone
DNA	deoxyribonucleic acid
E1S	estrone sulfate
EPL	eicosanoids, prostaglandins, and leukotrienes
EST	estrogen sulfotransferase
GSH	glutathione
GST	gluthathione-*S*-transferase
HIV	human immunodeficiency syndrome
HNF1	hepatic nuclear factor one
HNF4	hepatic nuclear factor four
Hr	hours
IFN-γ	interferon-gamma

IFNs	interferons
IL-1	interleukin-one
IL-1β	interleukin-one beta
IL-2	interleukin-two
IL-3	interleukin-three
IL-6	interleukin-six
LPS	lipopolysaccharide
MAPEG	membrane associated proteins in eicosanoid and glutathione metabolism
Min	minutes
mRNA	messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaOH	sodium hydroxide
NDS	normal donkey serum
NFκB	nuclear factor kappa B
NKs	natural killer cells
PAPS	3'phosphoadenosine 5'-phosphosulfate
PBS-T	phosphate buffered saline with Tween-20
PMNs	polymorphonuclear leukocytes
ROS	reactive oxygen species
SD	standard deviation
STS	steroid sulfatase

SULT	sulfotransferase
TGFβ	transforming growth factor beta
TNF-α	tumor necrosis factor-alpha
U/ml	unite per milliliter
UDPGA	uridine diphosphate glucuronic acid
UGT	UDP-glucuronosyltransferase
vHNF-1C	variant hepatic nuclear factor 1-C

Chapter 1: Introduction

This thesis explores the effects of the acute inflammatory response on the major Phase II metabolizing enzymes uridine diphosphate glucuronosyl transferase (UGT), sulfotransferase (SULT) and glutathione transferase (GST). We hypothesize that acute infection and inflammation affect enzyme expression and activity in the liver. Additionally, we hypothesize that regulatory co-promoters may play a pivotal role in altering UGT, SULT and GST expression and activity during acute infection and inflammation. The implications of this are that during inflammation and acute infection, phase II metabolism and clearance from the liver may be altered to such an extent that it may affect the safety and efficacy of some drugs.

Phase I vs. Phase II metabolism

Drug metabolism mechanisms fall into two categories: Phase I and Phase II. Phase I mechanisms involve structural alterations of the drug molecule while Phase II mechanisms consist of conjugation with another more water-soluble moiety (Benedetti, 2005). The Cytochromes P450 (CYP450s), one of the most well known and most important drug metabolizing families, are Phase I enzymes. The UGTs, SULTs and GSTs are the 3 major Phase II enzymes accounting for as much as 90% of drug, chemical, hormone and dietary detoxification. Phase II reactions, in general, are more difficult to determine since more than one enzyme is usually involved in the reaction pathway. Additionally, an intracellular conjugating agent (co-factor) usually needs to be available. Phase I reactions are responsible for the majority of metabolism in the body (Cassarte and Doull). In situations where Phase I reactions are down-regulated or suppressed, the

concern is that the burden of metabolism now falls onto Phase II reactions. Conversely, where Phase II reactions are altered (particularly when they are inhibited) clearance of reactive intermediate metabolites may be impaired resulting in toxic consequences (Cassaret and Doull).

UDP glucuronosyltransferases (UGTs)

The major function of glucuronidation is to make compounds more soluble and hydrophilic, allowing them to be detoxified and excreted. Rarely, this process can also synthesize derivatives that are biologically active and in some cases, demonstrate increased toxicity. For example benzo[α]pyrene is a polycyclic aromatic hydrocarbon found widely in the environment, including in tobacco smoke (Wells, 2004). It is eliminated primarily via glucuronidation and when this pathway is blocked or eliminated, the metabolites are found to bind to both protein and DNA, leading to increased oxidative DNA damage and micronucleus formation (Wells, 2004). Acetaminophen, the widely-used analgesic drug, is also eliminated primarily by glucuronidation. If glucuronidation of this substrate is decreased, enhanced CYP450-catalyzed bioactivation can occur resulting in enhanced hepatocellular and renal cellular necrosis (Guengerich, 2006). UGTs are the enzymatic catalysts for glucuronidation reactions. They are a family of enzymes which reside in the endoplasmic reticulum and catalyze the transfer of glucuronic acid to an electron rich atom on the substrate (typically hydrophobic chemicals, such as drugs, xenobiotics and products of endogenous catabolism) forming water soluble conjugates that are excreted in the urine and feces (Wells, 2004; Mackenzie 2003). Additionally, the UGTs are responsible for detoxifying a vast group of potentially

carcinogenic or teratogenic compounds that enter the body through airborne pollutants or through diet (de Wildt, 1999). Although they are also present and active in the alimentary tract, lung, brain, skin, prostate and kidneys (Radominska-Pandya, 1999), the UGT enzymes in the liver are the main source of glucuronidation reactions in the body.

Evidence suggests that of the nearly 50 mammalian UGT isoforms that exist, 12 of which are expressed in the human liver, although 3 of these have no known physiological or xenobiotic substrates (Radominska-Pandya, 1999; Levesque, 2001). The UGT isoforms have been separated into two main families: the UGT1 and the UGT2. Members of the UGT1A family are all encoded by a complex gene consisting of 16 exons localized on chromosome 2 and all share more than 50% sequence identity to one another. Each UGT1 protein is encoded by a transcript that is formed by alternate splicing of the first exon to a set of 4 downstream exons, designated 2-5. They have variable amino(n)-terminal halves and identical carboxy(c)-terminal halves (Radominska-Pandya, 1999; Mackenzie, 2003). Nomenclature is systematic and numbered with human isoforms using capital roman letters and other mammalian isoforms using lowercase letters respectively. For example, UGT1A4 is a human isoform and ugt1a12 is a rat isoform (Mackenzie 1997). Five of these isoforms, UGT1A1, UGT1A3, UGT1A4, UGT1A6 and UGT1A9 are expressed in the liver and have individual catalytic activity profiles (Radominska-Pandya, 1999). In contrast, the UGT2 enzymes do not share a common carboxy-terminal and are encoded by separate genes, clustered on chromosome 4, each containing 6 exons (Mackenzie, 2003). The UGT2 family is further subdivided into three groups: 2A, 2B and 2C. UGT2B isoforms are the most abundant and most studied and in

the liver they are represented by UGT2B4, UGT2B7, UGT2B10, UGT2B11, UGT2B15, UGT2B17 and UGT2B28 (Levesque, 2001; Mackenzie, 2003). The major endogenous substrates of the 2B isoforms are steroids and their metabolites and bile acids (Radominska-Pandya, 1999). UGT2A are only found in the nasal epithelium and are involved in olfaction (Barbier, 2000) while the UGT2C subfamily currently has only one representative isolated from a rabbit (Barbier, 2000). As UGT2A and UGT2C are not involved in metabolism in the liver, they are not subjects of this research and will not be discussed further.

Sulfotransferases (SULTs)

Sulfotransferase enzymes catalyze the conjugation of sulfate groups onto a wide number of xenobiotic and endogenous substrates, including drugs, toxic chemicals, hormones and neurotransmitters (Kauffman, 2004). The sulfonation pathway involves the transfer of a sulfonate group from the universal donor 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to a substrate that possesses an acceptor moiety such as a hydroxyl or amine group (Coughtrie, 2002). In general, sulfonation causes molecules to lose their biological activity, but in some circumstances, the addition of a sulfate can lead to highly reactive intermediates, such as minoxidil, and reactive electrophilic cations like N-hydroxy 2-acetylaminofluorene (McCall, 1983; Miller 1994). Sulfonation has a major role in regulating the endocrine system of an individual by modulating receptor activity in steroid biosynthesis, activity of estrogens and androgens, and metabolism of catecholamines and iodothyronines (Kauffman, 2004). Additionally, this pathway is

responsible for catecholamine inactivation (Buu, 1981) and is estimated to metabolize as much as 10% of the dopamine and norepinephrine in the brain (Rivett, 1982). Sulfonation is also a key response in the body's defense against toxic chemicals and may play a major role in early development since high levels of SULTs have been documented in the human fetus (Kauffman, 2004). For example, Barker *et al* (1994) found that production of DHEA sulfate through SULT2A1 by the fetal adrenal is established as a critical step in providing substrate for estrogen biosynthesis by the placenta during pregnancy.

Cytosolic sulfotransferases have been classified into five families with less than 40% similarity between them (Kauffman, 2004). SULT1 and SULT2 are the largest families and sulfonate the largest number of endogenous and foreign compounds. Currently there are 10 known sulfotransferases in humans and five of these (SULT1A1, SULT1A2, SULT1A3, SULT1E and SULT2A1) are expressed in the adult liver (Coughtrie, 2002). Inter-individual variances in the expression of SULT isoforms have pharmacological and toxicological significance. For example, SULT1A1, a broad spectrum sulfotransferase, has been linked with early-onset breast cancer as well as the occurrence of other tumors in individuals having higher amounts of the enzyme (Seth, 2000). High activity of SULT1A1 has also been linked with increased risk from dietary constituents, such as the consumption of charcoaled meats such as may be produced by barbequing (Zheng, 2001;

Nowell, 2004). A principle pathway for chemical carcinogenesis is through the formation of toxic and reactive sulfuric acid esters, which are then cleaved to generate sulfate ions and electrophiles that combine with nucleophilic groups in cellular DNA and proteins (Kauffman, 2004).

Glutathione transferases (GSTs)

Glutathione transferases catalyze the formation of thioether conjugates between glutathione (GSH) and reactive xenobiotics either by direct addition or through displacement of an electron-withdrawing group (Lin, 2007). Important substrates include polycyclic aromatic hydrocarbon epoxides produced from the catalytic actions of Phase I CPY450s as well as by-products of oxidative stress (Strange, 2001). There are three families of GSTs: cytosolic, mitochondrial and microsomal (Hayes, 2005). The mitochondria, and thus the mitochondrial GSTs, were removed during preparation of the cellular S9, so they will not be discussed further. Cytosolic GSTs are by far the most abundant family. In man and other mammalian species, 15-20 different cGST genes have been identified (Hayes, 2005). In humans, seven distinct gene families encode the cytosolic GSTs: Mu, Alpha, Theta, Pi, Zeta, Sigma and Chi (Strange, 2001; Hayes, 2005). GSTs are biologically active as dimers and share greater than 40% sequence identity within a class (Hayes, 2005).

The GST pathway is important for the metabolism of exogenous substrates such as drugs, and important examples include the cancer chemotherapeutic agents andriamycin, busulfan and chlorambucil, pesticides and herbicides like DDT and atrazine, and environmental pollutants found in cigarette smoke and exhaust fumes (Hayes, 2005). Similar to the other phase II enzymes, conjugation with GSH generally leads to the formation of less reactive, more readily excreted byproducts. However, in a few instances, the product is more reactive than the parents compound. Conjugation with the solvent dichloromethane results in the formation of highly unstable S-chloromethylglutathione, capable of modifying DNA. Conjugation with haloalkenes, which occurs primarily in the liver, can lead to the generation of reactive byproducts in the kidney (Hayes, 2001).

Microsomal GSTs, now designated as membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG), are less numerous that cytosolic GSTs and are far less characterized. Four MAPEG subgroups have been described (I-IV) and six human MAPEGs have been identified to date (Hayes, 2001). Most microsomal GST proteins are involved in the synthesis of eicosanoids, leukotrienes and prostaglandins, catalyzing GSH-dependent transferase and isomerase reactions (Frova, 2006). This is especially significant to our work since eicosanoids, leukotrienes and prostaglandins are commonly involved in inflammatory and pain reactions. These compounds have vast interactive networks with interleukins, which can influence pro-inflammatory cytokine production

and the time-course of physiological inflammation and its consequences. Therefore, through their synthesis of these compounds, microsomal GSTs may play an important role in the etiology of infectious and inflammatory morbidity, as well as contributing to chemical and hormone metabolism and removal.

Immune markers of infection and the acute inflammatory response

The immune response is a complex sequence of events that is triggered by the introduction of a stimulus and culminates in the elimination of the intrusive agent. There are two stages in the immune response: innate immunity and adaptive immunity. Innate immunity is present at birth and is characterized by the activation of macrophages, polymorphonuclear neutrophils (PMNs), eosinophils and Natural Killer (NK) cells (Goodman, 1991). If the innate immune system fails to clear the foreign agent then the adaptive immune system comes into play. This system is characterized by the activation of T and B cells and by the specificity of the activated cell for the invading antigen. The adaptive immune system also possesses memory cells that can mount an intensified attack on repeat invaders.

The inflammatory response, like the immune response, can be activated by cells, such as neutrophils and macrophages, or by circulating proteins including components of complement or coagulation (Broide, 2001). The inflammatory response is generated as a means of defending the body against infection and repairing damaged tissue. However, acute or prolonged inflammatory reactions can lead to disease states and morbidities,

including further tissue damage that can be detrimental to the host. In order to protect the host, cellular inflammatory mediators are stored either preformed in cytoplasmic granules or as phospholipids ready to be generated in the cell surface membrane (Broide, 2001).

Cytokines are mediators of immunologic, inflammatory and reparative host responses to invasion or injury. Cytokines function as intercellular signals that regulate local and systemic inflammatory responses (Oppenheim *et al*, 2001). They work by modulating the growth, mobility and differentiation of leukocytes and other cells. Although a battery of cytokines exist, this thesis will focus on interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α) which are mediators of acute infection and inflammation.

Interleukin 1 beta (IL-1β)

IL-1 is produced by 11 types of cells, namely: macrophages, keratinocytes, dendritic cells, astrocytes, microglial cells, normal B lymphocytes, cultured T cell clones, fibroblasts, neutrophils, endothelial cells and smooth muscle cells (Oppenheim *et al*, 2001). Production of IL-1 is stimulated by diverse adjuvants such as lipopolysaccharide (LPS), viral RNA and bacterial peptidoglycans. It acts on target cells through high-affinity receptors present on the plasma membrane. Activity can be regulated by endogenous factors that affect cytokine production by modulating either receptor expression or signal transduction after the signals are triggered (Oppenheim *et al*, 2001). Potent antagonists of IL-1β include transforming growth factor β (TGFβ), corticosteroids

and α melanocyte-stimulating hormone (αMSH). Pharmacologic inhibition of IL-1 activity through these antagonists may be useful in controlling some inflammatory reactions (Oppenheim *et al* 2001).

Injection of IL-1 induces local acute inflammatory responses that begin within 1 hour and peak within 3-4 hours (Oppenheim *et al* 2001). Initially, neutrophils adhere to endothelial cells and spread along the blood vessel walls. This is followed by neutrophil infiltration and edema from the flow of fluids into the tissues. IL-1 can also stimulate other cytokines such as IL-2 which stimulates the proliferation and maturation of T cells; the interferons, which induce the expression of major histocompatability complex proteins in macrophages and activate monocytes and macrophages; IL-3 which acts on pluripotent hematopoietic stem cells to promote cell development of granulocytes, macrophages, and mast cells, and IL-6 which promotes the proliferation and maturation of B cells (Rang, 1995).

Tumor necrosis factor alpha (TNF-α)

TNF-α is a pleiotropic cytokine that can induce proliferation, apoptosis, or inflammatory reactions in target cells. These different cellular reactions depend on the activation of distinct cellular signaling pathways that interact with each other through complex signaling networks, allowing for a wide range of cellular responses (Baud, 2001). TNF-α is a known activator of the apoptotic signaling cascade, but does not commonly induce

apoptosis in target cells due to the parallel activation of protective signaling pathways that interfere with the onset of apoptosis (Schwabe, 2002). TNF-α is also known to strongly activate the nuclear factor-κB pathway which prevents hepatocytes from undergoing TNF-α induced apoptosis during development, liver regeneration and other conditions associated with high levels of TNF-α (Scwabe, 2002).

TNF-α is produced by activated macrophages, lymphocytes and NK cells and has a broad spectrum of biologic actions on target cells. Although the high-affinity receptors for IL-1 and TNF are unrelated structurally, they possess a high degree of overlap in terms of their immunologic and nonimmunologic activities. For example, both act to enhance IL-2 receptor expression in T lymphocytes as well as inducing lymphokine expression in these cells, and both are known to enhance antibody production in B lymphocytes and promote B cell proliferation (Oppenheim *et al*, 2001). In vivo, however, TNF is known to have more potent effects on neutrophils, monocytes and endothelial cells and at higher concentrations (1000 U/ml) are chemotactic for neutrophils, activating neutrophil respiratory burst and degranulation (Oppenheim *et al*, 2001). Both IL-1 and TNF can cause monocytes to produce inflammatory mediators such as prostaglandins and IL-6 (Oppenheim *et al*, 2001). Both are also known to increase the adhesiveness of endothelial cells by inducing the expression of cell-adhesion molecules.

