THE PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR γ ANTAGONIST, GW9662, ALTERS UVB-INDUCED INFLAMMATORY RESPONSES, APOPTOSIS, AND DELAYED HYPERPROLIFERATION

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This is dedicated to my entire family. I wouldn't be who I am or where I am today, without all of you. Love, Kellie

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Abbreviations

UVB	Ultraviolet B
PPARγ	Peroxisome Proliferator-Activated Receptor γ
Cox-2	Cyclooxygenase-2

Introduction

Ultraviolet B light is a potent environmental carcinogen that elicits a characteristic response in skin following an acute exposure. This response includes: generation of reactive oxygen species; inflammation, marked by cyclooxygenase-2 (COX-2) induction; DNA damage resulting in an early phase of apoptosis; and the induction of a delayed hyperproliferative phase (Soter, 1990; de Gruijl, 1999) (Herrlich et al., 1994). Although mechanistic studies regarding inflammation, apoptosis, and proliferation have been widely investigated, regulation of these photobiological events in response to UVB is not well established. This investigation sought to explore the regulatory capabilities of the nuclear hormone receptor, Peroxisome Proliferator-Activated Receptor γ (PPARγ) in response to UVB irradiation.

The National Cancer Institute estimates more than 1,000,000 new cases of non-melanoma skin cancer (U.S. NIH, 2008b) and 62,480 new cases of melanoma (U.S. NIH, 2008a) will be diagnosed in 2008 with approximately 1,000 and 8,420 deaths reported from these cancers respectively. Chronic exposure to UVB can lead to insufficient repair of DNA damage resulting in clonal expansion of a mutated cell and ultimately malignancy. Although the inflammatory response, apoptosis, and delayed hyperproliferation are not usually carcinogenic processes individually, these UVB-induced events can act in concert to promote the initiation and development of cutaneous malignancies. It is because of this synergistic activity that it is crucial to identify the regulatory molecules and mechanisms associated with acute UVB exposure so that effective therapies that target these photobiological effects can be introduced prior to the

initiation and development of skin cancer. Therefore, in this study, we focused on the regulation of UVB-induced inflammation, apoptosis, and delayed hyperproliferation by the nuclear hormone receptor, Peroxisome Proliferator-Activated Receptor γ (PPARγ).

Establishing a knowledge base on the characteristics of UVB light, the biological results of acute UVB exposure, the relationship between these UVB-induced events and cancer initiation and promotion, and the possible therapeutic capacity of UVB light is warranted to better understand the primary focus of this study; the regulatory role of PPARγ in the epidermis after acute UVB exposure. Furthermore, an introduction on the characteristics of PPARγ, the known relationship of PPARγ to UVB light, and the characteristics of the known PPARγ antagonist, GW9662, as well as an explanation of the antagonist methodology used in this study, is necessary. All of the aforementioned information will be presented in the subsequent introduction.

The electromagnetic spectrum is a complete range of electromagnetic radiation that is measured by wavelengths. Ultraviolet light consists of wavelengths that are longer than X-rays on the electromagnetic spectrum, but shorter than visible light. The natural source of ultraviolet rays, the sun, emits electromagnetic radiation that can be divided into three categories of Ultraviolet light: Ultraviolet A (UVA), Ultraviolet B (UVB), and Ultraviolet C (UVC). UVA consists of wavelengths that fall between 400-320 nanometers (nm). UVB light consists of wavelengths that fall between 320-280 nm, and UVC consists of wavelengths between 280-100 nm. Although the most abundant form of UV irradiation to human exposure is UVA, UVB light is the most relevant source of ultraviolet light to the skin and can be both beneficial and dangerous depending upon the amount of exposure. Of particular interest to this study are the biological effects

following an acute exposure to UVB irradiation. More specifically to this study, the acute exposure resulted in a 1500 J/m^2 dosage of UVB with a one-time exposure to the radiation. Previous studies have shown unique biological consequences following acute UVB irradiation. These studies have indicated that UVB light results in biological effects in the epidermis including inflammatory responses, apoptosis, and delayed hyperproliferation and these photobiological events will be discussed in further detail.

The initial inflammatory response results in physiological changes that occur on both the macro and micro scale. The trigger of the basic inflammatory response includes invasion of a foreign object into the body such as bacteria. Depending on the invasion site, the immune response will vary as to which cell will be produced. Phagocytes will be released immediately upon invasion of the foreign object. Once this occurs, the appropriate signaling cascade is started. These signaling cascades begin with cytokine release such as interleukins, tumor necrosis factor, gamma interferon, transforming growth factor, lymphotoxin, prostaglandins, or histamine. These cytokines are produced by a host of inflammatory cells such as T-cells, macrophages, NK cells, or mast cells. The activation of these cytokines will result in certain physiological events. These events include induction of proliferation and differentiation of other inflammatory cells, induction of antibodies, and an induction of body temperature increase. On the larger physical scale, the release of cytokines will produce the classic inflammatory response which is characterized by swelling, redness, and pain in the infected area. This larger effect is a caused by the dilation of blood vessels in the infected area to increase blood circulation, which allows more of the inflammatory cells to reach the infected site at a faster rate.

When the skin, the body's largest organ, is invaded by a foreign object or is assaulted by an environmental element such as UVB, the inflammatory response occurs as described above. Upon UVB exposure, histamine is released and chemical mediators known as prostaglandins are produced. The histamine, produced by mast cells in the dermis, will increase vascular dilation and permeability to allow increased inflammatory cell infiltrate. Ultimately this signaling cascade is responsible for the acute inflammatory responses such as edema, redness, and pain in the epidermis that (Soter, 1990). Although histamine production is one inflammatory response to UVB exposure, UVB light creates reactive oxygen species, which in turn are the intermediates for prostaglandin formation, another cytokine that is responsible for an inflammatory response in the skin. Specifically, UVB creates oxidized phospholipids in the cell membrane. One such oxidized phospholipid produced is an oxidized arachidonic acid metabolite that is known to be an endogenous activator of PPARγ, 15-Deoxy-$\Delta^{12,14}$-Prostaglandin J_2 (Forman et al., 1995). This pathway could be related to the ability of PPARγ to regulate the inflammatory response post-UVB irradiation, due to the fact that PPARγ is known to regulate the inflammatory gene expression in other cells such as monocytes, macrophages, and T-cells (Jiang et al., 1998; Yang et al., 2000; von Knethen A., 2003). It is known that arachidonic acid is the precursor for production of prostaglandins. Specifically, arachidonic acid is metabolized to the important inflammatory mediator, Prostaglandin E_2 (PGE_2) (Samuelsson et al., 1975) and it was shown that induction of PGE_2 production occurred as a result of UVB irradiation (Davies et al., 1984). It is also known that PGE_2 induces vascular dilation, increased vascular permeability and an edema response (Nakanishi et al., 1995). However, an enzymatic

reaction must take place for the oxidized arachidonic acid to produce PGE_2. Cyclooxygenase-2 (COX-2) is the enzyme that catalyzes the reaction of arachidonic acid to PGE2 and has been shown to occur preferentially (Brock et al., 1999). More importantly, it has been shown that increased PGE_2 production is coupled with a marked increase in Cox-2 production upon UVB irradiation (Buckman et al., 1998). Hence, Cox-2 serves as excellent biomarker to observe inflammatory responses after UVB irradiation and is used as such in this study. Of particular importance to the current investigation is the previous study which indicates PPARγ directly or indirectly regulates Cox-2 expression (Zhang et al., 2005a). Therefore, PPARγ may be a regulatory molecule of the UVB-induced inflammatory response in the skin; an aspect for clarification in the current study. Although UVB-induced inflammation is a prominent photobiological effect, other molecular events take place in response to UVB light, such as apoptosis and proliferation.

