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The role of PPAR ligands in collecting duct growth and apoptosis

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The role of PPAR ligands in collecting duct growth and apoptosis

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Abstract

The collecting duct (CD) has considerable expression of PPARδ and PPARγ. While their roles are unknown in the CD, in other cells they have been shown to regulate both growth and apoptosis. We thus investigated the hypothesis that PPARs reduce apoptotic responses in the mouse collecting duct IMCD-K2 and M-1 cells and that while PPARγ has an inhibitory effect on cell growth, PPARδ stimulates growth in these cells. This study characterized the growth and apoptotic responses in the collecting duct cells, M-1 and IMCD-K2, in response to the PPARδ and PPARγ ligands, GW501516 and troglitazone. The extent of apoptosis in IMCD-K2 and M-1 cells, induced by anisomycin, was only affected by troglitazone, which reduced the protein levels of cleaved caspase-3 in M-1 cells. GW501516 treatment increased DNA synthesis in IMCD-K2 cells, decreased DNA synthesis in M-1 cells, and decreased protein synthesis in both IMCD-K2 and M-1 cells. Additionally, troglitazone reduced DNA and protein synthesis in M-1 cells, and this effect was unaltered in the presence of the PPARγ antagonist and ERK inhibitor. However, troglitazone treatment alone increased the levels of pERK. These results show PPAR ligands negatively regulate growth and apoptosis in CD cell lines, but more work is needed to ascertain the therapeutic potential of PPAR ligands in the prevention of CD injury.

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List of Abbreviations

AVP	Arginine-vasopressin
Bax	Bcl-2 associated X
Bcl-2	B-cell lymphoma 2
BrdU	5-bromo-2-deoxyuridine
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CCD	Cortical collecting duct
CCP	Cicaprost
CD	Collecting duct
CDK	Cyclin-dependent kinase
COX	Cyclooxygenase
CREB	cAMP response element-binding
cpm	Counts per minute
DAPI	4',6-diamidino-2-phenylindole
DBD	DNA binding domain
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
dpm	Disintegrations per minute
EP	PGE_2 receptor
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
ILP	Iloprost
IMCD	Inner medullary collecting duct
IP	PGI_2 receptor
LBD	Ligand binding domain
MAP	Mitogen-activated protein
NSAID	Non-steroidal anti-inflammatory drug
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pERK	Phospho-ERK
PG	Prostaglandin
PGE_2	Prostaglandin E_2
PGI_2	Prostaglandin I_2
PGJ_2	15-deoxy-Δ12,14-prostaglandin J_2
PPAR	Peroxisome proliferator-activated receptor
PPRE	Peroxisome proliferator response element
RT	Reverse transcriptase
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TBS-T	Tris buffered saline-Tween 20
TUNEL	Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling
TZD	Thiazolidinediones

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1.0: Introduction

1.1: Kidney

The human kidney is responsible for the excretion of waste from the body through the production of urine and also for maintaining the body's water and solute balance. Each kidney consists of roughly one million nephrons, which are the basic structural and functional unit of the kidney. It is made up of several segments, including the glomerulus, the proximal tubule, the loop of Henle, the distal tubule, and the collecting duct (CD). Each section has different functions concerning water and solute transport, due to, in part, the permeability of the cells, the presence of ion channels, and the concentration of the interstitum surrounding the cells (Knepper 1997).

1.2: Collecting duct

The CD is anatomically divided into three major segments: the cortical collecting duct (CCD), the outer medullary collecting duct (OMCD), and the inner medullary collecting duct (IMCD). The CD is made up of two types of cells: the principal, responsible for sodium and water reabsorption, and the intercalated cells, necessary for acid-base homeostasis. The proportion of principal cells increases towards the medullary end of the kidney. Since the CD is at the most distal part of the nephron, it is responsible for determining the final concentration of the urine and many hormones regulate its function, including: arginine-vaspressin, aldosterone, and prostaglandins (Stein, Kirschenbaum et al. 1975; Breyer and Ando 1994). The kidney is a major site of prostaglandin synthesis particularly in the collecting duct, the medullary interstitial cells,

and the glomeruli and may have essential responsibilities other than water and solute balance (Bonvalet, Pradelles et al. 1987; Campean, Theilig et al. 2003).

There are several CD cell lines; two of the more commonly studied are the IMCD-K2 and M-1 cell lines. IMCD-K2 is a well-characterized mouse cell line used for IMCD studies. The cell line is derived from the initial portion of the IMCD from a mouse that is transgenic for Simian Virus 40 (SV40) and it retains many characteristics of the intact IMCD, including amiloride-sensitive sodium absorption stimulated by aldosterone (Kizer, Lewis et al. 1995). M-1 is an immortalized CCD cell line that has also been developed from a mouse transgenic for the early region of SV40 (Stoos, Naray-Fejes-Toth et al. 1991). It too, retains many characteristics of the intact CCD including: a distinctive epithelial CCD appearance, made up of principal, α-, β-intercalated cells; and antigens specific for the CCD. However, unlike the IMCD-K2 cell line, the M-1 cells have had much greater use as a model in studies involving the cellular biology of the collecting duct.

The CD, especially the IMCD, survives in a harsh environment due to the hypertonic conditions in the interstitium and it is possible prostaglandins may contribute to this inherent resistance to stress.

1.3: Prostaglandins

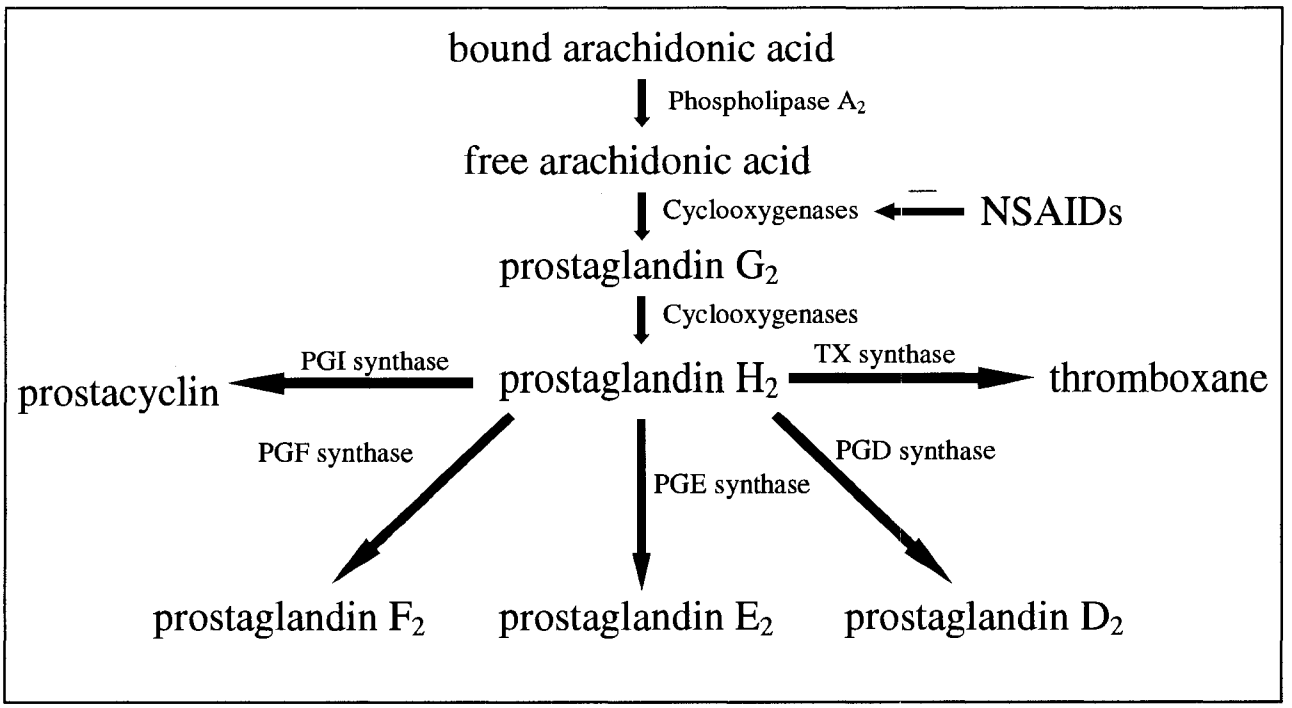
Prostaglandins (PGs), or prostanoids, are arachidonic metabolites, and their biosynthesis occurs in three well-defined steps through the cyclooxygenase (COX) pathway as depicted in **Figure 1**. Membrane-bound arachidonic acid is first released through the actions of phospholipase A_2 and the free arachidonic metabolites are quickly

converted to PGG_2 and then PGH_2 by COX enzymes (COX-1, COX-2). PGH_2 is the common precursor for all other prostanoids. Through the activity of several PG synthases a variety of PGs are then produced from PGH_2 (Smith 2008). Synthesis of PGs can be inhibited by the use of non-steroidal anti-inflammatory drugs (NSAIDs), which block the activities of the COX enzymes. The PG that is synthesized at the highest levels in the kidney is prostaglandin E_2 (PGE_2), followed by prostaglandin I_2 (PGI_2), also known as prostacyclin (Nasrallah, Clark et al. 2007).

1.4: Prostaglandin signaling

It has been established that PGs act on G-protein coupled receptors to alter cell signalling (Nasrallah, Clark et al. 2007). Both PGI_2 and PGE_2 can cause changes in cyclic adenosine monophosphate (cAMP) in many species and cell types including the mouse CD (Veis, Dillingham et al. 1990; Noland, Carter et al. 1992; Breyer and Breyer 2000; Nasrallah, Clark et al. 2007). PGI_2 produces it effects by binding to a G-stimulatory (G_s) protein coupled cell surface receptor, termed the I prostanoid receptor (IP) (Narumiya, Sugimoto et al. 1999). The binding of PGI_2 to IP causes an elevation of cAMP through the direct stimulation of adenylate cyclase. The IP receptor has been shown to be present in several areas of the kidney including the collecting ducts (Nasrallah, Zimpelmann et al. 2001; Nasrallah, Nusing et al. 2002). An extensive review by Nasrallah and Hébert from 2005 discussed this in detail.

Figure 1. Prostaglandin synthesis through the cyclooxygenase pathway. Prostaglandins are produced from arachidonic acid released from cell membrane phospholipids. The enzymes: phospholipase A_2, cyclooxygenase, and prostaglandin synthases control prostaglandin synthesis. The different synthases determine which prostaglandin is produced. Prostaglandin synthesis can be inhibited by non-steroidal anti-inflammatory drugs (NSAIDs), which block cyclooxygenase activity.


bound arachidonic acid
Phospholipase A2
free arachidonic acid
Cyclooxygenases
NSAIDs
prostaglandin G2
Cyclooxygenases
PGI synthase
prostacyclin
prostaglandin H2
TX synthase
thromboxane
PGF synthase
PGE synthase
PGD synthase
prostaglandin F2
prostaglandin E2
prostaglandin D2

PGE_2 also binds to G-protein coupled cell surface receptors known as the E prostanoid receptors (EP). Unlike PGI_2, however, PGE_2 binds to at least four subtypes of EP, designated EP_{1-4} (Coleman, Smith et al. 1994; Narumiya 1996). EP_1 is a G_q protein coupled cell surface receptor that activates phospholipase C. This enzyme then causes phosphatidylinositol-4,5-bisphosphate (PIP_2) to be cleaved creating inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). After its production, IP_3 transports to the endoplasmic reticulum where it stimulates calcium release. DAG, on the other hand remains in the membrane where it activates protein kinase C, resulting in varying effects. Both EP_2 and EP_4 are G_s protein coupled cell surface receptors, resulting in an increase in cAMP as previously described above with the IP receptor. Originally EP_2 and EP_4 were considered the same receptor. To distinguish them butaprost, a selective EP_2 agonist, can be used, as EP_4 is insensitive to it. Also, the EP_4 receptor has a longer C-terminal tail, resulting in increased internalization and desensitization (Desai, 2000). The final E prostanoid receptor is EP_3. This is a G-inhibitory (G_i) protein coupled cell surface receptor that, as it name implies, inhibits the activity of adenylate cyclase, resulting in a decrease of cAMP levels. Of the EP receptors, only EP_1, EP_3 and EP_4 are expressed in the collecting duct at any significant levels.

Traditional prostaglandin signaling in renal epithelial cells occurs through activation of the prostanoid receptors on both the basolateral and luminal surfaces of the cells (Hébert, Breyer et al. 1995; Breyer, Davis et al. 1996; Breyer and Breyer 2001). In recent years, however, a novel signaling pathway for prostaglandins has been found in which receptors are activated at the nuclear membrane; peroxisome proliferator-activated receptors (PPARs) (Lim and Dey 2000).

1.5: PPARs

PPARs are ligand-activated transcription factors (Ide, Egan et al. 2003) that are part of the steroid nuclear hormone receptor superfamily (Willson, Brown et al. 2000). The receptor contains a DNA binding domain (DBD) consisting of two zinc fingers, a ligand binding domain (LBD), a ligand-independent transactivation domain AF1 (activation function 1), and a ligand-dependent transactivation domain AF2. Although there are nuclear receptors that reside at the nuclear membrane and translocates into the nucleus after ligand-activation (Swales and Negishi 2004), PPARs are generally located inside the nucleus regardless of the presence of a ligand (Guan, Ishizuka et al. 2005). They exist as a heterodimer coupled with the retinoid X receptor, designated RXR, and can have several cofactors, coactivators or corepressors, bind to them, forming a complex. This activated complex then collectively binds to the peroxisome proliferator response element, or PPRE, found in the promoter of several target genes to regulate their expression (Mangelsdorf, Thummel et al. 1995).

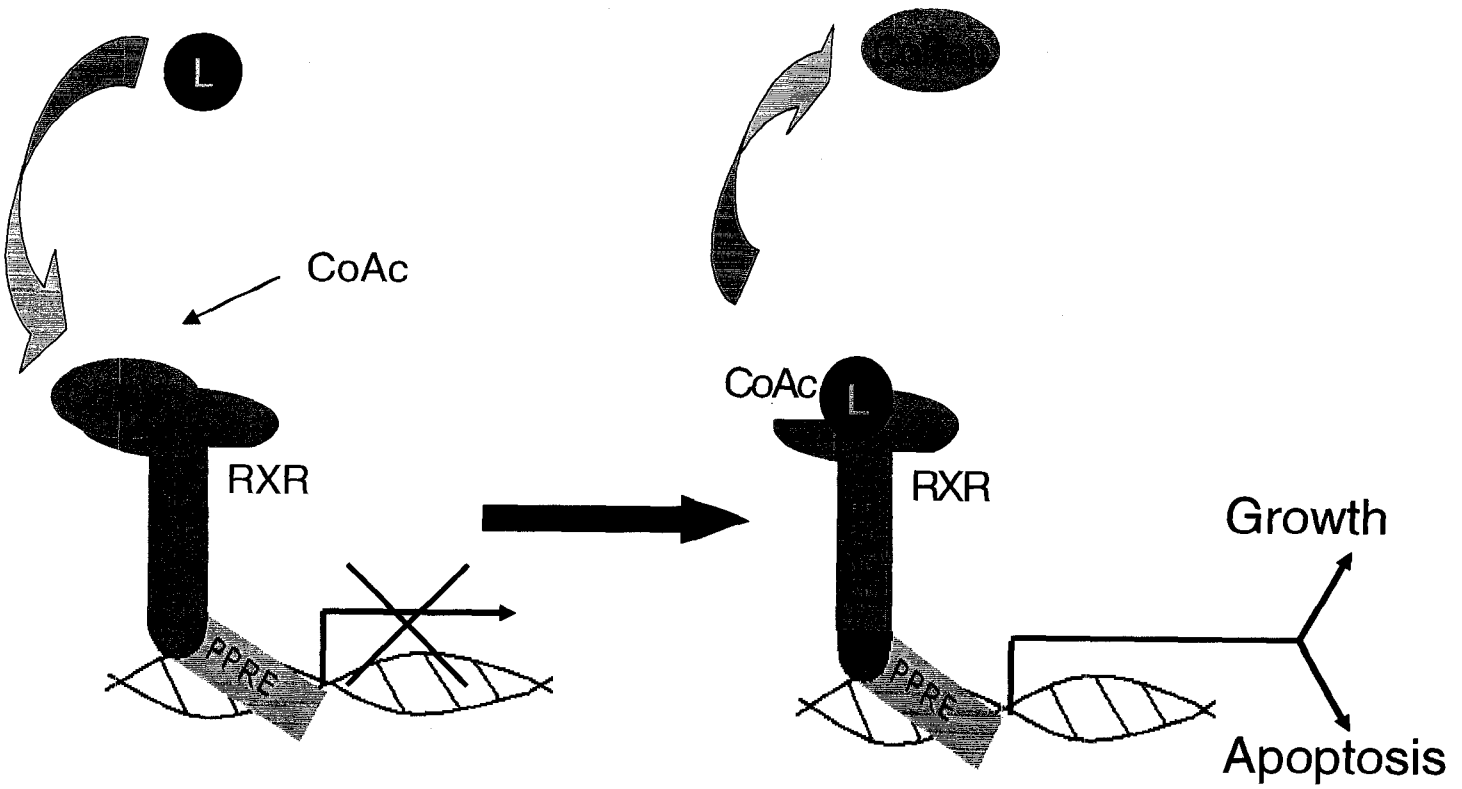
When bound to the PPRE, especially in its unliganded state, the complex can lead to the repression of several genes (Lee, Yang et al. 2006; Ng, Morisseau et al. 2006). It is believed that chromatin remodeling and histone deacetylation are believed to be involved since both N-CoR (nuclear receptor corepressor) and SMRT (N-CoR2) are recognized corepressors of the PPAR-RXR heterodimer (DiRenzo, Soderstrom et al. 1997; Chen, Kinyamu et al. 2006). These cofactors associate with histone deacetylases that, when active, condense the DNA and block transcription (Jepsen and Rosenfeld 2002). It should be noted that this repression can occur in the absence of a ligand and the unliganded state is actually preferred (Ordentlich, Downes et al. 1999). Often, once a

ligand binds to the complex there is a conformational change that promotes the dissociation of corepressors and the recruitment of coactivators. Examples of coactivator proteins are the steroid receptor coactivator 1 (SRC-1), PPARγ coactivator-1 (PGC-1), and the CREB-binding protein (Yang, Rachez et al. 2000; Kojo, Fukagawa et al. 2003; Puigserver and Spiegelman 2003). The mechanism of action for the majority of coactivators is either through histone acetylation, chromatin acetylation, or chromatin remodeling, all serving to open up the DNA to promote transcription (Yu and Reddy 2007).

One more level of control dictates the activity of the PPAR-RXR heterodimer: the state of phosphorylation by kinases (Zhang, Berger et al. 1996). It is believed to be through a mitogen-activated protein (MAP) kinase pathway, most likely Jun N-terminal kinase (JNK), and leads to a reduction in PPAR activation (Camp, Tafuri et al. 1999). In additional studies activation of ERK has resulted in increases in PPAR activation (Zhang, Berger et al. 1996). Thus, many factors need to be considered when determining the activity of any PPAR. **Figure 2** shows a simplified interaction of the complex with a PPRE.

There are three types of PPARs coded by different genes (PPARα, PPARβ/δ, and PPARγ), each with their own expression profile and actions. They share extensive homology with each other but each PPAR is responsive to a particular group of compounds (Ahmed, Ziouzenkova et al. 2007).

Figure 2. Mechanism of action for PPAR activation. In the repressed state, the peroxisome proliferator-activated receptor (PPAR) is unliganded, but usually heterodimerized with the retinoid X receptor (RXR), and near or on the peroxisome proliferator response element (PPRE) of PPAR-responsive genes. Upon binding of the ligand (L), a conformational change occurs, promoting the dissociation of corepressors (CoRep) and the recruitment of coactivators (CoAc), leading to the active state. The gene is now transcriptionally active, exerting many effects, including those concerning growth and apoptosis.



Repressed State

Activated State

1.6: PPARα

PPARα was the first to be cloned in 1990 (Issemann and Green 1990). It is highly expressed in the liver, renal cortex, intestinal mucosa, and heart where its effect is mainly to increase β-oxidation (Guan 2002). The class of drugs that activates PPARα is the fibrates, which is used as a hypolipidemic agent (Auwerx, Schoonjans et al. 1996). Endogenously, it has been discovered that unsaturated fatty acids can activate PPARα (Keller, Dreyer et al. 1993).

1.7: PPARβ/δ

Another type of PPAR is PPARδ also known as PPARβ. It is ubiquitously expressed in almost every tissue examined (Guan 2002). Even though it is widely distributed it was not until recently that much work was done at elucidating its roles in many systems. Since then PPARδ has been found to have a variety of effects. These include anti-inflammatory effects and roles in embryo implantation, tumor growth, cell survival, and metabolism (Gupta, Tan et al. 2000; Lim and Dey 2000; Youssef and Badr 2004; Kramer, Al-Khalili et al. 2005; Matthiessen, Pedersen et al. 2005). Another role of PPARδ may be preventing inflammation and atherosclerosis (Lee, Chawla et al. 2003). Unlike the other PPARs, as of yet, there is no specific drug class known to activate PPARδ, but there are several synthetic agonists, the most specific being GW501516 (Wei and Kozikowski 2003). Finally, the prostanoids PGI_2 and PGE_2 have been established as activators of PPARδ (Hao, Redha et al. 2002; Wang, Wang et al. 2004). PGI_2 activates the receptor directly whereas PGE_2 has been shown to activate it indirectly.

1.8: PPARγ

The final PPAR type, PPARγ, has two isoforms, PPARγ-1 and PPARγ-2, produced through alternative splicing (Zhu, Qi et al. 1995). PPARγ is present in many tissues, including adipose tissue and the kidney, where its main roles are to promote adipogenesis and increase cell proliferation (Guan 2002). PPARγ is the most studied of all of the PPARs with much of the research looking at cell growth and cell death in cancer tissues (Chou, Wang et al. 2007). PPARγ has also been examined with respect to lipid metabolism and the metabolic syndrome, a condition characterized by several metabolic risk factors including obesity, dyslipidemia, elevated blood pressure, and insulin resistance (Guan 2004; Ahmed, Ziouzenkova et al. 2007). The receptor is known to have a protective role in many cells. One paper suggested a cardioprotective effect of PPARγ in diabetic patients (Yu, Jin et al. 2007). The class of drugs that activates PPARγ is the thiazolidinediones (TZDs), a type of anti-diabetic agent (Auwerx, Schoonjans et al. 1996). Other drugs, such as NSAIDs have also been known to activate PPARγ. Indomethacin has been found to bind directly to PPARγ, causing its activation, in human colorectal cancer cells (Hawcroft, Gardner et al. 2003). Furthermore, 15-deoxy-Δ12,14-prostaglandin J2 (PGJ_2), an active metabolite of PGD_2, has been shown to interact with PPARγ in fibroblasts, promoting differentiation into adipocytes (Kliewer, Lenhard et al. 1995). Notably, this was the first prostaglandin known to activate a PPAR.

1.9: PPARs and the kidney

All three PPARs are found in the kidney (Guan 2002). PPARα is found mostly in the cortical regions, with the highest concentrations in the thick ascending limbs of the

loop of Henle and the proximal convoluted tubules. PPARγ is expressed in many areas of the kidney, with the greatest amounts in the collecting ducts, the thick ascending limbs, the proximal tubule, the glomeruli, and the podocytes. Not surprisingly, PPARδ is ubiquitously expressed in every part of the nephron, although the amount in the OMCD is relatively minute. Since only PPARδ and PPARγ are present in significant amounts in the CD, only these two PPARs were studied in this thesis.

There have been kidney-specific studies regarding PPARs, especially PPARγ. **Table 1** shows the localization of PPARδ and PPARγ in the kidney, as well as some of their observed roles. With respect to PPARδ, it has been implicated in the renal complications of metabolic syndrome (Guan 2004), however most studies involving the kidney and PPARδ examine its role in cell survival in proximal tubules by activating anti-apoptotic pathways (Letavernier, Perez et al. 2005). PPARγ has been studied quite extensively in the kidney with much of the research showing its involvment in the regulation of fluid retention and sodium balance (Yang, Michele et al. 1999). However, there are several studies that show that many of the effects of PPARγ in the kidney are protective. In the collecting duct, PPARγ has been shown to induce fluid retention, by promoting sodium absorption through increased transcription of the epithelial sodium channel, ENaC (Guan, Hao et al. 2005; Karalliedde and Buckingham 2007). One group has generated, using the Cre-loxP system, mice with a CD-specific deletion of PPARγ (Zhang, Zhang et al. 2005). The knockout resulted in the blockage of TZD-induced fluid retention. At this point, there is known role for PPARδ in CD function.

Table 1. Localization and function of PPARδ and PPARγ in the kidney.

	Renal Localization	Function
PPARγ	Collecting Duct	Sodium balance and blood pressure (Yang, Michele et al. 1999)
	Distal Tubule	Regulates fluid retention (Yang, Michele et al. 1999)
	Inner medulla (Yang, Michele et al. 1999), IMCD (Ito, Nakamura et al. 2006)	
	Renal interstitial cells (RMIC)	
	Kidney fibroblasts	Inhibits ECM production (Panchapakesan, Pollock et al. 2004)) Inhibits cell growth and reduces matrix production in human kidney fibroblasts (Zafiriou, Stanners et al. 2005
	Glomeruli	Reduces ECM proteins (fibronectin, type IV collagen) and TGF-β1, reduces proteinuria (Panchapakesan, Pollock et al. 2004)
	Proximal Tubule	Reduces ECM proteins (fibronectin, type IV collagen) and TGF-β1, reduces proteinuria (Panchapakesan, Pollock et al. 2004). Anti-proliferative and anti-inflammatory effects to high glucose (Panchapakesan, Pollock et al. 2004)
	Thick Ascending Limb (Ito, Nakamura et al. 2006)	
	Mesangial cells (Efrati, Berman et al. 2007)	Antihypertensive and anti-inflammatory properties
	Podocyte (Kanjanabuch, Ma et al. 2007)	Podocyte injuries and sclerotic conditions. Protective effect against apoptosis and necrosis. Effects mediated by p27 and TGF-β expression
PPARδ	Collecting Duct	
	Distal Tubule	
	inner medulla IMCD	
	Renal interstitial cells (RMIC) (Hao, Redha et al 2002)	Promotes cell survival following hypertonic stress
	Kidney fibroblasts	
	Glomeruli : podocytes and mesangial cells	
	Proximal Tubule	Protects against ischemic acute renal failure and tubular cell apoptosis (Letavernier, Perez et al. 2005)
	Medullary Thick Ascending Limb	

1.10: Cell survival/apoptosis

There are two major forms of cell death: necrosis and apoptosis. Apoptosis is programmed cell death as the cell itself undergoes several processes to destroy itself. Apoptosis can be identified by cell shrinkage, chromatin condensation, DNA fragmentation, and apoptotic body formation (Wang, Liu et al. 2005). The two major pathways, by which apoptosis occurs, are the death receptor pathway and the mitochondrial pathway. The death receptor pathway involves activation of a receptor by the FAS ligand which in turn activates caspase proteins, which are cysteine proteases that catalyze the apoptotic response (Hengartner 2000; Gupta 2003). When the mitochondrial pathway is activated the pro-apoptotic protein Bax (Bcl-2 associated X) is up-regulated, replacing the anti-apoptotic protein Bcl-2 (B-cell lymphoma 2), activating another caspase cascade (Hengartner 2000). Both pathways lead to the cleavage (activation) of effector caspases, such as caspase-3; the levels of which serve as a good measure of apoptosis (Gupta 2003).

There are several causes of apoptosis, including pharmaceuticals at toxic doses, and hypertonic stress. These generally activate the mitochondrial pathway, increasing Bax levels (Gupta 2003). In the kidney CD, osmolality is very important as many cells, specifically in the medullary regions, are exposed to hypertonic environments. The hyperosmalility is required for the counter-current exchange, which is critical in concentrating the urine. In most cells, apoptosis would occur at the levels of stress that is present in the environment surrounding the CD, but the CD manages to survive and resists any apoptotic activity (Zhang, Cai et al. 2002).

1.11: PPARs and cell survival/apoptosis

All three PPARs are involved in cell survival and a number of studies describe this role (Kim, Yoo et al. 2006; Muzio, Martinasso et al. 2006; Wang, Wang et al. 2006) particularly for PPARγ and PPARδ. Of these, several focus on the role of PPARδ in cancer. Several studies of PPARδ have shown conflicting results, especially regarding colon cancer growth (Gupta, Tan et al. 2000; Harman, Nicol et al. 2004). In one study by Wang *et al.* (2004) it was found that PGE_2 causes an increase in colon carcinoma cell survival through indirect activation of PPARδ. In another study it was shown that a product of linoleic acid down-regulated PPARδ, which in turn induced apoptosis (Shureiqi, Jiang et al. 2003). NSAIDs have been shown to increase apoptosis in cancer cells by suppressing the PPARδ pathway (Liou, Ghelani et al. 2007). Additionally, in neural cells activation of PPARγ produces an increase in an anti-apoptotic protein, protecting the cells from oxidative stress and apoptosis (Fuenzalida, Quintanilla et al. 2007). These studies indicate that both cell survival and apoptosis can be affected by PPARδ/γ.

This trend has also been seen in the kidney. A study by Hao *et al.* (2002) examined how PPARδ influenced the survival of renal medullary interstitial cells (RMICs) of New Zealand white rabbits. RMICs are routinely exposed to high levels of hypertonic stress and they wanted to determine if PPARδ was providing a protective effect, allowing for these cells to survive in these conditions. They discovered that PPARδ caused an increase in cell survival during high levels of stress and it was activated by PGI_2. In another study activation of PPARδ protected kidneys from renal failure after renal ischemia/reperfusion experiments via the anti-apoptotic Akt pathway

(Letavernier, Perez et al. 2005). Although, most of the studies have suggested a protective role of PPARδ, a few papers have shown that PPARδ can increase apoptosis (Hatae, Wada et al. 2001). However, PPARδ has been established as an important protein in the survival of many renal cell types.

As mentioned above, one of the major roles of PPARγ in the kidney appears to be to promote cell survival. One study showed that PPARγ reduced proteinuria and provided protection for both glomeruli and proximal tubules (Panchapakesan, Pollock et al. 2004). In another study, it was shown that insulin-sensitive mice that were PPARγ-deficient were protected against renal injury (Kume, Uzu et al. 2007). Further research illustrating that PPARγ protects many parts of the kidney from injury showed that pioglitazone, a synthetic PPARγ agonist and TZD, protected podocytes against injury in a puromycin aminonucleoside (PAN) model of renal injury in mouse cells (Kanjanabuch, Ma et al. 2007). The research indicates that PPARγ, along with PPARδ, is important in the survival of renal cells.

1.12: Cell cycle

Regulation of cell growth in the kidney is important for its normal function and disregulation can lead to several nephropathies (Nasrallah and Hébert 2005). In diabetes, an increase in mass of the proximal tubule is partly responsible for hyperfiltration (Thomson, Vallon et al. 2004). Another outcome of growth dysfunction is that it can lead to sodium retention and the development of hypertension (DiPetrillo, Coutermarsh et al. 2003). In parts of the kidney, especially the CD, overactive cell growth can lead to cyst formation, as in polycystic kidney disease (Nadasdy, Laszik et al. 1995). Without proper

control of the cell cycle, kidney function can fail, leading to some serious diseases. As PPARs can influence cell growth and survival it is likely that they are involved in the regulation of cell cycle. There are three main groups of proteins that affect the cell cycle: cyclins, cyclin-dependent kinases (CDKs), and CDK-inhibitors.

Cyclins are proteins that are needed for the progression of the cell cycle (Evans, Rosenthal et al. 1983; Minshull, Pines et al. 1989). There are many types of cyclins, the most important being cyclins A, B, D, and E, which act at different times during the cycle. There are also subtypes for some of the cyclins including cyclin D (1-3). The role of cyclins is to bind to CDKs, activating their kinase function to regulate cell cycle progression.