Infection, inflammation and drug metabolism

Infection and inflammatory reactions induce alterations in hepatic drug metabolism that may also cause significant increases or decreases in drug efficacy and toxicity. Because

little is known about the effects of infection and inflammation on Phase II metabolizing enzymes in human livers, cytokine effects on CYP450s can be used as an indication of potential consequences for cytokine effects on Phase II enzymes. The CYP450s share many similarities with UGTs, SULTs and GSTs including broad substrate specificity and the ability to catalyze metabolism of a variety of endogenous and exogenous substrates (Cassaret and Doull). The expression and metabolism in liver and extrahepatic tissues of the CYP450s can be significantly down-regulated during inflammation and it is likely that hepatic phase II metabolism is similarly affected. Alterations of Phase II metabolism could be important because it may change drug efficacy or enhance toxicity in patients that have infectious disease and/or are experiencing an inflammatory response (Morgan, 2006).

Effects of infection and inflammation on CYP450-mediated metabolism

As mentioned above, bacterial infections and/or inflammation have been shown to suppress hepatic and extrahepatic CYP450 expression and activity (Richardson, 2006; Morgan 2001; Renton, 2004). Work done by Richardson and Morgan (2005) demonstrated that administration of LPS significantly decreased the hepatic expression of CYP1A2, 2A5, 2C29, 2E1, 3A11, 4A10 and 4A14 mRNAs and proteins in mice. Richardson *et al.* used bacterial infection of *Citrobacter rodentium* in mice to study the effects on CYP450s. They found that *C. rodentium* infection down-regulated CYP4A10 and 4A14 mRNAs to 4% of the control. CYP3A11 was down-regulated to 16% of the control, and CYP2C29, 4F14, and 4F15 exhibited 50%, 55% and 45% reductions,

respectively. Protein expression was likewise reduced in CYP4A mice to 6% and 18% of the control (Richardson, 2006).

Cytokines have also shown to play an important role in CYP450 drug metabolism during inflammation and infection, when the levels of cytokines are dramatically increased. Again, in Richardson's study of bacterial infection in mice, hepatic mRNA levels of in IL-1β, interleukin-6 (IL-6) and TNF- increased significantly above control (Richardson, 2006.) Raised levels of TNF-α, IL-1α, IL-6 and interferons (IFNs), have been shown to depress CYP450 mRNA levels and enzyme synthesis. Monshouwer's study (1996) in pig hepatocytes showed that these pro-inflammatory cytokines inhibited CYCYP450 activities up to 41% (Monshouwer, 1996). There are three proposed mechanisms by which cytokines alter CYP450 gene expression; the destruction of the enzyme by a free radical (Parent, 1992), the inhibition of translation of CYP450 mRNA and reduction in CYP450 mRNA levels (Renton, 1990), and most recently is has been proposed that suppression of CYP450 activity is be mediated by nitric oxide (Stadler, 1994.) The suppression of CYP450s by nitric oxide is controversial (Monshouwer, 1996).

Cytokines may also control regulation of CYP450s at the genetic level. Morgan *et al.* (2002) have found that the Cyp2C11 promoter contains a NF-κB response element that allows down-regulation of the CYP gene by cytokines up to 60% of control. It was presumed that the binding of NF-κB inhibits transcription initiation (Morgan, 2002). Another regulator is the transcription factor is hepatic nuclear factor-1 (HNF-1), which is known to be involved in the regulation of CYP27, that converts cholesterol to 27

hydrocholesterol. During infection, LPS decreases the binding of HNF-1 in nuclear extracts, suggesting that the CYP27 promoter region is compromised (Renton, 2004). It is known that HNF-1 expression can be dependent on HNF-4 co-expression and both are decreased in response to LPS in rats, therefore; it was suggested that the loss of CYP450 in the liver caused by LPS was due to nitrosylation of the HNF-4 binding domain causing down-regulation of CYP450 transcription (Renton, 2004.) These results provide an interesting frame of reference for studying the effects of pro-inflammatory cytokines on Phase II metabolizing enzymes. Furthermore, they provide possible avenues of investigation to study the mechanisms by which enzyme activities are up- or down-regulated by pro-inflammatory cytokines; namely through the nuclear transcription factors.

Effects of infection and inflammation on UGTs

Although CYP450s provide a good model to begin from, the focus of these studies is the regulation of Phase II enzymes during inflammation and infection, which are far less well characterized. Most of the studies have been performed in animals and utilized injection of lipopolysaccharide (LPS) as a model of inflammation. Chen *et al* (1995) found that LPS administration to rat hepatocytes resulted in up to ~80% decrease in UGT activity, while Morgan *et al* (2006) have shown that LPS administration to mice reduced hepatic mRNAs for UGT1A1, UGT1A9 and UGT2B5 by approximately 40 % compared to control (untreated) mice and significantly down-regulated hepatic UGT1A and UGT2B

proteins by up to 60% of control. The difference between the ~40% change in mRNA and 60% change in protein levels is further indication of the importance of studying metabolizing enzymes at the protein/activity levels since, due to post-transcriptional and post-translational changes, alterations in mRNA message are rarely reflective of functional modification of enzyme activities.

Other studies using rats, with turpentine injection as a model of inflammation found that the UGT activity towards testosterone was significantly reduced to 65% of control along with a concurrent decrease in UGT mRNA levels (Strasser, 1998). This was consistent with studies in primary rat hepatocytes exposed to cytokines as a model of the inflammatory response (Strasser, 1998).

The effects on hepatic UGTs during inflammation are thought to be cytokine dependent, similar to CYP450 suppression after LPS administration. In Morgan's experiments with mice (discussed above), both LPS and *Citrobacter rodentium* infection increased hepatic expression of IL-1β, IL-6 and TNF-α (Morgan, 2006). This was confirmed by Strasser's experiments, where exposure of hepatocytes to IL-1β caused no significant changes in UGT1A1 or UGT2B3 mRNA levels, but administration of IL-6 significantly suppressed both isozymes (Strasser, 1998). Similar effects were reported by Monshouwer *et al* (1996) using pig hepatocytes which showed that TNF-α, IL-1α, IFN-γ and IL-6 all inhibit glucuronidation, but that different cytokines affected different UGT iso-enzymes to smaller and greater capacities.

Time dependency for the effects on UGT expression and activity was observed for IL-6 but not IL-1α and TNF-α which could mean that the latter two act via different mechanisms (Monshouwer, 1996). Alternatively, IL-1α and TNF-α could be degraded more rapidly in culture than IL-6, allowing for recovery in the hepatocytes. Alternatively decreases in glucuronidation caused by cytokines may be through by a lack of the UGT cofactor, UDP glucuronic acid (Monshouwer, 1996). This work gives insight into cytokine effects on UGT activity in pigs which may translate to human effects. For example, porcine IL-1α is about 80% similar to human IL-1α and monoclonal antibodies to human TNF-α cross-react with porcine TNF-α. However, important discrepancies exist between human and pig Phase II metabolism, including that porcine hepatocytes are unable to form sulfate conjugates of 1-naphthol and paracetamol (Monshouwer, 1996). This makes our aim to characterize the expression and secretion of pro-inflammatory cytokines in human liver cells during inflammation and acute infection and their relation to UGT expression and activity all the more important.

Effects of inflammation and infection on SULTs

Little work has been done on the effects of inflammation and infection on SULT enzymes in the liver. To date, research has focused on gastrointestinal, neuronal, bronchial and cardiovascular expression of these enzymes. Davies *et al* (2004) cultured neuronal and gastrointestinal cell lines with varying concentrations of TNF-α and TGF-β and found that the cytokines had relatively little effect on the neuronal cell line, but in the gastrointestinal cell line, TNF-α increased activity of SULT1A1 and SULT1A3 while

TGF-β decreased the activity of these SULTs. These results were significant because when diet-derived heterocyclic amines become bioactivated by sulfonation, they produce carcinogenic metabolites that may be a factor in the increased incidence of colorectal cancer in patients with inflammatory bowel disease.

Delmotte *et al* (2002) studied the effects of TNF-α on human bronchial mucosa where the cytokine is an important determinant in airway mucosa inflammation. It acts as an initial inflammatory cytokine that subsequently regulates both early neutrophil infiltraion and eosinophil recruitment into the lung and airspace (Lukacs, 1995). When TNF-α was applied to bronchial microsomes, elevated levels of galactose 3-O-sulfotransferase and N-acetylglucosamine 6-O-sulfotransferase were found. This is significant because this may increase the synthesis of epitopes on airway mucins, allowing the attachment of leukocytes to the mucus film which covers the airway lumen. These epitopes are largely expressed on cystic fibrosis mucins and are also possible sites of attachment for *Pseudomonas aeruginosa*, a frequent colonizer in the lungs of CF patients. Therefore, if TNF-α upregulate the synthesis of these epitopes, this may be contributing to the chronicity of airway infections in CF patients as well as other chronic bronchial diseases that involve severe inflammation (Delmotte, 2002).

The effects of IL-1β on SULTs in estrogen metabolism were also studies by Nakamura *et al* (2003). Specifically they assessed estrone sulfate (E1S), a major circulating plasma estrogen that is converted into the biologically active estrogen and estrone, by steroid sulfatase (STS) and estrogen sulfotransferase (EST). Their study found IL-1β markedly

inhibited the expression of STS mRNA and enzyme activity, but stimulated the expression of EST mRNA and enzyme activity. Since IL-1β is known to be expressed in atherosclerotic lesions, researchers believe that the expressions levels of both STS and EST mRNA and activity levels may be significantly associated with the degree of atherosclerotic changes in the female aorta (Nakamura, 2003).

Effects of inflammation and infection on GSTs

Glutathione transferases are of interest to pharmacologists and toxicologists because they provide targets for antiasthmatic and antitumor drug therapies (Hayes, 2005). Additionally, research has been conducted on the effects of cytokines in animal hepatocytes, but little has been done in human hepatocytes. Maheo *et al* (1997) treated rat hepatocytes with IL-1β and found GST activity levels to decrease to 50-60% of the control levels. GST mRNA were also found to be downregulated in the subunit rGSTP1 after 24 hours and similar effects were seen at the protein level. Further research found that the decrease in mRNA was due to mRNA destabilization which demonstrated that IL-1 regulates some major GST subunits in hepatocytes through a post-transcriptional mechanism (Maeho, 1997).

Cytokines are known to affect cells differently during acute and chronic infections. Desmots *et al* (2002) used cytokines TNF-α and IL-6 in murine hepatocytes and studied

their effects during liver regeneration. They found that introduction of these cytokines positively regulated mGSTA4 via survival signaling pathways and that during regeneration, several GST isoforms, including mGSTA1 and Pi, are induced by these cytokines.

Characterizing the expression and secretion of cytokines in human liver cells during inflammation and acute infection and their subsequent effect on UGT, SULT and GST activity and protein levels is important to understand events occurring in the liver during inflammation and infection. It may also provide insight into the metabolic capacity of the liver during chronic infection, where increased cytokine expression and altered UGT mRNA levels are not present. Congiu *et al.* compared mRNA levels from all UGT isoforms in human livers from patients with inflammation and compared these to UGT mRNA expression in patients with chronic liver fibrosis (Congiu 2002). Reduction in UGT mRNA levels was associated with the degree of inflammation, but the authors reported no correlation of UGT mRNA with the level of fibrosis. Previous to this, studies had indicated that levels of UGT enzymes are maintained or elevated in human and animal models of cirrhosis, thereby correlating with Congiu's findings (Congiu et al 2002, Debinsky, 1995).

Effect of reactive oxygen species on Phase II enzymes

Molecular oxygen is the premier biological electron acceptor and serves vital roles in multiple fundamental cellular processes (Scanalios, 2005). However, using oxygen produces reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, and

hydroxyl radicals. ROS can cause serious damage in aerobic cells by oxidizing lipids, proteins and DNA and can even cause cell death. To combat these ROS, organisms have evolved antioxidant defenses such as catalases, peroxidases, superoxide dismutases, and GSTs (Scandalios, 2005). The GSTs reduce phospholipids, fatty acid and cholesteryl hydroperoxides, curtailing the formation of downstream epoxides and reactive carbonyls (Hayes, 2005). GST accomplishes this by conjugating these compounds with the co-factor glutathione (GSH). This is significant because depletion of GSH decreases GST activity levels, preventing the metabolism of the ROS. Additionally, lower GSH levels conferring lower GST activities also enhances renal and hepatotoxicity caused by acetominophen, impairs survival in HIV diseases and has been clinically correlated with a number of human diseases including Alzheimer's, cancer, Parkinson's, cirrhosis, heart attack, stroke, diabetes ad infections caused by HIV and AIDS (Lin, 2007).

ROS have been also shown to activate immediate early genes such as NFκB and the jun Kinase pathway (JNK, Davis 2001). Classically, ROS activation of these genes and pathways has been demonstrated to be involved in apoptotic cell death and inappropriate proliferation that can lead to tissue necrosis and cancer respectively (Wang and Davis 2001). The depletion of GSH is believed to play a key role in the overproduction and prolonged action of ROS which tends towards the apoptotic pathway. Therefore, depletion of GSH and lowered GST activities may be responsible for tissue necrosis. In our studies, such a finding would be interesting, since it may imply a mechanism for liver failure caused by excessive inflammation or infection.

Nuclear regulation of Phase II enzyme expression and activity

Other than cytokine effects on protein synthesis, alterations in phase II enzyme expression and activity may occur through innate regulation of transcription by conserved sequences in the gene promoter regions. Inter-individual differences in expression of these phase II enzymes may contribute to an individual's response to pharmaceuticals and even possibly predisposition to cancer if exposed to a carcinogenic agent. Thus it is desirable to understand the mechanisms that drive transcription and attempt to determine the cause for their variable expression. Hepatocyte nuclear factor 1 (HNF1) is an important regulator of expression in UGT, SULT and GST in the liver (Wells, 2004). Deletion of HNF1 is lethal and a mutation of the HNF1 gene causes hyperbilirubinemia caused by inactivity of UGT enzymes and the inability to metabolize and excrete bilirubin (Usui, 2006). In the extreme this can lead to jaundice bilirubin accumulation in the ventricles of the brain, brain damage and/or death. The co-promoter HNF1 is highly dependent on the expression of HNF4 and, critically; this has been demonstrated to affect CYP450 metabolism through transcriptional regulation (Renton, 2004).

The presence of the HNF1 site is essential for expression of UGT2B7 and UGT2B17 in the liver (Gregory, 2002) and the HNF1 site is conserved in sequence in location in UGT2B4, UGT2B10, UGT2B11, and UGT2B15. HNF1α is a homeodomain-containing protein that forms homodimers, or heterodimers with HNF1β, and regulates transcription of liver and extrahepatic genes through a DNA binding site (Gregory, 2002). Gardner-Stephen and Mackenzie have also demonstrated that UGT1A3, 1A4 and 1A5 dependant on HNF1α in the proximal promoters of these UGTs. Additionally, mutation of the

HNF1 binding site in UGT1A3 promoter abolished promoter function (and thus transcription) in all cell types (Gardner-Stephen and Mackenzie, 2007). Furthermore, it has been shown that induction of UGT1A1 is not dependent on the elevation of HNF1 levels, but on nuclear glucocorticoid receptors that can induce UGT1A1 expression through intracellular signaling with the HNF1 site (Usui, 2006). HNF1α has also been shown to be an important factor in expression of UGT2B7 (Kamataki 2002). Kamataki *et al* (2002) showed that UGT2B7 expression was determined by HNF1α with the amount of mRNAs expressed for UGT2B7 being comparable to that of HNF1α. This suggests that HNF1α is a limiting factor for the expression of UGT2B7 and may be a causal factor for differential expression in individual human livers (Kamataki, 2002).

Similar to UGT, several studies have identified a role for HNF1 in GSTA regulation. For example, Whalen *et al* (2004) found that reduction of HNF1 in GSTA2 levels during the acute phase response where mediated by HNF1. Klone *et al* (1990) found that decreased GSTA2 expressions is human renal cell carcinomas is associated with reduced levels of HNF1. Additionally, it has been demonstrated that when IL-1β was introduced to Caco-2 cells, reduced GSTA1 mRNA levels were seen at all stages, but HNF1 mRNA levels were unaltered (Ng, 2007). It was determined that IL-1β repressed GSTA1 transcriptional activity and similar results were reported in HT-29 and HepG2 cells. The mechanism for this appears to be through the involvement of the transdominant repressor C isoform of variant HNF1 (vHNF-1C) (Ng, 2007). Other work performed in this area found that ceramide, a key regulator of apoptotic death, inhibits in GST gene transactivation through decreases in nuclear HNF1 (Park, 2004).

Importance of human studies and the suitability of HepG2 cells as a model

It is of major importance that little work on Phase II enzyme expression has been performed in humans. The majority of UGT studies to date have focused on rat hepatocytes (Strasser, 1998), mouse hepatocytes (Banhegyi, 1995; Morgan, 2006) or pig hepatocytes (Monshouwer, 1996). Clearly, other species are easier to manipulate (rodents) or may be more readily available, however; although metabolic activities of these laboratory animals may be similarly affected by cytokines, marked differences are observed in their ability to suppress or induce enzyme activities and in their time dependency (Monshouwer, 1996). Additionally, human cytokines vary in their similarity to other mammalian cytokines. In the case of pigs, IL-1α is about 80% similar to human IL-1α (Monshouwer, 1996). Work performed on SULT and GST enzymes has focused mainly on steroidogenic cell lines such as testis, breast and placenta (SULT) or neurological cell lines (glia, neurons; GST) and little is known about their effects in human hepatocytes. Likewise, study has been performed in primary human tumor tissues for all three enzymes, but little has been done for primary normal human tissues, especially the important sites of metabolism such as the liver, kidney and, to a lesser extent, intestine.