Apoptosis, or programmed cell death can be described as an intended metabolic process that occurs as a response to cellular damage. Apoptosis is a process that was initially observed in the mid 1800's by Carl Vogt, however the topic was not further investigated until 1965 when researchers at The University of Queensland in Brisbane used electron microscopy to observe this process (Kerr, 1965). Not long after the published report in 1965, the principle researcher, John Foxton Ross Kerr, from The University of Queensland, joined with researchers from the University of Aberdeen, where they continued to investigate the process of what they called "programmed cell necrosis". In the article in 1972, "Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics", found in the British Journal of Cancer, the term apoptosis was coined (Kerr, 1972). From the publication of the articles in 1965 and

1972, this biological concept has been an active area of study and it has been found that improper regulation of the process has implications in ischemic damage and cancer initiation and promotion.

More specifically, the process of apoptosis begins with a traumatic event to the cell that begins a signaling cascade to initiate programmed cell death. The events that lead to apoptosis can include viral infection, ionizing radiation, chemotoxic agents, irregular expression of apoptotic control mechanisms, and conditions of extreme cellular stress. Furthermore, if the DNA repair control mechanisms cannot be put into action fast enough, the cell will immediately begin the process of programmed cell death as a defense mechanism.

It has been demonstrated that UVB irradiation is a source of cellular toxicity. In particular, UVB irradiation causes cellular damage through mutagenic changes in the in the DNA such as cyclobutane pyrmidine dimer (CPD) formation and through oxidative stress through the production of reactive oxygen species (Sinha, 2002; Sinha RP, 2002). UVB light is a critical source of genotoxicity and reactive oxygen species that can cause irreparable damage in keratinocytes (de Gruijl, 1999). This damage leads to apoptotic keratinocytes, otherwise known as sunburn cells (Ziegler et al., 1994). Although there are many different signaling cascades that initiate the apoptotic response, depending upon the source of cellular stress or toxicity, the progression of apoptosis is the same. For example, the cellular events following a traumatic incident to the cell, including UVB irradiation, involve shrinkage of the cell, membrane blebbing, chromosomal condensation, and DNA fragmentation (Kulms and Schwarz, 2000).

The regulatory relationship between the activation of PPARγ and the process of apoptosis is not well established in keratinocytes. However, there have been studies in other cell types that indicate activation of PPARγ inhibits apoptosis. For example, a study involving activation of PPARγ with the thiazolidedione agonist, rosiglitazone, inhibited apoptosis in mesangial cells (Efrati et al., 2007). Root ganglion neurons have also been treated with rosiglitazone to activate PPARγ, which has shown to increase the anti-apoptotic protein Bcl-2 (Fuenzalida et al., 2007). However, studies in several cancerous cell lines have demonstrated that activation of PPARγ induces apoptosis (Vignati et al., 2004; Bonofiglio et al., 2006; Chen et al., 2006). Although the role of PPARγ in epidermal apoptosis has not been elucidated, there has been some preliminary evidence showing an indirect regulatory relationship may exist. Our previous studies have shown that blocking PPARγ, upon UVB irradiation, is associated with a significant decrease in Cox-2 production indicating PPARγ regulates Cox-2 production (Zhang et al., 2005a). Furthermore, recent studies have shown that Cox-2 inhibitors induce apoptosis (Basu et al., 2004; Fan et al., 2006; Liu et al., 2008). These studies are particularly interesting given that our studies indicate a regulatory role of PPARγ on Cox-2 production. Hence, PPARγ may be directly or indirectly involved in the regulation of apoptosis.

Although the complex interplay between the many apoptotic pathways remains the primary focus of many investigations, acute UVB exposure is also responsible for an increased proliferative phase observed and is observed in the epidermis 72-96 hours after UVB irradiation (Tripp et al., 2003b).

An increased proliferative phase is proceeded by a rapid increase in the number of cells entering the cell cycle. The cell cycle consists of five phases which include: G_0, G_1, S, G_2, and M phases. Cells in the G_0 phase are in considered to be in the quiescent phase or non-dividing stage. Cells that are in the G_1 and G_2 phases are not actually undergoing cellular division, however, these phases are important for biosynthesis of important enzymes and structures for the S phase and M phase of the cell cycle. The S phase is the cell cycle phase in which DNA replication occurs, whereas the M phase consists of nuclear and cytoplasmic division of the proliferating cell into two daughter cells. Cells that continually need to be renewed, such as keratinocytes, will undergo the cell cycle throughout the lifetime of the organism. In order for this to occur, there must be important regulatory mechanisms associated with the cell cycle.

Regulatory molecules associated with the cell cycle ensure that no uncontrolled cellular growth occurs as well as ensures any damage the cell incurs will be repaired at the appropriate cell cycle checkpoint or will be sent to undergo apoptosis. The regulatory molecules that do this are known as cyclins and cyclin dependent kinases (CDKs). Cyclins and CDKs work in heterodimer formation through a phosphorylation event by the CDK to enter the cell into the appropriate cell cycle phase. There are many cyclin-CDK heterodimers which interact to promote the production of target proteins. These target proteins will assist in the cell cycle phase that the cyclin-CDK heterodimer regulates. For example, Cyclin D will heterodimerize through phosphorylation with CDK4. This complex will then promote activation of genes that will produce proteins that are necessary for DNA synthesis in the S phase of the cell cycle. Although, this

example illustrates the induction of the cell into the S phase of the cell cycle, there are other molecules which are involved in cell cycle checkpoints and inhibition.

Cell cycle checkpoints prevent the cell from undergoing cell cycle phase changes, so that the cell may repair damaged DNA and to confirm the appropriate cell cycle operations are taking place. Once the cell carries out these checkpoints, the cell will proceed to the next cell cycle phase. The cell also possesses cell cycle inhibitors which prevent the cell from entering the cell cycle. These genes, in essence, prevent the cell from tumor formation and are known as tumor suppressor genes. In regards to cellular proliferation, it is extremely important that all regulatory mechanisms, whether they induce, check or inhibit the cell cycle, function properly. If the balance between these regulatory processes is askew, the possibility for tumor formation increases dramatically.

In this study, as found in previous studies, UVB irradiation induces gene expression of growth factors that regulate cellular proliferation (Herrlich et al., 1994). For example, UVB activates mitogenic signals such as mitogen activated protein kinase (MAPK) through signal transduction cascades. Additionally, epidermal growth factor receptor (EGFR) activation is an elicited UVB response (El-Abaseri et al., 2006). It has also been shown that UVB irradiation results in prymidine dimer formation that may lead to mutations occurring in the genes that regulate cellular proliferation (de Gruijl et al., 2001). Furthermore, it has been shown that if UVB irradiation mutates a cell at the time of proliferation and the damaged DNA is not repaired, this mutation may be passed on to the daughter cells resulting in unregulated cellular proliferation and possible subsequent tumor formation (de Gruijl, 1995). This increase in cellular proliferation due to UVB irradiation disrupts the balance of the cell cycle progression and plays an important role

in tumor promotion and development in epidermal carcinomas. More importantly, studies have shown that activation of PPARγ through agonistic activity mimics low dose UVB therapy in patients with psoriasis resulting in improvement of psoriatic lesions. This finding agrees with other studies that PPARγ may have antiproliferative characteristics. These results would make the hormone receptor a useful target in regards to cancer development and promotion events.

UVB has been shown to have therapeutic capabilities with cutaneous diseases such as psoriasis; possibly due to its immunosuppressive activity and its ability to inhibit proliferation and induce apoptosis at low doses (Piskin et al., 2004). Importantly, our previous studies in an epidermoid carcinoma cell line (KB) and in SZ95 sebocytes have indicated that UVB exposure activates the nuclear hormone receptor, PPARγ (Zhang et al., 2005b; Zhang et al., 2006b). This occurs via the ability of UVB and other oxidative stressors to produce oxidized glyercophosphocholines that act as potent agonists to PPARγ. Moreover, inhibition of PPARγ was shown to block UVB-induced COX-2 expression and Prostaglandin E_2 production, which is significant because COX-2 induction is thought to play a role in UVB-induced inflammation, apoptosis, and delayed hyperplasia (Tripp et al., 2003b). Thus, we reasoned that PPARγ is an important mediator of the acute photobiological responses in skin.