There are many CDKs and each CDK preferentially binds to certain cyclins. Cyclin A mostly associates with CDK1 and CDK2, cyclin B with CDK1, cyclin D (1-3) with CDK4 and CDK6, and cyclin E with CDK2. These too, are necessary for the progression of the cell cycle and would be up-regulated during cell growth.

The final major regulators of the cell cycle are the CDK-inhibitors. They work by inhibiting the action of the cyclin-CDK complexes by interfering with their interaction. Unlike the cyclins or CDKs, the inhibitors are down-regulated during cell growth and up-regulated when growth is inhibited. There are a variety of CDK-inhibitors each targeting specific cyclin-CDK complexes. The most common inhibitors include p21, p27 and p57.

In the kidney, cyclins A, B1, D1, and E are the most prevalent, with most of the attention being paid to cyclins D1 and E (Marshall and Shankland 2006). However, it is still not known which cyclins/CDKs are involved in the regulation of cell cycle progression in the collecting duct.

1.13: PPARs and cell cycle regulation

The involvement of PPARs in the cell cycle has been thoroughly examined in a variety of cells. CDKs, cyclins, and CDK inhibitors have wide-ranging effects and they vary from cell type to cell type, and also from species to species. However, many studies have shown that PPARs can affect the cell cycle.

In the literature, PPARγ, and/or its ligands, mainly inhibit the cell cycle. For instance, PPARγ induces cell cycle arrest for the purpose of differentiation (Shao and Lazar 1997). Also, withdrawal from the cell cycle by PPARγ has also been observed in cancer cells (Tontonoz, Singer et al. 1997). PGJ_2, a putative ligand of PPARγ, can reduce cyclin levels and increase levels of CDK inhibitors (Campo, Das et al. 2002; Cheng, Nakamura et al. 2004; Shen, Nishida et al. 2005). Other PPAR ligands, such as the TZDs, also block cell cycle progression by way of changing levels of cyclins and CDK inhibitors (Miwa, Sasaguri et al. 2000; Wang, Fu et al. 2001; Han, Sidell et al. 2004).

Unlike PPARγ, PPARδ activation mainly promotes cell growth. PGE_2, working through PPARδ, can stimulate proliferation of stem cells, increasing cyclin and CDK expression, while decreasing p21 and p27 (Kim and Han 2008). Over-expression of PPARδ has been shown to reverse inhibition of growth in cell lines and decrease levels of p21 (Jarvis, Gray et al. 2005). Furthermore, up-regulation of PPARδ in smooth muscle cells produces an increase in cyclin A and CDK2 levels, while lowering levels of a CDK inhibitor (Zhang, Fu et al. 2002).

It would appear in most cell lines, PPARγ and PPARδ have opposing effects on growth, each causing changes in the levels of cell cycle regulators. Their effect, however, in the CD has yet to be investigated.

1.14: Rationale

The CD is vital for water, sodium and urea balance in the human body, as well as acid-base homeostasis. Several events can cause the collecting duct to become damaged, such as ischemia-reperfusion injury and urinary tract obstruction (Cohen, Loutochin et al. 2007; Han, Kim et al. 2007). Other insults, such as diabetic nephropathy and reactive oxygen species, at earlier nehpron segments can lead to damage that can ultimately affect CD function (Rovin, Wurst et al. 1990; Chang, Wu et al. 2007). If left untreated, renal complications can occur and cells of the CD can undergo altered growth and apoptosis. The CD itself resides in an environment of high osmolality and the cells must have an inherent ability to survive in harsh situations. These innate properties may contribute to the resistance of the CD to pathological factors that otherwise affect other segments of the nephron. However, a disturbance in this inherent property could be associated with CD injury and corresponding changes in CD function that could contribute to disease processes, possibly leading to epithelial-to-mesenchymal transition and altered ion transport (Han, Kim et al. 2007; Hewitson 2007).

The kidney is a major producer of PGs, and the CD is one of the regions of the kidney with high levels of PG synthesis. Several prostanoid receptors, including EP and IP receptors can be activated by PGs. In addition to this, the CD has been shown to express both PPARδ and PPARγ, transcription factors that have both been associated with cell growth or survival and have been identified as possible targets of PGs. The roles of the PPARs in the CD are mostly unknown. Therefore, it is important to further our understanding of the role of these transcription factors in the CD, and clarify their potential as targets to prevent CD injury in renal disease.

1.15: Purpose

The purpose of this study was to characterize the growth and apoptotic responses in the collecting duct cells, M-1 and IMCD-K2, in response to the PPARδ and PPARγ ligands, GW501516 and troglitazone, and to determine if PPARs could regulate collecting duct properties.

1.16: Hypothesis

PPARs reduce apoptotic responses in the mouse collecting duct IMCD-K2 and M-1 cells. While PPARγ has an inhibitory effect on cell growth, PPARδ stimulates growth in these cells.

1.17: Objectives

Objective #1: To characterize the expression of COX, PGE_2/EP receptors, and PPARs in CD cells.

1) Examine COX protein in IMCD-K2 cells.

2) Study EP receptor mRNA expression in IMCD-K2 and M-1 cells.

3) Examine cAMP responses to different prostaglandins in IMCD-K2 cells.

4) Characterize PPARδ mRNA and protein expression in IMCD-K2 cells and PPARδ mRNA in M-1 cells.

5) Determine if PPARγ protein is expressed in M-1 cells.

Objective #2: To determine the effect of PPAR ligands on anisomycin-induced apoptosis of IMCD-K2 and M-1 cells.

1) Determine if GW501516 affects IMCD-K2 cell viability.

2) Study the effect of GW501516 (both cell lines) and troglitazone (M-1 only) on anisomycin-induced apoptosis in IMCD-K2 and M-1 cells. Apoptosis will be measured by caspase activity, TUNEL assays, cleaved caspase-3 protein levels and the ratio of Bax:Bcl-2 protein.

Objective #3: To determine the effect of PPAR ligands on cell growth in CD cells.

1) Determine the effect of PPARδ ligands, GW501516 and PGE_2, on DNA and protein synthesis in IMCD-K2 and M-1 cells through BrdU, thymidine, and leucine incorporation.

2) Examine the effect of PPARγ ligands, troglitazone and PGJ_2, on DNA and protein synthesis in M-1 cells through thymidine and leucine incorporation. A PPARγ antagonist, GW9662, will be used to verify if growth effects are mediated by PPARγ.

3) Study the effect of troglitazone on ERK activation (phosphoERK protein levels), and its role in troglitazone-mediated growth inhibition in M-1 cells. The ERK inhibitor, PD98059, will be used to confirm ERK involvement.

4) Characterize the effect of PPARδ ligands on cell cycle regulation in M-1 cells by examining protein levels of cyclins D1 and E, and CDK inhibitors p21 and p27.

2.0: Materials and Methods

2.1: IMCD-K2 cell culture: Cells were received from Dr. Bruce Stanton from Dartmouth Medical College (Hanover, U.S.A.). The cells had been isolated from the initial portion of the IMCD of a male mouse transgenic for Simian virus 40 (SV40; Tg(SV40E)Bri7). A cell line was then established by clonal dilution. The cells were maintained in media consisting of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 nutrient medium (DMEM/F-12; Gibco, Carlsbad, U.S.A.) containing 7.5 mM glucose, 2.5 mM L-glutamine, 15 mM HEPES, 0.5 mM sodium pyruvate, and 1.2 g/L sodium bicarbonate supplemented with 10% FBS (fetal bovine serum; Gibco, Carlsbad, U.S.A.), 1% penicillin/streptomycin (Gibco, Carlsbad, U.S.A.), ITS (5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium; Sigma; St. Louis, U.S.A.), 2 mM L-glutamine (Sigma, St. Louis, U.S.A.) and 5 µM dexamethasone (Sigma, St. Louis, U.S.A.). They were maintained in a 37°C incubator containing a 5% CO_2-95% air mixture. Cell culture medium was replaced every 48 hours. Twenty-four hours prior to experiments and stimulations the culture medium was replaced with serum-free medium (DMEM/F-12). This was done to remove any growth factors contained in the serum so they do not interfere with the experiments and to induce cell cycle exit, bringing all cells to the same point of the cell cycle.

2.2: M-1 cell culture: Cells were obtained from ATCC (Manassas, U.S.A.; CRL-2038). The cells had been established from the cortical collecting duct (CCD) of a mouse transgenic for the early region of SV40 (Tg(SV40E)Bri7). The cells were maintained in

DMEM/F-12 supplemented with 5% FBS and 1% penicillin/streptomycin. They were maintained in a 37°C incubator containing a 5% CO_2-95% air mixture. Cell culture medium was replaced every 48 hours. Twenty-four hours prior to experiments and stimulations the culture medium was replaced with serum-free medium (DMEM/F-12). This was done for the same reasons stated above.

2.3: mRNA expression (RT-PCR): RNA was isolated from confluent IMCD-K2 and M-1 cells grown on 100 mm plates of using 1 mL of the TRIzol reagent (Gibco, Carlsbad, U.S.A.) according to the manufacturer's instructions and was dissolved in 20 µL of diethylpyrocarbonate (DEPC)-treated water. The isolated RNA was then treated with 0.3U/µg of DNase I (Invitrogen, Carlsbad, U.S.A.) to remove any genomic DNA. The RNA was reverse-transcribed using MuLV (recombinant Moloney murine leukemia virus) reverse transcriptase and random hexamers from the GeneAmp RNA PCR Core Kit (Applied Biosystems, Foster City, U.S.A.) as per the manufacturer's instructions. Next, the RNA was amplified by polymerase chain reaction (PCR) using AmpliTaq DNA polymerase and primers for different prostanoid (ie. $EP_{1\text{-}4}$, IP) and PPARδ receptors. The primers were obtained from the University of Ottawa Biotechnology Research Institute (see Appendix I for primer information; Ottawa, Canada). The PCR parameters used were 40 cycles of: 95°C for 30 seconds, 59°C for 45 seconds and 72°C for 60 seconds, followed by 600 seconds at 72°C. The amplification was carried out using the GeneAMP PCR System 2400 (Applied Biosystems, Foster City, U.S.A.). The PCR products were run on an agarose gel and the bands were visualized using ethidium bromide under UV light. For internal control samples were run without reverse transcriptase.

2.4: ^{3}H-cAMP assay: Since prostanoids can cause changes in cyclic adenosine monophosphate (cAMP), their effect on cAMP production was determined. IMCD-K2 cells were serum-starved in 24-well plates with DMEM/F-12 for 24 hours and pretreated for 15 minutes with 0.5 mM isobutylmethylxanthine (Sigma, St. Louis, U.S.A.), a cAMP phosphodiesterase inhibitor, and 10^{-5} M indomethacin (Sigma, St. Louis, U.S.A.), a cyclooxygenase (COX) inhibitor. Next, the cells were stimulated in duplicate for ten minutes with control (serum-free media), 10^{-5} M forskolin (Sigma, St. Louis, U.S.A.) or 10^{-6} M arginine-vasopressin (AVP; Sigma, St. Louis, U.S.A.) as positive controls, cicaprost and iloprost (prostacyclin analogs; Bayer Schering Pharma AG, Berlin, Germany), or 10^{-9}-10^{-6} M of prostaglandin E_2 (PGE_2; Cayman, Ann Arbor, U.S.A.). The stimulation was then stopped by adding 300 µL of ice-cold 10% trichloroacetic acid (TCA, v/v) to each well and incubating the plate at 4°C for 30 minutes. Next, the samples were centrifuged for 10 minutes at 1413 x g and four ether extractions of TCA were carried out using 800 µL of H_2O-saturated diethyl ether per extraction. Following the ether extraction, a cAMP competitive binding assay was done using the ^{3}H-cAMP DPC radioassay (Intermedico, Markham, Canada) as per manufacturer's instructions. The samples were then incubated at 4°C for 90 minutes, and then 500 µL of a gelatin/charcoal solution was added to each tube. The tubes were again vortexed, incubated at 4°C for 10 minutes, and centrifuged at 12,718 x g for 5 minutes at 4°C. The supernatants were transferred into scintillation vials containing 10 mL of scintillation fluid, vortexed, and the amount of ^{3}H-cAMP in disintegrations per minute (dpm) was measured using a scintillation counter (Beckman Coulter; Fullerton U.S.A.). The percent stimulation of cAMP levels over control levels of each sample was calculated.

*2.5: **Western Blotting:*** The following is the protocol used for all Western blotting experiments described in this thesis. A list of antibodies used can be found in the appendix (Appendix II). Following 24-hour stimulations, protein was isolated from confluent IMCD-K2 and M-1 cells grown on 100 mm plates. The cells were scraped in ice-cold 1X phosphate buffered saline (PBS; 137 mM NaCl, 27 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4), and centrifuged at 500 x g for 5 minutes. The lysates were resuspended in RIPA lysis buffer containing: 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS, w/v), 4.5 mM NaCl, 2.5 mM Tris (pH 7.4), 8 μM EDTA, 0.2 mM sodium phosphate (pH 7.2), and fresh 0.5 mM PMSF, 1:100 protease inhibitor cocktail (Sigma, St. Louis, U.S.A), 1 mM sodium pyrophosphate, 10 mM sodium fluoride and 100 μM sodium orthovanadate. Next, the cells were sonicated, centrifuged at 9923 x g for 10 minutes at 4°C, and the protein in the supernatant was quantified using the Bradford reagent method (Bio-Rad, Hercules, U.S.A.). The lysates were then resolved by SDS-PAGE on a polyacrylamide gel consisting of a 4% stacking layer and a 10-15% resolving layer and electrophoresed at 180 V for 60-70 minutes using a Mini-PROTEAN II apparatus (Bio-Rad, Hercules, U.S.A.). The samples were transferred from the gel onto nitrocellulose membranes (Amersham, Piscataway, U.S.A.) at 100 V for ~60 minutes. After the transfer, the membranes were blocked for 90 minutes in 10% milk/TBS-T (137 mM NaCl, 20 mM Tris base, 0.1% Tween20) and incubated overnight with a primary antibody. After incubation with the primary, the membranes were washed with TBS-T three times fifteen minutes for a total of 45 minutes. The membranes were then incubated with their respective secondary antibodies (ie. anti-rabbit, anti-mouse, anti-donkey) for 90 minutes

and then washed for 60-90 minutes in TBS-T. The bands were then visualized by adding SuperSignal West Pico Chemiluminescent reagents (Pierce; Rockford, U.S.A.) onto the membranes for 5 minutes and then exposing the membranes to X-Omat Blue Kodak film (Perkin Elmer, Waltham, U.S.A.). Normalization of the protein samples was done with the detection of β-actin expression. Densitometric analysis of the bands was performed using the Kodak 1D Image Analysis software (Eastman Kodak Company, Rochester, U.S.A.).

2.6: Real-Time PCR: To determine the effect of PGE_2 on PPARδ levels in IMCD-K2 cells, RNA was isolated and DNase-treated as described in section ***2.3***. The cells were first pretreated with 10^{-5} M indomethacin. Next, the cells were treated for 2, 8, or 24 hours with serum-free media as control, 10^{-5} M indomethacin, 10^{-6} M PGE_2, or 10^{-5} M forskolin (stimulates cAMP independent of G-protein activation). The mRNA levels of PPARδ were quantified by real-time PCR using TaqMan One-Step RT-PCR master mix reagents (Applied Biosystems, Foster City, U.S.A.) and an ABI Prism 7000 sequence detection system. Reactions were carried out by using 50 ng of total IMCD-K2 RNA under the following conditions: 48°C for 30 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. The probe and primers for mouse PPARδ used were: probe, FAM-AGTTCAATGCGCTGGAGCTCGATGA-TAM; forward primer, 5'-GAGCCCAAGTTCGAGTTTGC-3'; reverse primer, 5'-TGAAGAGCGCCAGGTCACT-3'; (Sigma Genosys, Oakville, Canada). All values were normalized to GAPDH mRNA levels in the same sample, which was determined by the TaqMan Rodent GAPDH control reagent kit (Applied Biosystems, Foster City, U.S.A.).

*2.7: **BrdU labeling:*** To examine the effect of the PPARδ agonist, GW501516 (Alexis Biochemicals, San Diego, U.S.A.), on cell proliferation in IMCD-K2 cells, a BrdU incorporation assay was performed. IMCD-K2 cells were cultured onto glass coverslips in a 24-well plate and treated in duplicate with serum-free media (control), 10% FBS, or 10^{-6} M GW501516 for 24 hours. Once the cells had reached around 70-80% confluence they were incubated with a 5-bromo-2-deoxyuridine (BrdU) labeling reagent (which is incorporated into the cells during DNA synthesis) at 1:1000 for 2 hours as per the manufacturer's instructions (Roche, Basel, Switzerland). This was followed by three washes of 1X PBS and a 20-minute incubation of an ethanol fixative at 20°C. Another set of PBS washes were done and then the coverslips were incubated with an anti-BrdU reagent (1:25; Roche, Basel, Switzerland) at 37°C for 90 minutes. After another set of washes there was a co-incubation of an anti-mouse IgG fluorescein (1:25; Roche, Basel, Switzerland) and DAPI (1:1000; Roche, Basel, Switzerland) for 30 minutes in the dark at room temperature. After a final three washes the coverslips were inverted onto Fluoromount mounting media (Electron Microscopy Sciences, Hatfield, U.S.A.) on glass slides. The cells were visualized with the fluorescent microscope Axioskop Mot 2 (Zeiss, Jena, Germany) and captured with the camera AxioCam (Zeiss, Jena, Germany). For each coverslip 10 fields of view at 200x magnification were examined using pictures from both the DAPI filter (excitation: ~360 nm and emission: ~450 nm) and the green filter (excitation: ~480 nm and emission: ~540 nm). The pictures were processed using the Axiovision software (Zeiss, Jena, Germany). The DAPI-stained nuclei were automatically counted, using a macro created by Dr. Douglas J. Franks, and a mask of their position was applied to the picture from the green filter to determine the percent of

nuclei that were BrdU-positive. A total of 20 fields of view, or two coverslips, were used for each sample.

2.8: ^{3}H- thymidine incorporation: To study the effect of the PPAR ligands on cell proliferation of M-1 cells, DNA synthesis was measured using the incorporation of ^{3}H-thymidine. M-1 cells were cultured in 24-well plates, grown to ~50% confluence and then starved with serum-free media. Afterwards the cells were treated for 24 hours with: serum-free media (control), GW501516 (10^{-8}, 10^{-7}, 10^{-6} M), troglitazone ($5x10^{-7}$, 10^{-6}, $2.5x10^{-6}$, $5x10^{-6}$ M; Sigma, St. Louis, U.S.A), 15-deoxy-delta^{12,14}-prostaglandin J_2 (PGJ_2; 10^{-8}, 10^{-7}, 10^{-6} M; Cayman, Ann Arbor, U.S.A.), PGE_2 (10^{-8}, 10^{-7}, 10^{-6} M), 10^{-6} and $5x10^{-6}$ M rosiglitazone, pioglitazone, and ciglitazone (Cayman, Ann Arbor, U.S.A.); the PPAR antagonist GW9662 (10^{-6}, $5x10^{-6}$, 10^{-5} M; Cayman, Ann Arbor, U.S.A.); or the ERK inhibitor PD98059 (10^{-6}, $2.5x10^{-5}$, $5x10^{-5}$ M; Calbiochem, Madison, U.S.A.). The control cells were treated with a vehicle consisting of 1 µL/mL of dimethyl sulfoxide (DMSO) in DMEM/F-12. ^{3}H-thymidine (0.5 µCi/mL; Amersham, Piscataway, U.S.A.) was added during the final four hours of stimulation. After the stimulation the plates were washed four times in ice-cold PBS. Next, the cells were permeabilized in 500 µL of 1N NaOH at 37°C for ~30 minutes, transferred into scintillation vials containing 10 mL of scintillation fluid, and the amount of ^{3}H-thymidine in counts per minute (cpm) was measured using a scintillation counter measured the amount of ^{3}H-thymidine in counts per minute (cpm). Samples were done in triplicate and thymidine incorporation is expressed as fold control.

2.9: ^{3}H- leucine incorporation: To study the effect of the PPAR ligands on cell growth, protein synthesis was measured using the incorporation of ^{3}H-leucine. M-1 and IMCD-K2 cells were cultured in 24-well plates, grown to ~50% confluency and then starved with serum-free media for 24 hours. After the starvation the IMCD-K2 cells were treated for 24 hours with: serum-free media (control), 10% FBS, and GW501516 (10^{-8}, 10^{-7}, $5x10^{-7}$, 10^{-6}, $5x10^{-6}$, 10^{-5} M). The M-1 cells were treated for 24 hours with control, GW501516 (10^{-8}, 10^{-7}, 10^{-6} M), troglitazone ($5x10^{-7}$, 10^{-6}, $2.5x10^{-6}$, $5x10^{-6}$ M), PGJ_2 (10^{-8}, 10^{-7}, 10^{-6} M), PGE_2 (10^{-8}, 10^{-7}, 10^{-6} M), rosiglitazone (10^{-6}, $5x10^{-6}$ M), pioglitazone (10^{-6}, $5x10^{-6}$ M), ciglitazone (10^{-6}, $5x10^{-6}$ M), GW9662 (10^{-6}, $5x10^{-6}$, 10^{-5} M), or PD98059 (10^{-6}, $2.5x10^{-5}$, $5x10^{-5}$ M). The vehicle-treated control cells contained 1 µL/mL of DMSO in DMEM/F-12, as all compounds used were prepared in DMSO. ^{3}H-leucine (0.5 µCi/mL; Amersham, Piscataway, U.S.A.; Perkin Elmer, Waltham, U.S.A.) was added to each well during the 24 hour stimulation. The cells were then prepared as described in section ***2.8*** and the amount of ^{3}H-leucine was measured using the scintillation counter in cpm. Samples were prepared in triplicate and leucine incorporation is expressed as fold control. For all of the above experiments using the scintillation counter (sections ***2.4***, ***2.8***, ***2.9***) there was little variability between duplicates or triplicates and all results were within the limits of detection (40-10^{-7} dpm or 25-$65x10^{-7}$ cpm).

2.10: Cell viability: To evaluate the effect that GW501516 has on the viability of IMCD-K2 cells in both the presence and absence of an intiator of apoptosis a cell viability assay was performed. IMCD-K2 cells were grown to 70% confluence in 96-well plates and stimulated for 18 hours with: vehicle control (1 µL of ethanol and 1 µL DMSO in

DMEM/F-12), 10% FBS, GW501516 (10^{-8}, 10^{-7}, $5x10^{-7}$, 10^{-6}, $5x10^{-6}$, 10^{-5} M), anisomycin (10, 50, 100, 500, 1000, 5000 ng/mL; Sigma, St. Louis, U.S.A), or cisplatin (50 ng/mL; Sigma, St. Louis, U.S.A). Some cells were treated pretreated for 24 hours with GW501516 (10^{-6} M) and then treated with both anisomycin (100 and 1000 ng/mL) and GW501516 (10^{-6} M). Anisomycin and cisplatin were used to induce cell death. Following the stimulation, the media was aspirated from the wells and replaced with 100 µL of a 1x DNA dye-binding solution from the CyQAUNT NF kit (Invitrogen, Carlsbad, U.S.A.). The plate was incubated at 37°C for 1 hour and the fluorescence intensity, representing the number of cells, was measured in a FLUOstar Galaxy plate reader (BMG Labtechnologies, Offenburg, Germany) as per the manufacturer's instructions (excitation: ~485 nm and emission: ~530 nm). Samples were done in triplicate and fluorescence intensity is expressed as fold control.

2.11: Colorimetric caspase-3 activity assay: To determine if GW501516 is involved in apoptosis a caspase-3 activity assay was carried out using the colorimetric CaspACE Assay System kit (Promega, Madison, U.S.A.). IMCD-K2 cells were grown to ~50% confluency in 100 mm plates and starved for 24 hours. After starvation the cells were treated with control (1 µL of ethanol and 1 µL of DMSO in 1 mL of DMEM/F-12), 10% FBS, GW501516 (10^{-6} M), anisomycin (250 ng/mL), or Z-VAD-FMK ($2x10^{-5}$ M), a pan-caspase inhibitor supplied in the kit. Some cells were pretreated with GW501516 for 24 hours and then treated with both anisomycin and GW501516. Also, some plates were pretreated for 24 hours with Z-VAD-FMK and then treated with anisomycin and the inhibitor together. Following the stimulation, the cells were scraped, suspended in PBS,

and centrifuged at 500 x g. The pellets were resuspended in 100-200 µL of the caspase lysis buffer provided in the kit, subjected to two freeze-thaw cycles (at -20°C) and then incubated on ice for 15 minutes. After this step, the lysates were centrifuged for 20 minutes at 15,156 x g and the supernatants were transferred to new Eppendorf tubes. The protein was quantified by Bradford and 50 µg of each sample were incubated with the colorimetric caspase-3 substrate, Ac-DEVD-pNA ($2x10^{-4}$ M) in a 96-well plate at room temperature overnight, as per the manufacturer's instructions. The endogenous active caspase-3 binds to the substrate and a colored product is produced. The plate was then placed in the FLUOstar Galaxy plate reader and the absorbance of each well was measured at 405 nm. Samples were performed in duplicate and the caspase activity is expressed as relative absorbance. All duplicates showed little variability and were within the detection limits with absorbances between 0.03 and 0.45, representing 0 and 100 picomoles/µL of substrate metabolized, respectively.

2.12: TUNEL assay: To further evaluate the effect of GW501516 on apoptosis a TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) assay was performed. IMCD-K2 cells were grown to ~60% confluency on glass coverslips in a 24-well plate and treated in duplicate for 24 hours with: control (1 µL of ethanol and 1 µL of DMSO in 1 mL of DMEM/F-12), GW501516 (10^{-8}, 10^{-6} M), or anisomycin (250 ng/mL). Some cells were treated with both GW501516 (10^{-8}, 10^{-6} M) and anisomycin and these plates were pretreated with the respective concentrations of GW501516 for 3 hours. After the stimulation, the wells were washed with 1X PBS and placed at -20°C for 15 minutes with a fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.16

M sodium phosphate buffer (pH 6.9). The coverslips were then washed with PBS three times and incubated with 2 µL $CoCl_2$, 5 µL 5X TDT buffer, 0.1 µL terminal transferase (all taken from the Roche Terminal Transferase recombinant kit, Basel, Switzerland), 0.17 µL biotin-16-dUTP (Roche, Basel, Switzerland), and 17.73 µL of H_2O for 1 hour at 37°C in a humid chamber. The cells were then washed with 4X SSC (0.6 M NaCl, 76 mM Na Citrate, pH 7) and blocked for 30 minutes with 1% milk/4X SSC at room temperature. When the blocking was completed a streptavidin-Cy2 stock (1.0 mg/mL; Molecular Probes, Eugene, U.S.A.) was added to the blocking solution on the coverslips at 1:1000 and incubated for 45 minutes at room temperature in the dark. Next, the coverslips were subjected to a final wash with PBS and incubated with DAPI for 45 minutes at 1:1000. The coverslips were then mounted and visualized as previously described in section ***2.7***. The terminal transferase and biotin-16-dUTP target free DNA fragments of apoptotic cells producing a fluorescent green signal. The number of apoptotic cells was counted and by using the picture from the DAPI filter a percentage of TUNEL-positive cells was determined. A total of 16 fields of view (two coverslips) were used for each sample.

2.13: Statistics: The GraphPad Prism software for Windows (version 4.02) was used to analyze data. Results are expressed as means ± standard error of the mean (SEM). Either an unpaired t-test or a one sample t-test (with a hypothetical value of 1) was used to evaluate the statistical significance between data points. A p-value < 0.05 was considered statistically significant.

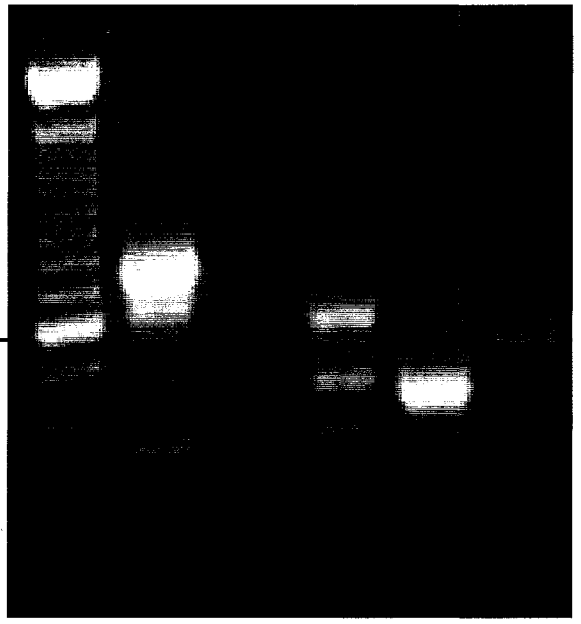
3.0: Results

3.1: EP receptor subtypes and PPARδ are expressed in IMCD-K2 cells. PGE_2 is known to activate prostanoid receptors, EP_1, EP_2, EP_3, and EP_4 as well as the PPAR receptor, PPARδ. Thus, the presence of these receptors in IMCD-K2 cells was determined. RNA was isolated from IMCD-K2 cells using the TRIzol method and then reverse transcribed and amplified using primers for the EP, IP and PPARδ receptors. The products were run on an agarose gel and viewed under an ultraviolet light. The band corresponding to each prostanoid and PPAR receptor is shown in **Figures 3A** and **3C**, respectively. The band seen between 700 and 800 bp in the EP_1 lane has been previously shown to represent protein kinase N (PKN), as the genes for PKN and EP_1 overlap (Batshake and Sundelin 1996). The bands seen in the EP_1, EP_3, EP_4, and PPARδ lanes at 336 bp, 437 bp, 423 bp, and 161 bp, respectively, are all of the expected sizes for each receptor. The other two bands seen in the EP_3 lane, at sizes of approximately 350 bp and 600 bp, are most likely due to alternative splicing of the EP_3 gene (Breyer, Emeson et al. 1994). Even though faint bands were seen in the EP_2 and IP lanes previous studies in our laboratory indicated that at higher stringency the band in the EP_2 lane disappears and that the product detected with IP primers did not correspond to the IP receptor, as determined by cloning and sequencing. The products were not present when the reverse transcriptase was omitted (negative control) for the prostanoid receptors and PPARδ as shown in **Figures 3B** and **3D**, respectively. PPARγ has been previously been shown to be expressed in these cells Hao, Redha et al. 1999). These results indicate that IMCD-K2 cells express EP_1, EP_3, EP_4, and PPARδ.

Figure 3. EP receptor subtypes and PPARδ are expressed in IMCD-K2 cells. RNA was isolated from IMCD-K2 cells using TRIzol, reverse transcribed, amplified by polymerase chain reaction using primers for the prostanoid receptors (A, B) and PPARδ (C, D), and visualized on an agarose gel using ethidium bromide under ultraviolet light. PCR was done A), C) with (RT+) and B), D) without (RT-) reverse transcriptase (negative control). As shown, specific bands for each PCR product was obtained at the predicted size for EP_1 (336 bp), EP_3 (437 bp; plus bands at ~350 bp and ~600 bp) and EP_4 (423 bp); as well as PPARδ (161 bp).

