# The Effects of 7β-OH Epiandrosterone on Cytokine Production by Human Immune Cells

**A Thesis Presented**

**By**

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## Declaration

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# Dedications

To the Soul of my Father

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# Abbreviations

| | |
|---|---|
| 15d-$PGJ_2$ | 15-deoxy-$\Delta^{12-14}$ prostaglandin $J_2$ |
| 7β OH-EpiA | 7β-hydroxy-epiandrosterone |
| AA | Arachidonic Acid |
| ACTB | Beta Actin |
| AP-1 | Activation Protein-1 |
| ATP | Adenosine Triphosphate |
| B cells | B-lymphocyte |
| BLAST | Basic Local Alignment Search Tool |
| BSA | Bovine Serum Albumin |
| cAMP | Cyclic Adenosine Monophosphate |
| CD14 | Cluster of Differentiation 14 |
| cDNA | Complementary Deoxyribonucleic Acid |
| CNA | Central Nervous System |
| COX | Cyclooxygenase |
| COX-1 | Cyclooxganase-1 |
| COX-2 | Cyclooxygenase-2 |
| $CPLA_2$ | Cytosolic Phospholipase A 2 |
| CRTH2 | Prostaglandin DP2 receptor |
| CT | Cycle Threshold |
| CV | Coefficient of Variance |
| DD | Death Domain |
| DHEA | Dehydroepiandrosterone |
| DMSO | Dimethyl Sulfoxide |
| DNA | Deoxyribonucleic Acid |
| DP2 | Prostaglandin DP2 receptor |
| dsRNA | Double stranded Ribonucleic Acid |
| DSS | Dextrin Sodium Sulphate |

| | |
|---|---|
| ECACC | European collection of Cell Cultures |
| ECL | Enhanced Chemiluminescence |
| EDTA | Ethylenediaminetetraacetic Acid |
| ELISA | Enzyme Linked Immunosorbent Assay |
| ERK | Extracellular Signal Related Kinase |
| ESB | Electrophoresis Sample Buffer |
| FADD | FAS Mediated Death Domain |
| FCS | Foetal Calf Serum |
| GAPDH | Glycerealdehyde-3-Phosphat Dehydrogenase |
| GCs | Glucocorticoids |
| gDNA | Genomic Deoxyribonucleic Acid |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| GR | Glucocorticoid receptor |
| GRE | Glucocorticoid response element |
| h-PGDS | Human Prostaglandin-D Synthase |
| HBSS | Hanks' Balanced Salt Solution |
| HCl | Hydrochloric Acid |
| HETEs | Hydroxy eicosatetraenoic Acid |
| HPA | Hypothalamic- Pituitary- Adrenal Axis |
| HPGDS | Human Prostaglandin-D Synthase |
| ID | Intermediate Domain |
| IDT | Integrated DNA Technologies |
| IFN-γ | Interferon Gamma |
| IKKs | IκB Kinases |
| IL-10 | Interleukin-10 |
| IL-12 | Interleukin-12 |
| IL-1β | Interleukin-1 Beta |
| IL-2 | Interleukin-2 |

| | |
|---|---|
| IL-4 | Interleukin-4 |
| IL1-R | Interleukin-1 Receptor |
| iNOS | Inducible nitric oxide synthase |
| IRAK | IL-1 Receptor-Associated Kinase |
| IRAK-1 | IL-1 Receptor-Associated Kinase- 1 |
| IRAK-2 | IL-1 Receptor-Associated Kinase- 2 |
| IRAK-4 | IL-1 Receptor-Associated Kinase- 4 |
| IRF3 | Interferon Regulatory Factor 3 |
| IRF7 | Interferon Regulatory Factor 7 |
| JNK | Jun- N Terminal Kinase |
| KCl | Potassium Chloride |
| KDO | Keto deoxyoctonoic acid |
| $KH_2PO_4$ | Monopotassium Phosphate |
| l-PGDS | Lipocalin-Type PGDS |
| LB | Luria-Bertani Broth |
| LBP | Lipid Binding Protein |
| LPS | Lipopolysaccharide |
| MAL | MyD88 Adaptor Like |
| MAPKs | Mitogen-Activated Protein Kinases |
| MD2 | Myeloid Differentiation Protein-2 |
| MEK | Mitogen-Activated Protein Kinase |
| MHC | Major Histocompatibility Complex |
| MKK3 | MAP Kinase Kinases-3 |
| MKK6 | MAP Kinase Kinases-6 |
| MMTV | Mouse Mammary Tumour Virus |
| mRNA | Messenger Ribonucleic Acid |
| MyD88 | Myeloid Differentiation Primary-Response 88 |

| | |
|---|---|
| $Na_2HPO_4$ | Disodium Hydrogen Phosphate |
| NaCl | Sodium Chloride |
| NAG | N-acetylglucosamine |
| NCBI | National Centre for Biotechnology Information |
| NF-κB | Nuclear Factor Kappa B |
| NK cells | Natural Killer Cells |
| NO | Nitric Oxide |
| NOS | Nitric Oxide Synthase |
| NSAIDs | Non-Steroidal Anti-Inflammatory Drugs |
| PAF | Platelet Activating Factor |
| PAMPs | Pathogen-Associated Molecular Patterns |
| PBMCs | Peripheral Blood Mononuclear Cells |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| $PGD_2$ | Prostaglandin $D_2$ |
| $PGE_2$ | Prostaglandin $E_2$ |
| $PGF_2\alpha$ | Prostaglandin $F_2\alpha$ |
| $PGG_2$ | Prostaglandin $G_2$ |
| $PGH_2$ | Prostaglandin $H_2$ |
| $PGI_2$ | Prostaglandin $I_2$ |
| PPARγ | Peroxisome proliferator–activated receptor gamma |
| PTGDR2 | Prostaglandin D2 Receptor 2 |
| RA | Rheumatoid arthritis |
| RIP-1 | Receptor interacting protein-1 |
| RPMI | Roswell Park Memorial Institute |
| RSV | Respiratory Syncytial Virus |
| RT | Reverse Transcription |

| | |
|---|---|
| SARM | Sterile α-and Armadillo-Motif-Containing Protein |
| SDS | Sodium Dodecyl Sulphate |
| siRNA | Small or Short Interfering Ribonucleic Acid |
| sTNFR | Soluble Tumor Necrosis Factor Receptor |
| T cells | T lymphocytes |
| TBE | Tris-Borate-EDTA |
| TE buffer | Tris EDTA Buffer |
| Th2 lymphocytes | T Helper Lymphocytes Type-2 |
| THP-1 | Human Leukaemia Monocytic Cell Line |
| TICAM-1 | Toll-Like Receptor Adaptor Molecule 1 |
| TIR | Toll-IL-1 Receptor |
| TIRAP | TIR-Domain-Containing Adaptor Protein |
| TLRs | Toll Like Receptors |
| Tm | Melting Temperature |
| TMB | Tetramethylbenzidine |
| TNF-α | Tumor Necrosis Factor-α |
| TNFRI | TNF Receptor Type I |
| TNFRII | TNF Receptor Type II |
| TRADD | TNF Receptor Associated Death Domain |
| TRAF3 | Tumor Necrosis Factor Receptor- Associated Factor 3 |
| TRAF6 | Tumor Necrosis Factor Receptor- Associated Factor 6 |
| TRAFs | Tumor Necrosis Factor Receptor- Associated Factors |
| TRAM | TRIF-Related Adaptor Molecule |
| TRIF | TIR-Domain Containing Adaptor Protein Inducing IFNβ |
| TXA2 | Thromboxane A2 |
| β-ME | Beta-Mercaptoethanol |

## Abstract

During inflammatory responses, tumour necrosis factor alpha (TNF-α) and interleukin-1 (IL-1β) are the major pro-inflammatory cytokines that can stimulate the production of secondary mediators such as prostaglandins (PGs) including $PGE_2$ which are responsible for the symptoms of inflammation. 7β-hydroxy epiandrosterone (7β OH-EpiA) is a naturally occurring steroid that has recently been reported to have cytoprotective actions and can prevent ischaemia-reperfusion induced cell death. This action appears to be related to the production of $PGD_2$ and its spontaneous metabolite 15-deoxy $PGJ_2$ (15-d-$PGJ_2$). However, previous studies have provided little information about the actions of this steroid on immune/inflammatory responses and whether any of its actions may be mediated via glucocorticoid receptors. Therefore, this study investigated the effects of 7β OH-EpiA on TNF-α and IL-1β in LPS-stimulated whole human blood, isolated monocytes and the human monocytic cell line THP-1 and also its effects on the production of anti-inflammatory cytokines such as IL-4 and IL-10.

Whole human blood was used directly, and responses were compared to monocytes isolated from whole human blood and to THP-1 cells which were cultured continuously. Levels of cytokines (TNF-α, IL-1β, IL-4 and IL-10) and prostaglandins ($PGE_2$, $PGD_2$ and 15d-$PGJ_2$), in plasma or cell supernatants, were measured by ELISA and enzyme immunoassay (EIA) respectively. In addition to this, the effect of 7β OH-EpiA on the expression of various genes (including TNF-α, TLR-4, PGDS and PTGDR2) in THP-1 cells involved in inflammatory responses was evaluated by quantitative real-time PCR.

7β OH-EpiA reduced TNF-α and IL-1β production at low concentrations (< 0.1 μM) in blood monocytes and THP-1 cells. In addition, the glucocorticoid analogue

dexamethasone also significantly reduced the LPS-induced production of both cytokines but at higher concentrations (≥ 50 μM). 7β OH-EpiA did not affect the level of the anti-inflammatory cytokines IL-4 or IL10 whereas dexamethasone significantly increased the quantity of IL-4 at concentrations ≥ 1 μM. The glucocorticoid and progesterone receptor antagonist mifepristone did not alter the inhibitory action of 7β OH-EpiA on LPS-induced TNF-α production but reversed the dexamethasone-induced suppression of TNF-α. The non-steroidal cyclooxygenase inhibitor, ketoprofen, reversed the 7β OH-EpiA-induced suppression of LPS-stimulated increases in the concentration of TNF-α. With respect to prostaglandin production, LPS increased concentrations of $PGE_2$, $PGD_2$ and 15d-$PGJ_2$ in both whole human blood and isolated monocytes. 7β OH-EpiA alone selectively increased concentrations of $PGD_2$ and 15d-$PGJ_2$ in contrast to decreasing the level of $PGE_2$. Dexamethasone reduced the LPS-stimulated increase in production of $PGE_2$, $PGD_2$ and 15d-$PGJ_2$. There were no effects of mifepristone on either the 7β OH-EpiA-induced increase in $PGD_2$ and 15d-$PGJ_2$ production or the decrease in $PGE_2$ production. Ketoprofen was confirmed to directly inhibit the production of all PGs in both blood and monocytes.

With respect to the actions of 7β OH-EpiA on gene expression, alone it did not affect expression of the TLR-4 gene in THP-1 cells, a small decrease in TLR4 expression was observed with both LPS and 7β OH-EpiA versus LPS alone. 7β OH-EpiA did, however, decrease the LPS-stimulated increase in TNF-α gene expression in THP-1 cells. With respect to $PGD_2$ processes, 7β OH-EpiA increased the quantity of mRNA for the PGDS (PGD synthase) gene and increased the concentration of PTGDR2 (DP2 receptor) expression.

The data indicates that the actions of 7β OH-EpiA on TNF-α production are highly unlikely to be mediated via a glucocorticoid or progesterone steroid receptor. They could be mediated via the selective upregulation of prostaglandin production, specifically $PGD_2$ or 15d-$PGJ_2$ which were increased by 7β OH-EpiA. Coupled with the observations that the cyclooxygenase inhibitor, ketoprofen reversed the suppressive actions of 7β OH-EpiA on TNF-α production and ketoprofen was directly confirmed to inhibit the production of all PGs, this implies that 7β OH-EpiA acts via the induction of PG biosynthesis. The identity of the PG is not certain but both $PGD_2$ or 15d-$PGJ_2$ are possible candidates as both were able to suppress LPS-stimulated TNF-α production. Nevertheless, at present the receptor for 7β OH-EpiA that initiates the increase in $PGD_2$ and 15d-$PGJ_2$ levels remains unknown.

# Chapter One: Introduction

# 1. Introduction

## 1.1 The Immune System and Immunity

The immune system plays a vital role in protecting the host against various potentially pathogenic microorganisms. The innate immune system can act through cells directed to the infected site such as phagocytic neutrophils and macrophages. It also acts via humoral mechanisms via circulating plasma proteins, particularly complement for direct killing of pathogens and cytokines as signalling molecules. Similarly, the adaptive immune response constitutes T and B lymphocytes, which can act against highly specific "learned" targets. The cells can then directly kill infected cells, as in the case of cytotoxic T-cells or provide antibodies to "recognised" targets by B-cells. In addition, T-helper cells produce cytokines to signal and coordinate further responses. The immune system is not only responsible for protecting the body but immune function disorders can lead to pathology (Mogensen, 2009). Disorders in the immune system could lead to, for instance, insufficient production of cytokines resulting in hypo-functioning of the immune system (Black, 1994). On the other hand, the overproduction of inflammatory cytokines can occur in autoimmune diseases such as inflammatory bowel disease and rheumatoid arthritis. This can occur when both innate and adaptive immune responses deviate from maintaining optimal immunity(Zhang & An, 2007).

## 1.2 Innate and Adaptive Immunity

The innate immune response acts as the first line of immune system defence. It is characterized by the recognition of microorganisms (Alam, 2007) via in-built receptors to various common components of microorganisms. Thus, this does not exhibit a memory effect with respect to subsequent encounters with same microorganisms, i.e.

the speed of the response is unchanged, but the response can be rapid, typically within seconds. The innate immune response can be activated by a broad range of molecular "patterns" in microorganisms termed pathogen associated molecular patterns (PAMPs) (Medzhitov & Janeway, 2002). These "patterns" can activate responses, which are mediated by pattern recognition receptors (PRRs). Toll- Like receptors (TLRs) are a family of these receptors that can be activated immediately within seconds to minutes of an encounter with a PAMP. There are many types of immune cells that are involved in innate immune responses such as phagocytic cells (neutrophils, monocytes, and macrophages), Natural killer cells (NK cells). In addition, these innate responses can also initiate and support adaptive immune responses by direct cell contact, in presenting antigen, and also by releasing cytokines directed to T cells etc. (Gonzalez *et al*., 2011).

Adaptive immunity is primarily mediated by antigen-specific receptors that are located on T-lymphocytes and B-lymphocytes. Cells of the adaptive response bind pathogen epitopes in a highly specific manner and this results in a "memory" to these characterised by a much faster response in a subsequent encounter (Medzhitov & Janeway, 1997). The adaptive immune response can be activated in response to innate immune cells, which are required to process and then present antigen to T-cells and also provide regulatory cytokines (Chaplin, 2010).

Although innate and adaptive immune responses differ in their responses and their reactions to eliminate pathogens, both are able to distinguish between self and non-self-molecules and also use cellular and humoral components to eradicate pathogens. Thus, innate and adaptive immune systems cooperate closely in response to pathogens (Borghans & De Boer, 2002).

## 1.3 Inflammation

Inflammation is a response to harmful stimuli such as pathogens, irritants and damaged cells (Sell, 1987). The inflammatory response can be defined as a protective attempt to remove harmful stimuli and initiate healing processes within the tissue (Gallin & Snyderman, 1999). This response usually occurs immediately after tissue damage. During the inflammatory response a series of actions take place including the following:

- Vasodilation, resulting in increased blood flow to the site of damaged tissue. This increase in blood flow is responsible for the redness and localised increase in temperature.
- Increased vascular permeability that results in exudation of fluid (plasma) into tissue leading to swelling.
- Increase in sensitivity of sensory nerves resulting in pain.
- Phagocytosis that result in the destruction and engulfment of microbes and dead cells by leucocytes, which leave the blood stream and migrate into the site of infection to become macrophages. Usually neutrophils are the first cells that migrate during inflammatory response followed by macrophages (Sherwood & Toliver-Kinsky, 2004).
- Tissue repair.

All these actions which occur during inflammation are first documented by the Roman scientist Celsus, who first described the four cardinal signs of inflammation:“ rubor, tumor, calor and dolor” (Plytycz & Seljelid, 2003).

### 1.3.1 Causes of Inflammation

The inflammatory response can be clinically divided into acute or chronic phases. Acute inflammation is the early response of the tissues to harmful stimuli, which lead to an increase in the movement of leucocytes and plasma from blood stream to the site of infection/ damage. This leads to the progression of an inflammatory cascade. When this resolves, typically within a few days there are no future consequences i.e. there is no spontaneous recurrence of the inflammation. However, chronic inflammation is characterised by progressive changing in cell types at the site of inflamed tissue and eventually shows adaptive immune cell involvement e.g. T cells. Chronic inflammation can be a consequence of a prolonged active immune response which might lead to cancer by inhibiting apoptosis, gene mutation as well as promoting cell proliferation (Rakoff-Nahoum, 2006). The pathology of chronic inflammation leads to diseases such as rheumatoid arthritis and inflammatory bowel disease.

### 1.3.2 Activation of Inflammatory Responses

There are many different types of inflammatory triggers (stimuli) such as environmental factors that include air pollution, immune reactions due to hypersensitivity, foreign bodies, which include splinters and dirt, and pathogenic factors such as infection by bacteria, viruses, fungi or parasites. Although all stimuli are different in their inflammatory action, they are similar in their inflammatory characteristics (Kumar *et al*., 2013). Common inducers of inflammation are bacterial products encountered during infection.

#### 1.3.2.1 Lipopolysaccharide

Lipopolysaccharide (LPS) is the major component that forms the outer membrane of gram-negative bacteria (Maldonado *et al*., 2016). It was originally discovered as an endogenous toxin from pathogenic bacteria, thus it was termed endotoxin (Rietschel *et al*., 1993). Richard Pfeiffer distinguished endotoxin from exotoxin, since he described endotoxin as toxin contained within bacterial cells and can be released following cell lysis or cell death (Caroff & Karibian, 2003). Meanwhile exotoxin was defined as toxin that is released into the surrounding environment by viable bacteria (Ramachandran, 2014). An important role of the outer membrane of gram-negative bacteria is to provide a permeability barrier that protects against harmful molecules such as antibiotics from entering into the bacteria cell (Nikaido, 2003). LPS molecules cover most of the space of the outer layer of the membrane of gram-negative bacteria, whereas glycerophospholipids are located in the inner layer of the membrane (Okuda *et al*., 2016; Vaara & Nurminen, 1999).

Lipopolysaccharides are amphipathic complexes, and the molecular weight is about (10 kDa-1000 kDa) that makes LPS different in their chemical structure between bacteria species (Sun & Shang, 2015). The chemical structure of LPS in general consists of three component regions (Fig.1.1). The first region is a lipid component known as lipid A (the hydrophobic portion of LPS), which is formed from phosphorylated N-acetyl glucosamine (Ebina et al.) that is attached to 6 saturated fatty acids. Most of the fatty acids of lipid A are directly connected to NAG, and others are esterified to 3 hydroxy fatty acids. The Lipid A moiety is directly responsible for the endotoxin activities of LPS that activate innate immune responses and result in inflammatory actions (Raetz et al., 2007). (See Fig 1.2)

The second region is a nonrepeating core or (R) antigen, also known as R polysaccharide. It is divided into an inner and outer core. The inner core attaches to lipid A and consists of oligosaccharides such as 2-keto-3-deoxyoctanoic acid (KDO) which is less variable and present in all LPS. Therefore, it has been used as a target indicator for endotoxin in many assays (Caroff & Karibian, 2003). The outer core is connected between R- antigen and O- antigen and consists of sugars such as heptoses and hexoses (Caroff & Karibian, 2003; Silipo & Molinaro, 2010).

The third region is a long repeating polysaccharide known as an O-specific chain or O-antigen. It consists of around 20 different types of sugars and can be up to 40 units in length. O-antigen is the hydrophilic domain of LPS and is responsible for immunogenic activity (specific antibody production) due to its high specificity within species and even strains. Therefore, the O-antigen has been used in the serological (specific antibodies present in serum) classification of gram-negative bacteria. This component of LPS is responsible for the smooth morphology of colonies on agar plates whereas the lack of this component makes the colonies appear rough (Lerouge & Vanderleyden, 2002; Raetz & Whitfield, 2002).

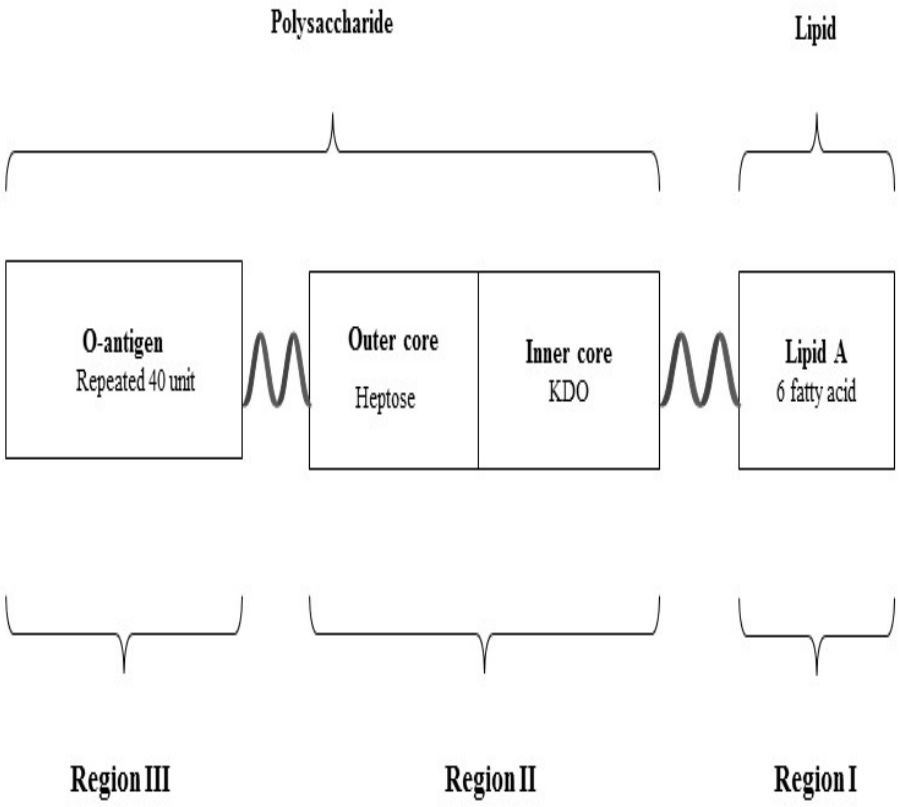



**Fig 1.1 General Structure of LPS Gram-Negative Bacteria Adapted from (Rietschel *et al*., 1994)**

Lipid A

Glucophospholipid

hydrophobic part

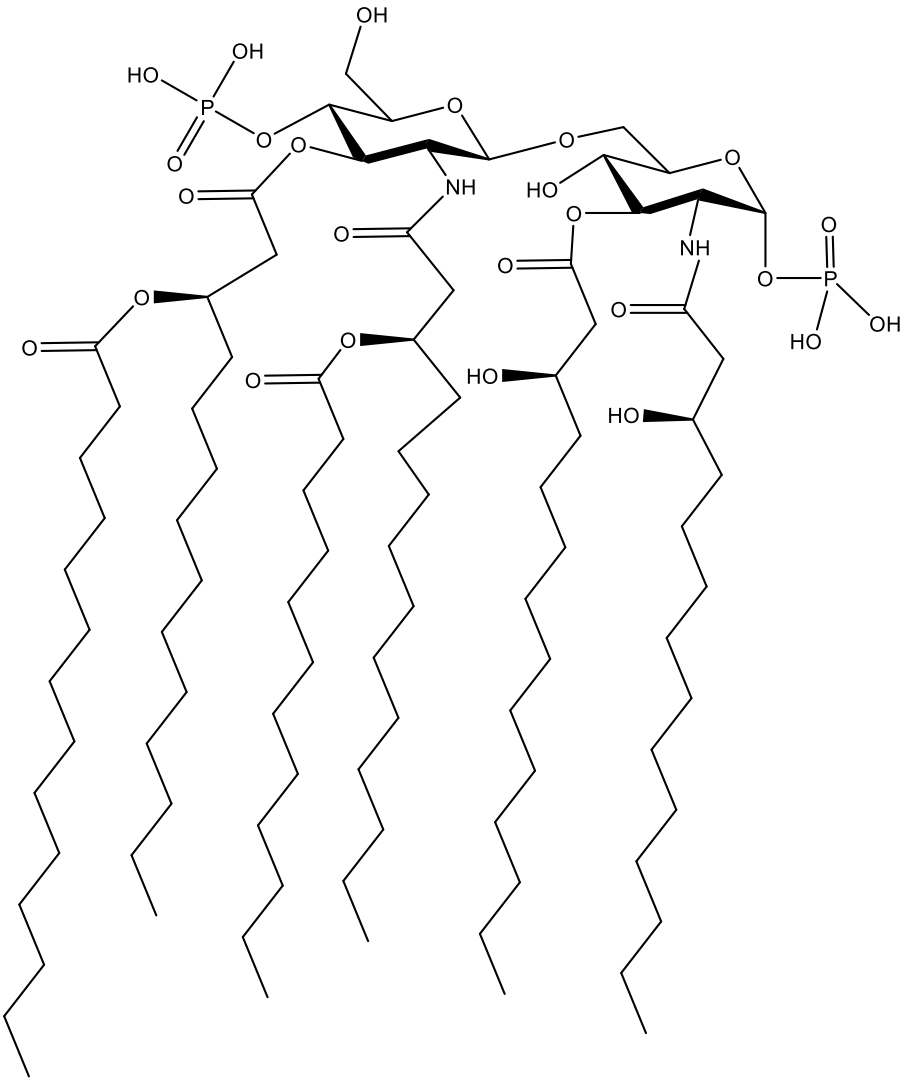



**Fig 1.2 Chemical Structure of lipid A(hydrophobic part of LPS) from E. coli (region I from Fig 1.1) (Scott *et al.*, 2017)**

In humans and other mammalian species, LPS triggers an innate immune response that leads to the production of inflammatory cytokines. Lipid A of LPS mostly documented to be trigger of diverse mediators of inflammation, such as production of TNF-α and IL1-β in macrophages and monocytes (Dinarello, 1991). This production of cytokines causes pathological pain of inflammation. LPS is the most effective stimuli for the induction and release of pro-inflammatory cytokines particularly TNF-α and IL-1β (Tang *et al*., 2006). Both cytokines in turn stimulate the production of LBP from liver, lung and others (Dentener *et al*., 2000).

In humans, LPS binds to a lipid binding protein (LBP), which is found to be increased in serum from 5-10μg/ml (normal range) to 200μg/ml during infection or LPS stimulation (Kopp *et al*., 2016). The role of LBP during innate immune responses to small amounts of LPS was clarified in two different studies. In the first study, where mice treated with monoclonal antibody against LBP binding to LPS, the mice were protected from septicaemia after intraperitoneal injection of LPS (Le Roy *et al*., 1999). In the second study, LBP gene deficient mice and wile -type mice that injected with E. Coli were used. LBP gene deficient mice showed increased mortality and high level of bacteria in the blood stream compared to wild-type mice (Knapp *et al*., 2003). All these studies confirmed the critical role of LBP in transferring the LPS to CD14. When LPS binds into LBP, it can bind to CD14 in the cell membrane of monocytes macrophages or endothelial cells which transfer it to another non-anchored protein (MD2) that connects with Toll-like receptor-4 (TLR4). This sequence of events initiates the production of inflammatory cytokines. Both CD14 and TLR4 are present in many immunological cells and can activate these cells to secrete pro-inflammatory cytokines such as TNF-α, IL-1, IL-6 as well as anti-inflammatory cytokines such as IL-10 (van der Bruggen *et al*., 1999).

These pro-inflammatory cytokines, in turn, stimulate the production of prostaglandins, particularly $PGE_2$, which are responsible for the symptomatic manifestations of the inflammatory response as shown in a study by Rotondo *et al* (1988) that showed an increase in the level of circulating PGE2 in response to LPS administration in rabbits. This increase is responsible for systemic inflammatory response particularly fever (Rotondo *et al*., 1988). The over-production of inflammatory cytokines can lead to mild systemic inflammatory responses such as fever or severe systemic inflammation that can be life-threatening resulting in septic shock and ultimately death (Dinarello, 2000). In vitro, gram-negative bacteria or purified LPS can be used to activate cells to produce pro-inflammatory and anti-inflammatory cytokines. Incubation of LPS with whole blood or cells isolated from the blood can stimulate these cells to release cytokines that further enhance inflammatory stimulation and release prostaglandins, reactive oxygen species and nitrogen species which exert cytotoxic effects against pathogens and tumour cells (Shibata *et al*., 2002). The production of these cytokines occurs in the first few hours but no longer than 24 hours due to their down regulation by prostaglandin production, mainly $PGE_2$ (see Fig. 1.3) (Kunkel *et al*., 1986a). The production of TNF-α in response to LPS stimulation mainly occurs via toll-like receptor 4 (TLR4) (Swantek *et al.*, 1997).

#### 1.3.2.2 Toll-Like Receptors

Recognition of different pathogenic stimuli is a vital role of macrophages and monocytes. This innate recognition takes place via Pattern Recognition. Receptors (PRRs) expressed on the surfaces of most immune cells (Takeuchi & Akira, 2010). Toll-like receptors are PRRs, which are involved in innate immunity and induce the secretion of pro-inflammatory cytokines and chemokines including TNF-α and IL-1 etc. (Kaisho & Akira, 2002). To date, 10 TLRs have been identified in humans,

whereas 13 TLRs have been identified within mice (Kaisho & Akira, 2006). There are two types of TLRs with different cellular localisations; plasma membrane receptors which are sensitive to microbial membrane components such as LPS and intercellular ones that sense microbial nucleic acid such as dsRNA and viral RNA/ DNA (Lester & Li, 2014).

Peptidoglycan and LPS, from the outer membrane of gram positive and negative bacteria respectively, activate immune cell responses by utilizing TLR receptor pathways. LPS was found to be recognised by TLR4 via detection of lipid A moiety of LPS that required LBP to bind firstly to lipid A to transform from multimers to monomer form that can be easily detected by CD4/TLR4 complex (as describe in details in 1.3.2.2.1) (Kaisho & Akira, 2006) (Miller *et al*., 2005). In addition, some types of virus such as respiratory syncytial virus (RSV) and mouse mammary tumour virus (MMTV) have been reported to activate cells via TLR4 (Kawai & Akira, 2005; Kumar *et al.*, 2009). Double stranded RNA (dsRNA) or poly I:C can be detected by TLR3 (Alexopoulou *et al*., 2001). A summary of TLR ligands is illustrated in Table (1.1)

**Table 1.1 TLRs and their Ligands (Kumar *et al*., 2009)**

| Receptor | PAMPs | species | Adaptor | Transcriptional factor |
|---|---|---|---|---|
| **TLR1&2** | Triacyl lipopeptide | Bacteria & Mycobacteria | TIRAP, MyD88 | NFκB |
| **TLR2** | Peptidoglycan, Lipoarabinomannan, Haemagglutinin, Phospholipomannan, Glycosylphosphatidyl-Inositol mucin | Bacteria, Mycobacteria, Viruses<br><br>Fungi, Protozoa | TIRAP, MyD88 | NF-κB |
| **TLR3** | ssRNA, dsRNA | Viruses | TRIF | NF-κB, IRF3 & IRF7 |
| **TLR4** | LPS, RSV & MMTV, Glycoinositolphospholipids, Glucuronoxylomannans | Bacteria, Protozoa, Viruses, Fungi | TIRAP, MyD88, TRAM & TRIF | NF-κB, IRF3 & IRF7 |
| **TLR5** | Flagellin | Bacteria | MyD88 | NF-κB |
| **TLR6** | Diacyl lipopeptide | Mycoplasma | TIRAP & MyD88 | NF-κB |
| **TLR7** | RSV | Viruses | MyD88 | NFκB, IRF7 |
| **TLR8** | ssRNA | Viruses | MyD88 | NF-κB, IRF7 |
| **TLR9** | dsDNA (HSV), unmethylated CPG motif (genomic DNA) | Viruses, Bacteria | MyD88 | NF-κB, IRF7 |

#### 1.3.2.2.1 Signalling Pathways of TLRs

TLRs can recognise different types of PAMPs which in turn initiate cytoplasmic signal proteins that are similar to the interleukin-1 receptor known as Toll-IL-1 receptor domain (TIR) (Takeda & Akira, 2004).The TIR domain contains proline residues which are conserved in all types of TLR except TLR3. Mutations in the structure of TIR lead to replacement of histidine instead of proline which result in negative effects on TLRs signalling (Hoshino *et al*., 1999). Signalling processes are mediated through the TIR domain, which contain cytoplasmic adaptor molecules for various TLRs. The adaptor proteins associated with the activation of TLRs signalling such as TLR-2, TLR-4 and TLR-9 is the adapter protein myeloid differentiation primary- response gene 88 (MyD88). TIR-domain-containing adaptor protein inducing IFN-β (TRIF) also known as (TICAM-1). TIR-domain-containing adaptor protein (TIRAP) also termed MyD88 adaptor-like (MAL), TRIF-related adaptor molecule (TRAM) and sterile α- and armadillo-motif-containing protein (SARM) (Kawai & Akira, 2010; Kumar *et al*., 2009; Takeuchi & Akira, 2010; Uematsu & Akira, 2006).

In order to produce cytokines, MyD88 is an essential cytoplasmic adaptor protein, which associates with all members of the TLR families except TLR3 (Kaisho & Akira, 2002). The initiation of MyD88 occurs when MyD88 binds to ligand on the TLR. This complex protein activates IL-1-receptor-associated kinase (IRAK) and tumor necrosis factor receptor-associated factor 6 (TRAF6). This in turn activates other protein kinases such as mitogen-activated protein kinases (MAPKs) and protein kinase C. These activated kinases result in the downstream translocation of transcription factor NF-κB and interferon INF regulatory factor 3 (IRF3) or 7 (IRF7), which in turn modulate expression and production of cytokines including TNF-α, IL-1β and chemokines. TLRs that use MyD88 in their signalling pathways are known as MyD88-

dependent TLRs (Kawai & Akira, 2010; Takeuchi & Akira, 2010). MyD88 can also, with TIRAP, mediate the signalling of TLR1, TLR2, TLR4 and TLR6 (Kawai & Akira, 2011; Takeuchi & Akira, 2010). MyD88 alone can mediate TLR5, TLR7, TLR9 and TLR11 signalling. In addition to MyD88 pathways, there is also a signalling pathway known as the TRIF-dependent pathway which is essential for expression and production of pro-inflammatory cytokines and IRF3 and IRF7 (Akira *et al.,* 2006; Hoebe *et al.*, 2003). TRAM is another molecular adaptor that can activate the TRIF-dependent pathway in response to ligands of TLR4 but not TLR3 (Yamamoto *et al*., 2003). Since TLR4 is important for responding to LPS, LPS activates TLR4 by the sequential interaction of a number of proteins including LPS binding protein (LBP), CD14 and myeloid differentiation protein-2 (MD2). LPS binds to LBP and promotes interaction between LPS and CD14; CD14 in turn facilitates binding of LPS to the TLR4/MD2 complex (Fitzgerald *et al.*, 2004). This complex binds to MYD88 which releases protein kinases such as IL-1-receptor associated kinase-4 (IRAK-4) and IRAK-1. These kinases recruit the adaptor- TRAF6. The IRAK-1-TRAF6 complex dissociates to permit activation of TAK1 and MAP kinase. This leads to IκB phosphorylation with the sequential disengagement and activation of the transcription factor NF-κB. This then leads to nuclear translocation of NF-κB for the expression of pro- inflammatory cytokine genes (Kaisho & Akira, 2002). See Fig.1.3

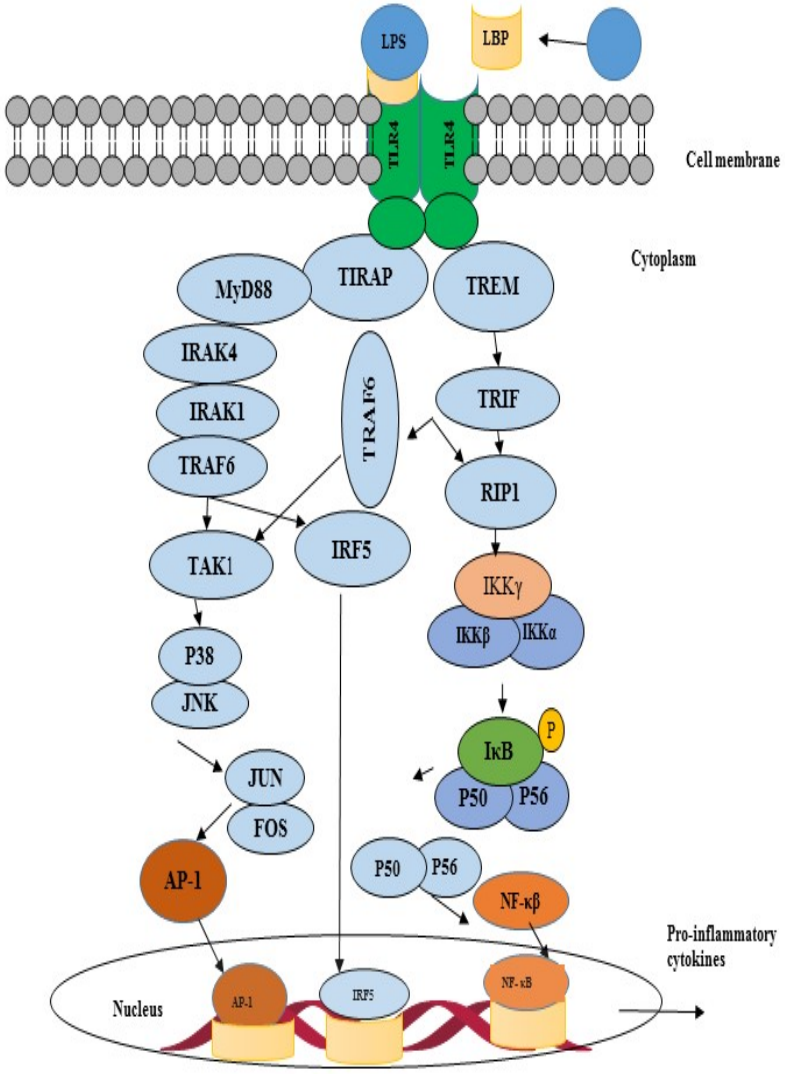



**Fig 1.3 LPS and TLR-4 signalling pathways to produce pro-inflammatory cytokines**

Firstly, LPS binds to LBP and this complex recognized by TLR-4 that in turn activates many pathways such as MyD88 and TRIF. The MyD88-dependent pathway activates nuclear factor-κB (Font-Nieves *et al*., 2012), activator protein-1 (AP-1) and interferon regulatory factor 5 (IRF5), which induce inflammatory cytokine expression, whereas the TRIF-dependent pathway activates IRF5, in addition to NF-κB and AP-1, which induce type I interferon (IFN) expression (adapted from Lee & Kim, 2007).

## 1.4 Pro-inflammatory Cytokines

Cytokines are small inter-cellular signalling proteins with a very wide spectrum of activity. They participate in the initiation of inflammatory responses and can be broadly grouped into two categories according to their activities during inflammation: pro-inflammatory and anti-inflammatory cytokines. TNF-α and IL-1β mainly orchestrate inflammation. These cytokines have the ability to activate the production of secondary mediators of inflammation, prostaglandins, which in turn are responsible for the symptoms of inflammation (see Fig 1.4).

### 1.4.1 Interleukin 1 (IL-1)

A variety of immune cell types can be affected by IL-1, which is classified as prototypic pro-inflammatory cytokine. IL-1 plays an important role in acute and chronic inflammation and autoimmune diseases (Ren & Torres, 2009). There are 11 members of the IL-1 family, which induce other pro-inflammatory cytokines and in turn initiate inflammatory responses via their receptors expressed on endothelial cells and leukocytes. IL-1α and IL-β are the most understood members of the IL-1 family because of their early discovery (Dinarello *et al*., 1990). There are many cells that can produce IL-1β such as monocytes, macrophages, dendritic cells and fibroblasts. IL-1β is released from cells after exposure to LPS via binding to TLR4. After stimulation by LPS, IL-1β is synthesised as a precursor which is released in its mature form following cleavage by caspase-1. IL-1β can stimulate responses via binding to either TLRs or to its specific IL-1 receptor. IL-1 β displays many pro-inflammatory activities, such as cytokine release, cell migration to inflamed tissue, increasing pain sensitivity and affecting the thermoregulatory region in the hypothalamus, which is responsible for the increased body temperature (fever) during infection (Contassot *et al*., 2012). IL-1

also increases the expression of cyclooxygenase (COX) resulting in the production of $PGE_2$ that can reduce the further production of IL-1 (Dinarello *et al*., 1986). Thus, $PGE_2$ may be able to suppress the production of IL-1 via a negative feedback mechanism (Kunkel *et al.*, 1986a). Reducing or neutralising IL-1 is a therapeutic approach for the treatment of many autoimmune diseases with an inflammatory component such as rheumatoid arthritis (Dinarello, 2011).

### 1.4.2 Tumour Necrosis Factor-alpha (TNF-α)

TNF-α is a pro-inflammatory cytokine, which appears to be mainly involved in the development of inflammatory processes (Apostolaki *et al*., 2010). It can be produced by different immune cells during the inflammatory course, such as activated monocytes and macrophages (Collins & Grounds, 2001) and NK cells (in response to CpG DNA) (Chow *et al*., 1999; Reefman *et al*., 2010). Neutrophils, mast cells, astrocytes and fibroblasts can also produce TNF-α in response to activation by bacteria or bacterial components mainly LPS (Fahey *et al.,* 1995). LPS is the most potent activator of TNF-α from blood monocytes although there are many other different stimuli of TNF-α production in addition to LPS such as oxygen radicals, viruses, nitric oxide, and parasites (Bedoui *et al*., 2005; Chow *et al.*, 1999).

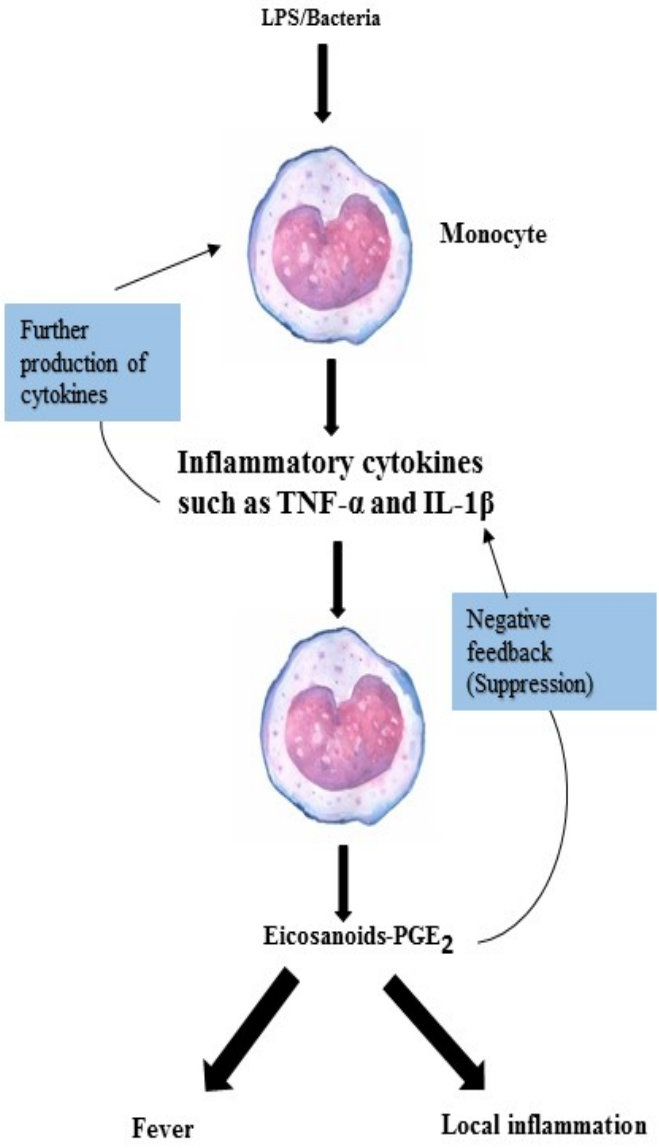



**Fig 1.4 LPS induction of cytokines, which are released by active monocytes and negative regulation by prostaglandin ($PGE_2$).**

### 1.4.2.1 TNF-α Receptors and Signalling

Two cell surface receptors for TNF-α have been described. Both are characterised by their extracellular domains which contain repeating cysteine-rich motifs. These receptors are classified as TNF receptor type I (TNFRI) and TNF receptor type II (TNFRII) (Naismith & Sprang, 1998). TNFRI is expressed in most tissues/ cells and can be activated by both membrane and soluble forms of TNF-α whereas TNFRII is expressed on immune cells (Vasanthi *et al.*, 2007). TNFRI receptors characteristically have circa 60 amino acid in their cytoplasmic tails known as the death domain (DD) that is responsible for pro-apoptotic signalling of TNF (Locksley *et al.*, 2001). Pro-inflammatory and cell death pathways, which are activated by TNF, are mediated by TNFRI due to the presence of the DD that is absent in TNFRII (van Horssen *et al.*, 2006). TNFRII signalling is still unclear but it might have a role in the regulation of TNFRI (Bradley, 2008). When TNF binds to TNFR, this interaction can activate many different responses leading to cell proliferation, apoptosis or inflammation (MacEwan, 2002).