PPARγ, like the related PPARα and PPARβ/δ, are members of the nuclear hormone receptor family. The three different PPAR proteins exhibit both distinct and overlapping functional roles, but exhibit differences in ligand specificity. The transcriptional activity of PPARs is dependent on their ability to heterodimerize with Retinoid X Receptors (RXR), which in turn bind to Peroxisome Proliferator Response

Elements (PPRE) in the promoter region of target genes (Gearing et al., 1993). Ligand binding acts to regulate the ability of these heterodimers to bind to coinhibitor or coactivator proteins that regulate transcriptional activity (Kuenzli and Saurat, 2003).

PPARγ is best known for its ability to regulate lipid and glucose homeostasis (Willson et al., 2001). However, more recent studies indicate a potential role for PPARγ in diverse pathologic processes, including atherosclerosis, inflammatory diseases such as colitis and psoriasis, and neoplastic development (Su et al., 1999; Kuenzli and Saurat, 2004; Li et al., 2004; Wang et al., 2006). These recent studies have indicated that PPARγ activation downregulates proinflammatory gene expression in patients with atherosclerosis, decreases the inflammatory response in patients with colitis and decreases proliferation in patients with psoriasis. The recent study by Wang et al. also demonstrated, in solid cancers and leukemia, that PPARγ activation decreases neoplastic development through a decrease in cellular proliferation coupled with an induction of apoptosis, an increase in terminal differentiation, and an inhibition of angiogenesis.

There is emerging evidence that PPARγ plays an important role in cutaneous biology through PPARγ agonists by inducing anti-inflammatory, pro-apoptotic, and anti-proliferative effects in epidermis and keratinocytes (Kuenzli and Saurat, 2003). But there is limited evidence demonstrating the biological outcome of these effects when PPARγ is activated through agonists produce by UVB irradiation. Because of the lack of information, the focus of this investigation was to clarify the role of PPARγ in regulating the inflammatory response, apoptosis and delayed hyperproliferation upon UVB irradiation. In order to proceed with this investigation, however, there was one issue that was of concern to this study and has plagued many studies of PPARγ function.

The concern that plagues many PPARγ investigations is that many PPARγ agonists exhibit PPARγ-independent functions (Galli et al., 2004; He et al., 2006). However, the ability to differentiate between PPARγ-dependent and independent effects has been markedly improved with the development of the potent, specific, and irreversible PPARγ inhibitor, 2-chloro-5-nitrobenzanilide (GW9662) (Leesnitzer et al., 2002). GW9662 was discovered through a competition binding assay to covalently bind to Cys285 of the PPARγ ligand binding domain and results in specific inhibition of PPARγ, but not PPARα or PPARβ/δ, transcriptional activity (Leesnitzer et al., 2002). In addition, GW9662 has been shown to inhibit PPARγ in many cell-based assays and animal models and is widely used to verify the specificity of PPARγ-ligand activity (Chen et al., 2004; Sivarajah et al., 2005; Nakano et al., 2006). Furthermore, our previous studies using two different cell lines, have shown that the use of both GW9662 and the dominant negative PPARγ (ΔPPARγ) construct produce equivalent results (Zhang et al., 2005a; Zhang et al., 2006a).

Given that we have shown that PPARγ is a target of UVB irradiation and thus is a potential regulator of the photoresponse, we sought to determine the role of PPARγ in acute UVB-induced epidermal inflammation, apoptosis, and proliferation. We examined this using the PPARγ antagonist, GW9662, in the epidermis of SKH1 mice for three reasons. First, we have shown that UVB-induces PPARγ ligand production, therefore the use of exogenous ligands would likely result in a minimal response. Second, previous studies have indicated a potential for PPARγ-independent effects with the use of agonists. Third, our previous studies with the ΔPPARγ approach have validated the use of GW9662 in blocking PPARγ-dependent effects following acute UVB irradiation *in vitro*.

In the current studies, we show that topical application of GW9662 inhibits UVB-induced inflammation and COX-2 expression, and augments both UVB-induced apoptosis and proliferation in hairless, albino mouse epidermis. These data provide clear evidence that UVB-induced PPARγ activation is an important regulator of the acute UVB-induced cellular response.

Materials and Methods

Materials

GW9662 (2-Chloro-5-nitrobenzanilide), (Cayman Chemical, Ann Arbor, MI). Murine COX-2 and GAPDH RT_2 primers, first-strand synthesis and SYBR Green qRT-PCR reagents were purchased from SuperArray Bioscience Corp (Frederick, MD). Unless otherwise indicated, all reagents and antibodies for immunohistochemistry were obtained from Biocare Medical (Concord, CA).

Animals

Hairless, albino, female SKH1 mice were purchased from Charles Rivers Laboratories (SKH1-Hr_{hr}; Wilmington, MA). The mice were housed in standard microisolator cages under a simulated 12 hour day/night cycle and were fed and watered *ad libitum*. All mouse studies were approved by the Indiana University-Purdue University Indianapolis Animal Care and Use Committee. SKH1 mice are immunocompetent mice that have a defect in hair follicle cycling leading to loss of hair production following the first hair cycle, but otherwise exhibit normal epidermal structure (Panteleyev et al., 1998). This mouse model is routinely used as the preferred mouse strain for acute and chronic photobiology and photocarcinogenesis experiments (Tripp et al., 2003a).

UV irradiation study

The dorsal skin of SKH1 hairless mice was pre-treated with 100 µl of GW9662 (0.01 mM, 0.1 mM, 1.0 mM) solubilized in 70% propylene glycol:30% ethanol for 1.5 hours

prior to UVB irradiation. Following pretreatment, the dorsal skin was wiped gently 3 times with cotton swabs soaked in 100% ethanol to remove non-absorbed reagents. The mice were then immobilized by intraperitoneal (ip) injection with 250 μl of a 1:10 dilution of Ketamine 1000 mg/Xylazine 142 mg in normal, sterile NaCl. Once immobilized, the mice were irradiated with 1500 J/m2 of UVB light using two Westinghouse FS40 sunlamps (National Biological Corp., Twinsberg, OH) (Fischer, 1999). A Kodacel filter (Kodak, Rochester, NY) was used to block UVC irradiation. UVB fluence was measured at a fixed distance equivalent to the distance between the UV lamps and the mouse dorsum prior to each experiment using an IL1700 radiometer equipped with a SED240 UVB detector (International Light, Newburyport, MA). The time of exposure for irradiation was calculated from the intensity (fluence) of the lamp and the required UVB dose. Immediately following irradiation, 50 μl of the appropriate GW9662 dose was reapplied to the dorsal skin of the mouse. Prior to excision, the dorsal midline was marked with an impermeable marker along the spinal axis. At each experimental time point, the dorsal skin was then excised and bisected along the midline and a portion was snap frozen for skin thickness measurements (see below). The remaining half was divided into two sections. The first section was placed in RNAlater (Qiagen, Valencia, CA), the second formalin-fixed in 10% neutral buffered formalin for paraffin-embedding and histologic assessment and immunohistochemical analysis. It should be noted that initial studies also included a dose of 10 mM GW9662. However, studies using 10 mM GW9662 were discontinued when it was determined that this dose had a modest sunblock effect, possibly as a result of incomplete solubilization, as GW9662 absorbs primarily in the UVC range (λmax = 261 nm). Doses of GW9662 at or

below 1 mM had no significant sunblock effect (< 5%), as determined by measuring the ability of 100 μl of GW9662 to block UVB transmission when it was applied to a Kodacel filter (using a surface area equivalent to the application area used in the mouse studies) (not shown). This lack of sunblock effect was also seen by measuring the inability of GW9662 at doses of 1 mM and below to block UVB-induced cyclobutane dimer formation (30 minutes post UVB) (not shown).

RNA isolation and COX-2 quantitative RT-PCR

RNA isolation was performed using the RNeasy kit (Qiagen) according to the manufacturer's protocol. Following first-strand DNA synthesis, quantitative RT-PCR (qRT-PCR) was performed using primers specific to murine COX-2 and GAPDH using a Cepheid Smart Cycler® real-time PCR instrument (Fisher Scientific, Pittsburgh, PA). COX-2 qRT-PCR results were normalized to GAPDH using the ΔΔCt method (ABI, 1997).

Skin thickness determination

The thickness of the snap frozen dorsal skin sections bisected at the midline was measured with a digital caliper by five random measurements taken along the midline cut edge.