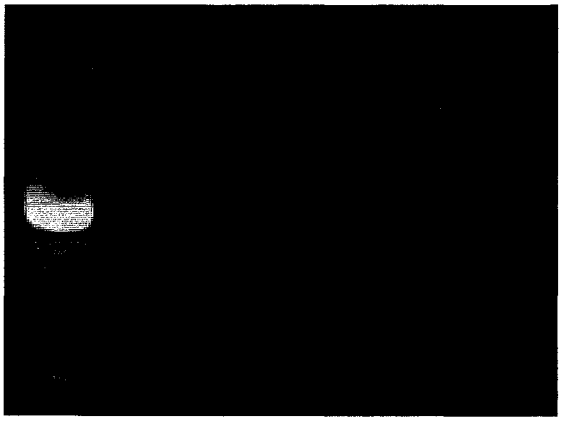
A)

L EP_1 EP_2 EP_3 EP_4 IP



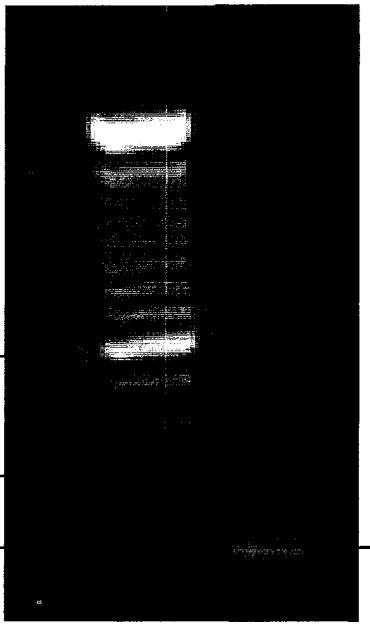
B)

L EP_1 EP_2 EP_3 EP_4 IP



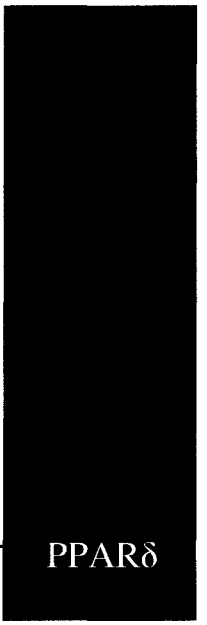
C)

L RT+



D)

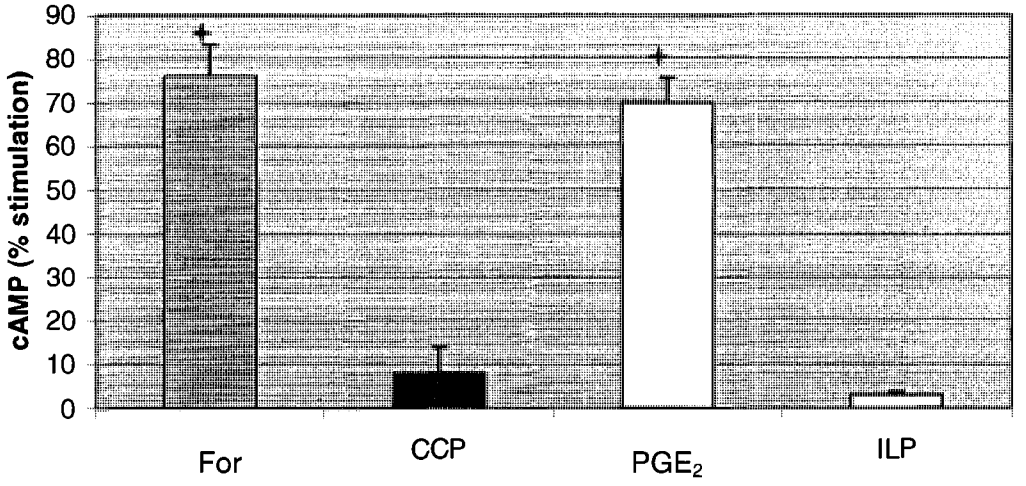
RT-



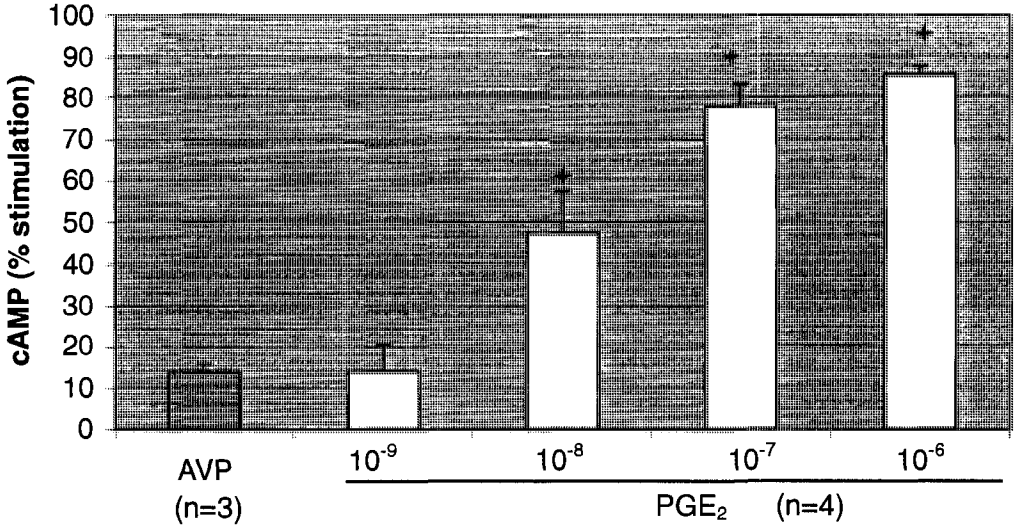
3.2: PGE_2 stimulates cAMP in IMCD-K2 cells. Both EP_3 and EP_4 receptors elicit changes in intracellular cAMP; therefore the effect of different prostanoids on cAMP stimulation was examined. Cells were treated with serum-free media (control), prostacyclin (PGI_2) analogs cicaprost (CCP) and iloprost (ILP), PGE_2, or forskolin (For). Forskolin is an adenylate cyclase agonist and causes a direct activation in cAMP production serving as a positive control. As shown in **Figure 4A,** CCP and ILP did not alter cAMP levels. PGE_2 however did cause a significant increase in percent stimulation, at about 70% above control, comparable to forskolin. Since PGE_2 produced a response, it was tested to see if the response was dose-dependent. As shown in **Figure 4B**, treatment with increasing amounts of PGE_2 resulted in an increase in the amount of cAMP stimulation. The percent stimulation for PGE_2 at concentrations of 10^{-9} M, 10^{-8} M, 10^{-7} M, 10^{-6} M were 14.0, 47.5, 78.0, and 85.5, respectively. Arginine-vasopressin (AVP) has been known to stimulate cAMP production in IMCD cells (Nadler, 1992), therefore 10^{-6} M AVP was used as a positive control. However, AVP only resulted in a 13.7 % increase in cAMP. This suggests that either the cell line does not retain this characteristic of the intact IMCD or the ability has been lost due to transformation in the cell line after several passages.

Figure 4. PGE_2 stimulates cAMP in IMCD-K2 cells. Serum-starved cells were stimulated for ten minutes with A) 10^{-6} M of different prostanoids [cicaprost (CCP), and iloprost (ILP), PGE_2] or 10^{-5} M forskolin (For); and with B) differing concentrations of PGE_2 (10^{-9} to 10^{-6} M) or arginine-vasopressin (AVP). A competitive binding assay with ^{3}H-cAMP was performed and the percent cAMP stimulation was determined using a scintillation counter. Values are means ± S.E.M.; n = 3-4. + $p < 0.05$ compared to control.

A)

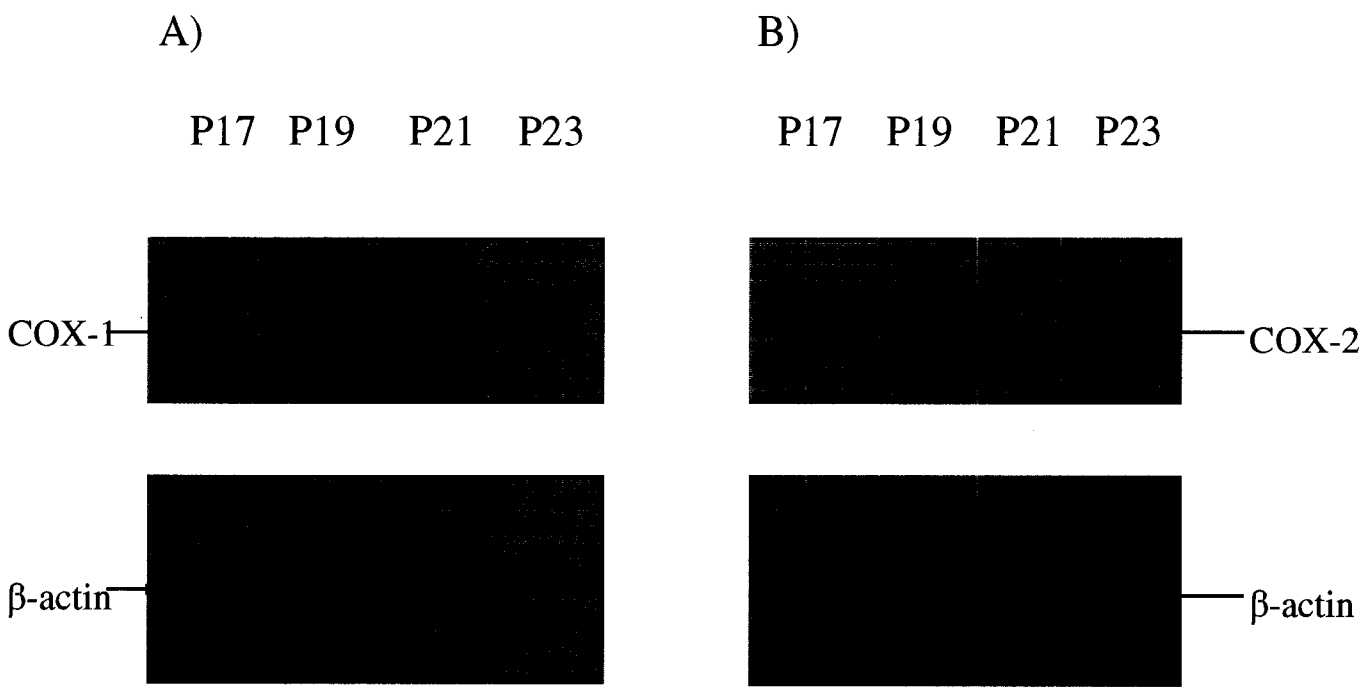

cAMP (% stimulation)
90
80
70
60
50
40
30
20
10
0
For
CCP
PGE2
ILP

B)


cAMP (% stimulation)
100
90
80
70
60
50
40
30
20
10
0
AVP
(n=3)
10-9
10-8
10-7
10-6
PGE2 (n=4)

3.3: COX-1 and COX-2 are expressed in IMCD-K2 cells. The production of PGE_2 is mainly dependent on cyclooxygenases (COX). To determine the presence of COX enzymes, and to ensure consistent expression, proteins from different passages of IMCD-K2 cells (P17, P19, P21, and P23) were isolated using a RIPA lysis buffer. The proteins were run on an SDS-PAGE gel, transferred, and were blotted with both COX-1 and COX-2 antibodies. As shown in **Figure 5,** all four passages contained a band in the proper position (70-72 kDa) indicating that both COX-1 (**Figure 5A**) and COX-2 (**Figure 5B**) proteins are present.

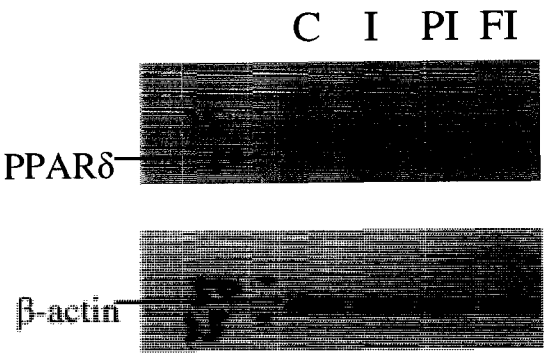
Figure 5. COX-1 and COX-2 are expressed in IMCD-K2 cells. Total protein was isolated from four passages of IMCD-K2 cells (P17, P19, P21, and P23) and run on an SDS-PAGE gel. Western blotting was performed using A) anti-COX-1 and B) anti-COX-2 antibodies (1:1000). Membranes were then stripped and blotted for β-actin (1:4000) for normalizing samples.


A)
P17 P19 P21 P23
COX-1
β-actin
B)
P17 P19 P21 P23
COX-2
β-actin

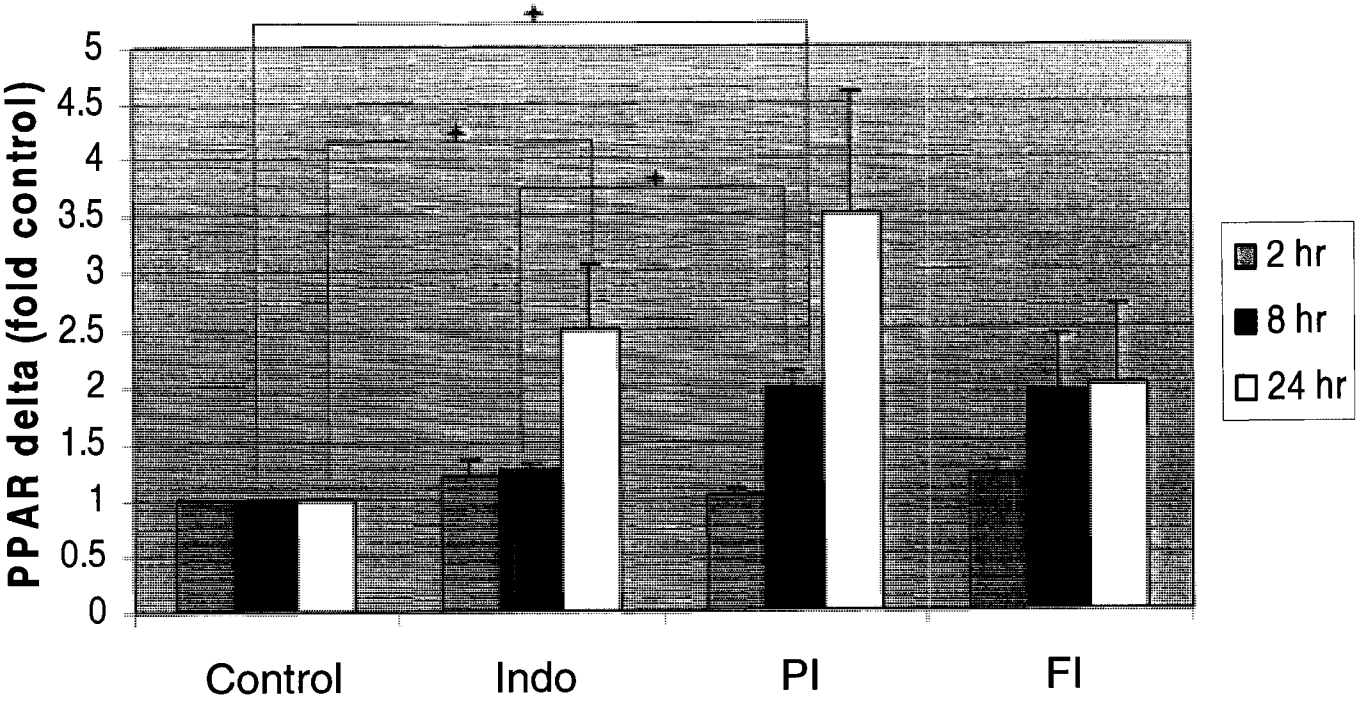
3.4: PGE_2 increases PPARδ protein expression. PGE_2 has been known to activate PPARδ to regulate several growth processes (Wang, Wang et al. 2004; Kim and Han 2008). So, it was ascertained how PGE_2 stimulation affected PPARδ protein levels. Indomethacin, a COX inhibitor, was used to inhibit endogenous PG synthesis. IMCD-K2 cells were treated for 2, 8, or 24 hours with serum-free media (control, C), 10^{-5} M indomethacin (I), PGE_2 in the presence of indomethacin (PI), or 10^{-5} M forskolin in the presence of indomethacin (FI). Western blotting was performed with a PPARδ antibody. As shown in **Figure 6A**, a band corresponding to PPARδ (52 kDa) was visualized in each lane. As seen in **Figure 6B**, the densitometry shows that eight-hour PI treatment in the cells resulted in increased PPARδ protein expression, compared to control or I alone. However two-hour stimulation did not alter PPARδ. Following 24-hour stimulation there was a tendency for an increase in PPARδ expression with stimulation of PI but this increase was not significant when compared with I alone. FI served as a positive control for cAMP activation.

Figure 6. PGE_2 increases PPARδ protein expression. Protein was isolated from IMCD-K2 cells that had been treated with serum-free media (Control), 10^{-5} M indomethacin (I), 10^{-6} M PGE_2 in the presence of I (PI), or 10^{-5} M forskolin in the presence of I (FI) for 2, 8 and 24 hours. The proteins were A) run on an SDS-PAGE gel and Western blotted using an anti-PPARδ antibody (1:500). The membranes were stripped β-actin was detected to normalize samples for B) densitometric analysis. Expression is presented as fold of control. Values are means ± S.E.M.; n = 3-6. + $p < 0.05$

A)

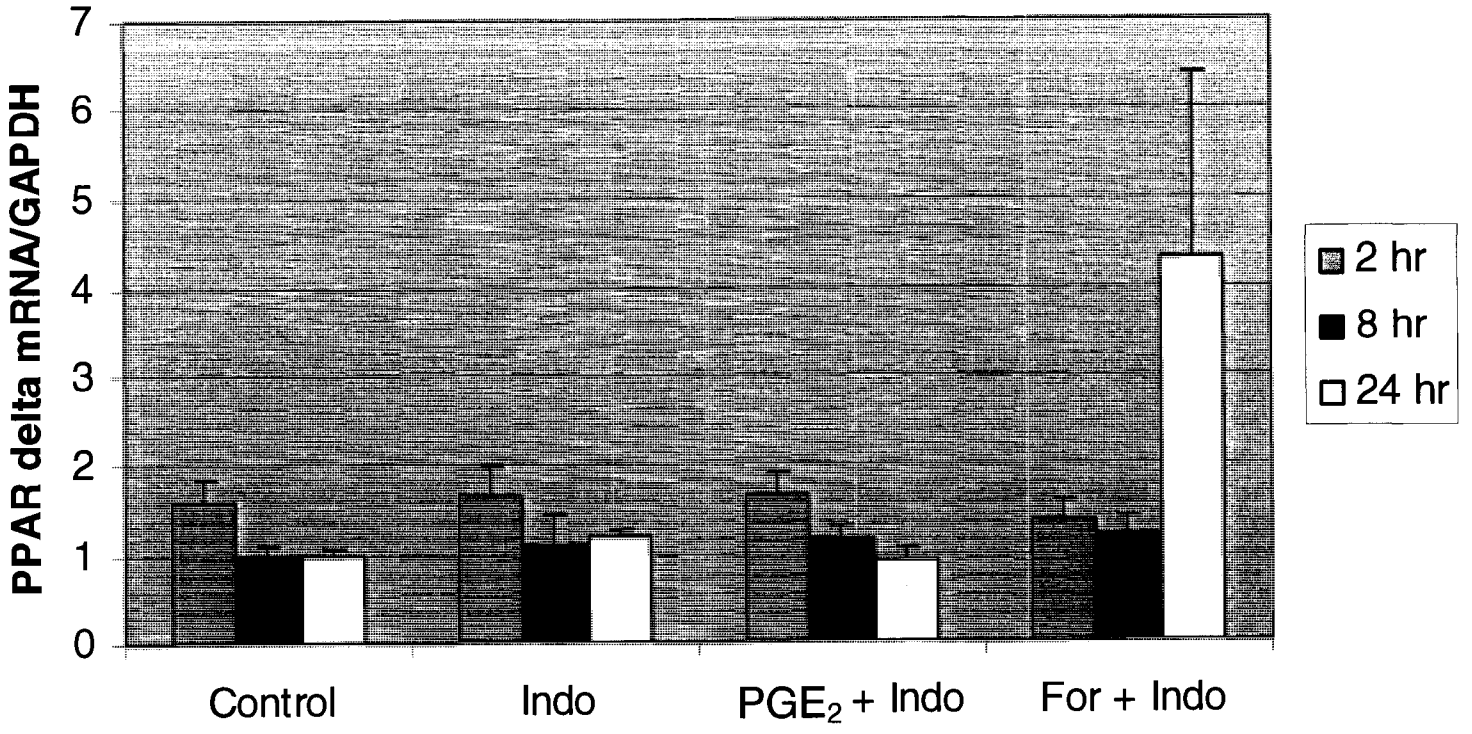

C I PI FI
PPARδ
β-actin

B)


PPAR delta (fold control)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Control
Indo
PI
FI
2 hr
8 hr
24 hr

3.5: PGE_2 does not affect PPARδ mRNA expression. The effect of PGE_2 on PPARδ mRNA was also examined. IMCD-K2 cells were stimulated as above with serum-free media (Control), indomethacin (Indo), PGE_2 and indomethacin (PGE_2 + Indo), or forskolin and indomethacin (For + Indo) for 2, 8, and 24 hours. RNA was isolated from these cells using the TRIzol method. The levels of PPARδ mRNA were then quantified by Real-time PCR. The Real-time analysis, as shown in **Figure 7**, indicated that PPARδ was unchanged in any treatment group or exposure time.

Figure 7. PGE_2 does not affect PPARδ mRNA expression. RNA was isolated from IMCD-K2 cells using the TRIzol method. The cells had been treated for 2, 8 and 24 hours with serum-free media (Control), 10^{-5} M indomethacin (Indo), 10^{-6} M PGE_2 in the presence of 10^{-5} M indomethacin (PGE_2+Indo), or 10^{-5} M forskolin in the presence of 10^{-5} M indomethacin (For+Indo). The RNA samples were quantified using Real-Time PCR using primers and probes for PPARδ. GAPDH was used as an internal control. Expression is presented as amount of PPARδ mRNA expression divided by GAPDH expression. Values are means ± S.E.M.; n = 5.


PPAR delta mRNA/GAPDH
0
1
2
3
4
5
6
7
Control
Indo
PGE2 + Indo
For + Indo
2 hr
8 hr
24 hr

3.6: GW501516 stimulates DNA synthesis in IMCD-K2 cells. PPARδ has been known to affect cell proliferation and as a result it was assessed what effect a PPARδ agonist, GW501515, would have on DNA synthesis. IMCD-K2 cells were stimulated with serum-free media (control, **Figure 8A**); 10 % fetal bovine serum (FBS, **Figure 8B**), which served as a positive control for growth stimulation; or 10^{-6} M GW501516 (GW, **Figure 8C**) for 24 hours. The cells were incubated with BrdU, which is incorporated into cells during the synthesis of DNA. A DAPI stain was used to establish the total number of cells that were present and the number of cells that were BrdU-positive (undergoing DNA synthesis) was represented as percent of the DAPI-positive cells. As shown in **Figure 8D**, the numbers of percent BrdU-positive cells were 16.2, 28.2 and 31.5 for control, FBS and GW-treated cells, respectively. The FBS- and GW-treated cells were approximately 1.7- and 1.9- fold control, respectively.

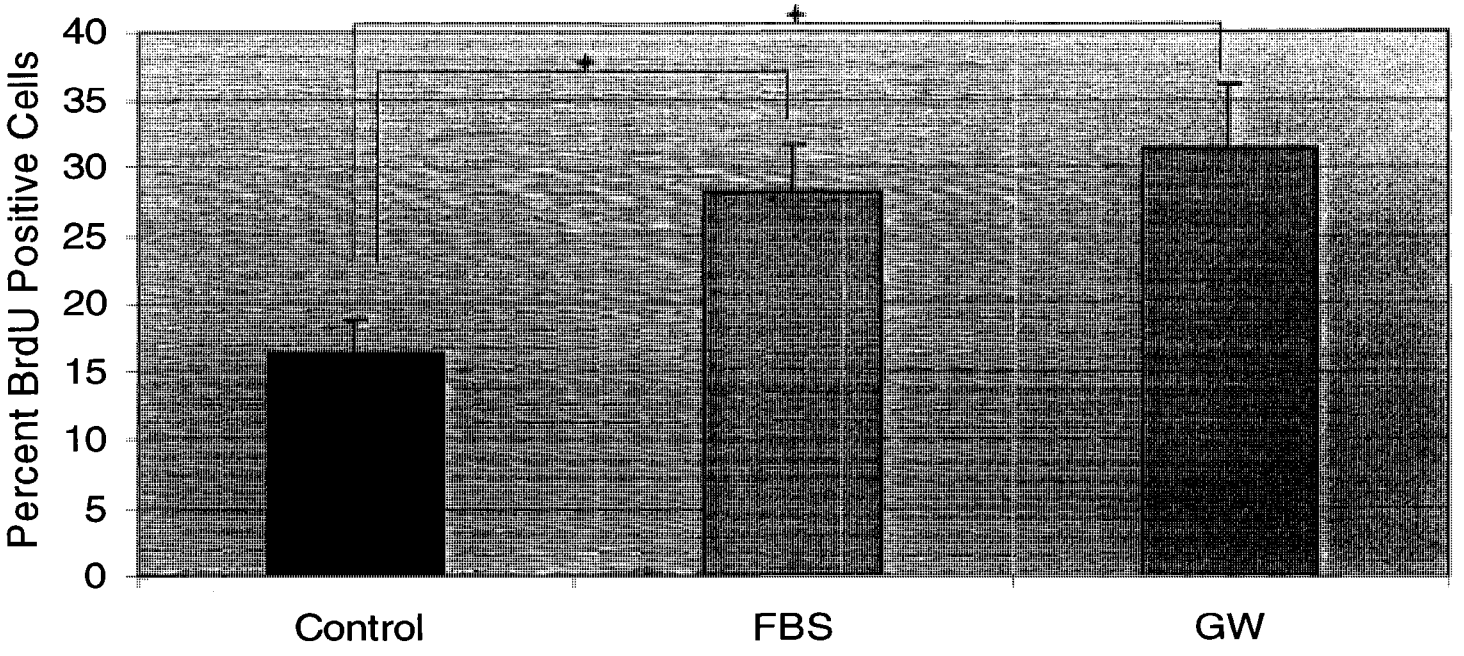
Figure 8. GW501516 stimulates DNA synthesis in IMCD-K2 cells. IMCD-K2 cells were treated with A) serum-free media (control), B) 10% FBS, or C) 10^{-6} M GW501516 for the 24 hours. The cells were then incubated with the 5-bromo-2-deoxyuridine (BrdU) labeling reagent for two hours, followed by a 90-minute incubation with anti-BrdU and 30 minutes with anti-mouse IgG fluorescein and DAPI. The cells were mounted on slides, visualized with a fluorescent microscope and captured with a camera. The DAPI-stained nuclei (blue) were counted and a mask of their position was applied to the picture from the green filter in the same field of view to determine the percent of nuclei that were BrdU-positive (green) as shown in D). Values are means ± S.E.M.; n = 4. $^{+}$ $p < 0.05$

A)

B)

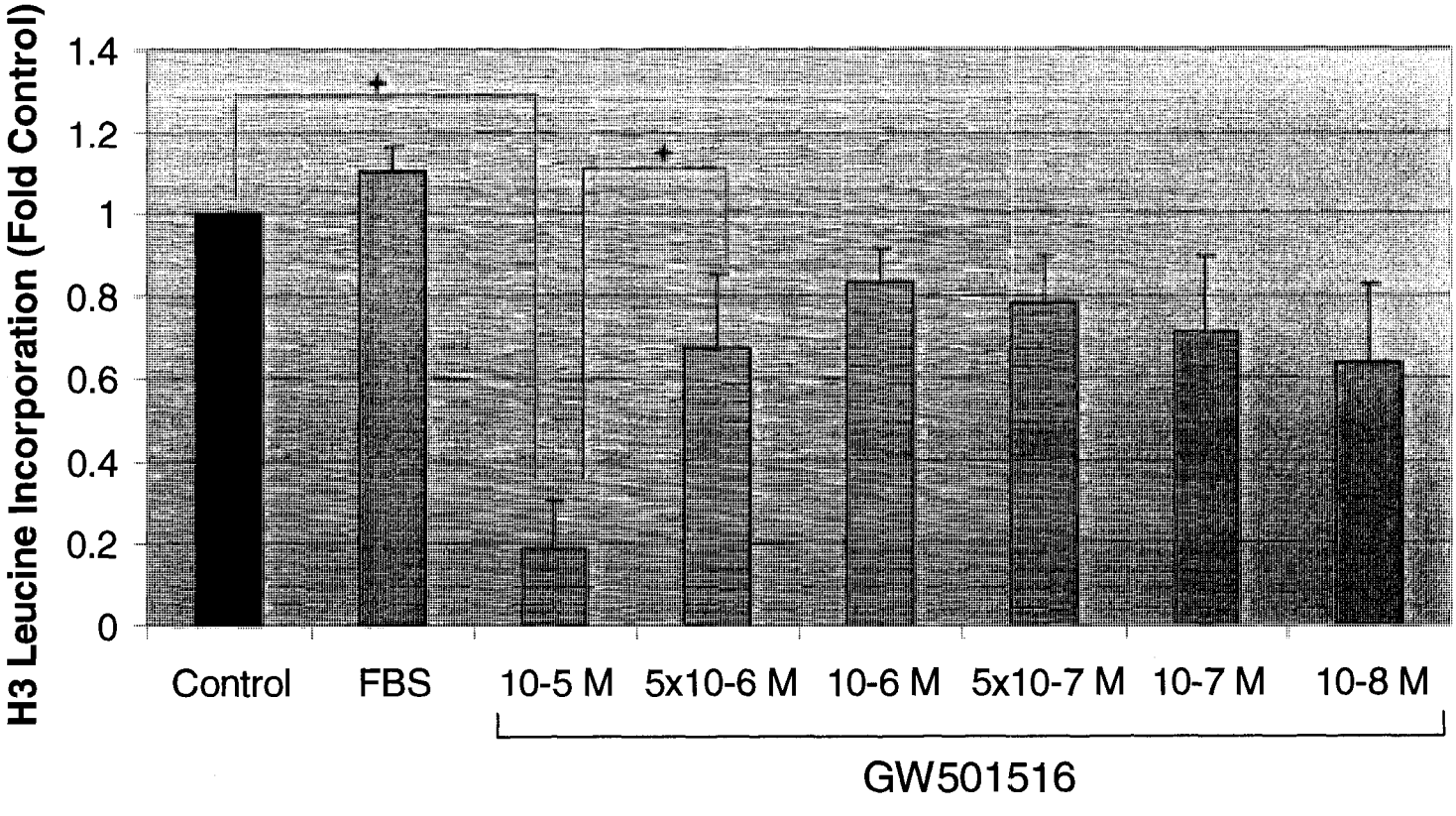
C)

D)


Percent BrdU Positive Cells
40
35
30
25
20
15
10
5
0
Control
FBS
GW

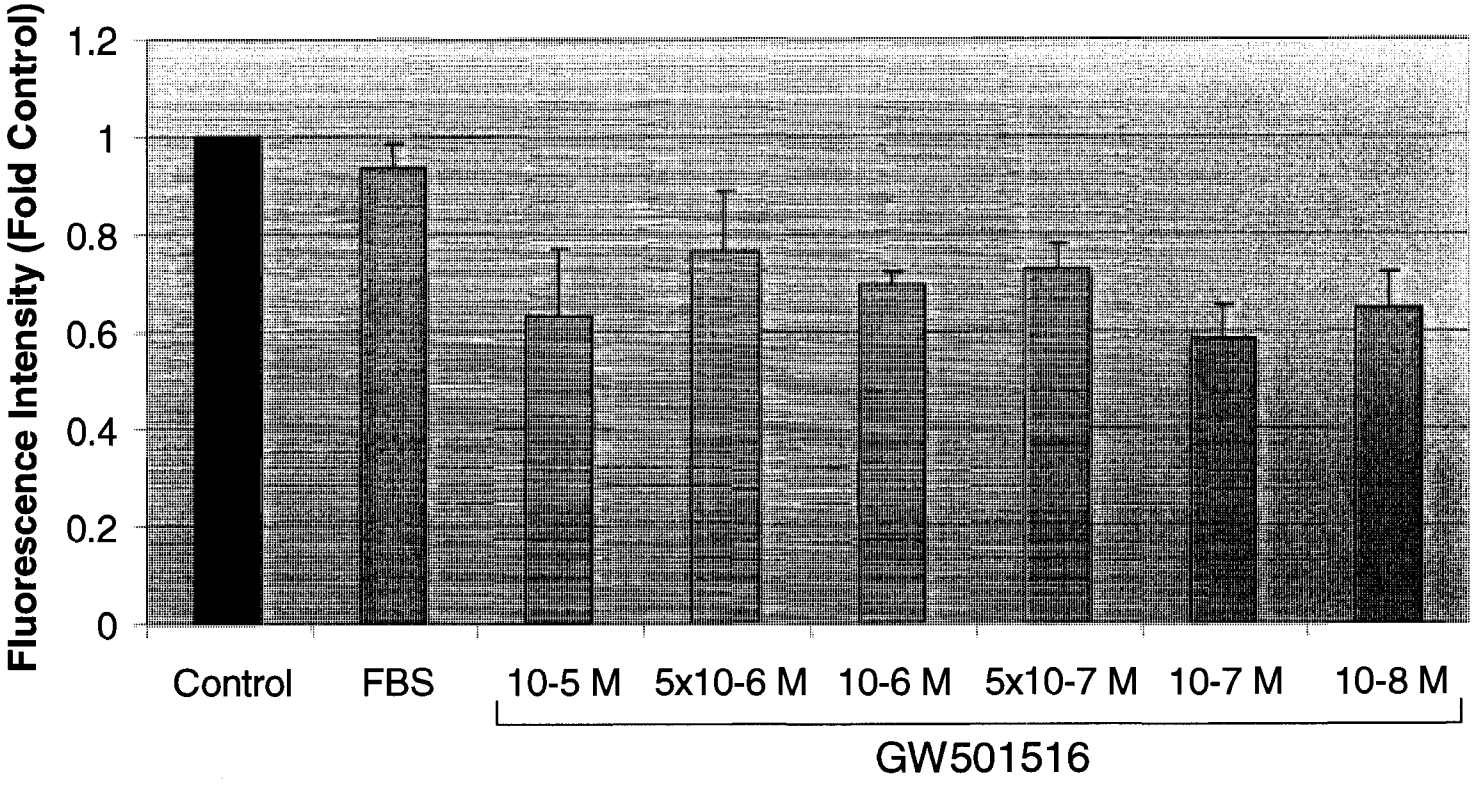
3.7: GW501516 causes a decrease in protein synthesis. The effect of GW501516, a PPARδ agonist, was also examined with regards to cell growth in IMCD-K2 cells. This was done through ^{3}H-leucine incorporation experiments, in which an increase in leucine incorporation corresponded to an increase in protein synthesis. IMCD-K2 cells were stimulated with serum-free media (control), 10% FBS, or decreasing concentrations of GW501516. As shown in **Figure 9**, treatment with 10^{-5} M GW501516 caused a significant decrease in protein synthesis to approximately 0.19-fold of control. The remaining concentrations of $5x10^{-6}$, 10^{-6}, $5x10^{-7}$, 10^{-7}, and 10^{-8} M were approximately 0.67-, 0.83-, 0.78-, 0.72-, and 0.64-fold of control, respectively.