The HepG2 cell line is an appropriate model for these experiments due to the fact that it is a liver cell line that expresses all of the human Phase II enzymes. This model also offers additional benefits due to the availability of cell: several experiments can be performed multiple times without the difficulties that come with obtaining human tissues.

This model also allows us to avoid any problems that may occur from *in vivo* studies such as metabolites being transformed via an alternate pathway (Phase I). These circumstances will result in more accurate and more reliable data. The drawbacks of using a cell line as opposed to actual human tissue or performing *in vivo* studies include the lack of other cell types normally present in the body that may alter the response as in the case of the combination of lymphoid tissue response and organ-level tissue response, or the lack of complexity in a cell line compared to a human subject, and even the artificiality of the cell line: alterations may have occurred when it was transformed creating an abnormal karyotype. However, on the balance of factors, HepG2 cells are clearly superior to an animal cell lines to their direct human derivation and may be superior to primary animal cells and *in vivo* animal studies due to their direct reflection of human enzymes and pathways, not homologues.

Summary

It is of great importance to understand the regulation of phase II enzymes during infection and inflammation. In situations where expression of enzymes is depressed, the metabolism and clearance of drugs and xenobiotics may be severely decreased. Alternatively, carcinogenic compounds and/or reactive intermediate metabolites (for example from Phase I metabolism) could accumulate, leading to toxic consequences such as DNA damage or oxidative stress.

However, if the enzymes can be induced during infection and inflammation, higher rates of drug metabolism and clearance would occur. This could prevent drugs from having their intended therapeutic effects since the concentration in the body will not be high enough.

We propose to study phase II enzyme expression in the liver by investigating UGT, SULT and GST enzyme activity during acute infection and inflammation. Our specific aims are to use individual cytokines TNF-α and IL-1α as a model of acute infection and inflammation to: study the subsequent effects on pro-inflammatory cytokine expression and secretion in the human liver cell line HepG2; to study the effects on UGT, SULT and GST expression and activity in the human liver cell line HepG2, and to study the effects on regulatory co-promoters HNF1 and HNF4 to see if this is a mechanism for altering UGT, SULT and GST expression and activity.

Chapter 2: Materials and Methods

Materials

1-chloro-2,4-dinitro benzene, 2', 7'-dichlorofluoresceine diacetate, 2', 7'-dichlorofluoresceine, 4-methylumbelliferone sodium salt (4MU), 4-methylumbelliferone glucuronide (4MUG), 4-nitorphenol, alamethicin (from *trichoderma viridae*), β-glucuronidase (from *E. coli*), glutathione, $MgCl_2$, sacchrolactone, Tris-HCl and uridine diphosphoglucuronic acid (UDPGA) were purchased from Sigma Chemical Company (St Louis, MO). The co-factor 3'phosphoadenopsine-5'-phosphosulfate (PAPS) was from EMD biosciences (San Diego CA). Glutamine, HamsF12 media 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT reagent) and Vectashield with propidium iodide were purchased from Van Waters (VWR, West Chester, PA), penicillin/streptomycin and fetal bovine serum (FBS) were purchased from Invitrogen (La Jolla, CA) , Primary antibodies to UGT1A, UGT2B, HNF1α and HNF4 were purchased from Santa Cruz Biotechnologies (Satna Cruz, CA) while primary antibodies to SULT1A1, 1A3 and 2B1 and total GST were from Abnova.com (Taipei City, Taiwan) Secondary donkey α rabbit-biotin, donkey α goat-biotin and donkey α mouse-biotin as well as normal sera were obtained from Jackson Immunolabs (West Grove, PA). Streptavidin-FITC was from GE Healthcare (Piscataway, NJ). All other salts, solvents and chemicals were of analytical grade or higher.

Methods

HepG2 Cell Culture Treatments

HepG2 cells were cultured in DMEM media with 10% fetal bovine serum, 2% penicillin/streptomycin, and 2% glutamine. They were maintained in a humidified atmosphere of 95% air, 5% CO_2. Cells were mycoplasma free as determined by routine testing in our laboratory using Hoescht 33342. HepG2 cells were seeded at a density of 100,000 cells/T-75 flask and cultured for 5 days until confluent then treated with TNFα or IL-1β for 0, 2, 4, 8, 12, 24 and 48 hours at concentrations of 1000, 100, 10, 1.0, 0.1 and 0 U/ml.

Preparation of cellular S9 and determination of protein content

Cells were harvested by trypsinization (0.05%), centrifuged and re-suspended in 200 µL Tris-HCl pH 7.4 containing 5 mM $MgCl_2$. The cell suspension was sonicated for 30 seconds, vortexed, then centrifuged at 10,000 x g for 20 minutes at 4°C. The resulting supernatant (S9) was removed and frozen at -80 °C until use.

Standardization of protein concentration

Protein content of samples was performed according to published biochemical methods (Smith, 1985). Briefly, 10 µL of protein at the native concentration was added to a clear microplate and then 90 µL of 0.1 M NaOH was added. Equal volume of bicinchoninic acid solution was then added and plate was allowed to incubate at room temperature for

15 minutes on a belly rocker. Absorbance was then read at 562 nm. Values were them compared to a standard curve and all concentrations were standardized to 0.25 mg/ml of protein for all subsequent assays.

Biochemical detection of total UGT activity

Detection of total UGT activity was performed using a fluorescent assay with the universal substrate 4-methyl umbelliferone as previously described (Collier *et al* 2000). Briefly, cellular S9 (10 μL at 0.25 mg/mL), 0.1 M Tris-HCl pH 7.4 containing $MgCl_2$ (69.5 μL at 5 mM), alamethicin (0.5 μL, final concentration 50 μg/mg protein) and 4MU (10 μL, final concentration 100 μM) were added to a microplate on ice. The plate was warmed to 37 °C for 2 minutes then UDPGA (10μL, final concentration 5 mM) added and the microplate returned to the Fluorescence Reader (Gemini XS, Molecular Devices, Sunnyvale CA). Fluorescence was monitored at 355 nm excitation and 460 nm emission wavelengths. Metabolism was assessed by substrate depletion (fall in fluorescence) over 10 minutes where initial rate of reaction (loss of less than 10% initial product) and transformed to a rate of nmol/min/mg protein using a standard curve of 4MU (n = 7, average r^2 =0.9984). Experiments were performed on 3 separate occasions and each aliquot was assessed in triplicate for separate HepG2 treatments.

Biochemical detection of total SULT activity

Detection of total SULT activity was assessed spectrophotometrically by a decrease in the color of the parent product (Mulder *et al*, 1975). The assay proceeded as follows: Cellular S9 (30μL at 0.25 mg/mL), 50 mM potassium phosphate buffer containing 5 mM

$MgCl_2$ (210 μL), *p*-nitrophenol (30μL, final concentration 400 μM) and PAPS (30 μL, final concentration 60 μM) were added to an eppendorf tube and incubated in a water bath at 37°C for 20 minutes. The reaction was terminated and developed by the addition of an equal volume of 1 M NaOH. Then 3 aliquots were transferred to a microplate and absorbance read once at 400 nm. Quantitation is by a comparison to a standard curve of 4-NP prepared in 1 M NaOH and is through depletion of absorbance (n=3, average r^2 =0.9998). Under these conditions, the assay detects the combined activity of SULT1A1, 1A3, 1B1, 1E1 and 2A1 although SULT1A1 and SULT 1B1 may show substrate inhibition with such high levels of 4-NP (Tabrett and Coughtrie 2003).

Biochemical detection of total GST activity

Detection of total GST activity was assessed spectrophotometrically (Gonzalez *et al*, 1989). The assay proceeded as follows: Cellular S9 (0.25 mg/mL), 0.1 M potassium phosphate buffer pH 6.5 (79μL), and glutathione (10 μL, final concentration 1 mM) were added to a microplate and warmed for 2 minutes. The substrate 1-chloro-2,4-dinitro benzene (CDNB, 1 μL at 0.5 mM final concentration) was added and the reaction is monitored every 10 seconds for 20 minutes at 340 nm. Specific activity is calculated using ε=9.6 mM/cm^2 (Habig, Pabst and Jakoby 1974)

Measurement of cell viability

HepG2 cells were plated in a clear microplate at a density of 10,000 cells per well and left to adhere overnight. Cells were then treated with TNFα or IL1β (1000, 100, 10, 1.0, 0.1, 0 U/mL) for 2-48 hours. At each time point, the cells were removed from the incubator and 2 μL of MTT reagent (10 mM) was added, then cells returned to the incubator. After 4 hours the cells were removed, the media and MTT reagent were aspirated, and 200 μL of 10% SDS solution were added to each well. Cells were then returned to the incubator overnight and then the optical density assessed at 595 nm. Results were calculated as percent of the zero dose control.

Detection of reactive oxygen species

HepG2 cells were plated in a clear microplate at a density of 10,000 cells per well and left to adhere overnight. Cells were then treated with TNFα or IL1β (1000, 100, 10, 1.0, 0.1, 0 U/mL) for 2-48 hours. At each timepoint, media was removed and fresh media containing 5 μM diclorofluorescene diacetate was added fluorescence was read every 30 seconds at 485 and 535 nm. Values were then compared to a standard curve of dichlorofluorescene.

Immunocytochemical staining for the presence of UGT, SULT and GST isoforms

HepG2 cells were cultured on sterile glass coverslips in 10 cm dishes. When confluent, cells were treated with TNFα or IL-1β (100U/mL) 24 hours. Subsequently, culture media was removed and cells were fixed by addition of 1:1 acetone-methanol fixative for 2 minutes. The fixative was then removed and discarded and PBS added. Cells were

stored at 4°C until immunocytochemistry was performed. After fixing, PBS was removed and 200μ/slide of biotinylated primary antisera (1:50 concentration for UGT1A and UGT2B, 1:100 for SULT1A1, 1A3, 2B1 and pan-specific GST antibodies) diluted in wash buffer (PBS-T) with 5% normal donkey serum (NDS) was added and incubated at room temperature for 1 hour. After incubation, antiserum was removed and wash solution added gently to avoid dislodging the cells from the slide. This was performed three times, then 200 μl/slide of secondary antisera (1:500 donkey α rabbit-biotin for UGT1A and GST, donkey α goat-biotin for UGT2B, donkey α mouse-biotin for all SULT enzymes) diluted in wash buffer (PBS-T) with 5% NDS was added and incubated at room temperature for 1 hour. After incubation, the cells were again washed in wash buffer three times and then streptavidin-fitc complex diluted 1:500 in wash buffer was added and incubated for 15 minutes in the dark. Subsequently cells were washed (1 time for 10 minutes) and then allowed to dry. Coverslips were then affixed to slides using Vectashield mounting medium containing prodidium iodide. Photomicrographs were taken with an Zeiss Axiovert 200 M fluorescence microscope using 63X oil immersion.

Immunocytochemical localization of HNF1 and HNF4 proteins

HepG2 cells were cultured on sterile glass coverslips in 10 cm dishes. When confluent, cells were treated with TNFα or IL-1β (100U/mL) for 0, 15, 30 and 60 minutes. Subsequently, culture media was removed and cells were fixed by addition of 1:1 acetone-methanol fixative for 2 minutes. The fixative was then removed and discarded and PBS added. Cells were stored at 4°C until immunocytochemistry was performed. After fixing, PBS was removed and 200 μ/slide of biotinylated primary antisera (1:50

concentration for HNF1 and HNF4) diluted in wash buffer (PBS-T) with 5% normal donkey serum (NDS) was added and incubated at room temperature for 1 hour. After incubation, antiserum was removed and wash solution added gently to avoid dislodging the cells from the slide. This was performed three times, then 200 μl/slide of secondary antisera biotin (1:500 donkey α rabbit-biotin) diluted in wash buffer (PBS-T) with 5% NDS was added and incubated at room temperature for 1 hour. After incubation, the cells were again washed in wash buffer three times and then streptavidin-fitc complex diluted 1:500 in wash buffer was added and incubated for 15 minutes in the dark. Subsequently cells were washed (1 time for 10 minutes) and then allowed to dry. Coverslips were then affixed to slides using Vectashield mounting medium with prodidium iodide. Photomicrographs were taken with an Zeiss Axiovert 200 M fluorescence microscope using 63X oil immersion.

Chapter 3: Results

Measurement of cell viability

Results were compared to internal controls meaning cells were grown for an equivalent amount of time as treated cells, but controls remained untreated. Thus, any increase or decrease seen in treated cells is reflective of the effects of the cytokine. When cells were treated with IL-1β, the results showed a nearly twofold increase in cell viability at 12 hours, compared to the zero dose control. After 12 hours, the viability began to decline (Figure 1). One-way ANOVAs, used to see if time was the cause of cell death, showed that between the 6 and 12 hour timepoints, for 1.0 U/ml and 0.1 U/ml, cell viability was significant ($P < 0.05$, Figure 2). ANOVA analysis also demonstrated that, for each time point, viability did not change when increasing amounts of IL-1β were administered. Together, these data indicate that viability of the HepG2 cells was time-dependent but not IL-1β-concentration dependent so this means that the cells are growing over time but not proliferating due to IL-1β effects.

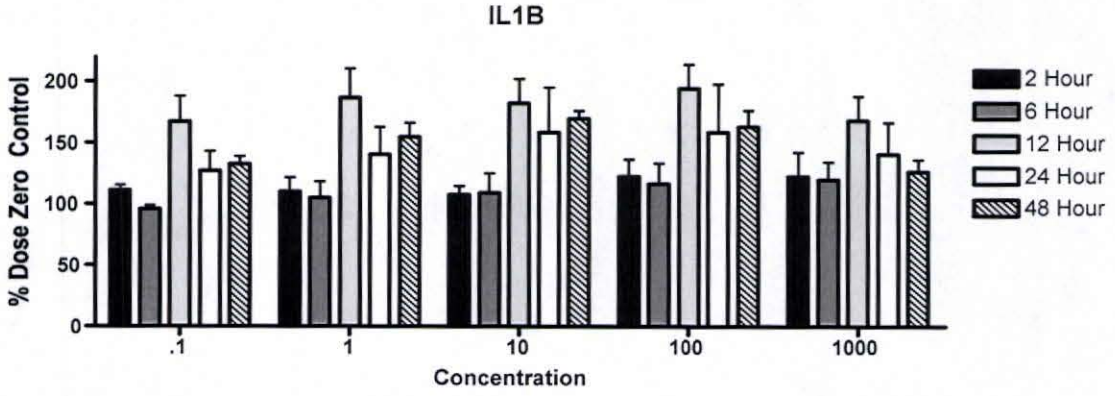

Figure 1: MTT assays demonstrate that an increase in cell viability occurs at 12 hours, thereafter; viability declines. Results were calculated as percent of the zero dose control. Bars are means of 3 separate experiments, performed in triplicate ± SD.

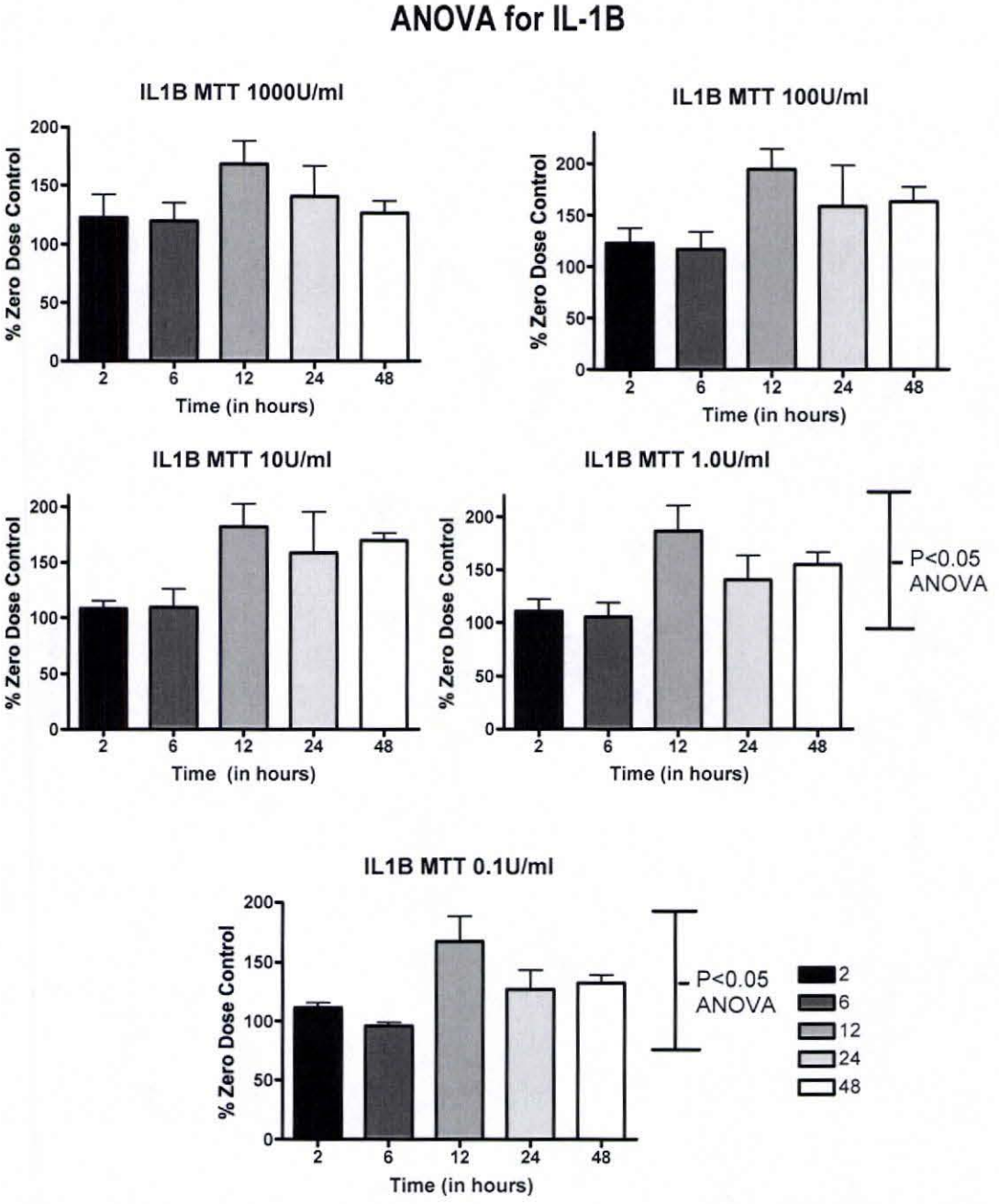

Figure 2: ANOVA performed on MTT assays to see the effect of IL-1β over time, show that changes in cell viability were only significant at 0.1 and 1 U/mL IL-1β. Results were calculated as percent of the zero dose control. Bars are means of 3 separate experiments, performed in triplicate ± SD.