Immunohistochemistry for activated Caspase-3, Ki-67, and phospho-Histone H3 (Ser 10)(pHH3)

Mouse dorsal skin sections were formalin-fixed in 10% neutral buffered formalin for 3 hours and changed to 70% EtOH, trimmed and placed in cassettes for further processing and embedding. Standard deparaffinization and hydration were performed followed by heat-induced antigen retrieval using Borg Decloaking Solution (Activated caspase 3) or 10mM citrate buffer (Ki-67 and pHH3), in a standard kitchen 8 qt programmable pressure cooker for 15 minutes using the high setting. Sections were incubated with Peroxoblock (Zymed Laboratories, San Francisco, CA) for two minutes at room temperature. For activated caspase 3 IHC, the sections were then washed with TBS and primary antibody (1:100 dilution of rabbit anti-human cleaved caspase-3) was applied in TBS with 1% bovine serum-albumin and incubated for 2 hours at room temperature. For alkaline phosphatase conjugation, the Mach 3 kit probe and polymer was added to the sections for 15 minutes at room temperature. Vulcan Fast Red was used for substrate detection. Sections were then counterstained and mounted. For Ki-67, sections were washed with PBS followed by incubation with Ki-67 (SP6) (Lab Vision, Fremont, CA) for thirty minutes at room temperature at a 1:1000 dilution in 0.1% bovine serum albumin/PBS. For pHH3, sections were washed with PBS and incubated overnight at 4^{o}C with Phospho-Histone H3 (Ser10) (Cell Signaling Technology, Danvers, MA) at 1:100 dilution in 5% normal goat serum/PBS. In both cases, the sections were then washed and incubated with Super Picture HRP Polymer Conjugate Rabbit Primary (Zymed Laboratories) for ten minutes at room temperature. DAB+ (Dako Cytomation) was used for substrate detection. Sections were counterstained and mounted.

Quantification of positive cells by IHC and statistical analysis

Ten random fields were counted in all slides, at either 200 or 400x total magnification, in a blinded manner for each condition using a Nikon Eclipse E400 microscope (Nikon Corp., Melville, NY). Data was plotted using GraphPad Prism 4.0. and statistics performed using the student *t*-test using a statistical significance cutoff of $p < 0.05$.

Results

Effects of GW9662 on inflammation:

Both Cox-2 and subsequent PGE_2 production are important mediators of the UVB-induced erythema and inflammatory response. Thus, UVB-induced COX-2 induction serves as an appropriate marker for UVB-induced inflammatory responses. We have previously determined that GW9662 treatment and the expression of a dominant negative construct both inhibit UVB-induced COX-2 expression and/or PGE_2 production in two different human cell lines. We therefore examined whether GW9662 pretreatment would alter UVB-induced COX-2 expression in murine skin. Previous studies by others have shown that UVB-induced COX-2 expression peaks at approximately 24 hours in SKH1 mouse epidermis after a similar UVB dose (Tripp et al., 2003a). Moreover, COX-2 induction is sustained through the period of peak hyperproliferation at 72 hours (Tripp et al., 2003a). We therefore examined COX-2 mRNA expression by qRT-PCR at 24 hrs (Fig. 1A) & 72 hrs (Fig. 1B) post-UVB irradiation. At 24 hours, UVB irradiation induced a marked increase in Cox-2 production in the UVB control mice, as would be expected. Importantly, there was a significant dose-dependent decrease among all GW99662 treated mice (0.01 mM, 0.1 mM, and 1.0 mM). The most dramatic decrease in Cox-2 mRNA expression was seen at the 1.0 mM GW9662 dose, with UVB-induced COX-2 induction suppressed by 80%. At this dose (1 mM), GW9662 had no significant effect on COX-2 expression in non-irradiated controls at 24 hrs. At 72 hours post-UVB, Cox-2 expression was again seen to be induced upon UVB irradiation, but only the 1.0mM GW9662 treated mice showed a significant 77% decrease in the amount of Cox-2

mRNA expression versus the vehicle control UVB irradiated mice. The lowest dose tested, 0.01 mM GW9662, resulted in an insignificant increase in UVB-induced COX-2 mRNA expression levels. The intermediate dose resulted in an intermediate reduction in UVB-induced COX-2 expression, although the decrease did not reach statistical significance. In non-irradiated controls, 1 mM GW9662 also suppressed COX-2 expression at 72 hrs, although this decrease did not reach statistical significance.

In addition to COX-2 induction, UVB irradiation of mouse epidermis results in a characteristic early edema response (24 hrs) followed by inflammatory cell infiltration of the dermal compartment that peaks at 48-72 hours post UVB irradiation (Hammerberg et al., 1996). In Fig. 2, we examined UVB-inflammation using an assessment of skin thickness. Twenty-four hours after irradiation (Fig. 2A), UVB induced an expected increase in skin thickness compared with the vehicle control mice. However, there were no significant differences seen between the UVB and GW9662 treated mice. At 72 hours post UVB irradiation (Fig. 2B), UVB again induced an increase in inflammation compared with non-irradiated control mice. Importantly, there was a significant 71% increase in skin thickness among the 0.01mM GW9662 and 0.1mM GW9662 treated mice over that seen with the UVB-irradiated control mice. In contrast, pretreatment with 1.0 mM GW9662 prior to UVB irradiation essentially reduced the skin thickness to non-irradiated control levels. It should be noted that no significant change in skin thickness was noted in non-irradiated mice treated with the highest dose of GW9662 (1 mM), either at 24 or at 72 hrs (not shown).

Topical GW9662 induces a dose-dependent augmentation of UVB-induced Apoptosis

UVB damage triggers numerous, downstream events which can lead to apoptotic Keratinocytes:

The UVB-induced apoptotic response in the epidermis was observed by quantification of activated caspase-3 positive cells as seen in Fig. 3. Caspase-3 is a protease that cleaves a specific substrate which induces DNA fragmentation in the apoptosis pathway (Liu et al., 1997). Peak caspase-3 activity, post-UVB irradiation, takes place at 24 hours (Assefa et al., 2000) thus, activated caspase-3 cells were quantified at this time point. As expected, there was a significant induction of UVB-induced apoptosis versus the non-irradiated controls. There was also a dose-dependent increase in the apoptotic response seen in the GW9662 treated epidermis versus the UVB control (22%, 112%, and 173% for 0.01, 0.1, and 1 mM GW9662 respectively). This augmented apoptotic response reached statistical significance for both the 0.1 and 1 mM doses. To rule out any physical sunblock effect, we also examined whether systemic administration of GW9662 could also augment UVB-induced apoptosis. Importantly, we show that there was also a dose-dependent increase in apoptotic cells in mice treated by intraperitoneal injection of GW9662 versus UVB control (61% and 173% for 1 & 5 mg/kg respectively); this increase in apoptosis relative to UV control mice reached statistical significance for the 5 mg/kg dose. Assuming 80% body water, 100% tissue bioavailability, and a weight of 25 gm, this dose is equivalent to approximately 25 μM GW9662. The presence of activated caspase-3 activity in the sebaceous gland(Akasaka et al.) served as a positive internal control (Fig. 3C).

Topical GW9662 treatment augments UVB-induced delayed hyperproliferation as assessed by epidermal thickness (hyperplasia), Ki-67 immunolabeling and Phospho-Histone H3 (Ser10) immunolabeling:

UVB results in delayed hyperproliferation in the epidermis of SKH1 mice, which is observed 72-96 hours after a similar dose of UVB (Tripp et al., 2003a). Moreover, UVB-induced COX-2 induction is necessary for this hyperplastic response (Tripp et al., 2003a). However, PPARγ agonists have been shown to have antiproliferative effects in human and mouse epidermis (Ellis et al., 2000). Given that we show that GW9662 blocks UVB-induced promitogenic COX-2 expression, we were particularly interested in examining the affects of GW9662 on this delayed hyperplastic response. We first evaluated the ability of GW9662 to alter UVB-induced epidermal hyperplasia by assessing epidermal thickness (Fig. 4A). Interestingly, GW9662 resulted in augmentation of UVB-induced hyperplasia. However, this augmentation exhibited an inverse dose-response effect, with 0.01 mM GW9662 exhibiting the greatest effect (64% increase in epidermal thickness compared with UV control skin), compared with a non-significant 22% and 9.6% increase for 0.1 and 1 mM GW9662 treated skin, respectively. Treatment with 1 mM GW9662 in the absence of UVB-irradiation had no significant effect on epidermal thickness (not shown).