Figure 9. GW501516 causes a decrease in protein synthesis. Serum-starved cells were stimulated for 24 hours with control (serum-free media), 10% FBS, or decreasing concentrations of GW501516 (10^{-5} to 10^{-8} M). ^{3}H-leucine was added to IMCD-K2 cells while they were being stimulated and leucine incorporation was measured in counts per minute using a scintillation counter, and expressed as fold control. Values are means ± S.E.M.; n = 3-5. $^{+}$ $p < 0.05$


H3 Leucine Incorporation (Fold Control)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
FBS
10-5 M
5x10-6 M
10-6 M
5x10-7 M
10-7 M
10-8 M
GW501516

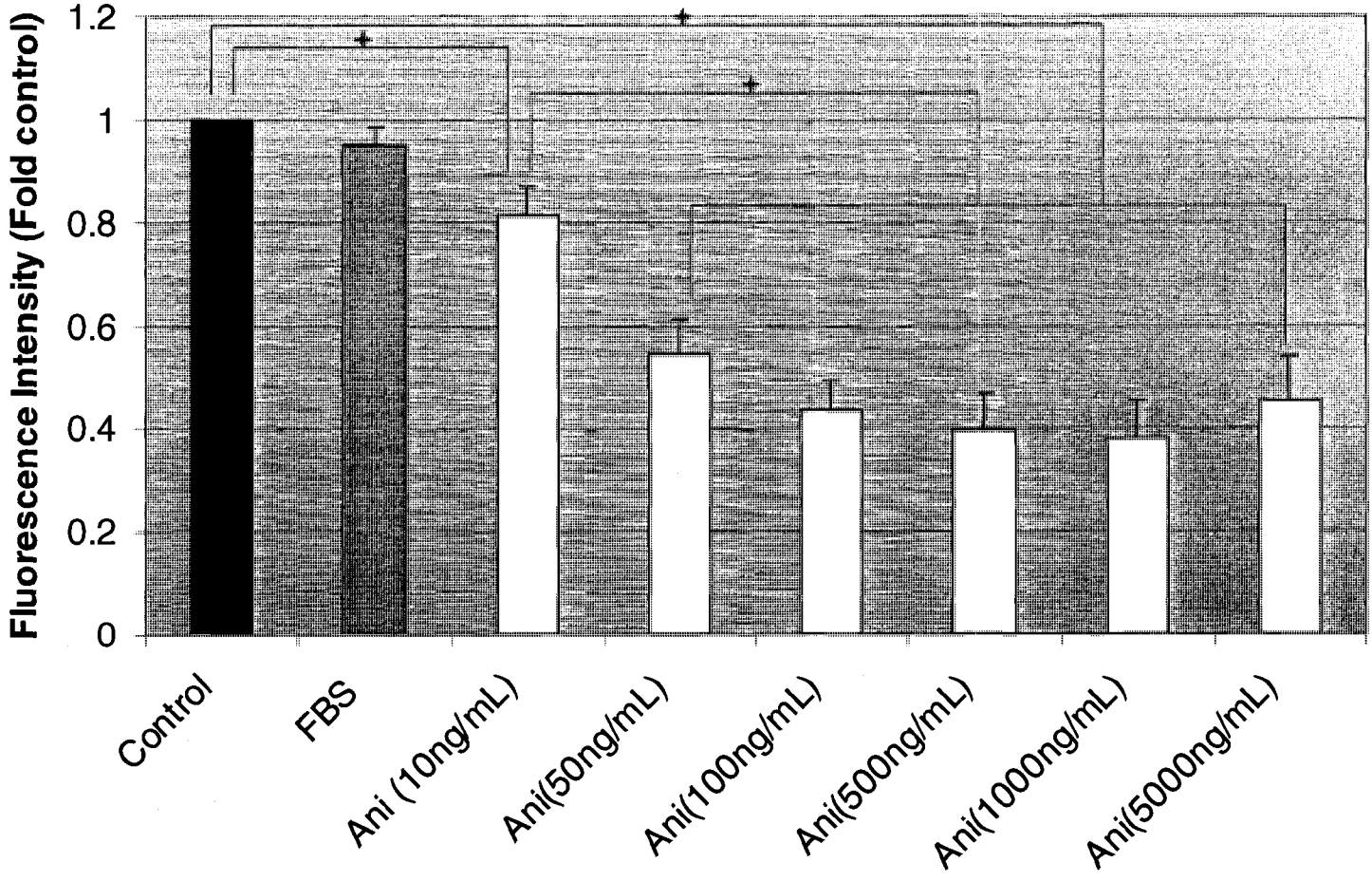
3.8: GW501516 does not significantly alter IMCD-K2 cell viability. To further characterize the effect of GW501516 on IMCD-K2 cells, its effects on cell viability were studied. Cells were stimulated with serum-free media (control), 10 % FBS, or decreasing amounts of GW501516 and a DNA dye was applied to the cells that caused the cells to fluoresce. Fluorescence intensity was then measured using a microplate reader. Since the CyQUANT NF assay measures cellular DNA content via the dye and because cellular DNA content is highly regulated the fluorescence values are closely proportional to cell number. All cells for each experiment were passaged from the same plate and each sample was done in triplicate to ensure even distribution and to control for the number of cells. As shown in **Figure 10,** all of the concentrations of GW501516 tended to decrease cell viability between 0.8- and 0.6–fold of control but the changes in cell viability were not significant.

Figure 10. GW501516 does not significantly alter IMCD-K2 cell viability. Serum-starved cells were stimulated for 24 hours with control (serum-free media), 10 % FBS, or decreasing concentrations of GW501516 (10^{-5} to 10^{-8} M). The cells were then incubated for one hour with the DNA dye binding solution from the CyQUANT NF assay kit (Invitrogen). Fluorescence intensity, representing number of cells, was then measured as per the manufacturer's instructions. Values (fold control) are means ± S.E.M.; n =3.


Fluorescence Intensity (Fold Control)
1.2
1
0.8
0.6
0.4
0.2
0
Control
FBS
10-5 M
5x10-6 M
10-6 M
5x10-7 M
10-7 M
10-8 M
GW501516

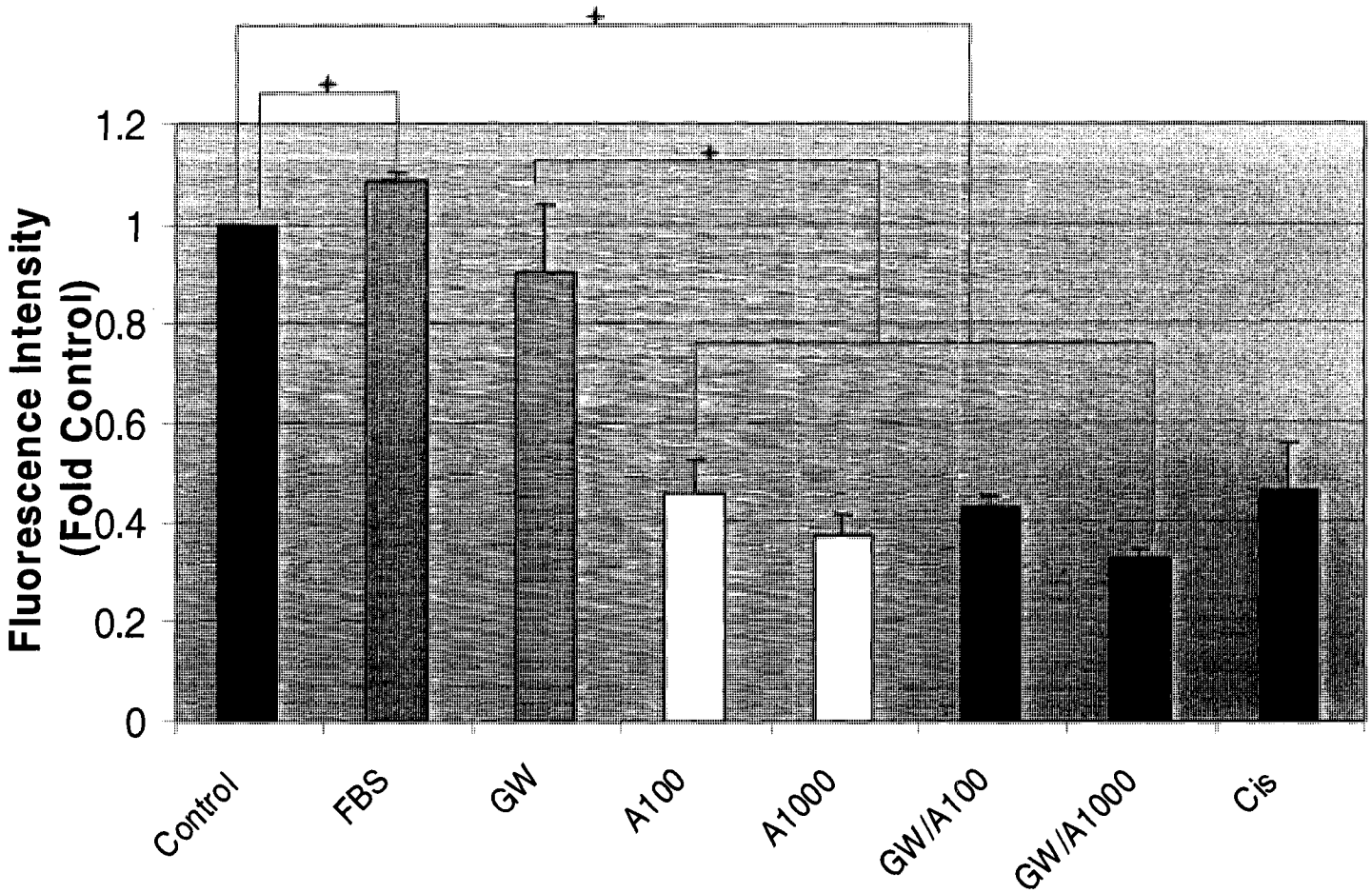
3.9: Anisomycin causes a decrease of IMCD-K2 cell viability. To establish a proper death response in IMCD-K2 cells it was determined if treatment with anisomycin, a known inducer of cell death through the inhibition of protein synthesis, could successfully produce a reduction in cell viability, and subsequent increase in apoptosis, in IMCD-K2 cells. The cells were treated with serum-free media (control), 10% FBS, or increasing concentrations of anisomycin. Cell viability was measured as described in section **3.8**. As shown in **Figure 11**, the anisomycin-treated cells (10-5000 ng/mL) had decreases in cell viabilities of approximately 0.83-, 0.55-, 0.44-, 0.40-, 0.38-, and 0.45-fold of control. All concentrations of anisomycin caused a significant decrease in the amount of cells compared to control.

Figure 11. Anisomycin causes a decrease of IMCD-K2 cell viability. Serum-starved cells were stimulated for 24 hours with control (serum-free media), 10 % FBS, or increasing concentrations of anisomycin (10- 5000 ng/mL) for 18 hours. The cells were then incubated for one hour with the DNA dye binding solution from the CyQUANT NF assay kit (Invitrogen). Fluorescence intensity, representing number of cells, was then measured as per the manufacturer's instructions. Values are means ± S.E.M.; n = 3-4. + $p < 0.05$


Fluorescence Intensity (Fold control)
1.2
1
0.8
0.6
0.4
0.2
0
Control
FBS
Ani (10ng/mL)
Ani(50ng/mL)
Ani(100ng/mL)
Ani(500ng/mL)
Ani(1000ng/mL)
Ani(5000ng/mL)

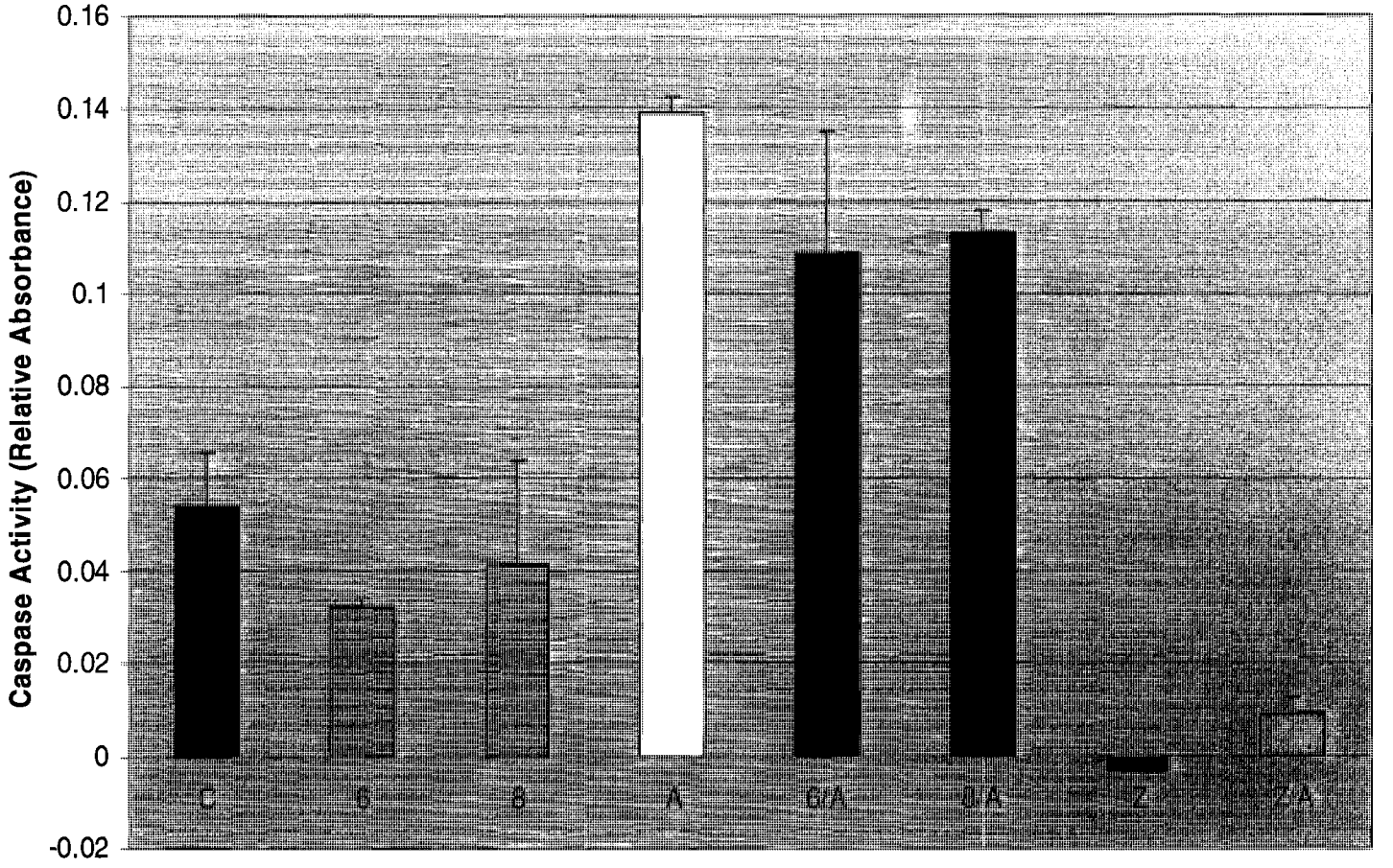
3.10: GW501516 has no effect on anisomycin-induced cell death. In an effort to examine if the PPARδ agonist could protect cells from an induced death, IMCD-K2 cells were treated with serum-free media (control), 10% FBS, anisomycin, GW501516 (in the presence or absence of anisomycin), or cisplatin (a positive control for cell death) and the effect of cell viability was examined. The cells that were co-treated with GW501516 and anisomycin were also pretreated with the agonist for 24 hours prior to anisomycin stimulation. As shown in **Figure 12**, anisomycin (100 and 1000 ng/mL) caused cell viability to decrease to approximately 0.46- and 0.37–fold of control, respectively. 10^{-6} M GW501516 did not alter cell viability alone nor did it significantly change the decrease in cell viability seen with the addition of either 100 or 1000 ng/mL of anisomycin.

Figure 12. GW501516 has no effect on anisomycin-induced cell death. Serum-starved cells were stimulated with control (serum-free media), 10% FBS, 10^{-6} M GW501516, or anisomycin (100, 1000 ng/mL) for 18 hours. Some of the cells treated with anisomycin were pretreated with 10^{-6} GW501516 for 24 hours and co-treated with the agonist and anisomycin for 18 hours. The cells were then incubated for one hour with the DNA dye binding solution from the CyQUANT NF assay kit (Invitrogen). Fluorescence intensity, representing number of cells, was then measured as per the manufacturer's instructions. Values are means ± S.E.M.; n = 3. $^{+}$ $p < 0.05$


Fluorescence Intensity
(Fold Control)
1.2
1
0.8
0.6
0.4
0.2
0
Control
FBS
GW
A100
A1000
GW/A100
GW/A1000
Cis

3.11: Anisomycin increases caspase activity in IMCD-K2 cells, independent of GW501516. Besides having effects in cell growth, PPARδ has also been linked to changes in apoptosis. Therefore, the ability of GW501516 to affect apoptotic levels was evaluated in IMCD-K2 cells. Caspase-3 activity was first examined because it is a downstream effector protease that is common to all apoptotic pathways. Cells were treated with serum-free media (control), different concentrations of GW501516, anisomycin (to promote apoptosis), and Z-VAD-FMK, a pan-caspase inhibitor. The cells were treated with anisomycin alone or were co-treated with 10^{-8} or 10^{-6} M GW501516. The co-treated cells were also pretreated with the agonist for 24 hours prior to anisomycin stimulation. As shown in **Figure 13**, anisomycin treatment resulted in an increase of caspase-3 activity levels of about 2.59-fold of control. Pretreatment with GW501516 showed a decrease that was 0.79-fold (10^{-6} M) and 0.81-fold (10^{-8} M) of anisomycin alone. The concentrations of GW501516 alone showed no considerable modification of caspase activity. The caspase inhibitor, Z-VAD, completely abolished caspase activity in unstimulated cells, and in anisomycin treated cells it decreased caspase activity to 0.06-fold of anisomycin alone.

Figure 13. Anisomycin increases caspase activity in IMCD-K2 cells, independent of GW501516. Serum-starved cells were treated with control serum-free media (C) for 24 hours, 250 ng/mL anisomycin (A) for 24 hours, 10^{-6} M GW501516 and 10^{-8} M GW501516 for 24 hours pretreatment followed by 10^{-6} M and 10^{-8} M GW501516 for 24 hours with (6/A and 8/A) or without anisomycin (6 and 8), Z-VAD-FMK, a pan-caspase inhibitor with (Z/A) or without (Z) anisomycin. Values are means ± S.E.M.; n = 2.


Caspase Activity (Relative Absorbance)
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
-0.02
C
G
S
A
G/A
Q/A
Z
Z/A

3.12: GW501516 pretreatment has no effect on anisomycin-induced apoptosis. In addition to looking at the effect of GW501516 on caspase activity, TUNEL assays were performed. IMCD-K2 cells were treated with serum-free media (control), two different concentrations of GW501516, or anisomycin. Cells were either treated with anisomycin alone or co-treated with anisomycin and 10^{-8} or 10^{-6} M GW501516 following a pretreatment with the agonist. After stimulation the cells were treated with terminal transferase and biotin-16-dUTP, allowing the free DNA fragments created during apoptosis to be labeled and visualized. DAPI was used to stain all cells. As shown in **Figure 14**, the number of TUNEL-positive cells tended to increase while the total number of cells appeared to decrease with the addition of 250 ng/mL anisomycin when compared to control or the PPARδ agonist (at both concentrations). Co-treatment of 10^{-6} M or 10^{-8} M GW501516 with anisomycin was comparable to anisomycin alone.

Figure 14. GW501516 pretreatment has no effect on anisomycin-induced apoptosis. Serum-starved cells were stimulated with control (serum-free media; C), 10^{-6} M GW501516 (6), 10^{-8} M GW501516 (8), 250 ng/mL anisomycin (A) for 24 hours, 10^{-6} M or 10^{-8} M GW501516 for 3 hours pretreatment followed by 10^{-6} M GW501516 for 24 hours with A (6/A and 8/A). The cells were incubated with terminal transferase and biotin-16-dUTP for one hour, followed by two 45-minute incubations with Streptavidin-Cy2 and DAPI. The cells were then mounted on slides, visualized with a fluorescent microscope and captured with a camera. Apoptotic cells are those with a nucleus that fluoresce green, while the blue represents DAPI-positive cells.

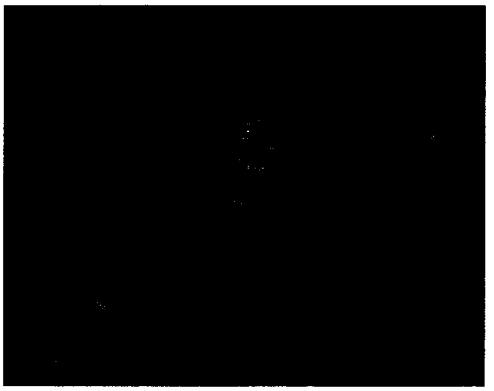
C

6

8

A

6/A

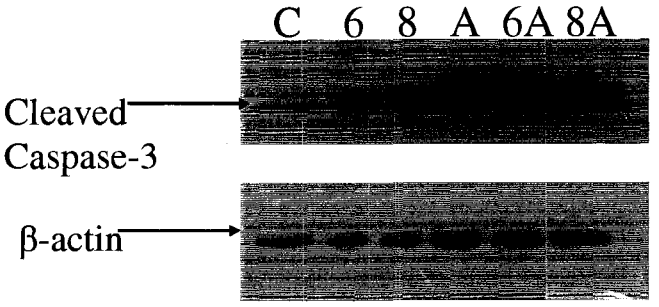


8/A

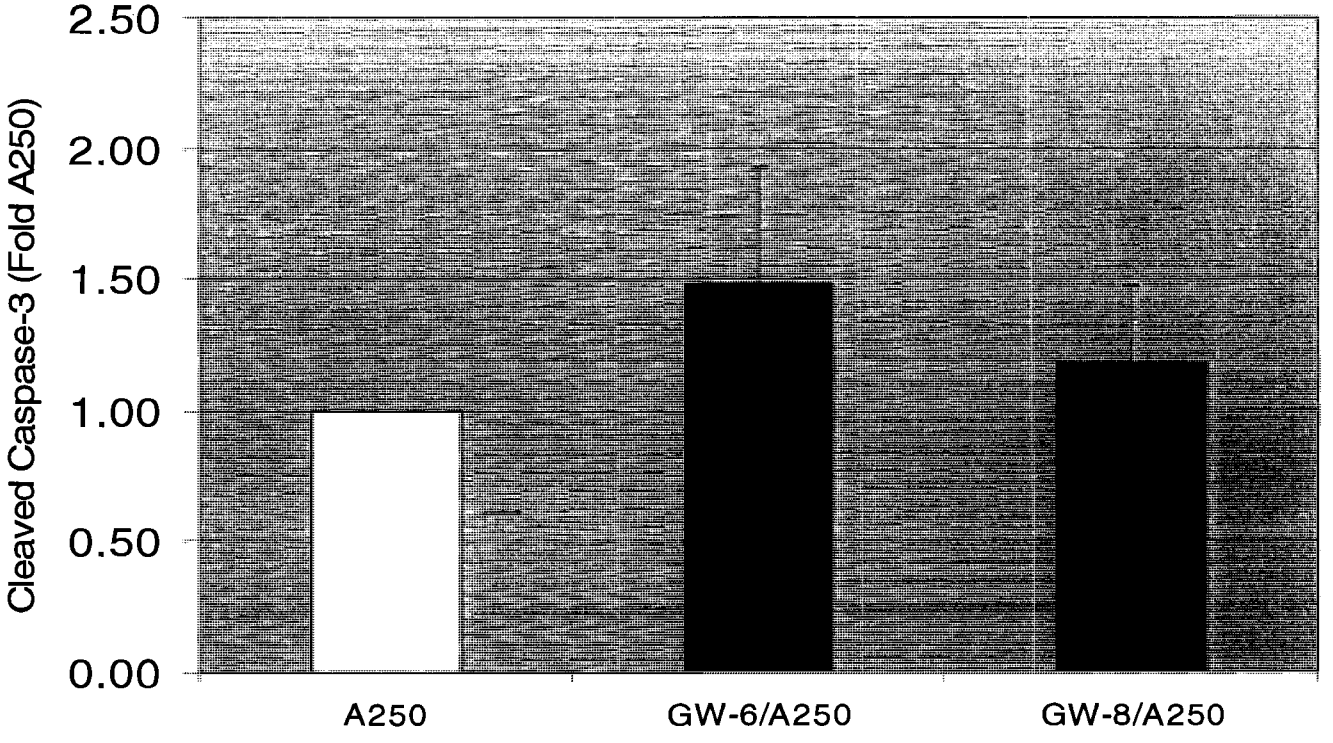
3.13: Pretreatment with GW501516 does not significantly alter anisomycin-induced cleaved caspase-3 protein in IMCD-K2 cells. The effect of GW501516 on apoptosis in cells treated with anisomycin was examined using Western blotting. IMCD-K2 cells were treated with serum-free media (control), two different concentrations of GW501516, or anisomycin in the presence or absence of GW501516. As shown in **Figure 15A**, a 17 kDa band corresponding to cleaved caspase-3 was observed. The intensity of the bands in the lanes representing control and GW501516 alone was too low to be analyzed in most of the blots; therefore densitometric analysis was not performed. The levels of active caspase-3 (**Figure 15B**) increased to 1.48- and 1.18-fold of anisomycin alone with both co-treatments (10^{-6} and 10^{-8} M GW501516, respectively) but the difference was not significant.

Figure 15. Pretreatment with GW501516 does not significantly alter anisomycin-induced cleaved caspase-3 protein in IMCD-K2 cells. Protein was isolated from IMCD-K2 cells that had been treated with control serum-free media (C), 10^{-6} M GW501516 (GW-6 or 6), 10^{-8} M GW501516 (GW-8 or 8), 250 ng/mL anisomycin (A250 or A) for 24 hours, GW-6 for 3 hours pretreatment followed by GW-6 for 24 hours with A250, or GW-8 for 3 hours pretreatment followed by GW-8 for 24 hours with A250. A) The lysates were run on an SDS-PAGE gel and incubated with an anti-cleaved caspase-3 antibody (1:1000). The membranes were stripped and incubated with anti-β-actin antibody (1:10000) to normalize for densitometry (B). Expression is presented as fold A250. Values are means ± S.E.M.; n = 8.