### 1.4.2.2 Functional/ Physiological Roles of TNF-α

TNF-α induces IL-1 production; therefore, IL-1 shares almost all of TNF-α biological actions (Mauviel *et al.,* 1991). Inducing B lymphocytes to proliferate and produce antibodies is one of the common actions of both TNF-α and IL-1. Among the functional roles of TNF-α in immune responses is the initiation of apoptosis (Naismith & Sprang, 1998). When TNF-α receptor undergoes a conformational change, TNF binding to TNFRI associates with TNF- receptor associated death domain (TRADD) this forms a complex with receptor interacting protein-1 (RIP-1) and TNF-associated

factor (TRAF-2). This activation can induce expression of growth factors and proliferation leading to cell survival.

In addition, interaction between TNF and TNFRI can also lead to apoptosis through the initiation of the caspase cascade (Wajant *et al.,* 2003, and Karin *et al.,* 1997). In target cells, apoptosis can occur when activated TNFRI stimulates TRADD (Fas-associated death domain) FADD which in turn shift inactive forms of caspase 8 and caspase 10 to active forms. Activation of caspase 8 and caspase 10 activate other caspases like 3, 6, and 7 that lead to subsequent apoptotic cell death (McIlwain *et al.*, 2013). Moreover, in blood circulation monocytes quickly undergo apoptosis (after two days). Therefore, in vitro, they are stimulated by LPS or pro-inflammatory cytokines such as TNF-α and IL-1β as well as reduction of caspase activation which can be prevented be differentiation factors (Mangan *et al.*, 1991). Till now, the molecular mechanisms of activation-induced survival signals in monocytes remain unclear (Perera & Waldmann, 1998).

#### 1.4.2.3 TNF-α as a Mediator of Chronic Diseases and their Treatment

Overproduction of cytokines appears to be the main cause of many chronic diseases with an inflammatory component. TNF-α is one of these cytokines, which are reported to be over-produced with high levels in plasma and synovial fluid of patient with chronic inflammatory diseases. It can lead to the pathology of many diseases such as rheumatoid arthritis (RA), arthrosclerosis, inflammatory bowel disease and others (Feldmenn & Ravinder, 2001). In RA, IL-1, IL-8, IFN-γ and TNF-α are the major pro-inflammatory cytokines released during the course of disease (Feldmenn &Ravinder, 2001). Therefore, the target of treating these diseases is to reduce the over-production

of cytokines. In a study that used cultures of dissociated rheumatoid synovial membranes, it was found that the inhibition of TNF- α by using TNF blockade or neutralising monoclonal antibodies (mAb) against TNF-α led to the reduction of other cytokines such as IL-1 and GM-CSF. This demonstrates the potential treatment of chronic diseases by blocking the actions of TNF-α by using anti-TNF-α antibodies (Brennan, 1994; Brennan *et al*., 1989). In clinical studies this has been confirmed in RA patients where blocking TNF-α has led to significant clinical improvement within a short time (Elliott & Maini, 1994).

TNF-α antagonists that are used in clinical practice are proteins, which are either antibodies against TNF-α or based upon TNF-α receptors, TNFRI and TNFRII. These biologic agents are Etanercept, Infliximab and Adalimumab which are also known as anti-TNF-α agents. These drugs act by suppressing the interaction of cytokines with their receptors such as Etanercept, a fusion protein of the sTNFRII and IgG, which specifically binds and effectively neutralises the actions of TNF-α. Other agents act in a similar manner by binding to both circulating levels of TNF-α and mTNF-α, such as Infliximab and Adalimumab (Flendrie *et al*., 2007). The best characterised example of these treatments is Infliximab.

#### 1.4.2.4 Anti-TNF antibodies as therapeutic agents

Anti-TNF antibodies such as adalimumab, infliximab and the soluble TNF receptor fusion molecule etanercept are the most commonly used inhibitors of TNF actions in chronic inflammatory diseases such as rheumatoid arthritis and Crohn's disease (Thalayasingam & Isaacs, 2011). The treatments have been found to reduce serum levels of other cytokines such as IL-1β, IL-6 and IL-8 within synovial tissue (Markham & Lamb, 2000). There are many disadvantages that have been reported to

be associated with using these treatments including a local reaction at the injection site such as swelling and itching (Vasanthi *et al*., 2007), infection (mainly tuberculosis reactivation), risk of malignancy, pneumonitis, hepatitis B and impairment of host defence against infectious pathogens (Saag *et al*., 2008; Tracey *et al*., 2008).

Recent studies carried out on various oxysterols, particularly 25-hydroxycholestrol have shown it to reduce LPS-induced TNF-α production (Englund *et al.*, 2001). Glucocorticoid analogues such as dexamethasone etc., are well established inhibitors of cytokines production, particularly TNF-α (Bleeker *et al*., 1997). There are also other inhibitors of inflammatory symptoms that act by suppressing the synthesis of prostaglandins, such as non- steroidal anti-inflammatory drugs (NSAIDs) (Joussen *et al., 2002)*. This is important to connect the negative feedback mechanism of PGs with the release of TNF-α and other pro-inflammatory cytokines (Kunkel *et al*., 1986a). In vitro, NSAIDs have been found to increase TNF-α production in rheumatoid synovial membrane cultures and human peripheral blood monocyte. This could have been due to inhibition effects of NSAIDs on COX and hence could have stopped negative feedback of PG on pro-inflammatory cytokines such as TNF-α (Page *et al*., 2010). This action of NSAIDs during RA cannot stop the progress of pathological symptoms (cartilage destruction) but can relief symptoms connected with high level of PGs such as pain and fever (Crofford, 2013).

Consequently, further study is required to identify safe and effective therapies to prevent the overproduction of TNF-α during many diseases including rheumatoid arthritis (Barsoum *et al*., 2009; Rao & Knaus, 2008).

## 1.5 Prostaglandins

Prostaglandins are small lipid molecules that derived from essential fatty acids. They were first isolated from the semen of sheep and human prostate glands from which their name is derived (Goldblatt, 1935). They are found in every tissue and body fluid and have many biological functions in the mammalian body with the potential to modulate almost every cellular response especially immune functions. Prostaglandins are classically the major factors that contribute to the complex course of an inflammatory response (Harris *et al.*, 2002). The production of prostaglandin can occur in response to mechanical trauma or inflammatory stimulators, which activate the production of cytokines and arachidonic acid from phospholipids membrane of cells. They are classified as pro-inflammatory mediators due to their high levels in inflamed tissues and their involvement in the symptoms of inflammation i.e. swelling and pain. Moreover, they also classified as anti-inflammatory due to high levels of it during the last stages of inflammation i.e. $PGD_2$ and its dehydration end product 15-deoxy-Delta-prostaglandin $J_2$ (15d-$PGJ_2$) (Scher & Pillinger, 2009).

Prostaglandins are derived from arachidonic acid (AA) released from plasma membrane phospholipids by phospholipase $A_2$ ($PLA_2$), and subsequently converted by cyclooxygenase (COX) to different types of prostaglandin (Balsinde *et al.*, 2002; Tilley *et al*., 2001).

### 1.5.1 Cyclooxygenase Enzymes

Cyclooxygenase (COX) is an enzyme that produces prostaglandins from arachidonic acid. COX is also known as prostaglandin H synthase (PGHS). To produce prostaglandins, arachidonic acid is converted by COX to the 9-11 endoperoxide, 15-hydroperoxide and endoperoxide -$PGG_2$ in two peroxidation steps. Then, the 15-

hydroperoxide undergoes peroxidase activity by the same COX enzyme to yield PGH2 (Williams *et al.,* 1999). $PGH_2$ is subsequently converted to different types of prostaglandins such as $PGE_2$, $PGD_2$, $PGF_{2\alpha,}$ and $PGI_2$ ( See Fig 1.5). In addition, there are two isoforms of COX known as COX-1 and COX-2 which have different location in the cells. COX1 is in the endoplasmic reticulum whereas COX-2 is in the nuclear envelope. Both types of COX are closely identical in their structure and substrate specificity. COX-1 is expressed in most cells, and constitutive COX-2 is induced by inflammatory stimuli. This can be demonstrated by the absence of the COX-2 enzyme from unstimulated cells.

Several studies found that COX-2 is induced by bacteria and LPS in immune cells via the induction of pro-inflammatory cytokines mainly IL-1β or TNF-α (Jeong & Jeong, 2010; Park *et al.*, 2004). This may explain the high levels of prostaglandins in inflamed tissues. The production of PGs also occurs in distinct phases. For example, in a study of carrageenan-induced pleurisy in rats, COX-2 had two peaks of high protein expression. The first one was 2 hours after the initial stimulation which was associated with the production of $PGE_2$ whereas in the second peak, at 48 hours the level of COX-2 expression was 350% that of expression at 2 hours and was associated with a high level of $PGD_2$ production which coincided with the start of the resolution of the inflammatory response. This late phase of inflammation is thought to result from an anti-inflammatory role of COX-2 (Gilroy *et al.*, 1999). Both COX isoforms contribute to inflammatory responses, but COX-2 is often induced during acute and chronic inflammation (St-Onge *et al.*, 2007). Anti-inflammatory cytokines like IL-4, IL-10, and IL-13 can decrease COX2 expression (Vane et al., 1998). Moreover, both COX-1 and COX-2 are associated with the conversion of arachidonic acid to $PGG_2$, then to $PGH_2$ and finely to a variety of specific prostaglandins during prostaglandin synthesis (Vane *et al.,* 1998).

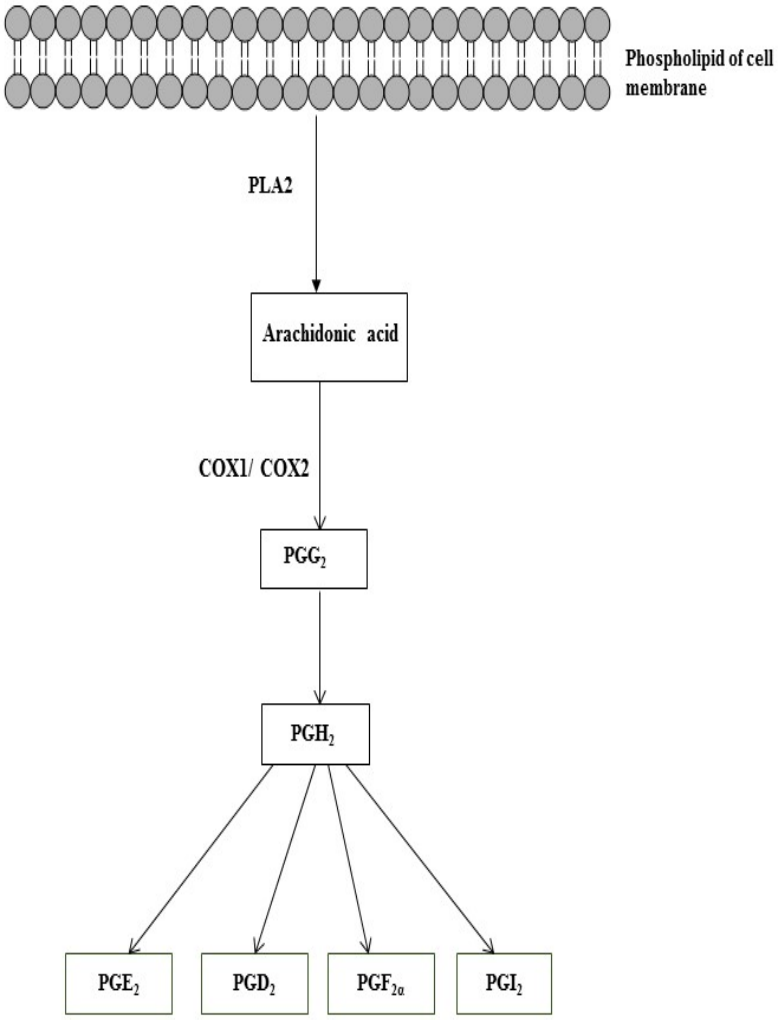



**Fig 1.5 Pathway of prostaglandins biosynthesis**

Arachidonic acid (AA) releases from phospholipids of cell membrane in the presence of PLA2.Then AA under the enzymatic activity of COX1 and COX2 converts it to $PGG_2$, which is quickly converted to $PGH_2$ (the common precursor for all PGs). Finally, $PGH_2$ is converted to the prostanoids $PGE_2$, $PGD_2$, $PGF_{2\alpha}$ and $PGJ_2$ depending on the presence of the appropriate isomerase (Simmons *et al*., 2004).

### 1.5.2 Prostaglandins and Inflammation

#### 1.5.2.1 Prostaglandin E $PGE_2$

$PGE_2$ is the major prostaglandin released during inflammatory responses, and it is regarded as the major pro-inflammatory eicosanoid (Funk, 2001). Studies found that $PGE_2$ can induce various symptoms of inflammation such as vasodilation, swelling and pain. In addition, Portanova *et al* (1996) found that specific anti-$PGE_2$ antibodies attenuated both swelling and pain which are associated with inflammation. In vivo, $PGE_2$ was found to be involved in the development of inflammatory symptoms especially oedema and pain. This was demonstrated by using rat paw oedema and hyperalgesia induced by carrageenan. Rat paw oedema and hyperalgesia were suppressed by an anti-$PGE_2$ monoclonal antibody compared to treatment with a control non-specific antibody (Portanova *et al.*, 1996). This provided very strong evidence that the symptoms of inflammation are induced by $PGE_2$. However, it has also been shown that $PGE_2$ can inhibit the effects of many immune cell responses such as the production of inflammatory cytokines or phagocytosis (Davidson *et al.*, 1998). Macrophages can be stimulated by LPS or TNF- to induce $PGE_2$ production, and the LPS response can be inhibited by adding exogenous of $PGE_2$ which inhibits the expression of mRNA of TNF-α and protein secretion (negative feedback loop) (Kunkel *et al*., 1986b; Kunkel *et al*., 1988). This negative feedback could also be mediated by inducing the production of IL-10 in response to $PGE_2$ (Strassmann *et al.*, 1994).

Cytokines released in response to LPS stimulation have been reported to be reduced by PGE2 particularly TNF-α and IL-1 (Hempel *et al*., 1996; Vassiliou *et al.,* 2003).

#### 1.5.2.2 Prostaglandin $D_2$ ($PGD_2$)

Like $PGE_2$, $PGD_2$ has also been found to modulate inflammatory responses. $PGD_2$ can be synthesised by the conversion of $PGH_2$ via two enzymes: hematopoietic PGD synthase (h-PGDS) and lipocalin- type PGDS (l-PGDS). Lipocalin- PGDS can be found constitutively in tissues, whereas h-PGDS can be induced during bacterial infections (Joo & Sadikot, 2012). In addition, $PGD_2$ acts by binding to the G protein–coupled receptors DP1 and CRTH2 (Chemoattractant receptor homologues molecule expressed on T-helper type 2 cells) which is also known as DP2. Moreover, $PGD_2$ can be produced by many cells including mucosal mast cells or by intestinal epithelial cells. The functional role of $PGD_2$ is mediated by binding to either its specific receptor or by conversion as it readily undergoes spontaneous dehydration to generate the metabolite 15-deoxy-$\Delta^{12,14}$-$PGJ_2$ (15d-$PGJ_2$) (Xue *et al.,* 2005). 15d-$PGJ_2$ can mediate anti-inflammatory responses by blocking pro-inflammatory NF-κB signalling cascades independently of peroxisome proliferator–activated receptor, PPARγ, via direct interactions with signalling molecules (Kim *et al*., 2007).

##### 1.5.2.2.1 15-Deoxy$^{12,14}$-prostaglandin $J_2$ (15d-$PGJ_2$)

$PGD_2$ is a natural precursor for $PGJ_2$ and can spontaneously convert to 15d-$PGJ_2$ (Hirata *et al.,* 1988). 15d-$PGJ_2$ has been identified in human body fluid (Hirata *et al*., 1988) as well as in inflammatory fluids during the resolution stage of inflammation (Gilroy *et al*., 1999). Production of 15d-$PGJ_2$ is thought to occur extracellularly via nonenzymatic conversion of $PGD_2$ (where it could act as an autocrine and paracrine agent during inflammatory process). In addition, it may be produced intracellularly where it could interact with PPARγ (Shibata *et al*., 2002). The anti-inflammatory effects of this novel prostaglandin in vivo have been identified in several studies (Shibata *et al.*, 2002).

Recent studies have shown that 15d-$PGJ_2$ is a ligand for PPARγ and contributes to the suppression of gene expression in activated macrophages, including iNOS, and TNF-α genes (Jiang *et al.*, 1998; Ricote *et al*., 1998). The anti-inflammatory actions of 15d-$PGJ_2$ have been suggested to occur via binding with PPARγ, which appears clear in the LPS or INF-γ activation of macrophages rather than in resting macrophages or circulating monocytes. This binding complex suppresses the expression of anti-inflammatory genes such as TNF-α, COX-2 and iNOS genes (Jiang *et al.,* 1998; Ricote *et al*., 1998). These suppressive effects could be mediated by the inhibitory actions on transcription factors including NF-κB and activator protein-1 (AP-1) which would decrease the production of TNF-α and IL-1 (Powell, 2003). It has also been shown that the production of 15d-$PGJ_2$ can be induced by novel cytoprotective steroids such as 7ß OH-epiandrosterone and inflammatory cytokines such as TNF-α and IL-1 from human monocytes and may be the activation of protective pathways (Davidson *et al*., 2008).

## 1.6 Anti-inflammatory Cytokines

### 1.6.1 Interlukin10 (IL-10) as an Anti-inflammatory Cytokine

IL-10 is an anti-inflammatory cytokine which is produced by different types of immune cells especially macrophages and monocytes. It appears to have a role in limiting inflammatory responses and regulating the proliferation and differentiation of many immune cells (Shao *et al.*, 2014). IL-10 can control inflammatory responses by reducing the production of pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6 and TNF-α. This was initially indicated in the study of de Waal Malefyt *et al*., (1991). Human monocytes were incubated with LPS, INF-γ or a combination of INF-γ and LPS with human IL-10 or viral IL-10 in the presence or absence of anti-IL-10 mAb for

24 hrs. The results showed that IL-10 inhibits the production of these pro-inflammatory cytokines in response to both methods of stimulation.

This was confirmed as being directly attributable to IL-10 as it was not possible to inhibit the production of these cytokines in the presence of anti-IL-10 antibody because of the neutralization of IL-10 (de Waal Malefyt *et al.*, 1991).

### 1.6.2 Interleukin 4 (IL-4) as an Anti-inflammatory Cytokine

Interleukin-4 (IL-4) is a pleiotropic glycoprotein cytokine with a molecular weight of 20,000. It can be produced by T lymphocytes, mast cells and bone marrow cells and was recently found to be produced by neutrophils (Brandt *et al*., 2000). IL-4 plays a central role in adaptive immune responses that control B-cell functions (particularly isotype switching of antibodies) and T-cell polarisation of helper cells (switching to TH2 responses) (Mukherjee *et al.*, 2009). It has also shown its ability to orchestrate innate immune by reducing production of TNF-α, IL-1β and $PGE_2$ in human macrophage and monocytes stimulated with LPS (Hart *et al*., 1991).

## 1.7 Anti-inflammatory Actions of Glucocorticoids

### 1.7.1 Endogenous Steroids

Glucocorticoids are steroid hormones synthesised by the adrenal cortex which occurs in response to stress or cytokine stimulation of the hypothalamic- pituitary- adrenal axis (HPA) (De Bosscher *et al*., 2008) ( See Fig 1.6). Cytokines such as TNF-α, IL-1β and IL-6 have been reported to stimulate the HPA to secrete glucocorticoids hormones (Tsigos & Chrousos, 2002). These pro-inflammatory cytokines induce expression and secretion of corticotrophin releasing hormone (CRH), which in turn stimulates

secretion of adrenocorticotropic hormone (ACTH), which directly targets the cells of adrenal cortex to release steroid hormones (Herman *et al*., 2016). These steroid hormones are involved in many biological actions such as their effects on metabolic, reproductive, endocrine and immune system in both health and disease. With respect to an immunological role, glucocorticoids act as endogenous anti-inflammatory agents (Liberman *et al*., 2007; Schleimer, 1993). Steroid hormones are a group of small lipophilic compounds, which are derived from cholesterol following a series of cytochrome P450-catalyzed reactions (De Bosscher *et al*., 2008; Liberman *et al*., 2007).

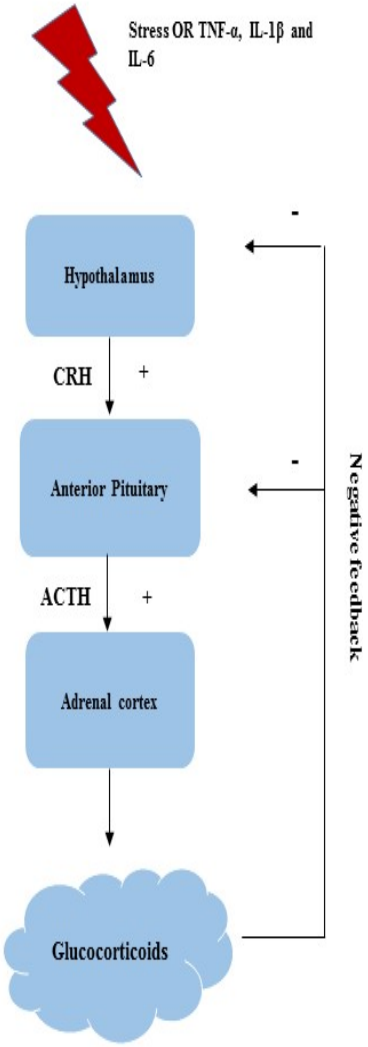



**Fig 1.6 Negative Feedback of Glucocorticoids on Their Secretion from Hypothalamic-Pituitary-adrenocortical AXIS (HPA)**

Under stress of inflammatory cytokines HPA can be activated and releases CRH, which in turn stimulates anterior pituitary. The anterior pituitary then releases ACTH, which stimulates adrenal cortex cells to synthesis and secrete glucocorticoids mainly cortisol (Herman *et al*., 2016).

Steroid hormones in the body can be divided into four major types: progestins, androgens, oestrogens and corticoids. There are two types of corticoids: mineralocorticoids, which act as regulators of ion transportation in the body and glucocorticoids (GCs), which control many biological activities including immunosuppressive and anti-inflammatory effects, processes and regulation of protein, carbohydrate and fat metabolism (Schleimer, 1993) (See Fig 1.7). Thus, GCs have an endogenous anti-inflammatory action which occurs through targeting many immune cells including monocytes-macrophages and T- lymphocytes in order to down regulate cytokine production in both normal immune responses and during inflammatory responses (Singh *et al*., 2004). In addition, GCs exert their effects via binding to the glucocorticoid receptor (GR) (Barnes, 2006).

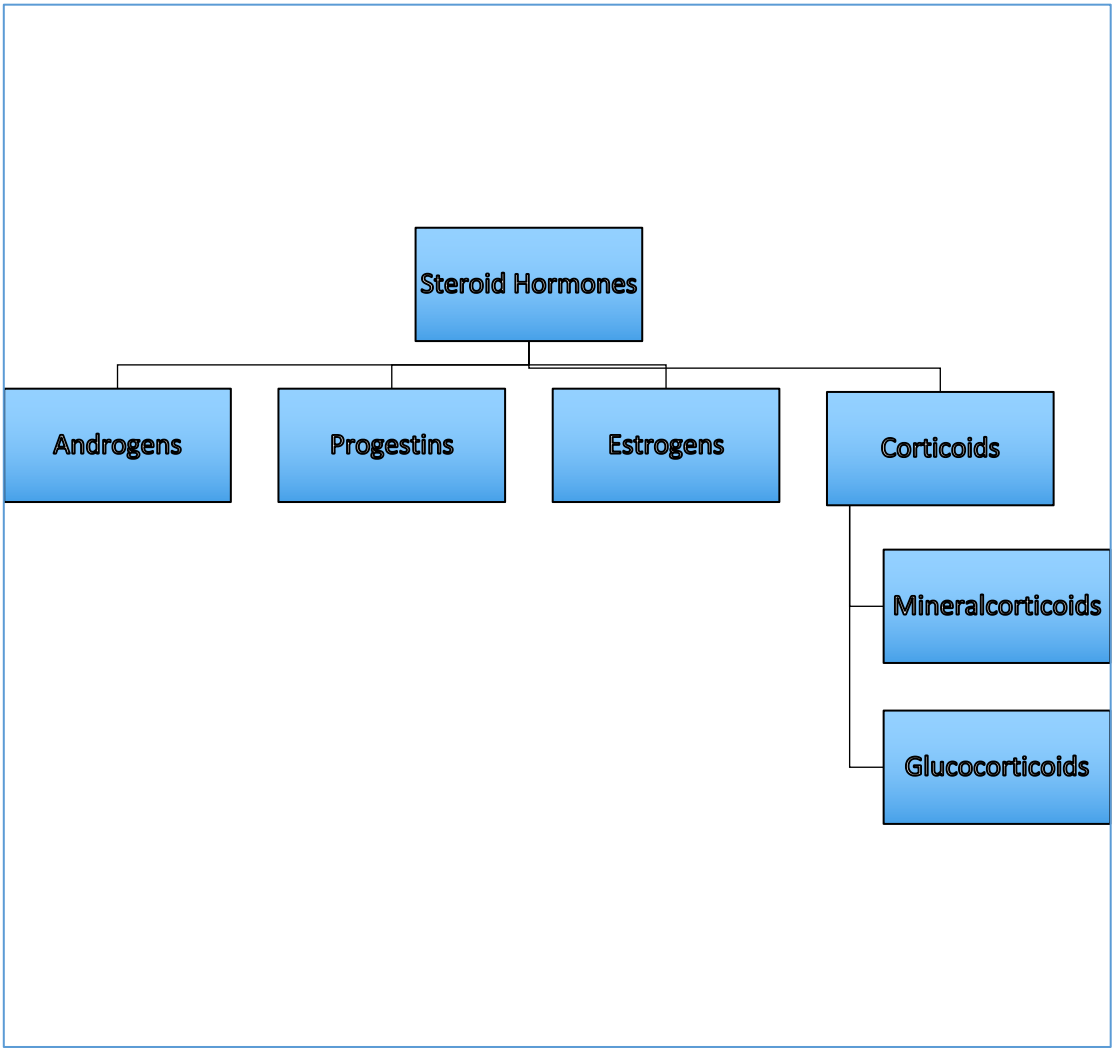



**Fig 1.7 Sub-Classes of Steroid Hormones**

### 1.7.2 The Action of Glucocorticoids during Inflammation

Inflammation triggers a negative feedback loop to the hypothalamic- pituitary- adrenal axis (HPA) to down regulate the natural production of glucocorticoids mainly cortisol, also known as hydrocortisone, which is the major glucocorticoid circulating in human blood and controls many physiological and regulatory functions in the body (van der Velden, 1998; Wang, 2005). This occurs through the binding of glucocorticoids to glucocorticoid receptors (GRs), which are nuclear receptors that are present in almost all cells in an inactive form where the GRs are "masked" by binding to heat shock proteins such as HSP-90. (Rhen & Cidlowski, 2005). Glucocorticoids orchestrate their effects on pro-inflammatory cytokines by transcriptional and non-transcriptional mechanisms controlled by GRs. The inhibition of pro-inflammatory cytokine expression occurs through the inhibition of transcription factors such as NF-κβ and AP-1 (Buttgereit *et al*., 2005). Exogenous GCs (dexamethasone) have showed activation effects on glucocorticoid induced leucine zipper (GILZ) gene which is suggested to interact with inhibition of NF-κβ and AP-1 (Coutinho & Chapman, 2011) (See Fig 1.8).

The anti-inflammatory properties of GCs may also be induced by transactivation mechanism, which can occur by enhancement of the synthesis of anti-inflammatory cytokines such as IL-10. This transactivation is a result of GRs binding to glucocorticoid response element (GRE) on DNA (Buttgereit *et al*., 2004; Miyata *et al*., 2015). Since GRs can disrupt the action of COX2, they down-regulate prostaglandins by transcriptional and non-transcriptional actins (Newton *et al*., 1998). Moreover, synthetic glucocorticoids also showed a decrease in the level of COX1 in pulmonary endothelium cells particularly dexamethasone (Jun *et al*., 1999).

Glucocorticoids have been well characterised for their inhibitory actions on pro-inflammatory prostaglandins such as $PGE_2$, which are thought to occur in response to lipocortin-1 induction (also known as annexin-1). There are many speculations that suggested that the anti-inflammatory activities of GCs occur by inducing the synthesis of annexin-1, which possesses antipyretic action in vivo (Davidson *et al*., 1991) as well as in vitro . Annexin-1 inhibits phospholipase $A_2$ ($PLA_2$) activity and this in turn stops the generation of prostaglandins from perfused lungs and activated macrophages (Flower, 1988). This action of annexin-1 was described as being inhibition of $PLA_2$, which in turn stops AA from being liberated from cell membranes, thus inhibiting its flow for PG synthesis (Malcher-Lopes *et al.*, 2008). This was described by Goppelt-Struebe, *et al*., (1989) who cultured isolated bone marrow macrophages with or without AA and dexamethasone overnight and determined $PGE_2$ level every two hours. The $PGE_2$ became reduced in supernatant that contained dexamethasone which was determined by a double antibody radioimmunoassay. In addition, they reported that the level of liberated AA was decreased in response to adding dexamethasone *(*Goppelt-Struebe *et al.*, 1989).

#### 1.7.2.1 Glucocorticoids and Their Derivatives as Treatments for Acute Inflammatory Diseases

Exogenous glucocorticoids are therapeutic agents for different types of inflammatory and immune disorders. They have a high lipophilic characteristic and are easily diffused through cell membranes and binding to GCR which makes them potential inhibitors of the production of inflammatory mediators. In fact, synthetic treatments of glucocorticoids mimic the natural steroids in their action (Goecke & Guerrero, 2006). These exogenous steroids have the same basic chemistry structure of endogenous

steroid (cortisol) cyclopentaneperhydrophenanthrene which is formed by the three rings (A, B, C) of phenanthrene plus a five membered cyclopentane ring (D) with differences in their functional side attached group (Diederich *et al*., 2002). One type of this steroid is prednisolone which consists of a double bond between ring A, C1 and C2 in the cortisol. Dexamethasone is another example of a steroid that is derived from cortisol by substitution of the fluorine group at 9-alpha position, double bond between C1 and C2 and addition of methyl group in C16 (Diederich *et al*., 2002). (See Fig 1.9). Dexamethasone is one of the synthetic glucocorticoids that has been established to be an effective anti-inflammatory drug since it has shown decreasing effects on LPS-stimulation of IL-1β and TNF-α production in vitro and vivo (Horton & Remick, 2010) via binding to the glucocorticoid receptor (Pandit *et al*., 2002). Hydrocortisone, a drug synthetic of cortisol, is used as an anti-inflammatory drug which has demonstrated the ability to activate and increase the production of anti-inflammatory cytokines (Goulding *et al*., 1990) as well as reduce the level of pro-inflammatory cytokines (Wirtz *et al*., 2004). However, prednisone is the most usable synthetic anti-inflammatory steroid in rheumatoid arthritis treatment (Beltrametti *et al*., 2016).

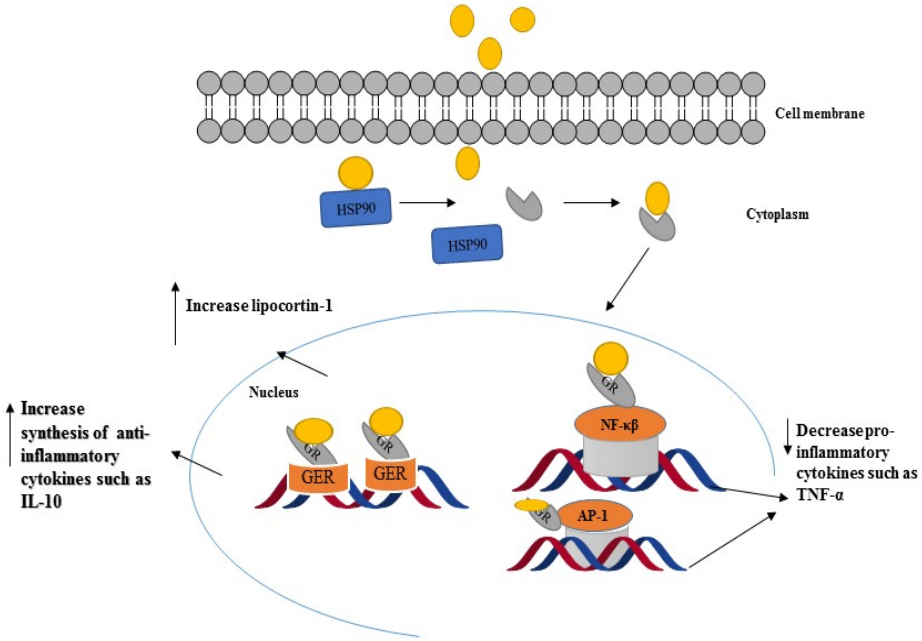



**Fig 1.8 Anti-inflammatory Action of Glucocorticoids**

Glucocorticoids easily cross the cell membrane into the cytoplasm where they bind to GR, which translocate to the nucleus after disengagement of HSP-90. GR complex binds to a GRE specific site on the gene, this binding can induce anti-inflammatory cytokine synthesis and lipocortin-1. GR complexes also deactivate pro-inflammatory cytokine synthesis by interaction with NF-κβ and AP-1 (Smoak & Cidlowski, 2004).

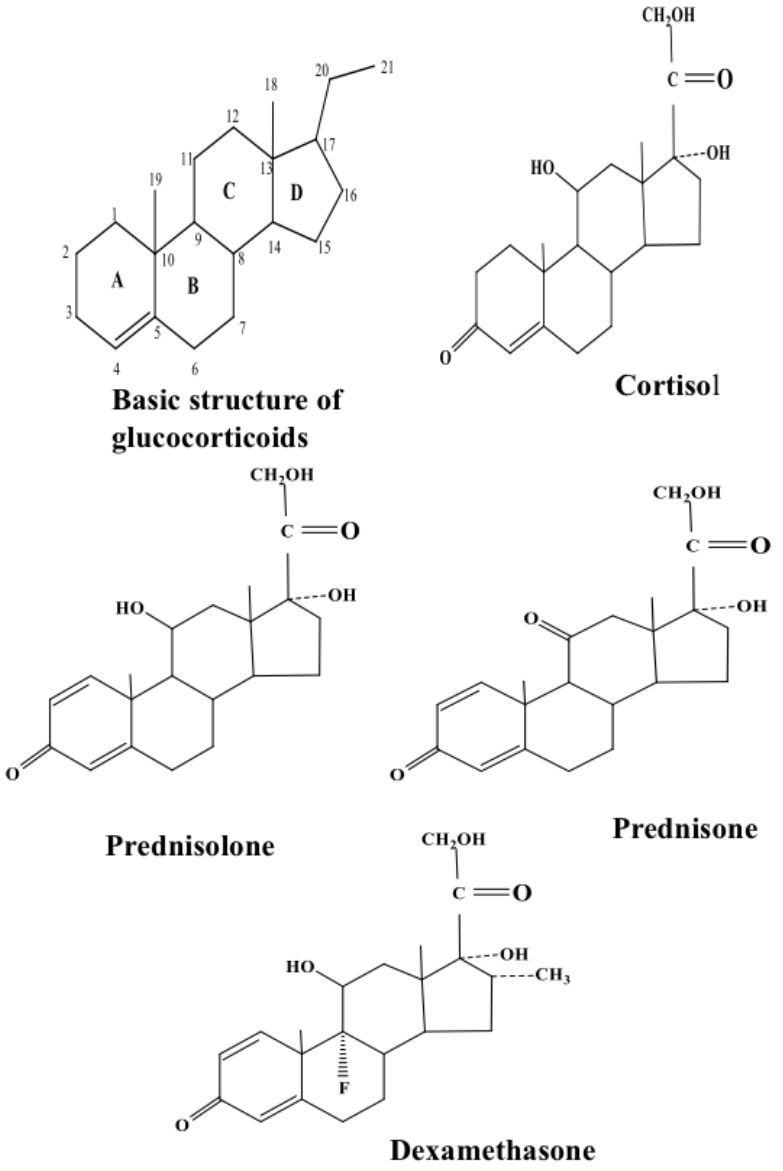



**Fig 1.9 Chemical Structure of Some Natural and Synthetic Glucocorticoids**

Neurodegeneration is a term used to describe the progressive damage which occurs in the function and structure of the brain and causes disorders such as Parkinson's disease and Alzheimer's disease. Using endogenous and synthetic neuroprotective steroids can reduce excitotoxicity and apoptosis of nerve system cells during acute injury in brain (trauma) or chronic diseases (Alzheimer's disease). Low levels of steroids in plasma and cerebrospinal fluid have been reported to be associated with neurodegeneration. Moreover, age-related declines in neurosteroids have been associated with some chronic pathogeneses of nerve systems such as Alzheimer's disease. One of these steroids is pregnenolone (precursor of estrogen, androgen and progesterone) of which its reduction plays an important role in the pathogenesis of chronic nerve system diseases (Luchetti *et al*., 2011).

Progesterone acts as a neurosteroid that can be synthesized in the nerve system. Progesterone is also biosynthesised in female ovaries and male testes and acts as a sex hormone. This steroid has been reported to be produced in glial cell cultures with the ability to express its receptor (progesterone receptor) (Jung-Testas *et al*., 1999) Moreover, it has been reported to have neuroprotective effects in a rat model with traumatic brain injury (TBI) where the level of progesterone in serum was increased with a reduction in the lesion of TBI (Stein, 2001) (Melcangi *et al*., 2012). Many recent studies have shown the cytoprotective role of steroids such as 7β-Hydroxycholesterol which has shown increased proliferation of human umbilical vein endothelial cells in culture as well as reduced apoptosis (Trevisi *et al*., 2010). Oestrogens appear to have roles in cardio- and neuroprotective effects such as 17β-estradiol which has been reported to reduce the infarct size in a rabbit model of reperfusion ischemia (Hale *et al*., 1996; Mahmoodzadeh *et al.*, 2014). In addition, estradiol has been shown by transcriptional and non-transcriptional mechanisms to

have neuroprotective actions on different nerve system disease models (Green & Simpkins, 2000; Peri, 2016). More recently, there are have been studies that focus on steroids for their potential cytoprotective actions such as dehydroepiandrosterone (DHEA).

## 1.8 Dehydroepiandrosterone (DHEA)

DHEA is a steroid hormone, which is synthesised in the adrenal gland as well as in the nervous system. This steroid is also known as a neurosteroid due to its high level of local biological secretion and effects on astrocytes, neurons and microglia which also have high levels of steroidogenic enzymes (Compagnone & Mellon, 2000; Mellon, 2007). It can also be found in high levels in the cerebrospinal fluid of patients with central nervous system disorders (Rammouz *et al*., 2011). A growing body of evidence indicates that DHEA and its sulphate ester DHEA-S are cytoprotective to nerve system cells and protects them from neurotoxic stimuli both in vivo and vitro. It has been reported that (in vivo) by using rats' models, DHEA has shown neuroprotective action in the cerebral ischemia. This was in a time- and dose-dependent manner. A study where feeding rats with DHEA at doses of 20 mg/kg during 3-48 hrs after ischemia reduced neuronal death in the hippocampal region whereas the same dose of DHEA 1 hr after ischemia increased neuronal death. Moreover, feeding rats with DHEA for 15 days before ischemia induction with doses of 25-100 mg/kg led to greater neuroprotective effects and reduced hippocampal neuronal injury (Li *et al*., 2009). In addition, the action of DHEA in vivo and in vitro prevented hippocampal neurons against toxicity induced by NMDA infusion observed by using different concentrations of DHEA in primary hippocampal cultures as models of nerve cells and

against glutamate excitotoxicity mediated by NMDA receptors (Kimonides *et al*., 1998). It has also been shown that DHEA reduces nitric oxide production in rat microglia (Barger et al., 2000). Accordingly, the neuroprotective action of DHEA may be related to inhibition of the NMDA-induced nitric oxide (NO) production in hippocampal cells or modulation of the calcium/NO signalling pathway. Mechanisms of the cytoprotective actions of DHEA are still not clear i.e. if it is due to its direct action or related to downstream metabolites. DHEA can undergo 7α-hydroxylation and 7β-hydroxylation in the presence of the enzyme cytochrome P450 7B1 (CYP7B1) which is found in many human tissues such as skin, liver and brain. One of the metabolites of this conversion of DHEA is 7β-hydroxy-epiandrosterone.

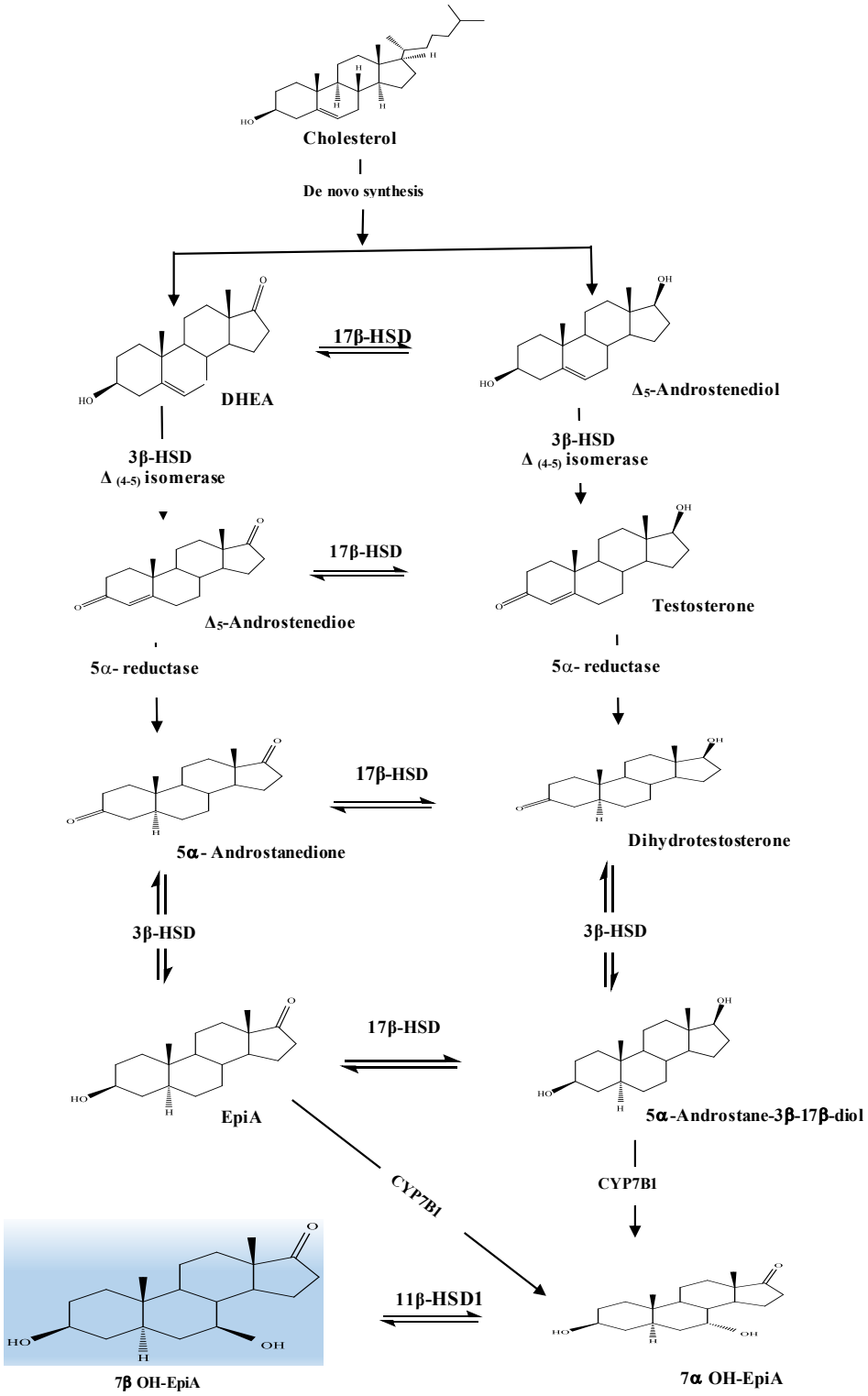



**Fig 1.10 The Pathway of Biosynthesis of 7β-OH-EpiA from Cholesterol**

### 1.8.1 7β-hydroxy-epiandrosterone (7β OH-EpiA)

7β hydroxy-epiandroestrone (7β OH-EpiA) is a steroid, which is naturally produced in animals and human tissues such as skin, brain and liver. 7β OH-EpiA is generated from DHEA, epiandrosterone (EPIA) and 5α-androstane-3β, 17β-diol by two enzymatic steps. These steps start from hydroxylation of three steroids by cytochrome P450 7B1 (CYP-7B1) to conversion into 7α-hydroxy-EpiA. 7α hydroxy-EpiA in turn is further converted into 7β OH-EpiA in the presence of the enzyme 11β Hydroxy-steroid dehydrogenase (Ricco *et al*., 2011) (see Fig 1.10).

In many studies, 7β-OH-EpiA has been demonstrated to have neuroprotective effects particularly in protection against ischemia induced neuronal cell death (Dudas *et al*., 2004). There are immune defence and neuroprotective effects associated with the use of this steroid in animal and in human tissues models. For example, in an in vivo rat model, 7β OH-EpiA treatment has been shown to significantly reduce dextrin sodium sulphate (DSS) induced colitis, which is suggested to occur in response to increased production of cytoprotective mediators. The neuroprotective effects of this steroid may be linked to the production of prostaglandins specially $PGD_2$ or its metabolite, 15d-$PGJ_2$ (Hennebert *et al*., 2008). Many studies have demonstrated the neuroprotective actions of prostaglandins such as $PGD_2$ and $PGE_2$ (Lee *et al*., 2004; Liang *et al*., 2005). In addition, in vitro, 7β OH EpiA has been found to induce human monocytes to produce $PGD_2$ and 15d-$PGJ_2$ in the presence or absence of TNF-α. While the level of $PGE_2$ did not change with 7β OH EpiA alone, $PGE_2$ was reduced in the incubation with a combination of TNF-α with 7β OH EpiA (Davidson *et al*., 2008). This indicates a very selective action of 7β OH EpiA on PG production.

