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Activation of platelet-activating factor acetylhydrolase by proinflammatory mediators

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ACTIVATION OF PLATELET-ACTIVATING FACTOR ACETYLHYDROLASE BY PROINFLAMMATORY MEDIATORS

by

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ABSTRACT

Activation of Platelet Activating Factor Acetylhydrolase by Proinflammatory Mediators
by

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Inflammation is a highly complex and beneficial response of the host innate immune system often characterized by redness or swelling of the infected area as a result of an injurious stimulus. The principal purpose of the inflammatory response is to rid the infected area of the noxious stimulus and hasten host recovery. Though the inflammatory response is both necessary and beneficial, if left unregulated, it can exact a devastating toll on the host. Thus, an understanding of the various mediators that are able to evoke an inflammatory response and the signaling of these mediators is crucial. One such mediator, central in initiating an inflammatory response, is the biologically active phospholipid platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine). This phospholipid is converted to its biologically inactive form by a calcium-independent phospholipase A_2 called PAF acetylhydrolase (PAF-AH). Thus, the action of PAF-AH provides a mechanism by which to hinder the propagation of an inflammatory response. In this study, the proinflammatory mediators, lipopolysaccharide (LPS), PAF, and tumor necrosis factor- α (TNF-α) were investigated for their ability to

upregulate PAF-AH expression. The cellular signaling pathways activated by these mediators were also investigated. We have demonstrated the ability of LPS, PAF, and TNF-α to upregulate PAF-AH levels. Further, we showed that the LPS-induced upregulation of PAF-AH levels is partially p38MAPK-dependent. The remaining LPS-induced signaling is mediated through the PAF receptor. The TNF-α and PAF-induced upregulation of PAF-AH levels is not p38MAPK dependent and the PAF acetylhydrolase induction observed may be the result of increased PAF production or signaling through separate pathway(s). We further demonstrate the ability of reactive oxygen species to induce the expression PAF acetylhydrolase.

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Foremost, I would like to thank the late Dr. Stephen Carper for his guidance which has helped bring me to where I am today. His willingness to listen, give sound advice and his sincere concern for his students is a testament to his strong character and has made a lasting impact on those who knew him. He is truly missed. I am also indebted to Dr. Katherine Howard for her strong leadership, patience and willingness to allow me to perform amazing research in her lab. I can say with confidence that any future success that I will come across, as I pursue a career in medicine, can be attributed to the knowledge and experience that I have gained while working under her guidance. Further, I would also like to thank Dr. Ronald Gary for offering to become a co-chair of my thesis committee during a time of need. The support and advice that Dr. Gary has given to both me and Dr. Howard for the last year is sincerely appreciated. I am also indebted to both Dr. Gillian Galbraith and Dr. Chulsung Bae for being valuable members of my thesis committee. I would also like to thank Dr. Karl Kingsley and Susan O'Malley for their technical assistance during my time of need and their kindness towards me in the past two years. I am also thankful for the willingness of Dr. Marcia Ditmyer to run the statistics on all my data. I also would like to thank Nipa Patel for being of invaluable assistance to me in lab. Further, I would like to thank my mother and father who have sacrificed so much for me to be in the position that I am today. Their support, advice and friendship during my life-time have shaped the person that I am today and I am forever thankful for their sacrifices.

CHAPTER 1

INTRODUCTION

1.1 *Background and Significance*

Inflammation is an elaborate, complicated, and highly integrated biological response of vascularized tissue to insult or injury. The principal and beneficial purpose of inflammation is to contain and remove offending microorganisms and necrotic tissue that may ensue as a result of some pathological trigger (1). While this is essential, the inflammatory process can also cause severe tissue injury and dysfunction via the same, but often accentuated, physiologic mechanisms (1). The inability to exact an appropriately regulated inflammatory response has led to 40 million inflammatory disease related cases in the United States today ranging from asthma to inflammatory bowel disease (2). Thus, a thorough understanding of the potent pro-inflammatory mediators that are essential in eliciting an inflammatory response is crucial. One such mediator, central in the inflammatory response, is the phospholipid platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine). The chemical structure of PAF is shown in Figure 1 (3).

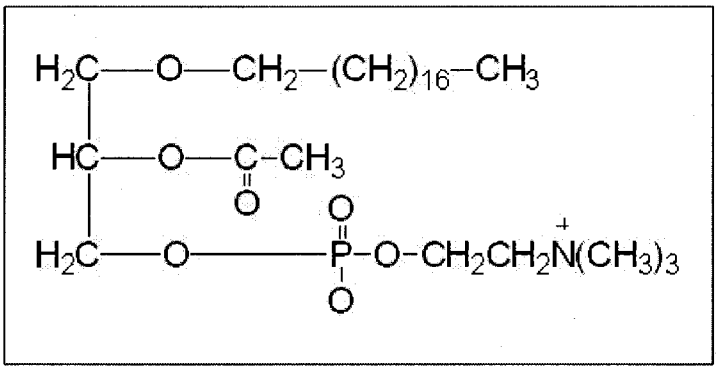
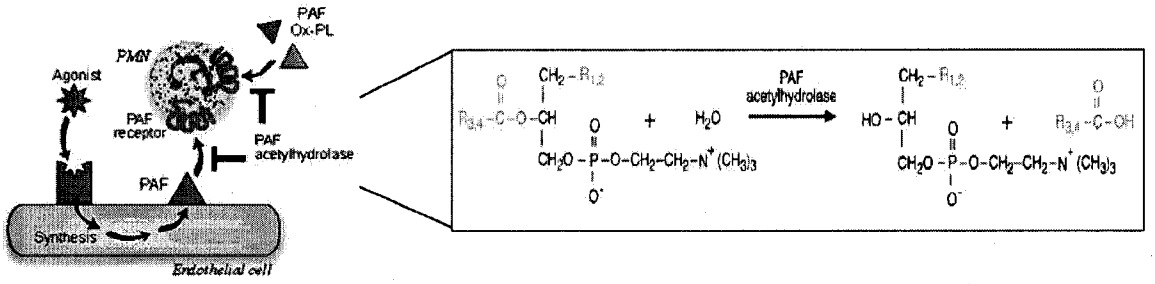


Figure 1: Structure of Platelet Activating Factor. Platelet Activating Factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a potent proinflammatory phospholipid that is central in exacting an inflammatory response. From: King, M. W. (2008) Lipid Metabolism. Illinois

Many isoforms of PAF exist with modifications in chain length and functionalities at both the *sn*-1 and *sn*-2 positions as seen in Figure 2. The most biologically potent form of PAF consists of a methylene chain length of sixteen carbons at the *sn*-1 position and an acetate moiety at the *sn*-2 position. PAF, itself, has been implicated in many inflammatory disease states affecting cardiovascular, gastrointestinal, pulmonary, hepatic and renal systems (2). PAF was originally identified through antigen stimulation of IgE sensitized rabbit basophils. The phospholipid was first isolated by its ability to aggregate platelets and was given the name platelet-activating factor (4). The structure of PAF was determined by Demopoulos *et al.* in 1979 (5).

A.



B.

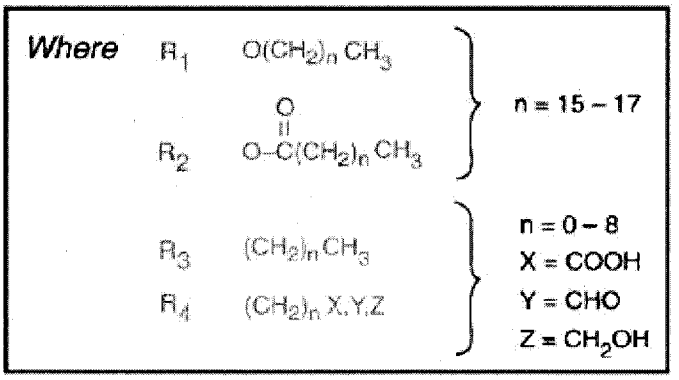


Figure 2: Enzymatic action of PAF acetylhydrolase and Variations in PAF Structure: *A.* Though the inflammatory response is both necessary and beneficial, if left unregulated, it can lead to the detriment of the organism. A class of enzymes categorized as calcium independent phospholipase A_2s called PAF acetylhydrolases help downregulate the inflammatory response. Upon exposure to an inflammatory agonist, endothelial cells are stimulated to generate PAF. PAF is then free to signal through its G-protein coupled receptor on various cell types of the hematpoietic cell lineage and induce an appropriate inflammatory response. By their action of PAF acetylhydrolases, the acetate moiety at the sn-2 position of PAF is cleaved generating the biologically inactive lyso-PAF thereby limiting the progression of the inflammatory response. *B.* Possible variations at the sn-2 position which are characteristic of PAF mimetics and are often induced through action of reactive oxygen species. Figure 2A is from Castro Faria Neto, H. C., Stafforini, D. M., Prescott, S. M., and Zimmerman, G. A. (2005) *Mem Inst Oswaldo Cruz* **100 Suppl 1**, 83-91. Figure 2B is from Tjoelker, L. W., and Stafforini, D. M. (2000) *Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids* **1488**, 102-123

PAF is produced primarily by cells of the hematopoietic cell lineage and common sources of PAF include macrophages, monocytes, neutrophils and eosinophils (6). Though not part of the hematopoietic cell lineage, endothelial cells are also able to produce PAF (2). In cells producing PAF, the primary means of synthesis is through the enzymatic remodeling pathway shown in Figure 3 (1). A *de novo* pathway for PAF synthesis exists; however, this pathway is less biologically relevant. The enzymatic synthesis of PAF is highly regulated and involves a two step mechanism— where the initial step involves the remodeling of choline containing membrane phospholipids. In response to inflammatory challenge, cytosolic phospholipase A_2 ($cPLA_2$) associates with the cell membrane and hydrolyzes the esterified fatty acyl moiety at the *sn*-2 position forming lyso-PAF. Lyso-PAF becomes acetylated by an acetyltransferase to produce bioactive PAF. PAF then signals through its G-protein coupled receptor on various cells of the hematopoietic cell lineage. PAF receptor activation stimulates numerous complex signaling pathways that culminate in producing PAF's diverse biological actions. Of note, PAF receptor activation results in the activation of cytosolic phospholipase A_2 and the generation of lyso-PAF. A cycle of PAF producing PAF receptor activation and more PAF synthesis is established. In many of the cells that produce PAF, the fatty acid that is esterified at the *sn*-2 position of membrane phospholipids is arachidonic acid (7).

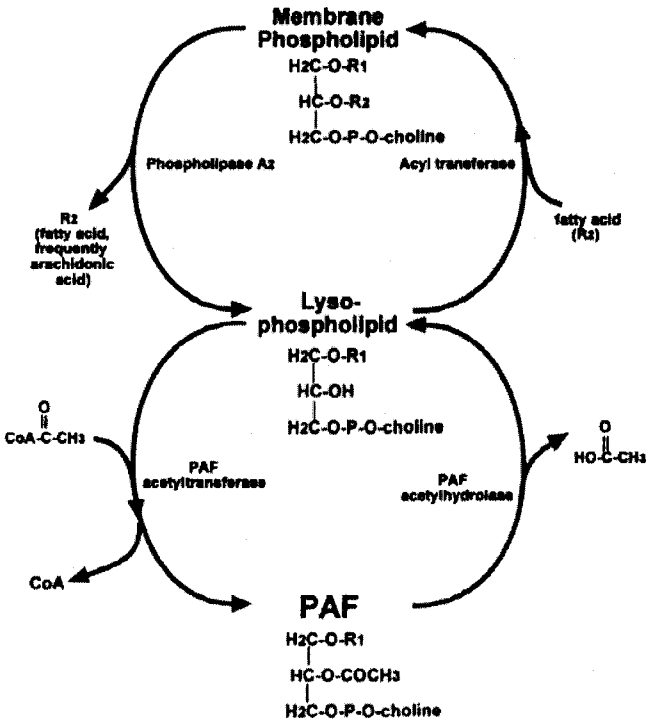


Figure 3: Enzymatic Remodeling Pathway in PAF Synthesis. The enzymatic remodeling pathway is the primary means by which PAF is synthesized. The sn-2 position of choline containing membrane phospholipids is esterified by a cytoplasmic phospholipase A2 upon exposure to an inflammatory stimulus. The action of the cytoplasmic phospholipase A2 generates lyso-PAF. After the formation of lyso-PAF, PAF acetyltransferase transfers an acetate group to the sn-2 position generating bioactive PAF. From: McManus, L. M., and Pinckard, R. N. (2000) *Critical Reviews in Oral Biology & Medicine* 11, 240-258

As shown in Figure 4, arachidonic acid is the precursor for formation of the eicosanoids (8). Because of this, the formation of various eicosanoids such as thromboxanes and leukotrienes is often concomitant with the synthesis of PAF. These lipids, working together, can augment the inflammatory response (1). In addition to the inflammatory response elicited by PAF, PAF-like phospholipids are also able to induce an inflammatory response. These PAF-like phospholipids are not derived enzymatically, but rather result from the oxidative fragmentation of membrane phospholipids. Unlike the

enzymatically derived PAF, whose synthesis is tightly regulated, the synthesis of these PAF-like phospholipids is not regulated (9) (10). Thus, a significant means to regulate the inflammatory response elicited by both PAF and PAF-like phospholipids is through the action of a family of enzymes known as PAF acetylhydrolases (PAF-AH).

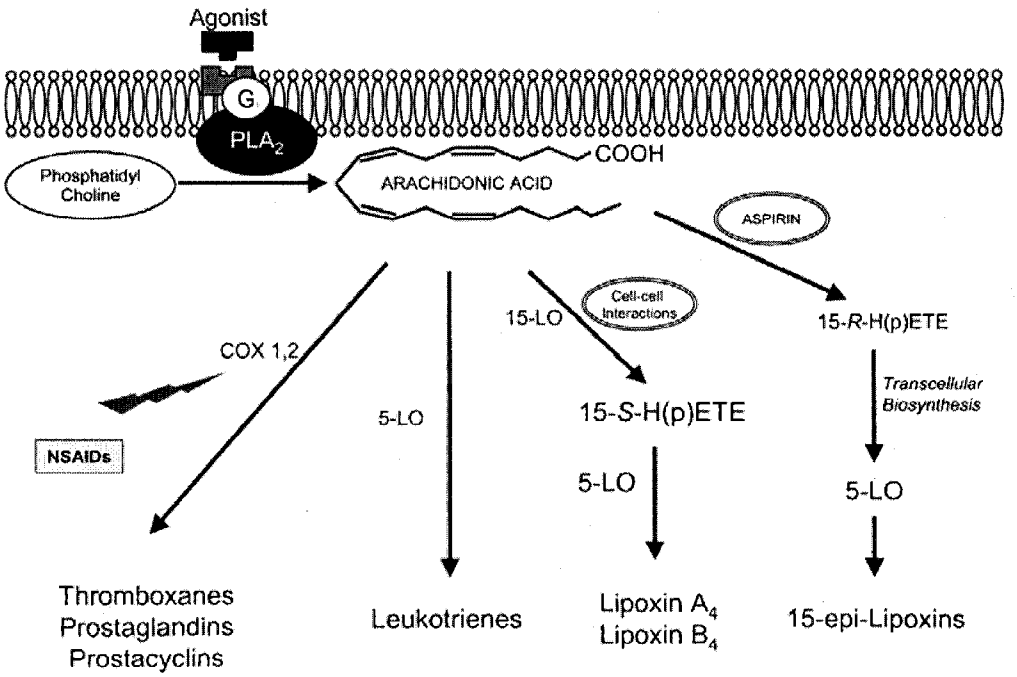


Figure 4: . **Synthesis of Various Eicosanoids via Arachidonic Acid**. Often, during the enzymatic synthesis of PAF, the fatty acid moiety that is esterified at the sn-2 position is arachidonic acid. This is of relevance because arachidonic acid is the precursor to various eicosanoids such as thromboxanes, prostaglandins and leukotrienes which can, in turn, augment the inflammatory response. From: Kantarci, A., and Van Dyke, T. E. (2003) *Crit Rev Oral Biol Med* **14**, 4-12

The various PAF acetylhydrolases are calcium-independent phospholipase A_2s which catalyze the removal of the *sn*-2 acetate moiety, or variations at this position from PAF or PAF-like phospholipids. Such variations, as shown in Figure 2, may consist of oxidized functionalities derived from fragmentation of the long acyl chain of membrane phospholipids. The PAF acetylhydrolases convert the bioactive PAF to its biologically

inactive form lyso-PAF—thereby providing a means to interrupt the progression of an inflammatory response. There are two categories of PAF acetylhydrolases: intracellular and secreted (11). The intracellular PAF acetylhydrolases are able to recognize a diverse array of PAF-like phospholipids and are found in various subcelluar locations. Additionally, the intracellular PAF acetylhydrolase has a wide range of structures and can be either monomeric, dimeric or heterotrimeric (2). There are three subcategories of the intracellular PAF acetylhydrolases which include PAF acetylhydrolase Ib, PAF acetylhydrolase II and the erythrocyte PAF acetylhydrolase. The PAF acetylhydrolase Ib is thought to control PAF levels in the brain and mutations in the β-subunit of this enzyme cause Miller-Dieker lissencephaly (12). PAF acetylhydrolase II is a 40 kDa protein found in various tissues and has similar function to the plasma form of the enzyme—the main difference being a substrate preference for short acyl groups at the *sn*-2 position (13). The primary function of PAF acetylhydrolase II is the protection from damage induced by lipid oxidation (14). The erythrocyte PAF acetylhydrolase is composed of two identical 25 kDa subunits (15). The function of the erythrocyte PAF acetylhydrolase is intriguing because erythrocytes do not produce nor are they stimulated by PAF. Possibly, the erythrocyte PAF acetylhydrolase hydrolyzes oxidatively fragmented phospholipids which are usually formed in high oxygen situations or as byproducts of free radical reactions (16). Though the intracellular PAF acetylhydrolases are diverse and important in function, the primary focus of this research concerns the action of the plasma PAF acetylhydrolase.