When cells were treated with TNF-α, an increase is also observed at 12 hours. However a sharp decrease in viability is observed at 24 hours followed by recover at 48 hours (Figure 3). When the graphs were statistically analyzed using one-way ANOVAs to see if variations in time were significantly different, for all concentrations, the decrease in cell growth from the 12 to 24 hour timepoints was statistically significant ($P < 0.01$, Figure 4). ANOVA analysis also demonstrated that, at each time point, there were no significant differences in viability caused by the increasing amounts of TNF-α administered. Together, these data indicate that viability of the HepG2 cells was time-dependent but not TNF-α-concentration dependent since the pattern of cell death was consistent across all doses of TNF-α. Alternatively, it could be that even as low as 0.1 U/mL, TNF-α is a potent pro-apoptotic agent that takes 24 hr to manifest.

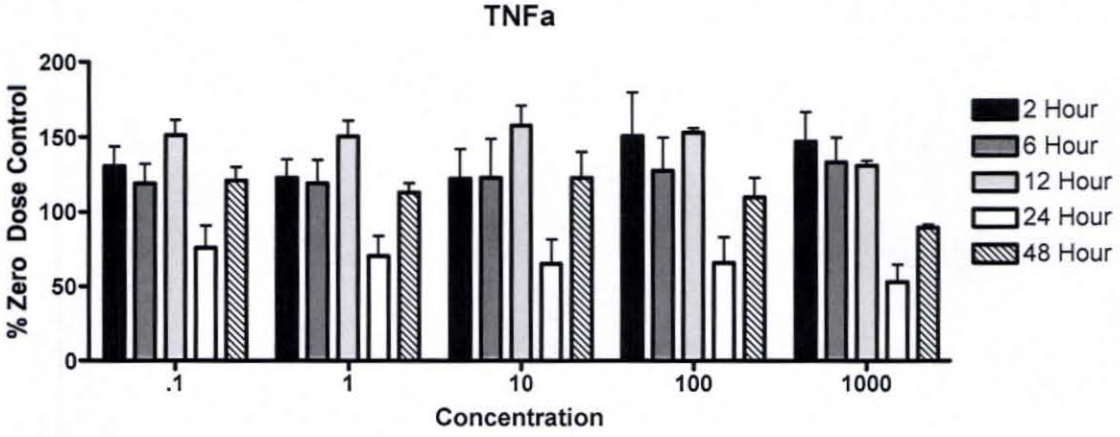

Figure 3: MTT assays demonstrate that an increase in cell viability occurs at 12 hours, thereafter; viability declines markedly at 24 and 48 hr. Results were calculated as percent of the zero dose control. Bars are means of 3 separate experiments, performed in triplicate ± SD.

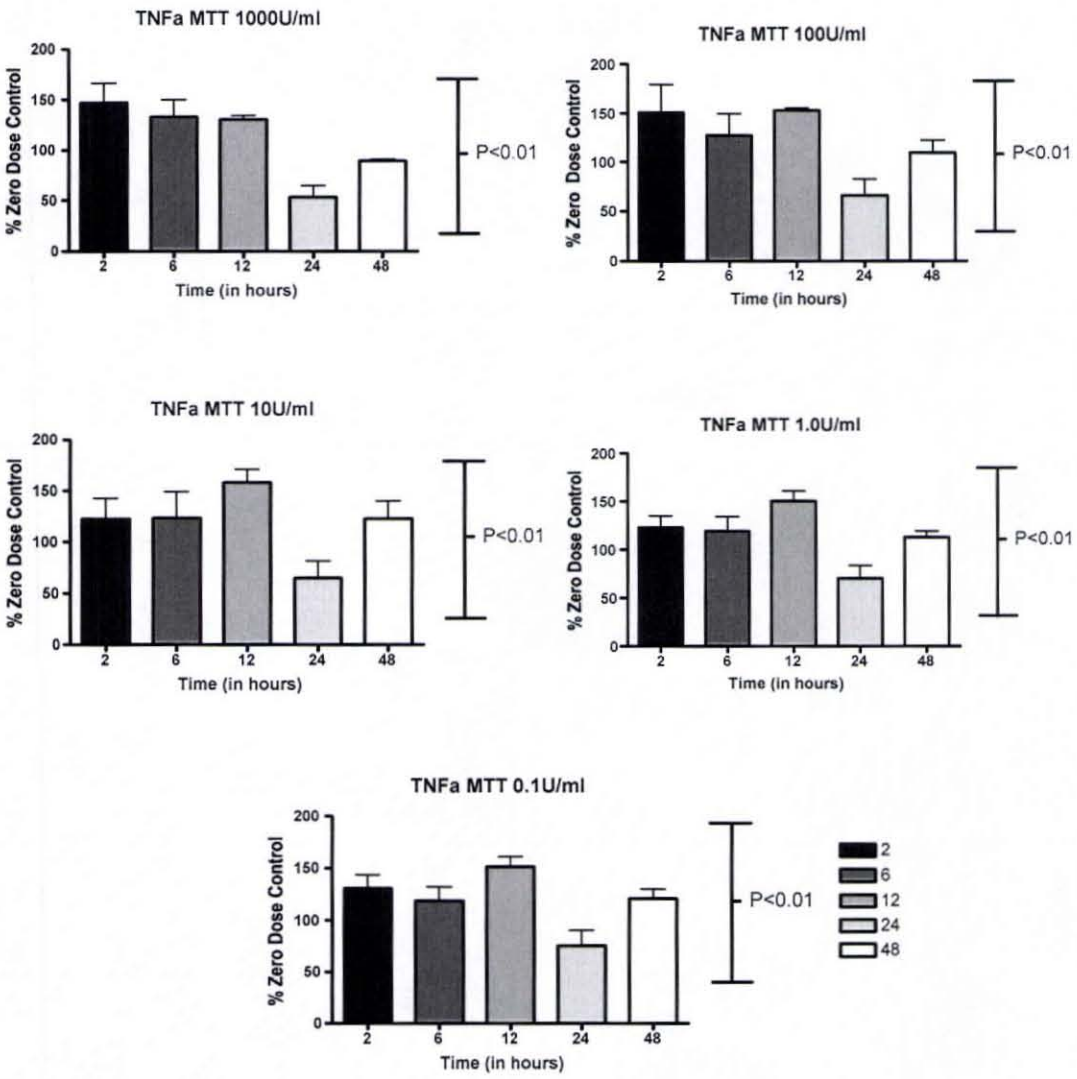

Figure 4: ANOVA performed on MTT assays to see the effect of TNF-α over time, show that changes in cell viability were significant at all concentrations of TNF-α. Since all concentrations were equally significant, this may indicate that cell death is a function of time rather than TNF-α concentration. Results were calculated as percent of the zero dose control. Bars are means of 3 separate experiments, performed in triplicate ± SD.

Effect of cytokines on reactive oxygen species

When HepG2 cells are treated with IL-1β for 0 – 48 hr, ROS increased at every concentration of IL-1β application. The levels of ROS recorded were consistent with the levels produced in control cells when no cytokine was administered. Therefore, increases in ROS are most likely caused by cell growth in the wells of the plate and not by IL-1β administration (Figure 5). When the graphs were statistically analyzed using one-way ANOVA to observe variation in ROS with time, all timepoints were found to be statistically significant ($P < 0.0001$, Figure 6). This analysis also demonstrated that, at each individual time point, there were no significant differences in ROS compared to control caused by increasing amounts of IL-1β administered. Together these data indicate that increases in ROS were time-dependent but not IL-1β concentration-dependent. Along with the known doubling time of HepG2 cells (around 16 hours) these data show that ROS levels are proportional to cell number and the increases observed likely represent over-crowding.

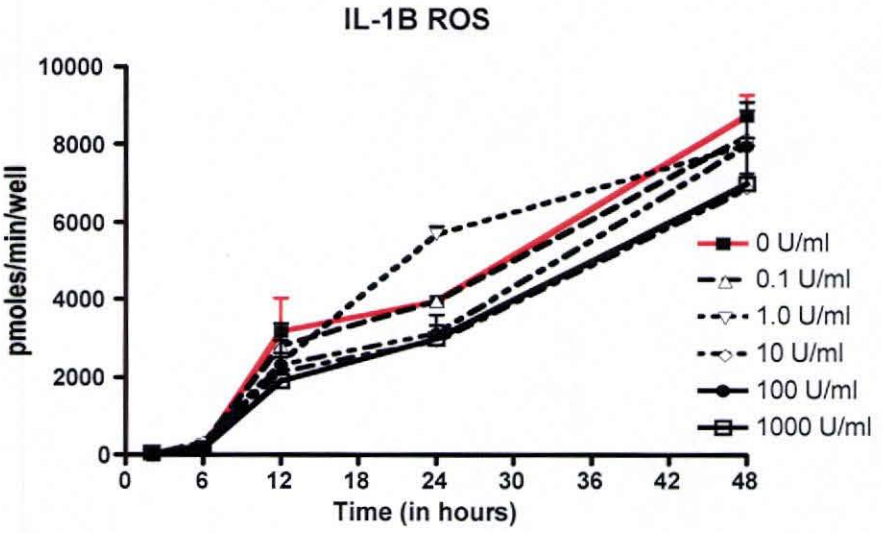

Figure 5: Increases in ROS over time when HepG2 cells are treated with IL-1β.

Results were calculated by comparison to a standard curve of dichlorofluoresceine. Points are means of 3 separate experiments, performed in triplicate ± SD.

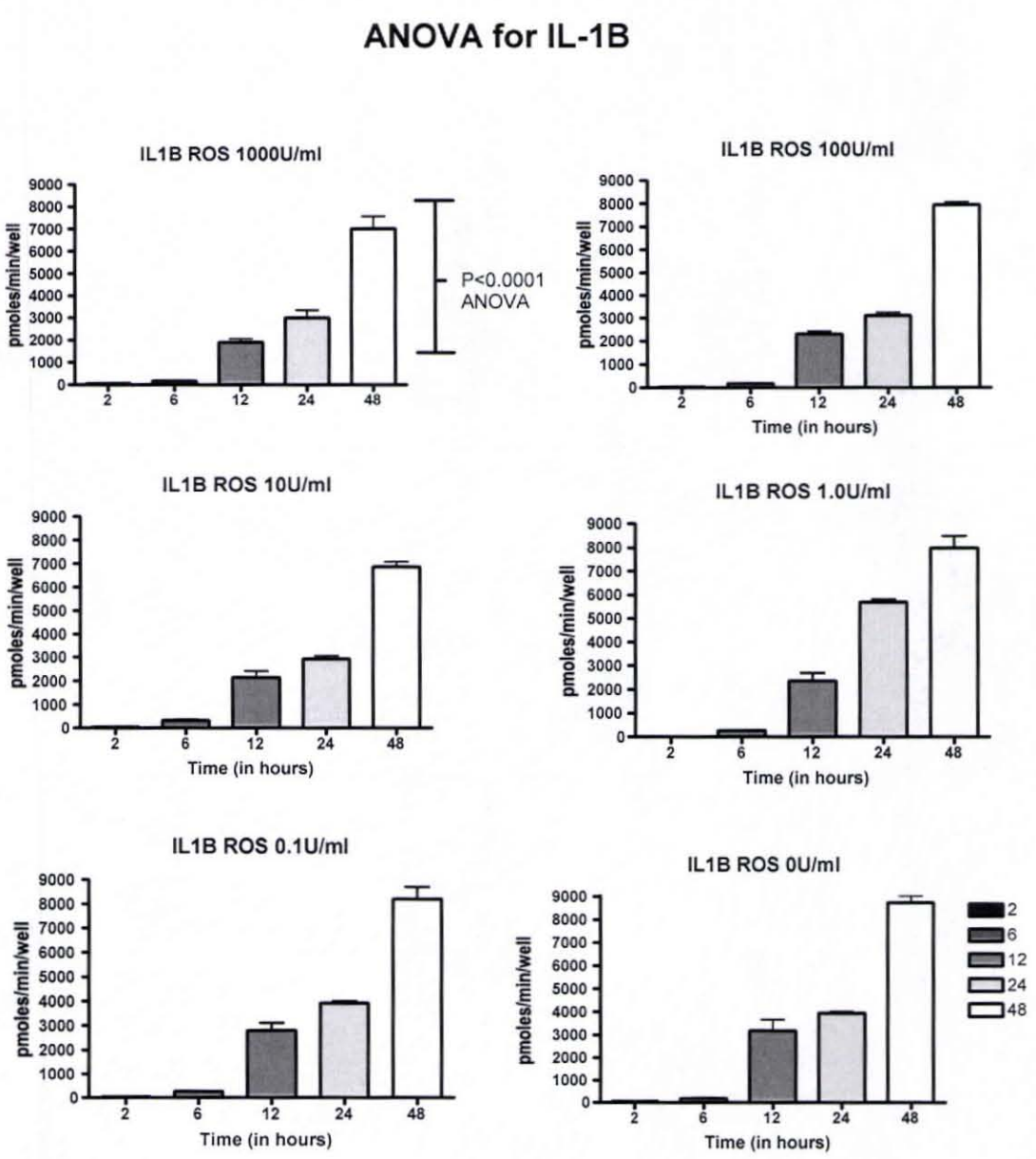

Figure 6: Increases in ROS over time when HepG2 cells are treated with IL-1β and analyzed by IL-1β time. Results were calculated by comparison to a standard curve of dichlorofluoresceine. Points are means of 3 separate experiments, performed in triplicate ± SD.

For TNF-α treatment, the amount of ROS increased rapidly and dramatically within 2 hr to levels equivalent to those observed after 24 - 48 hr in untreated cells. After this massive increase at 2 hr, ROS did not further increase over time with the amount of ROS for all concentrations except 10 U/ml consistently in the range of ~5000 pmoles/min/well over the entire timecourse. When 10 U/ml of TNF-α was administered, the amount of ROS was not dramatically raised and remained in the range of 1000-2000 pmoles/min/well over the entire timecourse. ROS variations over time were not significantly different for TNF-α administration, except at the 6 hour timepoint with 0.1 U/ml of TNF-α (Figure 8). When the graphs were analyzed with respect to TNF-α concentration, 10 U/ml TNF-α caused significantly lower ROS levels than any other concentration tested and significantly lower than control concentrations at all time points (Figure 9). These data indicate that when HepG2 cells are treated with TNF-α, ROS production is dependent on both time in culture and the concentrations of TNF-α applied to the cells.