## 1.9 Aims and objectives of the study

This introduction has outlined that during inflammatory responses, production of pro-inflammatory cytokines such as TNF-α and IL-1β induced by lipopolysaccharide (LPS) in immune cells especially monocytes is a fundamental action in inflammation. Accordingly, these cytokines can increase production of secondary mediators of inflammation such as prostaglandins including $PGE_2$. 7β-hydroxy epiandrosterone (7β OH EpiA) is a naturally occurring steroid that has recently been reported to have cytoprotective actions and can prevent ischaemia-reperfusion induced cell death (Dudas *et al.*, 2004). The cytoprotective action of this steroid could be due to an increase in the level of anti-inflammatory cytokines such as IL-4 or IL-10, which has been previously shown not to be produced in the presence of 7β OH EpiA (Davidson *et al*., 2008). Moreover, another possible cytoprotective action has been suggested to be mediated by increasing the level of cytoprotective prostaglandins such as $PGD_2$ and as a consequence its spontaneous metabolite 15d-$PGJ_2$ (Davidson *et al*., 2008; Hennebert *et al*., 2008). There is a lack of information regarding the action of this steroid during inflammatory responses. Therefore, this study aims to evaluate the effects of 7β OH-EpiA on the production of pro-inflammatory cytokines including TNF-α and IL-1β following stimulation by LPS in whole human blood and human monocytic THP-1 cells. It also aims to evaluate any actions of 7β OH-EpiA on the levels of different prostaglandins following inflammatory stimulation with LPS. This study will be carried out in the following steps:

- To determine the action of 7β hydroxy-epiandrosterone (7β OH-EpiA) on TNF-α and IL-1β production in response to stimulation by LPS.

- To determine the receptor system by which 7β hydroxy-epiandrosterone (7β OH-EpiA) induces its effects by using mifepristone as an antagonist, which interferes with the Glucocorticoid receptor system.
- Non-steroidal inflammatory drugs (i.e. cyclooxygenase inhibitors) will be used to prevent the production of prostaglandin in order to evaluate the effects of endogenous PGs on TNF-α production and the actions of 7β OH-EpiA.
- The effect of 7β OH-EpiA on the production of IL-4 and IL-10 in response to LPS.
- The effect of 7β OH-EpiA on the production of prostaglandins ($PGE_2$, $PGD_2$ and 15d-$PGJ_2$) in response to LPS.
- The effect of 7β OH-EpiA on gene expression (mRNA) of TNF-α, TLR-4 and IL-4 in the human monocytic cell line THP-1
- To determine whether 7β OH-EpiA has any effect on the synthesis and actions of $PGD_2$. This was determined by conducting a gene expression study of mRNA for the enzyme which synthesises $PGD_2$ (PGDS) and a PGD receptor (PTGDR2) by qPCR in THP-1 cells.

# Chapter Two: Materials and Methods

# 2. Materials and Methods

## 2.1 Materials

All materials used were of highest commercial purity available and were obtained from the following sources:

### 2.1.1 Human Blood and Cells

| | |
|---|---|
| Whole human blood | **Scottish National Blood Transfusion Service (SNBTS), (Glasgow,UK)** |
| Human Monocyte Leukaemia Cell Line THP-1, ECACC no. 8808120 | **European Collection of Cell Culture ECACC (Porton Down, UK)** |

### 2.1.2 Chemicals and Drugs

| | |
|---|---|
| 7β OH-EpiA | **A gift from Hunter-Fleming Ltd (Bristol, UK)** |
| 15deoxy Prostaglandin J2 (PGJ2) | **Cayman Chemical, Co. (Ann Arbor, Michigan, USA)** |
| 3,3',5,5'-Tetramethylbenzidine (TMB) | **Invitrogen, Life Technologies Ltd (Paisley, UK)** |

| | |
|---|---|
| Agarose Powder | **Sigma-Aldrich Co. Ltd (Poole, Dorset, UK)** |
| CAY10471 (DP2/CRTH2 antagonist) | **Cayman chemical., Co. (Ann Arbor, Michigan, USA)** |
| Dexamethasone | **Alfa Aesar, Thermo Fisher Scientific. (Heysham, Lancashire, UK)** |
| Dimethyl Sulfoxide (DMSO) (99.5%GC | **Sigma-Aldrich Co. Ltd (Poole, Dorset, UK)** |
| Disodium Hydrogen Phosphate ($Na_2HPO_4$) | „ |
| Disodium Hydrogen Phosphate ($Na_2HPO_4$) | „ |
| Ethanol | „ |
| Ethidium Bromide | „ |
| Ethylenediaminetetraacetic Acid (EDTA) | „ |
| Foetal Calf Serum (FCS) | „ |
| Ficoll Histopaque (1.077g/l) | „ |

| | |
|---|---|
| Hanks' Balanced Salt Solution (HBSS) | „ |
| HRP Chromogenic Substrate | **Invitrogen, Life Technologies Ltd (Paisley, UK)** |
| Ketoprofen | **Sigma-Aldrich Co. Ltd (Poole, Dorset, UK)** |
| Lipopolysaccharide (LPS from salmonella abortus equi) | ,, |
| Mifepristone | „ |
| Monopotassium Phosphate ($KH_2PO_4$) | „ |
| MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) | „ |
| Phosphate buffered saline (PBS) | „ |
| Potassium chloride, (KCl) | „ |
| Prostaglandin 15-deoxy-Δ12,14-$PGJ_2$ | **Cayman chemical., Co, (Ann Arbor, Michigan, USA)** |

| | |
|---|---|
| Prostaglandin D2 (PGD2) | **Cayman chemical., Co, (Ann Arbor, Michigan, USA)** |
| Prostaglandin $E_2$ ($PGE_2$) | **Cayman chemical., Co, (Ann Arbor, Michigan, USA)** |
| Roswell Park Memorial Institute Medium (RPMI-1640) | **Sigma-Aldrich Co. Ltd (Poole, Dorset, UK)** |
| Sodium Acetate 3M | „ |
| Sodium Chloride (NaCl) | „ |
| Sulphuric Acid ($H_2SO_4$) | „ |
| Tris-Borate-EDTA (TBE) | „ |
| Trypan Blue | „ |
| TWEEN-20 | „ |
| Virkon | **Antec International (Sudbury Suffolk, England)** |

### 2.1.3 Immunoassay Kits

| | |
|---|---|
| 15-deoxy-$\Delta^{12,14}$-$PGJ_2$ ELISA kit | **Enzo® Life Science (Exeter, UK)** |

| | |
|---|---|
| Human TNF-α ELISA Kit | **Invitrogen, Life Technologies Ltd (Paisley, UK)** |
| Human IL-1β ELISA Kit | **„** |
| Human IL4 ELISA Kit | **„** |
| Human IL10 ELISA Kit | **„** |
| Prostaglandin $D_2$-MOX ELISA Kit | **Cayman chemical (Ann Arbor, Michigan, USA)** |
| Prostaglandin $E_2$ Assay | **R&D Systems Europe, Ltd.(Abingdon, UK)** |

### 2.1.4 Consumables

| | |
|---|---|
| Micro Tubes with cap (0.5 ml) | **Starlab (Galway, Ireland)** |
| RNase/ DNase and Pyrogen Safe Tubes (0.6 ml) | **Corning Science Products (Tewksbury, USA)** |
| Cryo Tube vials (1 ml) | **Nunc (Roskilde, Denmark)** |
| Sterile Tubes (30 ml) | **Elkay Laboratory Products Ltd (Hampshire, UK)** |

| | |
|---|---|
| 6, 24, and 96-well Tissue Culture Plates | **TPP Techno Plastic Products (Switzerland, Europe)** |
| Eppendorf Microcentrifuge Tubes | **Trefflab, Scotlab Bioscience Ltd (Scotland, UK)** |
| Graduated Tips | **Corning Science Products (Tewksbury, USA)** |
| loose Immune Modules for ELISA | **Nunc (Roskilde, Denmark)** |
| Pipettes | **Gilson (Middleton, USA)** |
| Sterile 100 mm Petri-style Culture Dishes | **Corning Science Products (Tewksbury, USA)** |
| Tissue Culture Flasks (75 $cm^2$) | **Nunc (Roskilde, Denmark)** |
| Ultra-Pure Water | **Purite Water Purification System (Oxfordshire, UK)** |

### 2.1.5 Molecular biology (PCR) Materials

| | |
|---|---|
| Free-RNAase Water | **Sigma-Aldrich Co. Ltd (Poole, Dorset, UK)** |
| HyperLadder (DNA Molecular Weight marker) | **Bioline Reagents Ltd (London, UK)** |

| | |
|---|---|
| Isolate II RNA Kit | **Bioline Reagents Ltd (London, UK)** |
| PCR Fast Reaction MicroAmp Tubes with Cap | **Life Technologies Ltd (Paisley, UK)** |
| Power SYBR Green | **Bioline Reagents Ltd (London, UK)** |

### 2.1.6 Equipment and instruments

| | |
|---|---|
| $CO_2$ Incubator | **Sanyo (Loughborough, UK)** |
| DNA Thermal Cycler | **PerkinElmer (Beaconsfield, UK)** |
| Haemocytometer | **Weber Scientific (Hamilton, USA)** |
| Mini Centrifuge 5415D | **Eppendorf Ltd (Stevenage, UK)** |
| Multifuge Centrifuge | **Heraeus ( Hagen, Germany)** |
| Nano Drop Spectrophotometer (ND, 2000C) | **Thermo Scientific, Labtech International Ltd (East Sussex, UK)** |
| NMR Instrument | **Jeol JNM-LA400 (Peabody, MA, USA)** |
| Spectra Max 190 Absorbance Micro-Plate Reader | **Molecular Devices (San Jose, USA)** |

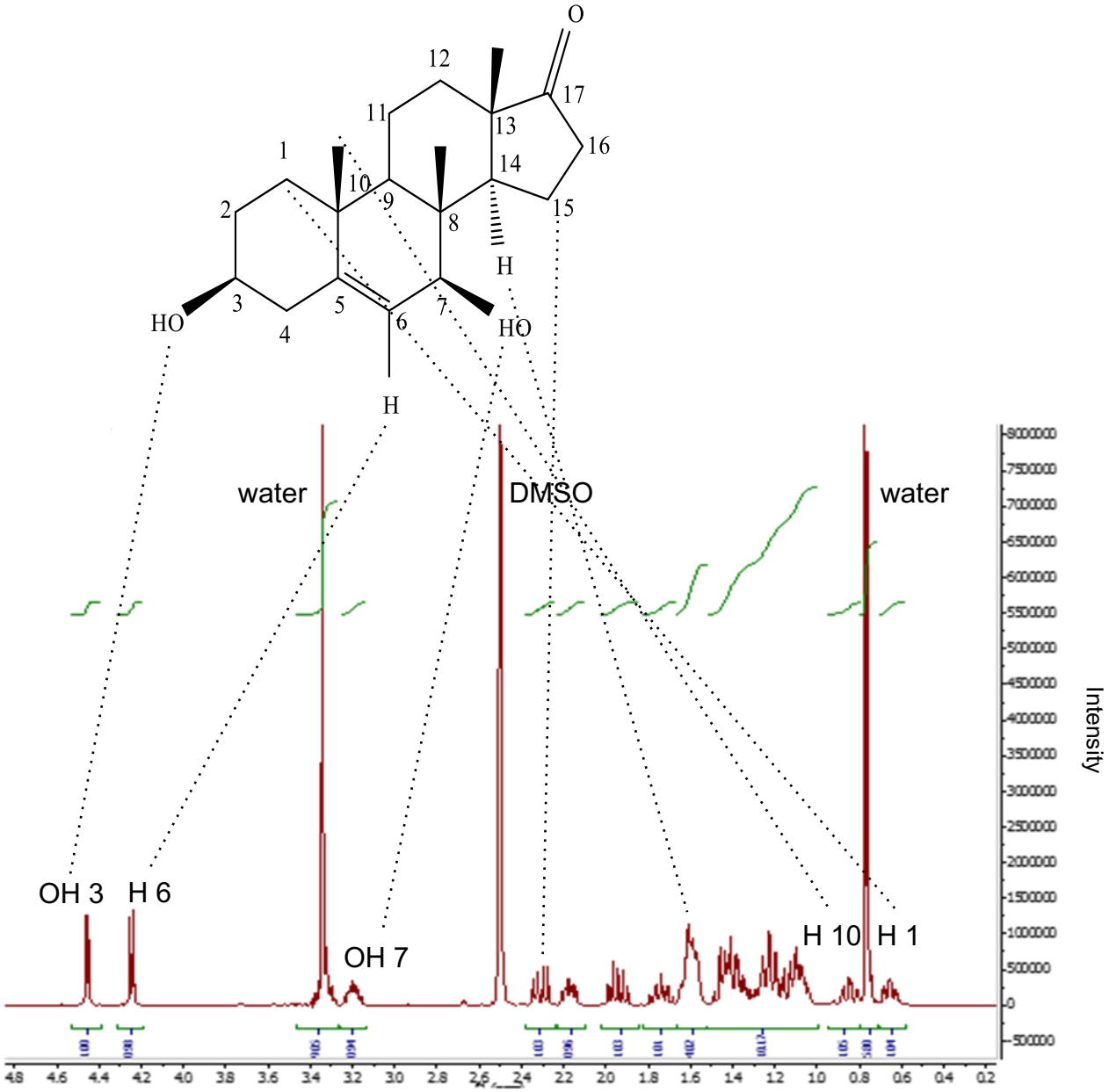
| | |
|---|---|
| StepOne Plus Real-Time PCR System | **Applied Biosystems, Life Technologies (Paisley, UK)** |

## 2.2 Methods

### 2.2.1 Preparation of Test Substances, Chemicals and Drugs

All drugs and chemicals were prepared in a Class II laminar air flow cabinet and sterile plastic containers. Stock solution of drugs and LPS were prepared immediately before use. LPS, Dexamethasone and Ketoprofen were dissolved directly in RPMI 1640. Dimethyl sulphoxide (DMSO) was used as a solvent for 7β OH-EpiA and Mifepristone. 7β OH-EpiA as supplied, was subjected to Nuclear Magnetic Resonance (NMR) spectroscopy to confirm the structure of the compound. 7β OH-EpiA was prepared as used in experiments and the solid powder was dissolved in deuterated DMSO (DMSO-d6) then directly subjected to NMR. The NMR analysis was kindly carried out by Prof. Sandy Gray's Lab (SIPBS, Strathclyde University). The NMR spectrum obtained is shown in Fig. 2.1. It confirms the main epiandrosterone steroid structure and also the 7-hydroxyl group in the ß-position.

**Fig 2.1 NMR Spectrum for 7β OH-EpiA**




**NMR Spectrum of 7β OH-EpiA (0.2-4.8) ppm**

NMR Spectrum for 7β OH-EPIA Dissolved in DMSO-d6 and the 2D NMR Spectrum Obtained on a Jeol JNM-LA400 Instrument at 400 MHz. The figure shows the spectrum of several hydrogens in the molecule including the hydrogen on the key hydroxyl at carbon atom 7 (OH 7).

### 2.2.2 Preparation of whole Human Blood for Experimental Use

Whole human blood was kindly provided by the Scottish National Blood Transfusion Service (SNBTS) Glasgow, from normal healthy donors, which had been screened and found to be, negative for HIV, hepatitis B and C and parasitic diseases. The blood was gently inverted 5 times and was then used directly from the donor bag.

### 2.2.3 Preparation of THP-1 Cells for Experimental Use

THP-1 cells obtained from the European collection of cell cultures (ECACC) were cultured as a continuous cell line in RPMI-1640 supplemented with 10% (v/v) FCS and maintained at 37°C, 5% CO2, and 100% humidity. The cells were transferred to sterilised 30 ml universal tubes from the cell culture flasks under aseptic conditions and centrifuged at 400g for 5 minutes at room temperature to produce a cell pellet. The supernatant was then removed, and the pellet was resuspended in RPMI-1640 and the re-suspended cells used in experiments.

### 2.2.4 Storage and Recovery of THP-1 Cells

When the THP-1 cells reached 80%-90% confluence and were not used directly in experiments, these cells were prepared for freezing and storage. The cells were centrifuged to form pellet as describe in previous section (2.2.3). This pellet was resuspended in 1 ml of freezing mixture which consisted of 50% foetal calf serum (FCS), 40% RPMI 1640 and 10% DMSO. The resuspended cells were transferred to Cryo vials and frozen at -20°C overnight before being transferred to storage at -80°C until further required. Recovery of cells from storage was achieved by removing vials

from the -80°C freezer and quickly thawing in a water bath at 37 °C by constant agitation until fully thawed. The cells were then placed directly into culture flasks with fresh medium, RPMI 1640 with 10 % FCS. All THP-1 cells did not used after 15 passages due to changes in their properties over time.

### 2.2.5 Monocyte Isolation from Whole Human Blood

Aseptic technique and sterile conditions were used throughout the preparation of cells. Whole human peripheral blood was used to isolate monocytes and was diluted (1:1) with Hank's balanced salt solution (HBSS). Tubes were prepared with 10ml of histopaque (1.077g/ml) at room temperature in sterile 30ml universal tubes and 10ml of the diluted blood was carefully layered/poured onto the surface of the histopaque solution in each tube. The tubes were then centrifuged for 90 minutes at 400g and 22°C. The mononuclear cell-layer in each tube (at the interface of the culture medium and histopaque) was aspirated carefully and collected into fresh sterile universal tubes. HBSS was added to a volume of 20ml to dilute any remaining histopaque and tubes were centrifuged for 40 minutes at 400g at room temperature. The supernatant was discarded, and the pellets were resuspended in 3ml of RPMI 1640 medium (without FCS). The mononuclear cell suspensions were placed into sterile 100 mm Petri-style culture dishes and incubated at 37°C in 100% humidity and with 5% CO2 for 4 hours. The culture medium (containing non-adherent cells) was discarded and culture dishes were washed 3 times with RPMI 1640. Cells were then gently scraped from the surface of petri dishes using a sterile rubber syringe plunger and washed with RPMI 1640 into fresh tubes. The cells were centrifuged for 20 minutes at 400xg at 22° C. The cell pellet was re-suspended in RPMI 1640 medium. These cells were then counted and used at a concentration of $1 \times 10^6$ cells/ml.

### 2.2.6 THP-1 Cells and Monocyte Cell Counting and Assessment of Viability

Cell viability was estimated concurrently with cell counting, using the Trypan blue exclusion test. An aliquot of the collected cells was diluted 1:10 (v/v) with RPMI-1640 and then diluted 1:1 with Trypan blue dye. The stained cells were then immediately transferred to the counting chamber of a Neubauer Improved Haemocytometer slide and counted under a phase contrast light microscope. The concentration of cells was calculated by multiplying the number of cells counted within the chamber, (0.1µl) by 1 x $10^4$ (this yielded the concentration as cells/ml) and then by the appropriate dilution factor. The cells then were diluted with RPMI 1640 to adjust for the required final concentration of cells for each experiment.

#### 2.2.6.1 THP-1 Cell Viability Using MTT Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye assay (measurement of viable cells) was used to determine the actions of 7β OH-EPIA and other compounds used in experiments on cell viability. THP-1 cells (1x106 cells/ml) were placed in 96-well plates and incubated with different concentrations of 7β OH-EpiA which were added in a volume of 2 µl (to yield the appropriate final concentrations indicated in the Results Section) in a final total volume of 200µl. RPMI 1640 alone and dead cells (boiled cells) were used as positive/ negative controls respectively. Plates were incubated at 37°C, 5% CO2 for 24h. Treatment media was removed from all wells and replaced with 10 µl of 5 mg/ml MTT solution (diluted in sterile PBS and covered with foil) and incubated for 4h at 37°C. MTT media was then removed and cells lysed with 100 µl/well DMSO. Absorbance was then measured at 570 nm using a spectrophotometer.

### 2.2.7 Blood Incubation Protocol

Aliquots of blood (700µl) were dispensed into sterile plastic 1.5ml Eppendorf tubes in triplicate. LPS (and other compounds) was added in 100µl aliquots while 7β OH-EpiA was added in 10µl aliquots at the appropriate concentrations to yield the required final concentrations. The volume was adjusted by adding RPMI-1640 or solvent to yield a final incubation volume of 1ml. Incubations were carried out for 20 hrs at 37°C, 5% CO2 and 100% humidity. After incubation, the samples were centrifuged at 13,000g for 10 seconds and the resultant plasma was collected. The plasma was transferred to fresh Eppendorf tubes and stored at -20° C until assayed.

### 2.2.8 THP-1 and Mononuclear Cells Incubation Protocol

THP-1 or mononuclear cells (300µl) were dispensed into 24- well cell culture plates, and LPS was added to each well in a volume of 50µl. 7β OH-EPIA, ketoprofen and mifepristone were added in 5µl aliquots whereas Dexamethasone was added in 50µl volumes. RPMI-1640 or solvent was added to each well to generate a final volume of 500µl. Plates were then incubated for 20 hr at 37°C, 5%CO2 and 100% humidity. At the end of the incubation period, the supernatants were transferred to fresh Eppendorf tubes and stored at -20°C until required for analysis.

### 2.2.9 Measurement of Cytokine Production

#### 2.2.9.1 ELISA Assay Protocol

The sandwich enzyme linked immunosorbent assay (ELISA) technique was used for the determination of cytokines. A typical flow diagram of the protocol is shown in Fig. 2.2. Microtitre plates (Nunc Immuno-modules) were used and were coated with 100µL/well of capture antibody diluted to 2µg/ml (for all assays) in a coating buffer

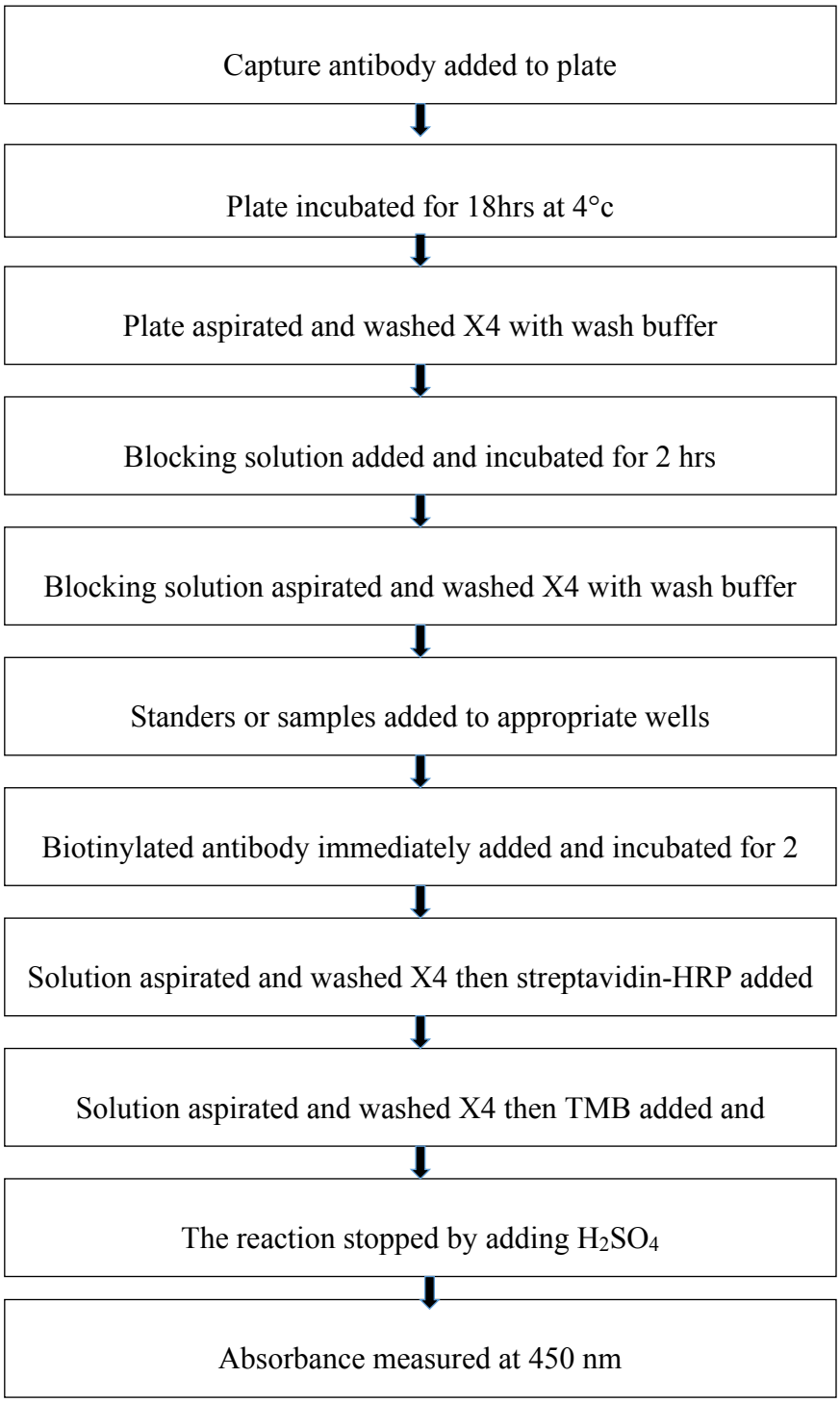
consisting of: NaCl 8.0g/L, Na2HPO4 2.H2O 1.42 g/L, KH2PO4 0.2g/L, KCl 0.2g/L, pH 7.4 and incubated for 18 hours at 4°C. Wells were then washed x4 with washing buffer (coating buffer described above) containing 0.5% (v/v) Tween-20. After washing, the plate was blotted dry. Immediately after this blocking buffer (coating buffer containing 0.5% (w/v) BSA) was added to wells (300µl/ well) and incubated for 2 hours at room temperature. Wells were then washed x4 using washing buffer as described previously. Cytokine standards (10ng/ml) were diluted in assay buffer (coating buffer containing 0.5% (w/v) BSA and 0.5 % (v/v) Tween-20) to yield 1ng/ml (1000 pg/ ml/ well). This was serially diluted to yield a range of standard concentrations. Plasma samples were diluted with assay buffer 1:3 and added to wells (100µl/ well). THP-1 cell and monocyte supernatants were added undiluted (100µl/ well).

Biotinylated detection antibody was diluted to achieve a concentration of 0.8 µg/ml (1/625 for TNF-α assay) with assay buffer and 50µl/well was added to all wells immediately after the addition of samples or standards. Plates were then incubated for two hours at room temperature then washed four times with wash buffer. Streptavidin-horseradish peroxidise (HRP) conjugate was diluted 1/5000 in assay buffer; 100µl was added to each well and incubated for 30 minutes at room temperature. Plates were then washed four times prior to adding the chromogenic substrate- tetramethyl benzidine (TMB). The TMB substrate solution was diluted 1/2 with distilled H2O, and 100µl was added to each well. The plates were then incubated at room temperature for 30 minutes. The enzymatic reaction was stopped by the addition of 100µl of 1M H2SO4 to each well and the absorbance was measured at 450 nm by using an ELISA 96-well plate spectrophotometer (see section 2.2.5.3).

#### 2.2.9.2 Measurement of IL-1β, IL-4 and IL-10

Quantities of these cytokines were achieved in an identical manner to the measurement of TNF-α. Only the concentrations of detection antibodies were different. The following concentrations were used: 0.16 µg/ml (1/1250) for IL-1β and IL-4, 0.35 µg/ml (1/950) for IL-10.

**Fig 2.2 Flow Diagram of ELISA Assay for cytokines**

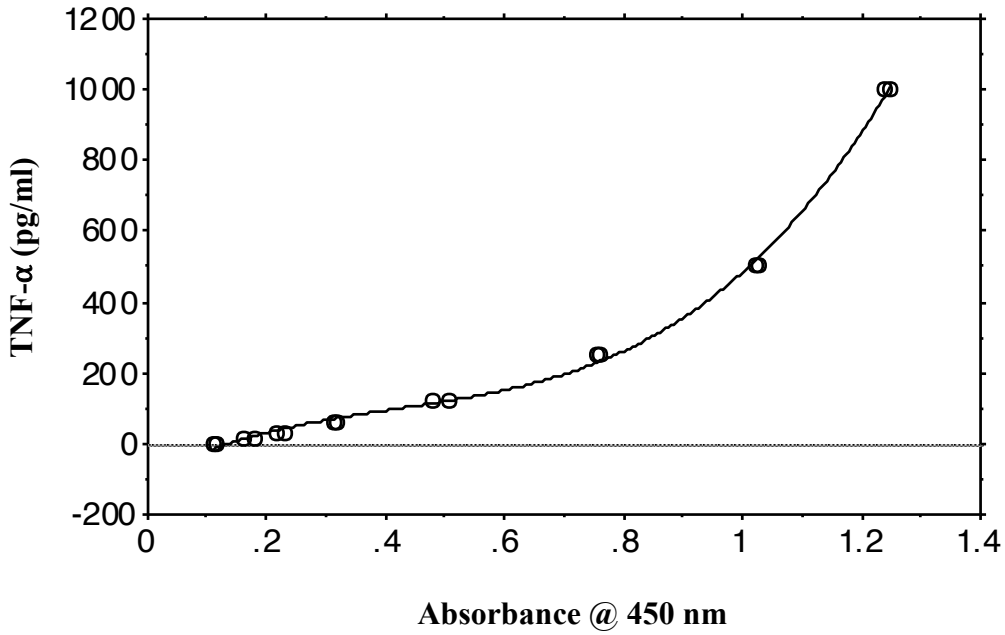


Standard curves for the respective cytokines were created for each assay (Figs. 2.3 - 2.6) using Statview software. 3rd Order polynomial regression analysis was used to calculate unknown concentrations of cytokines from the absorbance values. The following figures show typical examples of standard curves and the corresponding equation which was generated from each specific data set presented in each graph where X= absorbance at 450 nm and Y= concentration of cytokine (pg/ ml). Graphs were plotted this way as the independent (known) X variable was the absorbance from which the dependent (unknown) Y values (cytokine concentrations) were calculated.

### 2.2.10 Data Analysis

Data was analysed using Statview Student Software (Abacus Concepts Inc., Berkeley, USA). The cytokine (TNF-α, IL-4, IL-10, and IL-1β) or PG ($PGE_2$, $PGD_2$ and 15d-$PGJ_2$) concentration present within each sample was determined by 3rd order polynomial regression, enabling calculation of the respective standard curves. In each case the final values presented were adjusted for any appropriate dilution factors. All data were expressed as the mean of a number of incubations (n) ± the standard deviation (s.d.) of the mean and then assessed for statistical significance by using an ANOVA (Fisher's PLSD). The differences between control and treated groups were considered to be significant when the P value was less than 0.05 ($P=<0.05$).

**Fig 2.3 Typical TNF-α Standard Curve for ELISA Assay**

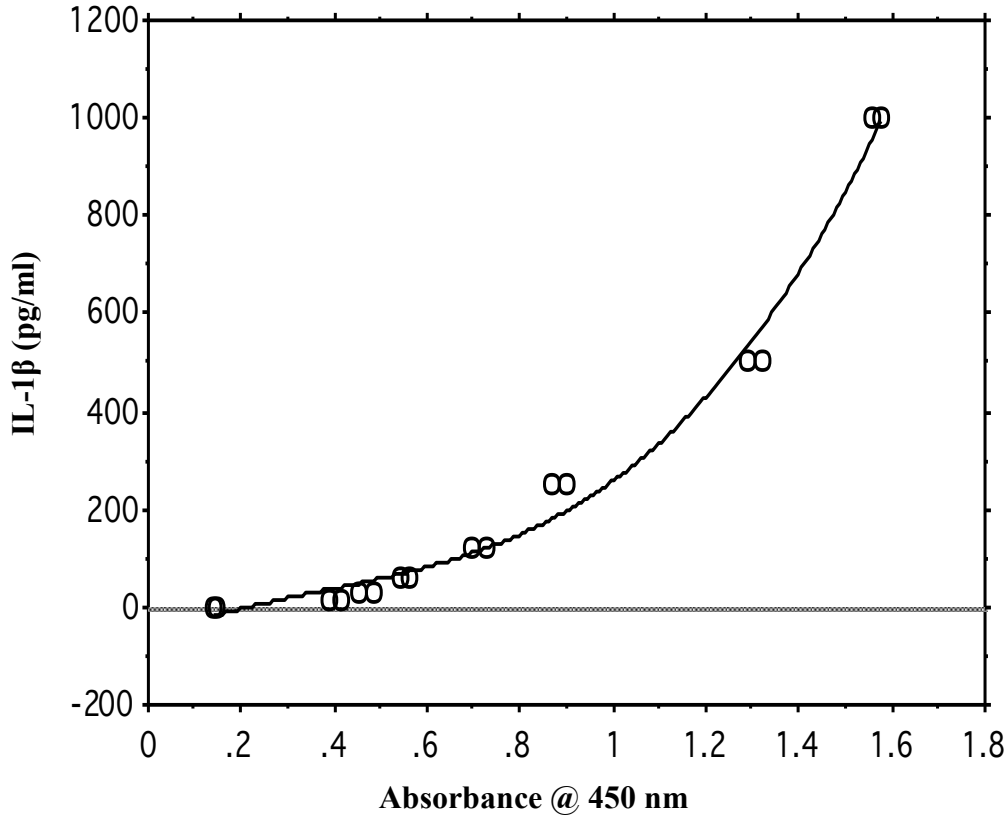



$$Y = -95.3 + 16.3x + 893.6x^2 + 1227.3x^3$$

$$r = 0.982$$

A standard curve for TNF-α was generated for each assay using human recombinant TNF-α standards that were run simultaneously with the samples during each analysis by ELISA as described in section 2.2.5.1. The equation below the figure shows the 3rd order polynomial linear regression algorithm generated by this particular data set and used by the software to calculate unknown concentrations of TNF-α (Y values) from the absorbance (independent variable) X values. The r value indicates the correlation coefficient for the linear relationship.

**Fig 2.4 Typical IL-1β Standard Curve for ELISA Assay**

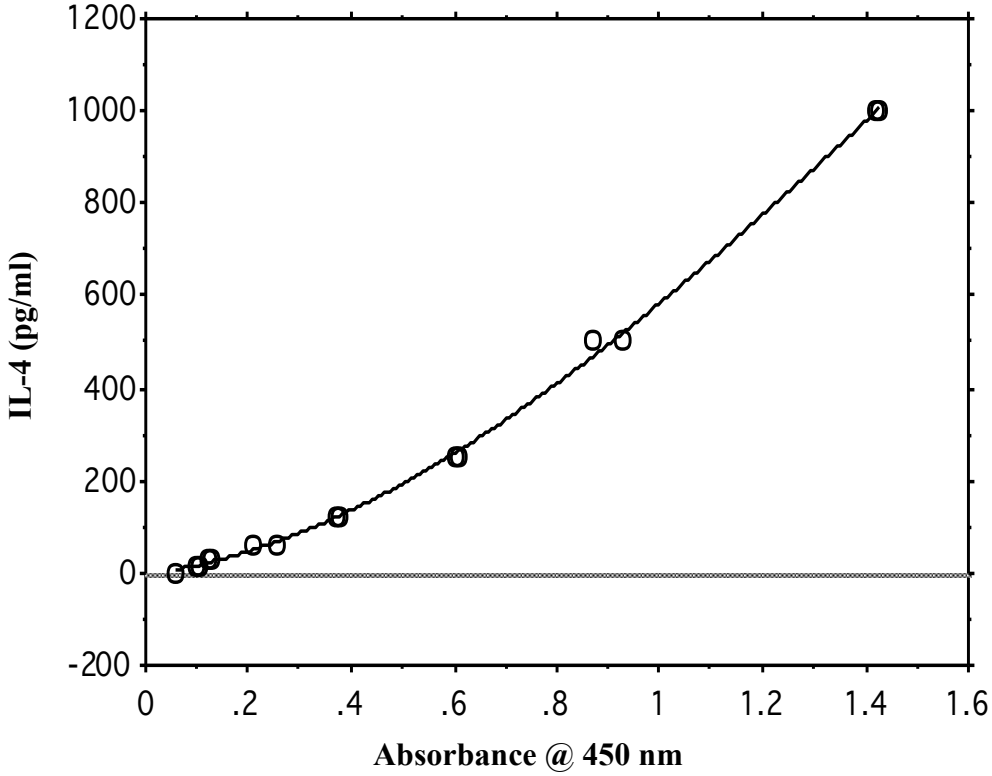



$Y = 61 + 367.1x - 462.9x^2 + 416.8x^3$

$r = 0.972$

Standard curve for IL-1β was generated for each assay using human recombinant IL-1β standards that were run simultaneously with the samples during each analysis, by ELISA as described in section 2.2.5.1. The equation below the figure shows the 3rd order polynomial linear regression algorithm generated by this particular data set and used by the software to calculate unknown concentrations of IL-1β (Y values) from the absorbance (independent variable) X values. The r value indicates the correlation coefficient for the linear relationship.

**Fig 2.5 Typical IL-4 Standard Curve for ELISA Assay**

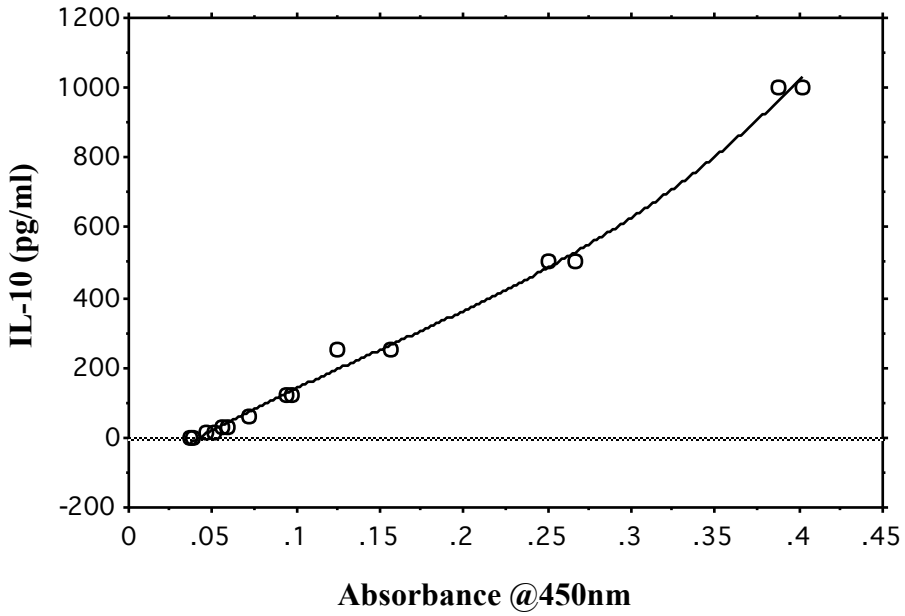



$$Y = 19.4 - 109.4x + 1086.1x^2 + 332.2\ x^3$$

$$r = 0.919$$

A standard curve for IL-4 was generated for each assay using human recombinant IL-4 standards that were run simultaneously with the samples during each analysis, by ELISA as described in section 2.2.5.1. The equation below the figure shows the 3rd order polynomial linear regression algorithm generated by this particular data set and used by the software to calculate unknown concentrations of IL-4 (Y values) from the absorbance (independent variable) X values. The r value indicates the correlation coefficient for the linear relationship.

**Fig 2.6 Typical IL-10 Standard Curve for ELISA Assay**




$$Y = -117.4 + 3042.2x - 5908.7x^2 + 13525.4x^3$$

$$r = 0.983$$

A standard curve for IL-10 was generated for each assay using human recombinant IL-10 standards that were run simultaneously with the samples during each analysis, by ELISA as described in section 2.2.5.1. The equation below the figure shows the 3rd order polynomial linear regression algorithm generated by this particular data set and used by the software to calculate unknown concentrations of IL-10 (Y values) from the absorbance (independent variable) X values. The r value indicates the correlation coefficient for the linear relationship.

### 2.2.11 Measurement of Prostaglandin Production

Prostaglandin production was determined in whole human blood (plasma) and supernatants from monocytes or THP-1 cells in response to different concentrations of 7β OH-EpiA or dexamethasone in either the presence or absence of stimulation with LPS (10μg/ml). Extracellular levels of prostaglandins were then determined by using commercially available competitive enzyme immunoassay kits (EIA).

#### 2.2.11.1 $PGE_2$ EIA

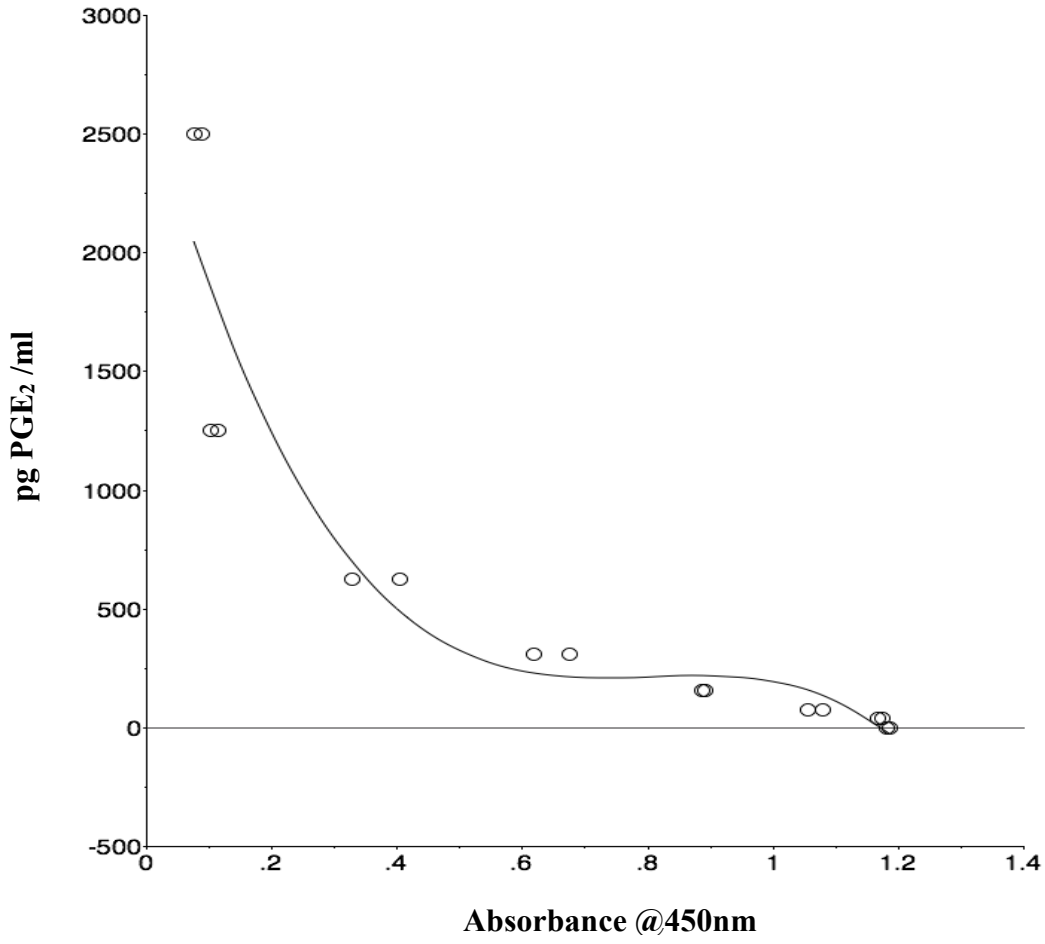
$PGE_2$ was determined in both plasma and supernatants as recommended in the manufacturer instructions. Each kit provided by the manufacturer included ready diluted assay buffer, washing buffer, HRP-labelled $PGE_2$ (as the competitive ligand for the mouse anti-$PGE_2$ antibody), 25,000 pg/ml of standard, as well as a pre-prepared goat anti-mouse microplate. All reagents were colour-coded to prevent errors in additions. Standard $PGE_2$ was serially diluted to yield concentrations of 2500 pg/ml, 1250 pg/ml, 625 pg/ml, 313 pg/ml, 156 pg/ml, 78 pg/ml, 39 pg/ml and assay buffer was also included (0 pg/ml).

150μl of control, standard, or sample (plasma or supernatant) was added to appropriate wells of the plate. Following this 50 μl of anti-$PGE_2$ antibody solution was added to the wells (except the non-specific binding wells). Then, the plate was covered with Clingfilm and incubated at room temperature for 1 hour on a microplate shaker. HRP-PGE2 conjugate (50 μl) was added to all wells. Then plate was re-covered and incubated at room temperature for a further 2 hours on a microplate shaker. The plate was then washed 4 times with wash buffer. TMB chromogen substrate (200 μl) was added to all wells and the plate was further incubated at room temperature protected from light for 30 minutes. At the end of this incubation 100 μl of stop solution was

added to each well. The absorbance was measured at 450 nm on a 96-well plate spectrophotometer/ reader.

Standard curves for the respective PGs were created for each assay (see Fig. 2.7 for a typical $PGE_2$ standard curve) using Statview software. 3rd Order polynomial regression analysis was used to calculate unknown concentrations of PGs from the absorbance values. A corresponding equation was generated from each specific data set which the software used to calculate levels of PGs in samples as described previously for cytokines (section 2.2.10).

**Fig 2.7 Typical $PGE_2$ Standard Curve for EIA**




$$Y = 2698 - 9477x + 11920x^2 - 4948x^3$$

$$r = 0.94$$

A standard curve for $PGE_2$ was generated for each assay using $PGE_2$ standards that were run simultaneously with the samples which were incubated in the presence of HRP-conjugated $PGE_2$ as the competitive ligand for anti-$PGE_2$ antibody in each assay as described in section 2.2.11.1. The equation below the figure shows the 3rd order polynomial linear regression algorithm generated by this particular data set and used by the software to calculate unknown concentrations of $PGE_2$ (Y values) from the absorbance (independent variable) X values. The r value indicates the correlation coefficient for the linear relationship.