The plasma form of PAF acetylhydrolase (referred to solely as PAF acetylhydrolase in subsequent use, unless otherwise noted) was isolated in 1985 by Farr

et al. and the cDNA was subsequently cloned in 1995 by Tjoelker *et al.* (17,18). The difficulty in purification of PAF acetylhydrolase was due to its association with LDL particles in the serum. Two-thirds of the PAF acetylhydrolase in serum is associated with LDL and the remaining one-third with HDL (19-21). The first seventeen residues of the protein encoded by the cDNA are hydrophobic and most likely direct the enzyme for secretion. The subsequent twenty-four residues are not found in the mature PAF acetylhydrolase and may represent a pre-pro-peptide. Additionally, the primary structure of PAF acetylhydrolase contains a GXSXG motif which is homologous to many lipases and serine esterases (22,23). The active sites of many serine esterases contain a serine, aspartate and histidine triad; a similar triad is found in the active site of PAF acetylhydrolase—indicating that the active site of both PAF acetylhydrolase and serine esterases may be similar.

Increases in PAF acetylhydrolase activity have been implicated in many disease states such as vascular disease, ischemic stroke and diabetes mellitus (24,25). Within the human population, the level of activity of PAF acetylhydrolase varies five-fold—60% of this variation is attributed to genetic factors where some of this variability can be attributed to varying levels of LDL-cholesterol (26,27). Because changes in activity of PAF acetylhydrolase may be linked to certain disease states, it is important to investigate whether PAF acetylhydrolase levels can be an indicator of disease—especially those with an inflammatory component. Numerous studies have shown strong correlations between PAF acetylhydrolase activity and LDL-cholesterol levels—with higher LDL-cholesterol levels accompanied by higher PAF acetylhydrolase levels. This is supported by patients with hypercholesterolemia treated with a HMG-CoA reductase inhibitor showing

decreased plasma PAF acetylhydrolase activity in conjunction with lower LDL-cholesterol levels (26,28). Individuals suffering from systemic lupus erythematosus also show decreased PAF acetylhydrolase levels alongside reduced LDL levels (29). Further, patients with lower pre-operative PAF acetylhydrolase enzyme levels were more prone to developing systemic inflammatory response syndrome after cardiac surgery as compared to patients with normal circulating PAF acetylhydrolase enzyme levels (30). Because the association of PAF acetylhydrolase activity and disease appears well correlated, it is important to investigate the ability of various proinflammatory mediators to regulate the expression of PAF acetylhydrolase and investigate the cellular pathways involved in such regulation. This research will aid in developing a clearer picture of the physiologic role played by this anti-inflammatory enzyme.

Lipopolysaccharide (LPS) is a component of the cell wall of gram negative bacteria and all of the pathophysiological responses consequent to bacterial sepsis can be attributed to LPS. Further, purified LPS is utilized in the laboratory as a potent stimulator of the inflammatory response to investigate specific cellular and biochemical processes. Recognition of LPS is accomplished through a complex interaction of cell-surface receptors and co-receptors. (Figure 5). Briefly, LPS will interact with the LPS binding protein (LBP) and activate toll like receptor 4 (TLR-4) via CD14—thus, initiating activation of the intracellular MAPK cascades (31). (See Figure 5). Work by Howard *et al.* demonstrated the *in vivo* upregulation of PAF acetylhydrolase mRNA levels in response to LPS challenge in rats. Specifically, the increased expression of PAF acetylhydrolase was detected in Kupffer cells, the resident macrophages of the liver (32). Further, previous literature has shown that in THP-1/CD14 cells, a human monocyte-like

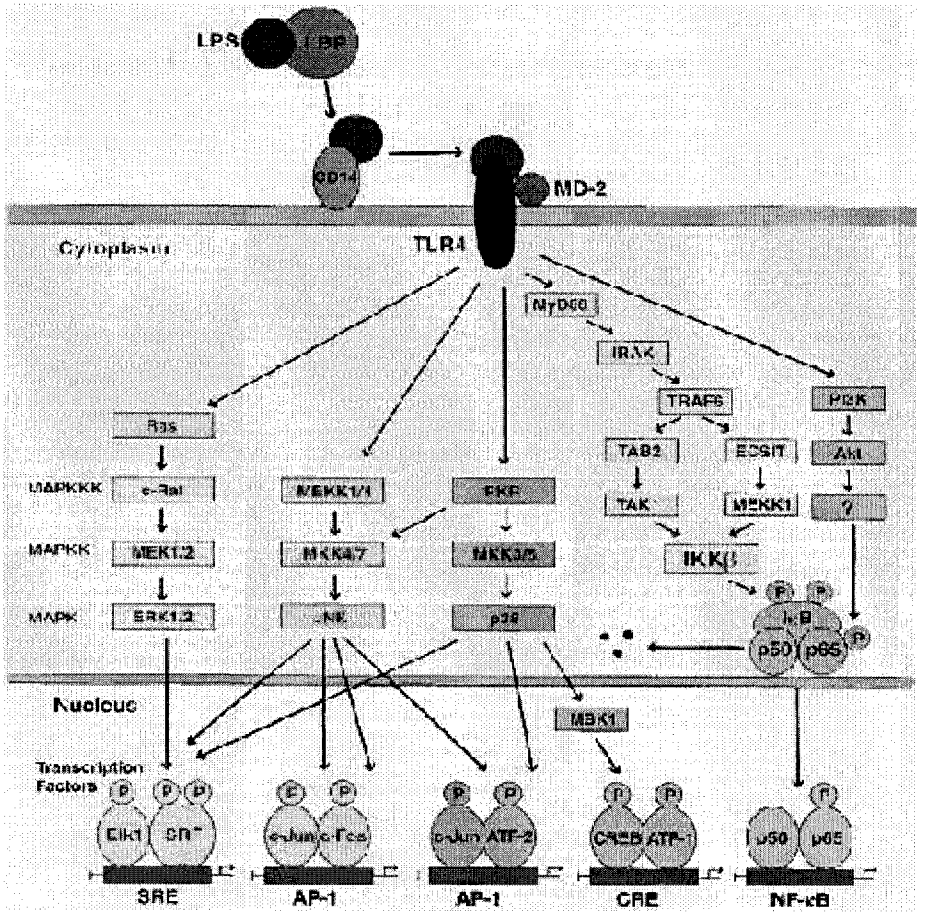


Figure 5: LPS Signaling via MAPK Pathway. Upon exposure to lipopolysaccharide (LPS) in serum, LPS will interact with the lipopolysaccharide binding protein (LBP). This new LPS-LBP complex is delivered to a cell surface receptor called CD14. CD14 then delivers LPS to a member of the toll-like receptor super family called toll like receptor 4 (TLR4). LPS binding to TLR4 activates the various portions of the MAPK cascades and the transcription of various genes. Adapted from:
Guha, M., et al. Cellular Signaling 13 (2001) 85-94.

cell line that over expresses CD14, the LPS-induced upregulation of PAF acetylhydrolase is solely p38MAPK dependent (33). In contrast, other researchers have documented that in cultured macrophages there is a decrease in PAF acetylhydrolase expression upon exposure to LPS (32,34). This observation may be related to the activation of these macrophages *in vitro* and adherence to the cell culture vessel. Because TNF-α and PAF are known pro-inflammatory mediators and activators of macrophages, an investigation

of their ability to upregulate PAF acetylhydrolase mRNA levels was deemed pertinent. PAF, as discussed above, is a potent pro-inflammatory phospholipid whose signaling is mediated by the seven membrane spanning G-protein coupled receptor. Additionally, TNF-α, a cytokine, primarily produced by macrophages, is involved in acute systemic inflammation (35).

In order to investigate the mechanism of increased PAF acetylhydrolase expression in response to inflammatory mediators, monocyte-macrophage 6 cells (MM6) were used because initial experiments demonstrated the MM6 cells increased PAF acetylhydrolase expression in response to LPS exposure (36)—a result identical to that detected in macrophages *in vivo*. This physiologically relevant cell line spans the monocyte macrophage differentiation lineage, is derived from a human leukemia patient, and grows in suspension. The latter fact is important because adherent macrophage cell lines, such as the RAW264.7 cell line, demonstrate opposite effects of LPS-induced PAF acetylhydrolase induction with respect to the response observed *in vivo* (i.e. LPS decreases PAF acetylhydrolase expression *in vivo*). Monocytes/macrophages are part of the non-specific innate immune system in which the inflammatory response is involved. In contrast, the slower, yet more specific, adaptive immune system is antigen dependent and does not directly involve macrophages/monocytes. However, macrophages are antigen-presenting cells. After an invading microbe is phagocytosed by a macrophage, the macrophage presents the peptide fragments of the microbe on it surface via class II MHC. Via CD4 co-receptor interaction, macrophages are able to activate helper T-cells which, via secretion of various interleukins, activate the antibody producing B-cells. Thus, macrophages able to provide a link to the innate immune response via helper T-cell

activation. Thus, because MM6 cells represent a physiologically relevant cell culture model, they were used in all subsequent experiments in this study.

To investigate the ability of PAF and TNF-α to alter PAF acetylhydrolase expression and to ascertain any similarities or differences to the LPS-induced signaling pathways Real-Time PCRs, ELISAs, and an inducer of reactive oxygen species, *tert*-butyl hydroperoxide, were all employed. In this study, we were able to demonstrate the ability of both TNF-α and PAF to upregulate PAF acetylhydrolase expression. In addition, we examined the various signaling pathways involved in the upregulation of PAF acetylhydrolase mRNA and were able to gain a greater understanding of the various MAPK cascades involved in PAF acetylhydrolase mRNA upregulation. Further, we demonstrated that reactive oxygen species may play a role in inducing PAF acetylhydrolase expression and that the LPS-induced upregulation of PAF acetylhydrolase mRNA levels also involves the PAF receptor.

CHAPTER 2

MATERIALS AND METHODS

2.1 *Materials*

Monocyte-Macrophage 6 cells (A generous gift from Shamim Mustafa), Fetal Bovine Serum (Atlanta Biological), Penicillin/Streptomycin (HyClone), Non-essential amino acids (HyClone), RPMI Media (Cellgro), Lipopolysaccharide (Sigma), Platelet Activating Factor (Sigma), TNF-α (Sigma), High capacity cDNA Reaction Kit (Applied Biosystems), 2x Universal PCR Master Mix (Applied Biosystems), TaqMan Primers (Applied Biosystems), Live Green ROS Detection Kit (Invitrogen), All ELISA kits (R&D Systems).

2.2 *Maintenance of the Human Monocyte-Macrophage 6 Cell Line*

Human Monocyte-Macrophage 6 (MM6) cells, derived from a Leukemia patient and grown in suspension, were obtained from Dr. Shamim Mustafa and were stored at -180°C. Upon use, 1mL of MM6 cells were quickly thawed from deep freeze and were resuspended in 10 mL RPMI media supplemented with the following components in their final concentrations: 10% fetal bovine serum, 10000 U/mL Penicillin, 10000 μg/mL Streptomycin, 1 mM oxaloacetate, 0.45 mM pyruvate, 0.2 U/ml insulin, and 1x non-essential amino acids. The MM6 cells were counted with the use of a hemocytometer, centrifuged and were seeded in a twenty-four well tissue culture plate at an initial density

of $5x10^5$ cells/mL with 2 mL/well of complete media and cultured overnight. Subsequently, MM6 cells were seeded at a density of $2x10^5$ cell/well in twenty-four well tissues culture plates (2 mL/well) for each subsequent passage. Cells were maintained at 37°C, in a humidified 5% CO_2 atmosphere for 2-3 days before use in any subsequent experiments. MM6 cells were split every two days, and the cells used in all experiments did not exceed sixteen passages.

2.3 *Isolation and Quantization of RNA*

All RNA isolation procedures were based on the method outlined by Sacchi and Chomczynski (37). MM6 cells were pelleted by centrifugation and were lysed in 1 mL TRIzol Reagent by repetitive pipetting. Lysates were transferred to 1.5 mL eppendorf tubes. For each 1 mL of TRIzol Reagent, 200 µL chloroform was added to each sample tube. Sample tubes were shaken vigorously for fifteen seconds and incubated at 15 to 30°C for 2-3 minutes. Samples were then centrifuged at 12,000xg for 15 minutes at 4°C. The clear upper aqueous phase was transferred to a new eppendorf tube and 500 µL isopropyl alcohol was added per 1mL of TRIzol Reagent initially used. The sample tube was shaken vigorously for fifteen seconds and centrifuged at 12,000xg for thirty-minutes at 4°C to pellet the RNA. The supernatant was removed and RNA pellets were washed with 1 mL cold 75% ethanol per mL of TRIzol Reagent initially used. Sample tubes were then mixed vigorously by vortexing and were centrifuged at 7,500xg for 5 min at 4°C. The supernatant was removed and the RNA pellet was allowed to air dry for five-minutes. RNA pellets were then resuspended in 40 µL diethyl pyrocarbonate treated

water. To quantify the isolated RNA, 3 μL of sample was diluted in 197 μL of Mili-Q water in a 96-well low-UV plate. The Spectra Max Plus microplate reader was used to obtain the optical density at 260nm and 280nm. The absorbance units at 260 nm provided the RNA concentration and the 260/280 ratio indicated the purity of the RNA.

2.4 *Performance of cDNA Synthesis*

Five μg RNA was reverse transcribed in 60 μL cDNA reactions with use of the High Capacity cDNA synthesis kit supplied by Applied Biosystems. Random Primers were used to initiate the cDNA synthesis and were supplied at 10x, Multiscribe for reverse transcription was supplied at 50 U/μL, the Reverse Transcriptase buffer was supplied as a 10x solution and 25x dNTP buffer was supplied at a 100 mM concentration. All samples were mixed in their appropriate concentrations to give a 1x solution. Multiscribe was diluted to a final concentration of 75 U/μL and the 25 dNTP buffer was prepared at a final concentration 4 mM. Prepared samples were heated to 25°C for ten minutes and then 37°C for two hours using the GeneAmp PCR systems 2400 machine.

2.5 *Performance of Real-Time PCR*

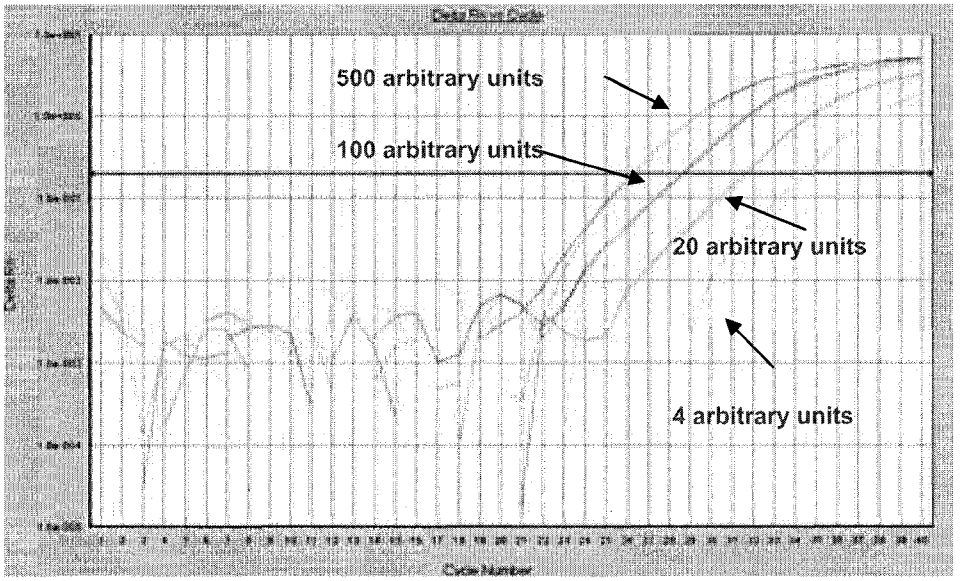
Thirty μL Real-Time PCR reactions were performed using 5 μL of cDNA created as described above. TaqMan primers (20x) specific for the human PAF acetylhydrolase and 18S ribosomal RNA and Universal PCR Master Mix (2x) were obtained from Applied Biosystems. Real-time PCRs were performed on the Applied Biosystems 7000 Real-

Time PCR machine to amplify the PAF acetylhydrolase cDNA. To make this method quantitative, four, five-fold serial dilutions of cDNA was reverse transcribed from 6 μg of RNA isolated from LPS treated MM6 cells and were run simultaneously with the experimentally derived cDNAs. Each dilution was assigned an arbitrary numerical value that was indicative of a five-fold serial dilution. In this way a quantitative numerical value is obtained where fold-induction in PAF acetylhydrolase levels over control can be ascertained. To correct for slight differences in RNA concentration, 18S levels were analyzed by Real-Time PCR as described above and the ratio of PAF-AH to 18S levels was determined. See Figure 6.