A)


C 6 8 A 6A 8A
Cleaved Caspase-3
β-actin

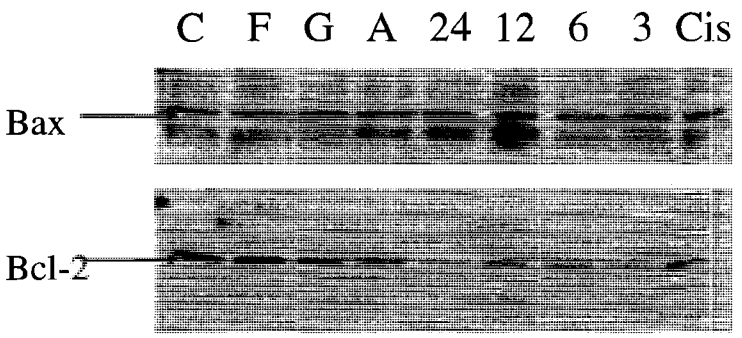
B)


Cleaved Caspase-3 (Fold A250)
2.50
2.00
1.50
1.00
0.50
0.00
A250
GW-6/A250
GW-8/A250

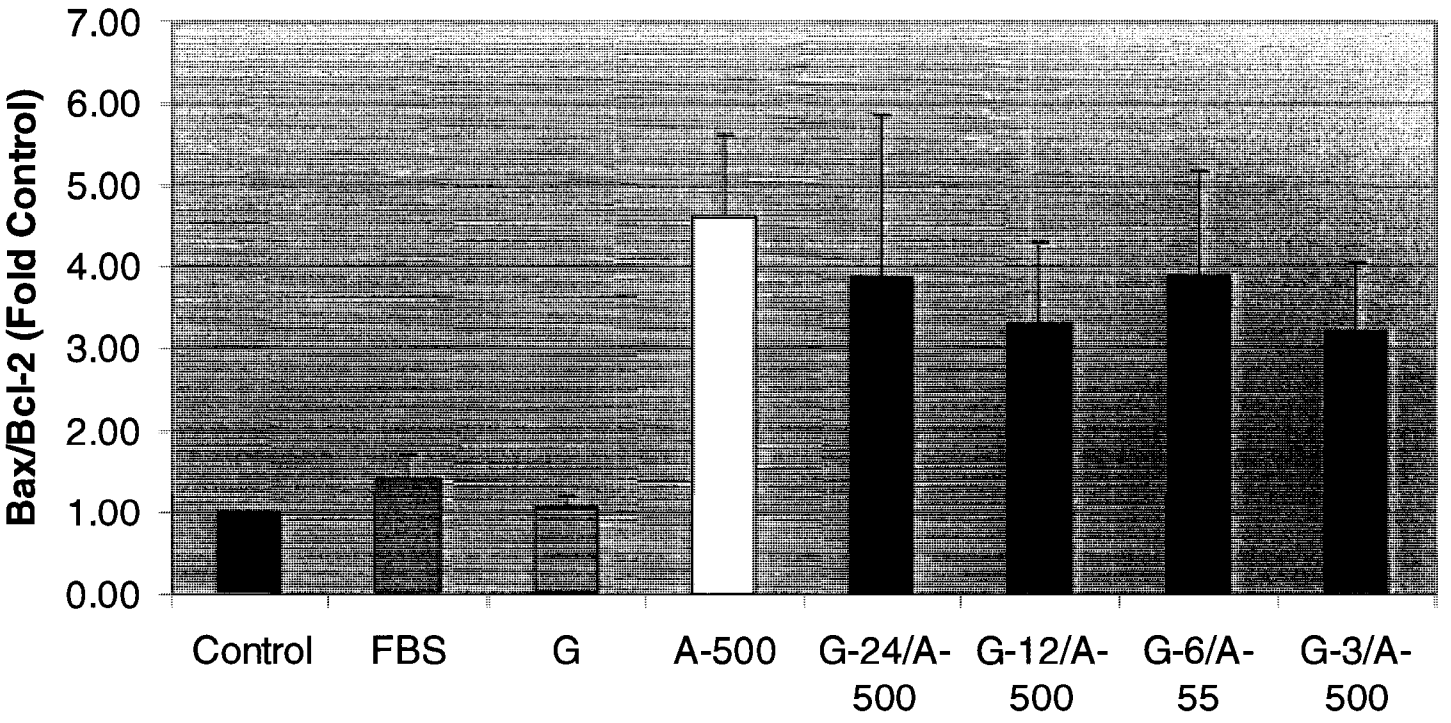
3.14: Pretreatment with GW501516 does not alter the Bax/Bcl-2 ratio. To further study the effect of GW501516 on specific apoptotic pathways, the levels of Bax (Bcl-2 associated X protein) and Bcl-2 (B-cell lymphoma 2) were investigated. Both proteins are from the Bcl-2 family and are involved in outer membrane permeabilization; however Bax is pro-apoptotic whereas Bcl-2 is anti-apoptotic. The proteins are involved in the mitochondrial pathway of apoptosis and an increase in the ratio of Bax:Bcl-2 indicates an activation of this pathway. IMCD-K2 cells were stimulated with serum-free media (control), 10% FBS, GW501516, cisplatin, or anisomycin in the absence or presence of 10^{-6} M GW501516. Those that were co-treated were also pretreated with the agonist prior to anisomycin stimulation for 24, 12, 6, or 3 hours. As shown in **Figure 16A**, 23 kDa and 25 kDa bands, representing Bax and Bcl-2 proteins, respectively, was observed. As shown in **Figure 16B**, stimulation of the cells with 500 ng/mL anisomycin caused an increase in the Bax/Bcl-2 ratio that was approximately 4.5-fold of control. Pretreatment with 10^{-6} M GW501516 did not significantly alter the ratio compared to anisomycin alone. The FBS or GW501516 alone had no effect on the ratio.

Figure 16. Pretreatment with GW501516 does not alter the Bax/Bcl-2 ratio. Protein was isolated from IMCD-K2 cells that had been treated with serum-free media (Control), 10 % FBS, 10^{-6} M GW501516 (GW), 500 ng/mL anisomycin for 24 hours, or 50 ng/mL cisplatin (Cis). Some of the cells treated with anisomycin were pretreated with 10^{-6} GW501516 for 24, 12, 6, or 3 hours. A) The proteins were run on an SDS-PAGE gel and Western blotted using an anti-Bax antibody (1:850). The membranes were stripped and blotting was done with an anti-Bcl-2 antibody (1:750). B) The intensity of the bands for Bax were taken and divided by the intensity of the Bcl-2 bands. Values are means ± S.E.M.; n = 3-4.

A)


C F G A 24 12 6 3 Cis
Bax
Bcl-2

B)

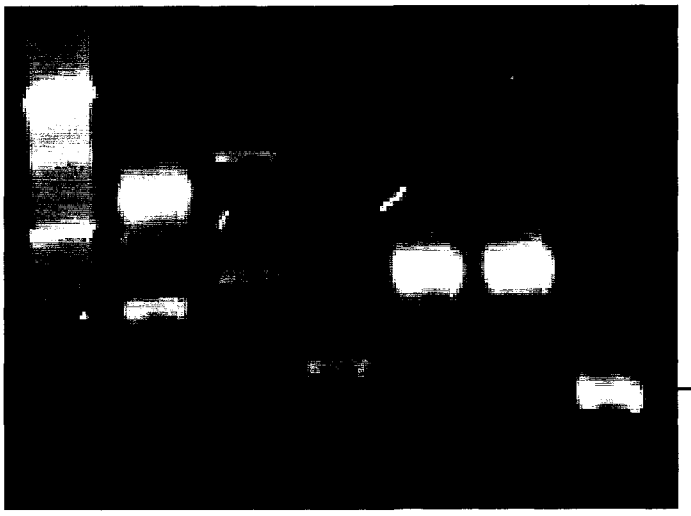

Bax/Bcl-2 (Fold Control)
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Control
FBS
G
A-500
G-24/A-500
G-12/A-500
G-6/A-55
G-3/A-500

3.15: PPARδ and PPARγ are expressed in M-1 cells. The IMCD-K2 cells appeared to be resistant to several treatments and showed much variability from passage to passage and because of this we wished to study another cell line. As a result, the expression of PPARs was also examined in another collecting duct cell line, the M-1 cells. As shown in **Figure 17A**, bands were present in all lanes, however only bands in the EP_1 (lower band of 336 bp), EP_3 (band of approximately 250 bp), EP_4 (band of 423 bp), and PPARδ (band of 161 bp) lanes actually represent the transcripts of the expected receptor. At higher stringency the EP_2 bands disappeared. The higher band in the EP_1 lane corresponds to PKN. Negative control (no reverse transcriptase used) produced no bands in any lanes (**Figure 17B**). In addition to PPARδ, PPARγ protein was detected in M-1 cells (**Figure 17C**). Western blotting produced a band that was consistent with the size of PPARγ (67 kDa) and this was also detected in the positive control (rat kidney cortex). Negative control (no reverse transcriptase used) produced no bands in any lanes.

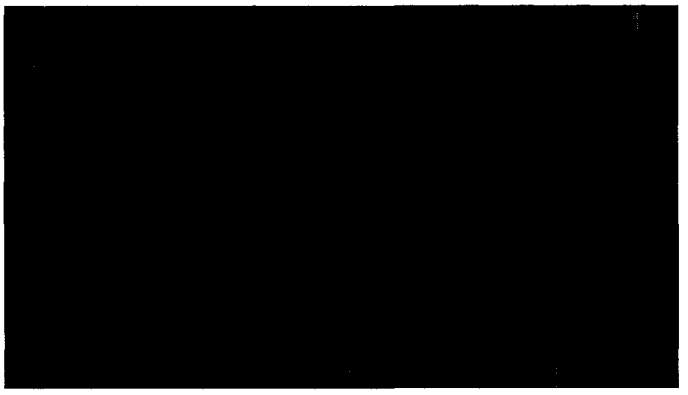
Figure 17. PPARδ and PPARγ are expressed in M-1 cells. RNA was isolated from M-1 cells using TRIzol, reverse transcribed, amplified by polymerase chain reaction using primers for the prostanoid receptors (EP_1, EP_2, EP_3, EP_4) and PPARδ, and visualized on an agarose gel using ethidium bromide under UV light. L is the DNA ladder. PCR was done A) with (RT+) and B) without (RT-) reverse transcriptase (negative control). C) Total protein was isolated from M-1 cells and run on an SDS-PAGE gel. Western blotting was performed using a PPARγ polyclonal antibody (1:1000). Rat kidney cortex (C) was used as a positive control.

A)

L EP$_1$ EP$_2$ EP$_3$ EP$_4$ PPARδ

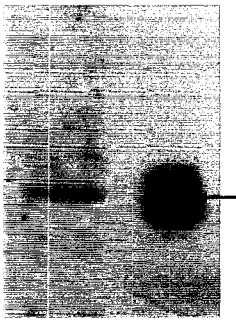


B)



C)

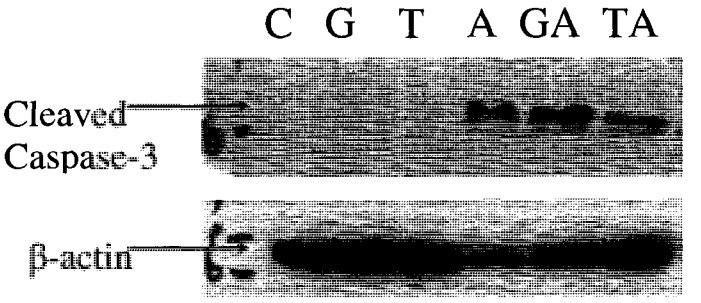
M-1 C



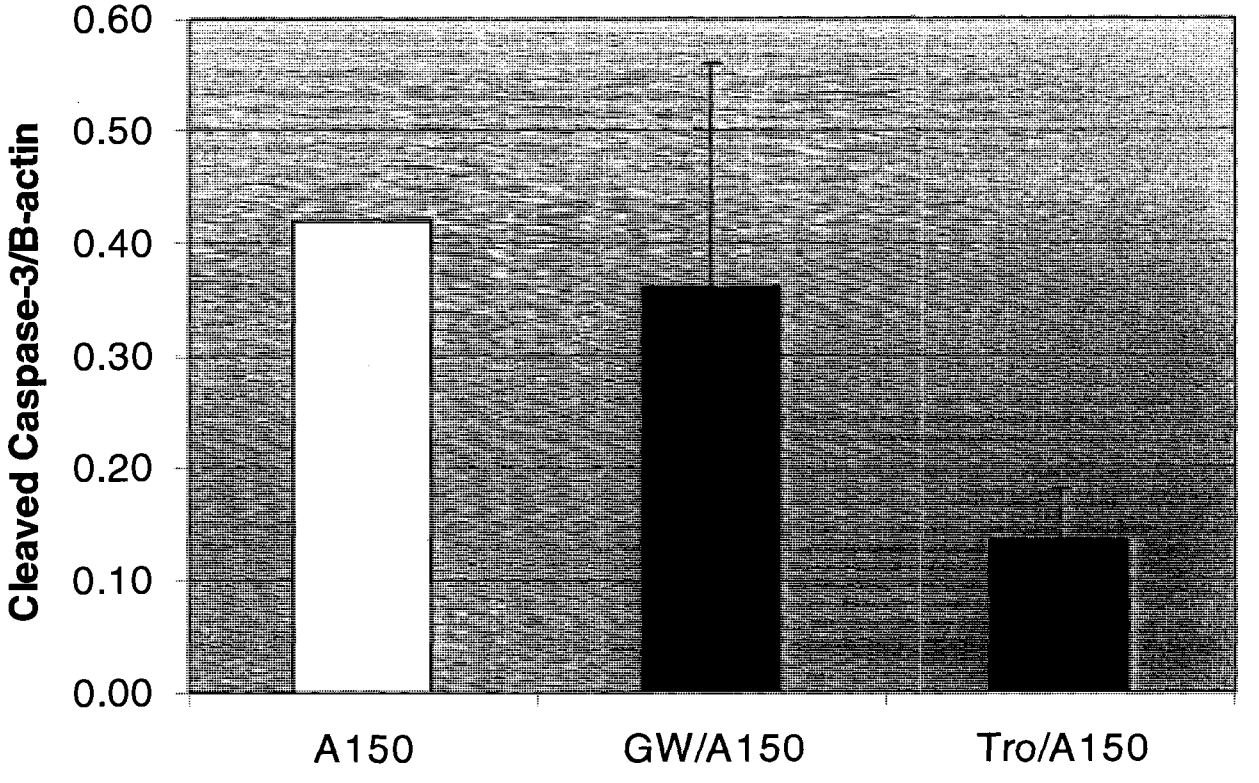
3.16: Troglitazone reduces anisomycin-induced cleaved caspase-3 protein in M-1 cells, whereas GW501516 has no effect. The role of PPARs in M-1 cells apoptosis was assessed. The cells were treated with serum-free media (control), GW501516, the PPARγ agonist troglitazone, or anisomycin. Of those cells that were treated with anisomycin some were co-treated with either GW501516 or troglitazone. The co-treated cells were also pretreated with their respective PPAR agonist. As shown in **Figure 18A**, a band corresponding to cleaved caspase-3 (17 kDa) was visualized. Stimulation of M-1 cells with GW501516 did not affect anisomycin-induced cleaved caspase-3 whereas co-treatment with troglitazone reduced the protein levels to approximately one third of the anisomycin only-treated cells (**Figure 18B**). The intensity of the bands in the lanes representing control GW501516, and troglitazone alone was too low to be analyzed.

Figure 18. Troglitazone reduces anisomycin-induced cleaved caspase-3 protein in M-1 cells, whereas GW501516 has no effect. Protein was isolated from M-1 cells that had been treated with vehicle control serum-free media (C) for 24 hours, 10^{-6} M GW501516 (GW or G) for 3 hour pretreatment followed by 24 hours, $5x10^{-6}$ M troglitazone (Tro or T) for 24 hours, 150 ng/mL anisomycin (A150 or A) for 24 hours, GW for 3 hours pretreatment followed by GW for 24 hours with A150, or Tro with A150. A) The proteins were run on an SDS-PAGE gel and Western blotted using a cleaved caspase-3 antibody (1:1000). The membranes were stripped and blotting was done with a β-actin antibody (1:10000), which was used for normalization for densitometric analysis (B). Values are means ± S.E.M.; n = 2.

A)


C G T A GA TA
Cleaved Caspase-3
β-actin

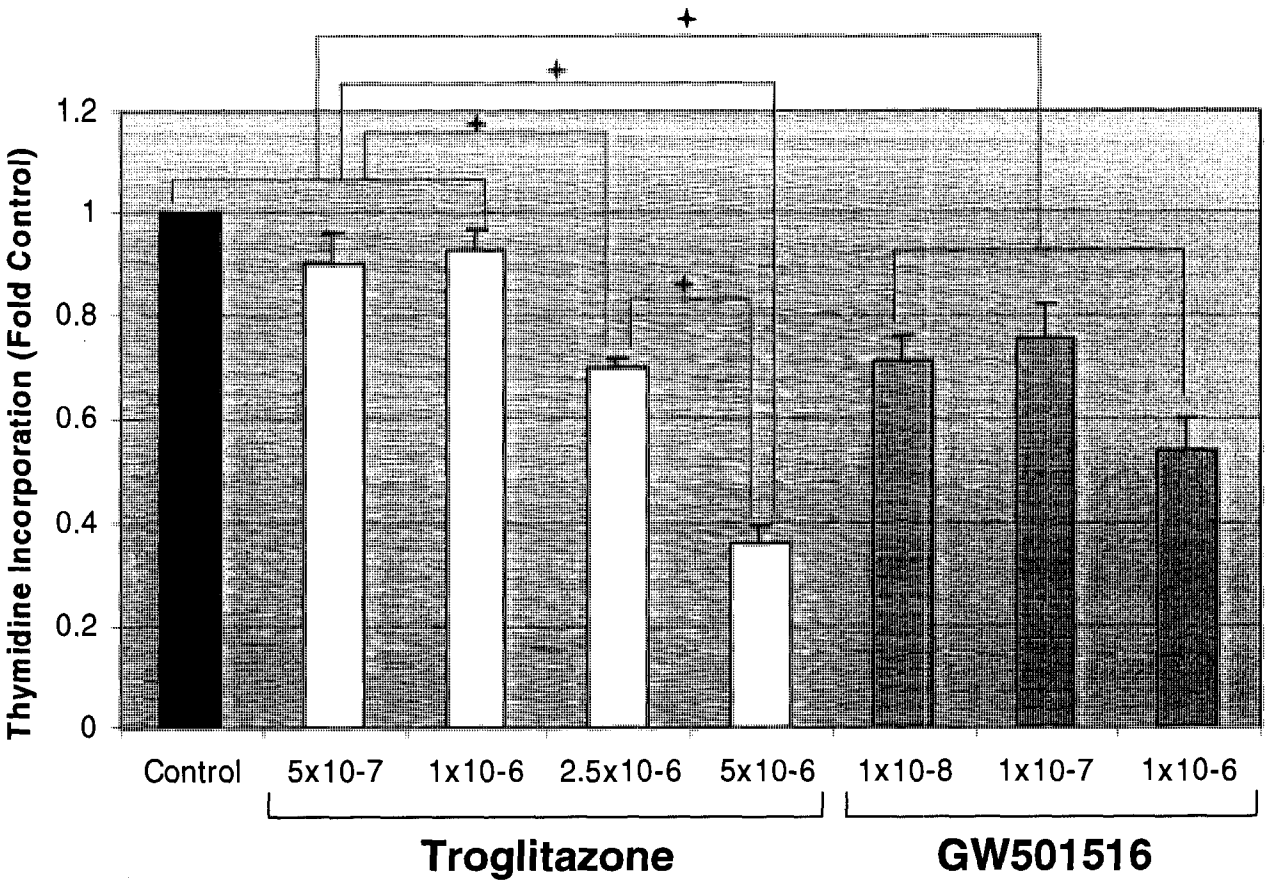
B)


Cleaved Caspase-3/B-actin
0.60
0.50
0.40
0.30
0.20
0.10
0.00
A150
GW/A150
Tro/A150

3.17: GW501516 and troglitazone treatment reduce DNA synthesis in M-1 cells.

The effect of the PPAR agonists on cell proliferation was determined through ^{3}H-thymidine incorporation experiments. An increase in thymidine incorporation corresponds to an increase in DNA synthesis. M-1 cells were stimulated with serum-free media (control), increasing concentrations of troglitazone, or increasing concentrations of GW501516. As shown in **Figure 19**, both GW501516 and troglitazone reduce thymidine incorporation in M-1 cells. For troglitazone, $2.5x10^{-6}$ and $5x10^{-6}$ M showed significant reductions that were 0.70- and 0.36-fold of control, respectively. The GW501516 doses of 10^{-8}, 10^{-7}, and 10^{-6} M showed significant reductions that were 0.72-, 0.76-, and 0.54-fold of control, respectively.

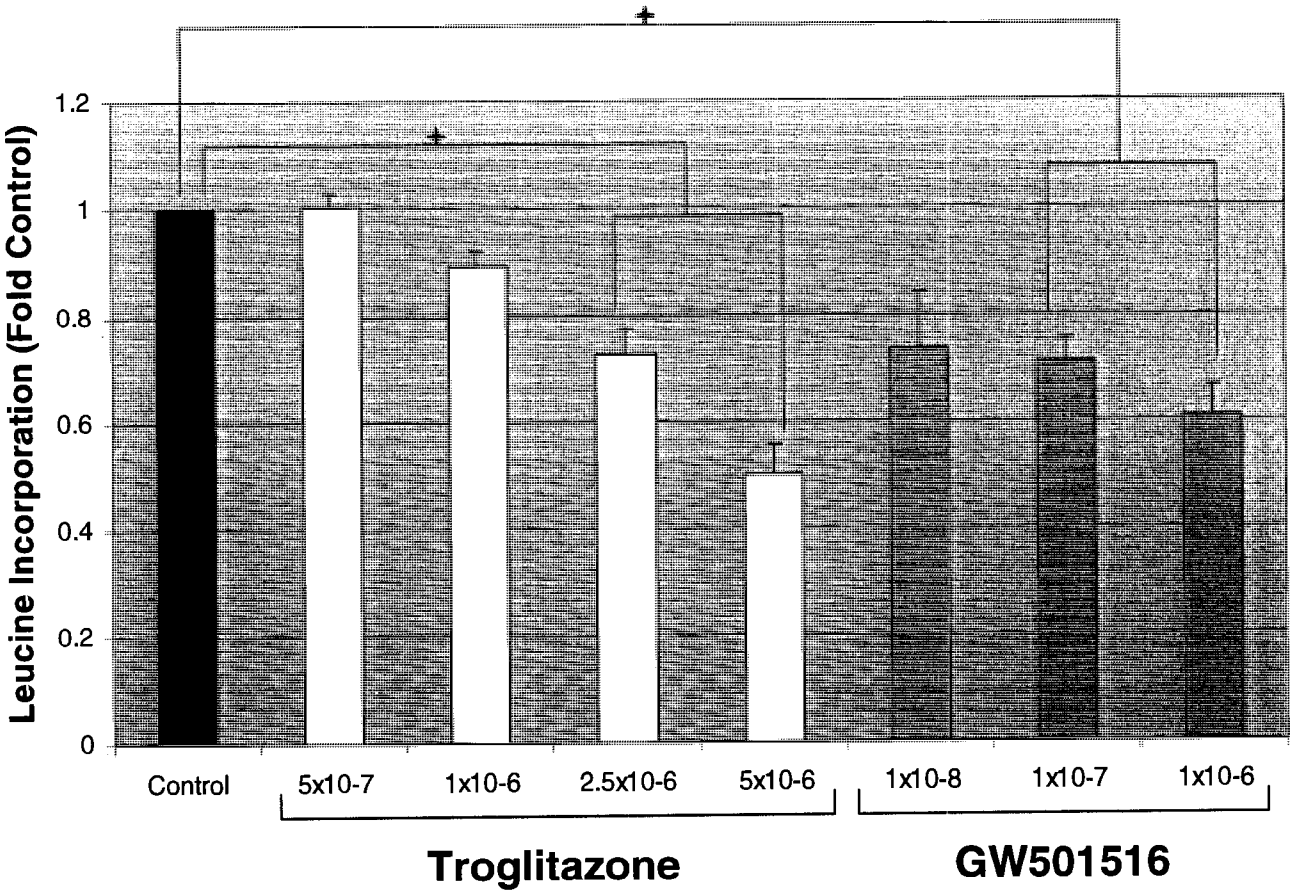
Figure 19. GW501516 and troglitazone treatment reduce DNA synthesis in M-1 cells. Serum-starved cells were stimulated for 24 hours with vehicle control (serum-free media), increasing concentrations of troglitazone ($5x10^{-7}$, 10^{-6}, $2.5x10^{-6}$, $5x10^{-6}$ M) or increasing concentrations of GW501516 (10^{-8}, 10^{-7}, 10^{-6} M). ^{3}H-thymidine was added to M-1 cells while they were being stimulated and thymidine incorporation was measured using a scintillation counter. Incorporation was measured in counts per minute and expressed as fold control. Values are means ± S.E.M.; n = 4. $^{+}$ $p < 0.05$


Thymidine Incorporation (Fold Control)
1.2
1
0.8
0.6
0.4
0.2
0
Control
5x10-7
1x10-6
2.5x10-6
5x10-6
1x10-8
1x10-7
1x10-6
Troglitazone
GW501516

3.18: GW501516 and troglitazone stimulation reduce protein synthesis in M-1 cells.

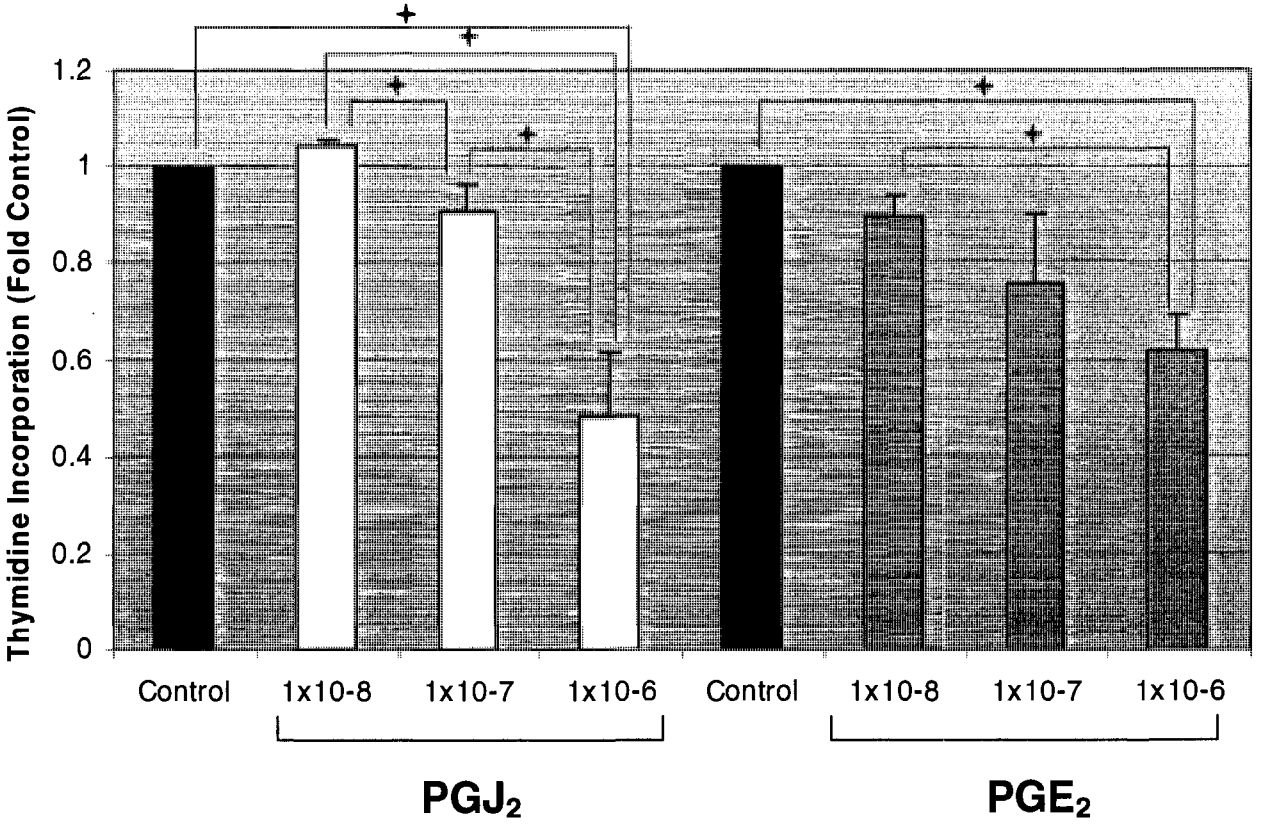
In addition to cell proliferation in M-1 cells, cell growth in response to the PPAR agonists was examined through ^{3}H-leucine incorporation. M-1 cells were stimulated with serum-free media (control), increasing concentrations of troglitazone, or increasing concentrations of GW501516. As shown in **Figure 20**, both GW501516 and troglitazone reduce thymidine incorporation in M-1 cells. For troglitazone, $2.5x10^{-6}$ and $5x10^{-6}$ M produced significant decreases in growth that were 0.73- and 0.50-fold of control, respectively. The GW501516 doses of 10^{-7} and 10^{-6} M showed significant decreases that were 0.71-, and 0.61-fold of control, respectively.

Figure 20. GW501516 and troglitazone treatment reduce protein synthesis in M-1 cells. Serum-starved cells were stimulated for 24 hours with vehicle control (serum-free media), increasing concentrations of troglitazone ($5x10^{-7}$, 10^{-6}, $2.5x10^{-6}$, $5x10^{-6}$ M) or increasing concentrations of GW501516 (10^{-8}, 10^{-7}, 10^{-6} M). ^{3}H-leucine was added to M-1 cells while they were being stimulated and leucine incorporation was measured using a scintillation counter. Incorporation was measured in counts per minute and expressed as fold control. Values are means ± S.E.M.; n = 4. + $p < 0.05$


Leucine Incorporation (Fold Control)
1.2
1
0.8
0.6
0.4
0.2
0
Control
5x10-7
1x10-6
2.5x10-6
5x10-6
1x10-8
1x10-7
1x10-6
Troglitazone
GW501516

3.19: PGJ_2 and PGE_2 treatment reduce DNA synthesis in M-1 cells. Since prostaglandins (PGE_2 and PGJ_2) have been known to activate PPARs, their effect on cell proliferation was investigated. M-1 cells were stimulated with serum-free media (control), increasing concentrations of PGJ_2, or increasing concentrations of PGE_2. As shown, PGJ_2 reduced thymidine incorporation in a dose-dependent manner (**Figure 21**). A significant reduction of 0.48-fold of control was obtained with 10^{-6} M PGJ_2. Similarly, PGE_2 reduced thymidine incorporation in a dose-dependent manner as well, up to 0.62-fold of control.

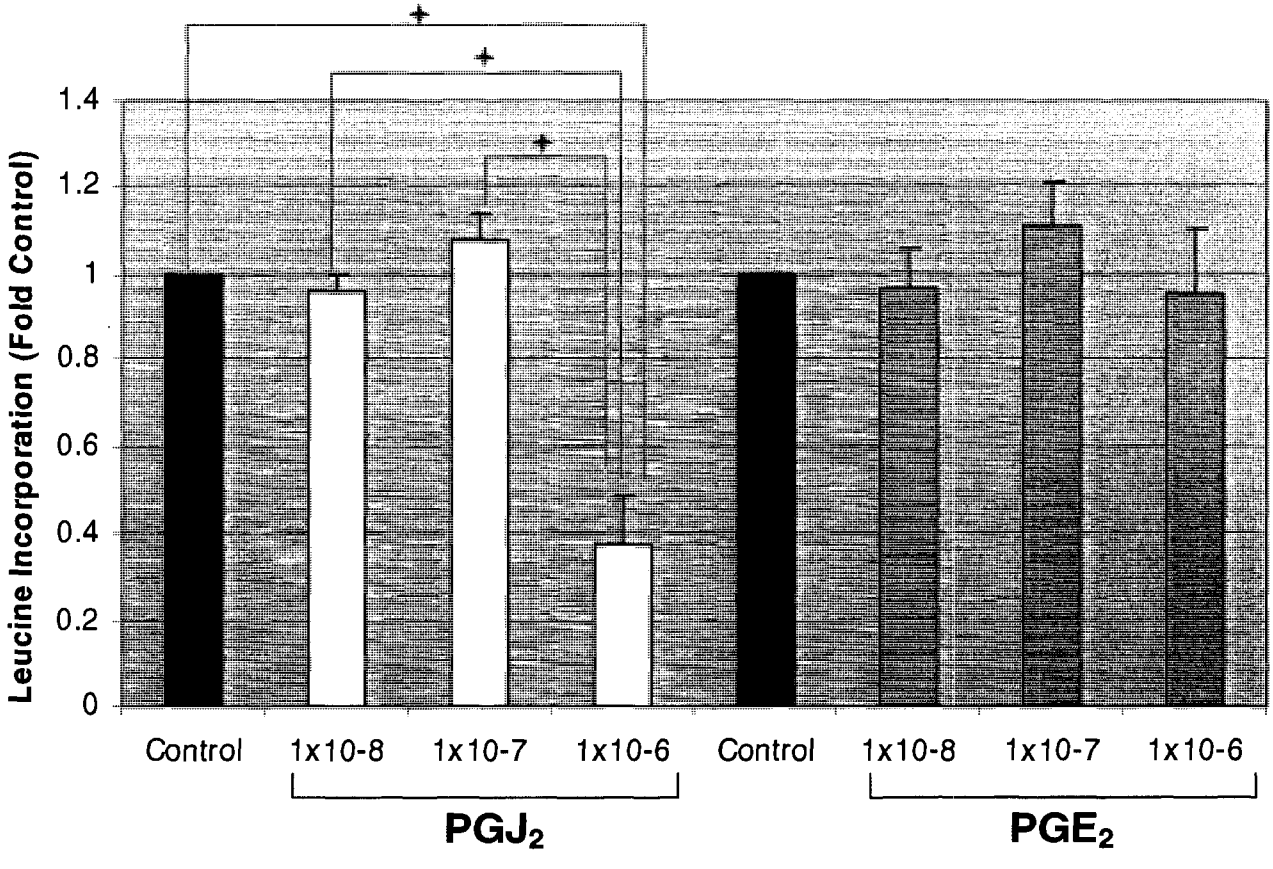
Figure 21. PGJ_2 and PGE_2 treatment reduce DNA synthesis in M-1 cells. Serum-starved cells were stimulated for 24 hours with vehicle control (serum-free media), increasing concentrations of PGJ_2 (10^{-8}, 10^{-7}, 10^{-6} M) or increasing concentrations of PGE_2 (10^{-8}, 10^{-7}, 10^{-6} M). ^{3}H-thymidine was added to M-1 cells while they were being stimulated and thymidine incorporation was measured using a scintillation counter. Incorporation was measured in counts per minute and expressed as fold control. Values are means ± S.E.M.; n = 3-4. + $p < 0.05$


Thymidine Incorporation (Fold Control)
1.2
1
0.8
0.6
0.4
0.2
0
Control
1x10-8
1x10-7
1x10-6
Control
1x10-8
1x10-7
1x10-6
PGJ2
PGE2

3.20: PGJ_2 reduces protein synthesis in M-1 cells. Additionally, the effect of PGJ_2 and PGE_2 on cell growth was examined. M-1 cells were stimulated with serum-free media (control), increasing concentrations of PGJ_2, or increasing concentrations of PGE_2. As shown in **Figure 22**, PGE_2 had no effect on protein synthesis in M-1 cells, but 10^{-6} M PGJ_2 reduced leucine incorporation to 0.38-fold of control.