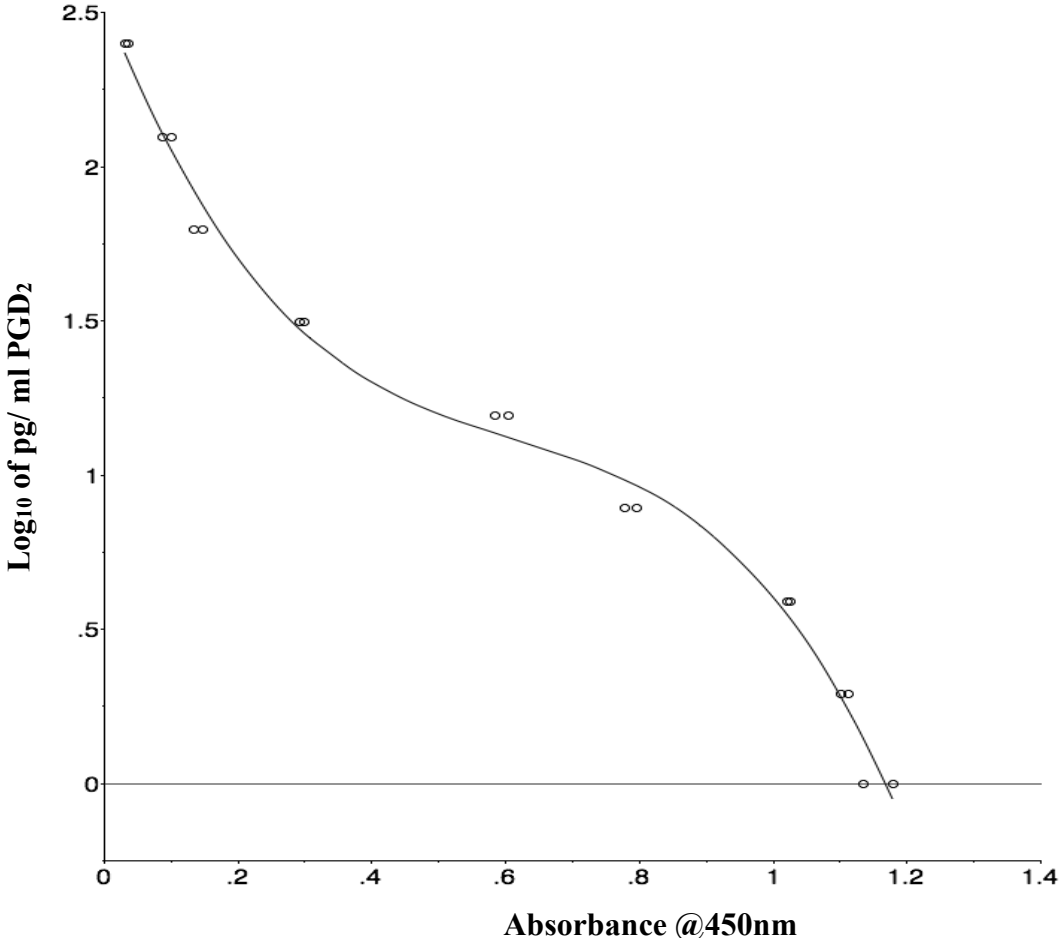
### 2.2.11.2 $PGD_2$ EIA

$PGD_2$ is chemically unstable and rapidly degrades to a number of J series prostaglandins including, $PGJ_2$, $\Delta$12-$PGJ_2$, and 15-deoxy-$\Delta$12,14-$PGJ_2$. In order to circumvent this problem unstable PGD2 can be chemically treated to give a stable derivative, in this case prostaglandin $D_2$–methoxime ($PGD_2$–MOX) which can be stored for subsequent analysis. This approach was used in the present study. Immediately following termination of incubations, 100 µl of sample plasma or supernatant was added to 1.5 ml tubes containing 100 µl of Methyl Oximating Reagent (methoxylamine hydrochloride, MOX-HCl) and sodium acetate dissolved in a 10:90 (v/v), ethanol/water solution. Tubes were then placed in a water bath and the reaction allowed to proceed for 30 min at 60 °C. At the end of this period samples were stored at -80 °C. The level of stable $PGD_2$-MOX was determined in both plasma or supernatant using an EIA kit. The use of this kit was similar to that described for the PGE2 EIA kit which contained the same components but also included the Methyl Oximating Reagents. Other differences were the provision of pre-methoximated $PGD_2$ ($PGD_2$-MOX) standard, mouse anti-$PGD_2$-MOX antibody and acetylcholinesterase-labelled/ conjugated $PGD_2$-MOX (as the competitive ligand for the mouse anti-$PGD_2$-MOX antibody). Standard $PGD_2$-MOX concentrations were prepared from the stock solution of 20,000 pg/ml to yield concentrations of 5000pg/ml, 250 pg/ml, 125pg/ml, 62.5pg/ml, 31.5pg/ml, 15.6pg/ml, 7.8pg/ml and assay buffer was also included (0 pg/ml).

150µl of control, standard, or sample (plasma or supernatant) was added to appropriate wells of the plate followed by acetylcholinesterase-conjugated $PGD_2$-MOX (50 µl) was added to all wells. Then, 50 µl of anti-$PGD_2$-MOX antibody solution was added to the wells (except the non-specific binding wells). Then the plate was covered in

clingfilm and incubated for 18 hr at 4° C. Plates were then washed x5 with the included wash buffer, after which 200 µl of Ellman's reagent (5,5'-dithio-bis-[2-nitrobenzoic acid], the chromogenic substrate for acetyl-cholinesterase) was added to each well. Plates were then incubated for a further 2 hours at room temperature. The plate was then directly taken for absorbance measurements which were conducted at 405 nm on a 96-well plate spectrophotometer/ reader. Standard curves were created for each assay using Statview software and levels of PG were calculated as described above for $PGE_2$ (see Fig. 2.8 for a typical $PGD_2$ standard curve).

**Fig 2.8 Typical $PGD_2$ Standard Curve for EIA**




$$y = 2.538 - 5.625x + 8.071x2 - 4.377x3$$

$$r = 0.98A$$

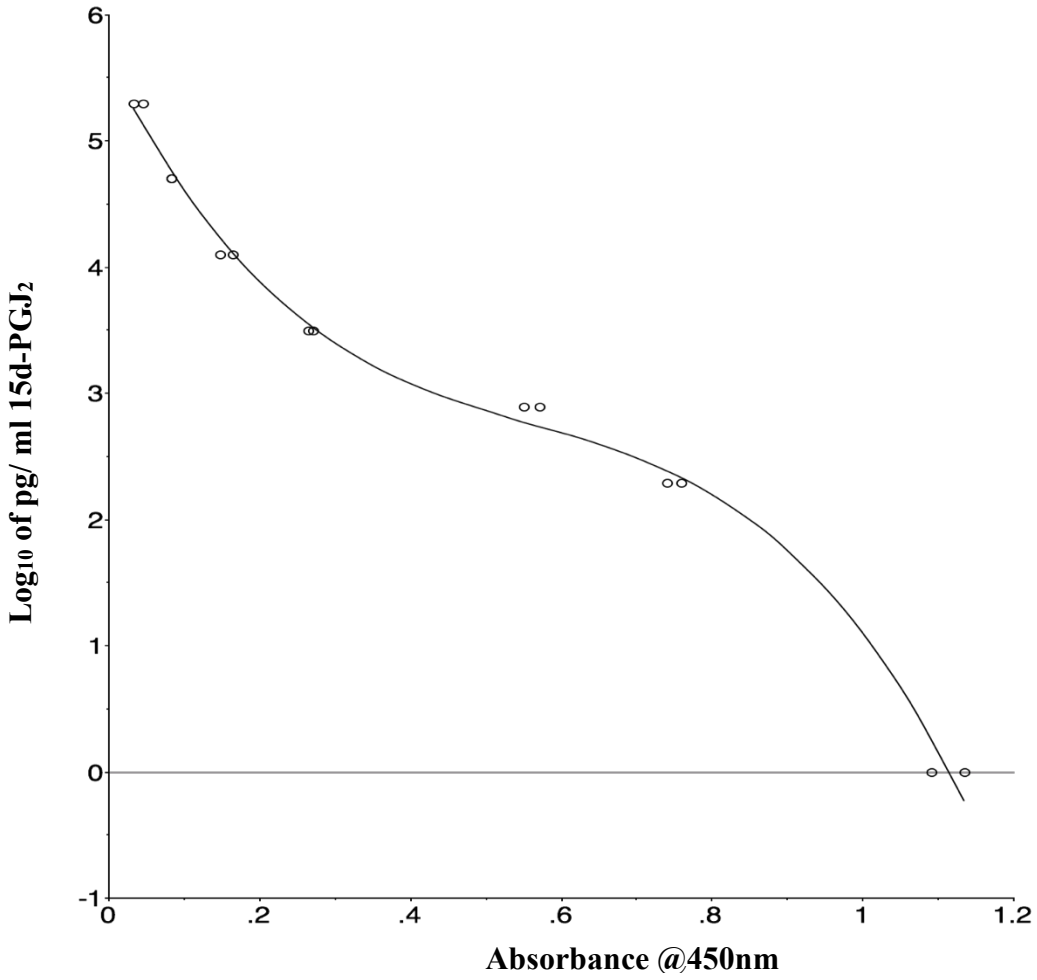
standard curve for $PGD_2$ was generated for each assay using $PGD_2$ standards that were run simultaneously with the samples which were incubated in the presence of acetylcholinesterase-conjugated $PGD_2$ as the competitive ligand for anti-$PGD_2$ antibody in each assay as described in section 2.2.11.2. The equation below the figure shows the 3rd order polynomial linear regression algorithm generated by this particular data set and used by the software to calculate unknown log10 concentrations (and subsequent concentration via anti-log) of $PGD_2$ (Y values) from the absorbance (independent variable) X values. The r value indicates the correlation coefficient for the linear relationship.

### 2.2.11.3 15d-$PGJ_2$ EIA

Levels of 15d-$PGJ_2$ were determined in both plasma and supernatant using an EIA kit. The use of this kit was similar to that of the $PGE_2$ EIA kit. The main differences were the provision of 15d-$PGJ_2$ standard, mouse anti-15d-$PGJ_2$ antibody and alkaline phosphatase-labelled/ conjugated-15d-$PGJ_2$ (as the competitive ligand for the mouse anti-15d-$PGJ_2$ antibody). Standard anti-15d-$PGJ_2$ concentrations were prepared from the stock solution of 1,000,000 pg/ml to yield concentrations of 200,000pg/ml, 50,0000 pg/ml, 12,500pg/ml, 3,125pg/ml, 781pg/ml, 195pg/ml, and assay buffer was also included (0 pg/ml).

100µl of control, standard, or sample (plasma or supernatant) was added to appropriate wells of the plate followed by alkaline phosphatase-conjugated-15d-$PGJ_2$ (50 µl) was added to all wells. Then, 50 µl of anti-15d-$PGJ_2$ antibody solution was added to the wells (except the non-specific binding wells). Then the plate was covered in clingfilm and incubated for 2 hr at room temperature on a microplate shaker. Plates were then washed x3 with the included wash buffer, after which 200 µl of p-Nitrophenyl phosphate (the chromogenic substrate for alkaline phosphatase) was added to each well. Plates were then incubated for a further 3 hours at room temperature. At the end of this incubation 100 µl of stop solution was added to each well. The plate was then taken for absorbance measurement which was conducted at 405 nm on a 96-well plate spectrophotometer/ reader. Standard curves were created for each assay using Statview software and concentrations of PG were calculated as described above for $PGE_2$ (see Fig. 2.9 for a typical 15d-$PGJ_2$ standard curve).

**Fig 2.9 Typical 15d-PGJ$_2$ Standard Curve for EIA**




$$y = 5.637 - 11.953x + 18.162x2 - 10.739x3$$

$$r = 0.99$$

A standard curve for 15d-PGJ$_2$ was generated for each assay using 15d-PGJ$_2$ standards that were run simultaneously with the samples which were incubated in the presence of alkaline phosphatase-conjugated 15d-PGJ$_2$ as the competitive ligand for anti-15d-PGJ$_2$ antibody in each assay as described in section 2.2.11.3. The equation below the figure shows the 3rd order polynomial linear regression algorithm generated by this particular data set and used by the software to calculate unknown log10 concentrations (and subsequent concentration via anti-log) of 15d-PGJ$_2$ (Y values) from the absorbance (independent variable) X values. The r value indicates the correlation coefficient for the linear relationship.

### 2.2.12 Gene Expression Studies

#### 2.2.12.1 Polymerase Chain Reaction (PCR) Amplification

##### 2.2.12.1.1 Extraction of Total RNA

THP-1 cells (2 x 106/ ml) were incubated in 6-wells plates at 37˚C in the presence of 5% CO2 (v/v) and 100% humidity with different concentrations of 7β OH-EpiA 0.001μM 1h prior to addition of LPS or RPMI 1640 or DMSO, as controls.

THP-1 total RNA was extracted by using an Isolate II RNA Mini kit (Bioline, UK) following the manufacturer's instructions. As THP-1 cells are suspension cells, culture medium and cells were transferred from each well to sterile microcentrifuge tubes and the well was washed with RPMI 1640 twice (to ensure as many cells as possible were collected from plates). The tubes were centrifuged at 400g for 7 minutes at 20˚C. The cell pellet was re-suspended in 350μl of lysis buffer, RLY, supplemented with 3.5μl β-mercaptoethanol (β-ME) and the sample vortexed vigorously. The lysate solution was transferred to an ISOLATE II (violet) filter column in a 2ml collection tube and centrifuged at 11,000 x g for 1 min. The filter column was discarded and the lysate flow-through was retained for extraction. One volume of 70% (v/v) ethanol was added to the lysate and mixed by pipetting before being loaded into another ISOLATE II RNA Mini spin column (Schumann et al.) in a 2ml collection tube and centrifuged for 30 seconds at 11,000 x g. The RNA from the lysate binds to the silica membrane of blue spin column so the flow through was discarded and 350μl of Membrane Desalting Buffer (MEM) was added to the column and centrifuged at 11,000x g for one minute. As genomic DNA carryover from the RNA isolation process can compromise the results from RT-qPCR assays, the sample was treated with DNase I to digest any DNA present. 95μl of the DNase I reaction mixture was pipetted onto the centre of the silica membrane (DNase I reaction mixture which was prepared by mixing 10μl of

reconstituted DNase I with 90µl of (RDN) Reaction Buffer for DNase I). The column with this mixture was incubated at room temperature for 15 minutes. A first wash was carried out to inactivate the DNase I by adding 200µl of washing buffer RW1 to the ISOLATE II RNA Mini Column followed by centrifuging for 30 seconds at 11,000x g. A second wash of the membrane was conducted by adding 600µl washing buffer RW2 into the ISOLATE II RNA Mini Column and the spin column centrifuged for 30 seconds at 11,000 x g. The elute was discarded and the column was placed back into the collection tube. A third wash with 250µl of wash buffer RW2 was added to ISOLATE II RNA Mini Column and the column centrifuged for 2 minutes at 11,000 x g. The dry column was placed into a new nuclease-free 1.5 ml collection tube. The RNA was eluted from the column into the collection tube by adding 40µl RNase-free water to the membrane and centrifuging it at11, 000 x g for one minute. The last step was repeated with the same eluate to improve the total yield of RNA.

#### 2.2.12.1.2 Re-purification of RNA

This step was recommended by manufacturer to help ensure all traces of DNA contamination were removed along with any salts and buffers from the samples. Re-purification was carried out as described in the following steps:

#### 2.2.12.1.3 DNA Digests

100µl of DNase (Bernard-Poenaru *et al*.) reaction buffer and 10µl DNase I were prepared in an RNase/DNase-free tube. The 1/10th volume of the mixed solution was added to one volume of RNA eluate and gently mixed by pipetting. DNase I digestion was carried out using a thermocycler (Applied Biosystems Model 480) at for 37°C for 10 minutes.

#### 2.2.12.1.4 RNA Re-Purification Using Ethanol Precipitation

1/10th volume of sodium acetate (3M, pH 5.2) was added in one volume of RNA eluate. Three volumes of 100% (v/v) ethanol was then added to this one volume of RNA sample and mixed thoroughly. The RNA sample was precipitated overnight at -20°C. Following this incubation, the sample was centrifuged at 13,000 x g for 10 minutes. The supernatant was aspirated carefully. The RNA pellet was washed with 20µl of ice-cold 70% (v/v) ethanol and centrifuged again at 13,000 x g for 10 minutes. The supernatant was again carefully aspirated, and the remaining ethanol was evaporated by keeping tube cap opened at room temperature and air-drying the pellet for 15-20 minutes. The pellet was re-suspended in 20µl of RNase/DNase free water. Concentration of RNA eluate readied and either stored at –80°C until required or start cDNA synthesis.

#### 2.2.12.1.5 Assessment of Extracted RNA Concentration

The concentrations of purified RNA from all of the samples were measured spectrophotometrically by using a NanoDrop ND 2000c spectrophotometer (Thermo, UK). RNase-free water (1 µl) was used as a blank and 1 µl volumes of the RNA sample were used in the determination of their concentrations. The Nanodrop software calculated RNA concentration and ratios (A260 nm/ A230 nm; A260 nm/ A280 nm) as well as displaying curves of the absorbance of each sample. An A260 nm/ A280 nm ratio of 2.0 is typical of a pure RNA sample. Salt carryover from the RNA isolation can affect the A260 nm/ A230 nm which should be around 2.0.

### 2.2.13 cDNA Synthesis (Reverse Transcription of mRNA)

For amplification and quantification by PCR, the messenger RNA transcripts in the isolated total RNA have to be reverse transcribed to complementary DNA (cDNA). This was done by using a Tetro cDNA Synthesis Kit (Bioline, London) The protocol in the manufacturer's manual was followed. 8 µl (1µg) of total RNA, One µl of Oligo (dT), 1 µl of 10 mM of dNTP mix, 4 µl of 5x RT buffer, and 1 µl of both RiboSafe RNase Inhibitor, and 200u/µl of Tetro Reverse Transcriptase were added to a RNase/DNase-free 500 µl tube and the final volume adjusted to 20 µl by adding DEPT-treated water. All these samples were labelled RT+. Two contamination controls were prepared as following:

1. First Control - samples that contain all cDNA synthesis components and total RNA, with the exception of the Tetro Reverse Transcriptase where DEPT-treated water was added instead of the Tetro Reverse Transcriptase, so these tubes were labelled as RT-.
2. Second Control - samples that contained all cDNA synthesis components with the exception of the total RNA where DEPT-treated water was added instead of the total RNA. These tubes were labelled as H2O.

Both controls ("RT-"& H2O) were run in parallel for each of the RNA samples. Each reaction was gently mixed by pipetting and incubated at 45˚C for 30 minutes. Then, a final termination incubation was done at 85˚C for five minutes and immediately chilled in ice. These samples were stored at -20˚C for long-term storage or used immediately for PCR without dilution.

### 2.2.14 PCR Primers Design for SYBR Green-based RT-qPCR

The specificity of RT-qPCR assays is dependent upon good primer design, ensuring that the PCR primers only bind to the intended target gene. It is important to avoid non-specific products in all SYBR® assays as well as primer dimerization which would compromise gene expression determination as SYBR-green will bind to any double-stranded amplicons generated in PCR regardless of whether they are derived from the intended target cDNA template or not (Thornton & Basu, 2011). A number of primers for target and reference genes were used to examine changes in their gene expression by quantitative real-time polymerase chain reaction amplification (qRT-PCR) for this study. All those primers were designed as described in the following steps:

- National Centre for Bioinformatics (NCBI) GQuery website (https:// https://www.ncbi.nlm.nih.gov/search/) was used to search the Gene database to obtain the necessary mRNA sequences or gene IDs (GenBank Accession numbers).
- The selected mRNAs sequence were imported into the PrimerQuest webtool (http://eu.idtdna.com/Primerquest/Home/Index) in the Integrated DNA Technologies (IDT) website (http://eu.idtdna.com/site) to identify potential primer pairs.
- The assay setting was chosen as "qPCR – 2 Primers and Intercalating dye" for use with SYBR-Green-based polymerase chemistries.
- For efficiency of PCR amplification, size of amplicon was chosen between 99-140 bp.
- Primer sequences with good specificity were identified. The primer specificity was validated using the NCBI Primer-BLAST (Basic Local Alignment Search

Tool) (Ye *et al*., 2012)) (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi). This step is important to ensure that the selected primers only bind to the target gene and to minimise the risk of mispriming on other targets, which could give rise to false positives.

- The melting temperature (Tm) of primers kept between 58–60°C; the ΔTm between forward and reverse primers was ≤1°C.
- Primer length: 19–29 bases.
- Primer GC content: 45%–58%.
- Primers were designed to span or flank intron-exon boundaries on genomic DNA (gDNA) sequences in order to prevent the false positive amplification of any contaminating genomic DNA present in the cDNA samples.
- The primers were synthesised by Integrated DNA Technologies (Belgium).

**Table 2.1Nucleotide Sequences of the Primers used for the Analysis of Gene Expression by Quantitative Real Time Polymerase Chain Reaction Amplification (qRT-PCR).**

| Primer Name | Primer sequence | PCR Product Size (bp) | Genbank ID |
|---|---|---|---|
| **RPL37A** | **Forward: 5'CCAAGATGAAGAGACGAGCTG-3' Reverse: 5'TGACAGCGGAAGTGGTATTG-3'** | **99** | **NM_000998.4** |
| **PPIB** | **Forward: 5'CACAGGAGGAAAGAGCATCTAC-3' Reverse: 5'CTGTCTTGACTGTCGTGATGAA-3'** | **130** | **NM_000942.4** |
| **PGDS** | **Forward: 5'-GCCGTCGCTAACTGGATAAA- 3' Reverse: 5'-ATTATCTGGCAGGCTGATGTAG-3'** | **129** | **NM_014485.2** |
| **PTGDR2** | **Forward: 5'-TGAGCAAAGGGAACAGTGAG-3' Reverse: 5'GGGAAGTGCTATGAGTAAAGGG-3'** | **107** | **NM_004778.2** |
| **ACTB** | **Forward: 5'GGACCTGACTGACTACCTCAT-3' Reverse: 5'CGTAGCACAGCTTCTCCTTAAT-3'** | **107** | **NM_001101.3** |
| **TNF-ALPHA** | **Forward: 5'CCCAGGGACCTCTCTCTAATC-3' Reverse: 5'AGCTTGAGGGTTTGCTACAAC-3'** | **105** | **NM_000594.3** |
| **TLR-4** | **Forward: 5' GCCCTAAACCACACAGAAGA-3' Reverse: 5'GGAACCACCTCCGTGATAAA-3'** | **123** | **NM_003266** |

### 2.2.15 Quantitative Real Time Polymerase Chain Reaction Amplification (RT-qPCR)

Dye-based real-time PCR (RT-qPCR) was performed by using an Applied Biosystems StepOne Plus real-time instrument and analysis software (Applied Biosystems, UK) along with Power SYBR Green Mastermix along with the appropriate primer sets. Each reaction was run in 100 µl fast reaction MicroAmp PCR tubes. The final volume of each reaction was 20µl, which contained: 10µl of Power SYBR Green, one µl of Forward (10 pmol/µl), one µl of Reverse primers (10 pmol/µl), one µl of cDNA and 7 µl of molecular grade water. The PCR protocol included a 15-minute pre-incubation at 95°C in order to allow heat activation of the Power SYBR Green DNA polymerase, followed by 40 cycles of two stages (denaturation stage at 94°C for 15 seconds and a combined annealing and extension stage at 60°C for 60 second). After the run, a melting curve analysis was conducted on the amplicons to assess the assay. PCR results were analysed, ΔCTs values were calculated to determine fold changes in gene expression quantities. Reference gene stability was calculated by using GeNorm, BestKeeper and NormFinder http://150.216.56.64referencegene.phptypereference. Control samples (Haynes et al.) and negative controls (H2O) were used to determine whether false positives were obtained from genomic DNA templates instead of cDNA. The amplified products were run on 2% (w/v) agarose gels and stained with ethidium bromide.

#### 2.2.15.1 The Relative Quantification [ΔΔCt] Method for Real-time PCR

The quantification method used with the PCR results was the relative quantification (ΔΔCt) method (Livak & Schmittgen, 2001). This method normalised Ct values of the target gene to Ct values of the endogenous reference gene in order to obtain the fold

changes in gene expression between the control and treated samples. The PCR efficiencies of both of the target gene and the reference gene primers under the cycling and reaction conditions was determined before using this quantification method and confirmed that they had the acceptable range of 90%–110% (see next Section for PCR efficiency). The following equations were used to compare expression levels between control and treated samples:

- Calculation of the difference between the Cts for the target gene and the reference gene for the treated and control samples (ΔCt):

$$\Delta Ct = Ct\ target - Ct\ reference\ gene$$

- Calculation of the difference between the ΔCts of the between the treated and control samples (ΔΔCt):

$$\Delta\Delta Ct = (Ct\ target - Ct\ reference)\ treated - (Ct\ target - Ct\ reference)\ control$$

$$\Delta\Delta Ct = \Delta Ct\ treated - \Delta Ct\ control$$

- Fold change (FC) in the treated sample was equal to $2^{-\Delta\Delta Ct}$

### 2.2.16 Selecting the qRT-PCR Reference Gene

The reference gene (housekeeping gene) is important for normalising differences in the cDNA concentration added in each PCR reaction. Therefore, it is important that the mRNA expression of the reference gene needs to be stably expressed between PCR samples. Reference genes such as ribosomal protein L37a (RPL37A), beta- actin (ACTB), glycereraldehyde-3-phosphate dehydrogenase (GAPDH), peptidylprolyl isomerase B (PPIB) and hypoxanthinephosphor-ribosyltransferase 1 (HPRT1) are

commonly used with selective THP-1 cells for qRT-PCR studies (Maeß *et al*., 2010; Vandesompele *et al*., 2002a). In this study (PPIB, RPL37A, HPRT1, ACTB and GAPDH) were chosen to be examined under the conditions of this study. Therefore, amplification of gDNA was detected using these reference genes in negative control samples even with an important DNase-digestion during RNA extraction. Genomic DNA-derived PCR products were predicted using NCBI Primer-BLAST with primers of these reference genes. To validate selection of only the most stably expressed reference gene for this study, candidate reference primers were used in qRT-PCRs with control (RPMI) and treated samples (LPS and 7β OH-EpiA) and the obtained Crossing Threshold (Apostolaki *et al.,* 2010) or Quantitation Cycle (Van Herreweghe *et al.,* 2010) values were validated using a comprehensive ranking of the stability of candidate reference genes via the free available program RefFinder (De Spiegelaere *et al.,* 2015). This was carried out by entering Ct values to RefFinder program. The RefFinder estimated result was collated from the three common analysis gene stability programs: geNorm, BestKeeper, and NormFinder (Andersen *et al.,* 2004; Pfaffl *et al.,* 2004; Vandesompele *et al.*, 2002b).

The RefFinder analysis suggested that PPIB was the most stable control gene under this study's experimental conditions as the PPIB gene showed no amplification with negative controls and mRNA expression of this reference gene was found to be the most stably expressed reference gene within all samples in the qRT-PCR assays. Therefore, the PPIB gene was selected to be the reference gene for the present study.

# Chapter Three: Results

# 3. Results

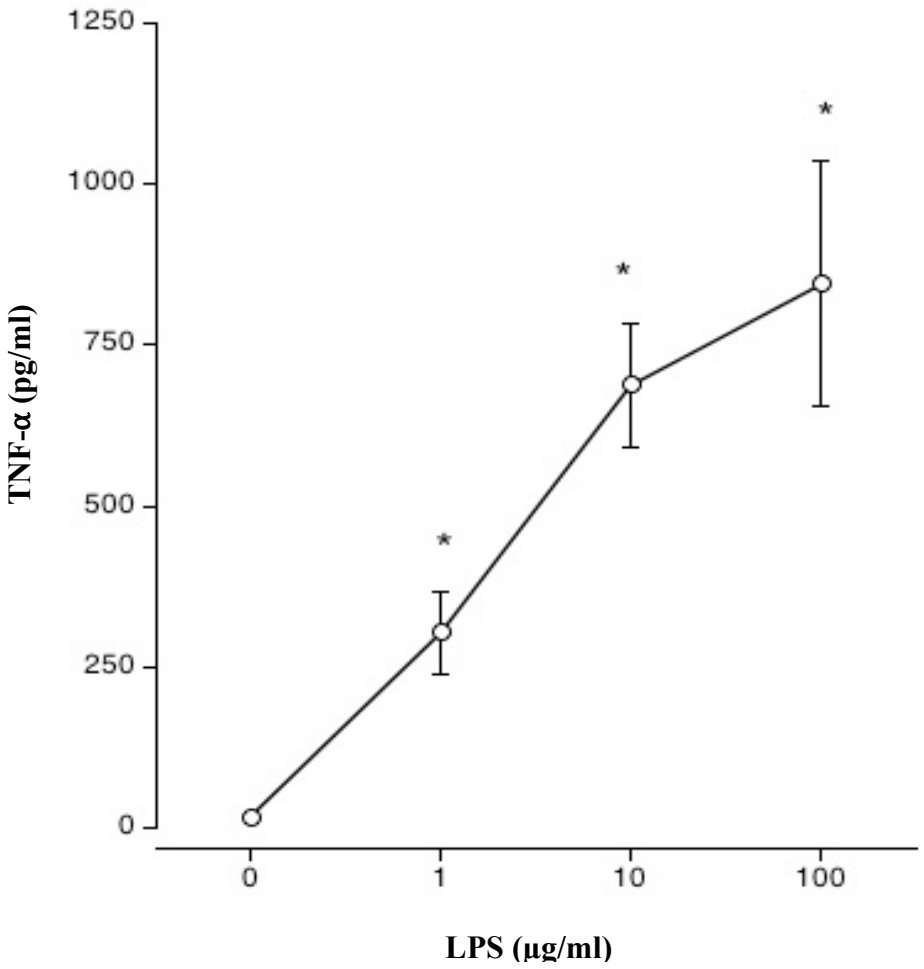
## 3.1 The effect of Varying LPS Concentrations on Pro-Inflammatory Cytokine Production in Whole Human Blood and THP-1 Cells

The effects of LPS are extensively documented in many experimental models for its ability to stimulate cytokine production especially TNF-α (Rietschel *et al.,* 1993). LPS in vitro has been shown to initiate inflammation responses in whole human blood (as a model of inflammation) and THP-1 cells (as a model of phagocytic cells) to produce cytokines such as TNF-α, IL-1β, and many others (Dorresteijn *et al.*, 2010; Zhang *et al.*, 1999) respectively. The first set of experiments was carried out to measure the capability of LPS to stimulate the production of TNF-α in human blood and from THP-1 cells.

### 3.1.1 The Effect of Different Concentrations of LPS on TNF-α Production in Human Blood and THP-1 Cells

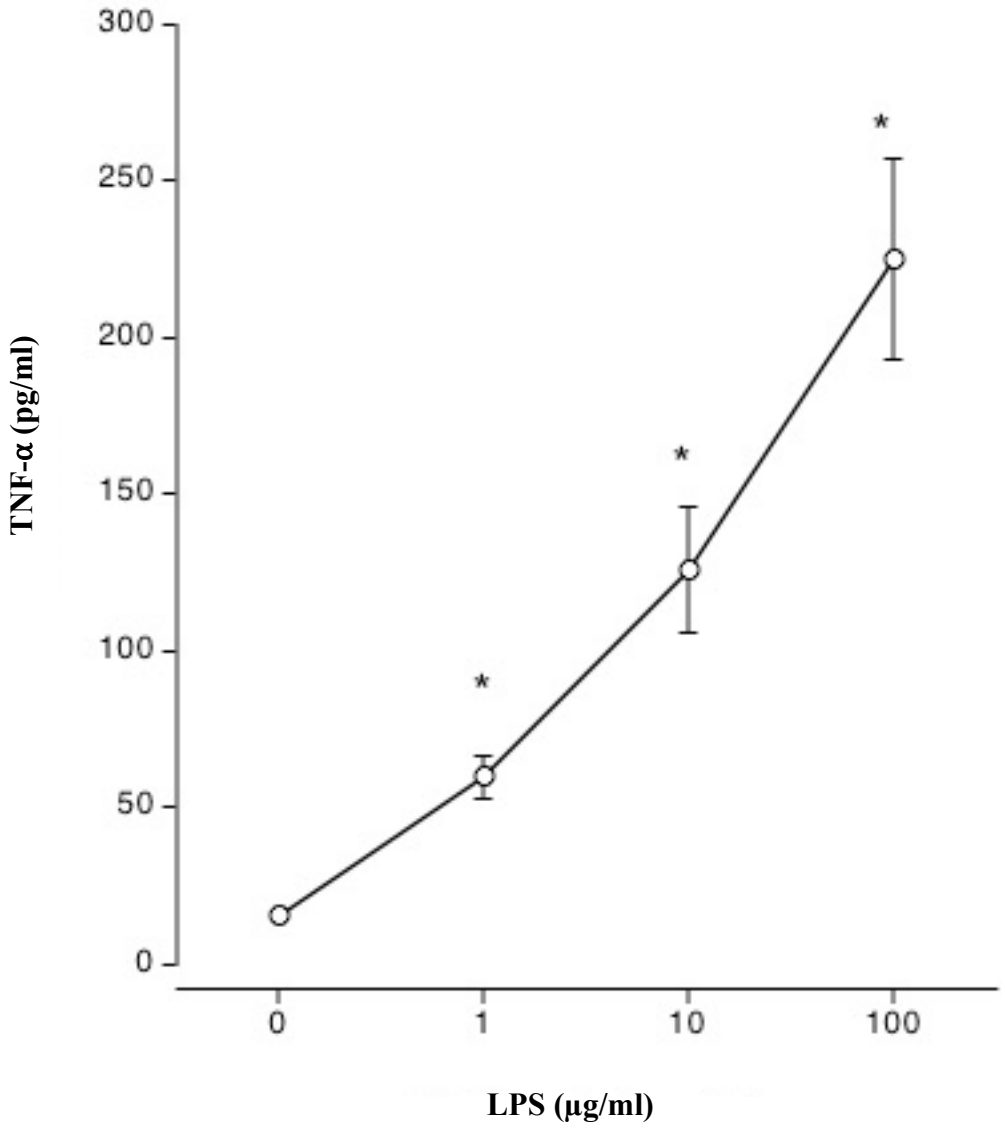
Initial experiments were carried out to determine the effect of LPS on the production of TNF-α in whole human blood and THP-1 cells. The concentration (amount) of TNF-α in both plasma and THP-1 cell supernatant was measured by ELISA assay as previously described above in the methodology section. In whole human blood, increasing concentrations of LPS (1-100μg/ml) resulted in a concentration-dependent increase in the levels of TNF-α compared to RPMI 1640 culture media only (Fig 3.1). At 1μg/ml LPS stimulated a small increase in the level of TNF-α, whereas 10μg/ml increased TNF-α levels in the order of 50-fold above the control quantity and approached a maximal increase at 100 μg/ml (Fig. 3.1). The effects of LPS on THP-1 were qualitatively similar with an increase in the level of TNF-α in the order of 12-fold with 10 μg/ml (Fig. 3.2).

**Fig 3.1 The Effect of Varying LPS Concentrations on TNF-α Production in Whole Human Blood**




Blood was incubated with varying concentrations of LPS. Incubations were carried out for 20 h at 37°C, 5% CO2 and 100% humidity, after which the concentrations of TNF-α in plasma was measured by ELISA. Values are the means of n = 3 ± s.d. *P < 0.01 versus incubations without LPS.

**Fig 3.2 The Effect of Varying LPS Concentrations on TNF-α Production in THP-1**

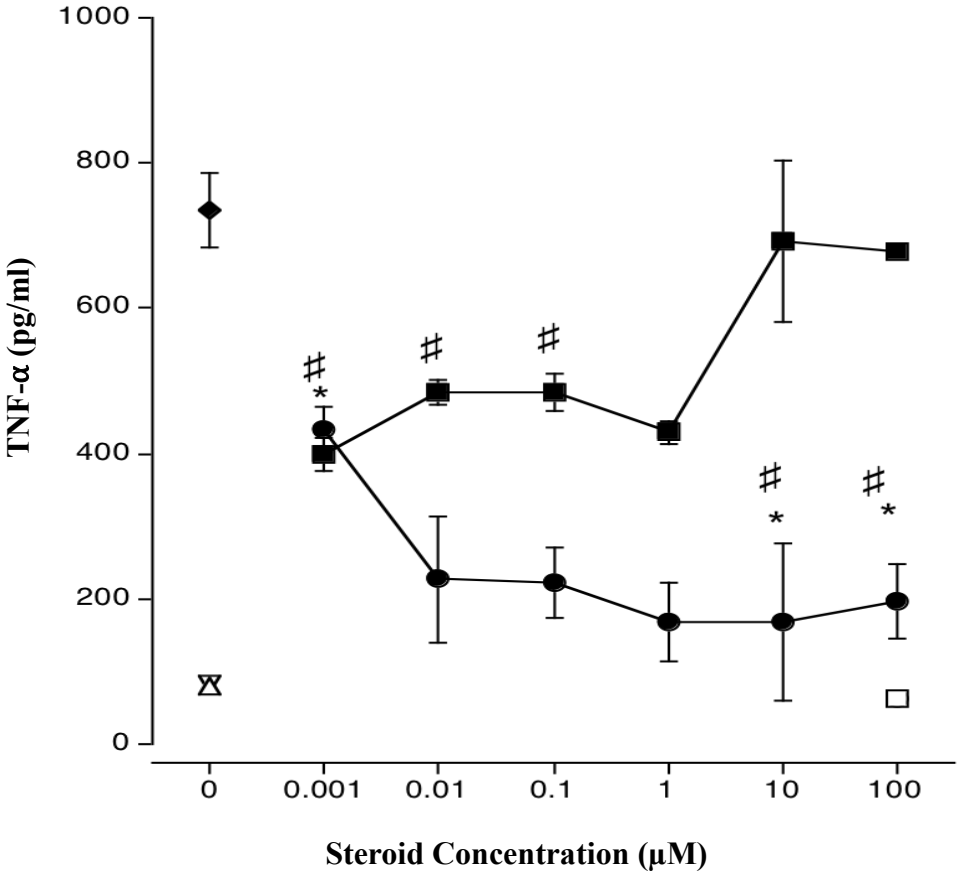



THP-1 cells (1x106) were incubated in 24 wells plate in triplicate at 37 °C, 5% CO2 for 20 h in the presence of gradual increase of LPS concentrations (0.1, 1, 10, and 100µg/ml or absence (culture media only) The TNF-α concentrations presented within the supernatants were determined by ELISA assay. Values represent the mean of n = 3 ± s.d. * P<0.001 versus control.

### 3.1.2 Effect of Varying Concentrations of 7β OH-EpiA and Dexamethasone on TNF-α Production in Whole Human Blood, THP-1 and Isolated Monocytes Cells in Response to LPS Stimulation

7β OH-EpiA was used to determine its effect on cytokine production (mainly TNF-α) in whole human blood. This was carried out by incubating various concentrations of 7β OH-EpiA and dexamethasone (positive control) in the presence of LPS (10μg/ml) for 20 h. The level of TNF-α was measured using ELISA as described in the methods section. Increasing concentrations of 7β OH-EpiA produced a variable response and the lowest concentration of this steroid used (0.001 μM) reduced TNF-α levels. Although this concentration was the most effective, higher concentrations, 0.01 - 1μM were also able to reduce TNF-α levels. The highest concentrations, 10μM and 100μM did not have any effect on TNF-α and the level remained close to that with LPS alone. On the other hand, with the positive control, dexamethasone, all concentrations were able to reduce TNF-α level in a concentration-dependent manner with 100μM (the highest concentration used) being the most effective.

**Fig 3.3 Effect of Varying Concentrations of 7β OH-EpiA and Dexamethasone on TNF-α Production in Whole Human Blood in Response to LPS Stimulation**

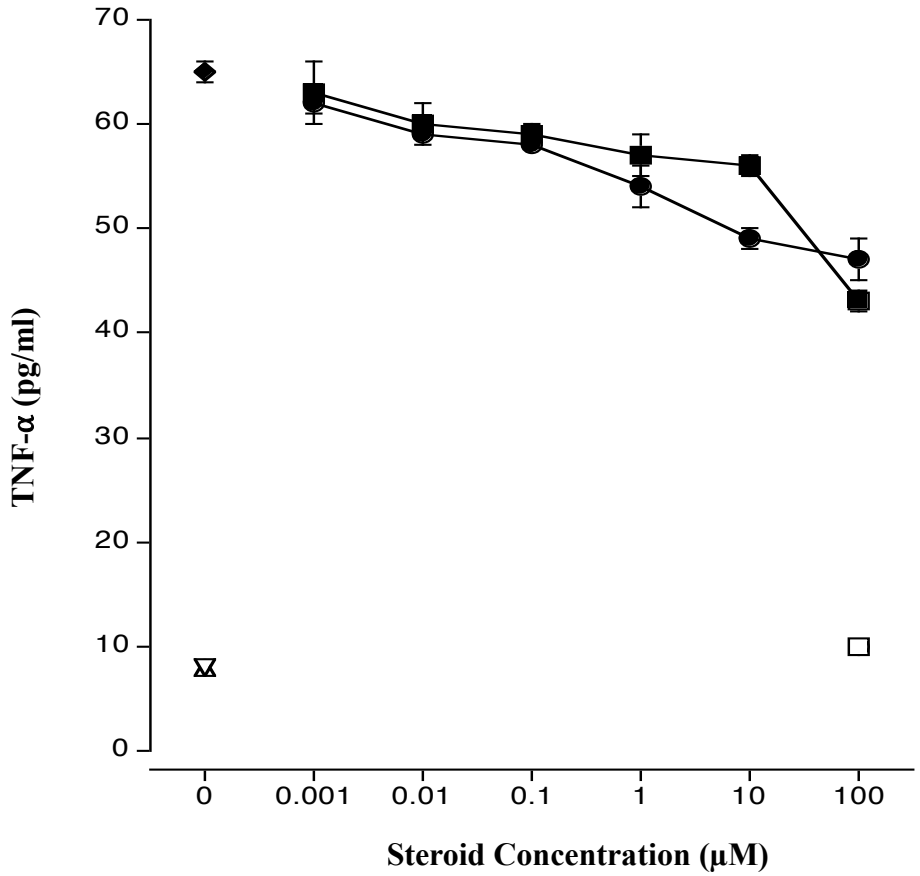



Blood was incubated with various concentrations of 7β OH- EpiA (square) or dexamethasone (circles) in absence (empty shape) or presence of LPS (10μg/ml) (filled shape) for 20 h at 37°C, 5% CO2 and 100% humidity. Incubations were then centrifuged, and the TNF-α concentrations in the plasma were determined by ELISA. Values are expressed as the means of 3 ± s.d. *Denotes $P < 0.05$ when compared to LPS alone, ♯Denotes $P < 0.05$ when compared to RPMI and DMSO.

### 3.1.3 Effect of Varying Concentrations of 7β OH-EpiA and Dexamethasone on TNF-α Production in THP-1 Cells in Response to LPS Stimulation

7β OH-EpiA was used to determine its effect on cytokine production, mainly TNF-α, in THP-1 cell supernatants. This was carried out by incubating various concentrations of 7β OH-EpiA and dexamethasone (positive control) in presence of (10 μg/ml) of LPS and for 20 h at 37ºC, 5% CO2 and 100% humidity. The level of TNF-α in cell supernatants was measured using ELISA as described in the methods section. As shown in Fig. 3.4, 7β OH-EpiA concentrations higher than 0.001 μM showed a small concentration-dependent decrease in LPS-stimulated TNF-α levels. Similarly, dexamethasone concentrations at 0.01 and above were able to reduce the TNF-α quantity. The magnitude of suppression of LPS-stimulated TNF-α production from THP-1 cells by both 7β OH-EpiA and dexamethasone was not as great as that observed in whole blood.