2.6 *Performance of Dose Response Experiments*

MM6 cells were seeded at a density of $2x10^5$ cells/mL in 2 mL of complete media, and were incubated in a humidified 5% CO_2 environment at 37°C for twenty-four hours prior to the start of any experiments. Subsequent to this, dose response experiments were performed for PAF, in its most potent form (1-O-Hexadecyl-2-O-acetyl-sn-glycero-3-phosphocholine), LPS (from E. coli, serotype: 0111:B4) and TNF-α. The PAF was prepared as a 100 mM stock solution dissolved in chloroform. Ten μL of the stock solution was dissolved in 990 μL anhydrous ethanol. The 1 mL solution was dried under a N_2 gas stream. The dried PAF was then resuspended in 240 μL phosphate buffered saline (PBS) supplemented with 1% Bovine Serum Albumin (BSA) and sonicated for ten-minutes before use in any experiments. A 2 mg/mL stock solution of LPS was dissolved in 1xPBS. Before use in experiments, 10 μL of the 2 mg/mL stock was dissolved in 90 μL 1xPBS.

A. PAF-AH Standard Curve



B. 18S Standard Curve

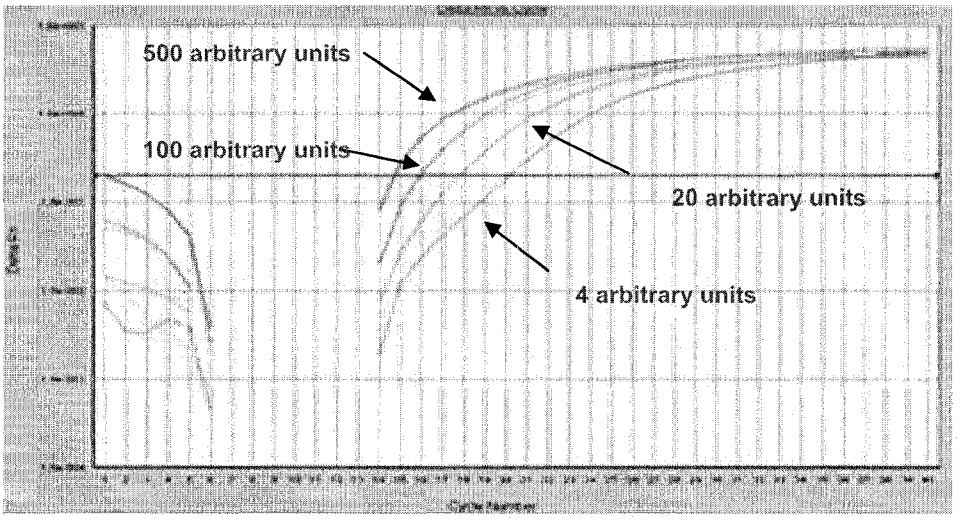


Figure 6: Real-Time PCR PAF-AH and 18S Standard Curves. A. PAF acetylhydrolase standards were prepared by reverse transcribing 6 μg of RNA to cDNA. Using the cDNA, four, five-fold serial dilutions were used to generate each standard. The undiluted standard was given an arbitrary value of 500 units. Each subsequent dilution was given a value indicative of a five fold serial dilution. B. 18s standards were generated by reverse transcribing 6 μg of RNA to cDNA. An initial 1:100 dilution was made of the cDNA. From this, a series of four, five-fold serial dilutions were generated and assigned values in the same manner as described in part A. above.

A 25 mM stock solution of TNF-α was prepared dissolved in 1xPBS. PAF was administered to MM6 cells at concentration of 0 μM, 0.125 μM, 0.25 μM and 0.5 μM. LPS was administered at concentration of 0 ng/mL, 50 ng/mL, 100 ng/mL and 200 ng/mL. TNF-α was administered at concentrations of 0 ng/mL, 5 ng/mL, 10 ng/mL and 20 ng/mL. At twenty-four hours after administration, the cells were harvested by centrifugation and RNA was isolated, reverse transcribed to cDNA and Real-Time PCR experiments were performed.

2.7 *Performance of Seventy-two Hour Time Course Experiments*

MM6 cells were seeded at a density of $2x10^5$cells/mL in 2 mL of complete media, cultured for twenty-four hours and were then treated with either 200 ng/mL LPS, 10 ng/mL TNF-α or 0.5 μM PAF. In each experimental condition cells were incubated with either of the aforementioned pro-inflammatory mediators for 0, 1, 3, 6, 12, 24, 48, and 72 hours. For LPS treated cells, seventy-two hour time course experiments were also run in the absence of 10% FBS by washing copiously with 1xPBS and resuspending in RPMI lacking serum. MM6 cells were also incubated for seventy-two hours in the absence of LPS for both serum containing and serum free RPMI. After each time point, RNA was isolated, reverse transcribed to cDNA and analyzed in real-time PCR experiments as described above.

2.8 *Performance of Synergistic PAF-AH Induction*

MM6 cells were seeded at a density of $2x10^5$ cells/mL in 2 mL of complete media, cultured for twenty-four hours and were then treated with either 200 ng/mL LPS, 10

ng/mL TNF-α or 0.5 μM PAF. In addition, various combinations of the aforementioned mediators were dually administered in each well. MM6 cells were cultured for twenty-four hours and RNA was isolated, reverse transcribed to cDNA and analyzed in Real-Time PCR experiments.

2.9 *Performance of MAPK inhibition experiments*

MM6 cells were seeded at a density of $2x10^5$cells/mL in 2mL of complete media, cultured for twenty-four hours and were then treated with either 15μM SB203580 (p38MAPK inhibitor) or 15μM PD980058 (ERK1/2 inhibitor) one hour prior to addition of either 200 ng/mL LPS, 0.5 μM PAF, or 10 ng/mL TNF-α. Cells were then harvested twenty-four hours later and RNA was isolated, reverse transcribed to cDNA and analyzed by real-time PCR experiments as described above.

2.10 *Preparation of MM6 cells for ELISAs*

MM6 cells were plated in serum-containing RPMI media for twenty-four hours as described above. MM6 cells were then switched into serum free media, after multiple wash steps with 1x PBS, and seeded at density of $4x10^5$ cells/mL and allowed to incubate overnight. Five-hundred ng/mL LPS were administered and MM6 cells were incubated for twenty-four hours. Cells were harvested at various time points and centrifuged. Conditioned media were collected and stored at -20°C. Cell pellets were lysed with lysis buffer containing phosphatase inhibitors and preservatives supplied by R&D Systems. Cells lysates were then supplemented with protease inhibitor cocktail from Sigma before use in subsequent experiments.

2.11 *Performance of ELISA for IL-1β*

ELISA kits were obtained from R&D Systems. The Quantikine Human IL-1β /IL-1F2 kit was used to assay for IL-1β. All supplied reagents were prepared according to the manufacturers instructions. Two-hundred μL of standard, sample or control were applied to antibody coated wells supplied by the manufacturer and allowed to incubate for two hours at room temperature. Wells were then aspirated and washed three times with wash buffer containing surfactant and preservatives four times. Two-hundred μL of a polyclonal antibody against IL-1β conjugated to horseradish peroxidase was then loaded into each well and incubated for one hour. Again, wells were aspirated and washed three times. A two-hundred μL mixture of stabilized horseradish peroxidase and chromogen (trimethylbenzidine) were then added to each well and incubated, protected from light, for twenty-minutes. Fifty μL of a 2N sulfuric acid stop solution was added to each well and the absorbance determined at 450 nm with wavelength correction at 540 nm within thirty-minutes of addition of the stop solution. A standard curve was generated using a four parameter logistic curve fit.

2.12 *Performance of ELISA for TNF-α*

ELISA kits were obtained from R&D Systems. The Quantikine Human TNF-α /SF1A kit was used to assay for TNF-α. All supplied reagents were prepared according to the manufacturers instructions. Fifty μL of the provided Assay Diluent RD1F was added to each antibody coated well. 200 μL of standard, control or sample were added to each well and allowed to incubate for two hours. Wells were then aspirated and washed with wash buffer containing surfactant and preservatives four times. Two-hundred μL of a

polyclonal antibody against TNF- α conjugate to horseradish peroxidase was added to each well and incubated for one hour. Wells were then aspirated and washed four times. A 200 μL mixture of stabilized hydrogen peroxide and chromogen (trimethylbenzidine) was then added to each well and incubated, protected from light, for twenty-minutes. Fifty μL of 2N sulfuric acid stop solution was added to each well and absorbance determined at 450 nm with wavelength correction at 540 nm within thirty-minutes. A standard curve was created using a four parameter logistic curve fit.

2.13 *Performance of ELISA for Phospho-p38α*

ELISA kits were obtained from R&D Systems. The Surveyor IC kit was used to assay for Phospho-p38α. All supplied reagents were prepared according to the manufacturers instructions. One-hundred μL of standard, sample or control were added to each antibody coated well and incubated for two hours. Wells were then aspirated and washed with wash buffer containing surfactant and preservatives four times. One-hundred μL of strepavidin-horseradish peroxidase was added to each well and incubated for twenty-minutes. A 100 μL mixture of stabilized hydrogen peroxide and chromogen (trimethylbenzidine) was then added to each well and incubated, protected from light, for twenty-minutes. Fifty μL of stop solution was added to each well and absorbance determined at 450nm with wavelength correction at 540nm within thirty-minutes. A standard curve was fit using a four parameter logistic curve fit.

2.14 *Performance of PAF Receptor Antagonist Experiments*

MM6 cells were cultured in serum-containing media at a density of $2x10^5$ cells/mL and used in experiments twenty-four hours later. The PAF receptor antagonist, WEB2170 was a generous gift provided by Merle S. Olson, University of Texas Health Sciences center. The p38MAPK inhibitor, SB203580, and the PAF receptor antagonist, WEB2170, were administered either one-hour or fifteen-minutes prior to administration of LPS or PAF respectively. In instances where WEB2170 and SB203580 were administered dually, WEB2170 and SB203580 were administered immediately together followed by LPS and PAF administration one hour later. Cells were harvested at twenty-four hours and RNA was isolated based on the method outlined by Sacchi and Chomczynski. Five μg of RNA, for each experimental condition, were reverse transcribed to cDNA and were used in Real-Time PCR reactions as described above.

2.15 *Performance of Reactive Oxygen Species Induction*

MM6 cells were cultured in serum-containing media at the appropriate density as described above. Two μL of a 100 mM *tert*- butyl hydroperoxide (TBHP) stock solution was administered to MM6 cells in the 2 mL cell culture plates, in complete media, to produce a final concentration of 100 μM TBHP on the cells. Cells were harvested at 0, 1, 3, 6, 12 and 24 hours and RNA was isolated as described above. Five μg of RNA, for each experimental condition, were reverse transcribed to cDNA and were used in Real-Time PCR reactions.

2.16 *Identifying Morphological Changes in MM6 Cells*

Various combinations of 0.5 μM PAF, 200 ng/mL LPS and 10 ng/mL TNF- α were administered to MM6 cells in serum-containing media. Twenty-four hours later, pictures of the MM6 cells were taken through use a digital camera attached to a Zeiss Axiovert inverted microscope (Gottingen, Germany). Pictures were taken at a total magnification of 50X.

2.17 *Performance of Statistical Analysis*

T-tests were conducted to determine if there were significant differences between selected variables (i.e. varying doses of LPS treatment vs. control) where comparisons were made between one control group and one treatment group. Analysis of variance (ANOVA) were conducted to determine if there were significant differences between selected variables (i.e. PAF-AH mRNA fold-induction over control in the presence of various inhibitors and inflammatory mediators) where comparisons were made between a control group and more than one treatment group. Repeated measures were conducted between selected variables (i.e. seventy-two hour time course experiments in the presence of various inflammatory mediators) where comparisons were made between one control group and one treatment group across several time periods. All analyses were conducted using SPSS 16.0 (SPSS, Inc., Chicago, IL).

CHAPTER 3

RESULTS

The goal of this research is to investigate whether TNF-α and PAF are able to upregulate PAF acetylhydrolase expression and, to ascertain any similarities or differences in signaling to that detected with LPS. Cultured MM6 cells were used in experiments for their ability to induce PAF acetylhydrolase expression in response to LPS *in vitro*—similar to the response demonstrated *in vivo*.

3.1 *Dose Response of Various Pro-inflammatory Mediators*

In order to determine if LPS, PAF and TNF-α are able to upregulate PAF acetylhydrolase expression, dose response experiments, measuring the fold induction of PAF acetylhydrolase mRNA, were performed. See Figures 7-9. MM6 cells were cultured in serum-containing media for twenty-four hours prior to administration of the proinflammatory mediators. After a twenty-four hour inflammatory challenge with the aforementioned mediators, PAF acetylhydrolase mRNA levels were ascertained through Real-Time PCR. For all doses of LPS, a 15-20 fold induction of PAF acetylhydrolase mRNA was observed over control levels as shown in Figure 7. One-hundred ng/mL LPS produced a maximal 20-fold induction in PAF acetylhydrolase levels over control. At 200 ng/mL LPS, PAF acetylhydrolase mRNA levels decreased slightly from the maximal values but still resulted in a sixteen-fold induction over control levels. For all of the

concentrations of PAF, a 3.5-4.5 fold induction of PAF acetylhydrolase message was observed. (See Figure 8). However, the upregulation of PAF acetylhydrolase levels did not appear to be dose-dependent—though, some variability was detected. The TNF-α induced PAF acetylhydrolase levels showed a 6.5-10 fold induction of PAF acetylhydrolase message over control for all concentrations of TNF-α administered. As shown in Figure 9, incremental increases in PAF acetylhydrolase mRNA were observed for increasing concentrations of TNF-α. Administration of 20 ng/mL TNF-α resulted in the largest increase in PAF acetylhydrolase (10-fold) however, higher concentrations were not tested.

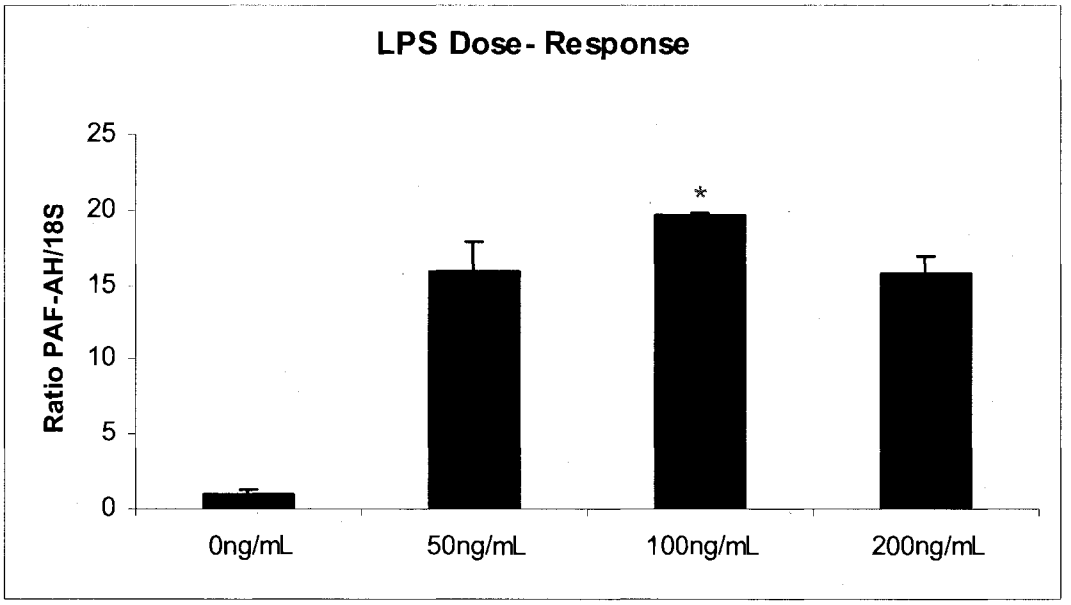


Figure 7: LPS Dose Response. MM6 cells were cultured in serum-containing media for twenty-four hours. Incremental increases in PAF-AH mRNA levels are observed up 100 ng/mL LPS administration. At 200 ng/mL LPS administration, MM6 cells appear to become desensitized to the increase LPS administration. The data reflect a value of two independent biological samples analyzed in triplicate; mean difference is significant at $p<0.05$. The single asterisk represents statistical significance from all other values. Error bars represented are +/- standard deviation.