We next assessed hyperproliferation through two different markers, Ki-67 and phosphohistone H3 (ser10) at 72 hours post-UVB irradiation. Ki-67 is a nuclear protein that serves as a proliferation marker and is only expressed during late G_1, S, M, and G_2 phases (Gerdes et al., 1984). In Fig. 4B, quantification of Ki-67 positive cells at 72 hours post UVB indicated that there was a significant increase in UVB-induced proliferation in

GW9662 treated epidermis. As for epidermal thickness, this response exhibited an inverse dose-response curve: the greatest response was seen at the lowest dose tested, 0.01 mM GW9662 (93% increase in Ki-67 immunolabeling compared with UV controls), while the higher doses were less effective (47% and 45% for the 0.1 and 1.0 mm doses, respectively). Treatment of mouse skin with 1 mM GW9662 had no significant effect on Ki-67 expression in the absence of UVB-irradiation (not shown).

Fig. 5 shows histological staining of positive Ki-67 cells in the epidermis. In the positive control gut tissue (Fig. 5A), Ki-67 immunolabeling was restricted to the proliferative crypts within the mucosa. Similarly, in the UVB control epidermis (Fig. 5C), the increase in the Ki-67 immunoreactivity was seen primarily in the basal layer. In contrast, 0.01 mM GW9662 treated epidermis demonstrated marked Ki-67 immunopositivity in suprabasal layers of the epidermis, demonstrating expansion of the proliferative basal compartment within the epidermis relative to UV controls. This expansion of the proliferative compartment was also seen to a lesser degree in the UVB-irradiated epidermis treated with the higher doses of GW9662 (not shown).

We also examined proliferation using phospho-Histone H3 (PHH3) immunolabeling. Phosphorylation of serine 10 of Histone H3 tightly correlates with the chromosomal condensation that occurs with mitosis (Hendzel et al., 1997).
A similar 78% increase in UVB-induced proliferation was noted in skin treated with 0.01 mM GW9662 by counting PHH3 immunopositive cells in the basal layer compartment (vehicle control = 3 ± 0.82; UV control = 11 ± 1.56; UV + 0.01 mM GW9662 = 17.22 ± 2.1 cells / 200 x field: mean and SEM for 4-5 mice). There was also no significant effect on PHH3 expression in the 1 mM GW9662 dose without UVB irradiation (not shown).

Discussion

In previous studies, we have shown that PPARγ is a target for UVB-induced ligand production through the production of oxidized glycerophosphocholines (Zhang et al., 2005a; Zhang et al., 2006a). In this study, we provide evidence that UVB-induced PPARγ activation plays a significant role in the acute cutaneous response to UVB irradiation. This is important, as a previous study has shown that exogenous PPARγ ligand exposure has no significant effect on UVB-induced photocarcinogenesis (He et al., 2005). In our initial survey, at 24 and 72 hours post-UVB irradiation, Cox-2 mRNA levels were significantly decreased when PPARγ was blocked with GW9662, with the most effective dose at 1 mM. This agrees with our previous results in an epitheliod carcinoma cell line and in immortalized sebocytes (Zhang et al., 2005a; Zhang et al., 2006a). A similar dose-response curve was seen for the induction of apoptosis, suggesting a potential COX-2 dependent mechanism. However, skin thickness studies and proliferation data indicate that UVB-induced PPARγ activation exhibits a more complex role in inflammation and delayed hyperproliferative responses.

In Fig. 2, we show that UVB-induced edema responses at 24 hrs are largely unaffected by GW9662. This is somewhat surprising, since 1 mM GW9662 effectively blocks UVB-induced COX-2 expression at the same time-point. Given that COX-2 inhibition is known to suppress UVB-induced edema responses in mouse epidermis, it is interesting that PPARγ antagonism had no significant effect on this early inflammatory response. If anything, PPARγ antagonism may slightly increase the edema response, suggesting that PPARγ has a modest anti-inflammatory effect. At 72 hours, a biphasic

dose-response effect was observed: lower doses of PPARγ significantly increased epidermal skin thickness, while the highest dose tested significantly suppressed UVB-induced inflammation. The decreased delayed inflammatory response seen at the highest dose (1 mM) could possibly be attributed to loss of pro-inflammatory COX-2 signaling. However, the increased inflammatory response seen with lower doses of GW9662 are likely attributed to anti-inflammatory activity of PPARγ. The idea that PPARγ acts as an anti-inflammatory signal is supported by studies of PPARγ in inflammatory skin disease as well as inflammatory diseases in other tissues. Thus, within the epidermis, PPARγ appears to mediate complex effects on inflammation, possibly as a result of COX-2 independent and COX-2 dependent signaling.

In the second aspect of our overview of photobiological responses to UV in SKH-1 mouse epidermis, we studied the effect of GW9662 treatment on UVB-induced apoptosis and found a significant dose-dependent induction of apoptosis in the GW9662 treatment groups. These results suggest that PPARγ activation acts to suppress UVB-induced apoptosis. Given that COX-2 induction is thought to suppress UVB-induce apoptosis (Liu et al., 1998; Akunda et al., 2007), it is possible that PPARγ regulates UV induced apoptosis through COX-2 dependent signaling.

In our next studies, we examined the role of PPARγ in the characteristic delayed hyperproliferative response that is observed following an acute UVB exposure. Previous studies in psoriatic epidermis have indicated that exogenous PPARγ agonist exposure exerts an antiproliferative effect, normalizing the hyperplasia associated with this inflammatory skin disease (Malhotra, 2005). However, other studies have shown that UVB-induced COX-2 expression is crucial for UVB-induced delayed hyperproliferative

response (Rundhaug, 2007). Thus, it is interesting that GW9662 had an inverse dose-response affect on delayed hyperproliferation, suggesting that the effects of PPARγ activation on proliferation is complex, likely involving COX-2 dependent and independent activity. However, at lower doses, it is clear that GW9662 treatment acts primarily to augment UVB-induced hyperproliferation. This is particularly interesting, given that recent studies in epidermal-specific PPARγ knockout mice provide evidence that PPARγ acts to suppress tumor promotion in a chemical carcinogenesis mouse model (Indra et al., 2007).

In conclusion, our studies show that topical application of the PPARγ antagonist, GW9662, results in variable dose-dependent effects on UVB-induced COX-2 induction, inflammation, apoptosis, and proliferation. Given the important role that UVB-irradiation plays in skin cancer, as well as its therapeutic use for inflammatory skin disease, it is crucial to elucidate the major factors that regulate UVB-induced inflammation, apoptosis, and proliferation. As demonstrated in our study, PPARγ provides such a target. Our findings that GW9662 treatment exhibits complex actions on UVB-induced inflammation, apoptosis and particularly proliferation suggest that additional mechanistic studies on the pharmacological activities of this compound are needed to address potential safety concerns.