**Fig 3.4 Effect of Varying Concentrations of 7β OH-EpiA and Dexamethasone on TNF-α Production in THP-1 Cells in Response to LPS Stimulation**

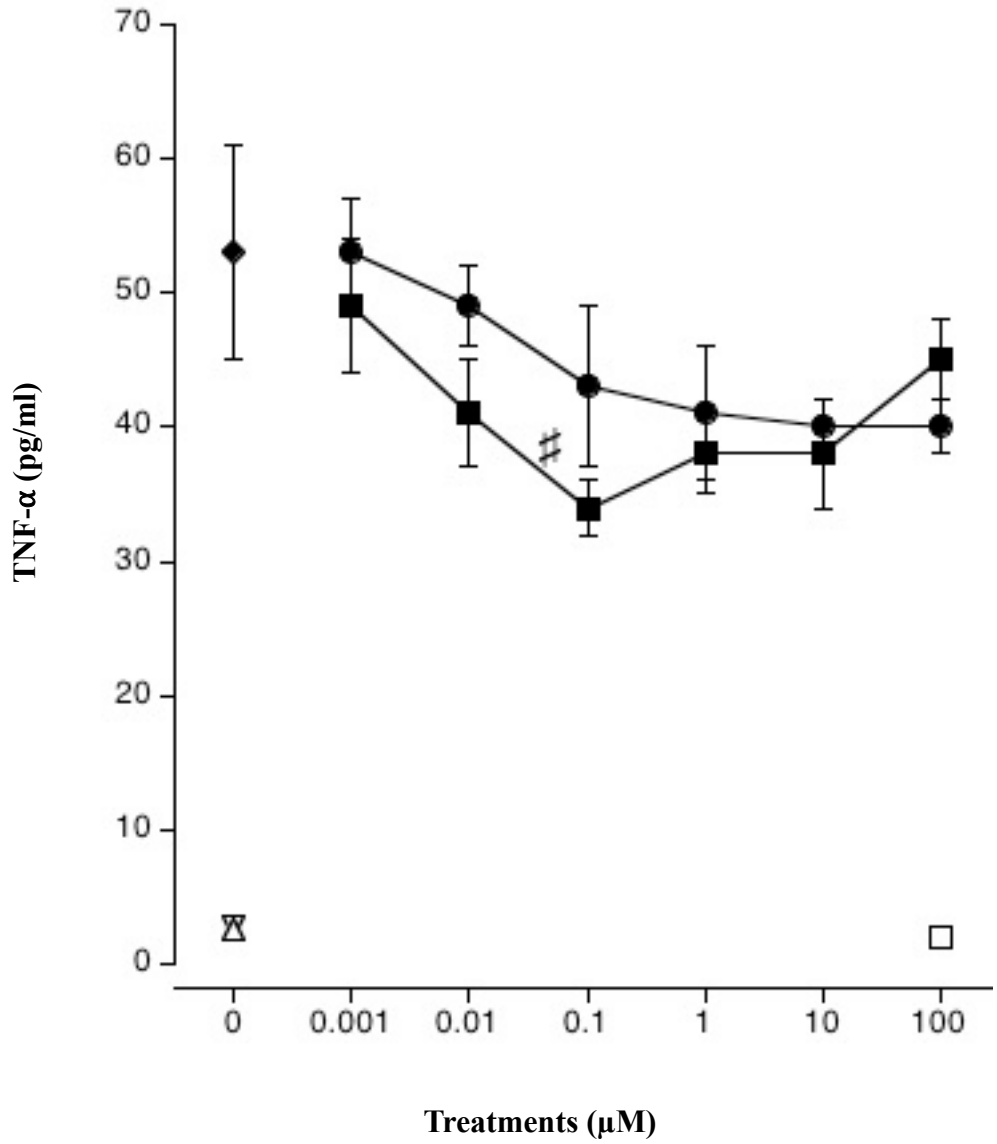



THP-1 cells ($1x10^6$ cells/ml) were incubated with varying concentrations of 7β OH EpiA (filled square) and dexamethasone (filled circle) in presence of (1µg/ml) of LPS (filled diamond). RPMI 1640 (up empty triangle), DMSO (down empty triangle) and 100µM of both treatments (empty shapes) were used alone as negative controls. Incubations were carried out for 20 h at 37°C, 5% CO2 and 100% humidity, after which the concentration of TNF-α in culture medium was measured by ELISA. Values are the means of n = 3 ± s.d. *P < 0.05 versus incubations with LPS alone.

### 3.1.4 Effect of Varying 7β OH-EpiA and Dexamethasone Concentrations on TNF-α Production in Isolated Human Monocytes

7β OH-EpiA was used to determine its effect on cytokine production in supernatants from isolated human monocytes. This was carried out by incubating various concentrations of 7β OH-EpiA and dexamethasone (positive control) in the presence of (1μg/ml) of LPS for 20 h. The level of TNF-α in supernatants was measured using ELISA as described in the methods section. As shown in Fig 3.5, 7β OH-EpiA reduced LPS-stimulated quantities of TNF-α only at higher concentrations (10μM and100 μM). Although the mean levels of TNF-α decreased between 0.001 μM and 1 μM of 7β OH-EpiA, this was not significantly different from the level of TNF- α production in presence of LPS alone. There was a similar action of dexamethasone where there was a decrease in concentrations of TNF-α with concentrations of dexamethasone between 0.1 and 10 μM (100μM did not reduce levels).

**Fig 3.5 Effect of Varying 7β OH-EpiA and Dexamethasone Concentrations on TNF-α Production in Isolated Monocytes in Response to LPS**



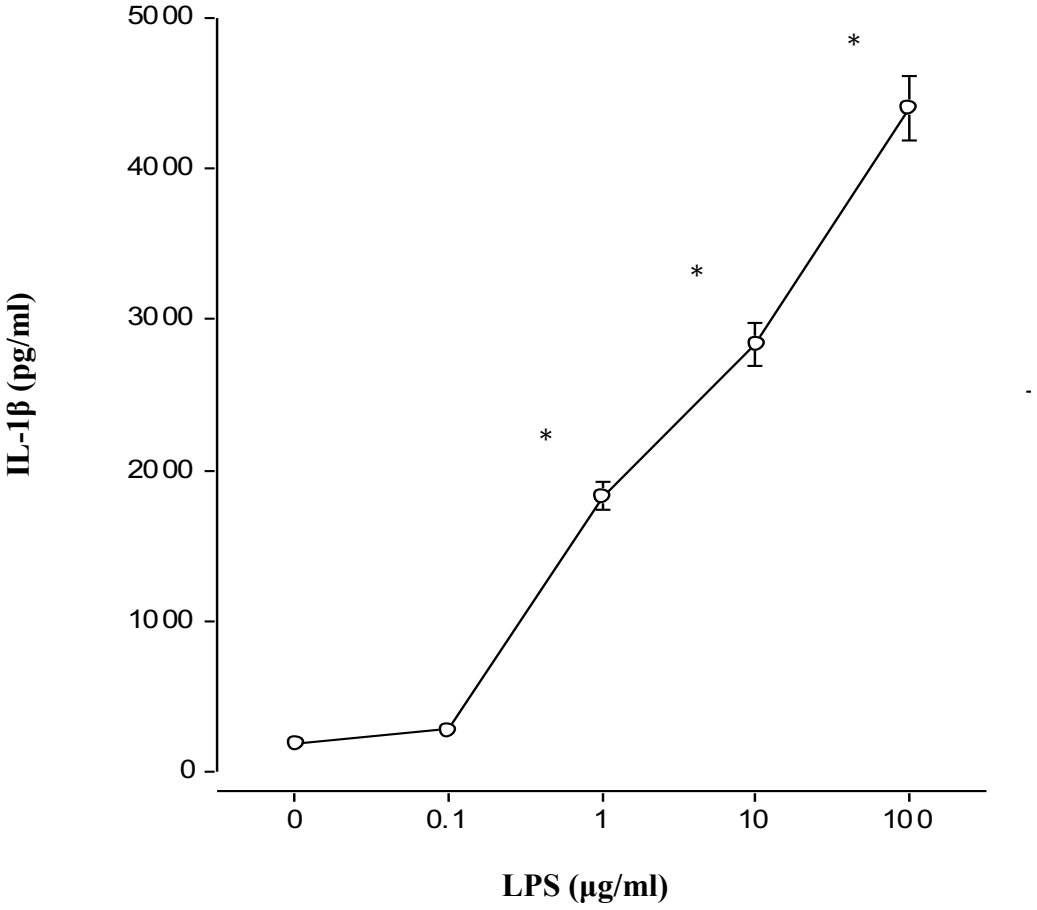

Monocytes ($1x10^6$ cells/ml) were isolated by Ficoll/Histopaque density gradient centrifugation and incubated with varying concentrations of 7β OH EpiA (filled square) and dexamethasone (filled circle) in present of (1µg/ml) of LPS (filled diamond). RPMI1640 (up empty triangle), DMSO (down empty triangle) and 100µM of both treatments (empty shapes) were used alone as negative controls. Incubations were carried out for 20 h at 37°C, 5% CO2 and 100% humidity, after which the concentration of TNF-α in culture medium was measured by ELISA. Values are the means of n = 3 ± s.d. *P < 0.01 versus incubations with LPS alone.

## 3.2 The Effect of Different Concentrations of LPS on IL-1β Production in Whole Human Blood and THP-1 Cells

As mentioned at the beginning of this chapter, LPS has been well documented to be an effective stimulus for production of pro-inflammatory cytokines especially TNF-α and IL-1β. Experiments were carried out to determine the effect of LPS on the production of IL-1β in whole human blood and THP-1 cells. The quantity of IL-1β in both plasma and THP-1 cell supernatants was measured by ELISA assay as described in the methodology section. In whole human blood, increasing concentrations of LPS between 1 and 100μg/ml of LPS resulted in a concentration-dependent increase in the concentrations of IL-1β (Fig. 3.6). The level of IL-1β with 100 μg/ml LPS was in the order of 40-fold higher than with culture medium alone.

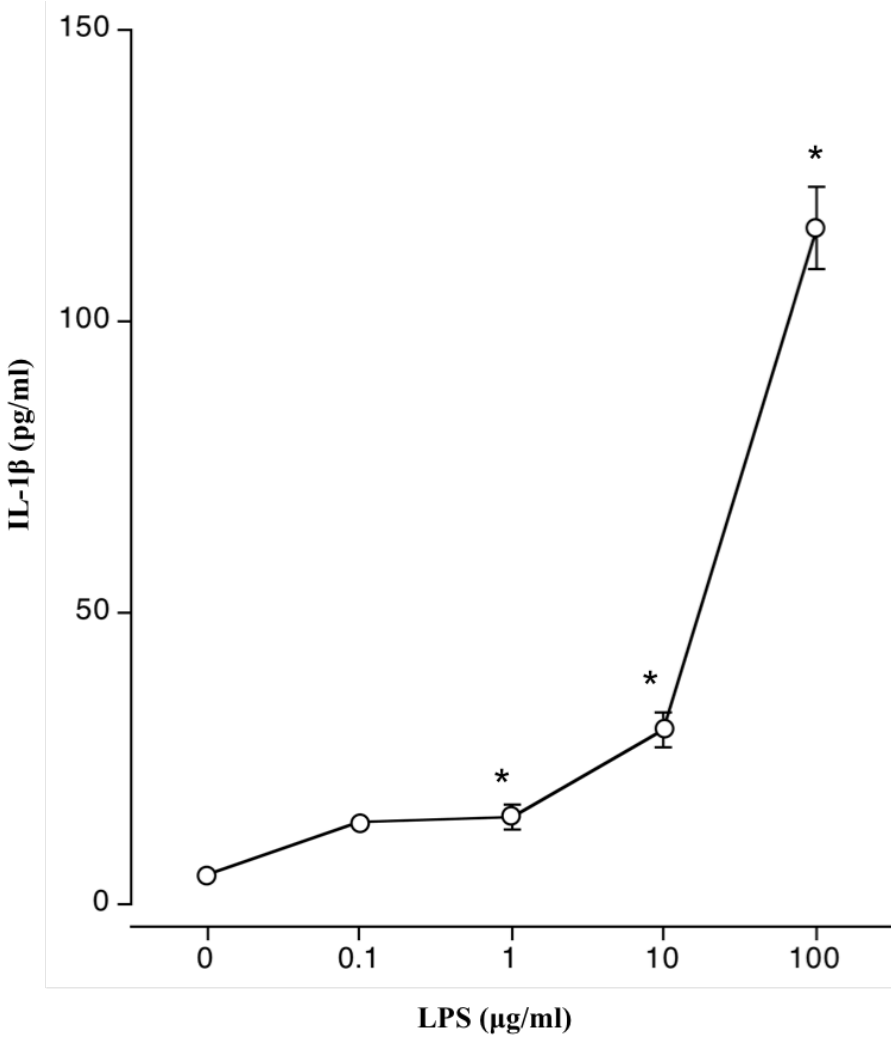
A similar effect of LPS on IL-1β concentrations was observed in THP-1 cells with a concentration-dependent increase between 1μg/ml and 100 μg/ml. LPS concentrations of 100μg/ml increased IL-1β concentration in the order of 15-fold above the control level (Fig. 3.7).

**Fig 3.6 The Effect of Varying LPS Concentrations on IL-1β Production in Whole Human Blood in Response to LPS Stimulation**




Whole Blood was incubated with varying concentrations of LPS. Incubations were carried out for 20 h at 37°C, 5% CO2 and 100% humidity, after which the concentration of TNF-α in plasma was measured by ELISA. Values are the means of n = 3 ± s.d. *P < 0.05 versus incubations without LPS.

**Fig 3.7 The effect of Varying LPS Concentrations on IL-1β Production in THP-1 Cells**




THP-1 cells ($1x10^6$) were incubated in 24 wells plate in triplicate at 37 °C, 5% CO2 for 20 h in the presence of a gradual increase in LPS concentrations (0.1, 1, 10, and 100µg/ml) (filled circles) or absence (culture media only) The IL-1β concentrations presented within the supernatants were determined by ELISA assay. Values represent the mean of n = 3 ± s.d. * $P<0.05$ versus control.

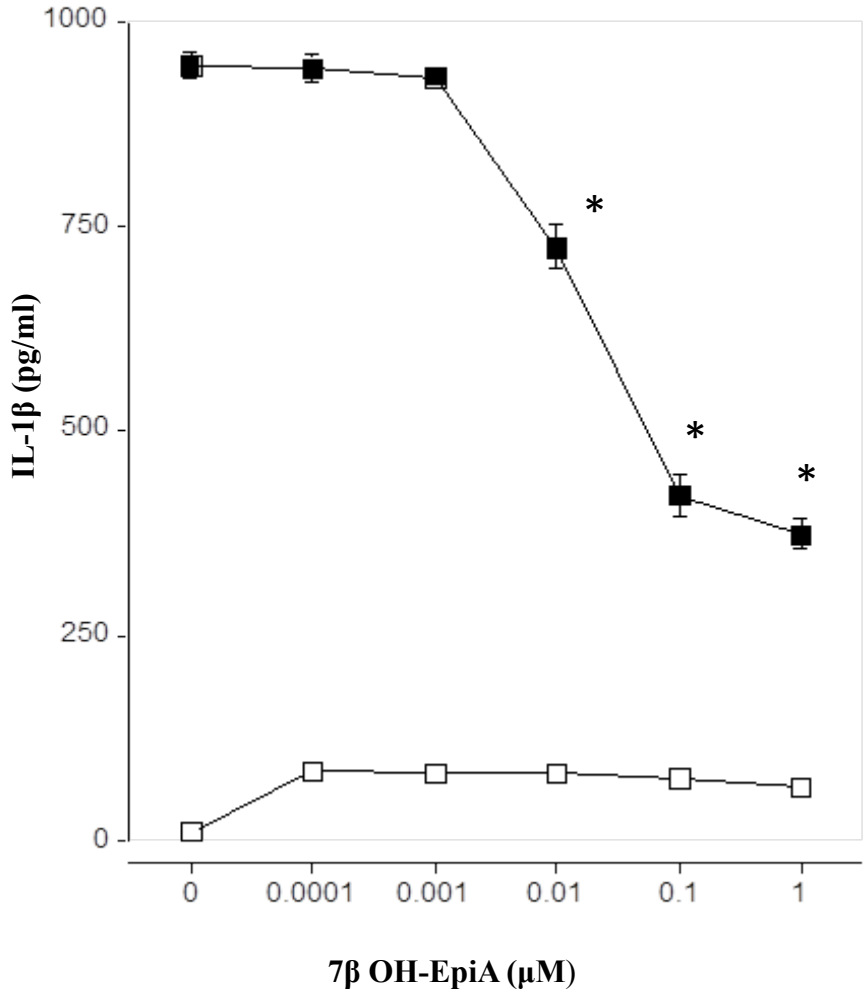
## 3.3 Effect of Varying Concentrations of 7β OH-EpiA and Dexamethasone on IL-1β Production in Whole Human Blood and from isolated monocytes and THP-cells

In previous experiments, 7β OH-EpiA at low concentrations was able to inhibit LPS stimulated TNF- α production in blood and monocytic cells. Experiments were also carried out to ascertain the effect of 7β OH-EpiA on IL-1β production in blood, human monocytes and human monocytic THP-1 cells. This was compared to the actions of dexamethasone. In blood, the concentrations of IL-1β were measured in plasma by ELISA as described in the methods section. 7β OH-EpiA resulted in a concentration-dependent decrease in IL-1β quantities. 7β OH-EpiA concentrations between 0.01 and 1 μM were able to reduce LPS-stimulated production of IL-1β in plasma Fig (3.8). Dexamethasone also reduced levels of IL-1β but at higher concentrations (10 and 100 μM) in plasma Fig (3.9).

In supernatants from isolated monocytes and THP-1 cells, both 7β OH-EpiA and dexamethasone yielded similar results as those in plasma. In monocytes, a decrease in LPS-stimulated IL-1β concentration was observed in response to 0.1 and 1 μM 7β OH-EpiA but higher concentrations of dexamethasone (10 and 100 μM) were required to achieve this (Figs. 3.10 and 3.11 respectively).

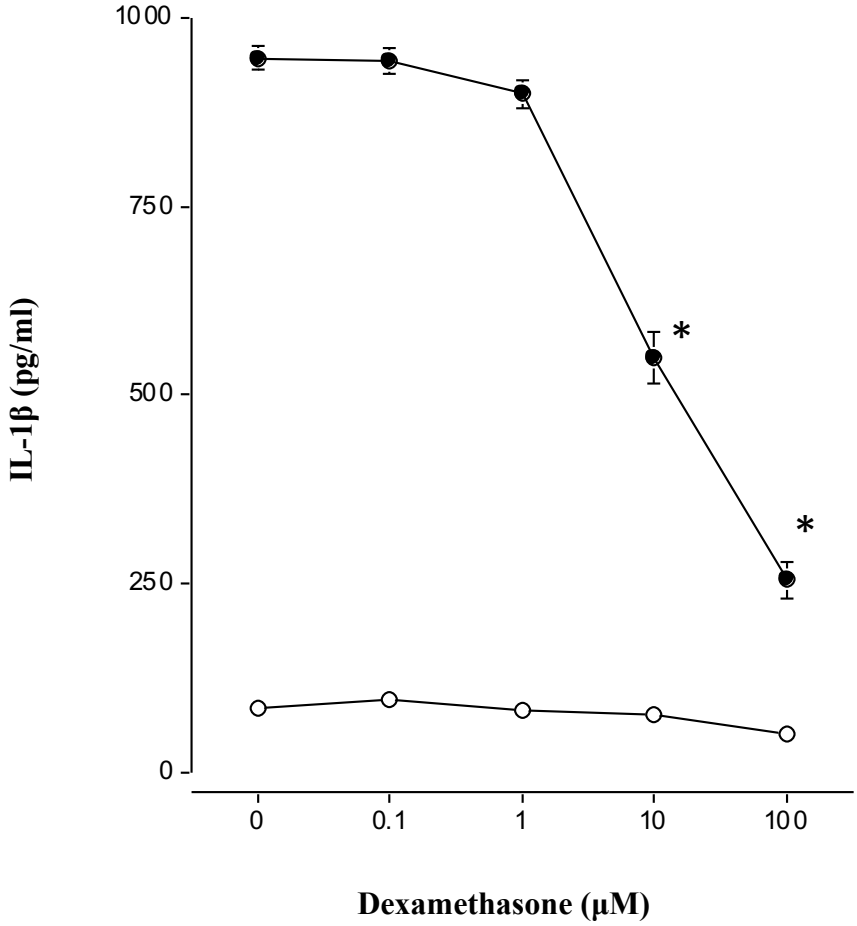
Qualitatively identical results were observed in THP-1 cell supernatants where a decrease in LPS-stimulated IL-1β levels was observed in response to 0.1 and 1 μM 7β OH-EpiA and higher concentrations of dexamethasone (10 and 100 μM) were required to achieve this (Figs. 3.12 and 3.13 respectively).

**Fig 3.8 The Effect of Different Concentrations of 7β OH-EpiA on IL-1β Production in Whole Human Blood in the Presence or Absence of LPS**



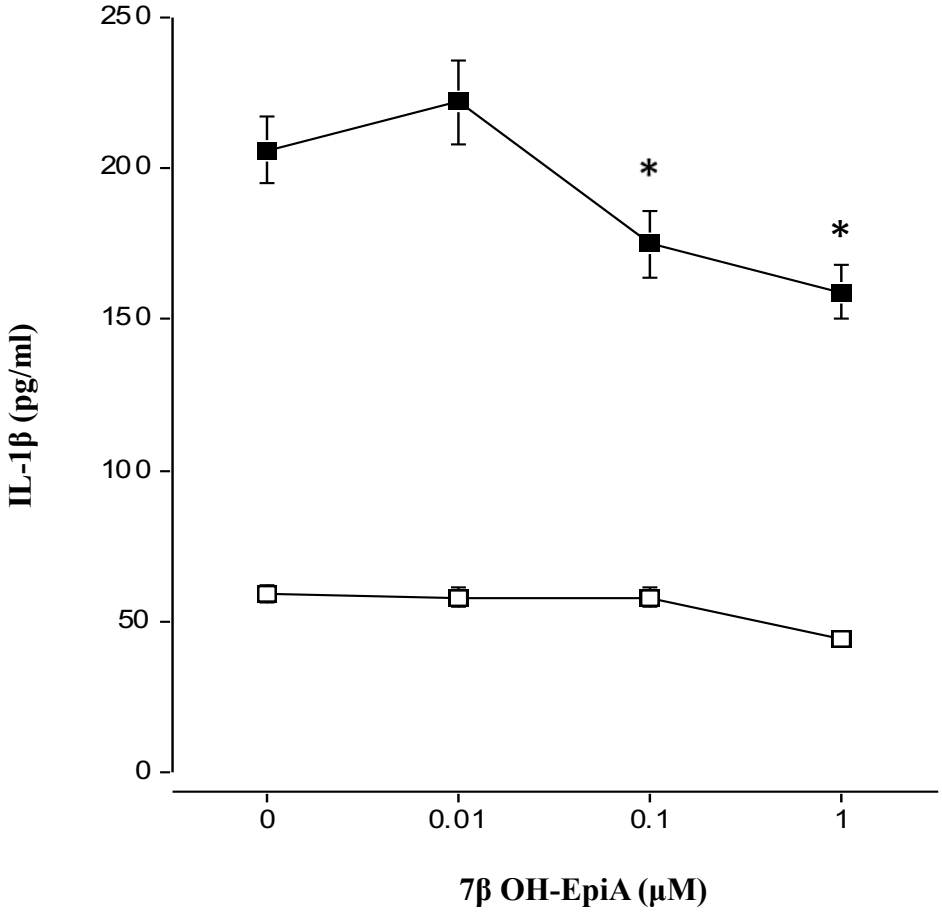

Whole human blood was treated with 7β OH-EpiA for 1 h after which either DMSO vehicle (control, open squares) or LPS (10 μg/ml, closed squares) was added and incubations carried out at 37 °C for 20 h; plasma was collected after centrifuging. Concentrations of IL-1β present in plasma were measured by ELISA. Values are the means of n = 3 incubations and are representative of at least three separate experiments. * $P<0.01$ versus LPS alone.

**Fig 3.9 The Effect of Different Concentrations of Dexamethasone on IL-1β Production in Whole Human Blood in the Presence or Absence of LPS**



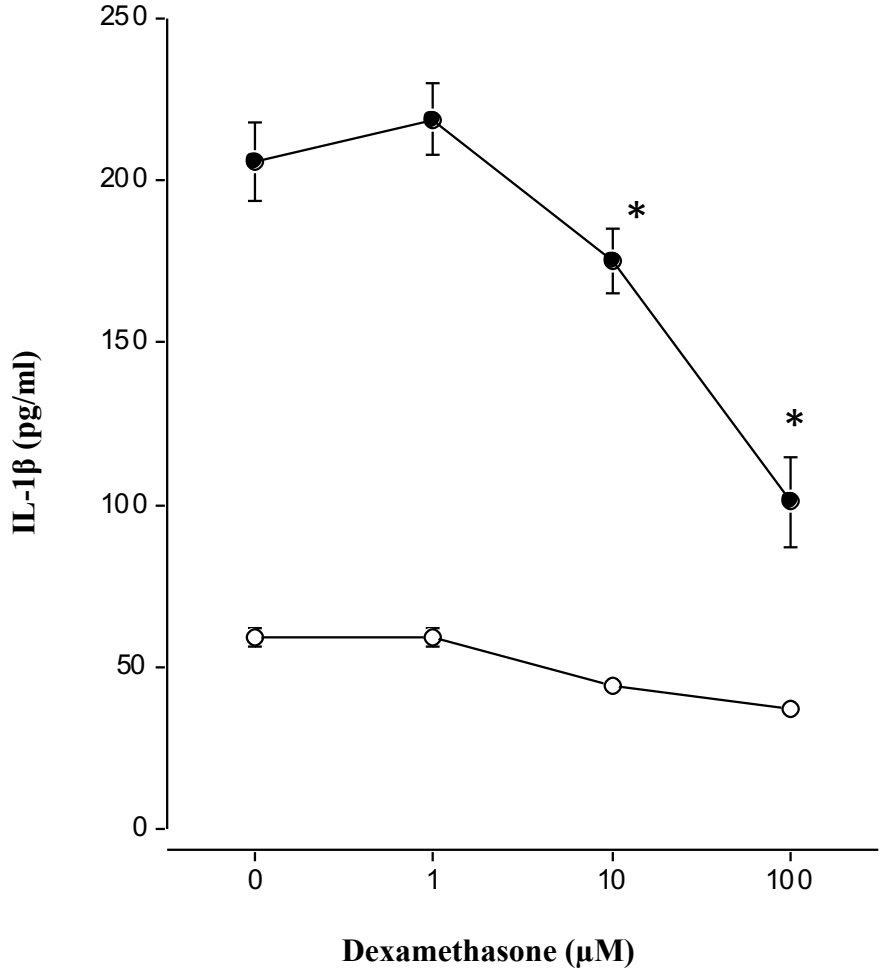

Whole human blood was treated with Dexamethasone for 1 h after which either RPMI vehicle (control, open circles) or LPS (10 μg/ml, closed circles) was added and incubations at 37 °C for 20 h. plasma was collected after centrifuging. Concentrations of IL-1β present in plasma were measured by ELISA. Values are the means of n = 3 incubations ± s.d. and are representative of at least three separate experiments* $P<0.01$ versus LPS alone.

**Fig 3.10 The Effect of Different Concentrations of 7β OH-EpiA on IL-1β Production in Isolated Monocytes in the Presence or Absence of LPS**



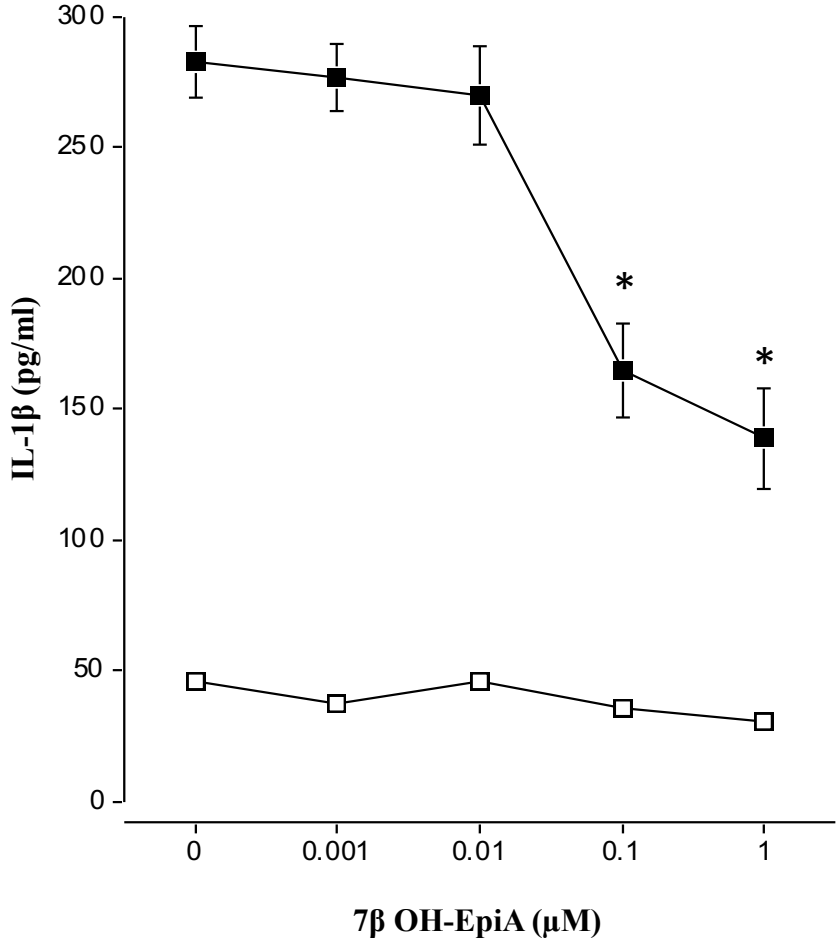

Monocytes were isolated by Ficoll/Histopaque density gradient centrifugation and incubated at 37 °C for 20 h. Cells were then treated with 7β OH-EpiA for 1 h after which either RPMI (control, open square) or LPS (1 μg/ml, closed square) was added and incubations continued for a further 3 h. Concentrations of IL-1β present in supernatants were measured by ELISA. Values are the means of n = 3 incubations ±s.d. and are representative of at least three separate experiments. * P<0.01 versus LPS alone

**Fig 3.11 The Effect of Different Concentrations of Dexamethasone on IL-1β Production from Isolated Monocytes in the Presence or Absence of LPS**



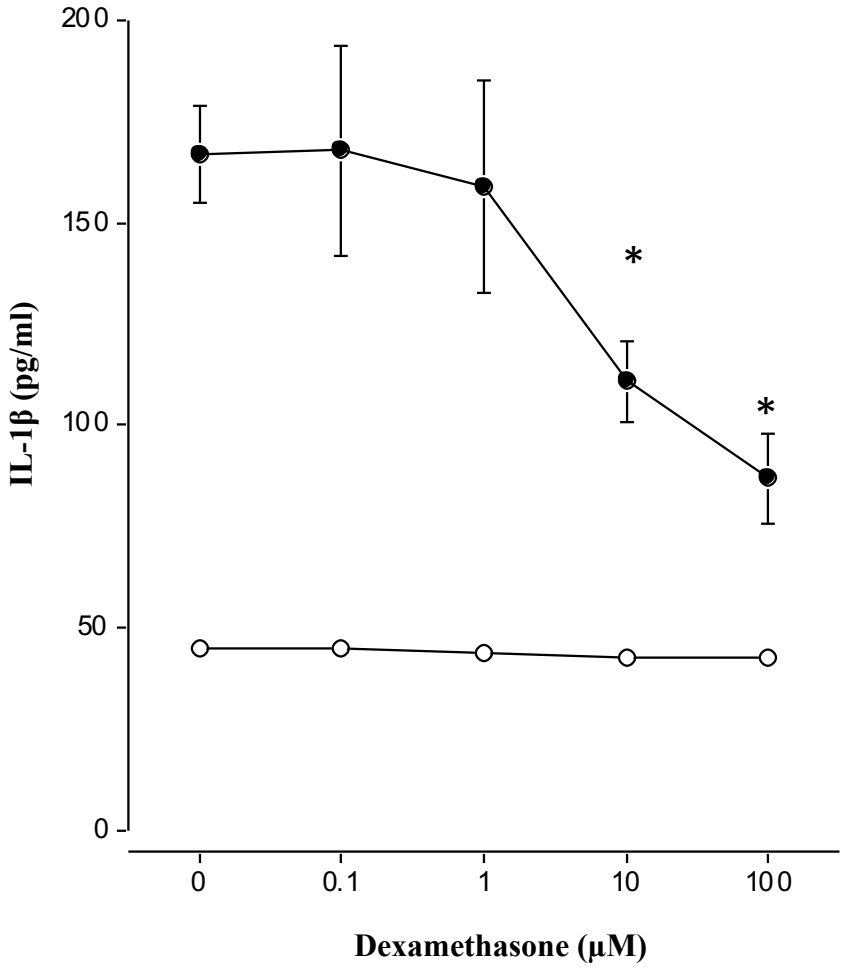

Monocytes were isolated by Ficoll/Histopaque density gradient centrifugation and incubated at 37 °C for 20 h. Cells were then treated with dexamethasone for 1 h after which either RPMI (control, open circles) or LPS (1 μg/ml, closed circles) was added and incubations continued for a further 3 h. Concentrations of IL-1β present in supernatants were measured by ELISA. Values are the means of n = 3 incubations ± s.d. and are representative of at least three separate experiments. * $P<0.01$ versus LPS alone.

**Fig 3.12 The Effect of Different Concentrations of 7β OH-EpiA on IL-1β Production from THP-1 Cells in the Presence or Absence of LPS**




THP-1 cells ($1x10^6$) were incubated in 24 wells plate in triplicate at 37 °C, 5% CO2 for 20 h with 7β OH-EpiA (square) in absence (empty shape) or presence of LPS (1μg/ml) (filled shape). Supernatants were then collected, and the IL-10 concentrations were determined by ELISA. Values are expressed as the means of 3 ± s.d. * $P<0.01$ versus LPS alone.

**Fig 3.13 The Effect of Different Concentrations of Dexamethasone on IL-1β in THP-1 Cells in the Presence or Absence of LPS**



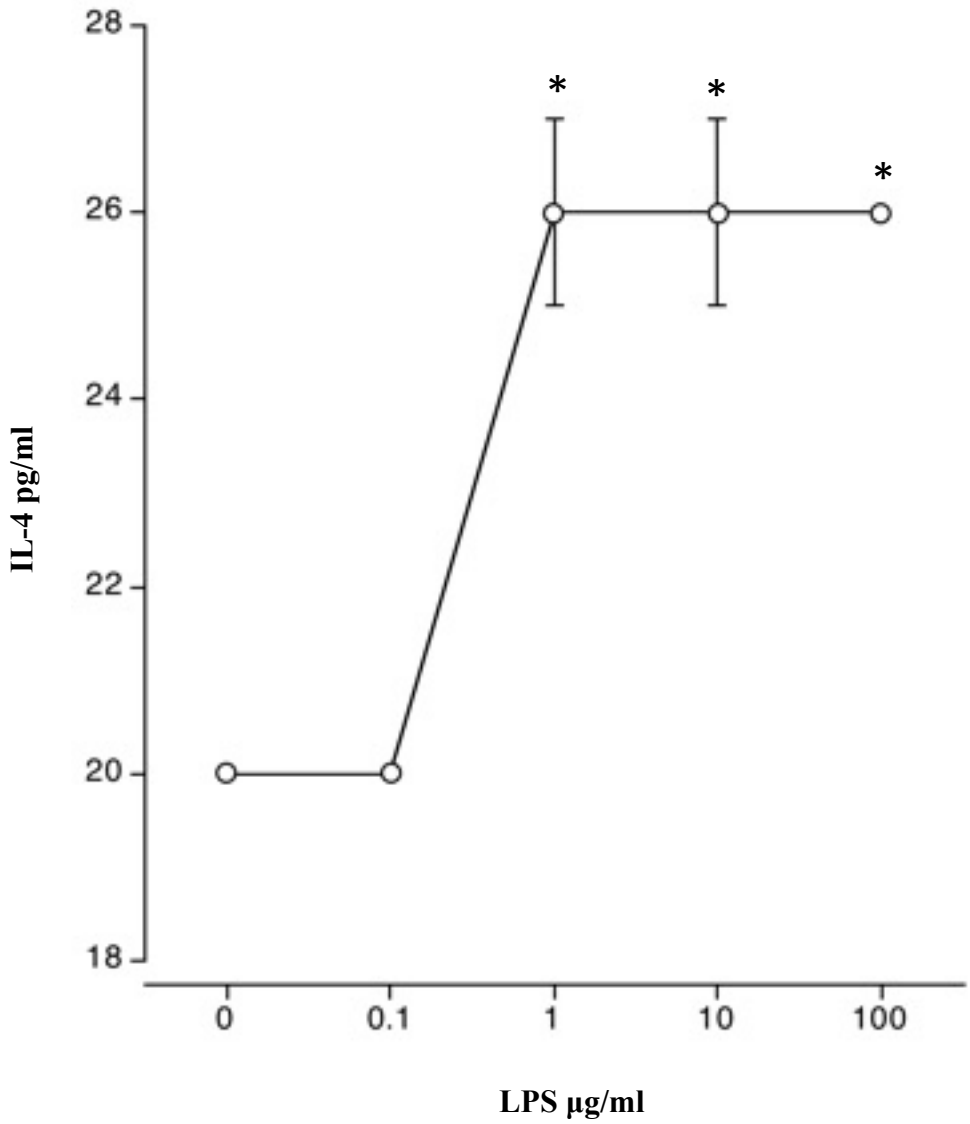

THP-1 cells ($1x10^6$) were incubated in 24 wells plate in triplicate at 37 °C, 5% CO2 for 20 h with Dexamethasone (circle) in absence (empty circle shape) or presence of LPS (1µg/ml) (filled circle shape). Supernatants were then collected, and the IL-10 concentrations were determined by ELISA. Values are expressed as the means of 3 ± s.d. (three separate observations) * $P<0.01$ versus LPS alone.

## 3.4 Effect of 7β OH-EpiA and Dexamethasone on IL-4 Production from Human Monocytic THP-1 Cells

### 3.4.1 The Effect of LPS on IL-4 Production in THP-1 Cells

THP-1 cells were incubated with different concentrations of LPS (0 - 100μg/ml) for 20 h after which the supernatants were collected and the level of IL-4 was determined using ELISA assay (as described in the methodology section). A small but clearly significant increase in levels of IL-4 occurred in response to LPS concentrations ≥ 1μg/ml. The effect was not concentration-dependent, and no further increase were observed with LPS concentrations above 1 μg/ml and up to 100 μg/ml (Fig. 3.14).

**Fig 3.14 The Effect of Varying LPS Concentration on IL-4 Production in THP-1 Cells**



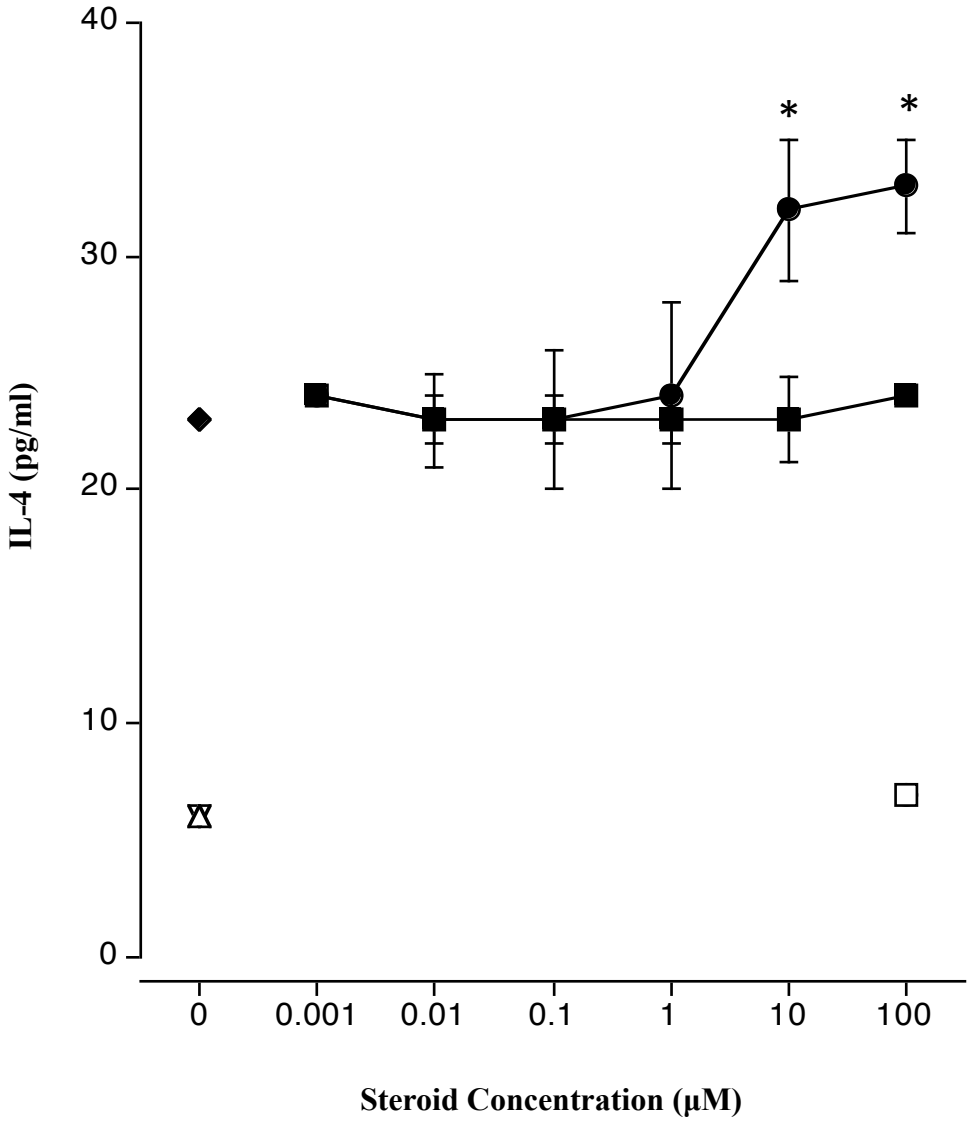

THP-1 cells ($1x10^6$) were incubated in 24 wells plate in triplicate at 37 °C, 5% CO2 for 20 h in the presence of a gradual increase in LPS concentrations (0, 0.1, 1, 10, and 100µg/ml) (circles) or absence (culture media only) The IL-4 concentrations present within the supernatants were determined by ELISA assay. Values represent the mean of n = 3 ± s.d. *Denotes $P < 0.05$ when compared to culture medium alone (0 LPS).

## 3.5 Effect of Varying the Concentration of 7β OH-EpiA and Dexamethasone on IL-4 Production in Whole Human Blood and THP-1 Cells

In previous experiments, 7β OH-EpiA showed its ability to reduce TNF-α and IL-1β all thinking were goes to observe its ability to increase anti-inflammatory cytokines such as IL-4. Fig (3.15) showed that all concentrations of 7β OH-EpiA had no effect on the production of LPS-stimulated IL-4. On the other hand, high concentrations of dexamethasone (10 and 100μM) in the presence of LPS, increased production of IL-4 in human blood compared to LPS alone.

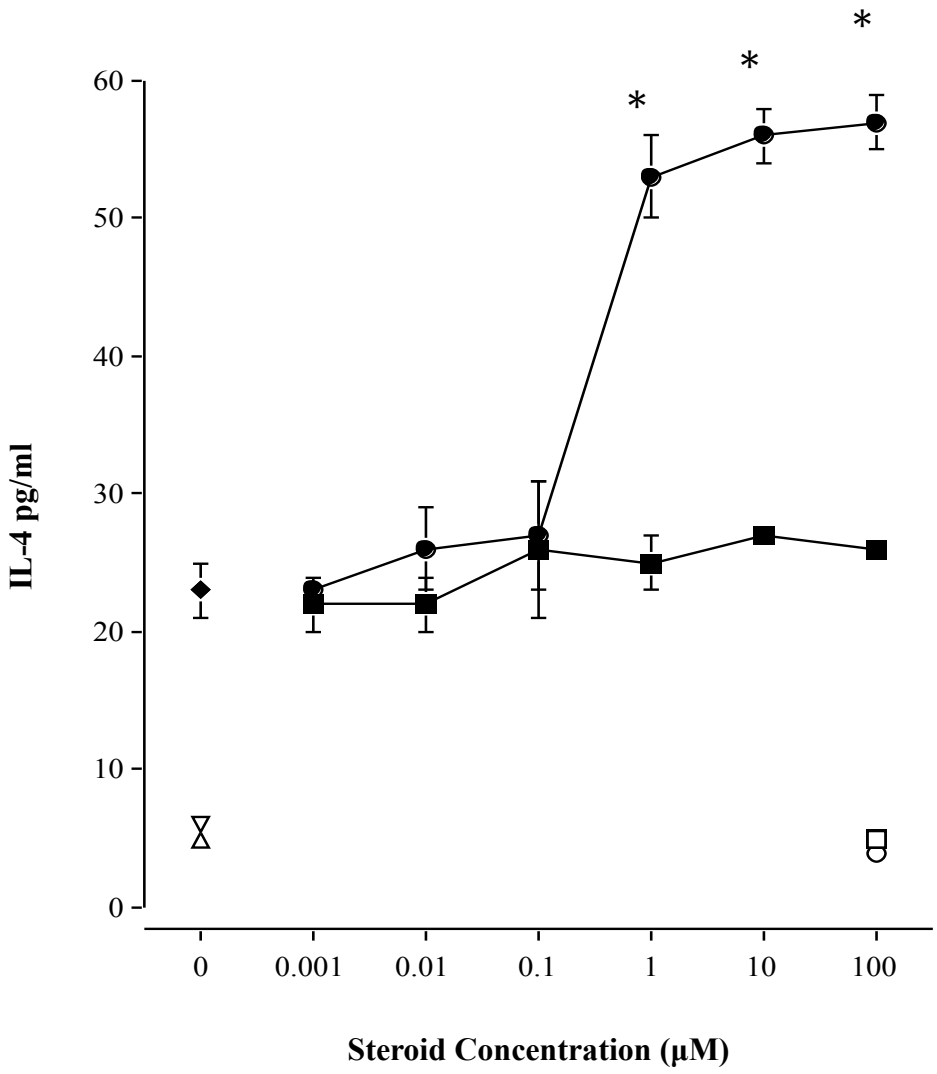
In THP-1 cells, 7β OH-EpiA, similarly, had no effect on the production of LPS-stimulated IL-4. Dexamethasone concentrations between 1 and 100μM significantly increased the LPS-stimulated production of IL-4 in THP-1 cells compered to LPS alone see Fig (3.16).

**Fig 3.15 Effect of Varying Concentrations of 7β OH-EpiA and Dexamethasone on IL-4 Production in Whole Human Blood in Response to LPS Stimulation**




Whole Blood was incubated with various concentrations of 7β OH- EpiA (square) or Dexamethasone (circle) in absence (empty shape) or presence of LPS (Diamond) (10µg/ml) (filled shape) for 20 h at 37°C, 5% CO2 and 100% humidity. Incubations were then centrifuged, and the IL-4 concentrations in the plasma were determined by ELISA. Values are expressed as the means of 3 ± s.d. (three separate observations) *Denotes $P < 0.05$ when compared to LPS alone.