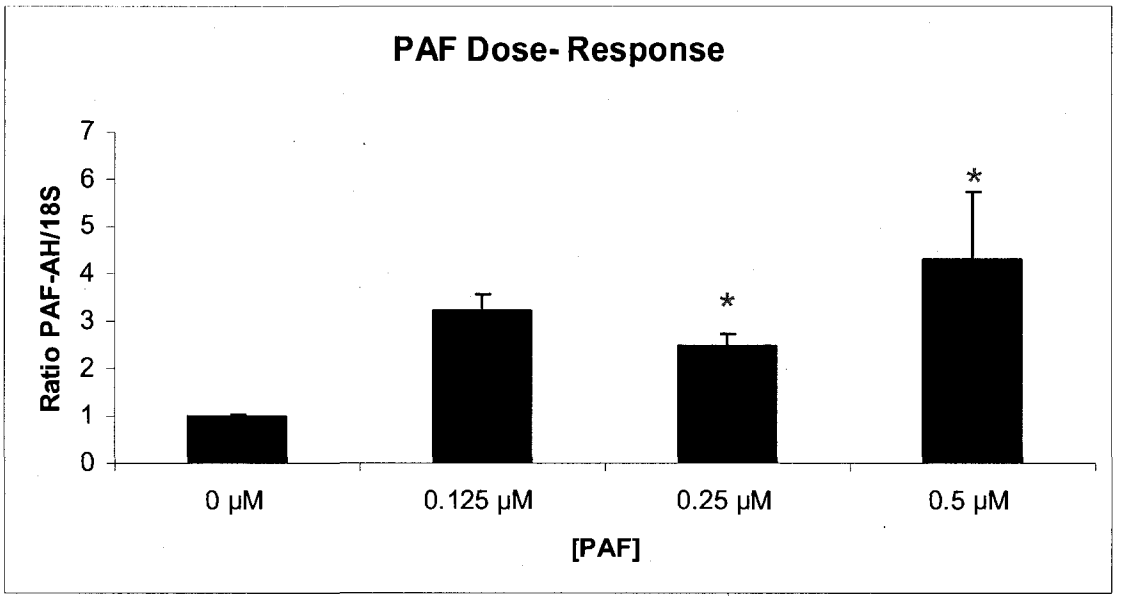


Figure 8: PAF Dose Response. MM6 cells were cultured in serum-containing media for twenty-four hours in the presence of various concentrations of PAF. The PAF induced PAF-AH mRNA levels do not appear to be dose dependent. This is observation is most likely due to the presence of PAF-AH in the 10% FBS. Fold-induction of PAF-AH levels is statistically different than control for all concentrations of PAF administered. The data reflect a value of two independent biological samples analyzed in triplicate; mean difference is significant at $p<0.05$. Single asterisk represents statistical difference between 0.25µM PAF and 0.5µM PAF. Error bars represented are +/- standard deviation.

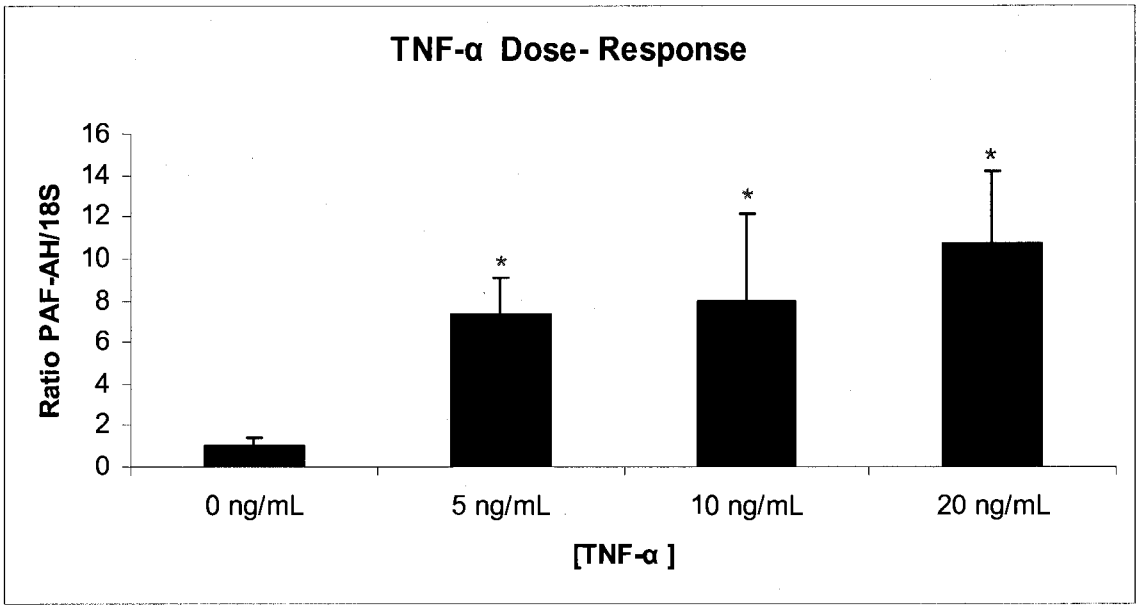


Figure 9: TNF-α Dose Response. MM6 cells were cultured in serum-containing media for twenty-four hours. The TNF-α induced upregulation of PAF acetylhydrolase shows increased expression of PAF acetylhydrolase with incremental increase in TNF-α dosage. The data reflect a value of two independent biological samples analyzed in triplicate; mean difference is significant at $p<0.05$. The single asterisk represents statistical significance from the control. Error bars represented are +/- standard deviation.

3.2 *Lipopolysaccharide Induced Expression of PAF-AH mRNA in MM6 Cells*

LPS is a component of the cell wall of gram-negative bacteria and is known to elicit pro-inflammatory responses that result in the upregulation of PAF Acetylhydrolase levels *in vivo*. In order to characterize the extent and time frame of the LPS induced PAF acetylhydrolase response, MM6 cells were cultured with 200 ng/mL LPS over a seventy-two hour period in either serum-containing or serum-free RPMI media. See Figures 10 and 11. Time course experiments for MM6 cells cultured in serum-containing media showed control levels of PAF Acetylhydrolase mRNA levels after one hour of LPS treatment. Subsequent to this, PAF Acetylhydrolase mRNA levels rose steadily—reaching a maximal 8-fold induction, compared to control levels, at twenty-four hours. At

forty-eight and seventy-two hours, PAF acetylhydrolase mRNA levels slightly decreased to a level that was approximately six-fold above control levels. Control cells, not receiving any LPS treatment, were incubated for the entire time course. Near control levels of PAF acetylhydrolase were detected in all samples.

In order to determine the importance of the lipopolysaccharide binding protein (LBP) in mediating the effects of LPS with respect to PAF Acetylhydrolase mRNA upregulation, MM6 cells were cultured in serum-free media with either 200 ng/mL LPS or in the absence of LPS for seventy-two hours as shown in Figure 11. In the absence of serum, PAF acetylhydrolase mRNA levels were similar to levels detected after exposure to 200 ng/mL LPS in serum-containing media for the initial twenty-four hours. Subsequent to this time, an approximately thirty and sixty fold increase in PAF acetylhydrolase mRNA levels was detected at forty-eighty and seventy two hours respectively in both MM6 cells cultured in serum-free media in the presence or absence of LPS.

3.3 *Platelet Activating Factor Induced Expression of PAF- AH mRNA in MM6 Cells*

PAF is a known potent phospholipid activator and mediator of many leukocyte functions, including platelet aggregation, inflammation, anaphylaxis and has been shown to be a central mediator in inducing an inflammatory response (38). The enzyme PAF acetylhydrolase, a calcium-independent phospholipase A_2, converts PAF to its biologically inactive form, lyso-PAF. This relationship between PAF and PAF acetylhydrolase suggests that the presence of PAF may upregulate PAF acetylhydrolase expression. To investigate the extent, if any, by which PAF is able to upregulate PAF

acetylhydrolase mRNA levels, MM6 cells were cultured for seventy-two hours in serum-containing RPMI media in the presence of 0.5 μM PAF. (See Figure 12). Near control

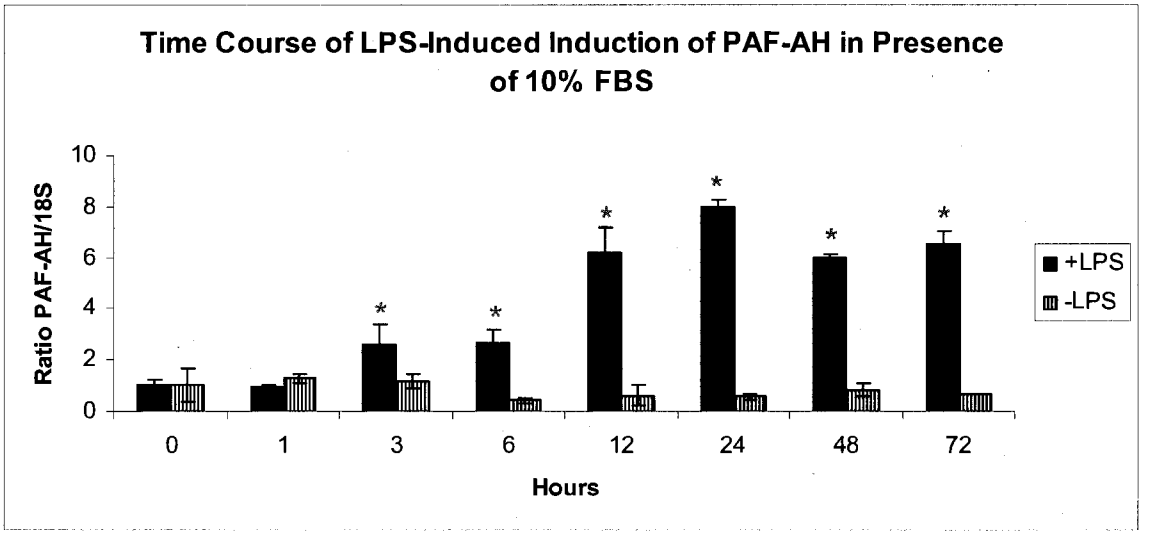


Figure 10: Serum-Containing Seventy-two Hour Time Course. MM6 cells were cultured for seventy-two hours in serum-containing RPMI media in the presence of either 200 ng/mL LPS or 0 ng/mL LPS. For MM6 cells incubated in the presence of serum, maximal fold induction of PAF acetylhydrolase levels occur at twenty-four hours. Levels drop by forty-eight hours and a slight increase in PAF acetylhydrolase induction is observed at seventy-two hours. MM6 cells cultured in absence of LPS show near control levels of PAF acetylhydrolase induction. The data reflect a value of two independent biological samples analyzed in triplicate; mean difference is significant at $p<0.05$. The single asterisks represent statistical significance from control. Error bars represented are +/- standard deviation.

levels of PAF acetylhydrolase mRNA expression were observed within six hours of 0.5 μM PAF exposure. PAF Acetylhydrolase mRNA levels increase five-fold over control levels at twelve hours of 0.5 μM PAF exposure. PAF acetylhydrolase levels fall to eight-fold over control by seventy-two hours.

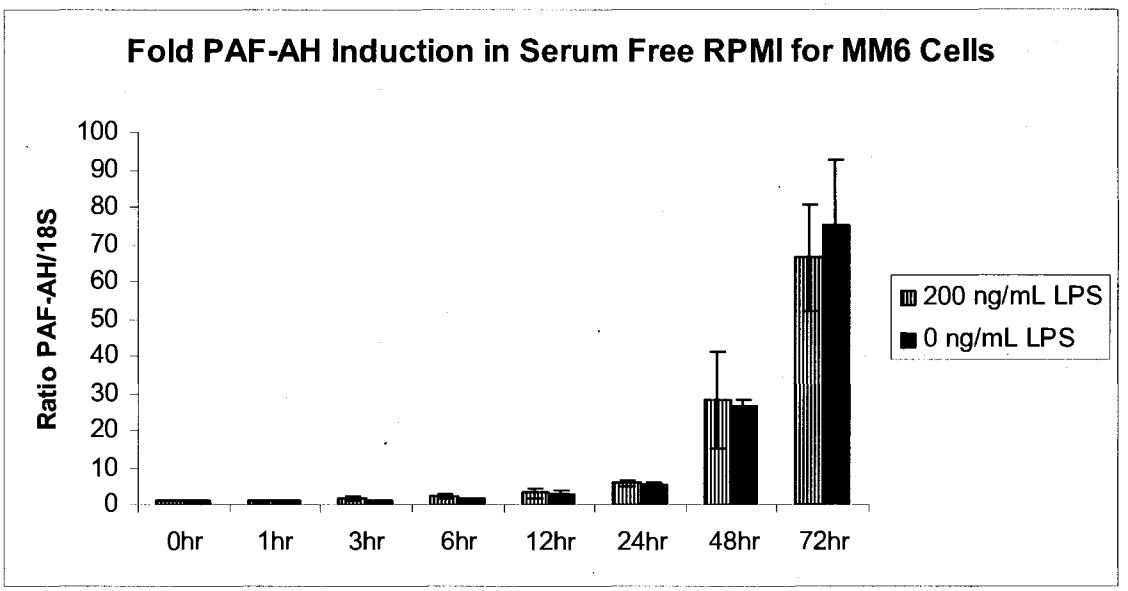


Figure 11: Serum-Free Seventy-two Hour Time Course. MM6 cells incubated in serum free RPMI with either 200 ng/mL LPS or 0 ng/mL LPS over a seventy-two hour time course. Fold induction of PAF acetylhydrolase between the two experimental conditions show no statistical difference. The data reflect a value of four independent biological samples analyzed in triplicate for LPS-induced cells; The data reflect a value of two independent biological samples analyzed in triplicate for control cells. There was no statistical difference between control and experimental conditions. Error bars represented are +/- standard deviation.

3.4 *Tumor Necrosis Factor- α Induced Expression of PAF-AH mRNA in MM6 Cells*

TNF-α is a 17 kDa cytokine involved in acute phase systemic inflammation. TNF-α is mainly produced by macrophages, but also by a broad variety of other cell types including lymphoid cells, mast cells, and endothelial cells. Large amounts of TNF-α are released in response to lipopolysaccharide, other bacterial products, and interleukin-1 (39) (35). To investigate the extent and time frame by which TNF-α is able to upregulate PAF acetylhydrolase mRNA levels in MM6 cells, cells were cultured for seventy-two hours in the presence of 10 ng/mL TNF-α in serum-containing media.

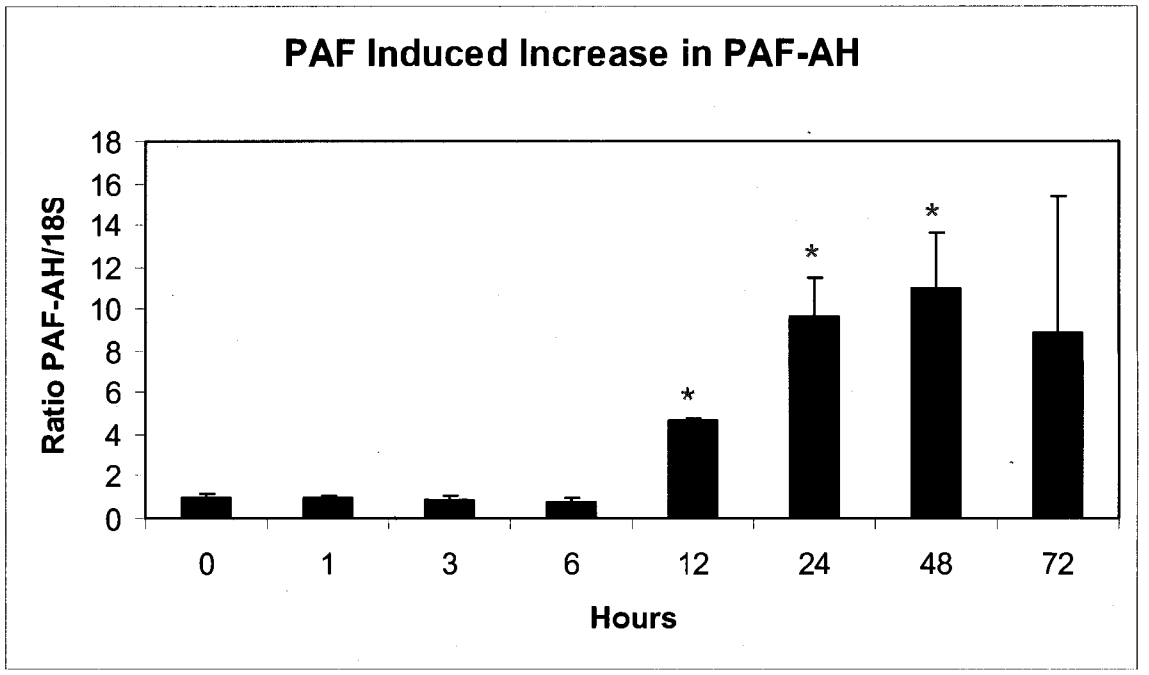


Figure 12: PAF Induced Serum-Containing Seventy-two Hour Time Course. MM6 were cultured in serum containing RPMI media for seventy-two hours in the presence of 0.5μM PAF. Levels of PAF acetylhydrolase induction remain relatively steady upon until 12 hours of PAF exposure. The data reflect a value of two independent biological samples analyzed in triplicate; mean difference is significant at $p<0.05$. Single asterisk represents statistical difference from the control. Error bars represented are +/- standard deviation.