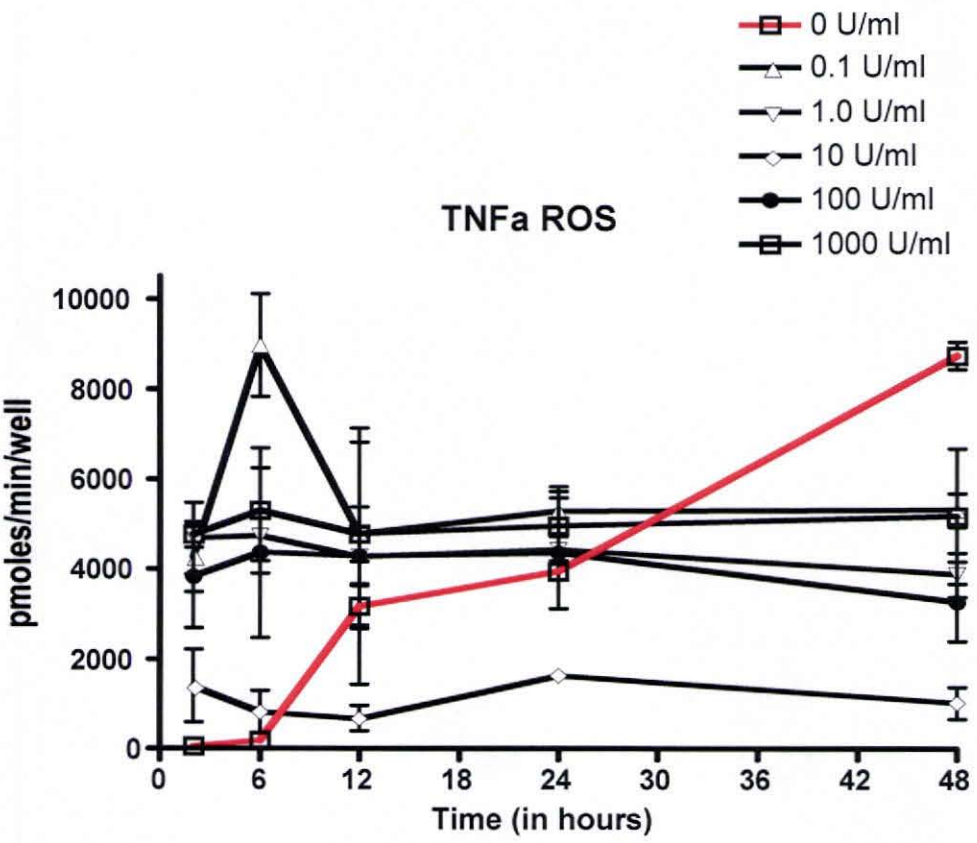

Figure 7: ROS do not increase over time when HepG2 cells are treated with TNF-α.

Results were calculated by comparison to a standard curve of dichlorofluoresceine. Points are means of 3 separate experiments, performed in triplicate ± SD.

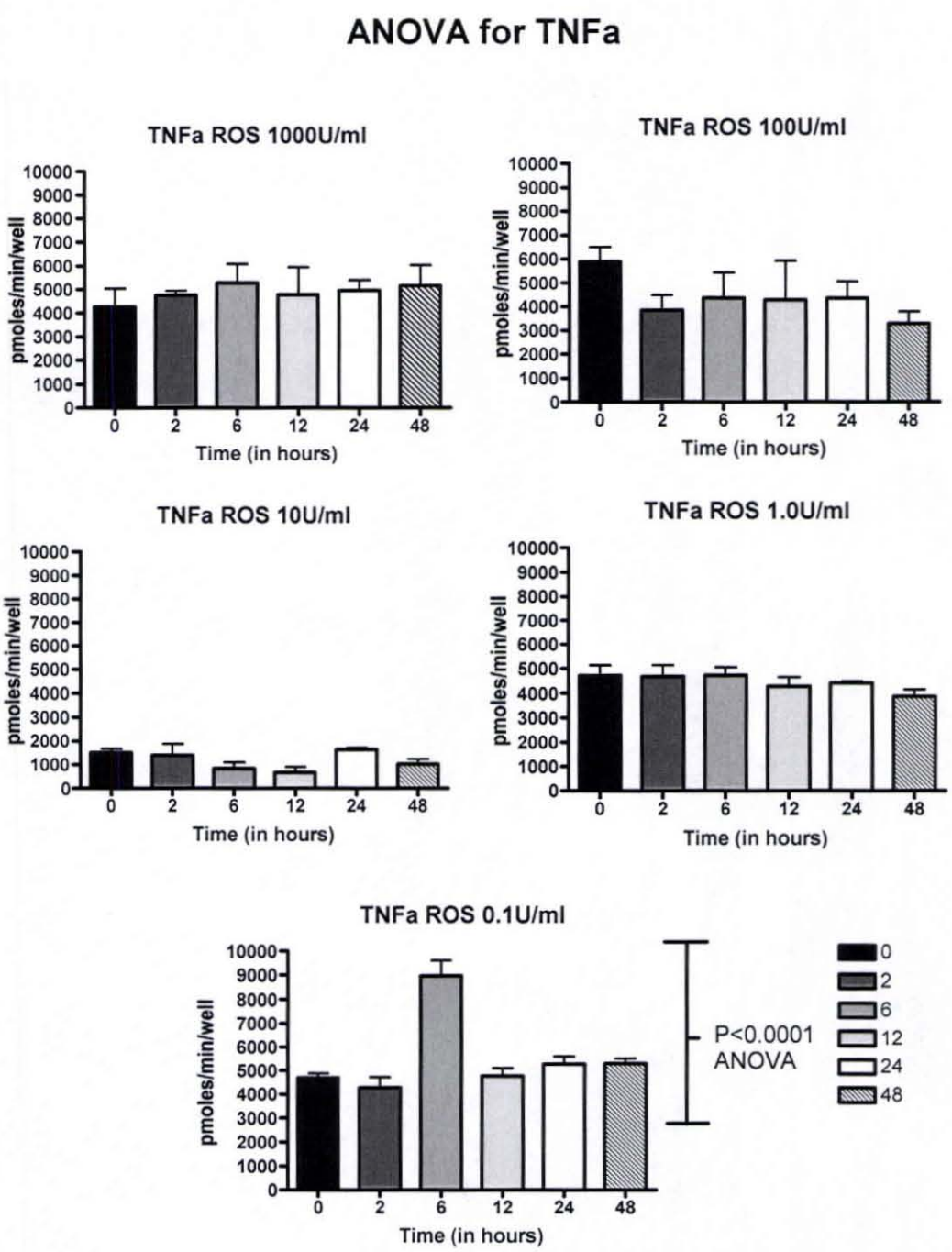

Figure 8: The lack of increase in ROS measured in HepG2 cells is consistent over time. Note especially the effects of TNF-α 10 U/mL. Results were calculated by comparison to a standard curve of dichlorofluoresceine. Bars are means of 3 separate experiments, performed in triplicate ± SD.

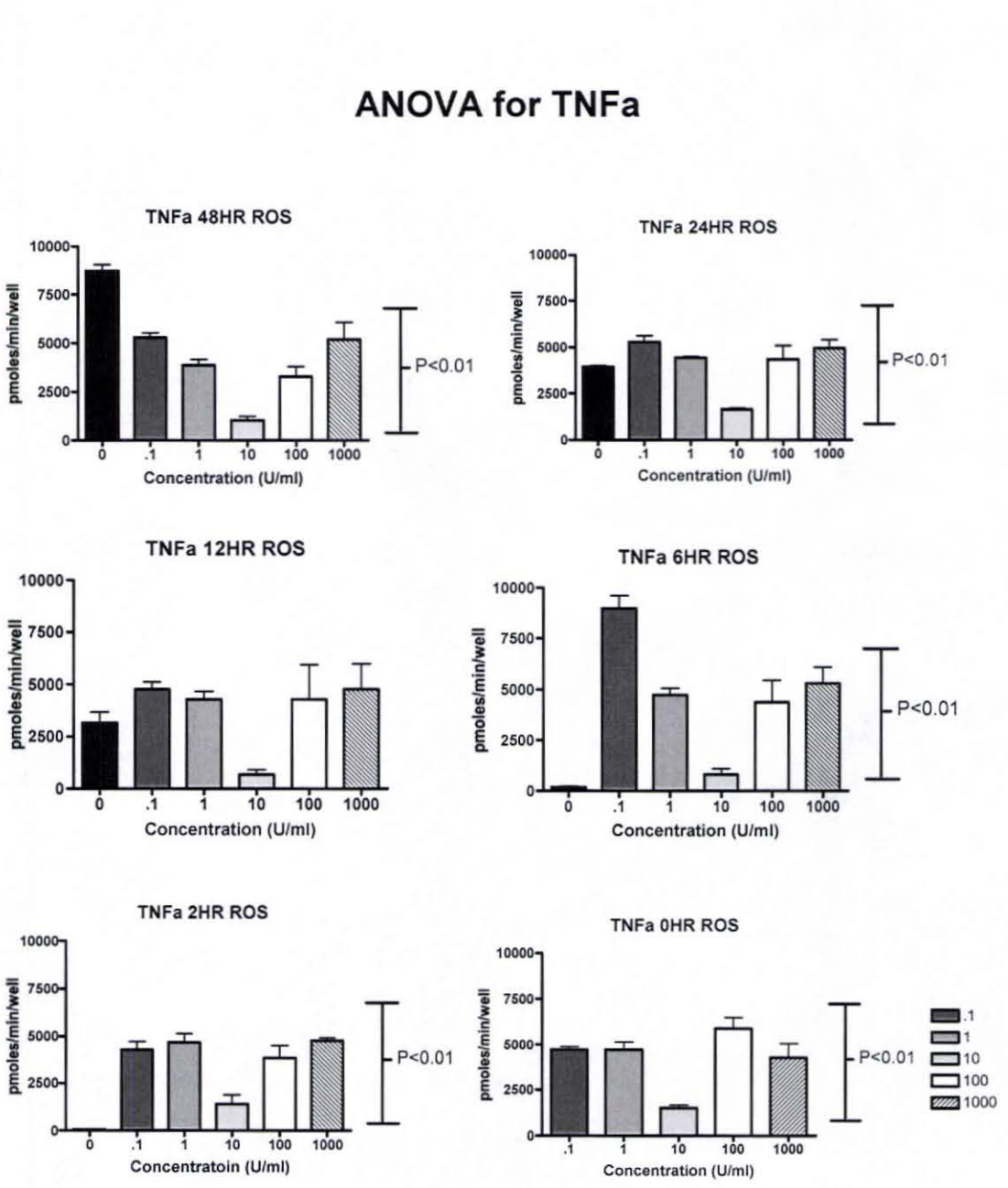

Figure 9: The lack of increase in ROS measured in HepG2 cells is consistent across all concentrations of TNF-α. Note especially the effects of TNF-α 10 U/mL. Results were calculated by comparison to a standard curve of dichlorofluoresceine. Bars are means of 3 separate experiments, performed in triplicate ± SD.

Biochemical detection of total UGT activity

The UGT assay results are shown in Figure 10. Each concentration has nearly the same shape both for IL-1β (Figure 10A) and TNF-α (Figure 10B). This shape is also seen in the control. We observed a consistent trend of decreasing UGT activity until the 12 hour timepoint, whereupon the level of UGT activity in the cells rebounded. When the graph was analyzed statistically only the 1.0 U/ml treatments reached significance ($P < 0.05$) for the decrease in UGT activity and this was significant for both IL-1β and TNF-α administration.

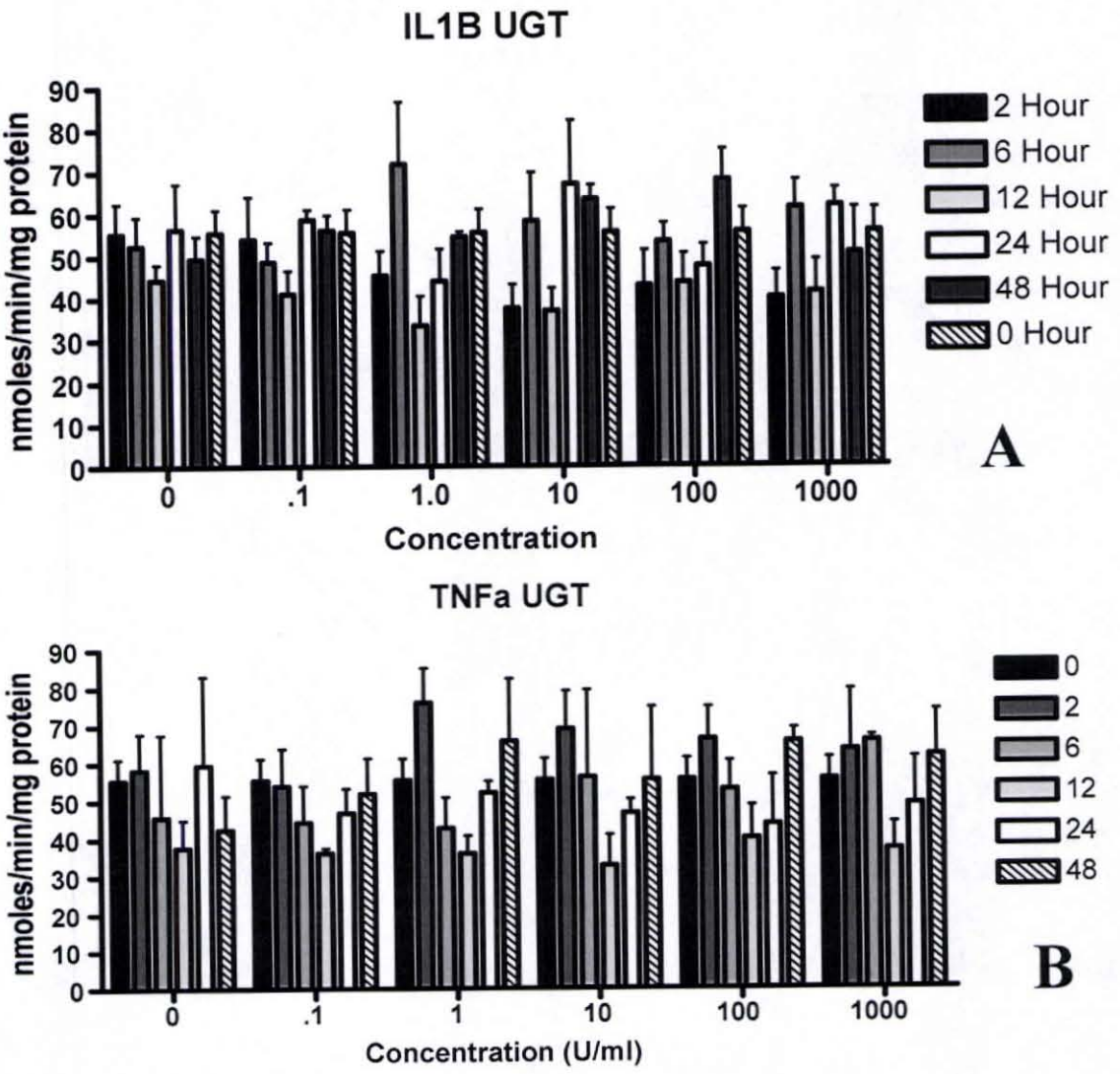

Figure 10: Effects of proinflammatory cytokines on UGT activity. 10A: IL-1β does not significantly alter UGT activity. **10B:** TNF-α causes a significant decline in UGT activity for up to 12 hrs at 1.0 U/ml, whereafter the enzymes recover to control levels. Bars are means of 3 separate experiments performed in triplicate.

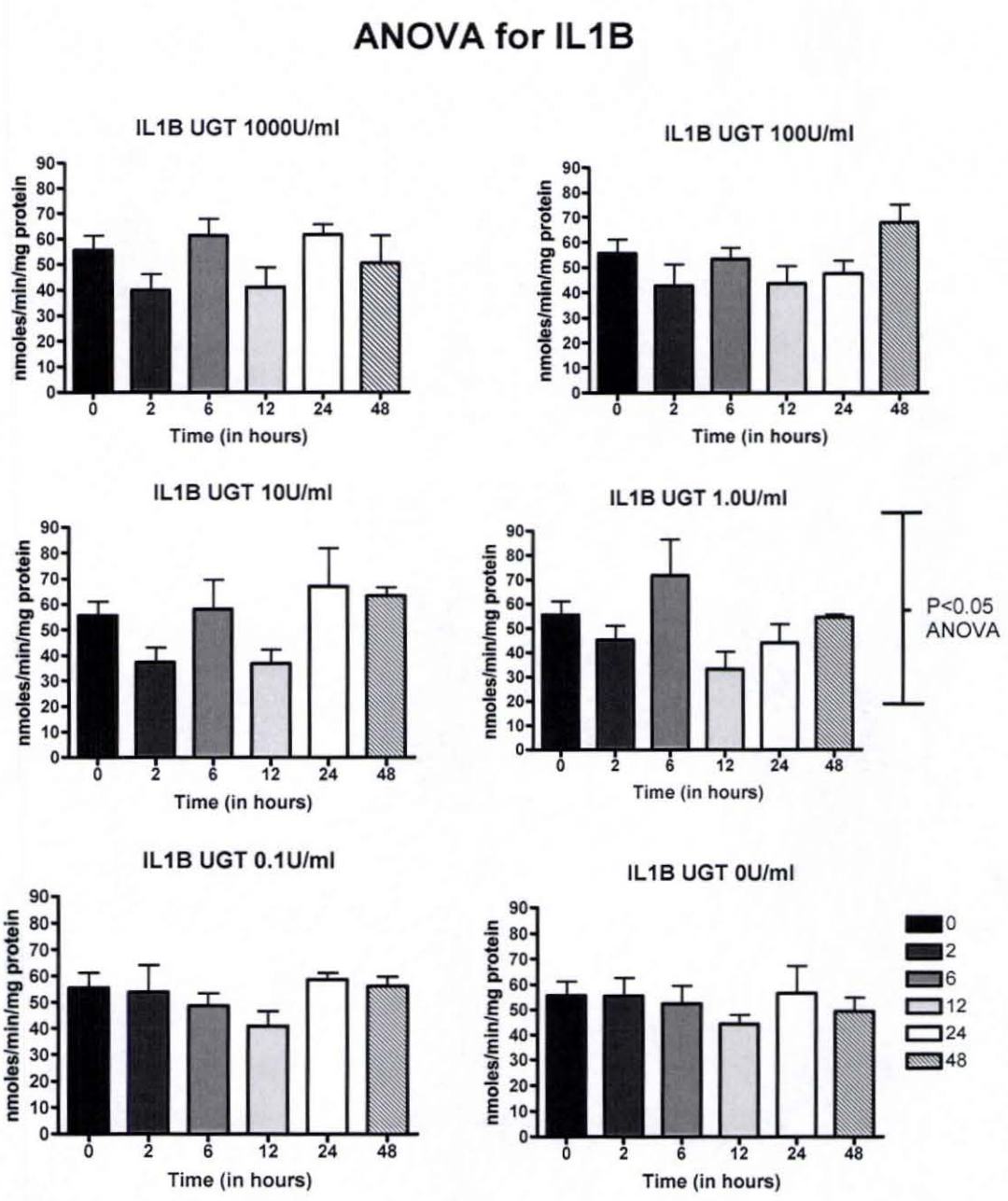

Figure 11: The trend of a fall in activity until 12 hours and then rebounding is consistent throughout and significant at 1.0U/ml for IL-1β. Bars are means of 3 separate experiments performed in triplicate.