**Fig 3.16 Effect of Varying Concentrations of 7β OH-EpiA and Dexamethasone on IL-4 Production in THP-1 Cells in Response to LPS Stimulation**




THP-1 cells ($1x10^6$) were incubated in 24 wells plate in triplicate at 37 °C, 5% CO2 for 20 h with 7β OH- EpiA (square) or Dexamethasone (circle) in absence (empty shape) or presence of LPS (Diamond) (1µg/ml) (filled shape). Supernatants were then collected, and the IL-4 concentrations were determined by ELISA. Values are expressed as the means of 3 ± s.d. (three separate observations) *Denotes $P < 0.05$ when compared to LPS alone.

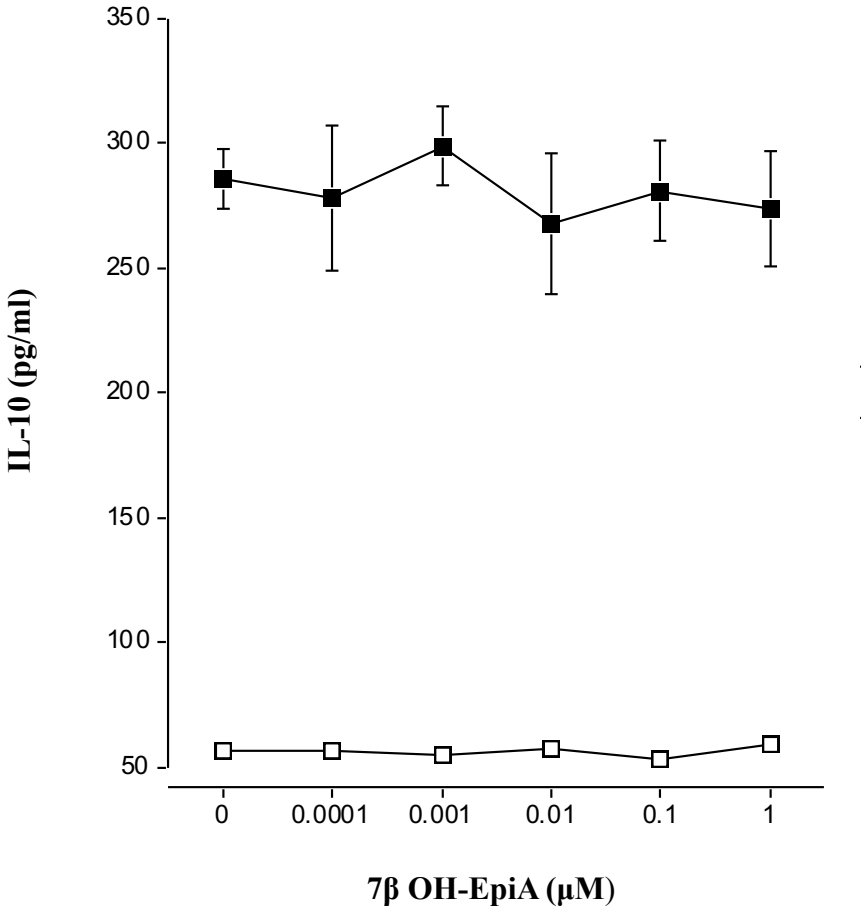
## 3.6 Effect of Varying Concentrations of 7β OH-EpiA and Dexamethasone on IL-10 Production in Whole Human Blood and THP-1 Cells

In previous experiments, 7β OH-EpiA did not have any effect on the anti-inflammatory cytokine IL-4 as shown in section 3.5. It was decided to evaluate whether this was restricted to IL-4 or that it may have had similar effects on other anti-inflammatory cytokines. Thus, the effect of 7β OH-EpiA (0.0001-1μM) was studied on the production of the major anti-inflammatory cytokine IL-10 in whole human blood, isolated monocytes and THP-1 cells and compared to dexamethasone as a positive control.

In human blood, LPS stimulated an increase in IL-10 levels in the order of 5-fold. 7β OH-EpiA (0.0001-1μM) did not affect the basal or LPS-stimulated concentration of IL-10 (Fig. 3.17), whereas dexamethasone concentrations of 10 and 100μM in the presence of LPS reducedIL-10 levels (Fig. 3.18).

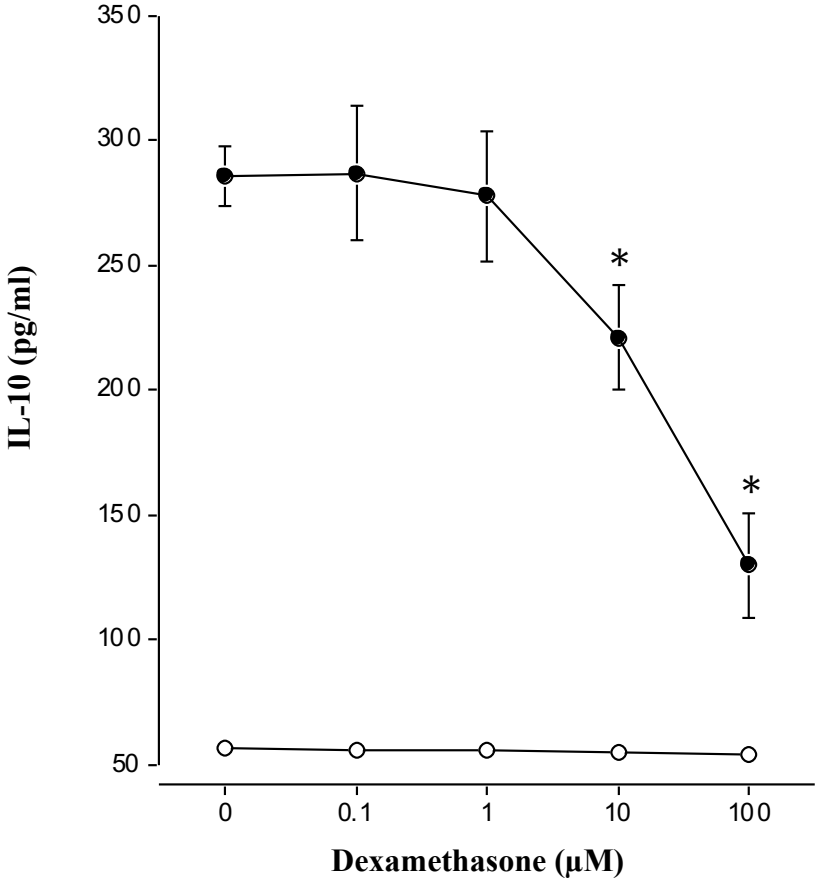
Qualitatively similar effects were observed in isolated monocytes and THP-1 cells. In both preparations, LPS-stimulated IL-10 levels were not altered in the presence of 7β OH-EpiA (Figs. 3.19 and 3.21). The quantity of LPS-stimulated IL-10 in supernatants from isolated monocytes and THP-1 cells was, however, reduced in the presence of dexamethasone concentrations of 10 and 100 μM (Figs. 3.20 and 3.22).

**Fig 3.17 The Effect of Different Concentrations of 7β OH-EpiA on IL-10 Production in Whole Human Blood in the Presence or Absence of LPS**



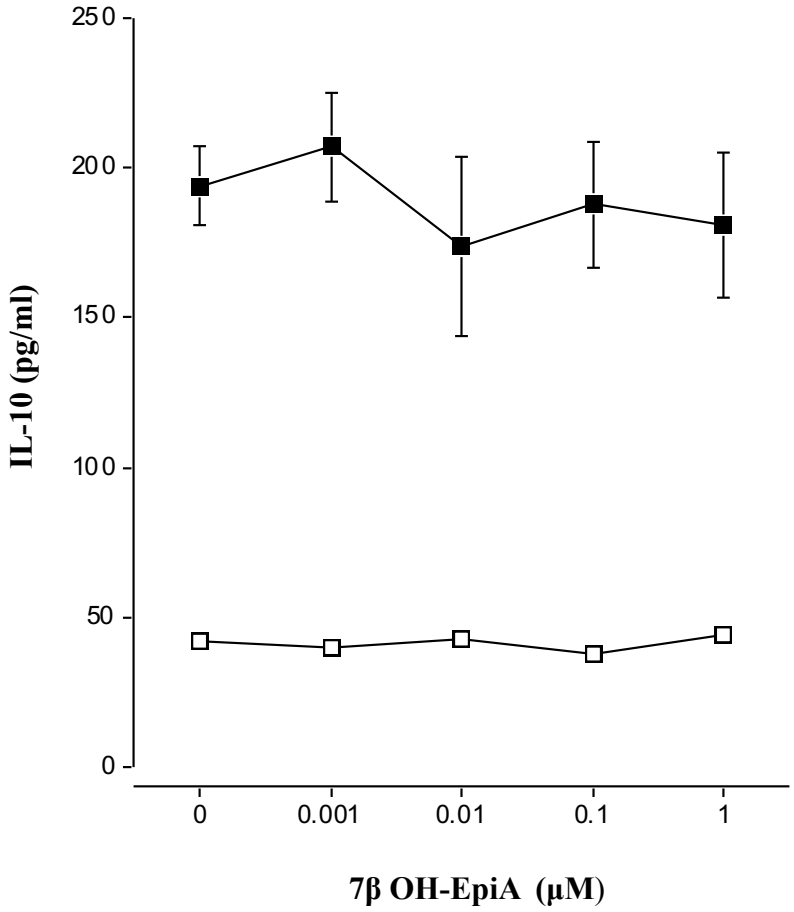

Whole human blood was treated with 7β OH-EpiA for 1 h after which either DMSO vehicle (control, open squares) or LPS (10 μg/ml, closed squares) was added and incubations at 37 °C for 20 h. plasma was collected after centrifuging. Concentrations of IL-10 present in plasma were measured by ELISA. Values are the means of n = 3 incubations ± s.d. and are representative of at least three separate experiments.

**Fig 3.18 The Effect of Different Concentrations of Dexamethasone on IL-10 Production in Whole Human Blood in the Presence or Absence of LPS**



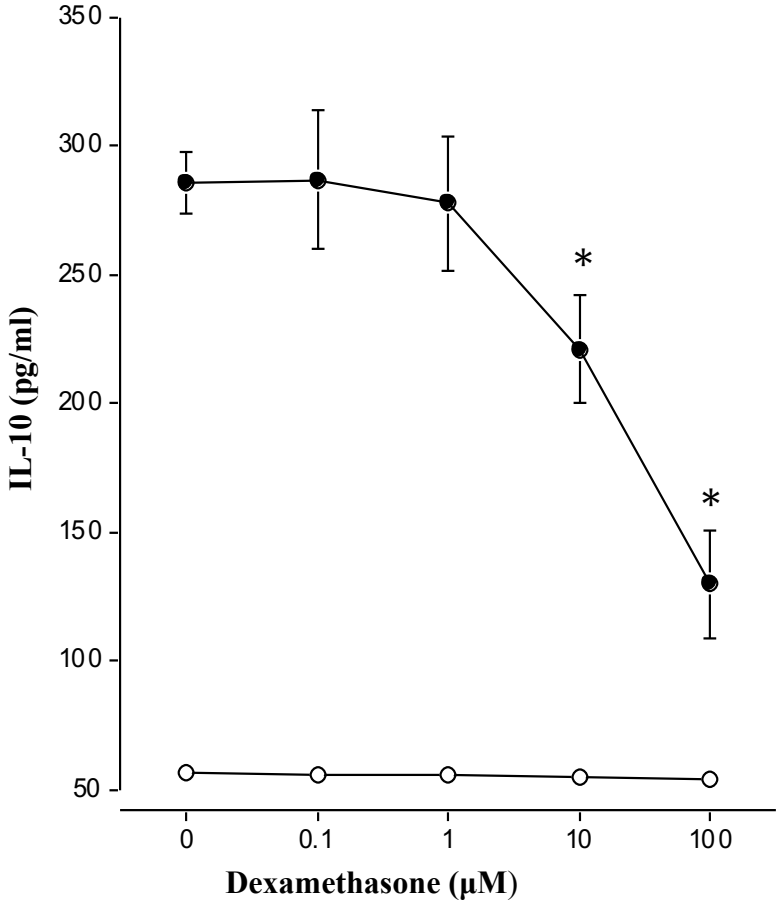

Whole human blood was treated with dexamethasone for 1 h after which either RPMI vehicle (control, open circles) or LPS (10 µg/ml, closed circles) was added and incubations at 37 °C for 20 h. plasma was collected after centrifuging. Concentrations of IL-10 present in plasma were measured by ELISA. Values are the means of n = 3 incubations ± s.d. and are representative of at least three separate experiments.

**Fig 3.19 The Effect of Different Concentrations of 7β OH-EpiA on IL-10 Production in Isolated Monocytes Cells in the Presence or Absence of LPS**



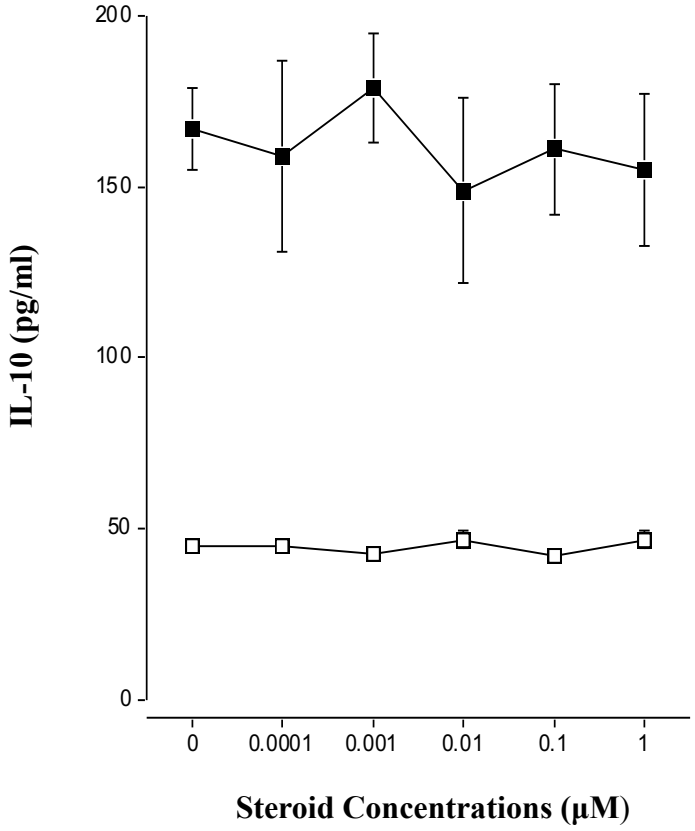

Monocytes were isolated by Ficoll/Histopaque density gradient centrifugation and incubated at 37 °C for 20 h. Cells were then treated with7β OH-EpiA for 1 h after which either RPMI (control, open square) or LPS (1 μg/ml, closed square) was added and incubations continued for a further 3 h. Concentrations of IL-10 present in supernatants were measured by ELISA. Values are the means of n = 3 incubations ± s.d. and are representative of at least three separate experiments.

**Fig 3.20 The Effect of Different concentrations of Dexamethasone on IL-10 Production in Isolated Monocytes in the Presence or Absence of LPS**



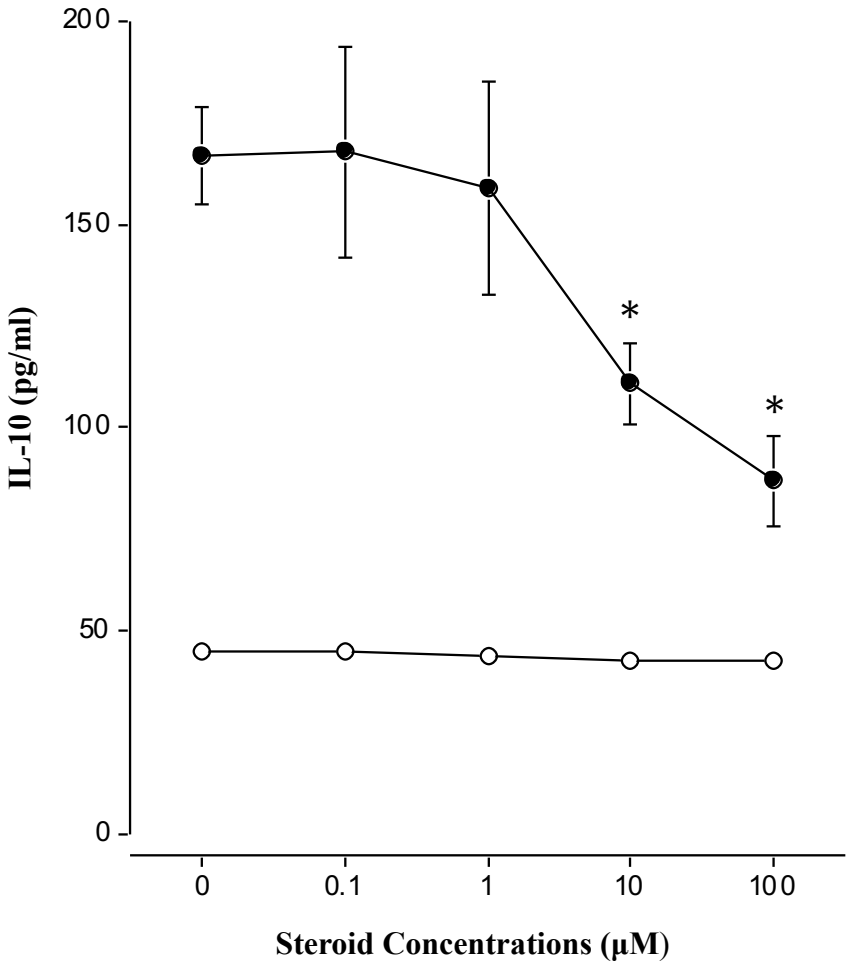

Monocytes were isolated by Ficoll/Histopaque density gradient centrifugation and incubated at 37 °C for 20 h. Cells were then treated with dexamethasone for 1 h after which either RPMI (control, open circles) or LPS (1 µg/ml, closed circles) was added and incubations continued for a further 3 h. Concentrations of IL-10 present in supernatants were measured by ELISA. Values are the means of n = 3 incubations ± s.d. and are representative of at least three separate experiments.

**Fig 3.21 The Effect of Different Concentrations of 7β OH-EpiA on IL-10 in THP-1 Cells in the Presence or Absence of LPS**




THP-1 cells ($1x10^6$) were incubated in 24 wells plate in triplicate at 37 °C, 5% CO2 for 20 h with 7β OH- EpiA (square) in absence (empty shape) or presence of LPS (1µg/ml) (filled shape). Supernatants were then collected, and the IL-10 concentrations were determined by ELISA. Values are expressed as the means of 3 ± s.d.

**Fig 3.22The Effect of Different Concentrations of Dexamethasone on IL-10 in THP-1 Cells in the Presence or Absence of LPS**




THP-1 cells ($1x10^6$) were incubated in 24 wells plate in triplicate at 37 °C, 5% CO2 for 20 h with dexamethasone (circles) in absence (empty shape) or presence of LPS (1µg/ml) (filled shape). Supernatants were then collected, and the IL-10 concentrations were determined by ELISA. Values are expressed as the means of 3 ± s.d. *P < 0.01 versus LPS alone.

## 3.7 Effect of the NSAID Ketoprofen on 7β OH EpiA-Induced Suppression of LPS-Stimulated TNF-α Production in Whole Human Blood, THP-1 Cells and Isolated Monocytes

In previous experiments, low concentrations of 7β OH EpiA inhibited LPS-stimulated TNF-α and IL-1β production in whole human blood, THP-1 cells and isolated monocytes (Figs. 3.3, 3.4 and 3.5). The following experiments were carried out to ascertain the role of endogenous cyclooxygenase products in the suppressive actions of 7β OH EpiA. This was achieved by studying the effect of ketoprofen (a COX inhibitor) on the 7β OH EpiA-induced reduction of TNF-α production in response to LPS.

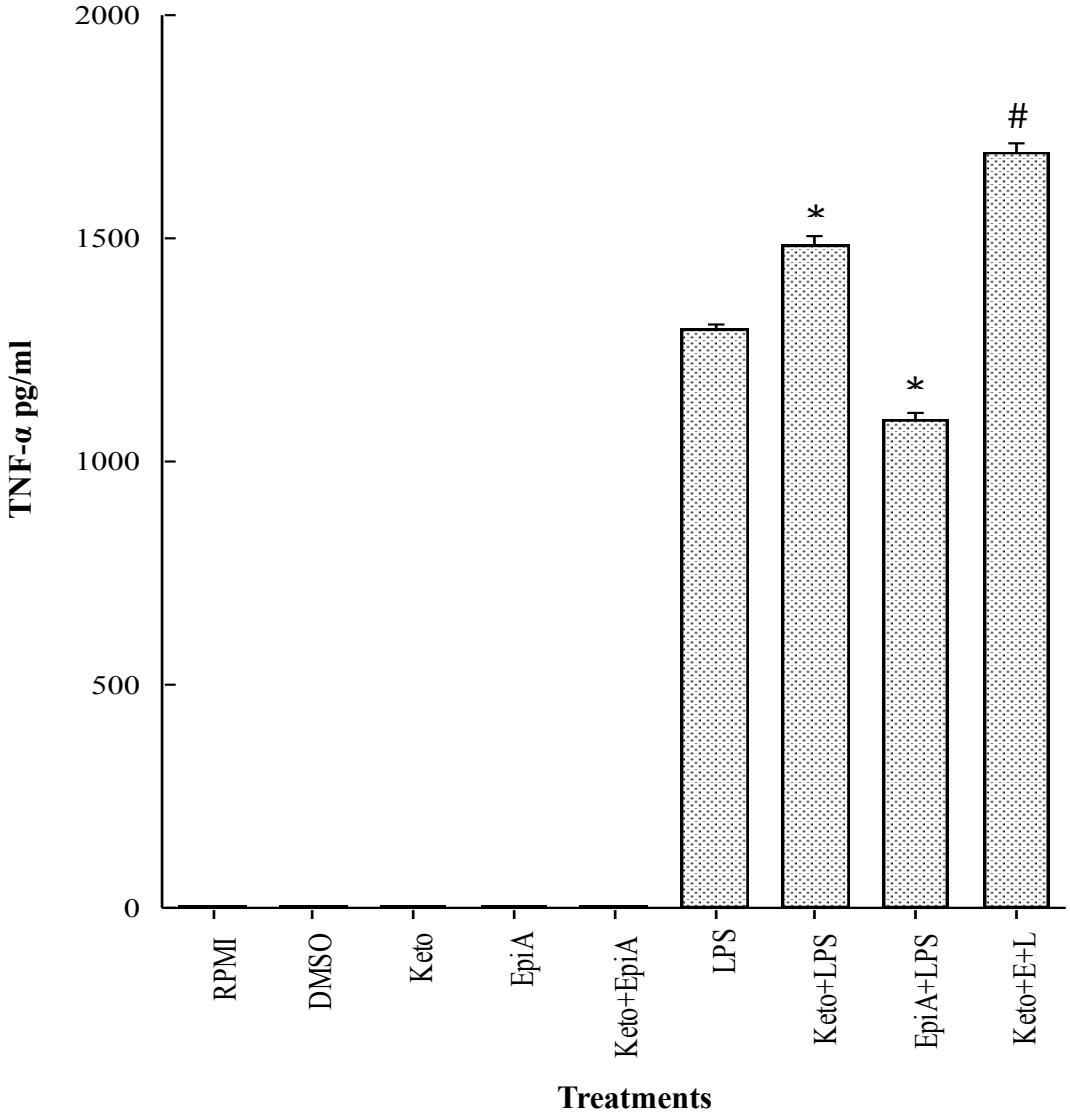
In blood, the LPS-stimulated increases in TNF-α levels were reduced by 7β OH-EpiA and in the presence of ketoprofen (50μM) this was reversed to a level higher than with LPS alone (Fig. 3.23). Although ketoprofen (50μM) in the absence of 7β OH-EpiA increased LPS-stimulated levels of TNF-α, this was not as high as that with ketoprofen and 7β OH EpiA in combination indicating an inhibitory action of ketoprofen on the 7β OH EpiA-induced suppression of TNF-α.

In isolated monocytes a similar result was observed. The LPS-stimulated increases in TNF-α levels were reduced by 7β OH-EpiA and in the presence of ketoprofen this was reversed to a quantity comparable to that with LPS alone (Fig. 3.24). Ketoprofen in the absence of 7β OH-EpiA did not have any effect on LPS-stimulated levels of TNF-α. This clearly indicated an inhibitory action of ketoprofen on the 7β OH-EpiA-induced suppression of TNF-α in isolated monocytes.

In THP-1 cells, the actions of ketoprofen (50μM) were qualitatively similar to its actions on the 7β OH-EpiA-induced suppression of TNF-α production. The LPS-stimulated increases in TNF-α concentrations were reduced by 7β OH-EpiA and this

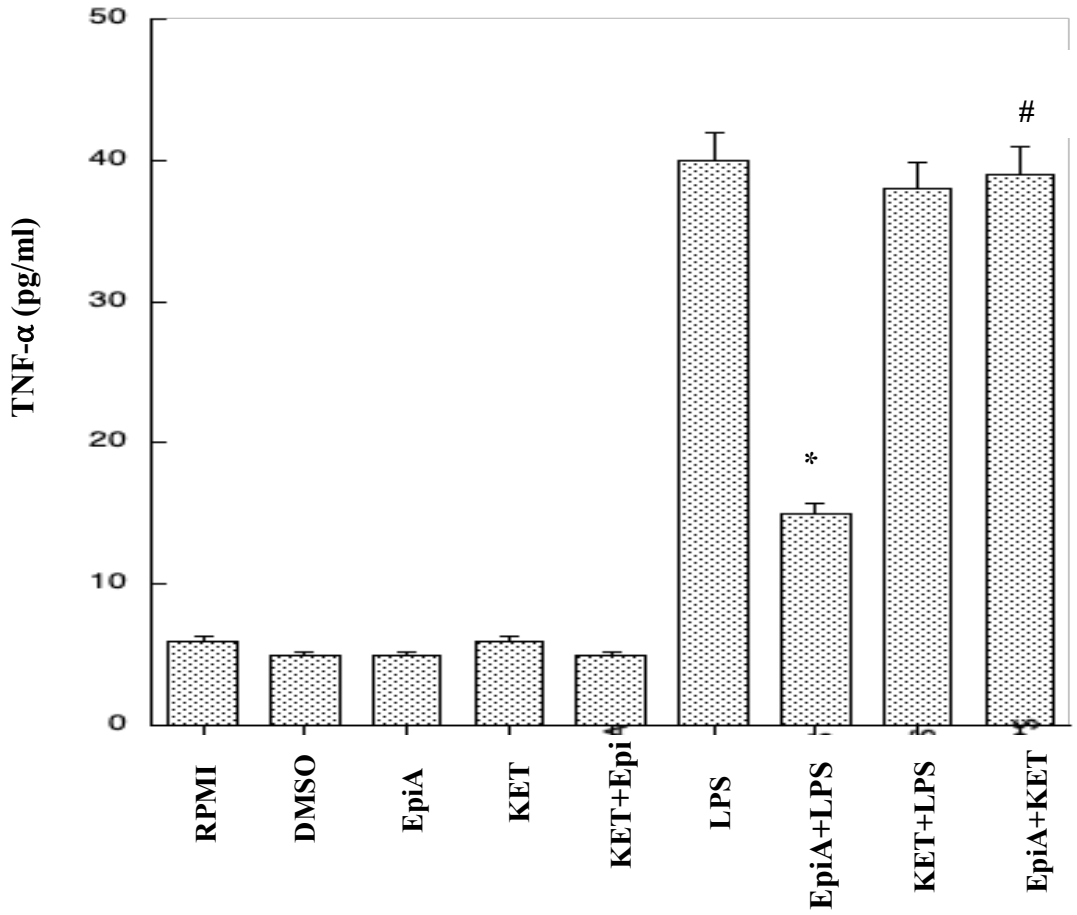
was reversed to the level found with LPS (1μg/ml) alone in the presence of ketoprofen (Fig. 3.25). Although ketoprofen in the absence of 7β OH-EpiA increased LPS-stimulated concentrations of TNF-α, this was not as high as that with ketoprofen and 7β OH-EpiA in combination indicating an inhibitory action of ketoprofen on the 7β OH-EpiA-induced suppression of TNF-α in THP-cells.

**Fig 3.23 Effect of the NSAID Ketoprofen on 7β OH EpiA-Induced Suppression of LPS- Stimulated TNF-α Production in Whole Human Blood**



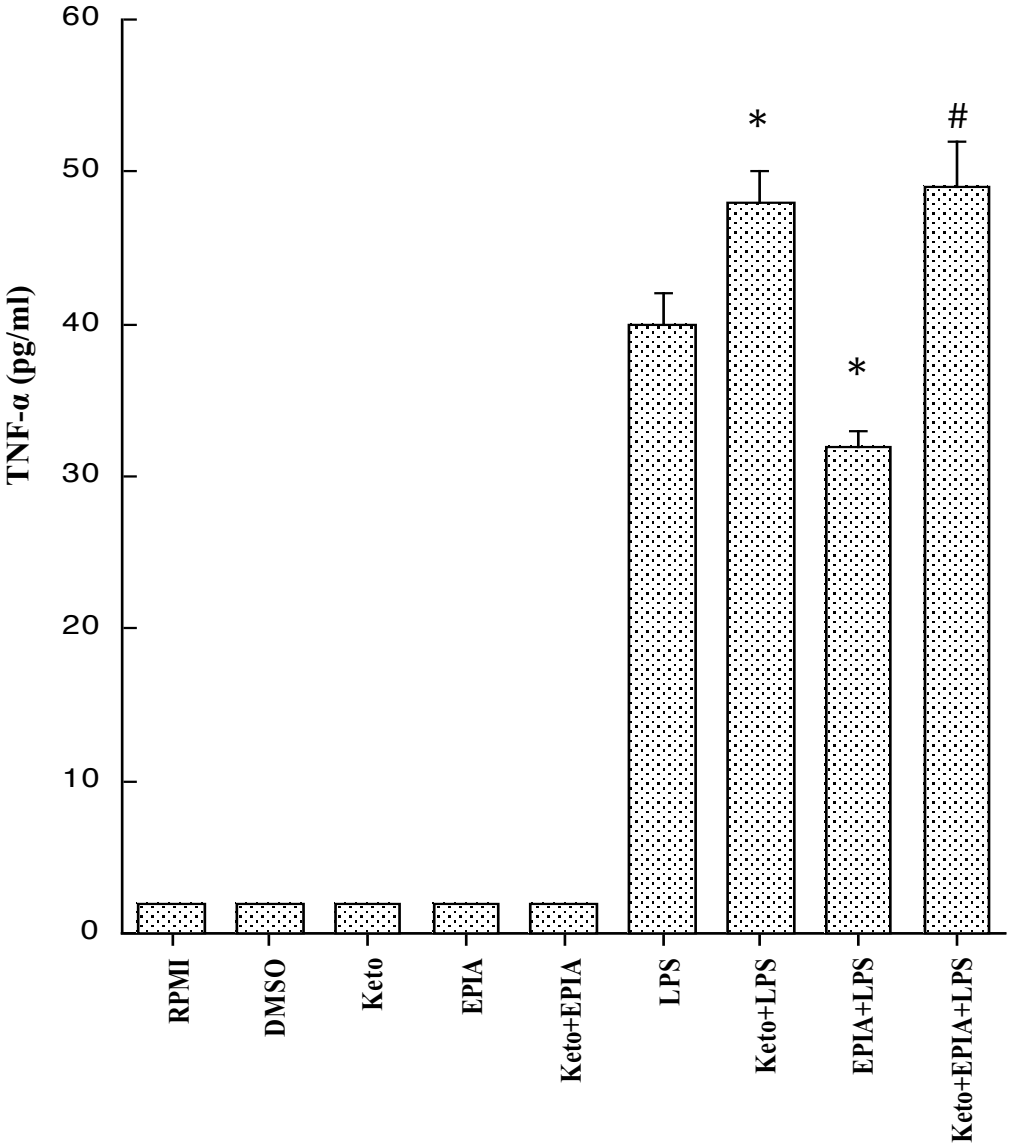

Whole human blood was incubated with various treatments; 7β OH-EpiA (0.001μM), ketoprofen (50μM) or a combination of both 7β OH-EpiA and Ketoprofen in presence and absence of LPS (10μg/ml) at 37 °C, 5%CO2 and 100% humidity for 20 h. The TNF-α concentrations in the plasma were determined by ELISA assay. Values represent the mean of n = 3 ± s.d. *P < 0.05 versus LPS alone, #P < 0.05 versus 7β OH-EpiA + LPS.

**Fig 3.24 Effect of the NSAID Ketoprofen on 7β OH EpiA-Induced Suppression of LPS- Stimulated TNF-α Production in Isolated Monocytes**




Monocytes ($1.3x10^6$) were incubated with various treatments; 7β OH-EpiA (0.001μM), Ketoprofen (50μM) or a combination of both 7β OH-EpiA and Ketoprofen in presence and absence of LPS (1μg/ml) at 37 °C, 5%CO2 and 100% humidity for 20 h. The TNF-α concentrations in the supernatants were determined by ELISA assay. Values represent the mean of n = 3 ± s.d. *P < 0.01 versus LPS alone, #P < 0.05 versus 7β OH-EpiA + LPS.

**Fig 3.25 Effect of the NSAID Ketoprofen on 7β OH EpiA-Induced Suppression of LPS- Stimulated TNF-α Production in THP-1 Cells**




THP-1 cells ($1x10^6$) were incubated with various treatments; 7β OH-EpiA (0.001μM), Ketoprofen (50μM) or a combination of both 7β OH-EpiA and Ketoprofen in presence and absence of LPS (1μg/ml) at 37 °C, 5%CO2 and 100% humidity for 20 h. The TNF-α concentrations in the supernatants were determined by ELISA assay. Values represent the mean of n = 3 ± s.d. *P < 0.05 versus LPS alone, #P < 0.05 versus 7β OH-EpiA + LPS.

## 3.8 Effect of Mifepristone (GR Antagonist) on the Ability of Dexamethasone and 7β OH-EpiA to Reduce Production of TNF- α

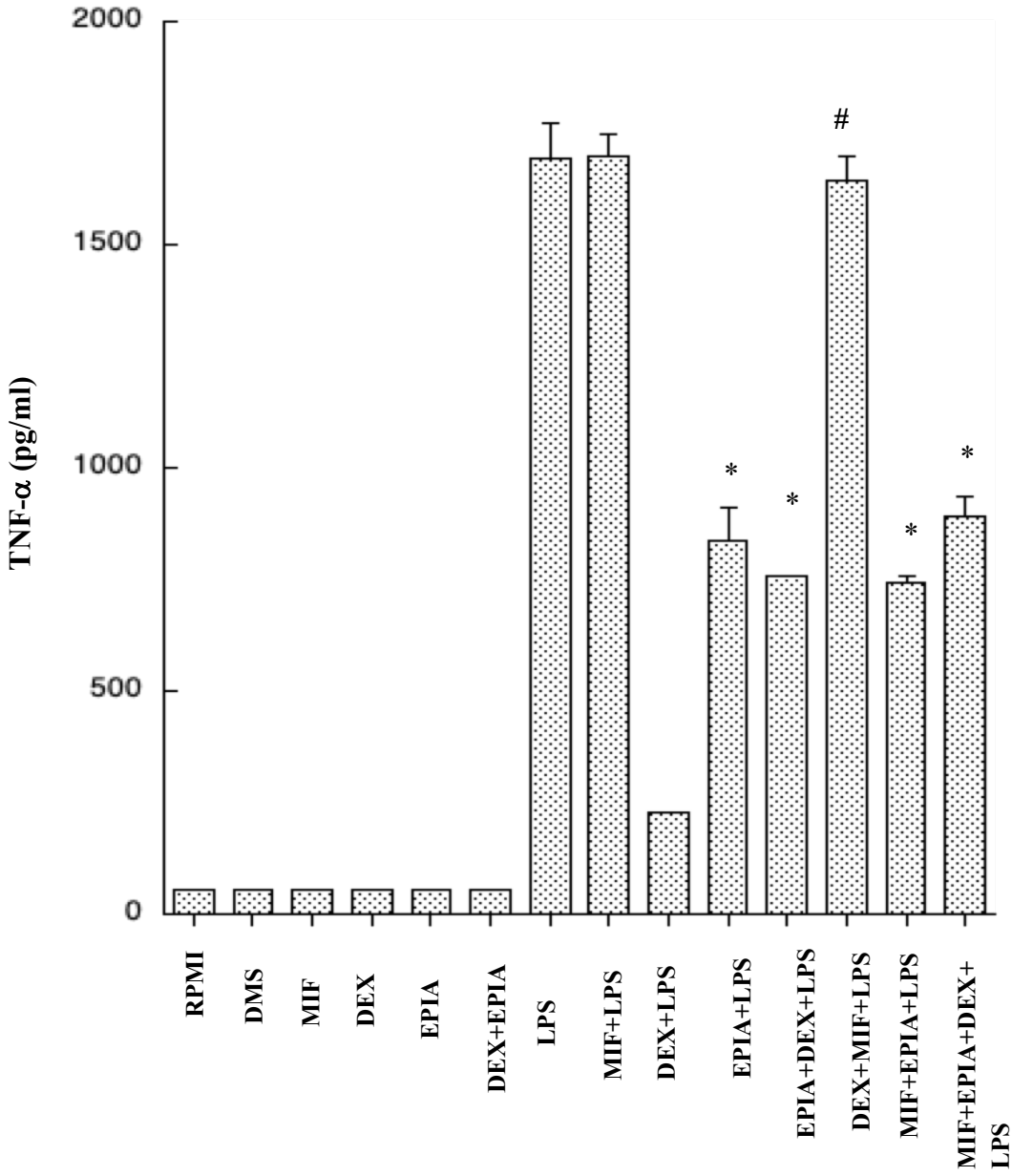
Mifepristone can inhibit the actions of steroids by binding to the glucocorticoid receptor (GR) and the progesterone receptor (PR) (Beck, 1993 #610). In previous experiments, as it was shown that both dexamethasone and 7β OH-EpiA were able to suppress the LPS-stimulated production of TNF-α, it was decided to ascertain if this effect was possibly mediated via a GR or PR by using the dual GR/ PR inhibitor, mifepristone.

Both 7β OH-EpiA (0.001 μM) and dexamethasone (100 μM) significantly reduced LPS-stimulated TNF-α production from whole human blood cells. Mifepristone (100μM) alone also reduced the level of TNF-α in response to LPS. The 7β OH-EpiA-induced reduction in the level of TNF-α was not affected in the presence of mifepristone. Conversely, mifepristone reversed the dexamethasone-induced reduction in the LPS-stimulated level of TNF-α (Fig. 3.26). This indicated a normal GR-mediated suppression of the LPS-stimulated quantity of TNF-α production by dexamethasone but the effects of 7β OH-EpiA were unlikely to be mediated via a GR or PR.

In isolated monocytes, 7β OH-EpiA (0.001 μM) and dexamethasone (100 μM) also significantly reduced LPS-stimulated TNF-α production from monocytes. Mifepristone (100μM) alone also reduced the level of TNF-α in response to LPS. The 7β OH-EpiA-induced reduction in the level of TNF-α was not affected in the presence of mifepristone. Equally, mifepristone reversed the dexamethasone-induced reduction in the LPS-stimulated level of TNF-α (Fig. 3.27). This indicated a normal GR-mediated suppression of the LPS-stimulated quantity of TNF-α production by dexamethasone

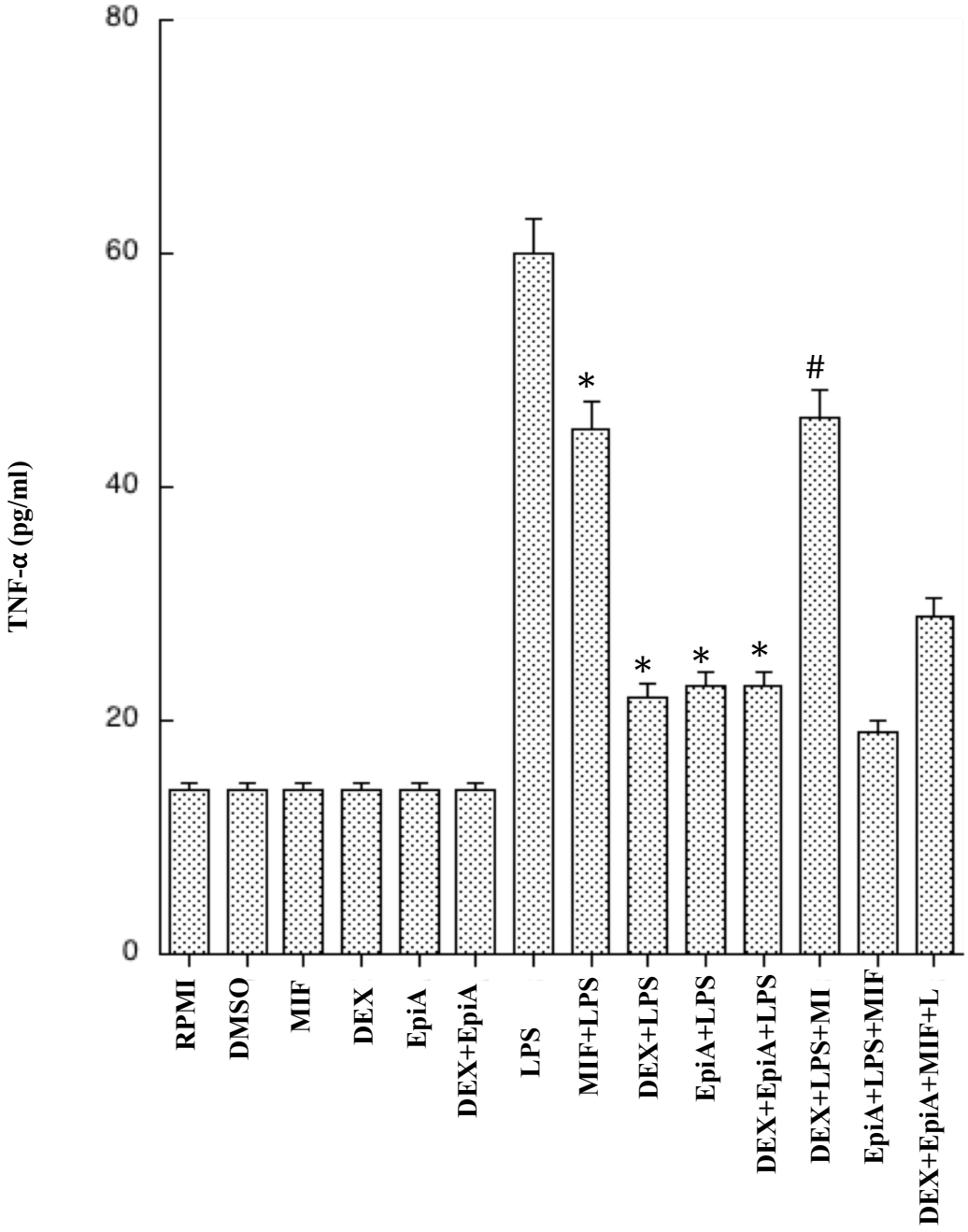
but the effects of 7β OH-EpiA were unlikely to be mediated via a GR or PR. A similar effect was observed in THP-1 cells. Both dexamethasone and 7β OH-EpiA suppressed LPS-stimulated levels of TNF-α production but only the dexamethasone-induced inhibition was reversed by mifepristone whereas the 7β OH-EpiA-induced inhibition was unaffected by mifepristone (Fig. 3.28).

**Fig 3.26 Effect of Mifepristone (GR/ PR Antagonist) on the Ability of Dexamethasone and 7β OH-EpiA to Reduce the Production of TNF-α in Response to LPS Stimulation in Whole Human Blood**



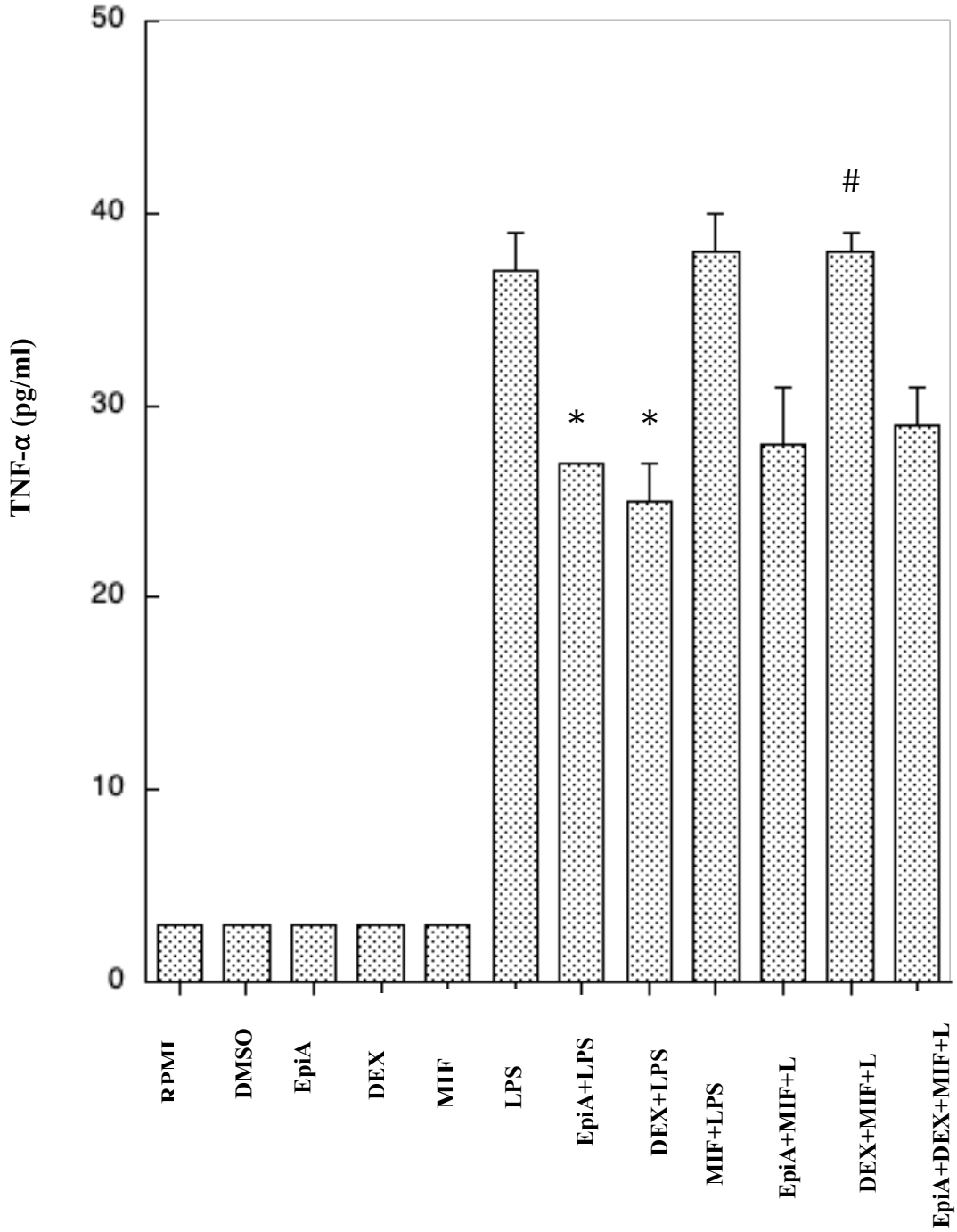

Whole human blood was incubated with various treatments; 7β OH-EpiA (0.001μM), dexamethasone (100μM), mifepristone (100 μM) or a combination of both 7β OH-EpiA and dexamethasone or mifepristone in the presence and absence of LPS (10μg/ml) at 37 °C, 5%CO2 and 100% humidity for 20 h. The TNF-α concentrations in the supernatants were determined by ELISA assay. Values represent the mean of n = 3. *P < 0.05 versus LPS alone, #P < 0.05 versus DEX + LPS.