As shown in Figure 13, levels of PAF acetylhydrolase mRNA remained steady, near control levels, up until six hours of exposure to TNF-α—where a two-fold induction, above control levels, was observed. PAF acetylhydrolase levels increased nine-fold, above control levels, at twenty-four hours of TNF-α exposure and abruptly returned to control levels by seventy-two hours.

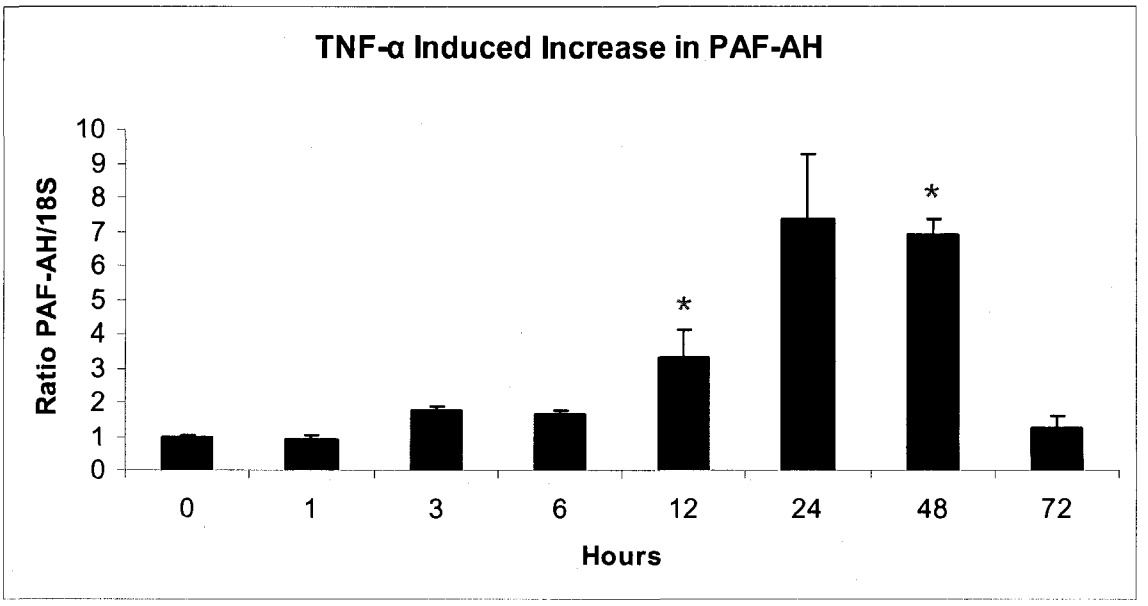


Figure 13: TNF-α Serum-Containing Seventy-two Hour Time Course. MM6 cells were cultured in serum-containing RPMI media for seventy-two hours in the presence of 10 ng/mL TNF-α. Levels of PAF acetylhydrolase induction begin to rise by three hours and reach maximal levels of induction by twenty-four hours. PAF acetylhydrolase levels then drop abruptly by seventy-two hours. The data reflect a value of two independent biological samples analyzed in triplicate; mean difference is significant at $p<0.05$. Single asterisk represents statistical difference from the control. Error bars represented are +/- standard deviation.

3.5 *PAF-AH mRNA Induction in Response to Dually Administered Pro-Inflammatory Mediators*

Recent work by Stafforini *et al.* has shown that LPS-induced PAF Acetylhydrolase upregulation is mediated entirely through p38MAPK in the monocyte-like cell line, THP-1/CD14 (40). In order to determine if PAF, TNF-α, and LPS can augment function in a synergistic manner, combinations of dually administered pro-inflammatory mediators were added to a MM6 cell culture and the levels of PAF acetylhydrolase mRNA were determined. Administration of either 200 ng/mL LPS, 10 ng/mL TNF-α, or 0.5μM PAF showed a 15, 12 and 4.5-fold induction over control PAF acetylhydrolase mRNA levels,

respectively. The effects of the dually administered pro-inflammatory mediators are shown in Figure 14.

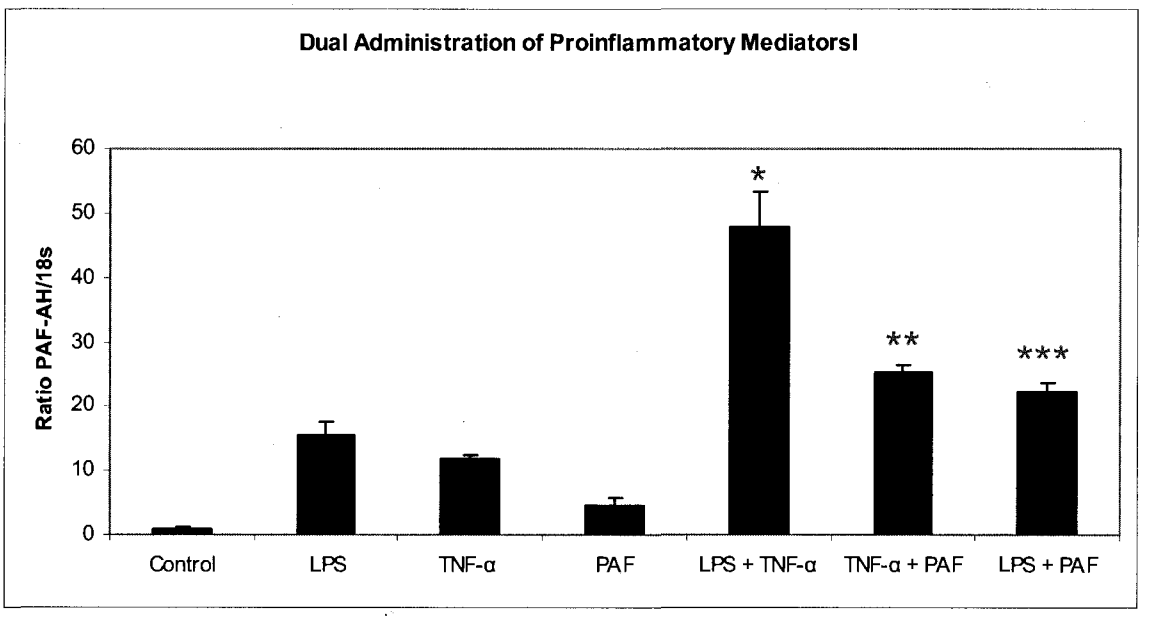


Figure 14: Dual Administration of Proinflammatory Mediators. MM6 cells were cultured in serum containing RPMI media and were incubated with various combinations of proinflammatory mediators. Strong fold induction of PAF acetylhydrolase mRNA levels were observed with LPS and TNF-α administered in combination and near additive effects in PAF acetylhydrolase induction were observed for TNF-α + PAF and LPS + PAF. The data reflect a value of two independent biological samples analyzed in triplicate; mean difference is significant at $p<0.05$. The single asterisk represents statistical difference from LPS-induced cells. The double asterisk represents statistical difference from TNF-α induced cells. The triple asterisk represents statistical difference from PAF-induced cells. Error bars represented are +/- standard deviation.

The combination of LPS and TNF-α showed a marked increase of forty-five fold induction over control levels at twenty-four hours. TNF-α + PAF and LPS + PAF showed a more modest synergistic twenty-five and twenty-two fold increase in PAF acetylhydrolase levels over control respectively. These latter results showed a near additive effect in PAF acetylhydrolase mRNA upregulation.

3.6 *Determination of Essential MAPK Signaling Pathways for LPS, PAF and TNF-α*

LPS is able to induce proinflammatory responses via activation of numerous intracellular signaling pathways. A recent review article summarized the major pathways activated by LPS. These pathways include the MAPK cascades—ERK1/2, JNK, and p38—as well as the NFkβ/Ikβ intracellular signaling pathway (31). (See Figure 5). In order to investigate if the MAPK pathways are essential for upregulation of PAF acetylhydrolase, and whether PAF and TNF-α also signal through similar mechanisms, use of various MAPK inhibitors were employed. The inhibitors, PD98059 and SB203580 were used to inhibit the ERK1/2 and p38MAPK portions of the MAPK pathway respectively as shown in Figures 15-17. MM6 cells were cultured in serum-containing media and were administered the inhibitors one-hour prior to administration of the proinflammatory agonist. Use of 15 μM SB203580 showed an approximately 50% decrease in PAF acetylhydrolase levels compared to MM6 cells treated with 200 ng/mL LPS alone as depicted Figure 15. A more modest 20% decrease in TNF-α mediated PAF Acetylhydrolase levels was observed with the inhibitor as compared to cells treated with 10 ng/mL TNF-α alone. (Figure 15). Administration of SB203580 also decreased the PAF-induced PAF acetylhydrolase levels by approximately 20%. (Figure 17). Paradoxically, inhibition of the ERK1/2 portion of the MAPK pathway with 15 μM PD98059 increased PAF acetylhydrolase expression by 50% relative to administration of the pro-inflammatory mediators alone for LPS, PAF and TNF-α. (Figures 16 and 17).

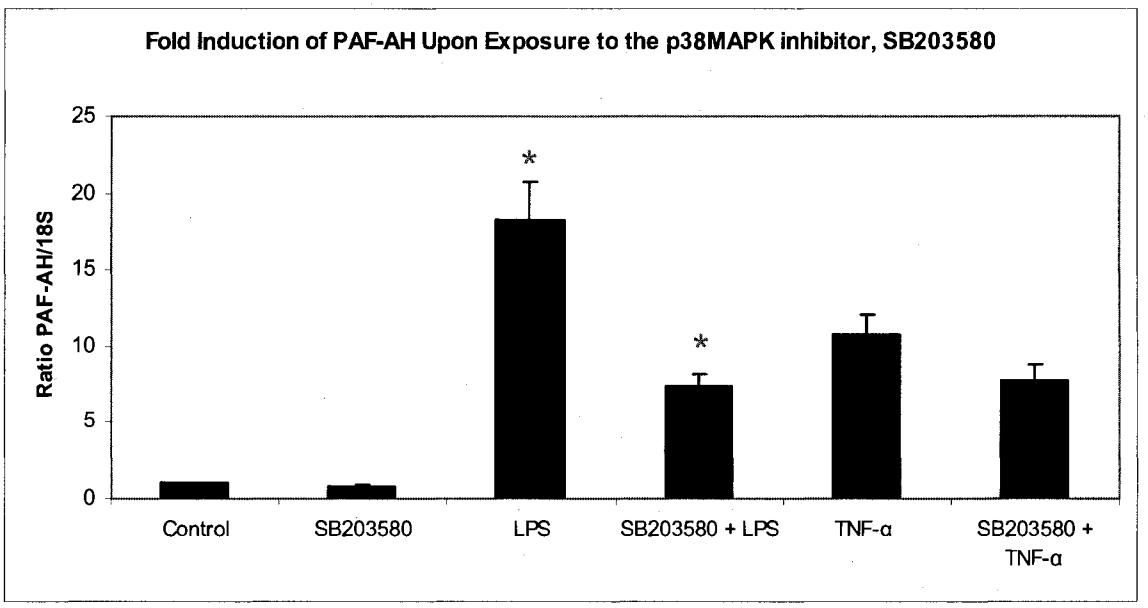


Figure 15: Inhibition of p38MAPK signaling via SB203580. MM6 cells treated with either 200 ng/mL LPS or 10 ng/mL TNF-α were cultured in serum-containing RPMI media for twenty four hours in the presence of the p38MAPK inhibitor, SB203580 at 15μM. A 50% decrease in LPS induced PAF-AH mRNA levels is observed in the presence of SB203580. An approximately 20% decrease in the PAF induced PAF-AH mRNA levels is observed in the presence of SB203580. Fold-induction of PAF-AH mRNA levels is statistically significant from control and SB203580 for all other experimental conditions. The data reflect a value of two independent biological samples analyzed in triplicate; mean difference is significant at $p<0.05$. The single asterisk represents a statistical difference between LPS and LPS + SB203580 treated cells. Error bars represented are +/- standard deviation.

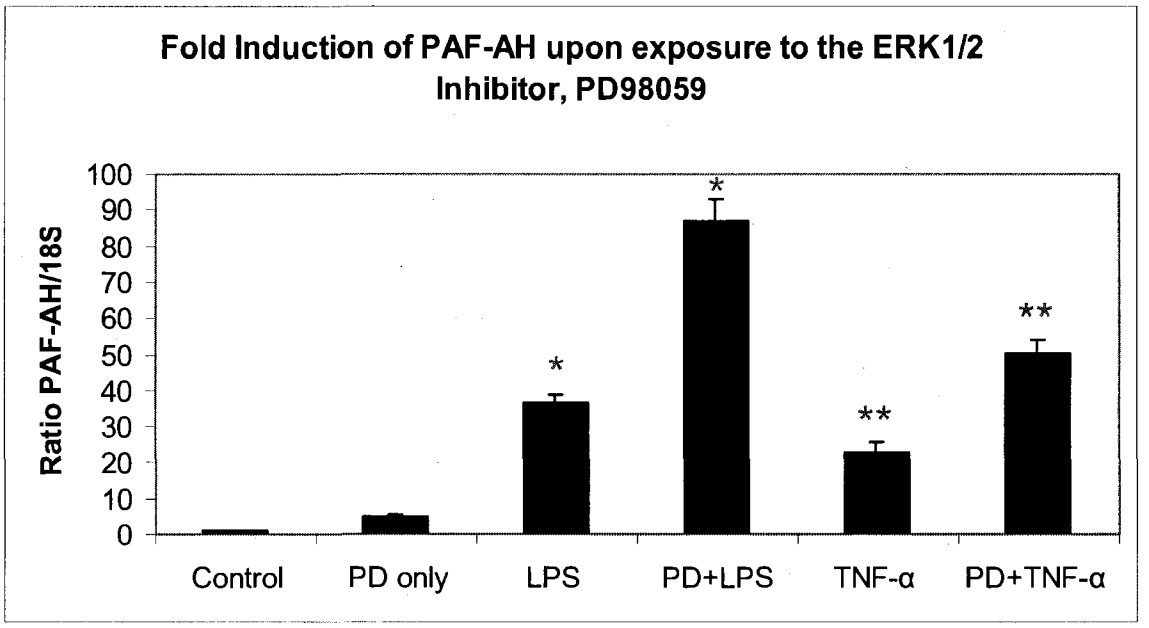


Figure 16: Inhibition of ERK1/2 Signaling via PD98059. MM6 cells, cultured in serum-containing media for twenty-four hours show a 50% increase in PAF-AH mRNA levels (with respect to the inflammatory mediator administered) upon administration of the ERK1/2 inhibitor PD98058 at 15 μM. LPS was administered at a concentration 200 ng/mL and TNF-α was administered at a concentration of 10 ng/mL. This result is most likely due to the stress imposed on the cell after inhibition of such a crucial pathway. See text. All experimental conditions are statistically different than control and PD98059 administration. The data reflect a value of two independent biological samples analyzed in triplicate; mean difference is significant at $p<0.05$. The single asterisk represents statistical difference between LPS and LPS + PD98059 treated cells. The double asterisk represents statistical difference between TNF-α and TNF-α + PD98059 treated cells. Error bars represented are +/- standard deviation.