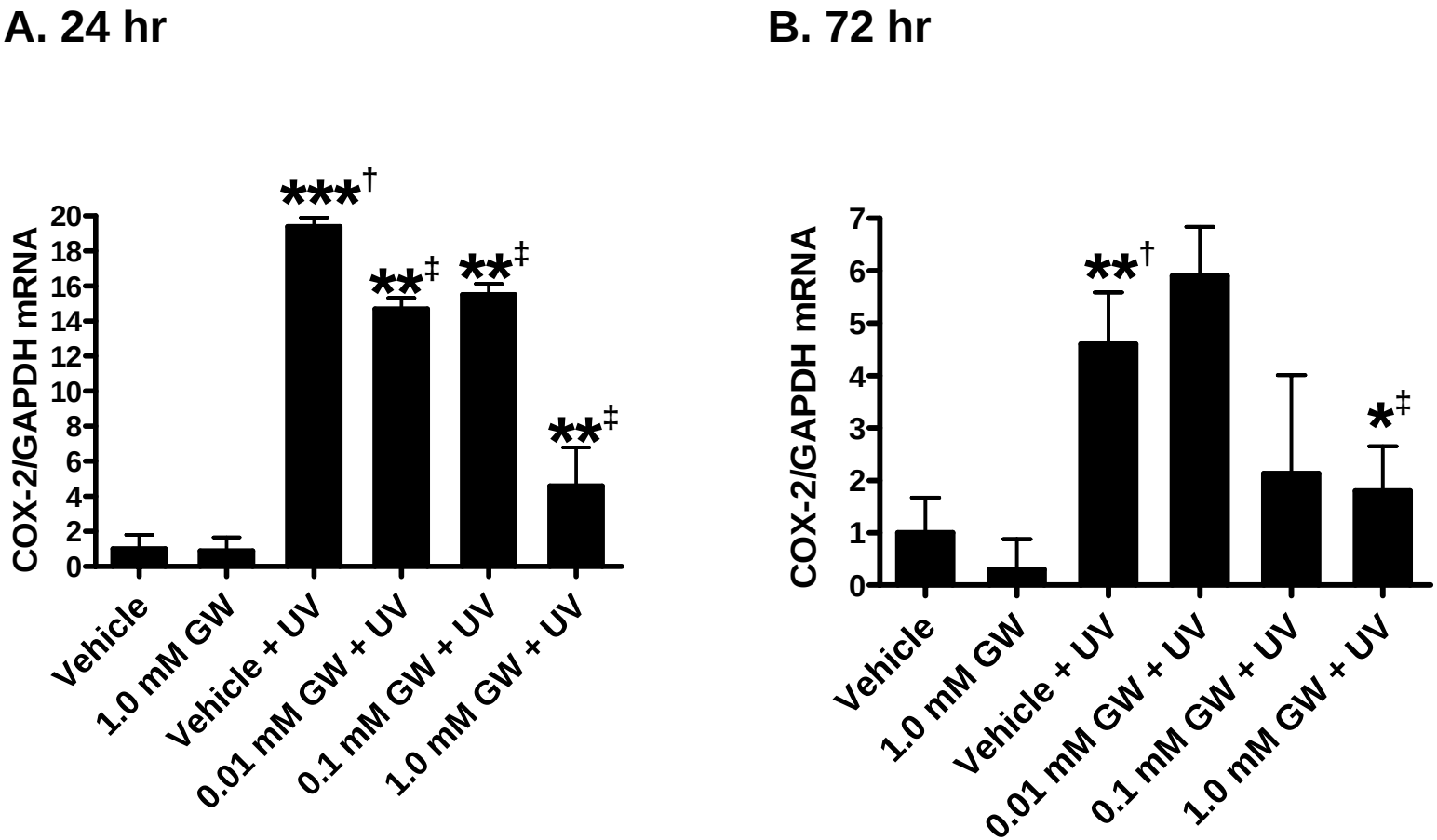

Fig. 1. Topical application of the PPARγ antagonist (GW9662) suppresses UVB-induced Cox-2 expression. A. SKH-1 mice were pretreated with 0.01mM, 0.1mM, 1.0mM GW9662 (GW) for 1.5 hours and irradiated with1500 J/m2 of UVB. A Kodacel filter was used to block UVC. Dorsal skin was excised and used for RNA isolation. Each sample for RT-PCR was run in duplicate. Results represent the mean and SEM of n = 3-5 mice. B. The same conditions as in section A apply to panel B. Results represent the mean and SEM of n=5-7 mice, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, † represents conditions compared to vehicle control, ‡ represents conditions compared to UVB control.

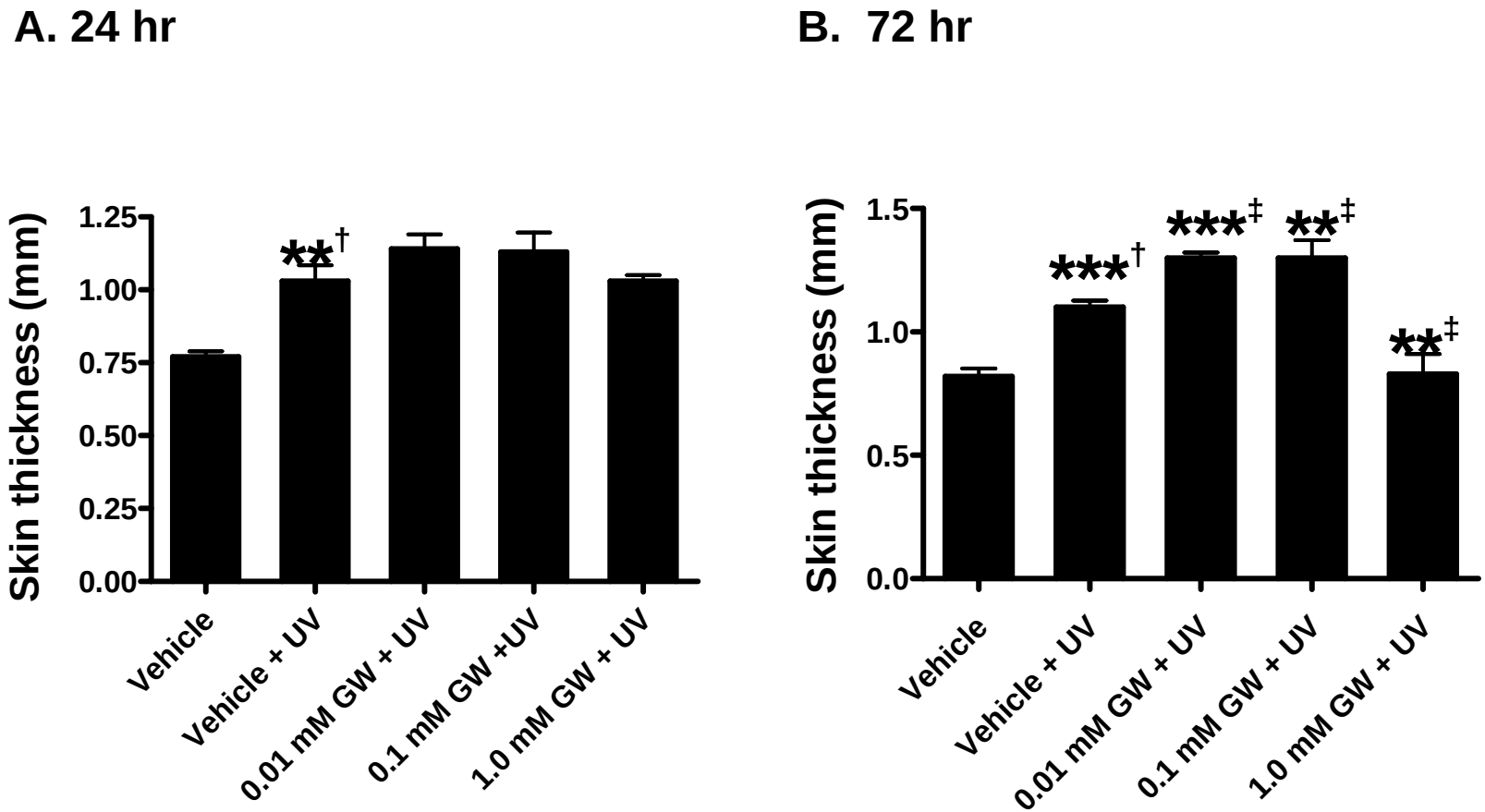

Fig. 2. Effects of topical GW9662 on UVB-induced skin thickness. A. After 1.5hr pretreatment with GW9662 (0.01mM, 0.1mM, 1.0mM), SKH-1 mice were irradiated with 1500J/m2 of UVB and a Kodacel filter was used to block UVC. The dorsal skin was excised and immediately snap frozen in Liquid N2. Five random measurements were taken along the midline section of the skin with a digital caliper. Results represent the mean and SEM of n = 6-10 mice, **p<0.01, † represents conditions compared to vehicle control. B. The same conditions and methods were used as in panel A. Results represent the mean and SEM of n = 3-7 mice, ** p <0.01; *** p<0.001, † represents conditions compared to vehicle control, ‡ represents conditions compared to UVB control.