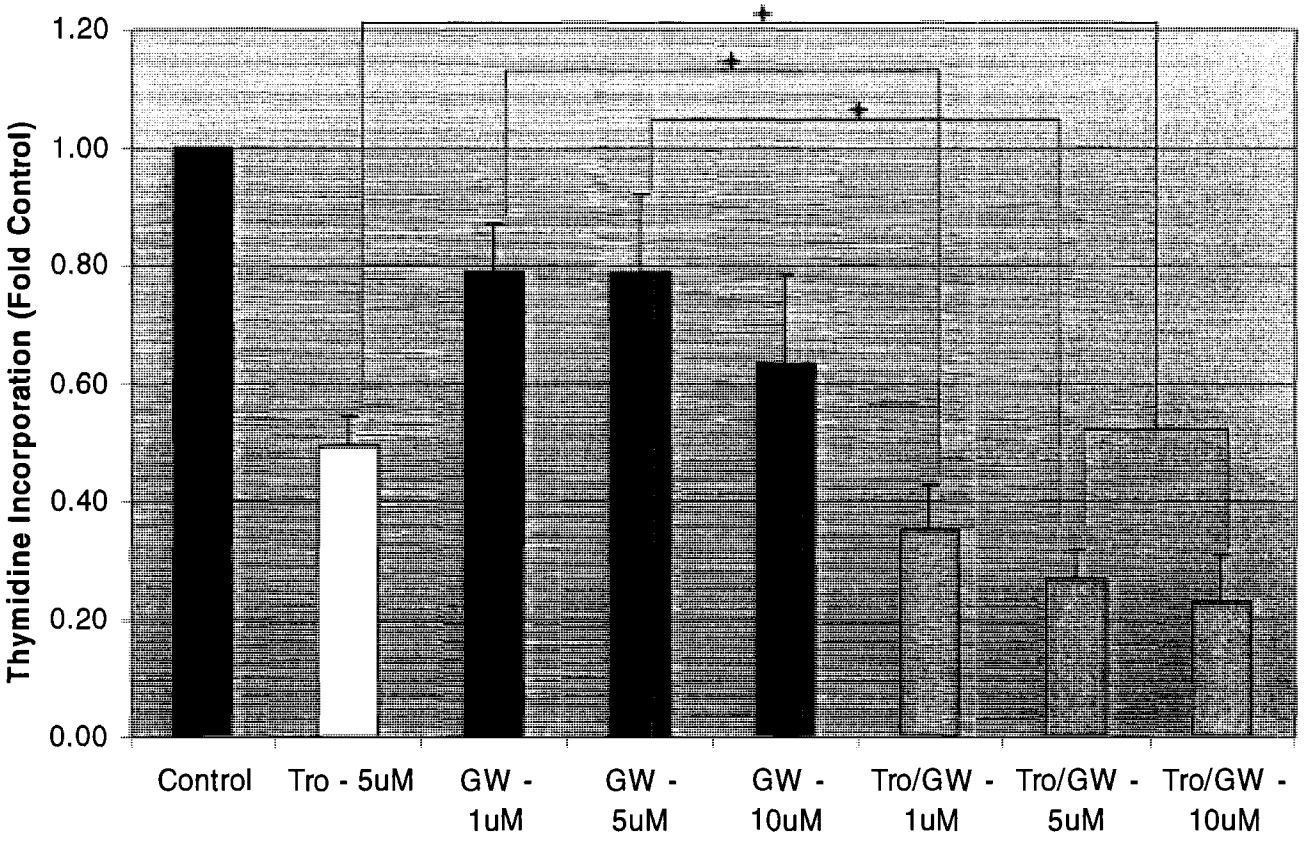
Figure 22. PGJ_2 reduces protein synthesis in M-1 cells. Serum-starved cells were stimulated for 24 hours with vehicle control (serum-free media), increasing concentrations of PGJ_2 (10^{-8}, 10^{-7}, 10^{-6} M) or increasing concentrations of PGE_2 (10^{-8}, 10^{-7}, 10^{-6} M). ^{3}H-leucine was added to M-1 cells while they were being stimulated and leucine incorporation was measured using a scintillation counter. Incorporation was measured in counts per minute and expressed as fold control of means ± S.E.M.; n = 3-4.

+ $p < 0.05$


Leucine Incorporation (Fold Control)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
1x10-8
1x10-7
1x10-6
Control
1x10-8
1x10-7
1x10-6
PGJ2
PGE2

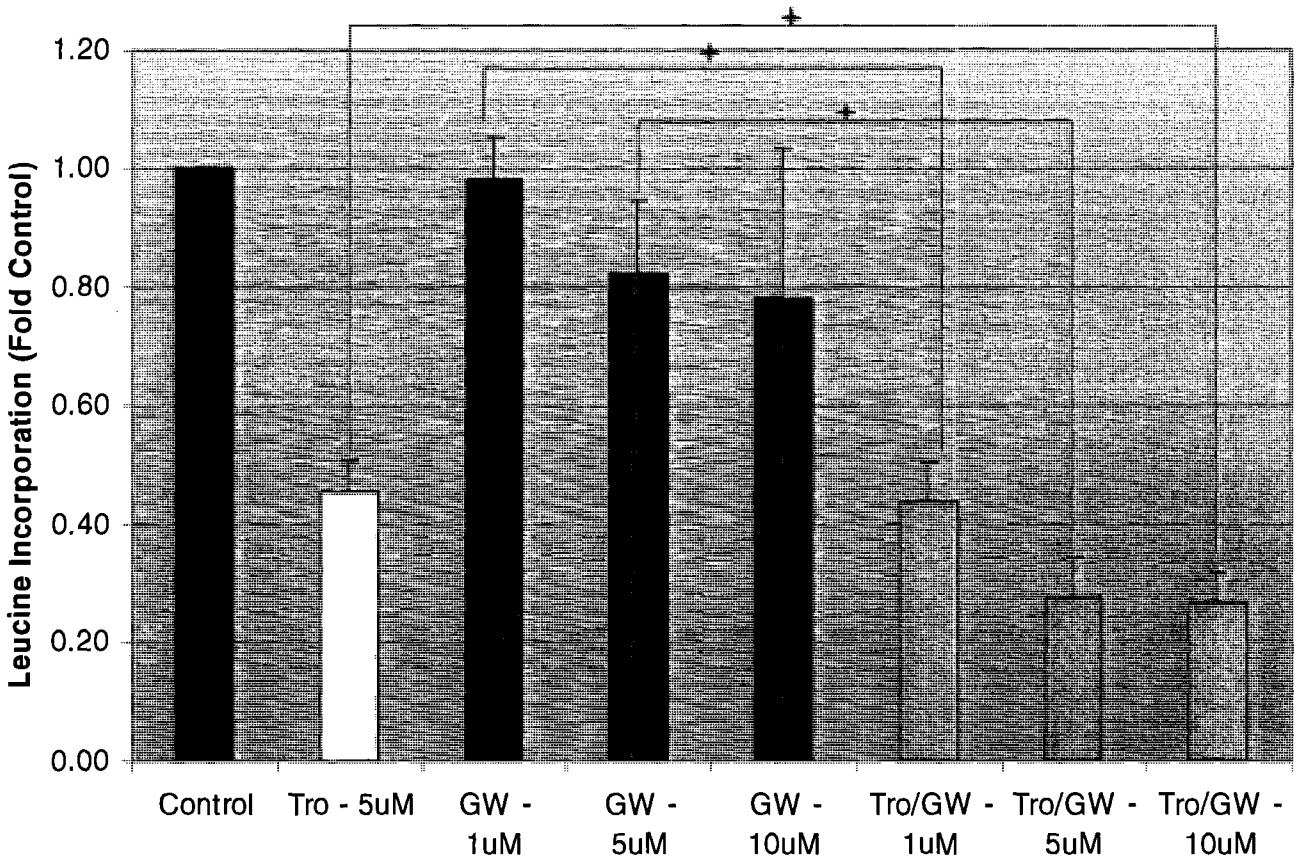
3.21: GW9662 further reduces DNA synthesis in M-1 cells. To determine if the growth effects of troglitazone are mediated by PPARγ, M-1 cells were treated with troglitazone in the presence of a PPARγ antagonist, GW9662. The cells were treated with serum-free media containing 2 μL of DMSO (vehicle control), troglitazone, in the absence or presence of increasing doses of the antagonist. As shown in **Figure 23**, troglitazone reduced thymidine incorporation to about 0.50-fold of control. However, when co-treated with GW9662 the DNA synthesis was further reduced to as low as 0.23-fold of control.

Figure 23. GW9662 further reduces DNA synthesis in M-1 cells. Serum-starved cells were stimulated for 24 hours with vehicle control (serum-free media); $5x10^{-6}$ M troglitazone (Tro); 10^{-6}, $5x10^{-6}$, 10^{-5} M GW9662 (GW); or 10^{-6}, $5x10^{-6}$, 10^{-5} M GW9662 and Tro preceded by a 1 hour stimulation of the antagonist. ^{3}H-thymidine was added to M-1 cells while they were being stimulated and thymidine incorporation was measured using a scintillation counter. Incorporation was measured in counts per minute and expressed as fold control of mean ± S.E.M.; n = 4. ✦ $p < 0.05$


Thymidine Incorporation (Fold Control)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Control
Tro - 5uM
GW - 1uM
GW - 5uM
GW - 10uM
Tro/GW - 1uM
Tro/GW - 5uM
Tro/GW - 10uM

3.22: GW9662 further reduces protein synthesis in M-1 cells. The effect of GW9662 on troglitazone-dependent protein synthesis was examined. M-1 cells were treated with serum-free media (control), troglitazone in the presence of increasing concentrations of the antagonist, GW9662. As shown in **Figure 24**, troglitazone reduced leucine incorporation to about 0.46-fold of control. As with the DNA synthesis, treatment with GW9662 resulted in further reductions of protein synthesis to 0.27-fold of control.

Figure 24. GW9662 further reduces protein synthesis in M-1 cells. Serum-starved cells were stimulated for 24 hours with vehicle control (serum-free media); $5x10^{-6}$ M troglitazone (Tro); 10^{-6}, $5x10^{-6}$, 10^{-5} M GW9662 (GW); or 10^{-6}, $5x10^{-6}$, 10^{-5} M GW9662 and Tro preceded by a 1 hour stimulation of the antagonist. ^{3}H-leucine was added to M-1 cells while they were being stimulated and leucine incorporation was measured using a scintillation counter. Incorporation was measured in counts per minute and expressed as fold control of mean ± S.E.M.; n = 5. + $p < 0.05$


Leucine Incorporation (Fold Control)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Control
Tro - 5uM
GW - 1uM
GW - 5uM
GW - 10uM
Tro/GW - 1uM
Tro/GW - 5uM
Tro/GW - 10uM

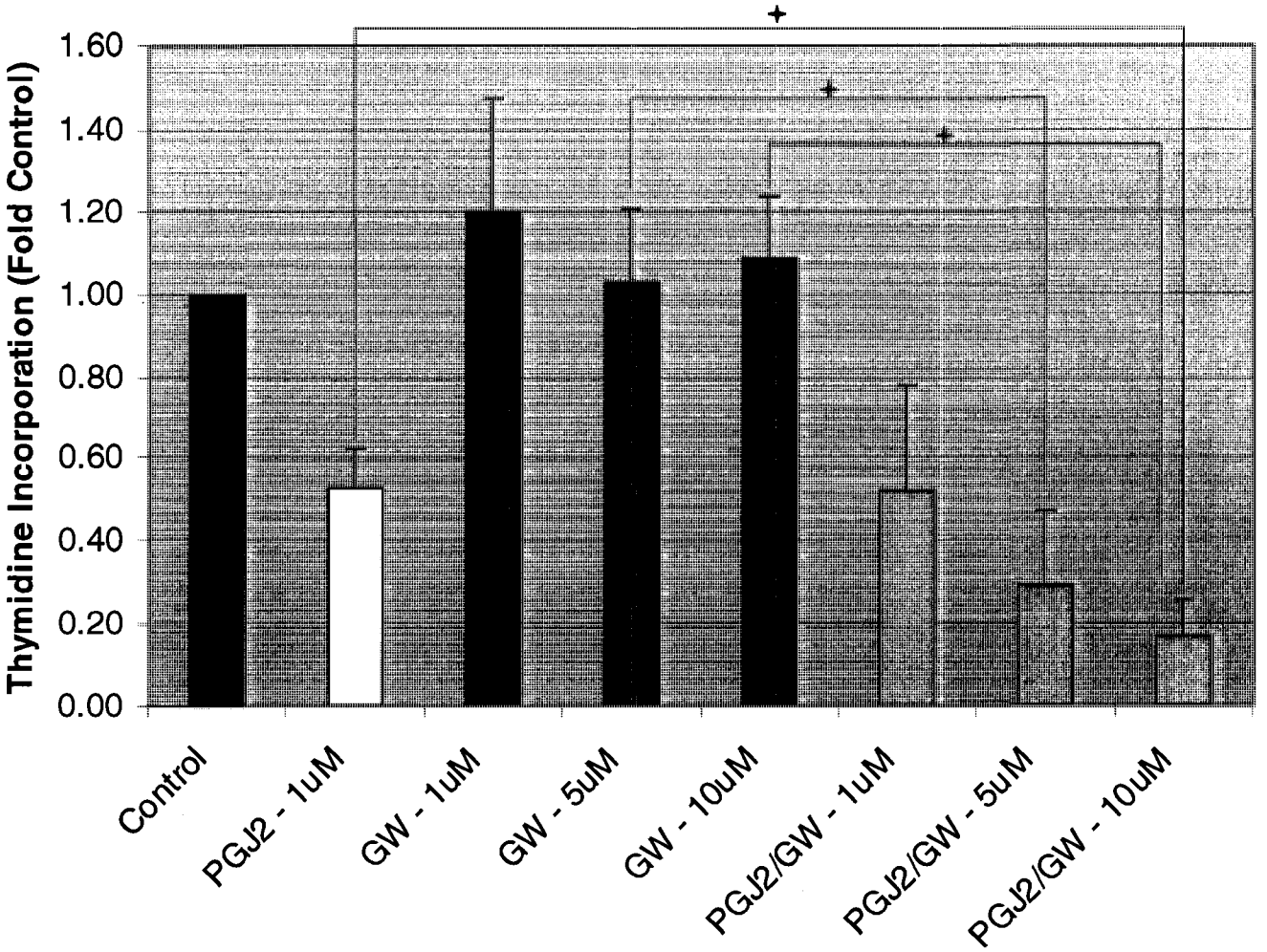
3.23: GW9662 does not reverse TZD-induced reductions in DNA and protein synthesis in M-1 cells. The effect of TZD treatment on protein and DNA synthesis was evaluated. M-1 cells were treated with serum-free media (control); troglitazone; other TZDs and putative PPARγ ligands, rosiglitazone, pioglitazone, or ciglitazone; or GW9662. The cells treated with TZDs were either treated with the TZD alone or co-treated with the antagonist. As shown in **Table 2**, troglitazone caused a reduction in thymidine and leucine incorporation to approximately 0.23- and 0.37-fold of control. Rosiglitazone, pioglitazone and ciglitazone reduced DNA synthesis by about 20% at 10^{-6} M. Again, rosiglitazone reduced protein synthesis modestly, at both 10^{-6} and $5x10^{-6}$ M. Pioglitazone reduced protein synthesis to 0.62- and 0.88-fold of control at 10^{-6} and $5x10^{-6}$ M, respectively. Ciglitazone reduced leucine incorporation levels to 0.67- and 0.42-fold of control at 10^{-6} and $5x10^{-6}$ M, respectively. The pretreatment with GW9662 did not produce any reversal in thymidine or leucine incorporation, but actually enhanced the reduction. This additive effect was most apparent with regards to leucine incorporation, with ciglitazone seeing a reduction of 64% with GW9662 treatment.

Table 2. GW9662 does not reverse TZD-induced reductions in DNA and protein synthesis in M-1 cells. Serum-starved cells were stimulated for 24 hours with vehicle control (serum-free media); 10^{-6} and $5x10^{-6}$ M troglitazone; 10^{-6} and $5x10^{-6}$ M rosiglitazone, 10^{-6} and $5x10^{-6}$ M pioglitazone, 10^{-6} and $5x10^{-6}$ M ciglitazone, or $5x10^{-6}$ M GW9662. For all of the co-treatments, the cells were treated with $5x10^{-6}$ M GW9662 for 1 hour prior to the stimulation. ^{3}H-leucine or ^{3}H-thymidine was added to M-1 cells while they were being stimulated and leucine incorporation was measured using a scintillation counter. Incorporation was measured in counts per minute and expressed as fold control of mean ± S.E.M.; n = 1-2; ND = not determined.

	Thymidine Incorporation		Leucine Incorporation	
	1 μM	5 μM	1 μM	5 μM
GW9662	ND	0.88 +/- 0.067	ND	0.75 +/- 0.005
Troglitazone	ND	0.23 +/- 0.041	ND	0.37 +/- 0.050
Rosiglitazone	0.83 +/- 0.160	ND	0.91	0.76
Pioglitazone	0.81 +/- 0.103	ND	0.62	0.88
Ciglitazone	0.80 +/- 0.158	ND	0.67	0.42
GW9662/Troglitazone	ND	0.14 +/- 0.020	ND	0.22 +/- 0.030
GW9662/Rosiglitazone	0.63 +/- 0.101	ND	0.39	0.53
GW9662/Pioglitazone	0.80 +/- 0.101	ND	0.48	0.64
GW9662/Ciglitazone	0.71 +/- 0.133	ND	0.37	0.15

3.24: GW9662 further enhances the decrease of PGJ_2 on DNA synthesis in M-1 cells. To determine if the effects produced by PGJ_2 on DNA synthesis involved PPARγ, PGJ_2-treated cells were co-treated with the antagonist. M-1 cells were stimulated with serum-free media (control), PGJ_2, or increasing concentrations of GW9662. The PGJ_2-treated cells that were co-treated with GW9662 were pretreated with the antagonist for 1 hour. As shown in **Figure 25**, PGJ_2 treatment resulted in a reduction in thymidine incorporation to 0.53-fold of control. The antagonist, having no effect on DNA synthesis alone, did not reverse the decrease in DNA synthesis caused by PGJ_2 but instead it produced a significant reduction in thymidine incorporation compared to PGJ_2 alone to as low as 0.17-fold of control.

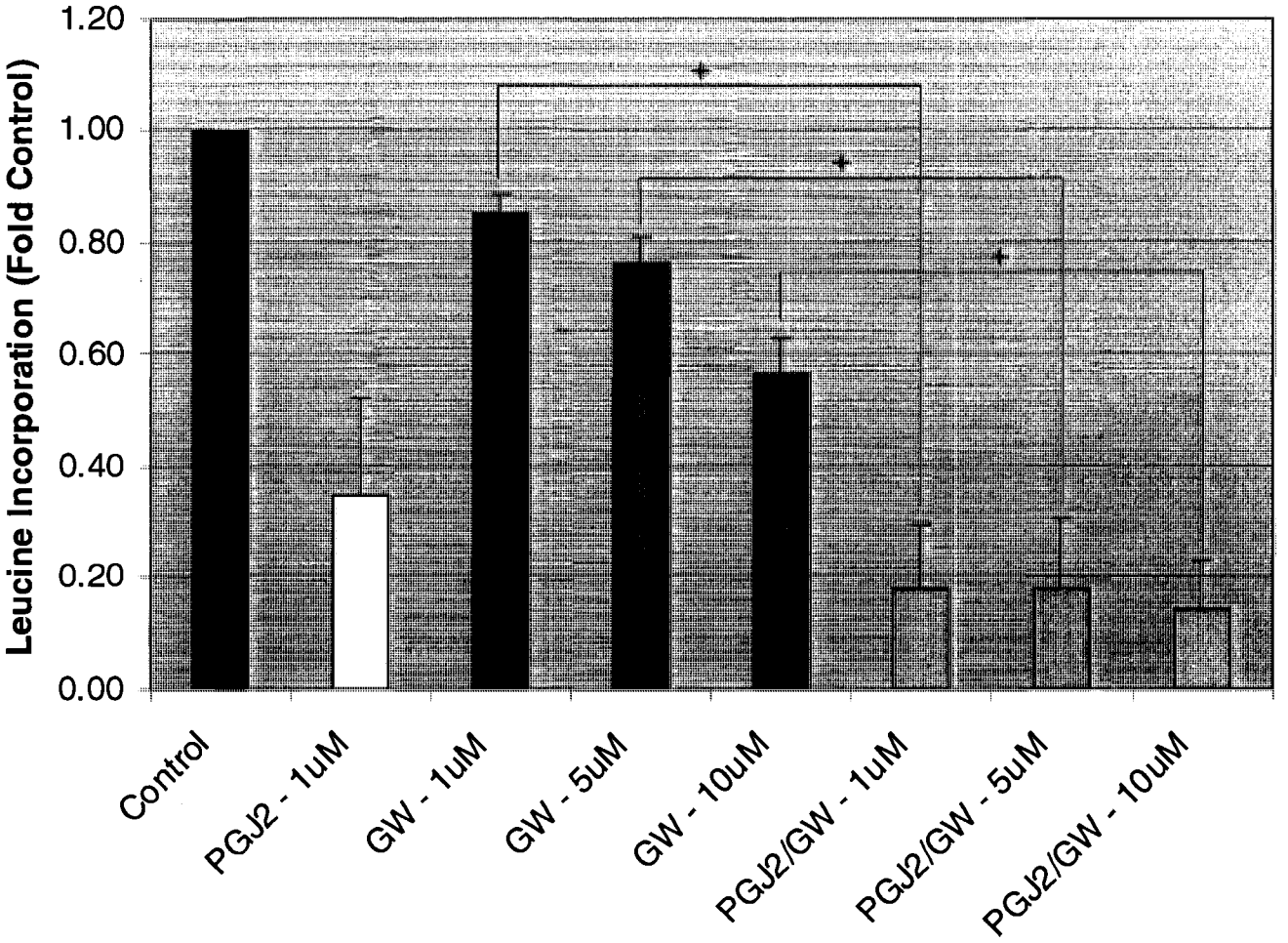
Figure 25. GW9662 further enhances the decrease of PGJ_2 on DNA synthesis in M-1 cells. Serum-starved cells were stimulated for 24 hours with vehicle control (serum-free media); 10^{-6} M PGJ_2; or 10^{-6}, $5x10^{-6}$, 10^{-5} M GW9662 (GW). For the co-treatments, the cells were treated with GW for 1 hour prior to the stimulation. ^{3}H-thymidine was added to M-1 cells while they were being stimulated and thymidine incorporation was measured using a scintillation counter. Incorporation was measured in counts per minute and expressed as fold control of mean ± S.E.M.; n = 3. + $p < 0.05$


Thymidine Incorporation (Fold Control)
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Control
PGJ2 - 1uM
GW - 1uM
GW - 5uM
GW - 10uM
PGJ2/GW - 1uM
PGJ2/GW - 5uM
PGJ2/GW - 10uM

3.25: GW9662 does not reverse the effect of PGJ_2 on protein synthesis in M-1 cells.

To determine if PGJ_2 protein synthesis effects could be abolished with the use of the PPARγ antagonist, M-1 cells were stimulated with serum-free media (control), PGJ_2, or increasing concentrations of GW9662. Similar to the thymidine experiments, PGJ_2 caused a 65% reduction in leucine incorporation (**Figure 26**), but GW9662 treatment did not reverse the PGJ_2-induced decrease in cell growth but there was a tendency for it to cause a greater decline compared to troglitazone alone.

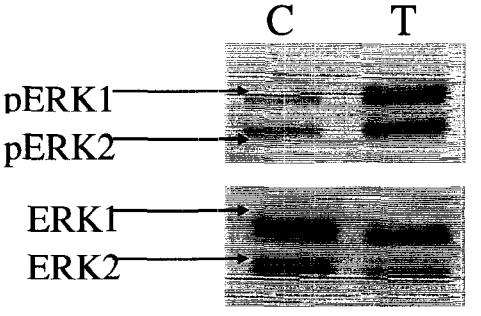
Figure 26. GW9662 does not reverse the effect of PGJ_2 on protein synthesis in M-1 cells. Serum-starved cells were stimulated for 24 hours with vehicle control (serum-free media); 10^{-6} M PGJ_2; or 10^{-6}, $5x10^{-6}$, 10^{-5} M GW9662 (GW). For the co-treatments, the cells were treated with GW for 1 hour prior to the stimulation. ^{3}H-leucine was added to M-1 cells while they were being stimulated and leucine incorporation was measured using a scintillation counter. Incorporation was measured in counts per minute and expressed as fold control of mean ± S.E.M.; n = 3. + $p < 0.05$


Leucine Incorporation (Fold Control)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Control
PGJ2 - 1uM
GW - 1uM
GW - 5uM
GW - 10uM
PGJ2/GW - 1uM
PGJ2/GW - 5uM
PGJ2/GW - 10uM

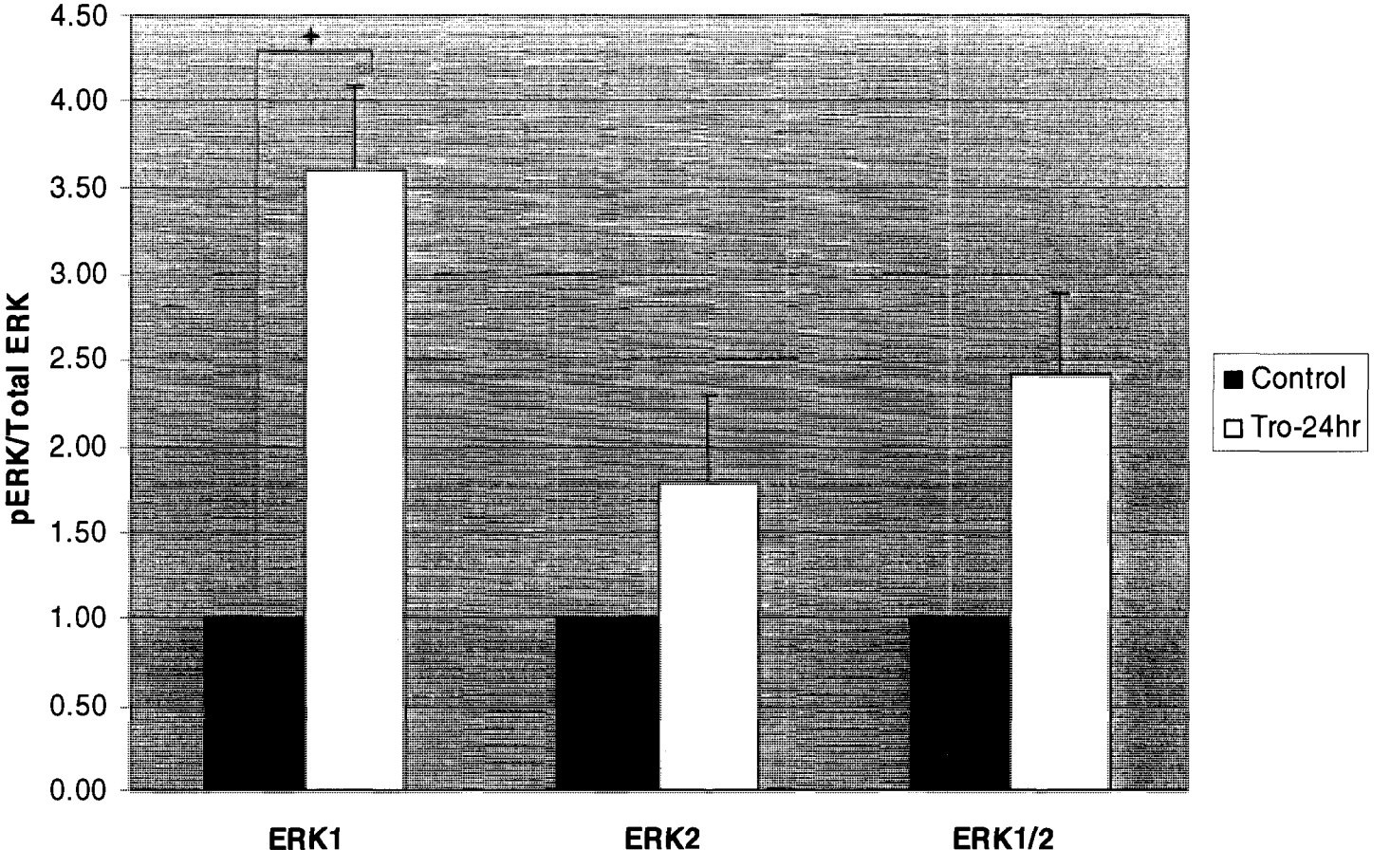
3.26: Troglitazone increases the pERK/ERK ratio in M-1 cells. To further explore the mechanisms by which troglitazone and PGJ_2 inhibit cell growth, we examined the activation of the extracellular signal-regulated kinase (ERK) pathway by troglitazone. As shown in **Figure 27A**, two bands corresponding to pERK1 (44 kDa) and pERK2 (42 kDa), the active forms of ERK, were visualized and on the same blots, two bands were produced that corresponded to Total ERK1 (44 kDA) and Total ERK2 (42 kDa). Activity was measured by dividing the density of the pERK bands by the density of their respective Total ERK bands. As shown in **Figure 27B**, twenty-four hour treatment with troglitazone resulted in increases in the ratio of pERK to ERK in ERK1/2 and its individual components (ERK1 and ERK2). The only response reaching significance was the troglitazone effect on ERK1 with levels ~3.5-fold of control.