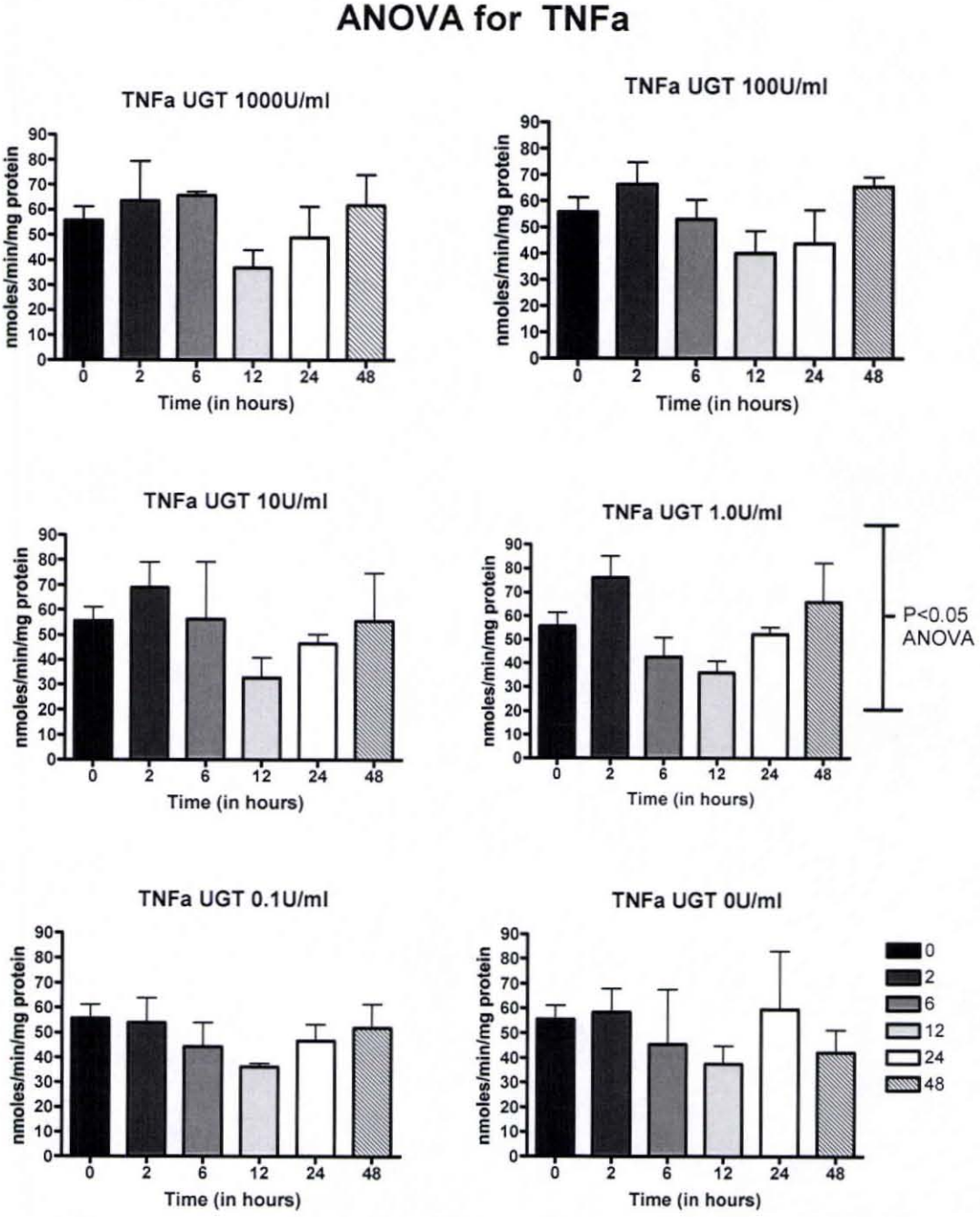

Figure 12: The trend of a fall in activity until 12 hours and then rebounding is consistent throughout and significant at 1.0U/ml for TNF-α. Bars are means of 3 separate experiments performed in triplicate.

Immunohistochemistry confirmed the presence of both UGT1A and UGT2B enzymes in HepG2 cells both before and after cytokine treatment (Figure 13). Staining for UGT1A and UGT2B isoforms was cytoplasmic, and punctate. For HepG2 cells treated with IL-1β, some nuclear staining for UGT1A was observed (Figure13A). The presence of UGTs in the nucleus or nuclear envelope has not previously been reported.

Additionally, despite our previously reported fall in viability (MTT assay) at 24 hr with TNF-α administration, no apoptosis was visible in cells treated with IL-1β or TNF-α (100 U/mL) for 24 hrs since no nuclear or cytoplasmic shrinkage was observed nor was any cytoplasmic blebbing recorded (Figure 13).

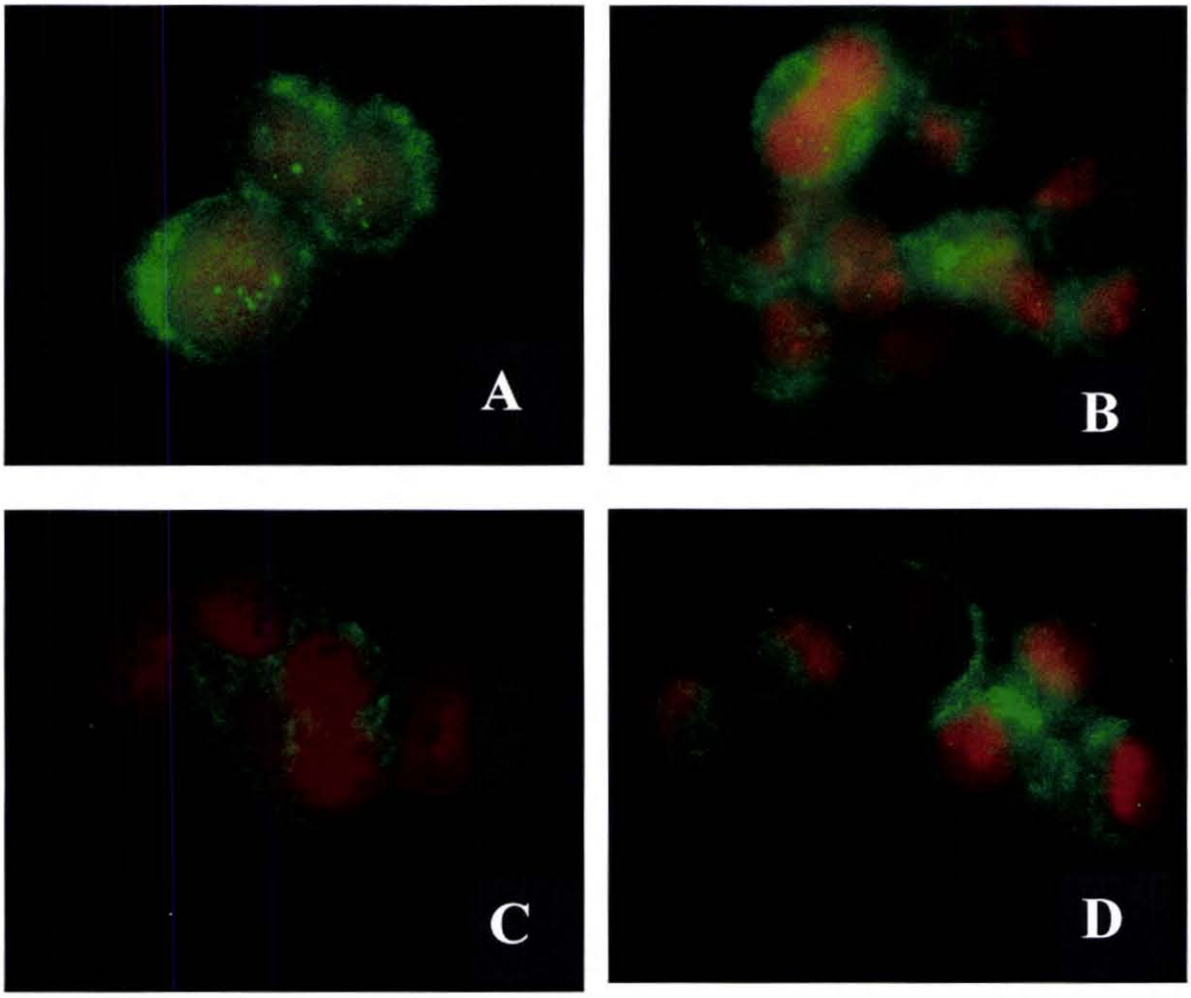

Figure 13: Immunohistochemical detection of the expression of UGT1A and UGT2B in Hep G2 cells shows that the isoforms are present and that apoptosis is likely not occurring (based on cell morphology). Figure 13A: The expression of UGT1A in cells treated with IL-1β. **Figure 13B:** The expression of UGT1A in cells treated with TNF-α. **Figure 13C:** The expression of UGT2B in cells treated with IL-1β. **Figure 1D:** The expression of UGT1A in cells treated with TNF-α. Magnification is 63X.

Biochemical detection of total SULT activity

The SULT assay revealed that there is apparent increase in SULT activity over time. This happens also in the control, so it appears to be independent of IL-1β administration. When these results were analyzed, the increase in SULT activity in controls and treated cells was not statistically significant (Figure 14A). In the case of TNF-α, again we observed an increase in SULT activity consistent with the zero dose control and again, none of the concentrations of TNF-α caused significant increases in SULT over the control. Therefore increases in SULT were time dependent only and likely reflective of increased numbers of cells as they proliferated (Figure 14B).

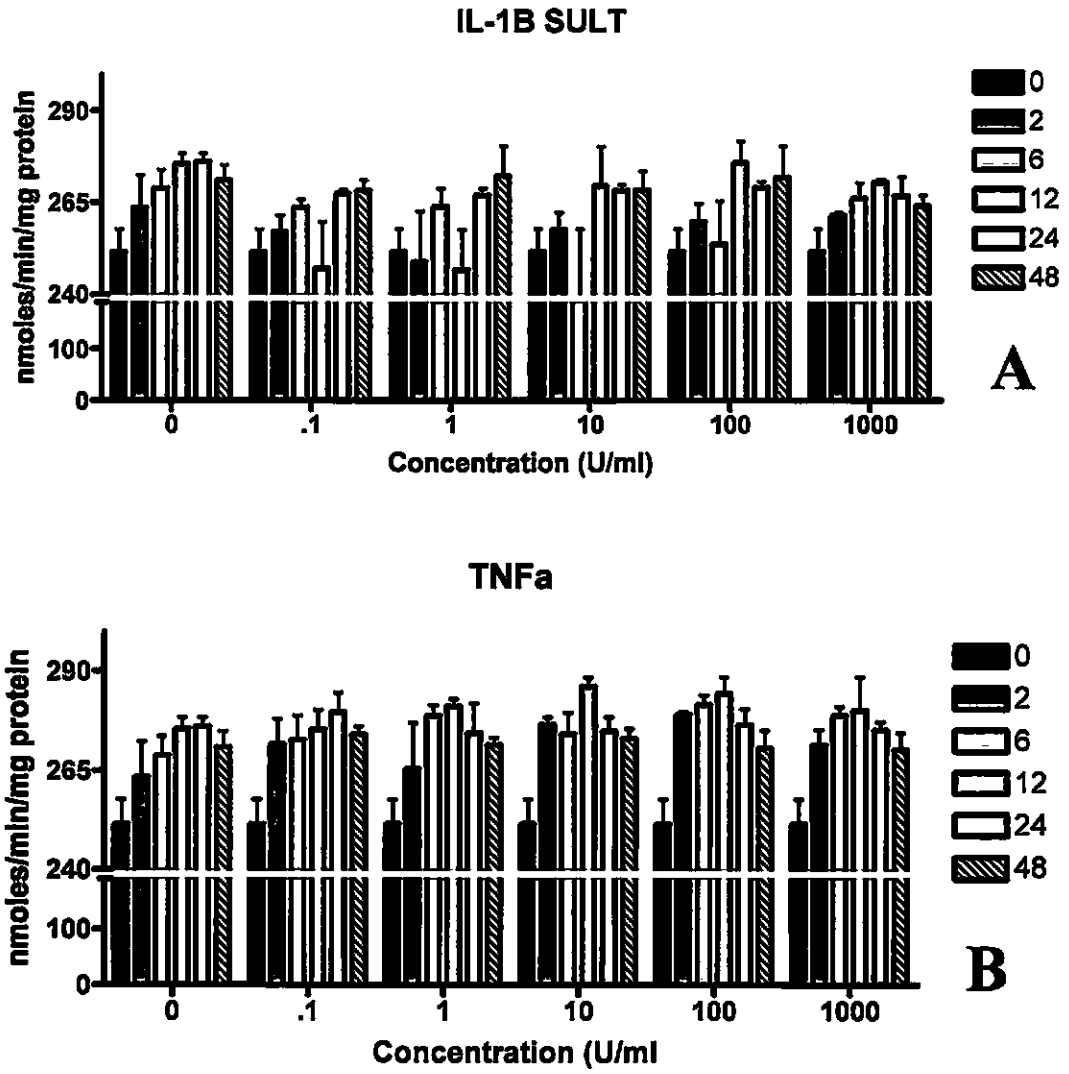

Figure 14: Effects of proinflammatory cytokines on SULT activity. Figure 14A: IL-1β does not significantly alter SULT activity although a consistently increased level of activity was observed. **Figure 14B:** TNF-α does not cause significant changes in SULT at any concentration of TNF-α. Bars are means of 3 separate experiments performed in triplicate ± SD.

Immunohistochemistry confirmed the presence of SULT1A1, 1A3 and 2E1 in HepG2 cells both before and after cytokine treatment (Figure 15). Staining for SULT isoforms was cytoplasmic. For HepG2 cells treated with IL-1β, some nuclear staining for SULT2E1 was observed (Figure 15E).

Similar to results reported for UGT immunohistochemistry (above), no apoptosis was caused by IL-1β or TNF-α for 24 hrs since no nuclear or cytoplasmic shrinkage was observed nor was any cytoplasmic blebbing recorded (Figure 13).