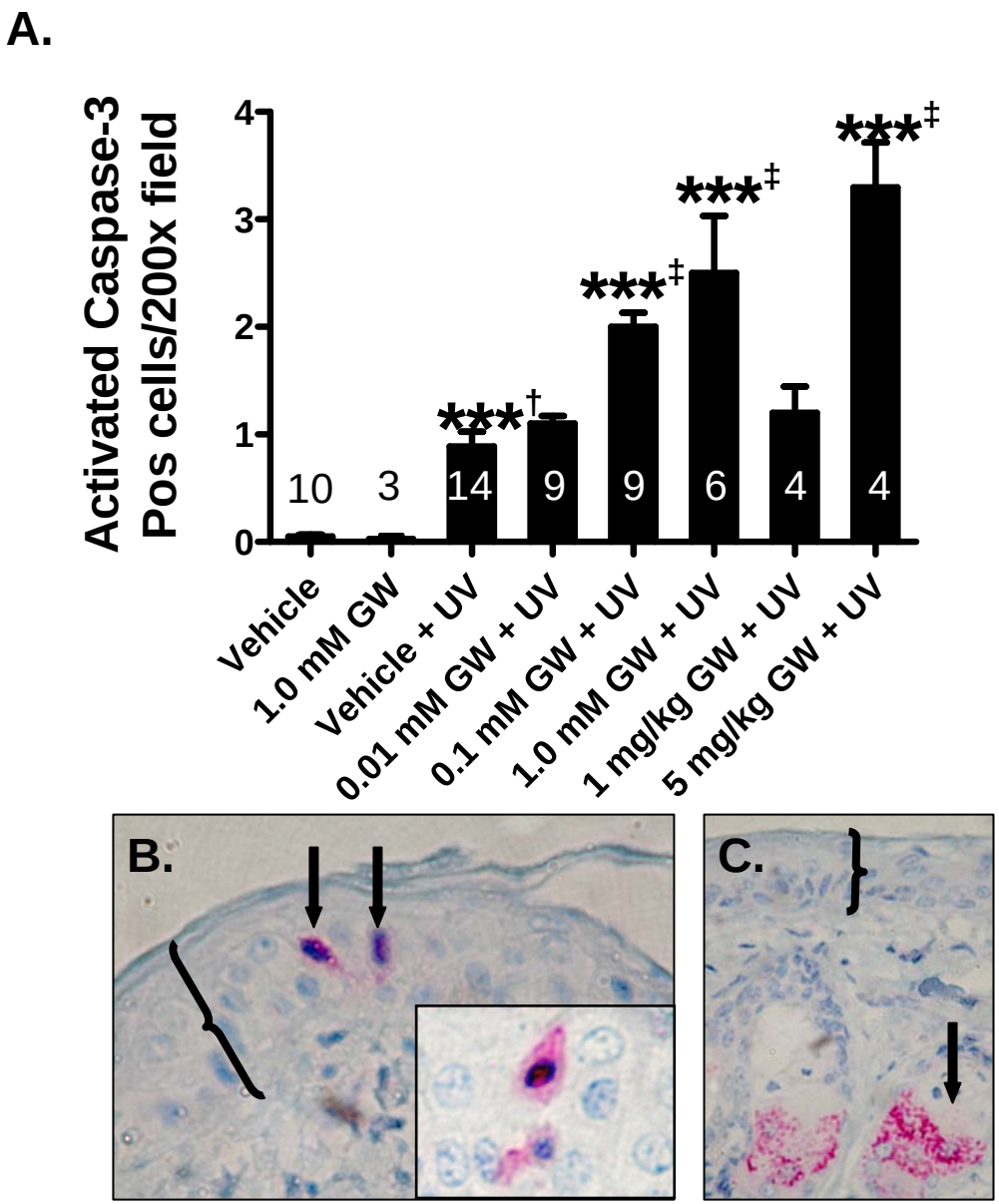

Fig. 3. The PPARγ antagonist, GW9662, augments UVB-induced apoptosis. A.SKH-1 mice were pretreated with GW9662 (0.01mM, 0.1mM, 1.0mM) for 1.5 hr and irradiated with 1500 J/m2. A Kodacel filter was used to block UVC. The dorsal skin was excised, fixed in 10% buffered formalin and paraffin-embedded. Results represent the mean and SEM of n = 3-14 mice for positive activated caspase-3 cells per 200x field and were counted in a blinded fashion, *** $p<0.001$, † represents conditions compared to vehicle control, ‡ represents conditions compared to UVB control. B. This represents a positive activated caspase-3 in a UVB irradiated SKH-1 mouse. The high power image of the positive cell is in the epidermis, note the pyknotic nucleus in the inset. C. The sebaceous glands in the skin were used as positive controls, note the positive pink stain in the sebaceous gland, shown by arrows, of a vehicle control mouse compared with the negative staining of the epidermis shown in brackets.