**Fig 3.27 Effect of Mifepristone (GR/ PR Antagonist) on the Ability of Dexamethasone and 7β OH-EpiA to Reduce the Production of TNF-α in Response to LPS Stimulation in Isolated Monocytes**




Monocytes (1.3 x$10^6$) were incubated with various treatments; 7β OH-EpiA (0.001μM), dexamethasone (100μM), mifepristone (100 μM) or a combination of both 7β OH-EpiA and dexamethasone or mifepristone in the presence and absence of LPS (10μg/ml) at 37 °C, 5%CO2 and 100% humidity for 20 h. The TNF-α concentrations in the supernatants were determined by ELISA assay. Values represent the mean of n = 3. *P < 0.05 versus LPS alone, #P < 0.05 versus DEX + LPS.

**Fig 3.28 Effect of Mifepristone (GR/ PR Antagonist) on the Ability of Dexamethasone and 7β OH-EpiA to Reduce Production of TNF-α in Response to LPS Stimulation in THP-1 Cells**



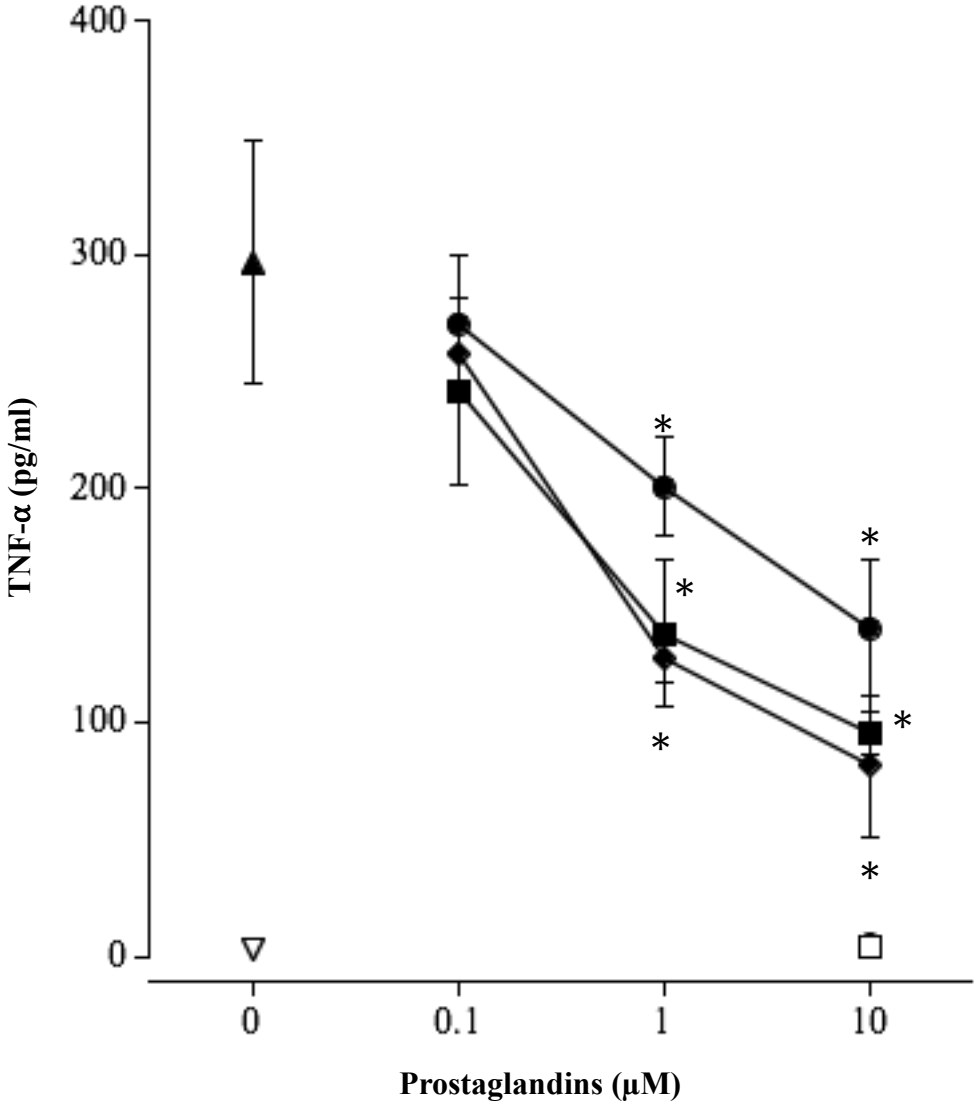

THP-1 cells ($1x10^6$) were incubated with7β OH-EpiA (0.001μM), dexamethasone (100μM), mifepristone (100 μM) or a combination of both 7β OH-EpiA and dexamethasone or mifepristone in the presence and absence of LPS (10μg/ml) at 37 °C, 5%CO2 and 100% humidity for 20 h. The TNF-α concentrations in the supernatants were determined by ELISA assay. Values represent the mean of n = 3 ± s.d. *P < 0.05 versus LPS alone, #P < 0.05 versus DEX + LPS.

## 3.9 The Effect of Different Concentrations of Prostaglandins on TNF-α Production in Monocytes

Many different prostaglandins (PGs) have been shown to reduce LPS-stimulated TNF-α production. As the effects of 7β OH-EpiA were reversed in the presence of ketoprofen this implies that a cyclooxygenase product, possibly a prostanoid, mediates it effects. Thus, three different related, potential candidate prostaglandins ($PGE_2$, $PGD_2$ and 15d-$PGJ_2$) were directly compared to confirm their comparative effects in suppressing TNF-α production in response to LPS. This was carried out by incubating cells with various concentrations of these prostaglandins in the presence of LPS.

All three prostaglandins reduced LPS-stimulated TNF-α production in a concentration-dependent manner between 0.1 and 10 μM in monocytes (Fig. 3.29). Both $PGE_2$ and $PGD_2$ appeared to be equipotent and 15d-$PGJ_2$ was slightly less potent than the other two. This confirmed their actions in the present experimental system are directly comparable to their actions in other studies. It also confirmed that they are potential mediators of the actions of 7β OH-EpiA on TNF-α production in monocytes.

**Fig 3.29 The Effect of Different Concentrations of Prostaglandins on TNF-α Production in Response to LPS Stimulation in Isolated Monocytes**




Monocytes were isolated by Ficoll/Histopaque density gradient centrifugation and incubated at 37 °C for 20 h. Cells were then treated with different concentrations of $PGE_2$, (filled circle) $PGD_2$ (filled square) and 15d-$PGJ_2$ (filled diamond) with LPS (1 μg/ml). LPS (1 μg/ml closed triangle) alone or three of PGs individually (alone) as control, open shapes) were added and incubations continued for a further 3 h. Concentrations of TNF-α present in supernatants were measured by ELISA. Values are the means of n = 3 incubations ± s.d. and are representative of at least three separate experiments. *P < 0.05 versus LPS alone.

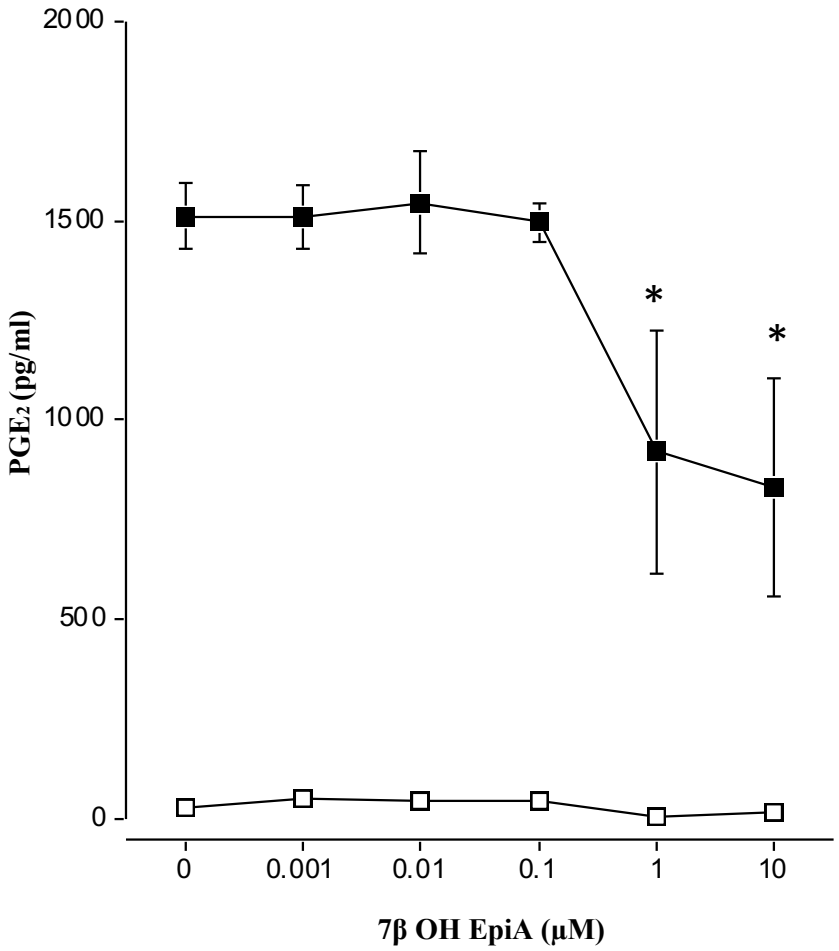
## 3.10 The Effect of Different Concentrations of 7β-OH-EpiA and 7α-OH-EpiA on the Production of various Prostaglandins in Whole Human blood and Isolated Monocytes

In previous experiments it was shown that the effects of 7β OH-EpiA were reversed by ketoprofen, indicating that a cyclooxygenase product, most likely a PG mediates its actions. It was also shown that three different (potential candidate) PGs; $PGE_2$, $PGD_2$ and 15d-$PGJ_2$ also suppressed LPS-stimulated levels of TNF-α. Thus, in the following experiments the effects of 7β OH-EpiA were studied on the production of these 3 PGs in whole human blood and human isolated monocytes. The actions of the 7β OH-EpiA 7α isomer, 7α OH-EpiA were also studied on the production of these PGs. The level of PGs in both plasma and cell supernatants was measured by EIA assay as described in the methodology section. Varying concentrations of 7β OH-EpiA or 7α OH-EpiA were used with or without LPS. The actions of both isomers of 7-OH-EpiA were compared to that of dexamethasone, used as positive control.

### 3.10.1 Effect of 7β OH-EpiA on $PGE_2$ Production

In whole blood incubations, 7β OH-EpiA (0.001-10μM) alone had no effect on unstimulated levels of $PGE_2$ detected in plasma. LPS (10 μg/ml) alone increased the level $PGE_2$ in the order of 50-fold. In the presence of 0.001, 0.01, and 0.1 μM 7β OH-EpiA, no effect was observed but at higher concentrations (1 and 10 μM), 7β OH-EpiA decreased LPS-stimulated levels of $PGE_2$ (Fig 3.30).

**Fig 3.30 The Effect of Different Concentrations of 7β OH-EpiA on $PGE_2$ Production in Whole Human Blood in the Presence or Absence of LPS**

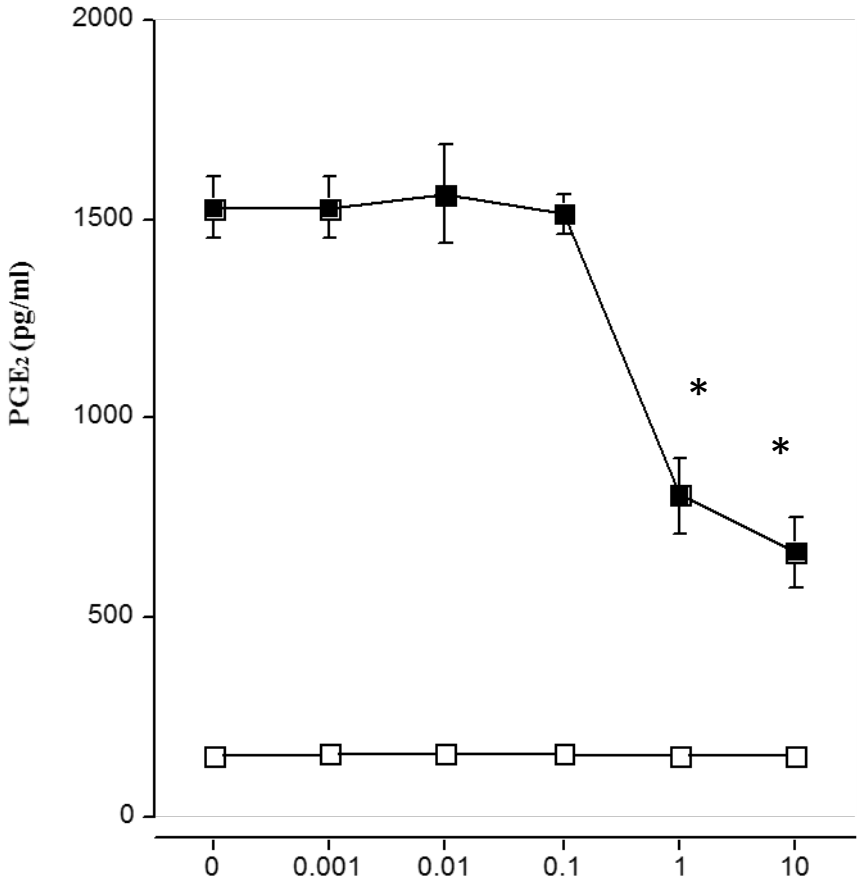



Whole human blood was treated with 7β OH-EpiA for 1 h after which either DMSO vehicle (control, open squares) or LPS (10 μg/ml, closed squares) was added and incubated at 37 °C for 20 h. Plasma was collected after centrifugation and concentrations of $PGE_2$ present in plasma were measured by EIA. Values are the means of n = 3 incubations ± s.d. *P < 0.05 versus LPS alone.

### 3.10.2 Effect of 7β OH-EpiA on $PGE_2$ Production in Human Isolated Monocytes

7β OH-EpiA (0.001-10μM) alone had no effect on the levels of $PGE_2$ detected in cell supernatants from unstimulated cells. LPS (10 μg/ml) alone increased the level of $PGE_2$ in the order of 8-fold. In the presence of 0.001, 0.01, and 0.1 μM 7β OH-EpiA, no effect was observed but at higher concentrations (1 and 10 μM), 7β OH-EpiA decreased LPS-stimulated levels of $PGE_2$ in monocytes (Fig 3.31).

**Fig 3.31 The Effect of Different Concentrations of 7β OH-EpiA on $PGE_2$ Production in Isolated Monocytes in the Presence or Absence of LPS**

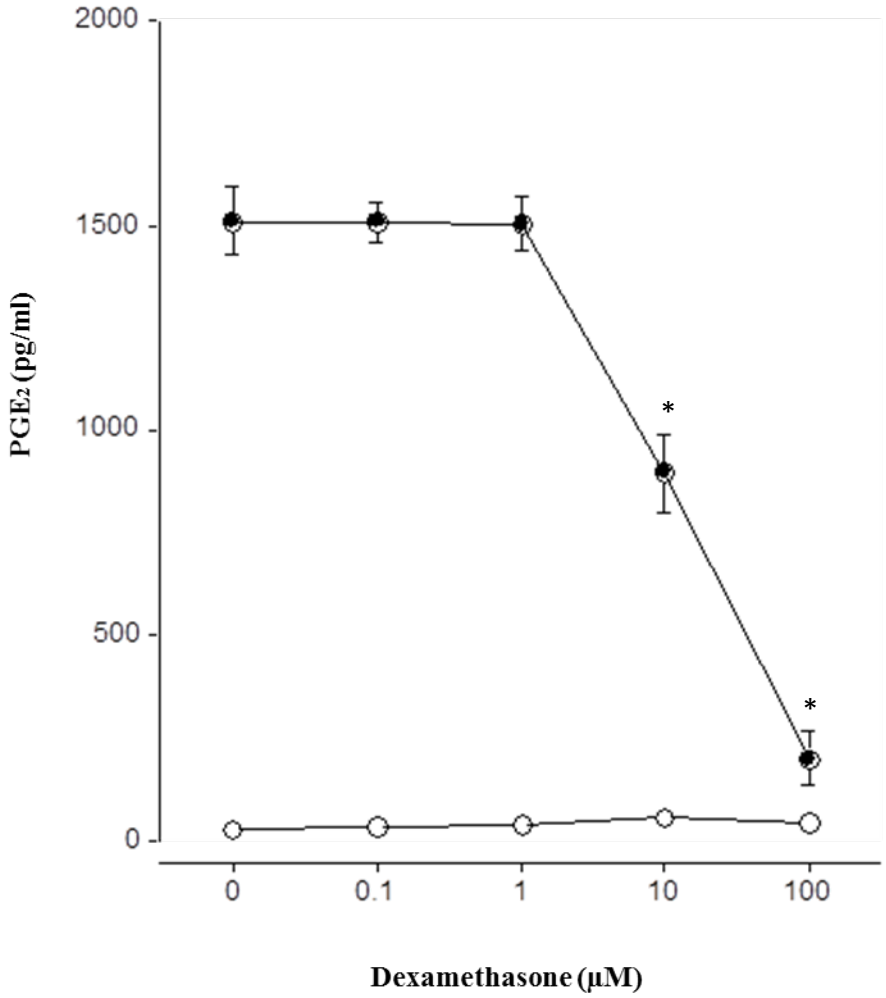



Monocytes were isolated by Ficoll/Histopaque density gradient centrifugation and incubated at 37 °C for 20 h. Cells were then treated with 7β OH-EpiA for 1 h after which either DMSO vehicle (control, open squares) or LPS (10 μg/ml, closed squares) was added and incubations continued for a further 3 h. Concentrations of $PGE_2$ present in supernatants were measured by EIA. Values are the means of n = 3 incubations ± s.d. *P < 0.05 versus LPS alone.

### 3.10.3 The Effect of Dexamethasone on $PGE_2$ Production in Whole Human Blood

In whole blood, dexamethasone (0.1-100μM) alone had no effect on unstimulated levels of $PGE_2$ detected in plasma. LPS (10 μg/ml) increased $PGE_2$ levels in the order of 50-fold compared to control incubations. Dexamethasone concentrations of 0.1 and 1 μM had no effect on LPS-stimulated levels of $PGE_2$ but higher concentrations lowered $PGE_2$ concentration and 100 μM almost abolished the increased levels (Fig 3.32).

**Fig 3.32 The Effect of Different Concentrations of Dexamethasone on $PGE_2$ Production in Whole Human Blood in the Presence or Absence of LPS**

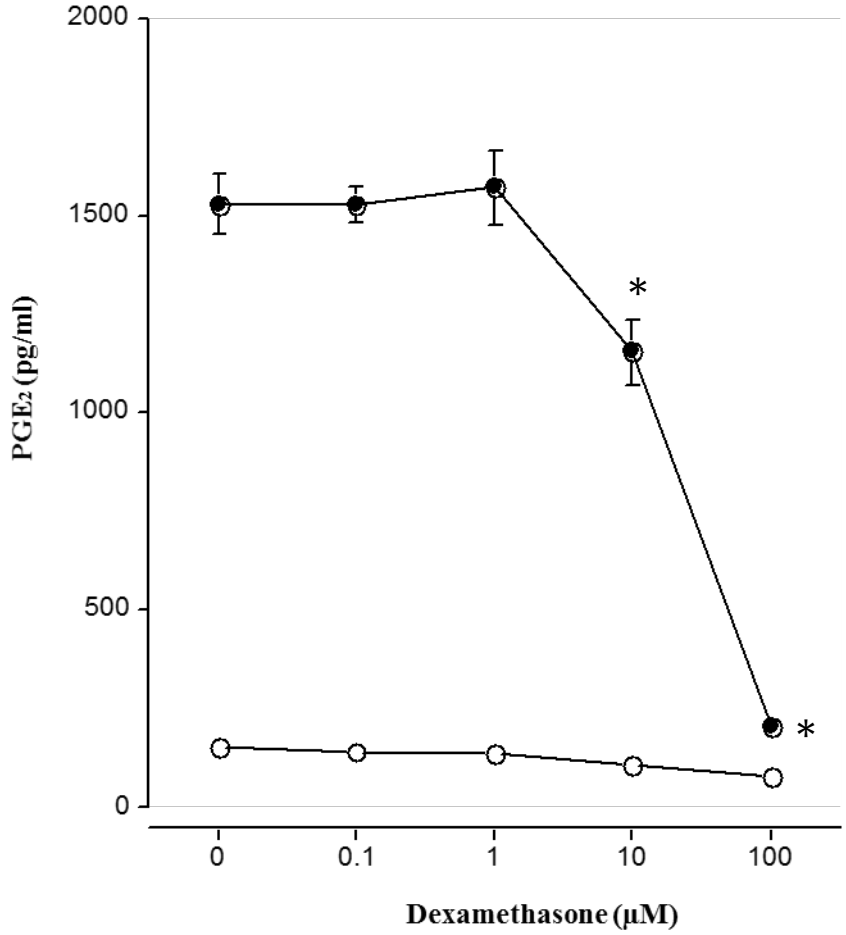



Whole human blood was incubated without (control, open circles) or with LPS (10 µg/ml, closed circles) in the presence of various concentrations of dexamethasone. Incubations were carried out at 37 °C for 20 h. Plasma was collected after centrifugation. Concentrations of $PGE_2$ present in plasma were measured by EIA. Values are the means of n = 3 incubations ± s.d. and are representative of at least three separate experiments. *P < 0.05 versus LPS alone.

### 3.10.4 The Effect of Different Concentrations of Dexamethasone on $PGE_2$ Production in Isolated Human Monocytes

In monocytes, dexamethasone had similar effects to those observed in whole human blood. In the absence of LPS, dexamethasone (0.1-100μM) alone had no effect on unstimulated levels of $PGE_2$ detected in plasma. LPS (10 μg/ml) increased $PGE_2$ levels in the order of 8-fold compared to control incubations. Dexamethasone concentrations of 0.1 and 1 μM had no effect on LPS-stimulated levels of $PGE_2$ but higher concentrations lowered $PGE_2$ concentrations and 100 μM almost abolished the increased levels (Fig 3.33).

**Fig 3.33 The Effect of Different Concentrations of Dexamethasone on $PGE_2$ Production in Human Monocytes in the Presence or Absence of LPS**



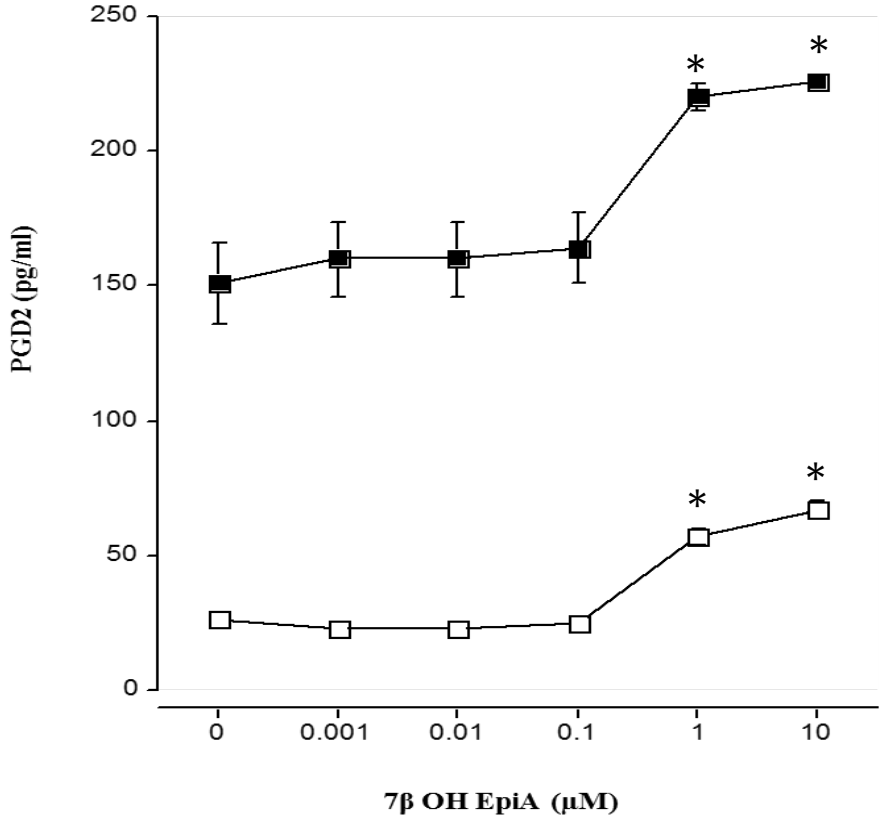

Monocytes were isolated by Ficoll/Histopaque density gradient centrifugation and incubated at 37 °C for 20 h. Cells were then treated with dexamethasone for 1 h after which either RPMI (control, open circle) or LPS (10 µg/ml, closed circle) was added and incubations continued for a further 3 h. Concentrations of $PGE_2$ present in supernatants were measured by EIA. Values are the means of n = 3 incubations ± s.d. and are representative of at least three separate experiments. *P < 0.05 versus LPS alone.

### 3.10.5 Effect of 7β OH-EpiA on $PGD_2$ Production in Human Blood and Monocytes

In contrast to the effects of 7β OH-EpiA on $PGE_2$ production in whole human blood and isolated monocytes, an opposite effect was observed on $PGD_2$ production. In the presence of 7β OH-EpiA (0.001 – 0.1 μM) alone, no effect on the level of $PGD_2$ was detected in plasma. However, higher concentrations (1 and 10 μM) resulted in an approximately 2-fold increase in $PGD_2$ concentrations (Fig 3.34). LPS alone stimulated an increase in $PGD_2$ in whole human blood in the order of 6-fold and 7β OH-EpiA had no effect on LPS-stimulated concentrations between 0.001 and 0.1μM but increased the LPS-stimulated level of $PGD_2$ at 1 and 10 μM (Fig 3.34).

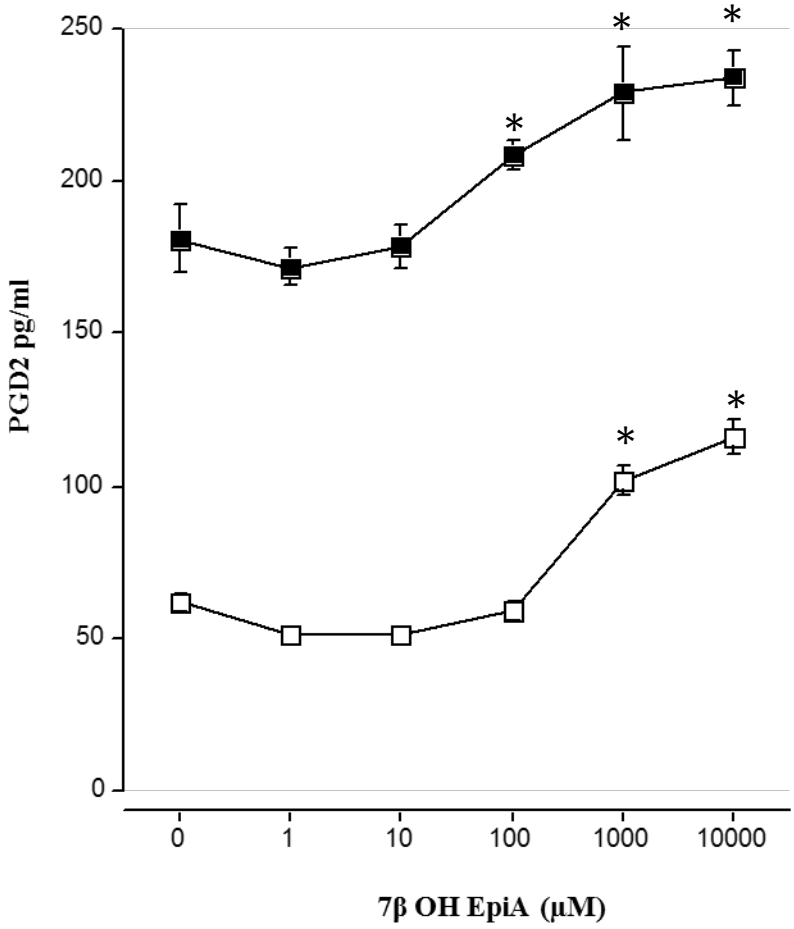
An almost identical action of 7β OH-EpiA on PGD2 production was observed in isolated monocytes. In the presence of 7β OH-EpiA (0.001 – 0.1 μM) alone, no effect on the level of $PGD_2$ was detected in cell supernatants. However, higher concentrations (1 and 10 μM) resulted in an approximately 2-fold increase in $PGD_2$ levels (Fig. 3.35). LPS alone stimulated an increase in $PGD_2$ in cells in the order of 3-fold and 7β OH-EpiA had no effect on LPS-stimulated levels at 0.001 and 0.01μM but increased the LPS-stimulated level of $PGD_2$ between 0.1 and 10 μM (Fig 3.35). These observations suggest that 7β OH-EpiA at higher concentrations selectively promotes the production of $PGD_2$ but suppresses $PGE_2$ production.

**Fig 3.34 The Effect of Different Concentrations of 7β-OH-EpiA on $PGD_2$ Production in Whole Human Blood in the Presence or Absence of LPS**




Whole human blood was treated with 7β-OH-EpiA for 1 h after which either DMSO vehicle (control, open squares ) or LPS (10 μg/ml, closed squares) was added and incubations at 37 °C for 20 h. plasma was collected after centrifuging. Concentrations of $PGD_2$ present in plasma were measured by EIA. Values are the means of n = 3 incubations ± s.d. and are representative of at least three separate experiments. *P < 0.05 versus respective controls in the absence of 7β-OH-EpiA.

**Fig 3.35 The Effect of Different Concentrations of 7β-OH-EpiA on $PGD_2$ Production in Isolated Monocytes in the Presence or Absence of LPS**

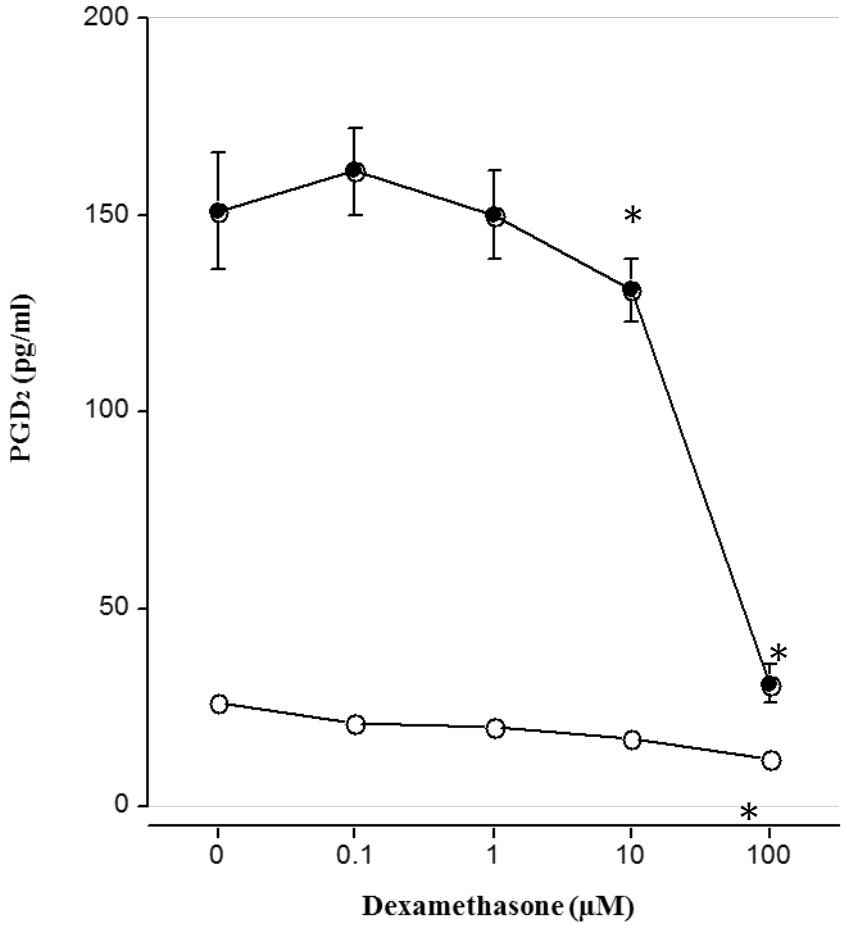



Monocytes were isolated by Ficoll/Histopaque density gradient centrifugation and incubated at 37 °C for 20 h. Cells were then treated with 7β OH-EpiA for 1 h after which either DMSO vehicle (control, open squares) or LPS (1 μg/ml, closed squares) was added and incubations continued for a further 3 h. Concentrations of $PGD_2$ present in supernatants were measured by EIA. Values are the means of n = 3 incubations ± s.d. *P < 0.05 versus respective controls in the absence of 7β OH-EpiA.

### 3.10.6 The Effect of Dexamethasone on $PGD_2$ Production in Whole Human Blood

In whole blood, dexamethasone (0.1-10 μM) alone had no effect on unstimulated levels of $PGD_2$ detected in plasma but at 100 μM, a small decrease was observed. LPS (10 μg/ml) increased $PGD_2$ levels in the order of 6-fold compared to control incubations. Dexamethasone concentrations of 0.1 and 1 μM had no effect on LPS-stimulated levels of $PGD_2$ but higher concentrations lowered $PGD_2$ levels and 100 μM almost abolished the increased concentrations (Fig 3.36).

**Fig 3.36 The Effect of Different Concentrations of Dexamethasone on $PGD_2$ Production in Whole Human Blood in the Presence or Absence of LPS**

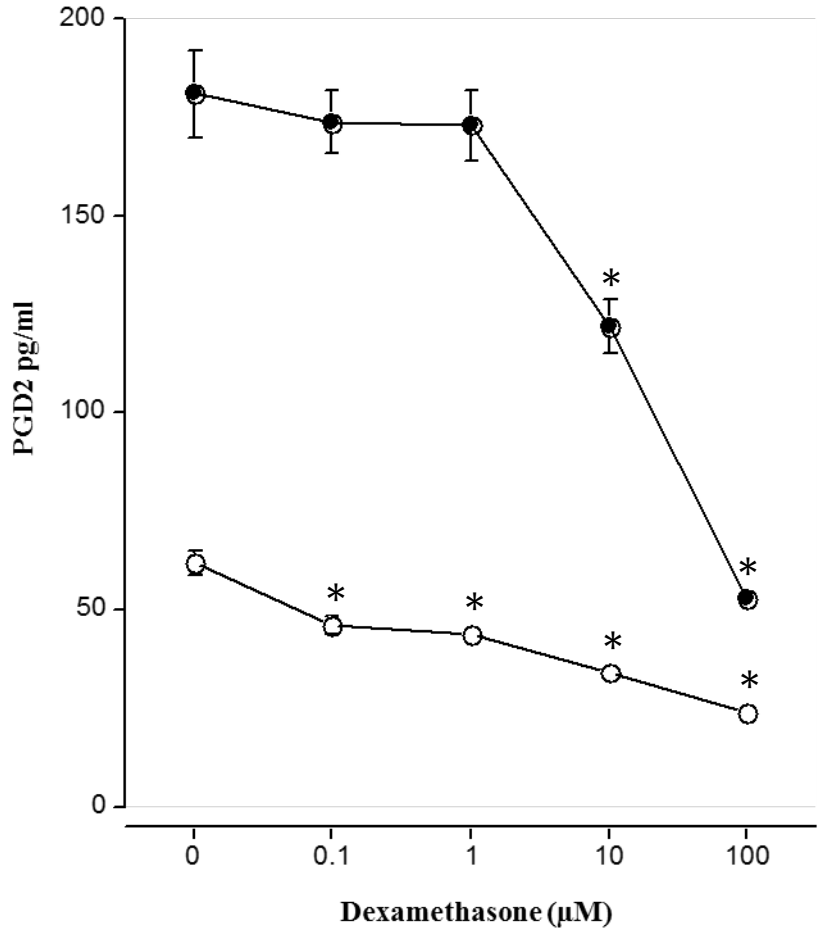



Whole human blood was treated with dexamethasone for 1 h after which either RPMI (control, open circles) or LPS (10 µg/ml, closed circles) was added and incubations at 37 °C for 20 h. plasma was collected after centrifuging. Concentrations of PGD2 present in plasma were measured by EIA. Values are the means of n = 3 incubations ± s.d. and are representative of at least three separate experiments. *P < 0.05 versus respective controls in the absence of dexamethasone.

### 3.10.7 The Effect of Dexamethasone on $PGD_2$ Production in Isolated Monocytes

In monocytes, dexamethasone had similar effects to those observed in blood. In the absence of LPS, dexamethasone (0.1-100 μM) produced a concentration-dependent decrease in unstimulated levels of $PGD_2$ detected in cell supernatants. LPS (10 μg/ml) increased $PGD_2$ levels in the order of 3-fold compared to control incubations. Dexamethasone concentrations of 0.1 and 1 μM had no effect on LPS-stimulated levels of $PGD_2$ but higher concentrations lowered $PGD_2$ levels and 100 μM almost abolished the increased levels (Fig 3.37).

**Fig 3.37 The Effect of Different Concentrations of Dexamethasone on $PGD_2$ Production in Isolated Human Monocytes in the Presence or Absence of LPS**



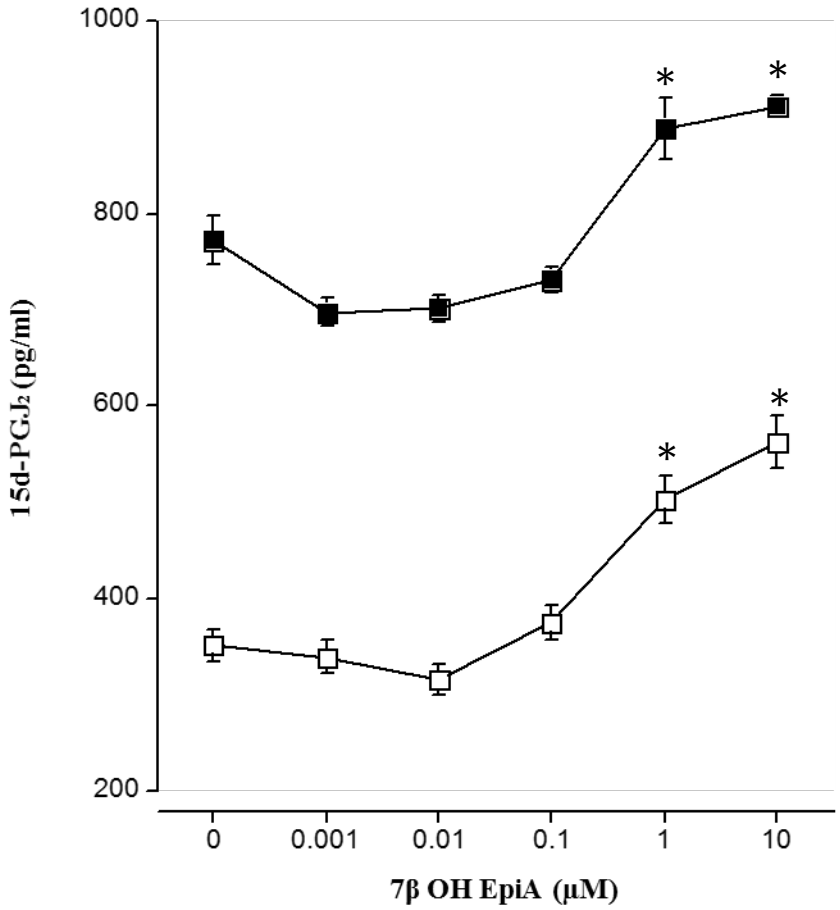

Monocytes were isolated by Ficoll/Histopaque density gradient centrifugation and incubated at 37 °C for 20 h. Cells were then treated with dexamethasone for 1 h after which either RPMI (control, open circles) or LPS (1 µg/ml, closed circles) was added and incubations continued for a further 3 h. Concentrations of $PGD_2$ present in supernatants were measured by EIA. Values are the means of n = 3 incubations ± s.d. and are representative of at least three separate experiments. *P < 0.05 versus respective controls in the absence of dexamethasone.

### 3.10.8 The Effect of 7β OH-EpiA on 15d-$PGJ_2$ Production in Whole Human Blood and Isolated Monocytes

7β OH-EpiA had a similar effect on 15d-$PGJ_2$ levels as that on $PGD_2$ levels. In the presence of 7β OH-EpiA (0.001 – 0.1 μM) alone, no effect on the level of 15d-$PGJ_2$ was detected in plasma. However, higher concentrations (1 and 10 μM) resulted in an approximately 1.5-fold increase in 15d-PGJ2 levels (Fig. 3.38). LPS alone stimulated an increase in 15d-$PGJ_2$ in whole blood in the order of 2-fold and 7β OH-EpiA had no effect on LPS-stimulated levels between 0.001 and 0.1μM but increased the LPS-stimulated level of 15d-$PGJ_2$ at 1 and 10 μM (Fig 3.38).

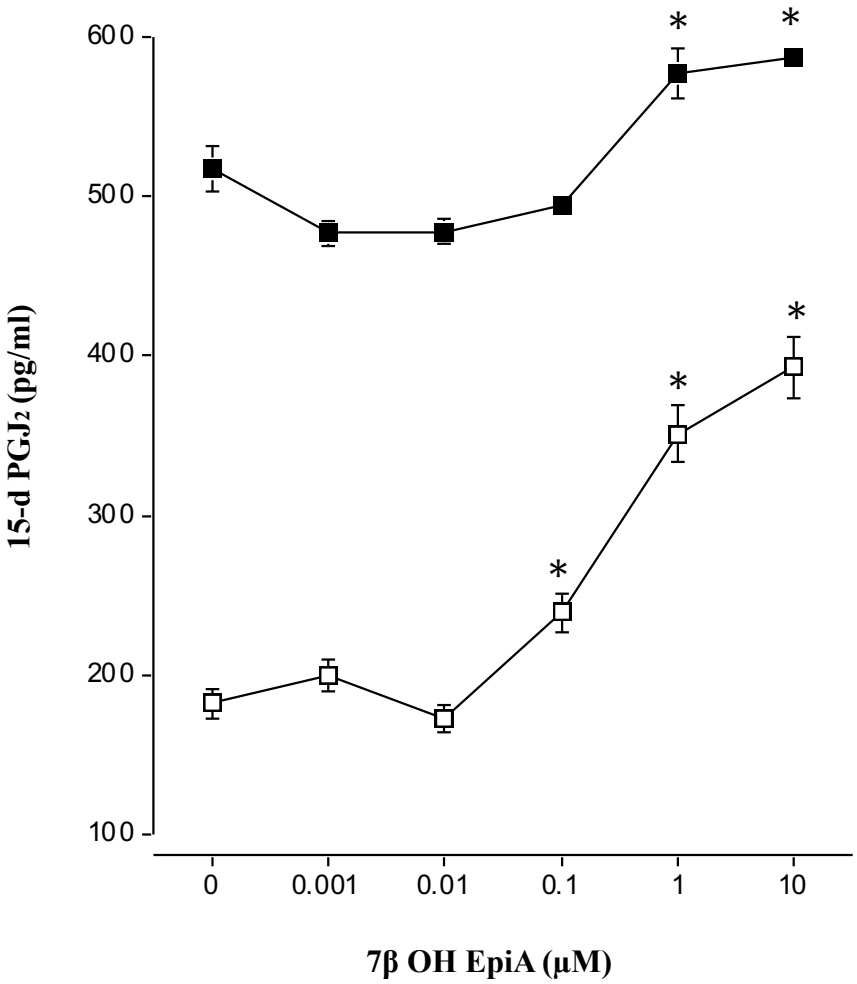
An almost identical action of 7β OH-EpiA on 15d-$PGJ_2$ production was observed in monocytes compared to its actions in blood. In the presence of 7β OH-EpiA (0.001 and 0.01 μM) alone, no effect on the level of 15d-$PGJ_2$ was detected in cell supernatants. However, higher concentrations (between 0.1 and 10 μM) resulted in an approximately 2-fold increase in 15d-$PGJ_2$ levels (Fig. 3.39). LPS alone stimulated an increase in 15d-$PGJ_2$ in cells in the order of 2.7-fold and 7β OH-EpiA had no effect on LPS-stimulated levels at concentrations between 0.001 and 0.1μM but increased the LPS-stimulated level of 15d-$PGJ_2$ at 1 and 10 μM (Fig 3.39). As with $PGD_2$, these observations indicate that 7β OH-EpiA selectively enhances the production of 15d-$PGJ_2$ whilst suppressing $PGE_2$ production.