Figure 27. Troglitazone increases the pERK/ERK ratio in M-1 cells. Protein was isolated from M-1 cells that had been treated with vehicle control serum-free media (C) or $5x10^{-6}$ M troglitazone (Tro) for 24 hours. A) The proteins were run on an SDS-PAGE gel and Western blotted using a pERK antibody (1:1000). The membranes were stripped and blotting was done with an ERK antibody (1:1000). B) Activity was measured by dividing the density of the pERK bands by the density of their respective Total ERK bands. Values are means ± S.E.M.; n = 3. + $p < 0.05$

A)

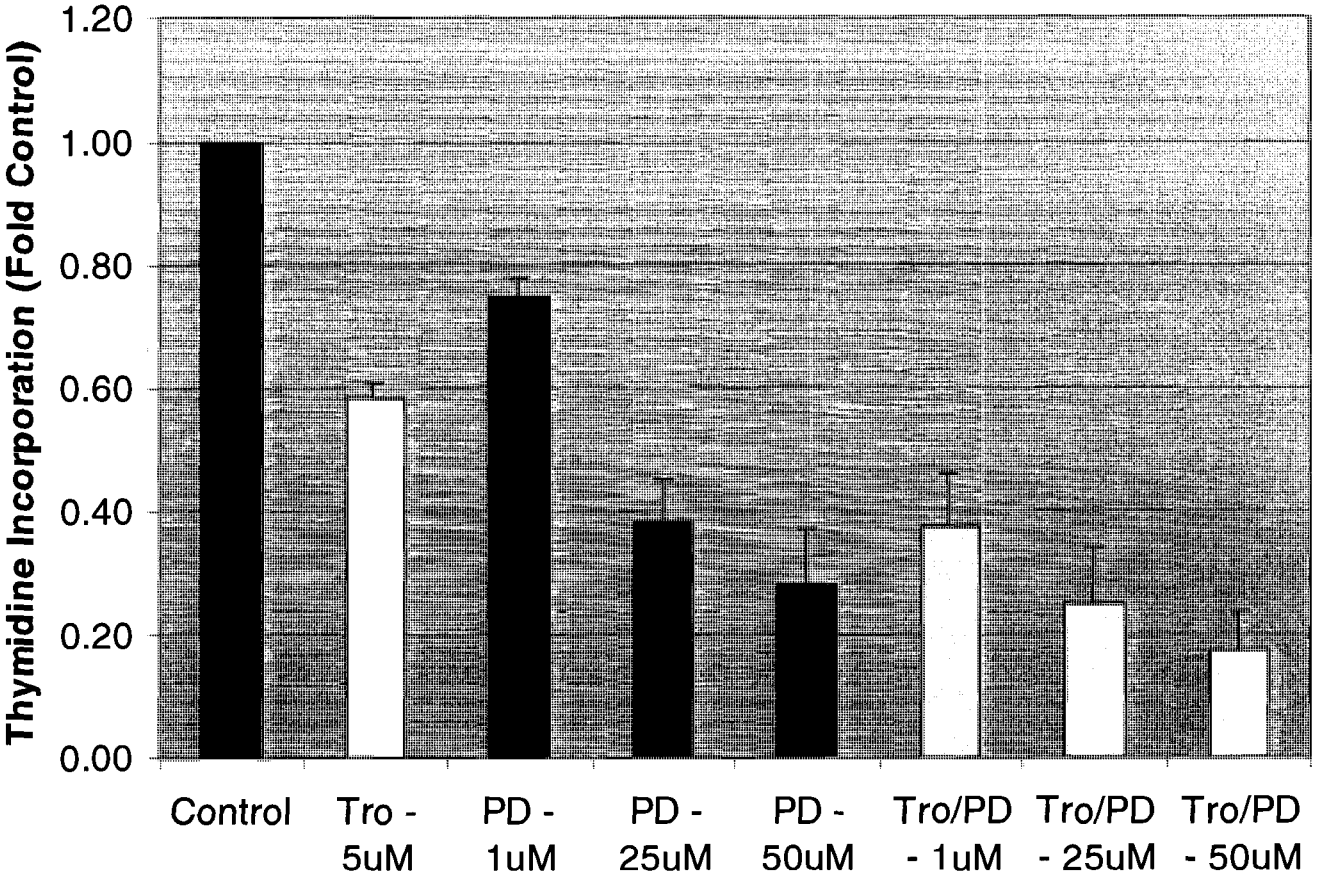

C
T
pERK1
pERK2
ERK1
ERK2

B)


4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
pERK/Total ERK
ERK1
ERK2
ERK1/2
Control
Tro-24hr

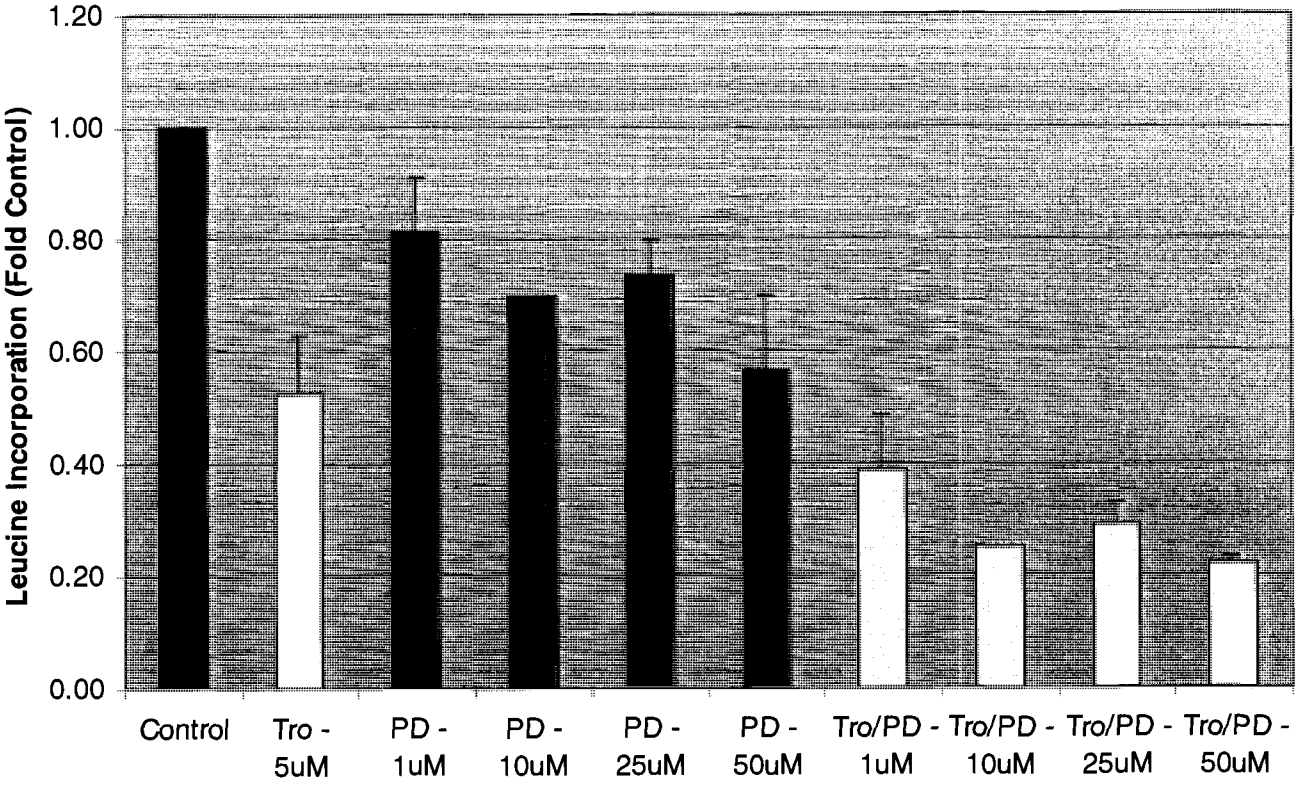
3.27: PD98059 does not reverse the effect of troglitazone on DNA synthesis in M-1 cells. Since troglitazone can increase the levels of active ERK, it was determined if the observed, troglitazone-induced, growth effect could be attributed to ERK, as a hyperactive ERK pathway can lead to cell cycle arrest (Meloche and Pouyssegur 2007). This was done by inhibiting the pathway with an ERK inhibitor, PD8059. M-1 cells were stimulated with serum-free media (control), troglitazone, or increasing concentrations of PD98059. As shown in **Figure 28**, troglitazone caused a reduction in thymidine incorporation to 0.59-fold of control but at all concentrations of PD98059 there was no reversal of the thymidine decrease caused by troglitazone. By itself, the inhibitor caused reductions of DNA synthesis to 0.75-, 0.39-, and 0.28-fold of control for the doses 10^{-6}, $2.5x10^{-5}$, and $5x10^{-5}$ M, respectively. There is also a trend showing an additive inhibition when troglitazone and PD98059 are administered together.

Figure 28. PD98059 does not reverse the effect of troglitazone on DNA synthesis in M-1 cells. Serum-starved cells were stimulated for 24 hours with vehicle control (serum-free media); $5x10^{-6}$ M troglitazone; or 10^{-6}, $2.5x10^{-5}$, $5x10^{-5}$ M of the ERK inhibitor, PD98059 (PD). For the co-treatments, the cells were treated with PD for 1 hour prior to the stimulation. ^{3}H-thymidine was added to M-1 cells while they were being stimulated and thymidine incorporation was measured using a scintillation counter. Incorporation was measured in counts per minute and expressed as fold control of mean ± S.E.M.; n = 2.


Thymidine Incorporation (Fold Control)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Control
Tro - 5uM
PD - 1uM
PD - 25uM
PD - 50uM
Tro/PD - 1uM
Tro/PD - 25uM
Tro/PD - 50uM

3.28: PD98059 does not reverse the effect of troglitazone on protein synthesis in M-1 cells. Next, the effect of PD98059 on leucine incorporation, in response to troglitazone, was determined. M-1 cells were stimulated with serum-free media (control), troglitazone, or increasing concentrations of PD98059. As can be seen in **Figure 29**, comparable to the results of the thymidine experiments, all concentrations of PD98059 did not alter the protein synthesis response to troglitazone, which produced a reduction to 0.53-fold of control. Although by itself the inhibitor caused a reduction in protein synthesis, it was not as great as what was seen with DNA synthesis. Furthermore, troglitazone and PD98059 showed an additive effect similar to that described in the thymidine studies above, resulting in a reduction to approximately 25% of control.

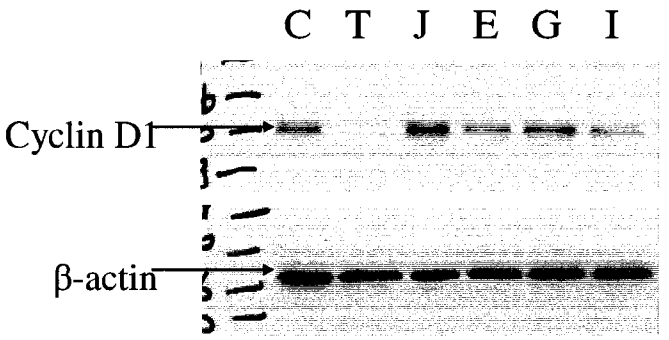
Figure 29. PD98059 does not reverse the effect of troglitazone on protein synthesis in M-1 cells. Serum-starved cells were stimulated for 24 hours with vehicle control (serum-free media); $5x10^{-6}$ M troglitazone; or 10^{-6}, 10^{-5}, $2.5x10^{-5}$, $5x10^{-5}$ M of PD98059 (PD). For the co-treatments, the cells were treated with PD for 1 hour prior to the stimulation. ^{3}H-leucine was added to M-1 cells while they were being stimulated and leucine incorporation was measured using a scintillation counter. Incorporation was measured in counts per minute and expressed as fold control of mean ± S.E.M.; n = 1-3.


Leucine Incorporation (Fold Control)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Control
Tro - 5uM
PD - 1uM
PD - 10uM
PD - 25uM
PD - 50uM
Tro/PD - 1uM
Tro/PD - 10uM
Tro/PD - 25uM
Tro/PD - 50uM

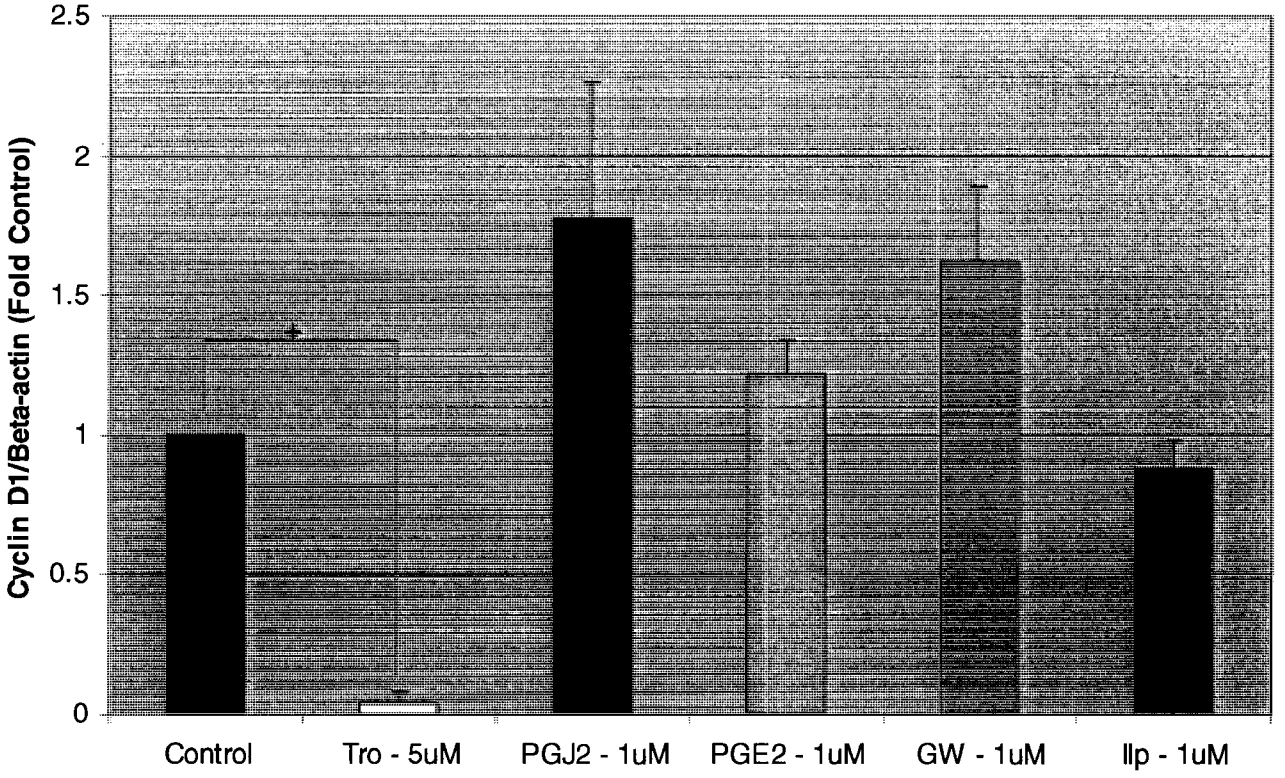
3.29: Troglitazone reduces cyclin D1 protein levels in M-1 cells. Since there was a noticeable decrease in protein synthesis in response to troglitazone treatment, different cell cycle proteins responsible for changes in cell growth were examined. One such protein is cyclin D1, which is crucial for the progression of the cycle, due to the fact that it is required for the activation of some cyclin-dependent kinases (CDK4, CDK6). M-1 cells were treated with serum-free media (control), $5x10^{-6}$ M troglitazone, 10^{-6} M PGJ_2, 10^{-6} M PGE_2, 10^{-6} M GW501516, or 10^{-6} M iloprost. A band corresponding to cyclin D1 (38 kDa) was visualized (**Figure 30A**). As shown in **Figure 30B**, troglitazone stimulation greatly and significantly lowered cyclin D1 protein levels to only about four percent of control. PGJ_2 and GW501516 raised levels to 1.77- and 1.62-fold of control but this change did not reach significance. PGE_2 and iloprost both had no effect on cyclin D1.

Figure 30. Troglitazone reduces cyclin D1 protein levels in M-1 cells. Protein was isolated from M-1 cells that had been treated with vehicle control serum-free media (C), $5x10^{-6}$ M troglitazone (Tro or T), 10^{-6} M PGJ_2 (J), 10^{-6} M PGE_2 (E), 10^{-6} M GW501516 (GW or G), or 10^{-6} M iloprost (Ilp or I) for 24 hours. A) The proteins were run on an SDS-PAGE gel and Western blotted using a cyclin D1 antibody (1:1000). The membranes were stripped and blotting was done with a β-actin antibody (1:20000), which was used for normalization for densitometric analysis (B). Values are means ± S.E.M.; n = 3. + $p < 0.05$

A)


C T J E G I
Cyclin D1
β-actin

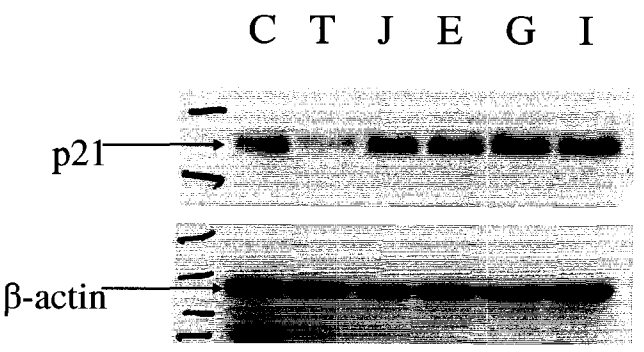
B)


Cyclin D1/Beta-actin (Fold Control)
2.5
2
1.5
1
0.5
0
Control
Tro - 5uM
PGJ2 - 1uM
PGE2 - 1uM
GW - 1uM
Ilp - 1uM

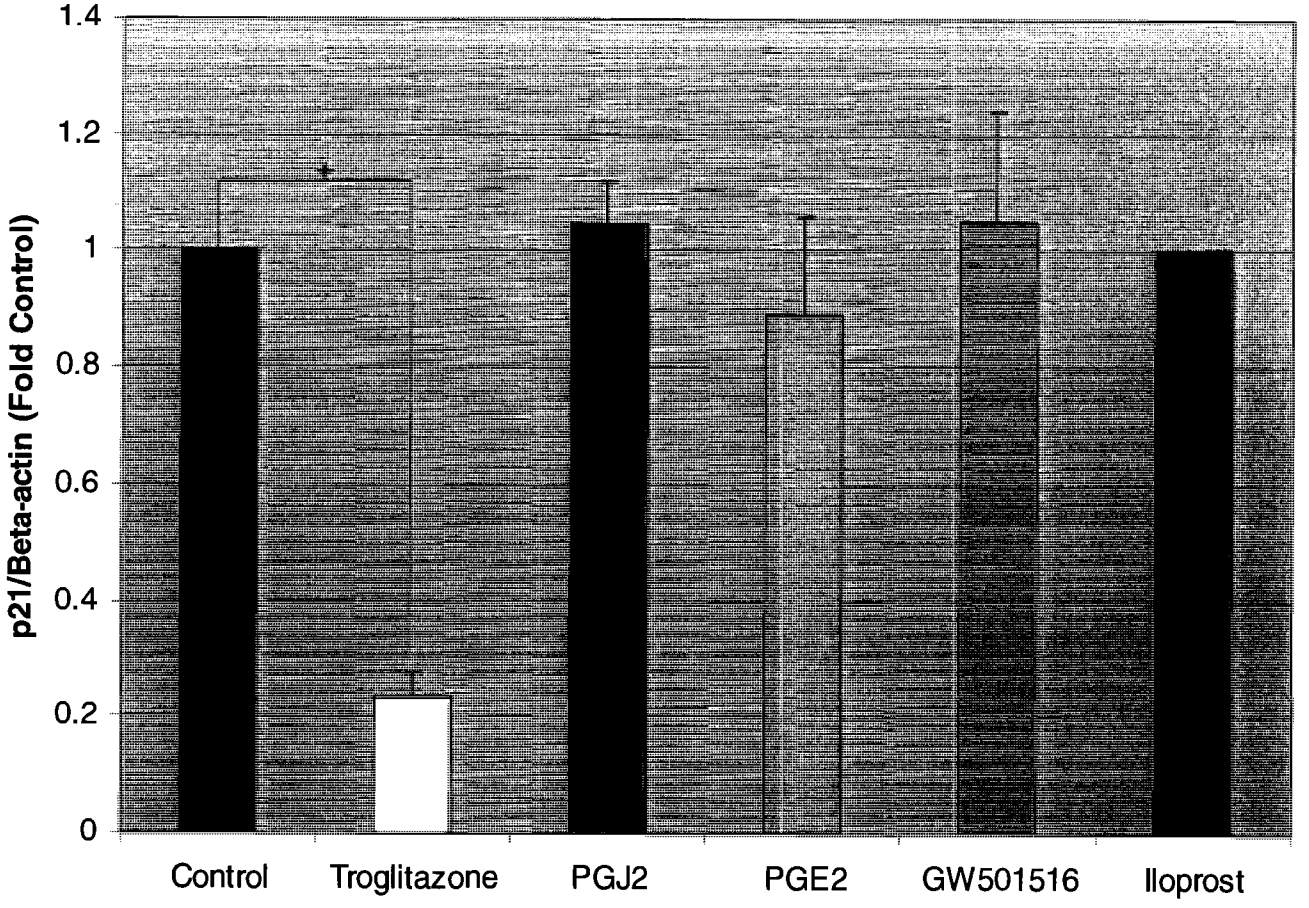
3.30: Troglitazone reduces p21 protein levels in M-1 cells. While cyclins promote the progression of the cell cycle, CDK inhibitors like p21 contribute to cell cycle arrest by preventing cyclin-CDK interaction. M-1 cells were treated with serum-free media (control), $5x10^{-6}$ M troglitazone, 10^{-6} M PGJ_2, 10^{-6} M PGE_2, 10^{-6} M GW501516, or 10^{-6} M iloprost. As shown in **Figure 31A**, a 21 kDA band corresponding to p21 was visualized. Treatment with troglitazone significantly reduced p21 protein levels to 0.27-fold of control whereas the other treatments had no discernible effect (**Figure 31B**).

Figure 31. Troglitazone reduces p21 protein levels in M-1 cells. Protein was isolated from M-1 cells that had been treated with vehicle control serum-free media (C), $5x10^{-6}$ M troglitazone (Tro or T), 10^{-6} M PGJ_2 (J), 10^{-6} M PGE_2 (E), 10^{-6} M GW501516 (GW or G), or 10^{-6} M iloprost (Ilp or I) for 24 hours. A) The proteins were run on an SDS-PAGE gel and Western blotted using a p21 antibody (1:500). The membranes were stripped and blotting was done with a β-actin antibody (1:20000), which was used for normalization for densitometric analysis (B). Values are means ± S.E.M.; n = 3. + $p < 0.05$

A)


C T J E G I
p21
β-actin

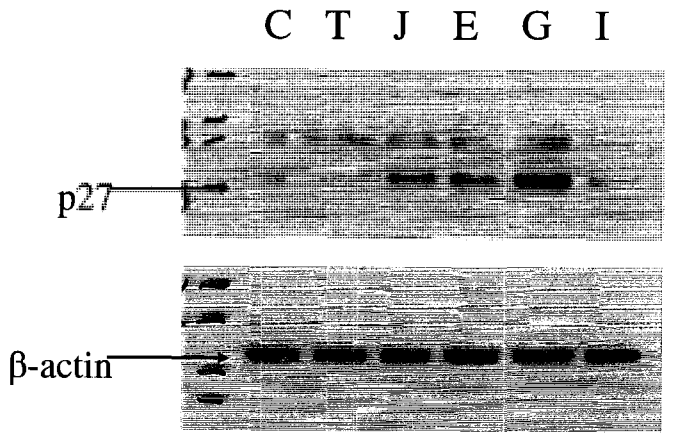
B)


p21/Beta-actin (Fold Control)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
Troglitazone
PGJ2
PGE2
GW501516
Iloprost

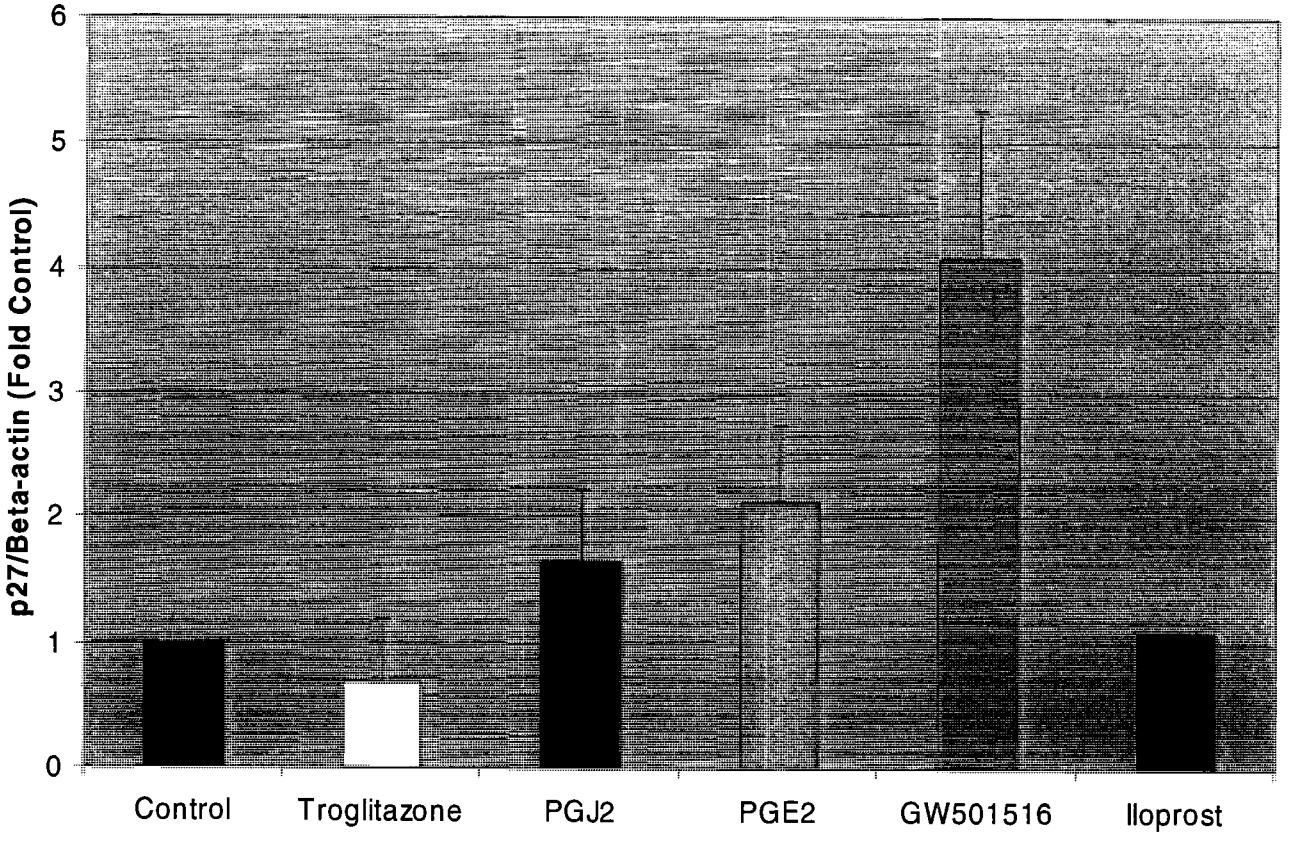
3.31: GW501516 induces $p27^{kip-1}$ protein levels in M-1 cells. In addition to p21, another cell cycle inhibitor $p27^{kip-1}$ is an important regulator of renal cell growth. M-1 cells were treated with serum-free media (control), $5x10^{-6}$ M troglitazone, 10^{-6} M PGJ_2, 10^{-6} M PGE_2, 10^{-6} M GW501516, or 10^{-6} M iloprost. As shown in **Figure 32A**, a 27 kDa band corresponding to p27 was visualized. However, treatment with troglitazone, PGJ_2, PGE_2, and iloprost had no significant effect on p27 levels (**Figure 32B**). The PPARδ agonist, GW501516, produced a large increase in p27, to approximately 4-fold of control, but this did not reach significance.

Figure 32. Troglitazone reduces $p27^{kip-1}$ protein levels in M-1 cells. Protein was isolated from M-1 cells that had been treated with vehicle control serum-free media (C), $5x10^{-6}$ M troglitazone (Tro or T), 10^{-6} M PGJ_2 (J), 10^{-6} M PGE_2 (E), 10^{-6} M GW501516 (GW or G), or 10^{-6} M iloprost (Ilp or I) for 24 hours. A) The proteins were run on an SDS-PAGE gel and Western blotted using a $p27^{kip-1}$ antibody (1:1000). The membranes were stripped and blotting was done with a β-actin antibody (1:20000), which was used for normalization for densitometric analysis (B). Values are means ± S.E.M.; n = 4.

A)


C T J E G I
p27
β-actin

B)


p27/Beta-actin (Fold Control)
0
1
2
3
4
5
6
Control
Troglitazone
PGJ2
PGE2
GW501516
Iloprost

4.0: Discussion

The goal of our study was to determine if PPARδ and PPARγ regulate growth and apoptosis in M-1 and IMCD-K2 cells. Both PPARδ and PPARγ are found in the CD but their role is not clear. Our results indicate that PPAR ligands regulate growth in M-1 and IMCD-K2 cells. The PPARδ ligand, GW501516, showed a noteworthy increase in DNA synthesis in IMCD-K2 cells but significant decreases in protein synthesis in both IMCD-K2 and M-1 cells, and DNA synthesis of M-1 cells. We also show that reductions in synthesis were consistent with the up-regulation of p27 protein levels. The effect of troglitazone on growth also demonstrated significant decreases in DNA and protein synthesis in M-1 cells, likely by decreasing cyclin D1 levels. Through the use of a PPARγ antagonist and an ERK inhibitor, it was concluded that the troglitazone growth effects are likely PPAR-independent and not through the ERK pathway. With regards to apoptosis, there was no effect of GW501516 treatment in cells that were treated with anisomycin in either the IMCD-K2 cells or the M-1 cells. However, troglitazone treatment did tend to reduce active caspase levels in M-1 cells.

4.1: Characterization of the PGE_2/EP system. Since 1995, prostaglandins have been known to target PPARs (Yu, Bayona et al. 1995). These include PGJ_2, PGE_2, and PGI_2, promoting differentiation, cell growth, and apoptotic pathways, respectively (Hatae, Wada et al. 2001; Nosjean and Boutin 2002; Xu, Han et al. 2006). In the collecting duct, PGs, specifically PGE_2, are major homeostatic regulators of transport, increasing water permeability and inhibiting sodium absorption (Hebert, Breyer et al. 1995). The presence

of PG receptors and the enzymes partially responsible for their production was studied to determine if PGs could affect PPAR expression/activation. Receptors for PGE_2 and PGI_2 were examined because these PGs are the most highly expressed in the kidney (Bonvalet, Pradelles et al. 1987). As expected, COX enzymes are present in the IMCD-K2 cells. COX expression in the collecting duct has previously been shown in our lab in the mouse M-1 and rabbit RCCD cortical collecting duct cell lines as well as in cultured rat IMCD cells (Ferguson, Hebert et al. 1999; Nasrallah, Landry et al. 2003). The expression of the COX enzymes is not only species-dependent but also differs from cell type to cell type (Nasrallah, Clark et al. 2007). It has been shown in other labs that COX-1 is usually the predominant COX enzyme found in the mouse CD however our lab has previously demonstrated that COX-2 is a major contributor to PG synthesis (Ferguson, Hebert et al. 1999; Campean, Theilig et al. 2003; Ye, Zhang et al. 2006). It is unclear how each COX contributes to different pools of PGs in the CD, but the levels of PGE_2 and PGI_2 are both high in the CD (Nasrallah, Clark et al. 2007). Using PCR, it was also demonstrated that the prostanoid receptors EP_1, EP_3, and EP_4 are expressed in both cell lines. This too has been previously reported (Hebert, Breyer et al. 1995; Nasrallah, Laneuville et al. 2001; Nasrallah, Zimpelmann et al. 2001) and it is not surprising due to the fact there is great deal of PGE_2 synthesis in the CD and that the EP_2 receptor is mainly found in the renal vasculature (Morath, Klein et al. 1999). In addition, IP receptor expression was not detected in IMCD-K2 cells in our study nor in M-1 cells, which was previously reported in our lab (unpublished data). To further characterize the PG responses in IMCD-K2 cells we determined what effect PGE_2 and PGI_2 analogs had on cAMP levels. Prostanoids can affect cAMP and treatment with PGE_2 produced a considerable increase

in cAMP. This is most likely through EP_4 since, as mentioned, EP_2 could not be detected. In contrast, there was no response to the IP ligands, CCP and ILP.