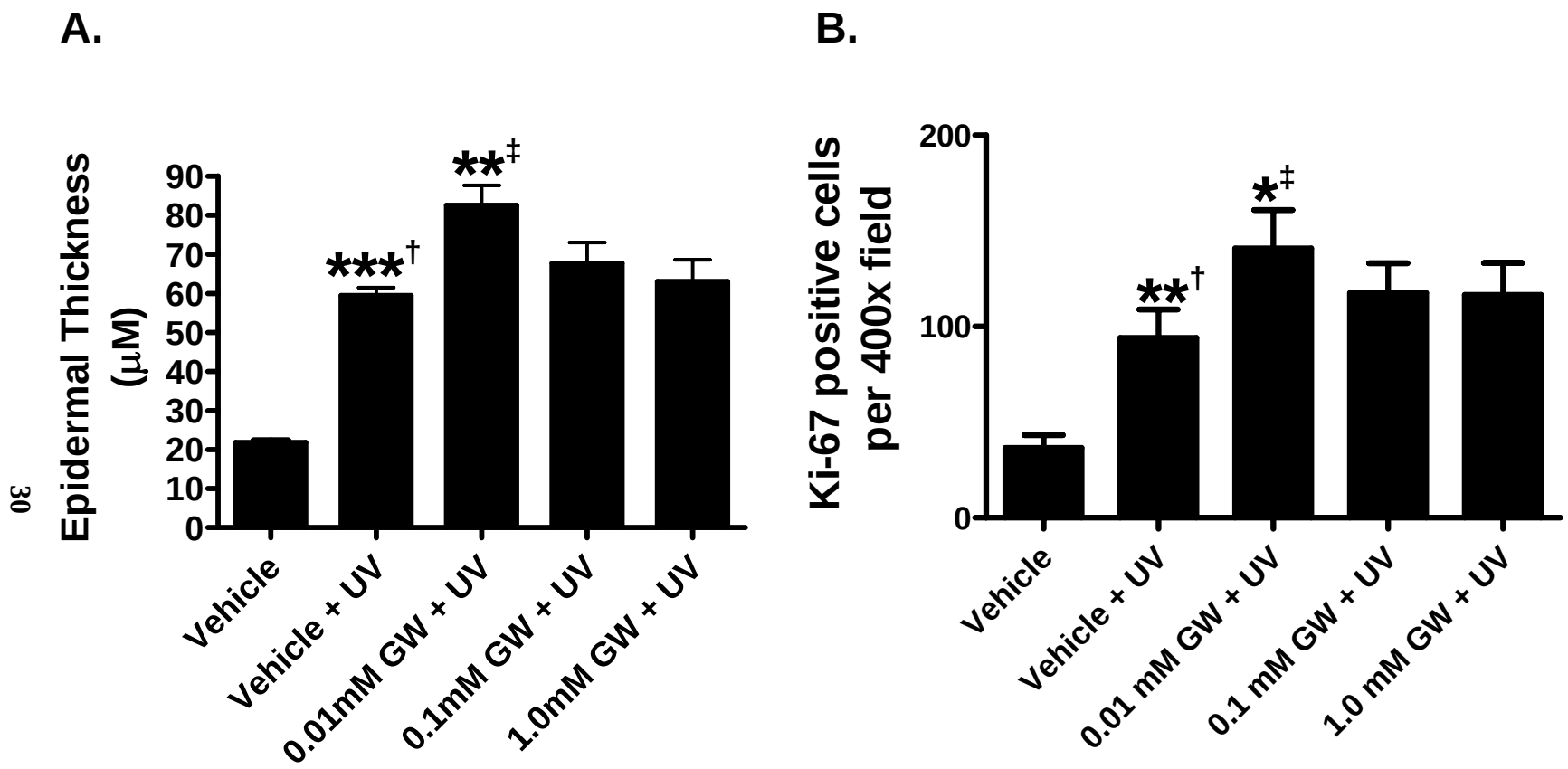

Fig. 4. UVB-induced delayed hyperplasia is augmented by GW9662 pretreatment. A. GW9662 augments UVB-induced epidermal thickness. SKH-1 mice were irradiated with 1500 J/m2 of UVB after a 1.5 pretreatment with GW9662 (0.01mM, 0.1mM, 1.0mM). After 72 hrs, the dorsal skin was excised, fixed in 10% buffered formalin, and paraffin-embedded. Epidermal thickness was then measured at 72 hrs in H&E stained sections by light microscopy using a micrometer, ** p <0.01; *** p<0.001, † represents conditions compared to vehicle control, ‡ represents conditions compared to UVB control. B. GW9662 pretreatment augments UVB induced proliferation as measured by Ki-67 immunolabeling. Paraffin-embedded mouse skin excised 72 hrs following UVB irradiation were subjected to immunohistochemical analysis of Ki-67 as detailed in the methods sections. For each treatment group, Ki-67 positive cells were counted in 10 randomly chosen high power fields (400x). Results represent the mean and SEM 28 of Ki-67 + cells/400x field (n = 5 mice). In both A & B, slides were blinded prior to analysis, *p<0.05; ** p <0.01, † represents conditions compared to vehicle control, ‡ represents conditions compared to UVB control.

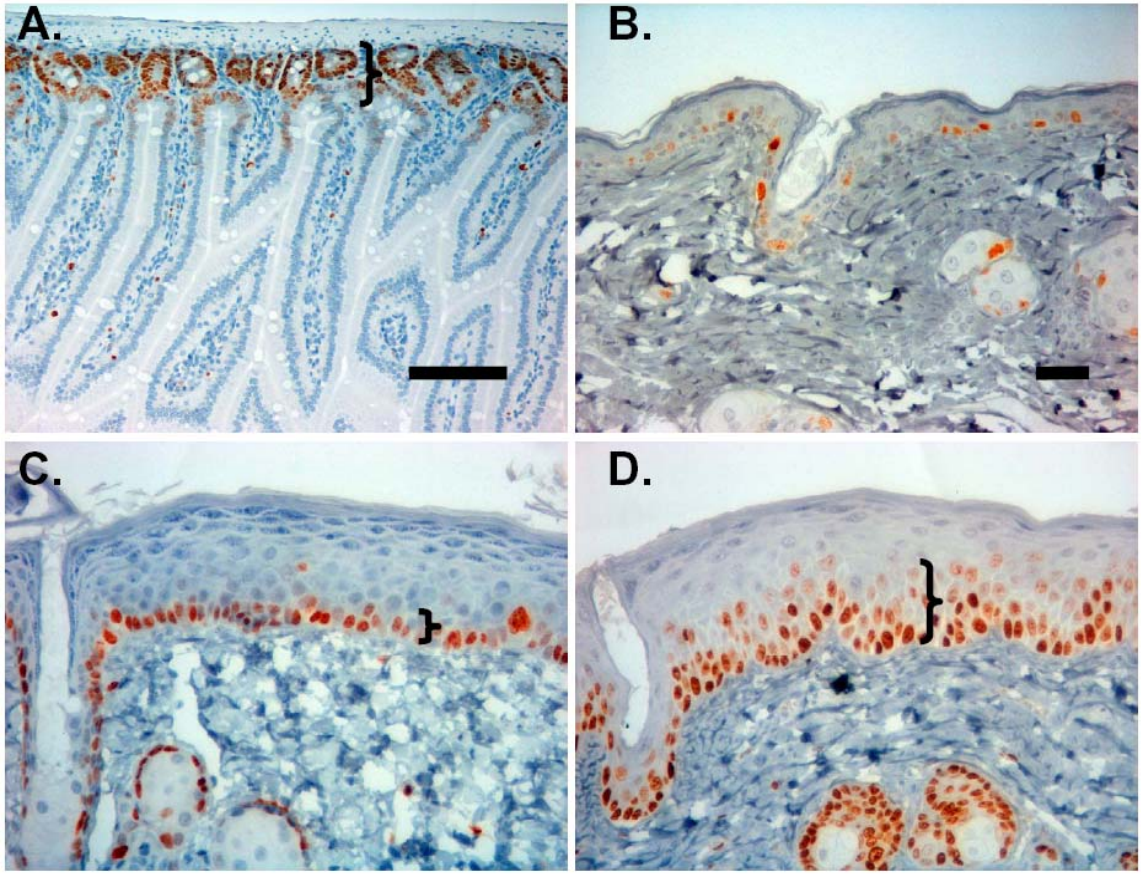

Fig. 5. GW9662 pretreatment results in proliferative zone expansion in UVB-irradiated SKH-1 epidermis. Photomicrographs of mouse epidermis 72 hrs post UVB were taken after Ki-67 immunohistochemical staining as described in Fig 4 above. A. Positive control gut tissue demonstrates strongly positive staining in the proliferative compartment in the deep crypts (brackets). Lack of epithelial staining is noted in the differentiated cilial luminal epithelium. 200x, bar = 25 μm. B. Vehicle sham control. Note occasional basal layer immunopositive cells for Ki-67. 400x, bar = 25 μm. C. UVB-irradiated vehicle control skin. Note the prominent epidermal hyperplasia and prominent Ki-67 immunolabeling of basal cells lining the epidermal dermal junction (bracket). 400x. D. UVB-irradiated skin treated with 0.01 mM GW9662. Note the expansion of the proliferative basal zone and further increase in epidermal thickness. 400x.

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Bachelor of Arts, Biology-Chemistry, Manchester College, North Manchester, IN, May 2005, G.P.A. 3.36

Research and Training Experience

***In vitro* pharmacologic studies** including UVB irradiation and transfection studies
Cell culture maintenance including aseptic technique, media preparation, archiving and revival of primary human keratinocytes, immortalized keratinocytes, and epidermoid carcinoma cells
***In vivo* pharmacology studies** in mice using receptor specific agonists and antagonists with both topical and intraperitoneal administration
Biomolecular Assays on tissue from *in vivo* studies including RNA isolation, cDNA synthesis, quantitative RT-PCR analysis, and immunohistochemical analysis
Animal Husbandry – implementation of breeding strategy to produce tissue specific knockout mice, routine colony maintenance including detailed record keeping of each colony, weaning, genotyping using standard PCR method, and continuation of each colony through backcross breeding strategy
Western Blot analysis including protein isolation and Bradford Assay quantitation

Professional Experience

Graduate Research Assistant, *I.U. School of Medicine/Department of Pathology and Laboratory Medicine, Laboratory of Dr. Raymond Konger, April 2008-June 2006*
The responsibilities of this position not only included those listed in the skill summary but also protocol development, presentation of data in both written and oral format, proficiency in Microsoft Office and GraphPad Prism 4.0, management of laboratory equipment and supplies, procurement, employee training, and interlaboratory coordination. Project participation included: Involvement of PPARγ in UVB-induced inflammation, apoptosis, and hyperproliferation *in vivo*, Regulation of ΔNp63α through PPARγ upon UVB irradiation *in vitro*, Transfection studies to determine agonistic activity of potential PPARγ activator.

Summer Student Research Assistant, *I.U. School of Medicine/Department of Nephrology Laboratory of Dr. Simon Atkinson, Summer 2004* The summer research assistant responsibilities entailed isolation and purification of plasmid DNA, tissue subculturing, collection of retrovirus, and image analysis. This position also required presentation of research findings in a scientific seminar format.