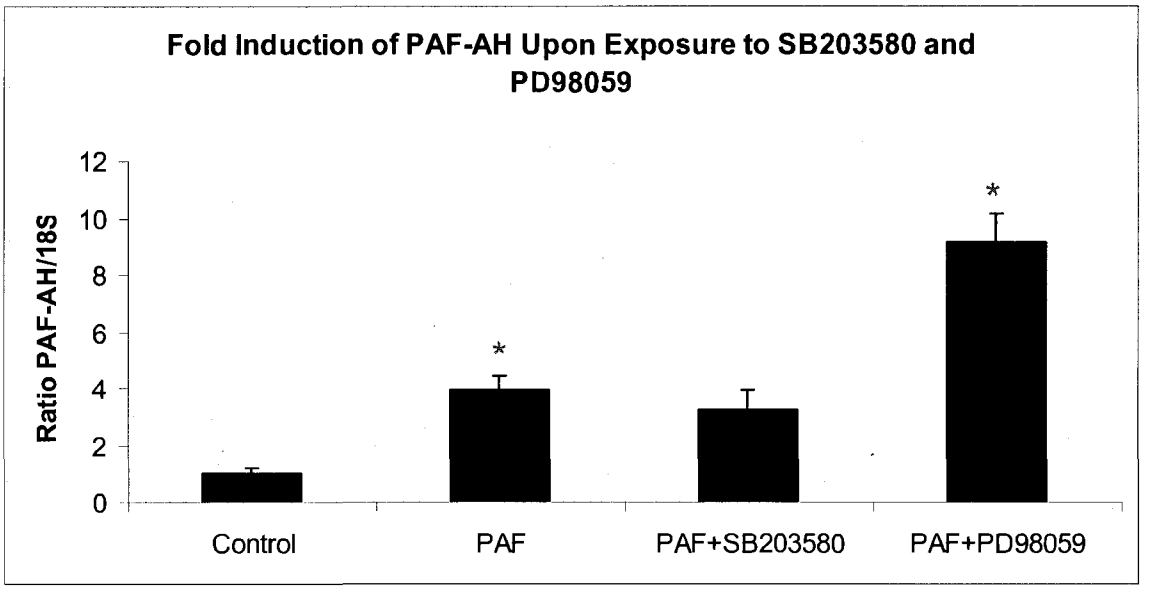


Figure 17: Inhibition of p38MAPK and ERK1/2 Signaling via SB203580 and PD98059 Respectively. A.MM6 cells cultured in serum containing RPMI media were exposed to 15 μM SB203580 and 15 μM PD98059. The PAF induced PAF acetylhydrolase mRNA upregulation was decreased by approximately 20% in the presence of the p38 MAPK inhibitor. Paradoxically, inhibition of the ERK1/2 portion of the MAPK pathway caused an approximately 50% increase in fold induction of PAF acetylhydrolase levels over the induction elicited by PAF alone. PAF was administered at a concentration of 0.5 μM. The data reflect a value of two independent biological samples analyzed in triplicate; mean difference is significant at $p<0.05$. The single asterisk represents a statistical difference between PAF and PAF + PD98059 treated cells. Error bars represented are +/- standard deviation.

3.7 *PAF- AH mRNA induction in Response to Dual Administration of SB203580 and WEB2170*

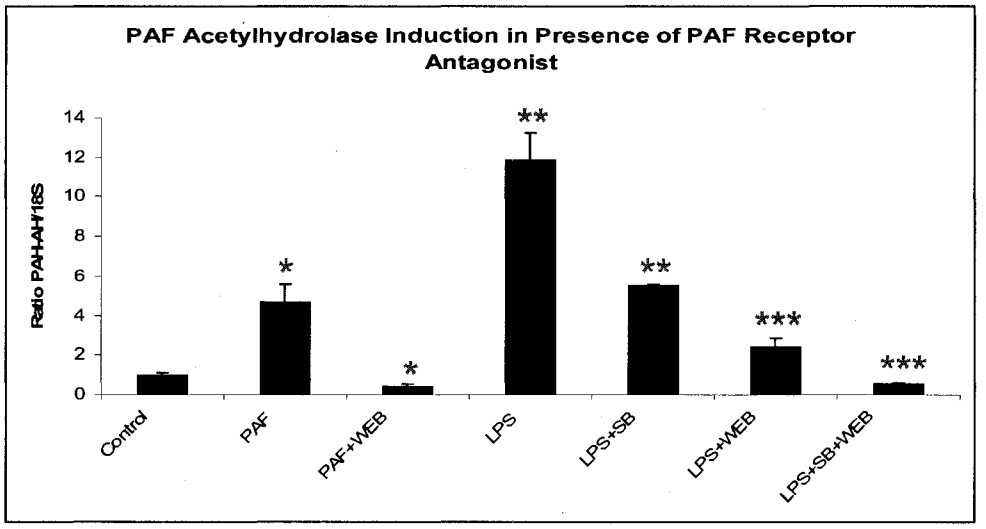
Recent work by Howard *et al.* suggested that 50% of the LPS-induced PAF acetylhydrolase response in rats is due to increased signaling through the PAF receptor as a result of increased PAF production due to LPS challenge (32). Further, our work showed only a 50% inhibition of the LPS-induced PAF acetylhydrolase mRNA upregulation upon administration of SB203580. In order to investigate if increased PAF signaling through its receptor contributes to the upregulation of PAF acetylhydrolase,

MM6 cells were either singly or dually administered WEB2170 (50 μM) and SB203580 (15 μM) prior to stimulation with PAF or LPS in serum-containing media for twenty-four hours. Figure 18 showed WEB2170 as an effective PAF receptor antagonist and inhibited the PAF-induced increase in PAF acetylhydrolase. Both LPS and PAF, alone, showed the typical fold induction of PAF acetylhydrolase levels over control. As seen previously, a 50% decrease in the LPS-induced PAF acetylhydrolase mRNA, upon administration of SB203580, was observed. Administration of WEB2170, inhibited the LPS-induced increase in PAF acetylhydrolase resulting in a two-fold induction over control levels. Dual administration of both SB203580 and WEB2170, completely abolished the LPS-induced PAF acetylhydrolase levels and depressed PAF acetylhydrolase mRNA to half of that detected in control cells.

3.8 *IL-1β Induction in Response to LPS Treatment*

IL-1β, primarily produced by both monocytes and macrophages, is a known cytokine that aids in the induction of an inflammatory response (41). Because IL-1β production is increased in response to bacterial endotoxin, it was necessary to ascertain if the administration of LPS was able to induce IL-1β production in our MM6 cell culture model. In preparation for use in ELISAs, MM6 cells were grown in serum-containing media for twenty-four hours and then switched to serum-free media. After seven-hours in serum-free media, a 500 ng/mL dose of LPS was administered. MM6 cells were centrifuged; supernates were harvested and then used in subsequent ELISAs. As shown in Figure 19A, IL-1β protein levels increased to 115 pg/mL by three-hours after LPS exposure. The levels of IL-1β dramatically rose by six and twelve hours to 791 pg/mL IL-1β for both time points (extrapolated values).

A.



B.

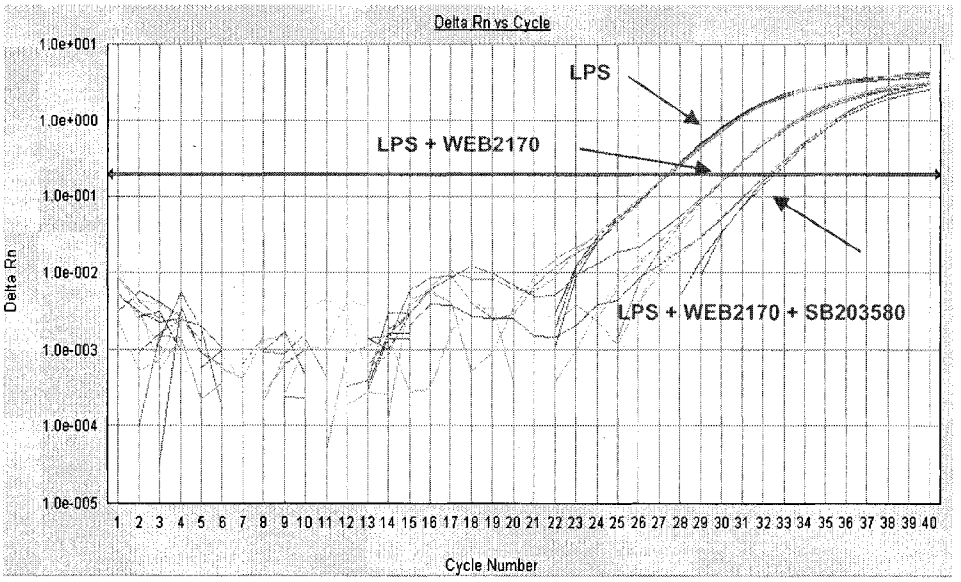


Figure 18: Effects of the PAF Receptor Antagonist, WEB2170, on Fold PAF-AH Induction and PAF-AH Standard Curve. A. MM6 cells were cultured in serum-containing media for twenty-four hours with 0.5 μM PAF or 200 ng/mL LPS, alongside either 50 μM WEB2170 or 15 μM SB203580. WEB2170 was an effective PAF receptor antagonist and was also able to inhibit the LPS-induced PAF-AH mRNA upregulation. Administration of LPS, SB203580 and WEB2170 together decreased PAF-AH mRNA levels to half those of control levels. B. Real-Time PCR curves showing differences in PAF-AH mRNA levels for each noted experimental condition. The data reflect a value of two independent biological samples analyzed in triplicate; mean difference is significant at $p<0.05$. The single asterisk represents statistical significance between PAF

and PAF + WEB2170 administration. The double asterisk represents statistical significance between LPS and LPS + SB203580 administration. The triple asterisk represents statistical significance between LPS + WEB2170 and LPS + WEB2170 + SB203580 administration. Error bars represented are +/- standard deviation.

By twenty-four hours, IL-1β protein levels fell to control levels with 169 pg/mL of IL-1β protein present. In control cells, IL-1β levels remained relatively constant with a slight trend of increasing IL-1β over the twenty-four hour incubation period (15-200 pg/mL).

3.9 *TNF-α Induction in Response to LPS Treatment*

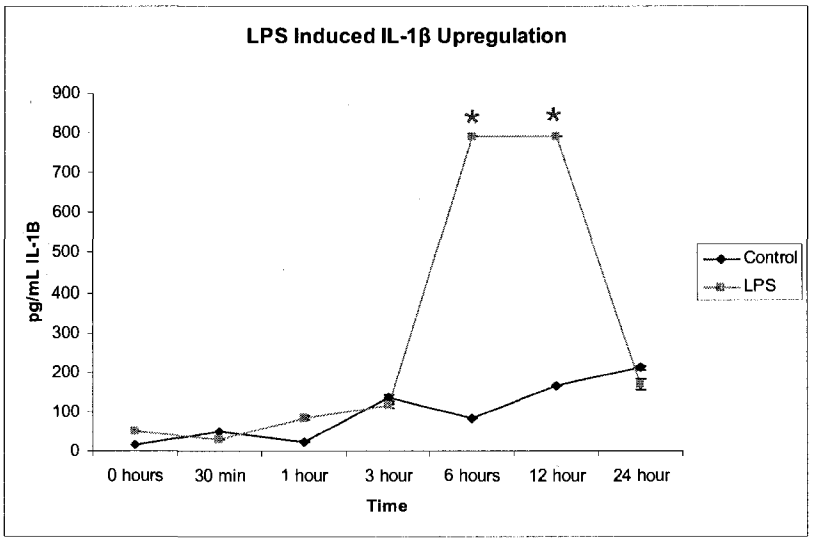
As mentioned previously, TNF-α is a cytokine involved in acute phase systemic inflammation. In order to investigate increases in TNF-α protein production as a result of LPS administration, MM6 cells were cultured as described in the previous section. As shown in Figure 20, control cells not receiving LPS produced very little TNF-α and levels detected ranged from 17 pg/mL (time 0) to 43 pg/mL (24-hours) Supernates from the LPS-induced cells showed a sharp increase by one-hour. TNF-α protein production reached a maximal 577 pg/mL. After six hours, TNF-α levels began fell to 413 pg/mL and 253 pg/mL by twelve and twenty-four hours respectively.

3.10 *Phospho-p38α Induction in Response to LPS Treatment*

Phosphophorylated-p38 (p-p38α) is produced in the cell upon activation of the p38MAPK portion of the MAPK cascades. This portion of the MAPK cascade is activated upon exposure to inflammatory mediators, and various cellular stresses and this pathway helps to regulate cell differentiation and apoptosis. To determine if the p38MAPK pathway is indeed activated in MM6 cells, ELISAs against the p-p38α were

preformed. (See Figure 21). As described above, MM6 cells were grown in serum-containing media for twenty-fours then switched to serum-free media.

A.



B.

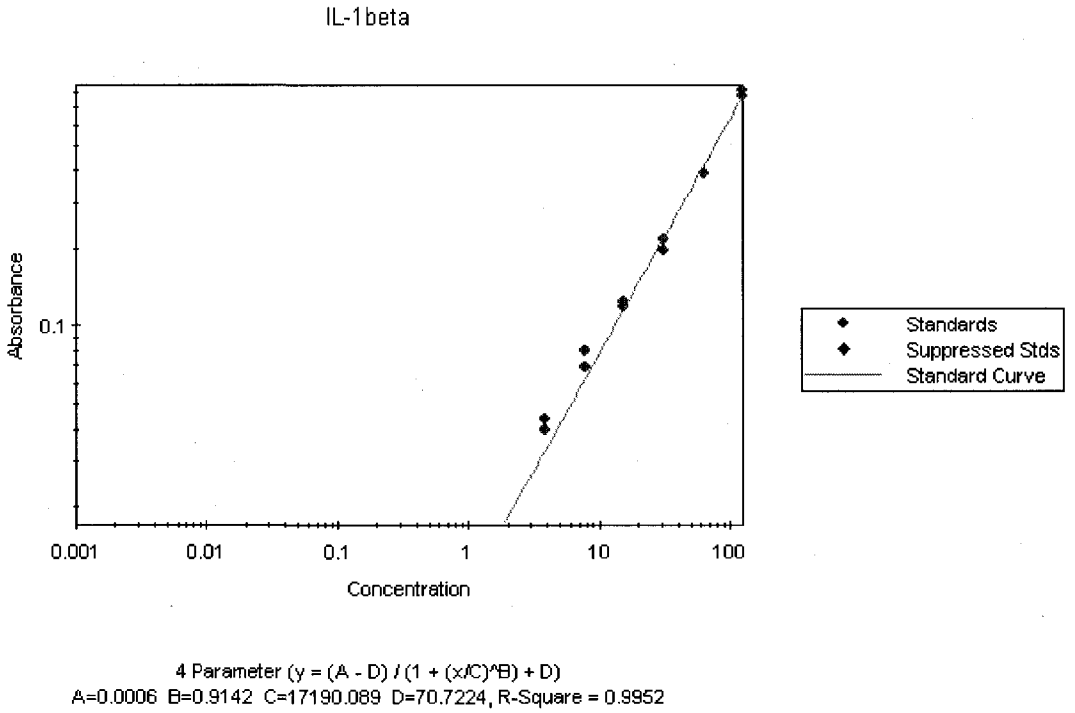
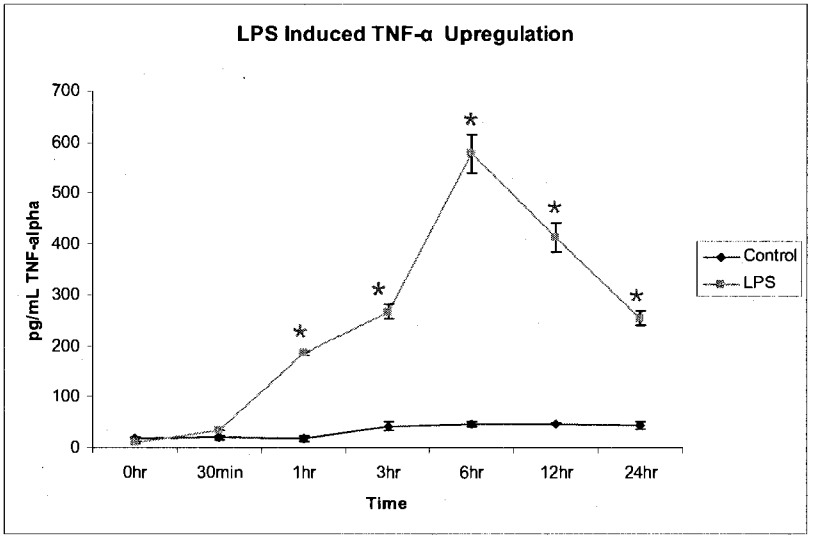


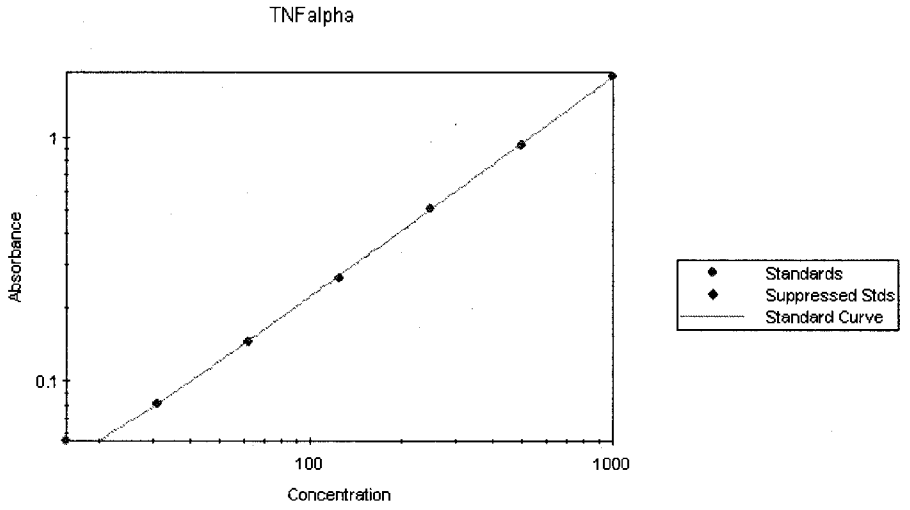
Figure 19: IL-1β ELISA. A. MM6 cells were cultured in serum-free media in the presence of 500 ng/mL LPS and the conditioned media was collected twenty-four hours later to be used in ELISAs. IL-1β protein levels begin to increase by thirty-minutes of

LPS exposure and levels rise substantially by six and twelve hours. Values at six and twelve hours are extrapolated due to the large induction in IL-1β protein levels at those time points. IL-1β protein levels begin to increase by three-hours in control conditions and continue to rise by twenty-four hours. B. Computer generated four-parameter logistic curve fit showing correlation. The data reflect a value of two independent biological samples analyzed in duplicate; mean difference is significant at $p<0.01$. The single asterisk represents statistical difference between the six and twelve hour time points compared to the corresponding control time points. Error bars represented are +/- standard deviation.