It has been shown previously that PGE_2 and PPARδ influence the activity of each other. They have been shown to be involved in a positive feedback loop in cholangiocarcinomas (Xu, Han et al. 2006). Another study demonstrated that activation of PPARδ could increase expression levels of EP_4 in human non-small cell lung carcinomas (Han, Ritzenthaler et al. 2005). In our studies, experiments were performed to ascertain if PGE_2 could also produce changes in the levels of PPARδ. Treatment with PGE_2 for eight hours initiated an increase in PPARδ protein levels, whereas the two and twenty-four hour stimulations had no effect. The change in protein levels did not correspond with any change at the mRNA level, as PPARδ mRNA remained unaltered. There are two likely explanations for this. The first reason is that the PPARδ protein could be stabilized. A previous study in a prostate cancer cell line has shown that PGE_2 can stabilize hypoxia-inducible factor 1 alpha (HIF-1α) protein levels, without affecting mRNA levels (Liu, Kirschenbaum et al. 2002). This would most likely be due to the PGE_2 preventing proteolysis of PPARδ. Interestingly, it has been revealed that, unlike most nuclear receptors, degradation of PPARδ does not occur upon ligand binding but is in fact is inhibited by it (Genini and Catapano 2007). This could indicate that PGE_2 could be causing an increase in PPARδ activation. The second possible role of PGE_2 is that it could be enhancing the translation of basal PPARδ mRNA levels or stabilizing it. The ability of PGE_2 to stabilize interleukin 8 mRNA has been previously described in a study by Yu *et al.* (Yu and Chadee 1998). Therefore, from the results reported here and the literature, the effect of PGE_2 on PPARδ is most likely a post-transcriptional event.

4.2: PPARδ agonist and cell growth in IMCD-K2 cells. It has been previously reported that activation of PPARδ and the use of PPARδ ligands promotes cell survival and proliferation. One recent study, using both PPARδ short interfering RNA (siRNA) and a PPAR antagonist, showed that down-regulation or blocking the activation of PPARδ, respectively, inhibited the PGE_2-induced proliferation of mouse embryonic stem cells (Kim and Han 2008). Another study revealed that a PPARδ agonist can stimulate propagation in both human and mouse aortic endothelial cells (Piqueras, Reynolds et al. 2007). However, studies directed at the kidney involving PPARδ and cell proliferation are not available. Because of this, we investigated the growth effect of an agonist of PPARδ in the IMCD-K2 cells. Cell proliferation, was measured in response to treatment with the PPARδ agonist GW501516, first described by Oliver *et al.* (Oliver, Shenk et al. 2001), which resulted in an increase in DNA synthesis. This result is consistent with the literature, in which PPARδ has been known to have positive roles in the proliferation of a variety of cells, such as human colorectal cancer cells, mouse stem cells, and rat vascular smooth muscle cells (Zhang, Fu et al. 2002; Kim and Han 2008; Yang, Zhou et al. 2008). However, the increase in DNA synthesis, see in our results, is contradictory to the rest of the results that we obtained for both the IMCD-K2 and M-1 cells. This may have been due to inconsistencies in the procedure (BrdU fluorescent analysis) in which there is an inherent bias, as it was not completed with a blind approach. Also, FBS treatment only resulted in a stimulation of approximately 10%, indicating the cells may be resistant to some treatments and that growth may be at a maximum. Further studies (i.e. thymidine incorporation experiments) should be performed to confirm the observed effect, and this would serve as a better comparison for the thymidine studies performed in the M-1 cells.

Unlike the changes in DNA synthesis, our leucine experiments showed that protein synthesis was reduced by GW501516 but only at higher concentrations. Although most studies indicate that PPARδ activation results in stimulation of cell growth, mostly in human and mouse colon cancer cells (Wang, Wang et al. 2004; Yang, Zhou et al. 2008), there have been previous findings showing that PPARδ agonists, including GW501516, can hinder or have no effect on cell growth in certain human breast cancer cells and melanomas (Hollingshead, Killins et al. 2007; Girroir, Hollingshead et al. 2008). These differences may be due to species differences or diversity among cell lines.

4.3: PPARδ agonist and anisomycin-induced apoptosis in IMCD-K2 cells. As mentioned in the introduction, PPARδ has been associated with cell survival and apoptosis. With regards to cell survival PPARδ has been shown to protect the heart of fatty rats from ischemia/reperfusion injury as well as promote cell survival in renal medullary interstitial cells (Hao, Redha et al. 2002; Yue, Nerurkar et al. 2008). It has also been demonstrated that PPARδ activation can reduce levels of apoptosis (Liou, Lee et al. 2006; Pesant, Sueur et al. 2006) and that down-regulation of PPARδ can lead to increased apoptosis (Shureiqi, Jiang et al. 2003). The results in this thesis show that treatment with GW501516 does not reverse the reduction in cell survival due to exposure to anisomycin. These results are inconsistent with the literature as it had been shown in many papers that activation of PPARδ, or the use of a PPARδ agonist, protects many different cell types, including renal cells. It is possible that the agonist may not actually be activating PPARδ in IMCD-K2 cells, as its efficacy in this cell line has never been

tested. In addition, PPARδ activation may not be affecting cell survival if the death response is mostly through necrosis because studies only show a survival role for PPARδ with regards to apoptosis. To have an effect on anisomycin-induced apoptosis PPARδ has to be involved in protecting the cells, through the possible up-regulation of cell survival proteins, before treatment as anisomycin blocks protein synthesis. To evaluate if the agonist is having an effect on PPARδ activation, experiments can be performed with cells transfected with a PPRE-driven reporter plasmid (Sumanasekera, Tien et al. 2003). To completely assess death pathways activated by anisomycin, we could perform flow cytometry using the Annexin V-FITC and propidium iodide stains (Chen, Cheng et al. 2008).

Even though the use of the agonist, in our experiments, elicits no response in overall cell survival in IMCD-K2 cells the effect on apoptosis had yet to be investigated. Using a variety of experiments assessing apoptosis (caspase activity assay, Western blotting, TUNEL) we found little to no change in the levels of apoptosis in cells treated with GW501516 compared to those stimulated with just anisomycin. Again these results were contradictory to previous studies, as the majority of studies saw PPARδ having anti-apoptotic activities. The variation between our data and the majority of the literature could be due to differences in cell types and the condition of the cells, as most studies have focussed on colorectal cancer tissues (Gupta, Tan et al. 2000; Shureiqi, Jiang et al. 2003; Wang, Wang et al. 2004; Liou, Ghelani et al. 2007). It seems unlikely that the discrepancy in PPARδ effects is due to species differences as most of the cancer studies were done using mouse tissues. There have been some studies though that clearly indicates PPARδ may not provide any prevention in certain types of apoptosis, such as in

the López *et al.* (Lopez, Fernandez et al. 2004) study where over-expression of PPARδ did not protect aspirin-induced apoptosis in Jurkat cells. Thus, it is possible that PPARδ may only protect cells from apoptosis in certain conditions and cell types. From the data we collected, GW501516 has no effect on cell survival nor can it prevent or lessen anisomycin-induced apoptosis. The IMCD, and possibly the IMCD-K2 cells, are resistant to cell death because their environment *in vivo* is one of hypertonicity (Rauchman, Nigam et al. 1993). This inherent resiliency, due to in part by osmolyte transport, up-regulation of heat shock proteins, and activation of pro-survival genes, may make them resistant to different treatments, like what has been seen in a mouse IMCD cell line (Maroni, Koul et al. 2004).

4.4: PPARδ/γ agonists and anisomycin-induced apoptosis in M-1 cells. Apoptosis was measured in M-1 cells that had been treated with anisomycin and GW501516 or troglitazone, a PPARγ agonist (Lambe and Tugwood 1996). It was hypothesized that GW501516 would provide a protective role for the cells and reduce levels of apoptosis for the same reasons discussed in the previous section. PPARγ agonists have been shown to provide cardioprotective effects (Yu, Jin et al. 2007) as well as well as prevent or reduce injury in a variety of kidney tissues, including human proximal tubules, mouse glomeruli, and mouse podocytes (Panchapakesan, Pollock et al. 2004; Kanjanabuch, Ma et al. 2007; Kume, Uzu et al. 2007).

Although the experiments presented in this thesis did not demonstrate any noticeable change in cleaved caspase-3 levels with GW501516 treatment, troglitazone did significantly reduce its expression. The effect of the GW501516 was consistent with

that which was obtained in the IMCD-K2 cells but it did not correlate with the majority of the literature, which examined apoptosis and PPARδ in cancer cells. There is only one study that dealt with PPARδ, apoptosis, and the kidney and it showed that the use of a PPARδ agonist reduced apoptosis in the medullary region of mice (Letavernier, Perez et al. 2005). The response to troglitazone, however, was similar to what has been previously published, with the agonist having protective effects. Similar to the findings in the IMCD-K2 cells, the PPARδ agonist had no effect on anisomycin-induced apoptosis, while the PPARγ agonist decreased active caspase levels in M-1 cells. When the collecting duct is in a disease state due to urinary tract obstruction, ischemia-reperfusion injury, or other insults apoptosis levels tend to increase (Cohen, Loutochin et al. 2007; Han, Kim et al. 2007). In at least one study a PPARγ ligand has provided protective effects after renal ischemia-reperfusion injury and has lowered cleaved caspase-3 levels (Doi, Masaki et al. 2007). Thus, our data, and that of other groups, indicate that PPARγ can aid in the prevention of cellular loss due to injury in collecting duct cells by way of reducing apoptosis.

4.5: PPARδ/γ agonists and cell growth in M-1 cells. The growth effects of GW501516 and troglitazone were studied. Similar to the effect on cell survival many studies have utilized various TZDs or PPAR agonists which generally have mixed effects on DNA and protein synthesis. PPARγ agonists have been shown to increase cell survival and growth in bovine renal tubular epithelial cells and human colon cancer cells (Sommer and Wolf 2007; Choi, Kim et al. 2008). Furthermore, silencing of PPARγ in Jurkat and HeLa cells indicates an important role for the receptor in promoting both cell survival and

proliferation (Ferreira-Silva, Rodrigues et al. 2008). Despite these positive effects on growth, there have been a number of reports that have indicated that PPAR agonists, especially TZDs, can halt cellular growth (Kim, Choi et al. 2008; Zhou, Wen et al. 2008). The majority of studies that illustrate PPARγ as a negative regulator of cell growth are in cell lines or tissues that have abnormally high levels of growth due to cancer or hypertrophy caused by diabetic nephropathy (Okada, Wada et al. 2006; Kim, Choi et al. 2008; Zhou, Wen et al. 2008). Coupled with the evidence that PPARγ tends to protect cells, it would appear that PPARγ might attempt to maintain homeostasis in several cell types, especially immortalized cells, by lowering the rate of growth. TZDs acting on PPARγ have been shown to induce withdrawal from the cell cycle in an immortalized murine cell line (adipocytes) that was transgenic for SV40 (Altiok, Xu et al. 1997). The contradictions in the PPARγ effects on cell growth is most likely not species-specific as most of the studies looking at PPARγ as affecting cell growth, either promoting or inhibiting it, were in human cells (Choi, Kim et al. 2008; Zhou, Wen et al. 2008). However, there are PPARγ studies using mouse cells but these only show inhibition of cell growth (Kim, Choi et al. 2008). The effects seen in the M-1 cells could be cell specific but there is no evidence supporting this since there have not been any studies of PPARδ/γ regulating growth in M-1 cells or the CD. PPARγ has also been identified as a target for the differentiation of adipocytes and to undergo differentiation the cells must withdraw from the cell cycle (Altiok, Xu et al. 1997). Thus, PPARγ could be inhibiting the cell growth of several cell lines to promote differentiation. One other possibility for the differences in growth effects by PPAR ligands is that TZDs can have both PPAR-dependent and –independent actions. This is difficult to evaluate using the literature as

the majority use PPAR ligands and do not test if the growth effects are mediated through PPARγ. Of the studies mentioned above, only Sommer *et al.* (2007) and Ferreira-Silva *et al.* (2008) showed a direct link to PPARγ, which supported PPARγ as a promoter of cell growth. Nothing definitive can be said of the research of Choi *et al.* (2008), Zhou *et al.* (2008), and Kim *et al.* (2008), as none showed the ligands activating PPARγ.

The data in this thesis show that both GW501516 and troglitazone treatment results in reductions in both cell proliferation and growth. The effect of troglitazone was seen at doses that were relatively close together, between 10^{-6} and $5x10^{-6}$ M. The decrease in DNA and protein synthesis was very similar for troglitazone, as the concentration of $5x10^{-6}$ M produced a decline that was approximately forty to fifty percent of control in both experiments. GW501516 did not produce as great of an effect. Unlike troglitazone, the outcome of GW501516 treatment was mostly the same over several concentrations and it seems not to be as dependent on the dose. From these results the use of PPARδ and PPARγ agonists inhibit cell growth in M-1 cells. A possible explanation for the inhibition of cell growth by troglitazone could be that the G_0/G_1 switch gene 2 (G0S2), a gene with a functional PPRE that is involved in the cell cycle, has been activated (Zandbergen, Mandard et al. 2005). However, we have not examined this in these cells. All PPARs appear to trigger the gene, although PPARγ seems to be the most potent activator. It should be noted that troglitazone is considered one of the most effective TZDs with regards to activating PPARγ, but at higher concentrations it is also one of the more toxic TZDs and some of our observed effects with troglitazone may be, in part, due to toxicity (Lambe and Tugwood 1996). At this

point, we cannot exclude the possibility that these effects are toxic response, and studies with over-expression of PPARγ or siRNA might address this.

4.6: Troglitazone with GW9662 and cell growth in M-1 cells. The growth effect of troglitazone was examined in the presence of a reversible PPARγ antagonist, GW9662 (Huang, Welch et al. 1999; Willson, Brown et al. 2000). In view of the fact that troglitazone is a PPARγ agonist, it was expected that treatment with the antagonist would reverse or lessen any effects of troglitazone. In contrast, the antagonist did not reverse the troglitazone-induced decrease in cell growth but actually caused a greater reduction than with troglitazone alone. This was true for both DNA and protein synthesis. Since there was no reversal in troglitazone effects with GW9662, it would appear that troglitazone's changes in growth are through a PPAR-independent pathway. This is consistent with several studies in the literature as it has been reported in numerous occasions that there are many PPAR-independent effects of troglitazone, including the activation of ERK in porcine renal tubules, reducing apoptosis in human glioma cells, and increasing nucleoside transporters in human smooth muscle cells (Leung, Man et al. 2005; Schultze, Bock et al. 2006; Turturro, Oliver et al. 2007). The further reduction in cell growth with the antagonist indicates that any activation of PPARγ may actually be causing an increase in cell growth but this response is hidden due to the PPAR-independent effects. However, there is a notable study that shows that treatment with GW9662 did not reverse rosiglitazone-induced growth inhibition but enhanced it, as is seen here (Seargent, Yates et al. 2004). It has also been demonstrated that GW9662 can cause cell cycle arrest via a PPAR-independent pathway (Schaefer, Takahashi et al.

2007). To further explore this possibility and determine if the effects are PPAR-dependent or –independent, we could transfect a PPARγ over-expression plasmid into our cells and observe the outcome with troglitazone treatment. If troglitazone treatment results in an even greater reduction in growth in the cells containing the plasmid compared to cells with a control plasmid, it can be assumed that the effects are PPAR-dependent. If there is a reversal in the decrease in growth, then the original troglitazone effects were PPAR-independent. PPARγ siRNA could also be used to determine if troglitazone is acting through PPARγ. In this case, an increase or reversal in cell growth would correspond to PPAR-dependent effects, whereas a further reduction or no change in growth would indicate a PPAR-independent response.

In addition to troglitazone, we tested other TZDs, which have been used as PPARγ agonists (Lehmann, Moore et al. 1995; Lambe and Tugwood 1996), to see if the antagonist would prevent any response produced by them. GW9662 did not abolish any of the response seen with the TZDs: rosiglitazone, pioglitazone, or ciglitazone. The glitazone effects had indications that they may themselves be responsible for some inhibition of cell growth. These data support a PPAR-independent effect of TZDs in M-1 cells. Again, there was a tendency for a further reduction in cell growth, when the antagonist was used with the TZDs. In the literature, TZDs have been shown to most likely result in an inhibition of cell growth in human leukemia cells lines, human colon cancer cells, and human renal carcinoma cells (Yoshizumi, Ohta et al. 2004; Yang, Zhang et al. 2005; Saiki, Hatta et al. 2006). It would appear that the inhibition of cell growth by TZDs mostly occurs in human cancer cells, and this has been previously seen with treatment of troglitazone in human liver cancer cells (Zhou, Wen et al. 2008). Like with

troglitazone, there are many studies examining PPAR-independent actions of the other TZDs and a good review by Feinstein *et al.* (Feinstein, Spagnolo et al. 2005) chronicles these. The growth results of the TZDs and troglitazone, when taken together, seem to confirm that the antagonist may be arresting cell growth as by Schaefer *et al.* (2007).

4.7: Prostaglandins and cell growth in M-1 cells. We wanted to determine if PGs could be regulating growth responses through PPARs in M-1 cells. The vast majority of PG studies in the kidney have been focussed on body fluid homeostasis, ion transport, and blood pressure (Hao and Breyer 2008). A few studies have shown the growth effects of PGs in cells, like mouse chondrocytes, and some have supported a positive growth effect in other tissues, such as human colon cancer (Aoyama, Liang et al. 2005; Cherukuri, Chen et al. 2007). Those papers dealing with PGs, growth, and the kidney are very limited and tend to show PGs having varied effects on growth and proliferation (Suganami, Tanaka et al. 2001; Rovin, Wilmer et al. 2002). The PGs we used were PGE_2 and PGJ_2 and they both have been known to activate PPARδ and PPARγ, respectively (Kliewer, Lenhard et al. 1995; Wang, Wang et al. 2004). In our studies PGJ_2 produced a decrease in both DNA and protein synthesis, suggesting that PGJ_2 and troglitazone could be acting through the same pathway, possibly via PPARγ. It had also been revealed in several cancer cells that PGJ_2 is able to inhibit cell growth and in other tissues induce cell cycle arrest to promote differentiation (Nakahata, Abe et al. 1990; Shao and Lazar 1997; Nikitakis, Siavash et al. 2002; Chen, Zhong et al. 2003). PGE_2 treatment reduced DNA synthesis significantly but had no effect on protein synthesis, suggesting an increase in cell growth. This differed from the effects of GW501516, and so it is likely that PGE_2 is

not acting through PPARδ in M-1 cells. Further experiments, as discussed below were performed to verify if the troglitazone and PGJ_2 growth effects are through PPARγ.

4.8: PGJ_2 with GW9662 and cell growth in M-1 cells. Although we did not show GW9662 affecting the growth response seen with troglitazone, it was possible that PGJ_2 acted through PPARγ and that the antagonist would reverse any effects. However, in our studies, the antagonist did not reverse the decrease in either cell proliferation or growth. It looks as if the antagonist could be enhancing the effect of PGJ_2 as it is doing with troglitazone. Again this indicates that the inhibitory growth effects are PPAR-independent and if there are any PPAR-dependent growth effects, they are hidden. To assess if any PPAR-dependent effects exist, we could use either a PPAR over-expression plasmid or PPARγ siRNA, as mentioned above. As with the glitazones, there have been several papers that have shown that PGJ_2 has effects independent of PPARγ, through prostanoid receptors, NFκB, or others (Nosjean and Boutin 2002).

4.9: Troglitazone and the ERK pathway. Since the results imply that the inhibition of cell growth is due to pathways that are PPAR-independent, the involvement of the ERK pathway was examined. There have been many articles that have shown that TZDs, including troglitazone, can activate ERK (He, Sung et al. 2006; Kempna, Hofer et al. 2007; Kim, Cho et al. 2007; Kang, Choi et al. 2008). This has even been shown in renal tubule-derived cell lines (Turturro, Oliver et al. 2007). The ERK pathway has also been well described pertaining to growth and the cell cycle. ERK, being a MAPK (mitogen-activated protein kinase), is growth promoting and is normally associated with the

progression of the cell cycle but when the pathway becomes hyperactive it is known to cause cell cycle arrest (Meloche and Pouyssegur 2007). In our studies, activation of the ERK pathway, as shown by the phosphorylation of the ERK1/2, occurred in response to a 24-hour troglitazone treatment, with activation of ERK1 exceeding 3.5-fold control. These results are consistent with studies that have shown that troglitazone can activate ERK in porcine renal tubule-derived cell lines, stimulating cellular growth (Turturro, Oliver et al. 2007).

Due to the ERK activation that was observed in response to troglitazone, growth experiments were performed in the presence of an ERK inhibitor, PD98059 (Dudley, Pang et al. 1995). Since troglitazone was activating the ERK pathway, it is possible that inhibition of ERK activation would reverse some of the troglitazone-induced growth inhibition. In spite of this, the use of the inhibitor produced no reversal of growth inhibition with regards to both DNA and protein synthesis and PD98059 caused further reductions in growth. It is likely that the inhibitor is in fact inhibiting the ERK pathway resulting in an inhibition of cell growth, which is well known and has been shown in renal carcinoma cells (Huang, Ding et al. 2008). All of the data collected signifies that although troglitazone is activating ERK in M-1 cells, this activation is not responsible for the observed decrease in cell growth.

4.10: Cell cycle regulators. The reduction in both DNA and protein synthesis suggest there is inhibition of the cell cycle, thus, regulators of the cell cycle, including cyclins and cyclin-dependent kinase (CDK) inhibitors, were analyzed in response to troglitazone. The levels of cyclins, which are necessary for the progression of the cell cycle, are

expected to be lower in response to troglitazone treatment. Conversely, an increase in CDK inhibitor levels would contribute to cell cycle arrest. The role of cell cycle regulators in renal cells is complicated but it should be noted that the expression profile of cell cycle proteins may differ from cell type to cell type (Shankland and Wolf 2000).

Cyclin D1 is one of the most important cyclins and because of this we examined its protein expression. Troglitazone reduced cyclin D1 protein to barely detectable levels. This was anticipated since it is known that cyclins are down-regulated in cells that are undergoing an inhibition of the cell cycle. Cyclin E was also explored but protein levels were undetectable. Treatments with the PGs or a GW501516 did not produce any significant change compared to control. It is unknown if the effect of troglitazone on cyclin D1 is through a PPAR-responsive gene or not but the results indicate that the decrease in cell growth could be due to this reduction. We would have to examine the mRNA levels of cyclin D1 to confirm a direct effect on gene expression. The inhibition of cyclins by all TZDs, including troglitazone, has been well studied and many papers are available on the subject (Huang, Shiau et al. 2005; Wei, Lin et al. 2007; Kim, Choi et al. 2008), including studies involving renal cells (Yang, Zhang et al. 2005). Similar effects of troglitazone on cyclin levels have been reported in most studies, regardless of species or cell type, as seen in mouse skin keratinocytes, and human renal carcinoma cells (He, Thuillier et al. 2004; Yang, Zhang et al. 2005).

p21 and $p27^{kip-1}$, which are among the most prominent of the CDK inhibitors, were investigated to determine how the expression was affected by troglitazone, PGJ_2, PGE_2, GW501516, or iloprost treatment. The levels of p21 were reduced by troglitazone stimulation but were unchanged with all other treatments (PGJ_2, PGE_2, GW501515, and

iloprost). This differed from what was expected and does not correspond with the majority of literature (Yang, Zhang et al. 2005; Ming, Yu et al. 2006). There has been some evidence, though, that has shown that troglitazone can reduce both cyclin and p21 levels blocking cell cycle progression (Wakino, Kintscher et al. 2000). The reduction in cyclin D1 in response to troglitazone was greater than the reduction of p21, thus inhibiting cell growth. It is also possible that, since there is a reduction in overall protein, the decrease in p21 levels is due to a general inhibition of protein synthesis. However, this seems unlikely due to the increase we saw in ERK protein levels. The protein levels of $p27^{kip-1}$ were not significantly changed with troglitazone or iloprost treatment. However, PGJ_2, PGE_2, and GW501516 all produced increases in p27, with GW501516 producing the largest increase. In the literature both PGJ_2 and PGE_2 have been shown to produce increases in p27 levels in human breast carcinomas and human $CD4^+$ T cells (Chemnitz, Driesen et al. 2006; Suzuki, Hayashi et al. 2006). Concerning the kidney our lab previously had shown that a PGI_2 analog can decrease levels of p27 in rat mesangial cells (Nasrallah and Hebert 2004). With regards to GW501516, these results differ from most studies that show that activation of PPARδ usually produces a reduction in p27 (Kim and Han 2008). The tendency towards an increase in p27 could be partially responsible for the decrease in cell growth seen with GW501516, PGJ_2, and PGE_2. No change in p27, as seen with troglitazone, can also contribute to a decrease in cell growth when coupled with a decrease in cyclin levels. As with the cyclins, mRNA levels of the CDK inhibitors need to be measured to confirm the direct involvement of PPARs.

The reduction in cell proliferation and growth in M-1 cells treated with troglitazone is likely due to a decrease in cyclin D1 levels, whereas the growth effects seen with GW501516, PGJ_2, and PGE_2 presumably corresponds to increases in $p27^{kip-1}$.

*4.11: **Summary.*** Our hypothesis for this study was to determine if PPARδ and PPARγ reduce apoptotic responses and if PPARδ stimulates while PPARγ inhibits cell growth in M-1 and IMCD-K2 cells. Both PPARδ and PPARγ are highly expressed in the CD, and thus may be responsible for the protection of the CD. We showed that growth responses in these cells are affected by PPARδ and PPARγ ligands, mostly reducing DNA and protein levels significantly. This had previously been shown in several cell lines but the effect in CD cells was unknown. The actions of the PPARδ ligand, GW501516 could be mediated through the CDK inhibitor, p27, as its protein levels were increased with GW501516 treatment. In addition, the actions of the PPAR ligand, troglitazone, may be through cyclin D1, as treatment with troglitazone lowered cyclin D1 protein to barely detectable levels. This drop in cyclin D1 is consistent with cell cycle arrest, previously seen in human renal carcinoma cell lines that had been treated with troglitazone (Yang, Zhang et al. 2005). With regards to apoptosis, GW501516 had no effect on anisomycin-induced apoptosis in both cell lines, differing from what was hypothesized. The PPARγ ligand, however, showed a tendency to reduce apoptotic levels. This was consistent with the majority of the literature, which show both PPARs as having protective effects, but none had examined the role of PPARs in CD cell survival. The use of antagonists and inhibitors illustrated that the growth effects seen by the PPARγ ligand may be independent of PPARγ or ERK activation. It should be noted that our work was

performed in immortalized cells and does not necessarily reflect the growth responses in the intact CD and that future studies should comprise isolating the IMCD or CCD from mice for primary cultures.

To expand our understanding of the effects of PPAR ligands on growth and apoptosis, further studies are required. Also, to confirm if the observed effects are mediated by PPARs, PPAR over-expression plasmids and siRNA should be utilized. The mRNA levels of the cell cycle regulators (i.e. cyclins, CDK inhibitors) should be examined to determine if the effects on them are direct or indirect. Furthermore, possible gene targets of the PPAR ligands should be assessed, including the G_0/G_1 switch gene 2 (Zandbergen, Mandard et al. 2005). By completing these studies we may gain new insights that would support targeting PPARs in the CD to regulate its function in health and disease.

5.0: References

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6.0: Appendix

This section contains two figures detailing the primers used for RT-PCR and antibodies employed for Western blotting.

Table 3. Primer sequences for EP, IP and PPARδ receptors used for RT-PCR.

Receptor	Position	Size	Direction	Primer Sequence
EP_1	865-1201	336 bp	Forward	5'-CGCAGGGTTCACGCACACGA-3'
			Reverse	5'-CACTGTGCCGGGAACTACGC-3'
EP_2	757-1158	401 bp	Forward	5'-AGGACTTCGATGGCAGAGGAGAC-3'
			Reverse	5'-CAGCCCCTTACACTTCTCCAATG-3'
EP_3	538-975	437 bp	Forward	5'-CCGGGCACGTGGTGCTTCAT-3'
			Reverse	5'-TAGCAGCAGATAAACCCAGG-3'
EP_4	941-1364	423 bp	Forward	5'-TTCCGCTCGTGGTGCGAGTGTTC-3'
			Reverse	5'-GAGGTGGTGTCTGCTTGGGTCAG-3'
IP	856-1263	407 bp	Forward	5'-GGCACGAGAGGATGAAGTTTACC-3
			Reverse	5'-GTCAG-AGGCACAGCAGTCAATGG-3'
PPARδ	1294-1455	161 bp	Forward	5'-TGGAGCTCGATGACAGTGAC-3'
			Reverse	5'-GTACTGGCTGTCAGGGTGGT-3'

Product	Primary Antibody		Secondary Antibody	
	Details	Dilution	Details	Dilution
β-actin	Sigma, 5% milk	1:10000	Promega, anti-mouse IgG HRP	1:2000
Bax	Santa Cruz, 10% milk	1:850	Promega, anti-mouse IgG HRP	1:2000
Bcl-2	Santa Cruz, 10% milk	1:750	Promega, anti-mouse IgG HRP	1:2000
Caspase-3	Santa Cruz, 10% milk	1:1000	Promega, anti-mouse IgG HRP	1:2000
Cleaved Caspase-3	Cell Signaling, 10% milk	1:1000	Promega, anti-rabbit IgG HRP	1:2000
COX-1	Cayman, 10% milk	1:1000	Promega, anti-rabbit IgG HRP	1:2000
COX-2	Cayman, 10% milk	1:1000	Santa Cruz, anti-goat IgG HRP	1:2000
Cyclin D1	Santa Cruz, 10%milk	1:1000	Promega, anti-mouse IgG HRP	1:2000
Cyclin E	Santa Cruz, 10% milk	1:200	Promega, anti-mouse IgG HRP	1:2000
ERK (Total)	Cell Signaling, 5% BSA	1:1000	Promega, anti-rabbit IgG HRP	1:2000
p21	Santa Cruz, 10% milk	1:500	Promega, anti-mouse IgG HRP	1:2000
$p27^{kip-1}$	Santa Cruz, 10% milk	1:1000	Promega, anti-mouse IgG HRP	1:2000
pERK	Cell Signaling, 5% BSA	1:1000	Promega, anti-rabbit IgG HRP	1:2000
PPARδ	Santa Cruz, 10% milk	1:500	Santa Cruz, Anti-goat IgG HRP	1:2000
PPARγ	Santa Cruz, 10% milk	1:1000	Promega, Anti-mouse IgG HRP	1:2000

Table 4. List of antibodies. Included in the list are the companies from which the antibodies were purchased, the blocking solution they were incubated with, their dilution and the secondary used.