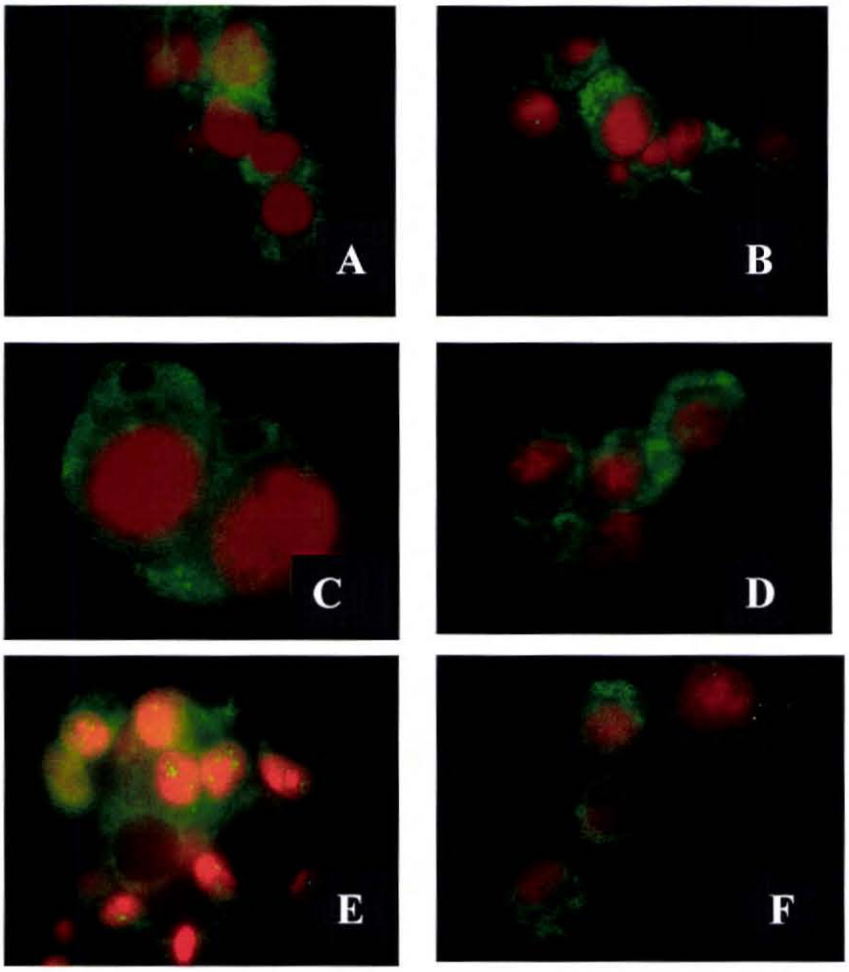

Figure 15: Immunohistochemical detection of the expression of SULT isoforms in Hep G2 cells. Figure 15A: The expression of SULT1A1 in cells treated with IL-1β. **Figure 15B:** The expression of SULT1A1 in cells treated with TNF-α. **Figure 15C:** The expression of SULT1A3 in cells treated with IL-1β. **Figure 15D:** The expression of SULT1A3in cells treated with TNF-α. **Figure 15E:** The expression of SULT2E1 in cells treated with IL-1β. **Figure 15F:** The expression of SULT2E1 in cells treated with TNF-α. Magnification is 63X.

Biochemical detection of total GST activity

Results from the GST assay showed no change in the amount of GST activity when IL-1β was applied at all concentrations and timepoints compared to the zero dose control (Figure 16). When TNF-α was administered, initial increases in GST activity were observed at the 2 hr and then by 12 hours, GST activity returns to normal, control levels. These increases were analyzed using a t-test and were found to be statistically significant with $P < 0.05$ for all concentrations.

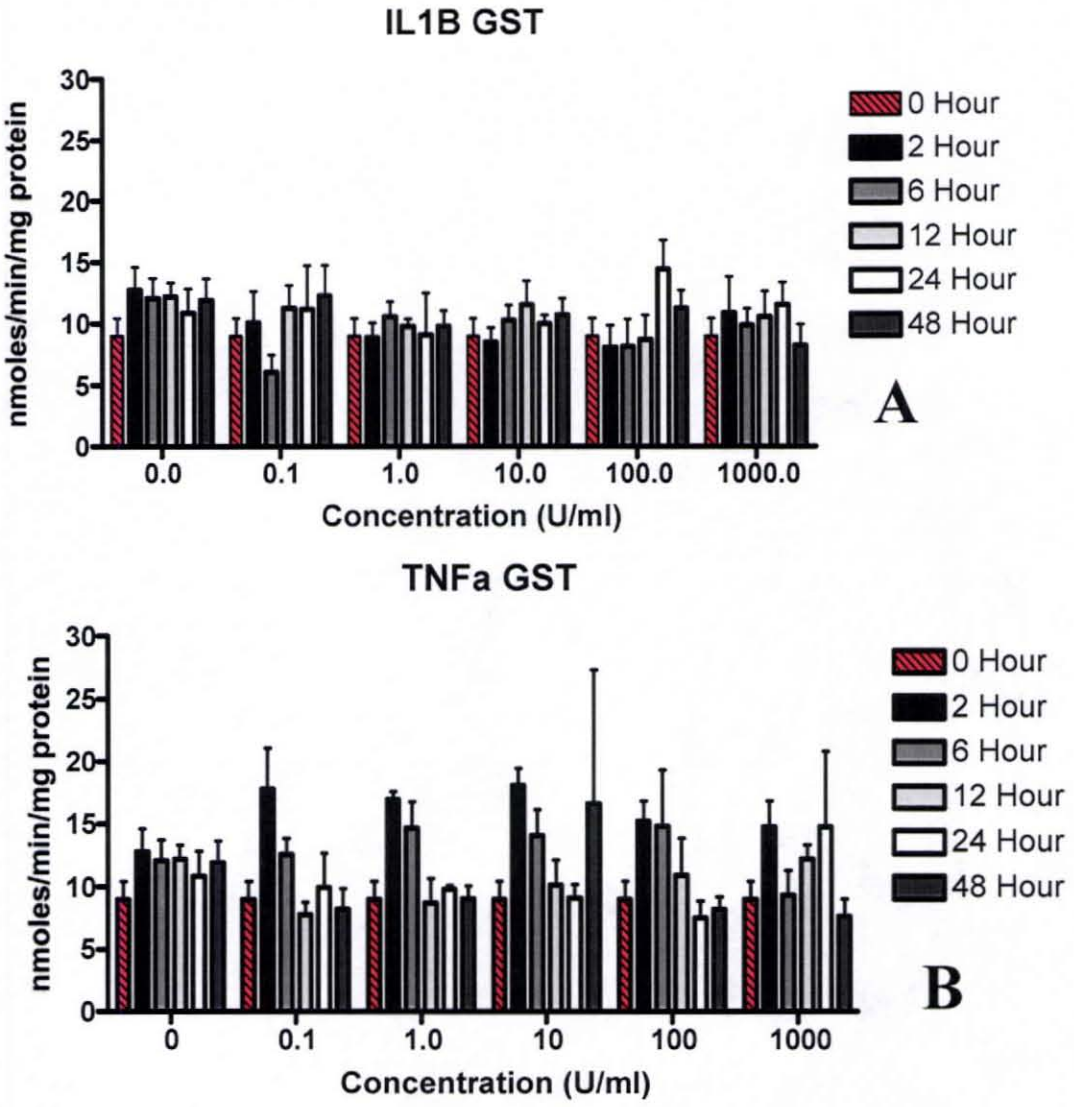

Figure 16: The proinflammatory cytokine IL-1β does not affect GST activity. TNF-α significantly increases GST activity initially and then levels return to normal by 12 hours. With IL-1β treatment, GST activities are unaffected (Figure 16A) but for TNF-α, GST activities significantly increase at 2 hours and then recover by 12 hours (Figure 16B).

Immunohistochemistry confirmed the presence of GST in HepG2 cells both before and after cytokine treatment (Figure 17). Staining for GST was cytoplasmic and very strong. This parallels the known high abundance of GST proteins in the cytosol.

Similar to those data reported above, no apoptosis was caused by IL-1B or TNF-α for 24 hrs since no nuclear or cytoplasmic shrinkage was observed nor was any cytoplasmic blebbing recorded (Figure 17).

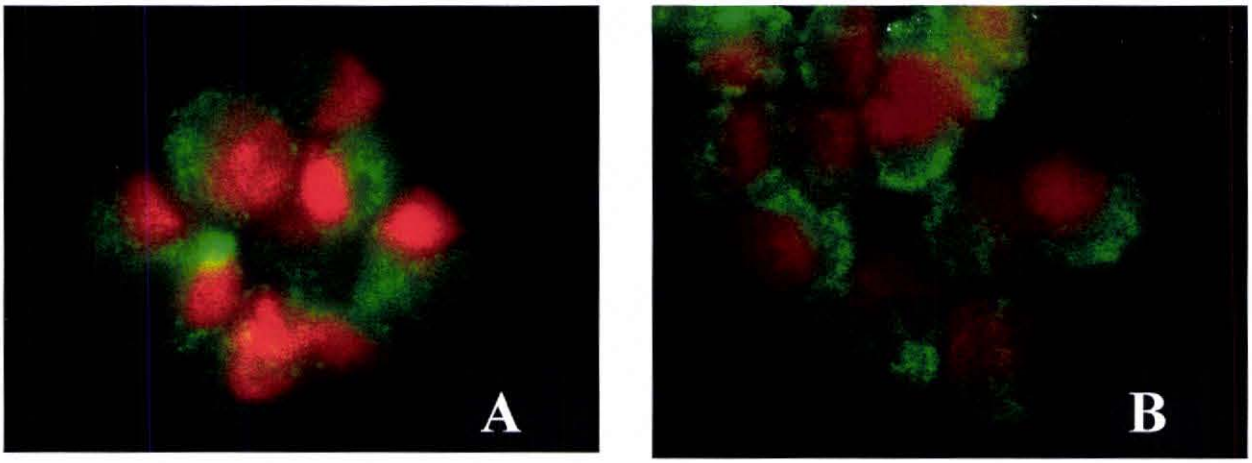

Figure 17: Immunohistochemical detection of the expression of total GST in HepG2 cells. Figure 17A: The expression of GST in cells treated with IL-1β. **Figure 17B:** The expression of GST in cells treated with TNF-α. Magnification is 63X.

Immunocytochemical localization of HNF1 and HNF4 proteins

Immunocytochemistry for HNF1 and HNF4 showed no translocation to the nucleus within 60 minutes when HepG2 cells were treated with either IL-1β or TNF-α at 100 U/ml (Figure 18 and Figure 19 respectively). Staining was punctate and cytoplasmic for controls and did not change after 30 min or 60 min of TNFα and IL-1β exposure. This indicates that it is unlikely that HNF1 or HNF4 are nuclear signaling mechanisms utilized by IL-1β and TNF-α. These data also indicate that the significant changes in enzyme activities observed where UGT activity was decreased for up to 12 hours and GST activity was dramatically increased within 2 hours, were not caused by HNF1 or HNF 4 nuclear signaling.

Additionally, similar to the results reported for the enzyme immunohistocehmistry, no apoptotic morphology was observed. None of the micrographs taken showed significant apoptotic morphology in HepG2 cells after treatment with wither IL-1β or TNFα at 100 U/mL (28 micrographs are shown although since all experiments were performed on 3 separate days, they are representative of over 100 slides). No nuclear shrinkage was observed in any slide (Figures 13, 15, 17, 18 or 19). The cytoplasm of treated cells was not fragmented or stellate, with the lone exception of Figure 16E that demonstrates slightly stellate and extended cytoplasm in HepG2 cells treated with TNFα for 60 min. Blebs were not routinely observed. Blebbing was never observed in IL-1B treated cells (Figures 13, 15, 17, 18 or 19), although some suspicion of blebbing is observed in

Figures 18D and 18E (TNFα 30 and 60 min treatments, indicated by white arrow) and Figure 19B (TNFα 60 min, white arrow).

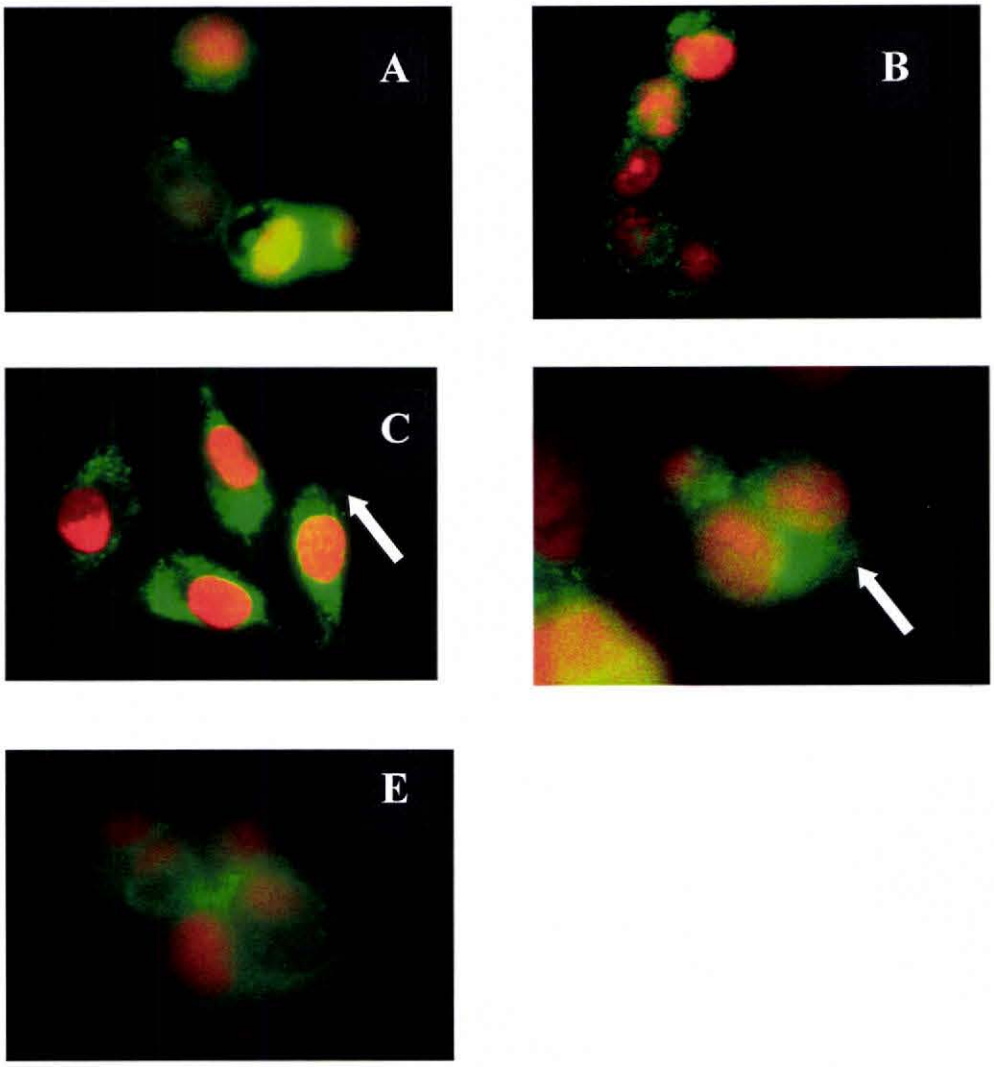

Figure 18: Immunohistochemical localization of HNF1 proteins in HepG2 cells.

Figure 18A: The localization of HNF1 in the cytoplasm of cells treated with IL-1β after 30 minutes. **Figure 18B:** The localization of HNF1 in the cytoplasm of cells treated with IL-1β after 60 minutes. **Figure 18C:** The localization of HNF1 in the cytoplasm of cells treated with TNF-α after 30 minutes. **Figure 18D:** The localization of HNF1 in the cytoplasm of cells treated with TNF-α after 60 minutes. **Figure 18E:** The localization of

HNF1 in the cytoplasm of cells untreated at time zero. Arrows indicate possible areas of blebbing. Magnification is 63X.

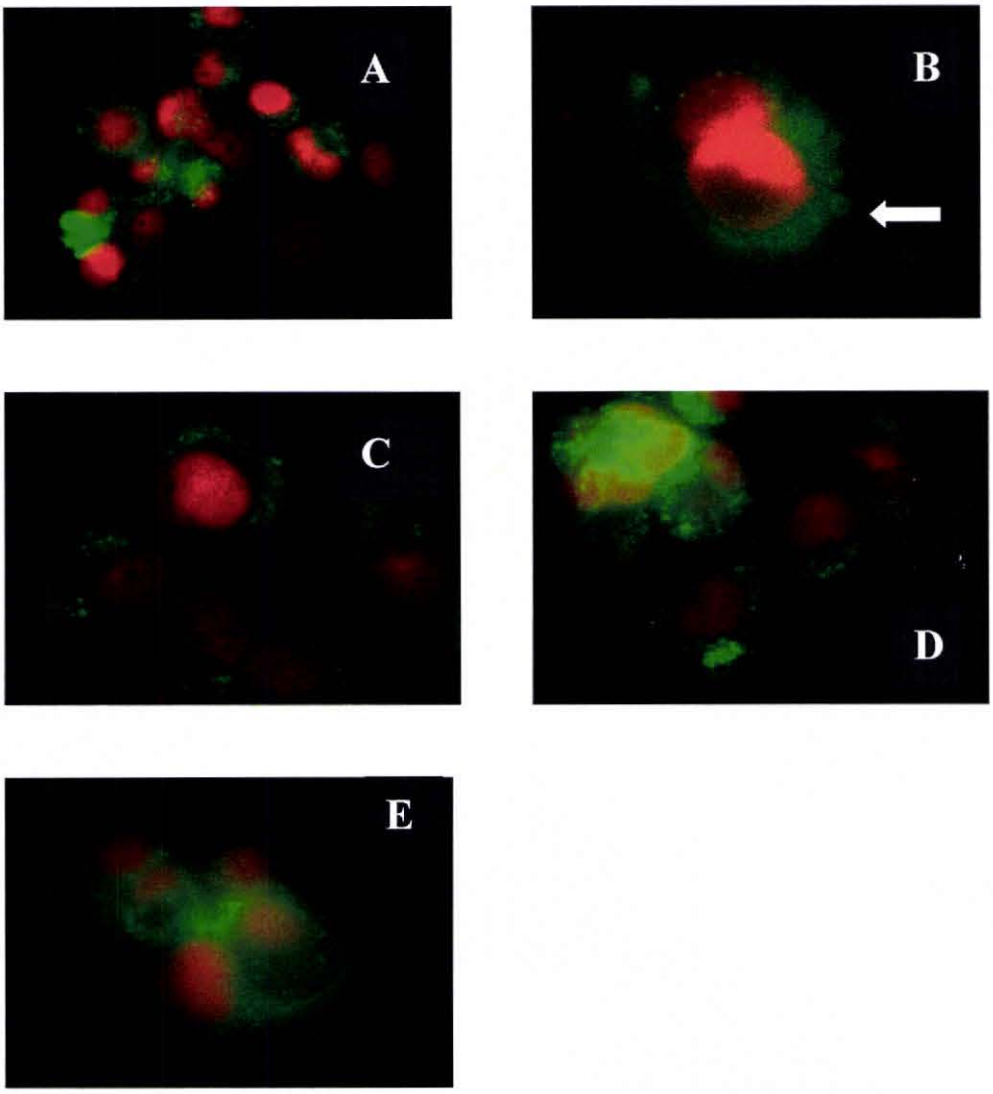

Figure 19: Immunohistochemical localization of HNF4 proteins in HepG2 cells.