A.



B.

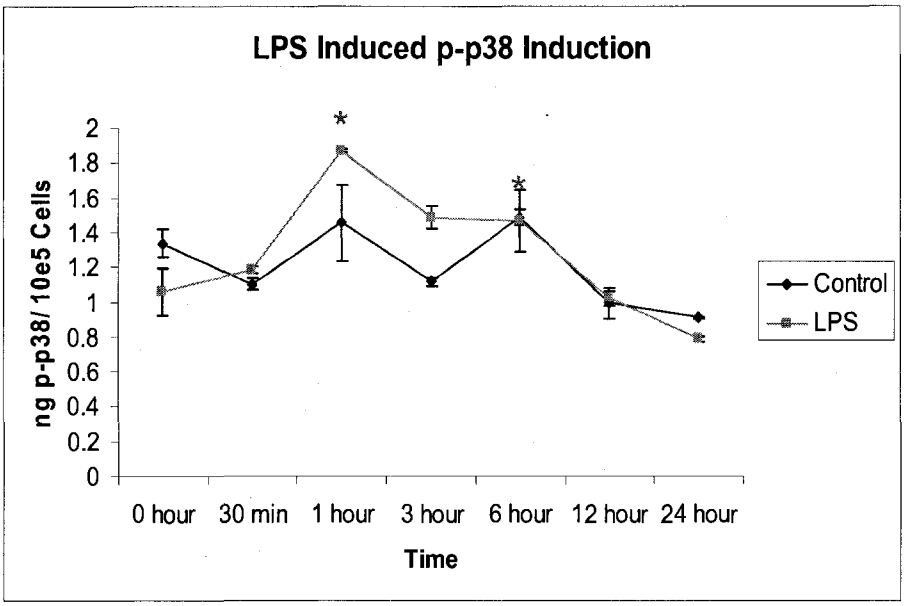


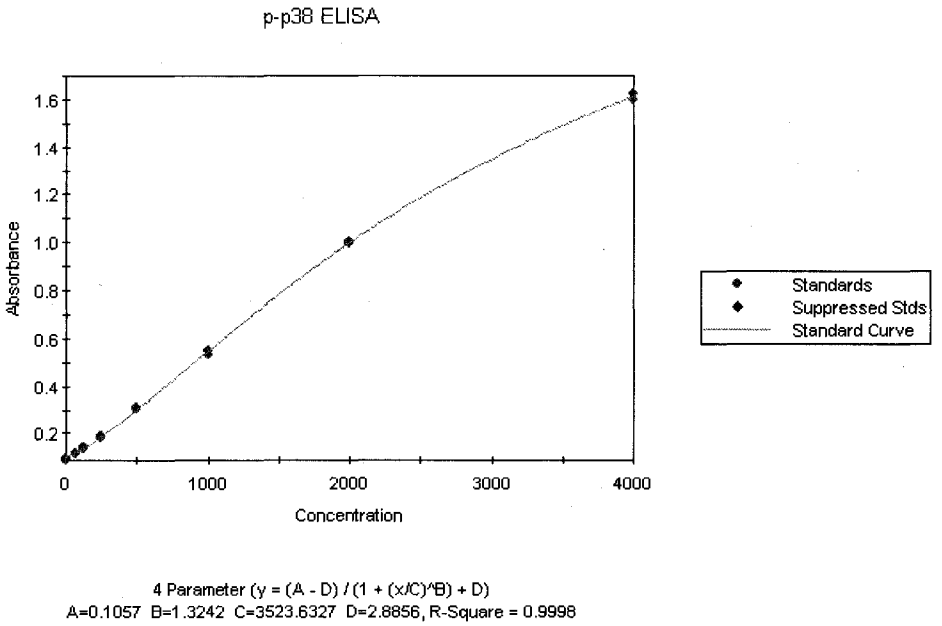
4 Parameter (y = (A - D) / (1 + (x/C)^B) + D)
A=1207.7284 B=-0.9137 C=1260744.9 D=0.0061, R-Square = 0.9998

Figure 20: TNF-α ELISA. A. MM6 cells were cultured in serum-free media in the presence of 500 ng/mL LPS and the conditioned media was collected twenty-four hours later to be used in ELISAs. Incremental increases in TNF-α protein were observed, upon administration of 500 ng/mL LPS, with maximal protein levels present at six-hours. Subsequent to this time point, levels begin to fall steadily. Control levels remain relatively steady throughout the time course. B. Computer generated four-parameter logistic curve fit showing correlation. The data reflect a value of two independent biological samples analyzed in duplicate; mean difference is significant at $p<.001$. The single asterisk represents a statistical difference between the one through twenty-four hour time points compared to the corresponding control time points. Error bars represented are +/- standard deviation.

Cell pellets were resuspended in cell lysis buffer to give a density of 10^7 cells/mL. Before use in ELISAs, a series of two, six-fold dilutions were prepared. An analysis of the p-p38α ELISA demonstrated a statistically significant increase in the amount of p-p38α protein at one and three hours compared to controls. (1.3 ng p-p38α/ 10^5 cells vs. 1.0 ng p-p38α/ 10^5; 1.1 ng p-p38α/ 10^5 vs. 1.4 ng p-p38α/ 10^5) Subsequent to this time, p-p38α levels decrease to .78 ng p-p38α/10^5 cells by twenty-four hours. Control levels of p-p38α fluctuate between .99 and 1.4 ng p-p38α/10^5 cells over the entire twenty-four hour time course.

A.





B.

Figure 21: p-p38α ELISA. A. MM6 cells were cultured in serum-free media for twenty four hours in the presence of 500 ng/mL LPS. Cells were harvested, centrifuged and cell pellets were lysed in cell lysis buffer to give a density of 10^7 cells/mL to be used in ELISAs. A statistical difference in p-p38α protein levels is observed between the one and three hour time points. B. Computer generated four-parameter logistic curve fit showing correlation. The data reflect a value of two independent biological samples analyzed in duplicate; mean difference is significant at $p<0.05$. The single asterisk represents statistical difference between the one and three hour time points compared to the corresponding control time points. Error bars represented are +/- standard deviation.

3.11 *Reactive Oxygen Species Induced Upregulation of PAF-AH mRNA*

Because of the results depicted in Figures 10 and 11, we hypothesized that reactive oxygen species (ROS) may induce PAF acetylhydrolase mRNA production. To investigate this possibility, 100 μM *tert-* butyl hydroperoxide (TBHP), an inducer of ROS, was administered to MM6 cells in serum-containing media for twenty-four hours. The cells were harvested, RNA isolated, reverse transcribed to cDNA and analyzed via Real-Time PCR. Real-Time PCR showed near control levels of PAF acetylhydrolase mRNA for the initial twelve hours; however, at twenty-four hours TBHP exposure produced a 2.5-fold increase in PAF. (See Figure 22).

3.12 *Morphological Changes in MM6 cells in Response to Singly and Dually Administered Proinflammatory Mediators*

While inducing inflammatory responses in MM6 cells, various morphological changes, specifically cell clumping, were observed during the incubation times. These morphological changes may have physiological relevance. Since infiltration from the vasculature to the injured tissues is a necessary action to mediate an inflammatory response, expression of various adhesion molecules may play a beneficial role in this regard. As shown in Figures 23A and 23B, the degree of cell clumping roughly parallels the fold induction of PAF-acetylhydrolase mRNA levels in response to these mediators. LPS-treated MM6 cells showed the greatest extent in cell clumping compared to TNF-α and PAF alone. Dual administration of proinflammatory mediators showed greater cell clumping compared to the singly administered proinflammatory mediators. LPS + TNF-α

appeared to demonstrate the greatest degree of cell clumping followed by PAF + LPS and TNF-α + PAF.

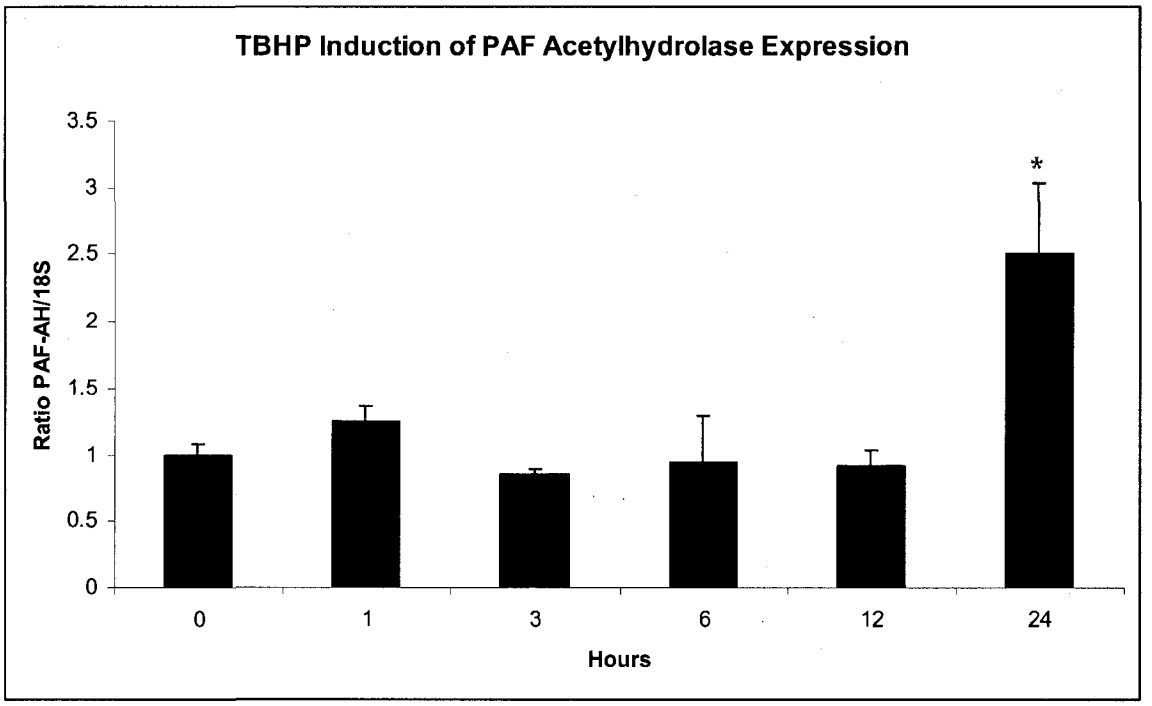
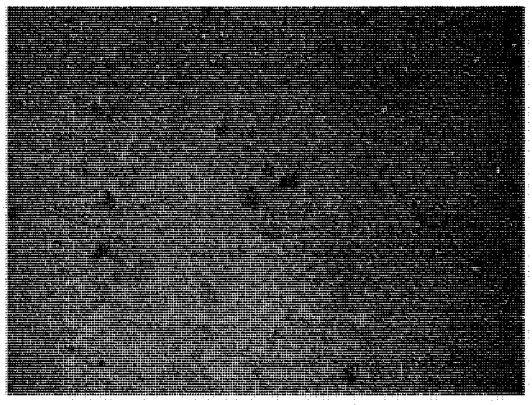
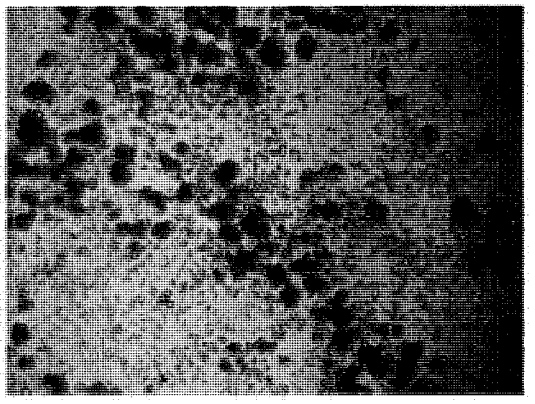


Figure 22: ROS induced Upregulation of PAF-AH mRNA. MM6 cells were cultured in serum-containing media in the presence of 100 μM *tert*- butyl hydroperoxide for twenty-four hours. Near control levels of PAF acetylhydrolase mRNA induction are observed for the first twelve with a 2.5 fold increase in PAF acetylhydrolase mRNA levels at twenty-four hours. The data reflect a value of two independent biological samples analyzed in triplicate; mean difference is significant at $p<0.05$. The single asterisk represents statistical significance from the control. Error bars represented are +/- standard deviation.

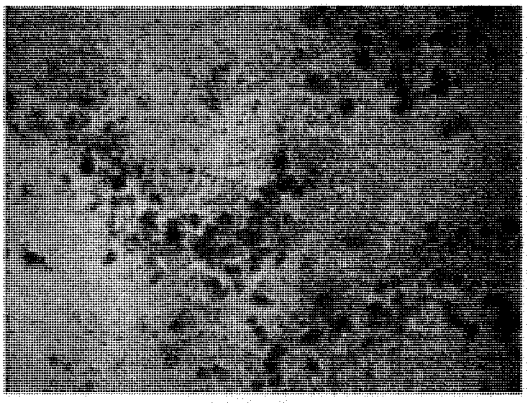
A.



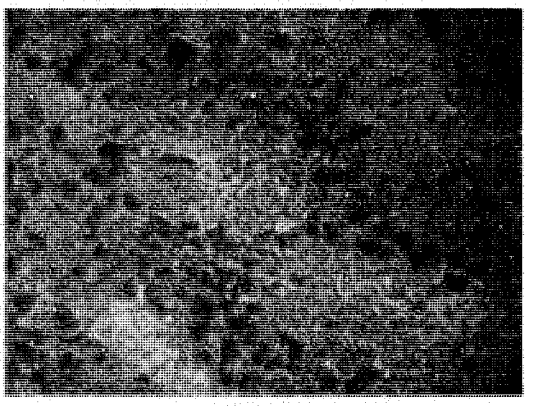
Control



LPS



PAF



TNF-α

B.

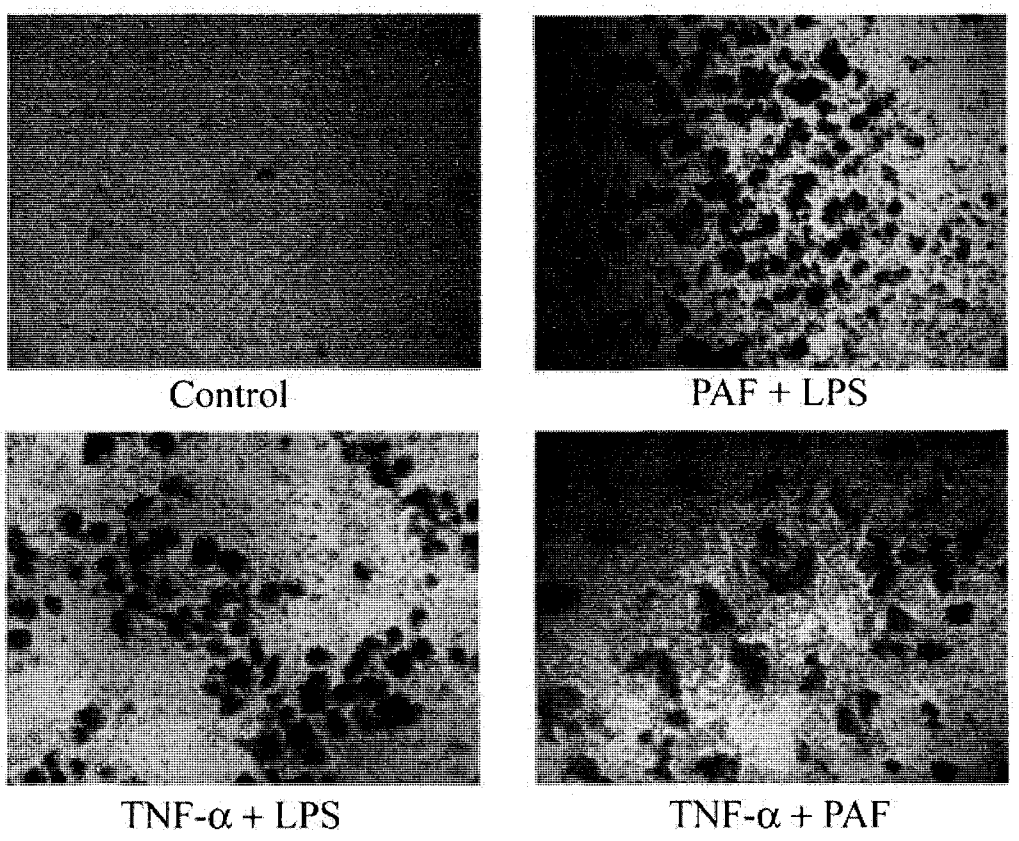


Figure 23: Morphological Changes in MM6 Cells After Administration of Various Proinflammatory Mediators. A. Effects of various proinflammatory mediators on the morphological changes in MM6 cells. B. Effects of dually administered proinflammatory mediators on MM6 cells. MM6 cells were incubated with the above listed proinflammatory mediators and combinations therein for twenty-four hours. LPS was administered at 200 ng/mL. PAF was administered at a concentration of 0.5μM. TNF-α was administered at a concentration of 10 ng/mL. The degree of cell clumping for the singly and dually administer mediators roughly parallels the extent of PAF acetylhydrolase mRNA upregulation. Total magnification shown is at 50X.

CHAPTER 4

DISCUSSION

4.1 Analysis of Results

The goal of this study was to investigate PAF acetylhydrolase expression in response to both TNF-α and PAF and elucidate any similarities or differences that this response may have with respect to the LPS-induced response. TNF-α and PAF are known inflammatory mediators; however, it was not known whether these mediators would be able to affect PAF acetylhydrolase expression. In order to investigate this possibility, dose response experiments were performed. With increasing concentrations of LPS, PAF or TNF-α, MM6 cells were exposed to the various concentrations for twenty-four hours in serum-containing media and the level of PAF acetylhydrolase mRNA was analyzed through Real-Time PCR experiments. Dose response experiments indeed showed the ability for both TNF-α and PAF (like LPS) to upregulate PAF acetylhydrolase expression. (See Figures 7-9). LPS showed the greatest ability to induce PAF acetylhydrolase mRNA levels followed by TNF-α and PAF. Increasing concentrations of both LPS and TNF-α resulted in increasing levels of PAF acetylhydrolase mRNA. However, the PAF dose response did not appear dose dependent. For all concentrations of PAF administered, an approximately three-fold increase over PAF acetylhydrolase levels was observed. This observation is most likely the result of the presence of PAF acetylhydrolase in the fetal bovine serum (FBS) that rapidly inactivates PAF. Thus, only

a portion of PAF is available to bind the receptor and signal. The wide variation observed in PAF acetylhydrolase expression levels (2-20-fold), for all presented dose responses, induced by the various mediators, demonstrates that the PAF acetylhydrolase gene is highly regulated rather than "on or off." This observation hints that varying levels of PAF acetylhydrolase are induced contingent upon the degree of inflammation present in the host. Previous work by Howard *et al.* demonstrated upregulation of PAF acetylhydrolase in rats exposed to LPS (32). Furthermore, the increased expression was detected solely in Kupffer cells, the resident macrophages of the liver. We have successfully reproduced the *in vivo* macrophage response to LPS in our cultured MM6 cells. Since PAF and TNF-α are capable of inducing PAF acetylhydrolase expression in the MM6 cells, this likely represents a physiologically relevant response that would be seen *in vivo*.

Our data in MM6 cells are in good agreement with that by Howard *et al.* that demonstrated maximal induction of PAF acetylhydrolase mRNA and protein via *in vivo* LPS administration in the liver sinusoidal cells of rats at twenty-four hours (32) (42). Further, in our work, LPS, PAF and TNF-α were able to upregulate PAF acetylhydrolase mRNA expression beginning at around 12-hours, with both TNF-α and LPS-induced PAF acetylhydrolase mRNA levels peaking at twenty-four hours. (See Figures 10-13). However, the PAF induced response continued to increase at both forty-eight and seventy-two hours. This time frame for PAF acetylhydrolase induction agrees with what is seen *in vivo (42)*. The LPS-induced upregulation of PAF acetylhydrolase mRNA is mediated by interaction of LPS with LPS binding protein (LBP) present in the serum. LPS and LBP form a complex which delivers the LPS to CD14, a cell surface receptor.

The new LPS-CD14 complex then interacts with a member of the toll-like receptor super family called TLR-4. The activation of TLR-4 initiates signaling through the MAPK cascades as shown in Figure 5 (31). Experiments were preformed to determine if LBP is required for the LPS-induced expression of PAF acetylhydrolase. In the presence of 10% FBS, and hence LBP, a 10-20 fold increase in PAF acetylhydrolase expression was detected after the addition of LPS. Control cells not receiving LPS, demonstrated low constitutive levels of PAF acetylhydrolase mRNA as shown in Figure 10. In the absence of serum, PAF acetylhydrolase levels also increased with time. Surprisingly, this increase was detected in cells that did and did not receive LPS. (See Figure 11). This LPS-independent response was particularly robust at forty-eight and seventy-two hours where an over thirty and sixty-fold increase in PAF acetylhydrolase mRNA levels was detected respectively.

Because PAF acetylhydrolase mRNA expression was induced under the serum-free conditions, regardless of LPS administration, we were unable to equivocally determine if LBP is required for LPS signaling. However, no statistically significant differences were detected in the magnitude of induction between LPS-treated and control cells. This either represents the fact that LPS requires the presence of LBP for signaling or that the LPS-independent induction is so large that it is masking any solely LPS-mediated effects.

MM6 cells cultured under serum-free conditions begin to deteriorate in morphological appearance and viability beginning around twenty-four hours (Data not shown). By seventy-two hours, over 50% of the cell population is non-viable. Clearly, environmental stress induced by cell starvation for prolonged periods, dramatically

induced PAF acetylhydrolase mRNA expression. During times of environmental stress, ROS levels can increase dramatically. Therefore, it is possible that the large extent of PAF acetylhydrolase induction in serum-free conditions at forty-eight and seventy-two hours may be the result of generation of ROS. Kulisz *et al.* have shown that ROS increase the amount of p-p38α (43). Thus, an induction in ROS may be responsible for the large increase in PAF acetylhydrolase mRNA levels observed both at the forty-eight and seventy-two hour time points in serum-free media. To a slight extent, these ROS may also generate an uncontrolled production of PAF-like phospholipids that can also upregulate PAF acetylhydrolase mRNA levels.

To investigate if ROS are capable of inducing the PAF acetylhydrolase message, we administered a ROS inducer, *tert*-butyl hydroperoxide (TBHP) at 100 μM. TBHB administration resulted in a 2.5-fold increase in PAF acetylhydrolase expression at twenty-four hours This result clearly demonstrated that ROS can indeed induce expression of PAF acetylhydrolase. However, additional experiments are required to assay for induction at the forty-eight and seventy-two hour time points. Further, the 2.5-fold induction upon 100 μM TBHP did not recapitulate the thirty and sixty-fold induction observed at the forty-eight and seventy-hour time points for cultured MM6 cells in serum-free media. This may be due to insufficient production of ROS. Dose response experiments with TBHP may help determine the effective concentrations to recapitulate the serum-free RPMI result. Further, LPS is able to induce the formation of superoxide radicals in alveolar macrophages and other phagocytes as part of the immune system mechanism of killing pathogens (44). The fact that ROS are able to induce the expression

of PAF acetylhydrolase makes logical sense to hydrolyze any oxidized phospholipids that can be formed.

To elucidate any similarities in signaling between LPS, TNF-α, and PAF, the proinflammatory mediators were administered in various combinations. Administration of TNF-α and LPS together stimulated PAF acetylhydrolase expression far greater than TNF-α or LPS alone. This observed effect was greater than what would be predicted from just an additive response. The synergism detected between TNF-α and LPS suggests TNF-α activates signaling pathways that influence the response to LPS (i.e. cross-talk). In contrast, the PAF acetylhydrolase induction in response to LPS plus PAF and TNF-α plus PAF was approximately additive. This observation suggests the activation of parallel but independent pathways that result in stimulated PAF acetylhydrolase expression. Because of these observations, and the importance of the MAPK pathways in inflammatory responses, the roles of the various portions of the MAPK pathways were investigated.

Examination of the MAPK pathways that were important in the LPS-mediated upregulation of PAF acetylhydrolase showed predominant signaling through the p38 portion of the MAPK pathway marked by 50% inhibition from SB203580. (Figure 15). For TNF-α- treated MM6 cells, inhibition of the p38MAPK pathway showed a 20% decrease in PAF acetylhydrolase induction that was found not to be statistically significant. This indicates only a minor role for the p38MAPK pathway in TNF-α signaling. Similar to the TNF-α response, the PAF-induced induction of PAF acetylhydrolase was also decreased by approximately 20% in the presence of SB203580; however, this decrease was not statistically significant (Figure 17). These data show a

significant difference in the signaling between LPS compared to TNF-α/PAF signaling. In these presented experiments, inhibition with SP600125, an inhibitor of the JNK portion of the pathway was not investigated—although the JNK inhibitor does not alter the LPS-induced expression (data not shown). To further elucidate the predominant signaling pathways involved in TNF-α and PAF signaling with the dual administration of inhibitors must be performed

Interestingly, inhibition of the ERK1/2 MAPK showed a 50% increase in PAF acetylhydrolase production over the induction elicited by the pro-inflammatory mediators alone (LPS, TNF-α, and PAF). (See Figures 16 and 17). Additionally, inhibition of ERK1/2 by itself resulted in a small but significant increase in PAF acetylhydrolase levels. This result may be explained by the fact that the MAPK pathways play diverse roles in signaling that regulate cell proliferation, differentiation, gene expression and cell survival (45). Specifically, the ERK1/2 portion of the MAPK pathway regulates cell differentiation and proliferation. Thus, inhibition of this pathway may impose a further stress on the cell which results in slight activation of p38MAPK and thus increased PAF acetylhydrolase levels.

Stafforini *et al.* have shown that LPS-induced PAF acetylhydrolase induction is solely mediated through the p38 portion of the MAPK pathway in THP1/CD14 cells (40). Our work in MM6 cells showed 50% inhibition of PAF acetylhydrolase mRNA induction upon exposure to SB203580—the p38MAPK inhibitor. Interestingly, Howard *et al.* demonstrated the PAF receptor antagonist, WEB2170, was able to downregulate the *in vivo* LPS-induced upregulation of PAF acetylhydrolase in rats by 50% (36). Thus, these separate observations prompted experiments to determine the effects of dually

administered SB203580 and WEB2170. The PAF receptor antagonist, WEB2170, was administered prior to stimulation with LPS or PAF. As expected, WEB2170 is an effective PAF receptor antagonist and able to inhibit the PAF-induced response. LPS-induced upregulation of PAF acetylhydrolase mRNA was inhibited by approximately 50% by the PAF receptor antagonist. Importantly, dual administration of SB203580 in conjunction with WEB2170 completely abolished PAF acetylhydrolase induction. Our data strongly support our contention that the remaining LPS-induced PAF acetylhydrolase mRNA levels present after SB203580 administration are the result of increased PAF production and signaling instigated by LPS administration. Our findings are in contrast with the work of Stafforini *et al.* in that the LPS-mediated upregulation of PAF acetylhydrolase is solely p38MAPK dependent. Although not examined, it would be interesting to determine if the effects of TNF-α are also partly mediated via PAF biosynthesis and PAF-receptor activation. TNF-α is capable of stimulating PAF synthesis in monocytes (46).

In order to gain a more thorough understanding of the inflammatory response in MM6 cells, ELISAs for IL-1β, TNF-α and p-p38α were performed. (See Figures 19-21). hese experiments were performed in serum-free media to assay for the secreted cytokines. Because of the absence of serum in the media and thus a lack of LBP, a 500 ng/mL LPS dose was administered to ensure signaling through TLR-4. Cell lysates and conditioned media exposed to LPS were harvested and used in various ELISAs. The ELISAs showed the ability of MM6 cells to mount an inflammatory response to LPS and secrete various cytokines such as TNF-α and IL-1β. Because the PAF receptor antagonists partly inhibited the LPS-induced upregulation of PAF acetylhydrolase, we

concluded that the proinflammatory mediator PAF is also being produced. Maximal induction of IL-1β or TNF-α protein occurred between six and twelve hours. In our experiments examining PAF acetylhydrolase mRNA, maximal levels were observed by twenty-four hours (Figures 10, 12 and 13). This delayed responsiveness in PAF acetylhydrolase induction most likely reflects the need to control and limit PAF and PAF-like mediator production at this later stage. In other words, initial PAF production is a beneficial response, but left unchecked, can lead to unwanted effects. The induction of PAF acetylhydrolase at twenty-four hours most likely serves to limit the damage that can occur during an inflammatory response and helps promote recovery of the host.

Statistically significant differences in p-p38α by one hour and three hours show that LPS is indeed signaling through the p38 portion of the MAPK pathway—further advancing the hypothesis that LPS signals at least in part through p38MAPK. However, at time points other than one and three hours, no statistical differences were seen. A repeat of this ELISA is necessary in order to clarify this result and gain a more thorough understanding.

There are numerous signaling pathways that are linked to the PAF receptor activation and this may explain the many PAF-induced biological responses of various cell types. PAF stimulates the expression of adhesion molecules and various cell surface receptors, when the PAF receptor is stimulated (47,48). In our experiments, administration of LPS, PAF and TNF-α and combinations thereof showed marked morphological changes in the MM6 cells, specifically cell clumping. (See Figures 23A and 23B). This observation may have physiological relevance. Monocytes express the ligand for P selectin, P selectin glycoprotein ligand-1, which aids in adhesive interactions

with both endothelial cells and platelets (47). Such expression is relevant since infiltration from the vasculature into the tissue is necessary in an inflammatory response to rid the infected area of invading microorganisms or necrotic tissue. Exactly how LPS, TNF-α and PAF are producing MM6 cell clumping remains to be demonstrated.

4.2 *Conclusions and further Work*

This research has shown the various signaling pathways that are important in inducing the upregulation of the plasma form of PAF acetylhydrolase. We have demonstrated that in addition to LPS, TNF-α and PAF are able to upregulate PAF acetylhydrolase expression with LPS being the most potent inducer followed by TNF-α and PAF. We have also demonstrated that 50% of the observed LPS-induced upregulation of PAF acetylhydrolase is the result of PAF signaling through its receptor. Though the LPS-induced response is partly p38MAPK dependent, the induction observed for both TNF-α and PAF is not. The TNF-α and PAF-induced induction of PAF acetylhydrolase levels may be the result of increased PAF synthesis upon administration of these mediators or signaling through a separate pathway(s). In these studies, the role of the JNK portion of the MAPK pathway was not investigated. Further work is necessary to elucidate the role that this pathway may have with TNF-α or PAF signaling.

Additionally, the 50% induction in PAF acetylhydrolase mRNA levels (relative to the inflammatory mediator administered) after administration of the ERK1/2 inhibitor requires further investigation. Performing ELISAs, specific for p-p38α protein, and assays for ROS will help answer the question whether cellular stresses, increased activation of p38MAPK, or both occur in the presence of PD98059. Work by Kulisz *et al.*

supports our hypothesis that cellular stresses, specifically ROS, can increase p38MAPK activation (43). Our work with TBHP has shown the ability of ROS to induce PAF acetylhydrolase levels. Whether this is p38MAPK-dependent in MM6 cells still requires further elucidation.

In conclusion, we have demonstrated that LPS, TNF-α, and PAF are able to induce PAF acetylhydrolase expression and have gained a greater understanding in regulation of PAF acetylhydrolase expression. The increases in PAF acetylhydrolase levels, in response to numerous mediators, likely represent the physiological requirement to curb the progression of the inflammatory response and maintain the viability of the host. Thus, the work presented here furthers our knowledge concerning the roles played by the plasma PAF acetylhydrolase in the inflammatory process.

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