Figure 19A: The localization of HNF4 in the cytoplasm of cells treated with IL-1β after 30 minutes. **Figure 19B:** The localization of HNF4 in the cytoplasm of cells treated with IL-1β after 60 minutes. **Figure 19C:** The localization of HNF4 in the cytoplasm of cells treated with TNF-α after 30 minutes. **Figure 19D:** The localization of HNF4 in the cytoplasm of cells treated with TNF-α after 60 minutes. **Figure 19E:** The localization of

HNF4 in the cytoplasm of cells untreated at time zero. Arrow indicates possible area of blebbing. Magnification is 63X.

Chapter 4: Discussion

In summary, the MTT assay showed that cytokine administration causes an increase in cell viability at 12 hours and then viability declines while ROS assays demonstrated that increases in ROS occur over time with application of IL-1β in a manner that is proportional to cell number. In contrast ROS levels are immediately and dramatically increased by TNF-α to high levels that persist over time, regardless of cell growth. The levels of UGT activity exhibited a consistent trend when cells were treated with both cytokines by decreasing until 12 hours and then rebounding. For both cytokines, 1.0 U/ml was found to decrease significantly. SULT enzymes displayed no change in activity caused by IL-1β or TNF-α. Finally, GST activity increased twofold within two hours when cells were treated with TNF-α but did not change due to IL-1β administration. All enzymes were detected using immunocytochemistry before and after cytokine treatment, but neither TNF-α nor IL-1B cause translocation of HNF1 and HNF4 to the nucleus minutes.

To demonstrate that the pro-inflammatory cytokines IL-1β and TNF-α may be responsible for changes in phase II enzyme activity, we first had to demonstrate that administration of these cytokines did not cause cell death. If IL-1β and TNF-α cause significant cell death in themselves, decreases in enzyme activities could be attributed to decreases in cell number. Since the cells were not dying (in fact MTT assays indicated

cells were proliferating) changes in enzyme activity were likely through another mechanism.

A caveat to using MTT to report cell viability is that the may be inaccurate since intracellular components other than the mitochondria are able to reduce the formazan salt used for the assay. Although MTT reduction is usually considered a measure of mitochondrial viability, the actual location of the enzymes responsible for reduction is unknown. The original proof that MTT reduction occurred primarily through conversion in the mitochondrial electron transport chain was published by Slater *et. al.* (1963) using rat liver homogenates and respiratory inhibitors. Subsequently, it has been demonstrated that mitochondrial-dependent reduction of MTT accounts for some of the formazan produced, but that nicotinamide-dependent MTT reduction occurs outside of the mitochondrial membrane. The extra-mitochondrial reduction of MTT associated with the cytosolic compartment was equal to the reduction associated with the mitochondria in rat livers (Gonzalez, 2001). Most important to this thesis, it has previously been shown that that only ~ 45% of reduced MTT reagent in the human cell line HepG2 is performed within the mitochondrial membrane (Bernas, 2002).

Essentially these published studies indicate that MTT may report greater viability than is actually present when HepG2 cells are under conditions of oxidative stress or when reductive cytoplasmic enzymes are induced (Collier *et al*, 2003). In the context of this project, even though the MTT assay provided evidence that HepG2 cells were

proliferating and not dying, this may be inaccurate since changes in ROS were observed in our cell.

Since ROS have been shown to alter enzyme expression and activity due to signaling pathways of their own (Hayes, 1995) and also to ensure ROS had not confounded our MTT data, we assessed the levels of intracellular ROS in HepG2 cells treated with pro-inflammatory cytokines. Our data indicate that ROS levels increase as cells proliferate but not due to IL-1B treatment for up to 48 hr, therefore, at earlier timepoints, MTT data is likely accurate. However after 24 and 48 hr in culture (when HepG2 cells have doubled once and twice respectively) the viability reported may be falsely elevated. When TNF-α was administered, extremely high levels of ROS were recorded as early as 2 hr at all concentrations of TNF-α except 10U/ml. Thus for TNF-α, viability reported by MTT is likely to be overestimated, except for cells treated with 10 U/mL. Additionally, high levels of intracellular ROS and ROS signaling pathways may confound our enzyme activity data for TNF-α at all concentrations except 10 U/ml and for IL-1B after 24 h. Examples of how this may be occurring are discussed below.

Microscopy consistently showed no apoptotic morphology in HepG2 cells treated with IL-1β and TNF-α. This may indicate that the cells were not dying due to the cytokines administered or due to ROS-initiated apoptotic pathways however, since HepG2 cells are an adherent cell line and apoptotic cells lift from slides, it is also possible that cells

showing apoptosis were lifting from slides during washing steps. This is mitigated somewhat by the observation that slides treated with cytokines did not appear (visually) to have markedly less cells than untreated slides. Over 30 micrographs were taken and in only 3 cases (< 10%) were possible signs of blebbing or cytoplasmic shrinkage observed. Since apoptosis does occur at a low background rate in all cells, it is likely that this is what was observed and not an effect of the cytokines. Therefore, we believe that any significant changes in enzyme activity reported can be attributed to cytokine effects on the cell when IL-1β is administered before 12 hours and when TNF-α is administered at 10 U/ml. For all other circumstances, changes in enzyme activity may be due to ROS effects.

UGT levels declined consistently within the first 12 hours of cytokine administration. This was significant at 1.0 U/ml. After 12h UGT activities recovered to control levels. These data answer a very important question related to earlier work in this area where cytokines were found to depress UGT mRNA levels acutely but not chronically in the human liver (Congiu, 2002). Our acute decreases in UGT activity that do not persist after 12 hours correlate extremely well to the acute decreases in mRNA but lack of chronic effects observed by Congiu *et al* (2002) in studying UGT mRNA levels in acutely and chronically damaged human livers.

Additionally, we report that SULT activities were not significantly altered when IL-1β or TNF-α were administered which is interesting in light of other results reported for UGT and GST activities. There is considerable overlap and redundancy in phase II

metabolism, so if changes in enzyme are occurring, it is likely that changes in another enzyme will be observed in an attempt to maintain homeostasis. However, even though there is a consistent decrease in UGT activity at 12 hours and a significant increase in GST activity at 2 hours, SULT activity remains unaltered. There are several possible reasons for this, although two are of likely importance. Firstly, UGT enzymes are high abundance enzymes that are high capacity, low affinity metabolizers. They are seldom saturated by physiological levels of substrates. Therefore, a reduction of UGT activity by ~30% within 12 hr may not meaningfully affect the clearance of substrates and hence the cell does not to upregulate other mechanisms. Alternatively, significant increases in GST activity of up to 2-fold are observed within 2 hr. Hence, the greater GST activity may be compensating for decreases in UGT activity. Of the three enzyme families studied, the UGT and SULTs have the most similar substrate affinity profiles, thus; this complementary UGT/SULT regulation might be expected. Therefore, increases in GST activity to compensate for decreased UGT activity would be a surprising compensatory mechanism.

GST activity increases nearly twofold within two hours of TNF-α administration. These levels return to normal levels by about 12 hours. Studies performed using 10 U/ml TNF-α are the only reliable results for the affects of this cytokine on enzyme activity because this is the only concentration that did not cause significant levels of ROS. Although a similar trend is evident with all concentrations of TNF-α administered to HepG2 cells,

the increase in GST activity at other concentrations may be due to the high levels of ROS that were induced by the cytokine. The main way in which ROS affect GST enzymes is to upregulate their activity through increased transcription of GST protein using the Antioxidant Response Element nuclear receptor (Hayes, 1995). However, since increases in GST activity occur within 2 hours (before transctiption and translation can occur) even when high levels of ROS are not present, we can conclude that TNF-α does increase GST activity independent of ROS-mediated transcription.

By 12 hours, GST levels have returned to their normal levels. There could be several explanations for this. Depletion of glutathione likely occurs since the co-factor itself is a key player in antioxidant defenses by binding and deactivating ROS which protects the cell from oxidative injuries (Winiarska, 2003; Whitekus, 2002). If glutathione may is reacting with ROS it becomes oxidized and is not available for the GST reaction. Another possible explanation involves the microsomal GSTs known as MAPEGs which are involved directly in inflammatory responses.

MAPEGs are known to be involved in the production of eicosanoids, prostaglandins and leukotrienes (EPLs), which are all known to increase propagation of inflammation and pain. An upregulation of MAPEGs, like the one seen at 2 hours, would coincide with an increase in EPLs and acute inflammation. Thus if GSTs are decreased, like the decreased seen at 12 hours, it could be as a protective mechanism to prevent over-production of EPLs, preventing inflammation and pain. Thus, the body maintains homeostasis.

We also performed immunocytochemistry studies to determine whether TNF-α and IL-1β can cause translocation of the nuclear transcription factors HNF1 or HNF4. Since all of our Phase II enzymes of interest can be regulated by these transcription factors, this was a logical direction to study enzyme activation. These studies demonstrated that TNF-α and IL-1β do not induce translocation of the HNF1 or HNF4 transcription factors to the nucleus of HepG2 cells. Thus, the activation of GST enzymes and apparent suppression of UGT enzymes by TNF-α and IL-1β must occur through a mechanism independent of HNF1 or 4 signaling.

Since a twofold increase of GST activity is very high, these results have potential clinical significance. As an example, patients on long term drug therapy may be at risk of altered drug metabolism if they contract an acute infection. Additionally, since the body maintains homeostasis, this is an issue during the initial stages (first 2-6 hours) of infection. An example of how this could have serious consequences for certain drugs would be to consider the use of nitroglycerin on a long term basis for the treatment of angina, a chronic heart disease characterized by chest pain and an irregular heartbeat. Nitroglycerin is known to have an extremely rapid onset of action and is also metabolized and removed rapidly. Since nitroglycerin also has a very narrow therapeutic index (meaning there is a small dosage range between effectiveness and toxicity for this drug), it is conceivable that if a drastic increase in the metabolism and removal of nitroglycerin

occurred, a dose of the drug in the blood would not reach clinically effective levels at the desired site of action . Nitroglycerin is primarily metabolized by GST enzymes (Cassaret and Doull) so increases in GST activity, such as those reported; might result in enhanced clearance of nitroglycerin. The mechanism of action for nitroglycerin is to reduce pressure on the heart, lowering heart rate, and dilate the blood vessels, lowering blood pressure. If the drug is metabolized too quickly, high levels of pressure on the heart and elevated blood pressure may not be relieved.

Suggestions for Further Research

Further research possibilities in studying the effects of pro-inflammatory cytokines on detoxification defenses may include investigating which transcription factors are responsible for the changes observed in UGT and GST detoxification enzymes when TNF-α and IL-1β are administered. Since we have shown no apparent translocation of the HNF1 and HNF4 transcription factors, simple gel-shift assays may be useful to further confirm that TNF-α and IL-1β do not bind these transcription factors. Secondly, we could pursue other candidate gened that may be responsible for changes in Phase II enzyme expression and activity, one possible gene is the RXR:RAR gene which is also known to be present in the promoter region of all three enzymes (Li, 2002).

Additional research may center on the role of ROS. Much study has been performed using TNF-α and IL-1β as promoters of apoptosis and it is known that ROS can deplete

glutathione, the co-factor necessary for the GST reaction. Biochemical assays to determine glutathione depletion are readily available and performing these studies would be extremely helpful in confirming depletion of the glutathione co-factor as a mechanism for lowered GST activity after the initial 2 hr spike. Furthermore, in order to determine if the changes in enzyme activity levels are due to changes in the amount of protein available to metabolize substrates, Western blots on all the enzymes could be used to compare protein levels at different timepoints when IL-1β and TNF-α are applied to normal, control HepG2 cells.

Summary

In summary, acute infection and inflammation do cause significant changes phase II detoxifying enzymes. The SULT enzymes are unaffected and the UGT enzymes may decline slightly but, within 12 hours return to their pre-inflammatory levels. In contrast GST activity increased twofold within a very short period (2 hours). These results, particularly for GST enzymes, have potential clinical and biological relevance. It would be useful to expand upon them in a more physiological paradigm such as primary human hepatocytes or an animal model to assess their applicability to acute infection and inflammation *in vivo*. Additionally, these studies are important in demonstrating that TNF-α and IL-1β do not induce translocation of HNF1 or HNF4 to the nucleus, thereby presenting evidence that increases in GST activity and and suppression of UGT activity caused by proinflammatory cytokines likely do not occur through HNF signaling pathways.

Appendix A

Biological Safety Program
June 12, 2007

MEMORANDUM

TO: Dr. Abby C. Collier
T3MP JABSOM
BSB 320

FROM: Dr. James T. Douglas, Chair
Institutional Biosafety Committee

Hubert Olipares
Biological Safety Officer

LETTER of AUTHORIZATION No.: 423O0607G

SECTION III. EXPERIMENTS COVERED BY THE *NIH GUIDELINES*
Section III-F. Exempt Experiments

APPROVED: "Detoxification is altered by inflammatory cytokines: HepG2 cells as a model for immunotoxicology studies"

The Environmental Health and Safety Office, Biological Safety Program (BSP) and the UH Executive Committee of the Institutional Biosafety Committee (IBC), have reviewed your biological activities on Friday, June 8, 2007. The IBC and BSP have approved the use as described in your protocols as submitted on the BSP- Registration. Your facility at the John A. Burns School of Medicine, Department of Tropical Medicine, Medical Microbiology and Pharmacology, Biosciences Research Building, Room 320 been certified and rated a minimum as a Biological Safety Level 2 according and as appropriate to the National Institutes of Health, Centers for Disease Control and Prevention, US Department of Agriculture (USDA) and/or as required by Hawaii Occupational Safety and Health. The biological safety guidelines for use of biological materials, including recombinant activities at the University of Hawaii are being met, to its satisfaction.

This memorandum and signed copy of your BSP form is your authorization as approved by the UH IBC. Upon conducting a risk assessment of the facility, procedures, practices, training and expertise of personnel of this protocol and containment levels required by the guidelines, it meets a Biosafety Level 2. Laboratory inspected in April 2007. This authorization is good for five years (through June 2012). However, if there are major changes from this registration and authorization, please complete an updated form. Full review must be done in 2012.

This project conforms with UH Biosafety Guidelines as applicable to the NIH - "Guidelines for Research Involving Recombinant DNA Molecules" (DHHS, NIH April 2002), USDA (7 CFR Parts 340), or the CDC-NIH Biosafety in the Microbiological and Biomedical Laboratories (5th edition, Feb 2007).

All facility that houses regulated biological commodities must have a written security plan and wastes management must be properly treated (preferably autoclaved), including end of project destruction. All necessary transportation requirements must be adhered with according to US Department of Transportation. There is an UH policy on moving local isolates. Local isolates are not considered regulated biological commodities; however, standard biosafety practices (good laboratory practices) must be adhered with during active manipulation.

Remember that all imported "live biological commodities," come under the jurisdiction of State Departments of Agriculture and Health "Import and Use" permits, including, live biological materials such as *E. coli*, animals, plasmids, bacteriophage, DNA, and cell lines. All staff, faculty and students manipulating biological commodities must be initially and annually trained and documents of their training and assurances of project kept for a minimum of three years. There may be additional requirements from the (1) UH Veterinarian- Laboratory Animal Services and the Compliance Office of the Institutional Animal Care and Use Committee, (2) Committee on Human Subjects, (3) Diving Safety Control Board for scientific diving, (4) Radiation Safety Committee for radioactive usage and (5) the Workplace Safety Committee.

A final report should be provided, once the project is completed. Report immediately any injury, theft, lost, unanticipated problems and damage to the experiments or to workers. This requirement is necessary as an assurance from the National Institutes of Health and US Department of Agriculture on recombinant activities.

If there are further questions or request, more information please calls me at 956-3197.

c: Karen Quinn

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