**Fig 3.38 The Effect of Different Concentrations of 7β-OH-EpiA on 15d-$PGJ_2$ Production in Whole Human Blood in the Presence or Absence of LPS**




Whole human blood was treated with 7β OH-EpiA for 1 h after which either DMSO vehicle (control, open squares) or LPS (10 μg/ml, closed squares) was added and incubations at 37 °C for 20 h. plasma was collected after centrifuging. Concentrations of 15d-$PGJ_2$ present in plasma were measured by EIA. Values are the means of n = 3 incubations ± s.d. and are representative of at least three separate experiments. *P < 0.05 versus respective controls in the absence of 7β-OH-EpiA.

**Fig 3.39 The Effect of Different Concentrations of 7β OH-EpiA on 15d-$PGJ_2$ Production in Isolated Monocytes in the Presence or Absence of LPS**




Monocytes were isolated by Ficoll/Histopaque density gradient centrifugation and incubated at 37 °C for 20 h. Cells were then treated with 7β OH-EpiA for 1 h after which either continued for a further 3 h. In presence of LPS (10μg/ml) (close squares) or absence (open squares). Concentrations of 15d-$PGJ_2$ present in supernatants were measured by EIA. Values are the means of n = 3 incubations ± s.d. and are representative of at least three separate experiments. *P < 0.05 versus respective controls in the absence of 7β-OH-EpiA.

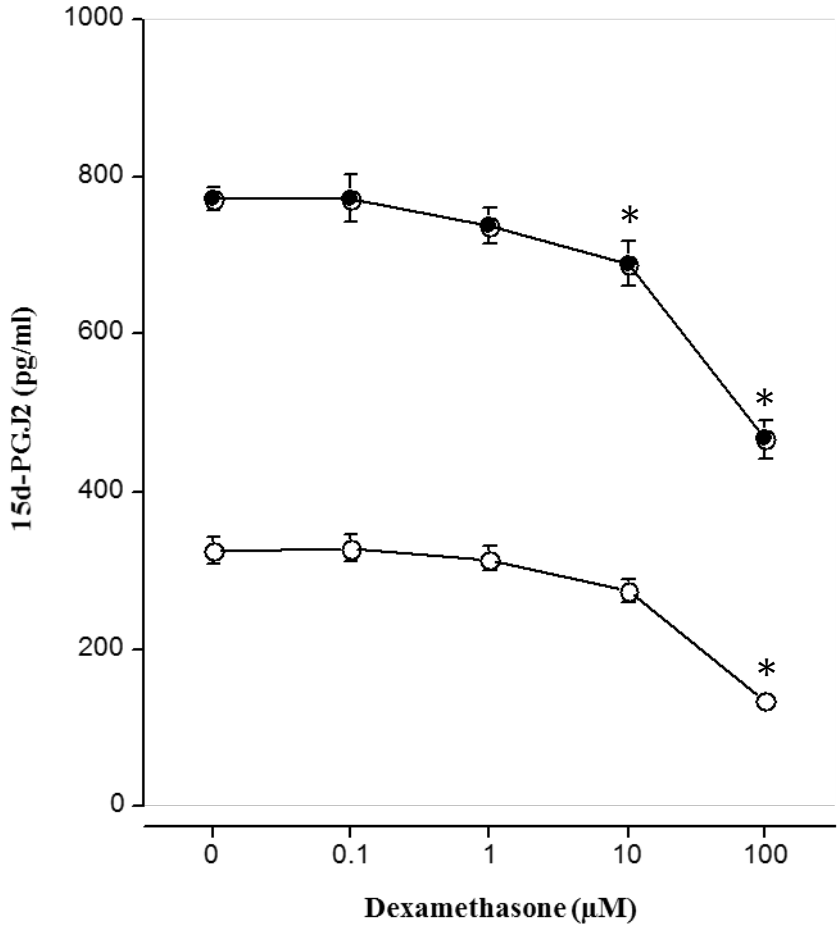
### 3.10.9 The Effect of Dexamethasone on 15d-$PGJ_2$ Production in Whole Human Blood and Isolated Monocytes in the Presence or Absence of LPS

Dexamethasone had similar suppressive effects on 15d-$PGJ_2$ production as it had on $PGD_2$ and $PGE_2$. This was observed in both whole blood and isolated monocytes.

In whole blood, dexamethasone (0.1-1 μM) alone had no effect on unstimulated levels of 15d-$PGJ_2$ detected in plasma but at 100 μM, a small decrease was observed. LPS (10 μg/ml) increased 15d-$PGJ_2$ levels in the order of 2-fold compared to control incubations. Dexamethasone concentrations of 0.1 and 1 μM had no effect on LPS-stimulated levels of 15d-$PGJ_2$ but higher concentrations (10 and 100 μM) lowered 15d-$PGJ_2$ levels (Fig. 3.40).

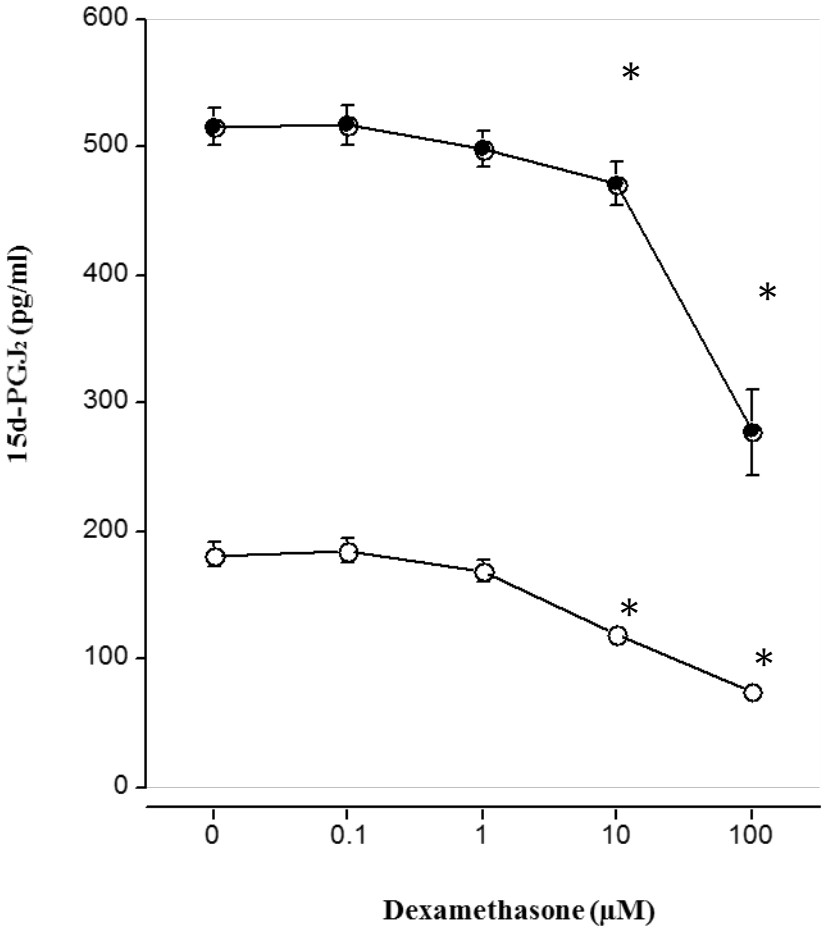
In monocytes, dexamethasone had similar effects to those observed in whole blood. In the absence of LPS, dexamethasone at 0.1 and 1 μM did not show any effect but at 10 and 100 μM, produced a decrease in unstimulated levels of 15d-$PGJ_2$ detected in cell supernatants (Fig 3.41). LPS (10 μg/ml) increased 15d-$PGJ_2$ levels in the order of 2.7-fold compared to control incubations. Dexamethasone concentrations of 0.1 and 1 μM had no effect on LPS-stimulated levels of 15d-$PGJ_2$ but higher concentrations lowered 15d-$PGJ_2$ levels (Fig 3.41).

**Fig 3.40 The Effect of Different Concentrations of Dexamethasone on 15d-PGJ$_2$ Production in Whole Human Blood in the Presence or Absence of LPS**




Whole human blood was treated with dexamethasone for 1 h after which either RPMI (control, open circles) or LPS (10 µg/ml, closed circles) was added and incubations at 37 °C for 20 h. plasma was collected after centrifuging. Concentrations of 15d-PGJ$_2$ present in plasma were measured by EIA. Values are the means of n = 3 incubations ± s.d. *P < 0.05 versus the respective control in the absence of dexamethasone.

**Fig 3.41 The Effect of Different Concentrations of Dexamethasone on 15d-$PGJ_2$ Production in Human Isolated Monocytes in the Presence or Absence of LPS**




Monocytes were isolated by Ficoll/Histopaque density gradient centrifugation and incubated at 37 °C for 20 h. Cells were then treated with dexamethasone for 1 h after which either RPMI (control, open circle) or LPS (1 µg/ml, closed circle) was added and incubations continued for a further 3 h. Concentrations of 15d-$PGJ_2$ present in supernatants were measured by EIA. Values are the means of n = 3 incubations ± s.d. and are representative of at least three separate experiments. *P < 0.05 versus respective controls in the absence of dexamethasone.

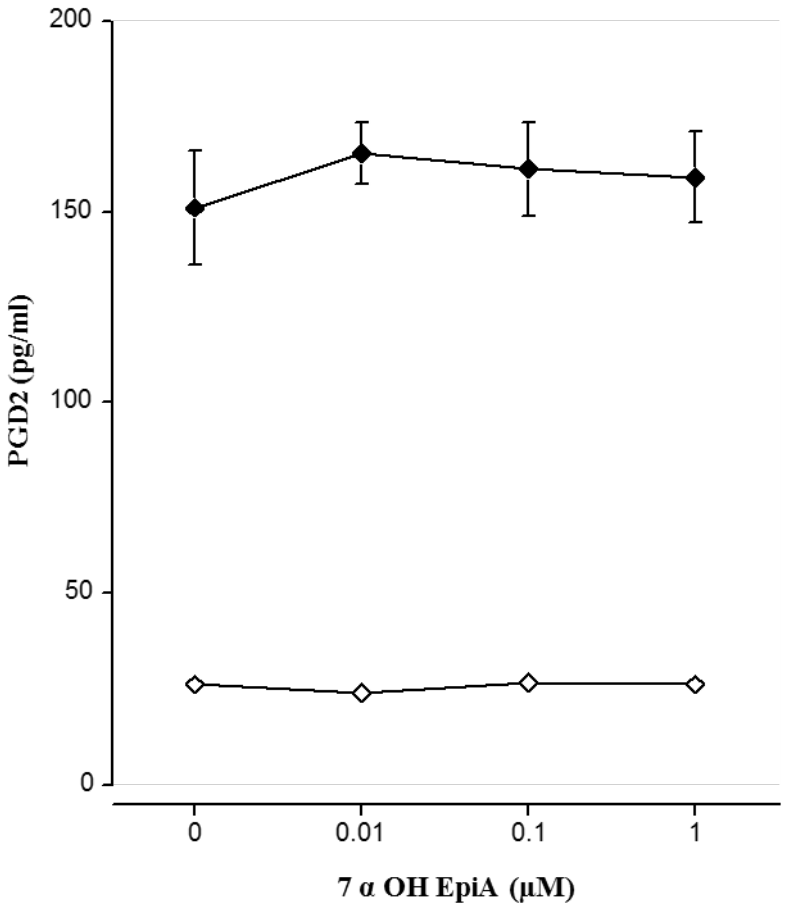
## 3.11 The Effect of 7α-OH-EpiA on $PGD_2$ and 15d-$PGJ_2$ Production in Whole Human Blood

Following the data obtained with 7β OH-EpiA which showed that it enhanced $PGD_2$ and 15d-$PGJ_2$ levels it was decided to evaluate the actions of the 7α isomer of 7β OH-EpiA. Normally, isomers have different effects on receptor systems with only one of the isomers having activity. Therefore, the actions of 7α-OH-EpiA were also studied on the production of $PGD_2$ and 15d-$PGJ_2$ in whole blood incubations with a view to providing an indication of a specific receptor action.

In whole blood, 7α-OH-EpiA (0.01-1μM) alone had no effect on levels of $PGD_2$ detected in plasma. LPS (10 μg/ml) increased levels of PGD2 6-fold compared to control (Fig. 3.41). 7α-OH-EpiA (0.01-1μM) had no effect on the LPS-stimulated levels of $PGD_2$ (Fig. 3.42).

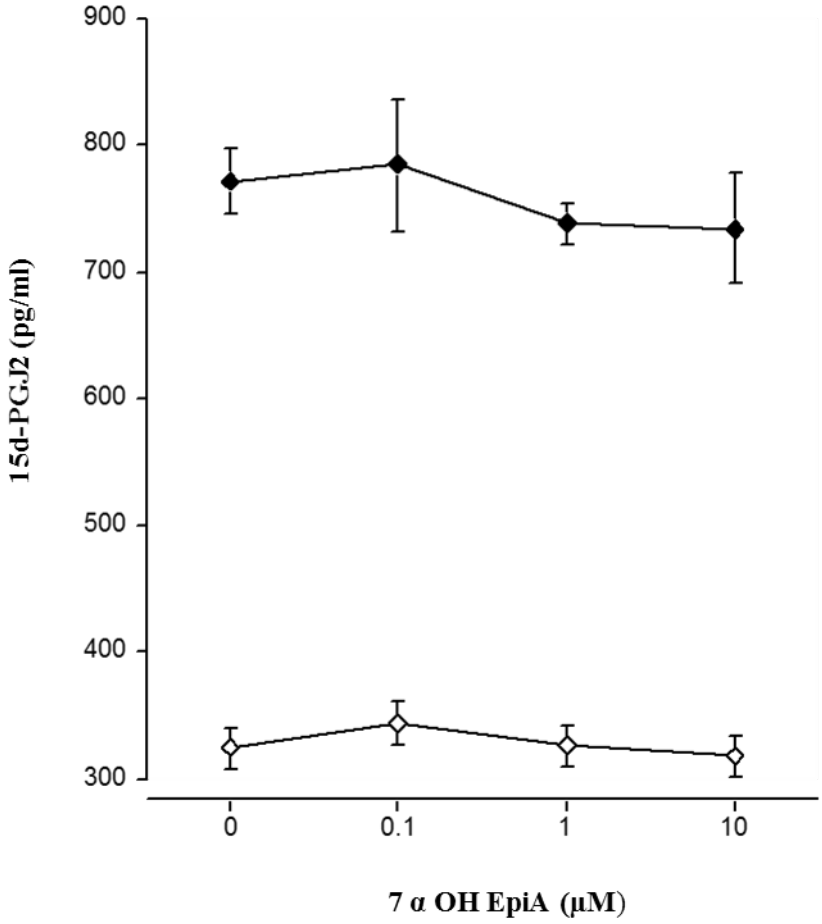
A similar effect of 7α-OH-EpiA was observed on 15d-$PGJ_2$ levels in blood. 7α-OH-EpiA (0.1-10μM) alone had no effect on levels of 15d-$PGJ_2$ levels detected in plasma. LPS (10 μg/ml) increased levels of 15d-$PGJ_2$ levels 2.5-fold compared to control (Fig. 3.42). 7α-OH-EpiA (0.1-10 μM) had no effect on the LPS-stimulated levels of 15d-$PGJ_2$ (Fig. 3.43).

**Fig 3.42 The Effect of Different Concentrations of 7α-OH-EpiA on $PGD_2$ Production in Whole Human Blood Cells in the Presence or Absence of LPS**




Whole human blood was treated with 7α-hydroxyepiandrosterone for 1 h after which either DMSO vehicle (control, open diamond) or LPS (10 μg/ml, closed diamond) was added and incubations at 37 °C for 20 h. plasma was collected after centrifuging. Concentrations of $PGD_2$ present in plasma were measured by EIA. Values are the means of n = 3 incubations ± s.d. and are representative of at least three separate experiments.

**Fig 3.43 The Effect of Different Concentrations of 7α-OH-EpiA on 15d-$PGJ_2$ Production in Whole Human Blood Cells in the Presence or Absence of LPS**




Whole human blood was treated with 7α-hydroxyepiandrosterone for 1 h after which either DMSO vehicle (control, open diamond) or LPS (10 μg/ml, closed diamond) was added and incubations at 37 °C for 20 h. plasma was collected after centrifuging. Concentrations of 15d-$PGJ_2$ present in plasma were measured by EIA. Values are the means of n = 3 incubations ± s.d. and are representative of at least three separate experiments.

## 3.12 Effect of Mifepristone (GR/ PR Antagonist) on the Actions of 7β OH-EpiA on Prostaglandin Production in Whole Human Blood and Isolated monocytes

Previous work in this study also showed that the effects of 7β OH-EpiA on cytokine production were not affected by the dual steroid receptor (GR/ PR) antagonist mifepristone. It was, therefore, decided to also evaluate the effect of the GR/ PR receptor antagonist on the actions of 7β OH-EpiA on PG production in order to ascertain if the GR/ PR receptor involved in control of PGs modulation by 7β OH-EpiA.

In whole blood 7β OH-EpiA did not have any effect on unstimulated levels of $PGE_2$ in plasma but reduced the LPS-stimulated increase in $PGE_2$ levels (Fig. 3.44). Mifepristone did not affect the 7β OH-EpiA-induced suppression of LPS-stimulated $PGE_2$ levels. Similarly, mifepristone did not affect the basal level of $PGE_2$ or the increased level in response to LPS alone (Fig. 3.44).

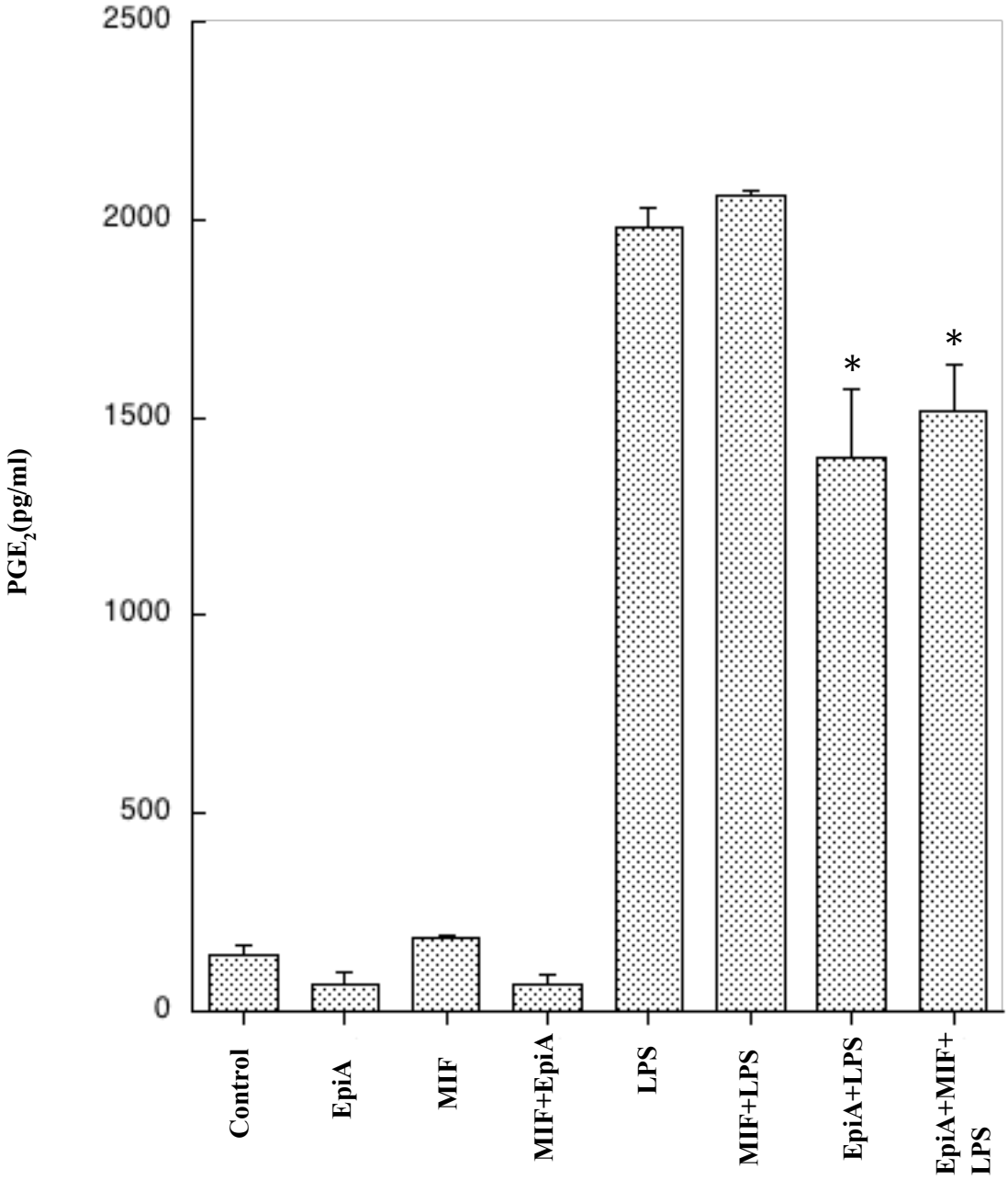
A similar action of mifepristone was observed in isolated monocytes. Mifepristone did not have any effect on the 7β OH-EpiA-induced suppression of LPS-stimulated $PGE_2$ levels in cell supernatants. Similarly, mifepristone did not affect the basal level of $PGE_2$ or the increased level in response to LPS alone (Fig. 3.45).

In whole blood 7β OH-EpiA increased the unstimulated and LPS-stimulated levels of 15d-$PGJ_2$ in plasma (Fig. 3.46). Mifepristone did not affect the 7β OH-EpiA-induced elevation of unstimulated or LPS-stimulated 15d-$PGJ_2$ levels. Similarly, mifepristone did not affect the basal level of 15d-$PGJ_2$ or the increased level in response to LPS alone (Fig. 3.46).

In isolated monocytes a similar pattern was observed where Mifepristone did not have any effect on the 7β OH-EpiA-induced elevation of LPS-stimulated 15d-$PGJ_2$

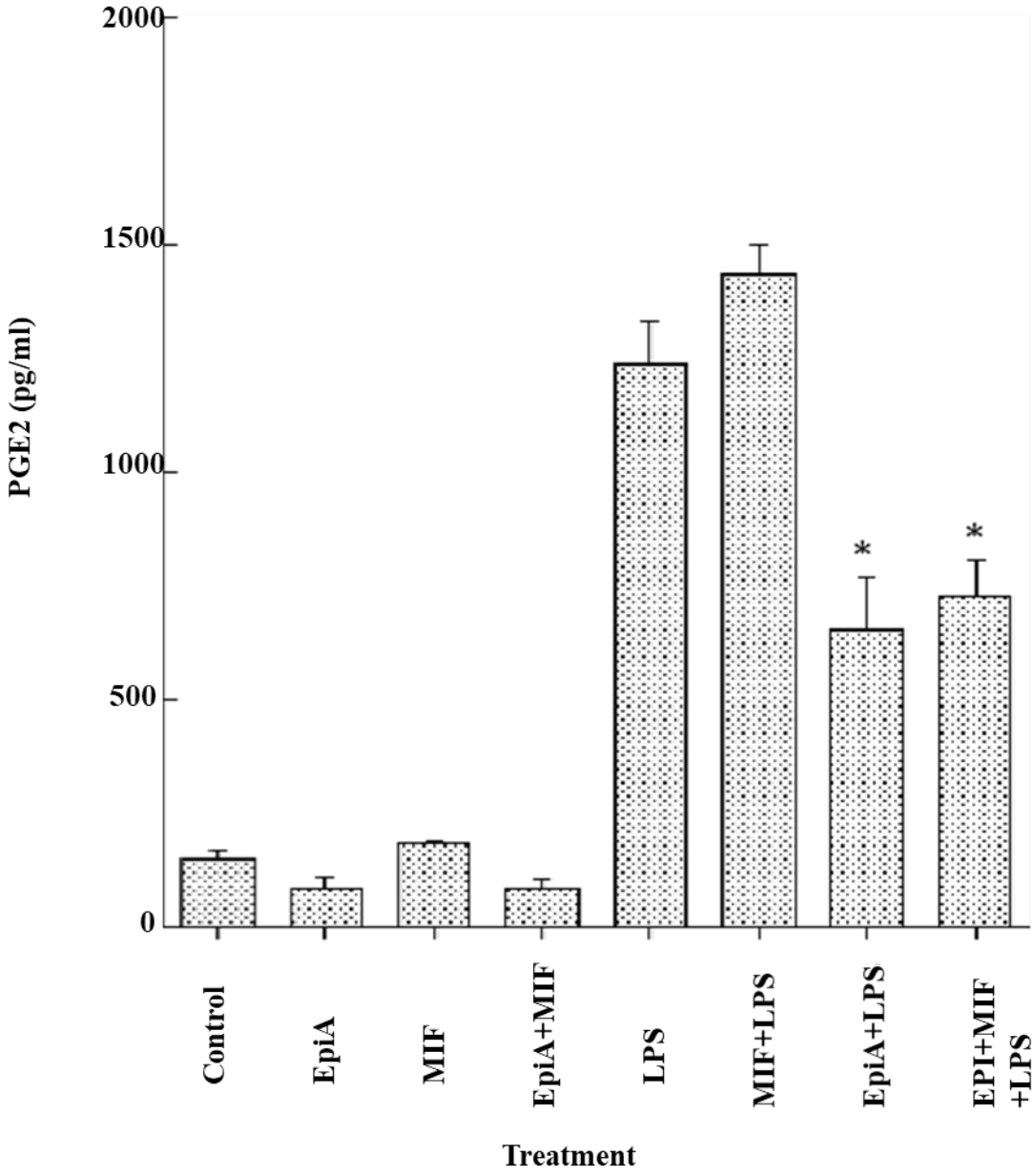
concentrations in cell supernatants. Similarly, mifepristone did not affect the basal 15d-$PGJ_2$ levels or the increased level in response to LPS alone (Fig. 3.47). This showed that mifepristone did not affect any of the 7β OH-EpiA-induced responses on PG production whether they were suppressive ($PGE_2$) or enhancing (15d-$PGJ_2$). This implies that the effects of 7β OH-EpiA on PG production are not mediated via a GR or PR.

**Fig 3.44 Effect of Mifepristone (GR/ PR Antagonist) on the Ability of 7β OH-EpiA to Reduce Production of $PGE_2$ in Response to LPS Stimulation in Whole Human Blood**



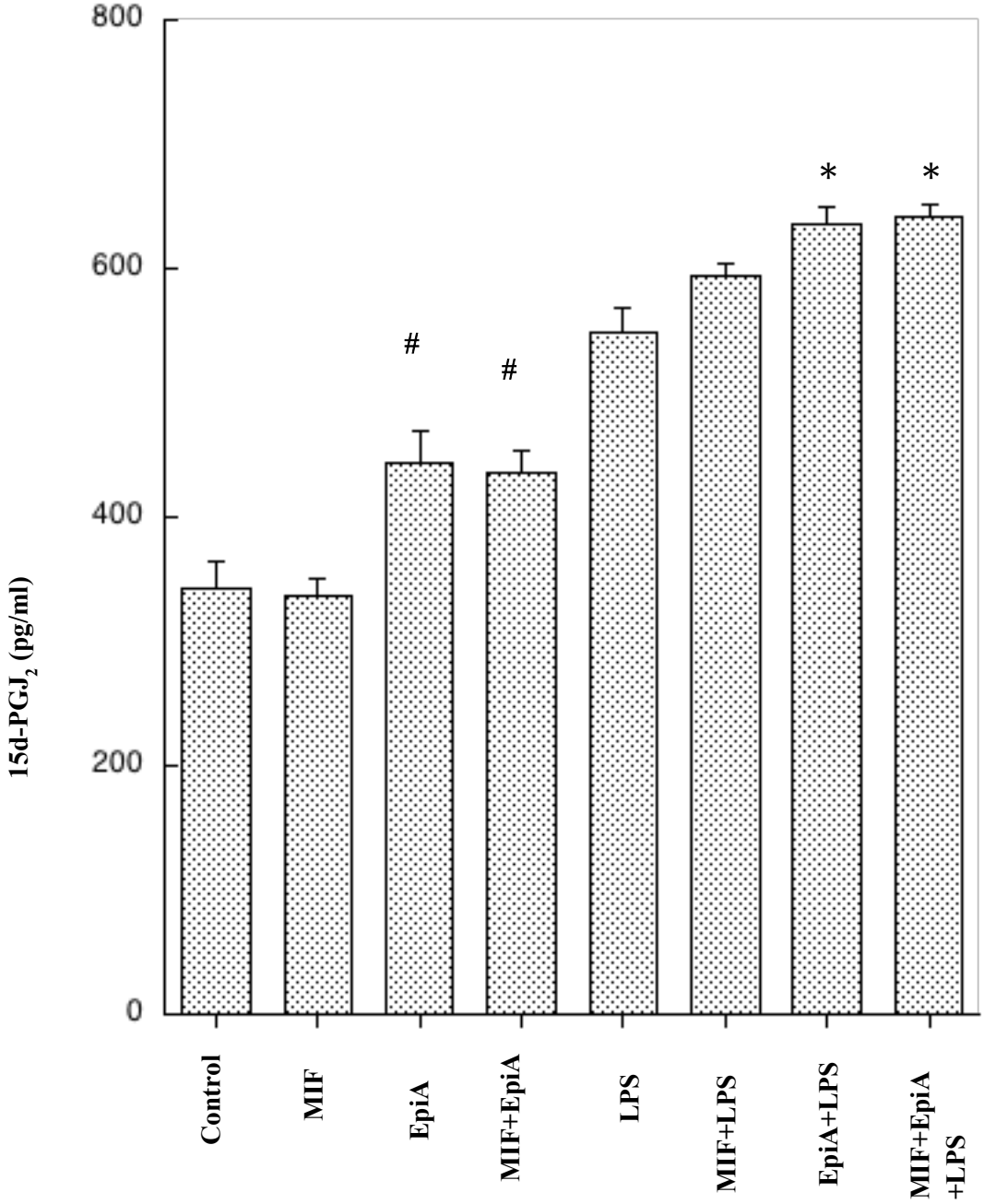

Whole human blood was incubated at 37 °C, 5% CO2 in air for 20 h with 1% DMSO (v/v) in media (control), 100µM of mifepristone or 0.001 µM 7β OH-EpiA alone or in companied with 10µg/ml LPS. Concentrations s of $PGE_2$ present in plasma were measured by EIA. Values are the means of n = 3 ± s.d. *P < 0.05 versus LPS alone.

**Fig 3.45 Effect of Mifepristone (GR/PR Antagonist) on the Ability of 7β OH-EpiA to Reduce Production of $PGE_2$ in Response to LPS Stimulation in Human Isolated Monocytes**



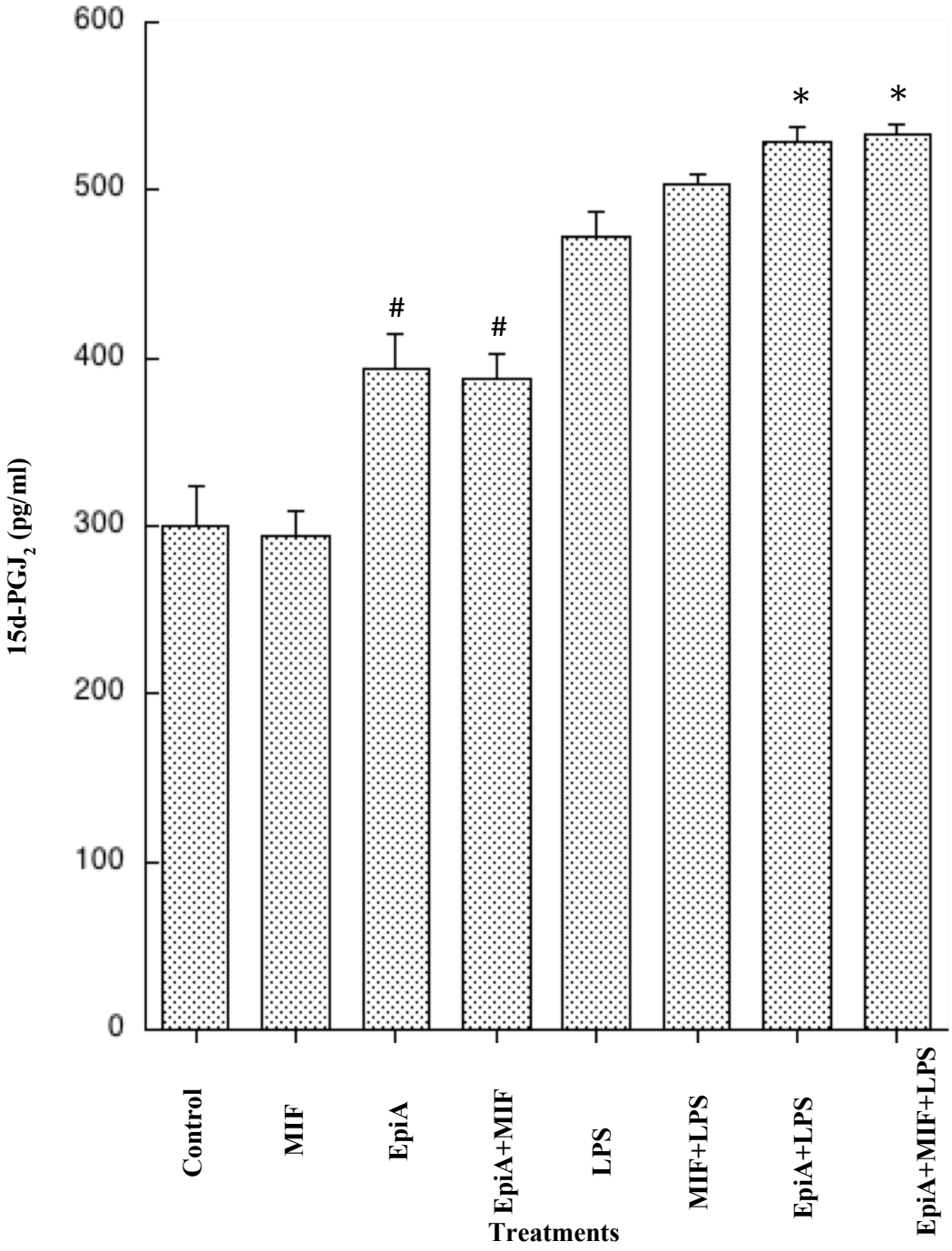

Monocytes were isolated by Ficoll/Histopaque density gradient centrifugation and maintained at 37 °C, 5% CO2 in air for 20 h. Cells were then incubated for 4 h with either 1% DMSO (v/v) in media (control), 100μM of mifepristone or 0.001 μM 7β OH-EpiA alone or in companied with 10μg/ml LPS. Concentrations of $PGE_2$ present in supernatants were measured by EIA. Values are the means of n = 3 ± s.d. *P < 0.05 versus LPS alone.

**Fig 3.46 Effect of Mifepristone (GR Antagonist) on the Ability of 7β OH-EpiA to Enhance Production of 15d-$PGJ_2$ in Response to LPS Stimulation in Human Blood**




Whole human blood was incubated at 37 °C, 5% CO2 in air for 20 h with 1% DMSO (v/v) in media (control), 100μM of mifepristone or 0.001 μM 7β OH-EpiA alone or accompanied with 10μg/ml LPS. Concentrations of 15d-$PGJ_2$ present in plasma were measured by EIA. Values are the means of n = 3 ± s.d. *P < 0.05 versus LPS alone, and #P < 0.05 versus control incubations.

**Fig 3.47 Effect of Mifepristone (GR/PR Antagonist) on the Ability of 7β OH-EpiA to Enhance Production of 15d-$PGJ_2$ in Response to LPS Stimulation in Isolated Monocytes**




Monocytes were isolated by Ficoll/Histopaque density gradient centrifugation and maintained at 37 °C, 5% CO2 in air for 20 h. Cells were then incubated for 4 h with either 1% DMSO (v/v) in media (control), 100μM of mifepristone or 0.001 μM 7β OH-EpiA alone or accompanied with 10μg/ml LPS. Concentrations of 15d-$PGJ_2$ present in supernatants were measured by EIA. Values are the means of n = 3 ± s.d. *P < 0.05 versus LPS alone, and #P < 0.05 versus control incubations.

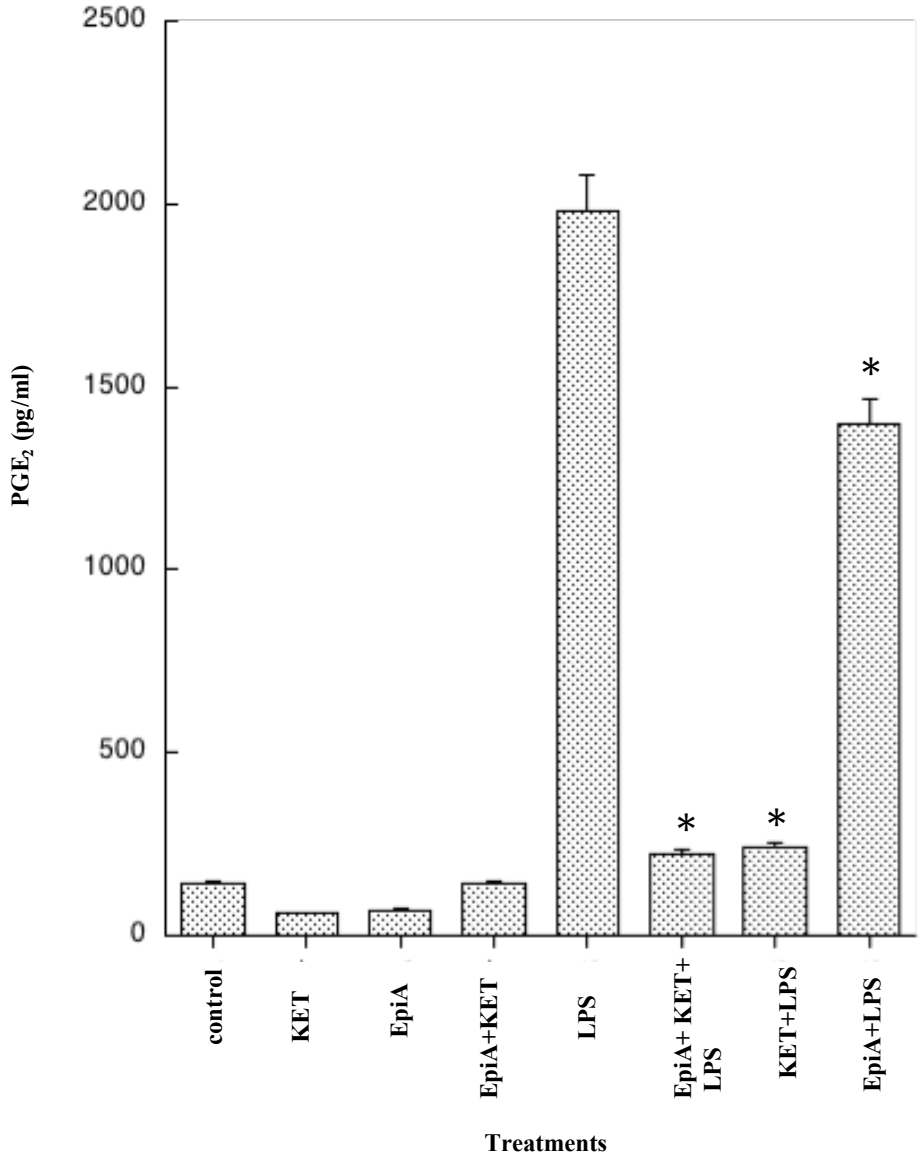
## 3.13 Effect of the NSAID Ketoprofen on 7β OH-EpiA-Induced Modification of $PGE_2$ and15d-$PGJ_2$ Production in Whole Human Blood and Isolated Monocytes

Previous experiments in this study indicated that the inhibition of TNF-α by 7β OH-EpiA was suppressed ( alleviated) by ketoprofen, a NSAID cyclooxygenase inhibitor. This implied that a PG was most likely involved in the inhibitory actions of 7β OH-EpiA. Thus, experiments were carried out to directly confirm the actions of ketoprofen on the production of $PGE_2$ and 15d-$PGJ_2$ in whole human blood and isolated monocytes in the current experimental conditions.

In whole blood, 7β OH-EpiA alone decreased $PGE_2$ levels in plasma and also decreased the LPS-stimulated increase in $PGE_2$ concentrations (Fig. 3.48). Ketoprofen alone reduced $PGE_2$ levels and also abolished the LPS-stimulated increase in $PGE_2$ levels both in the absence and presence of 7β OH-EpiA (Fig. 3.48).

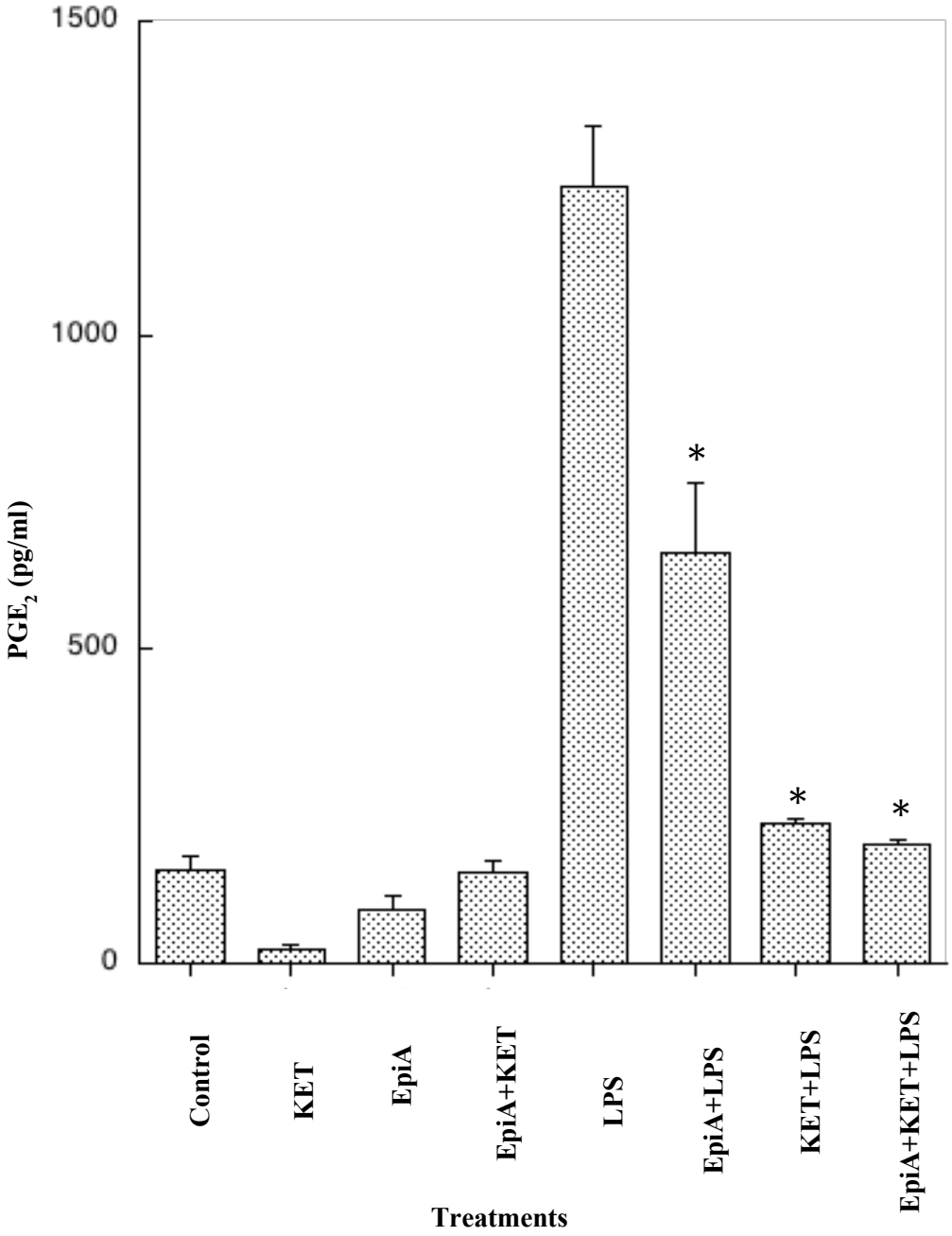
In isolated monocytes similar results were observed to those in whole blood. 7β OH-EpiA alone decreased $PGE_2$ levels in cell supernatants and also decreased the LPS-stimulated increase in $PGE_2$ levels (Fig. 3.49). Ketoprofen alone reduced $PGE_2$ levels and also abolished the LPS-stimulated increase in $PGE_2$ levels both in the absence and presence of 7β OH-EpiA (Fig. 3.49).

**Fig 3.48 Effect of the NSAID Ketoprofen on 7β OH-EpiA Induced Suppression of LPS Stimulated $PGE_2$ Production in Whole Human Blood**




Whole human blood was incubated at 37 °C, 5% CO2 in air for 20 h with 1% DMSO (v/v) in media (control), 50μM of ketoprofen or 0.001 μM 7β OH-EpiA alone or in companied with 10μg/ml LPS. Concentrations of $PGE_2$ present in plasma were measured by EIA. Values are the means of n = 3 ± s.d. *P < 0.05 versus LPS alone.

**Fig 3.49 Effect of NSAID (Ketoprofen) on 7β OH EpiA Induced Suppression of LPS Stimulated PGE2 Production in Human Isolated Monocytes**



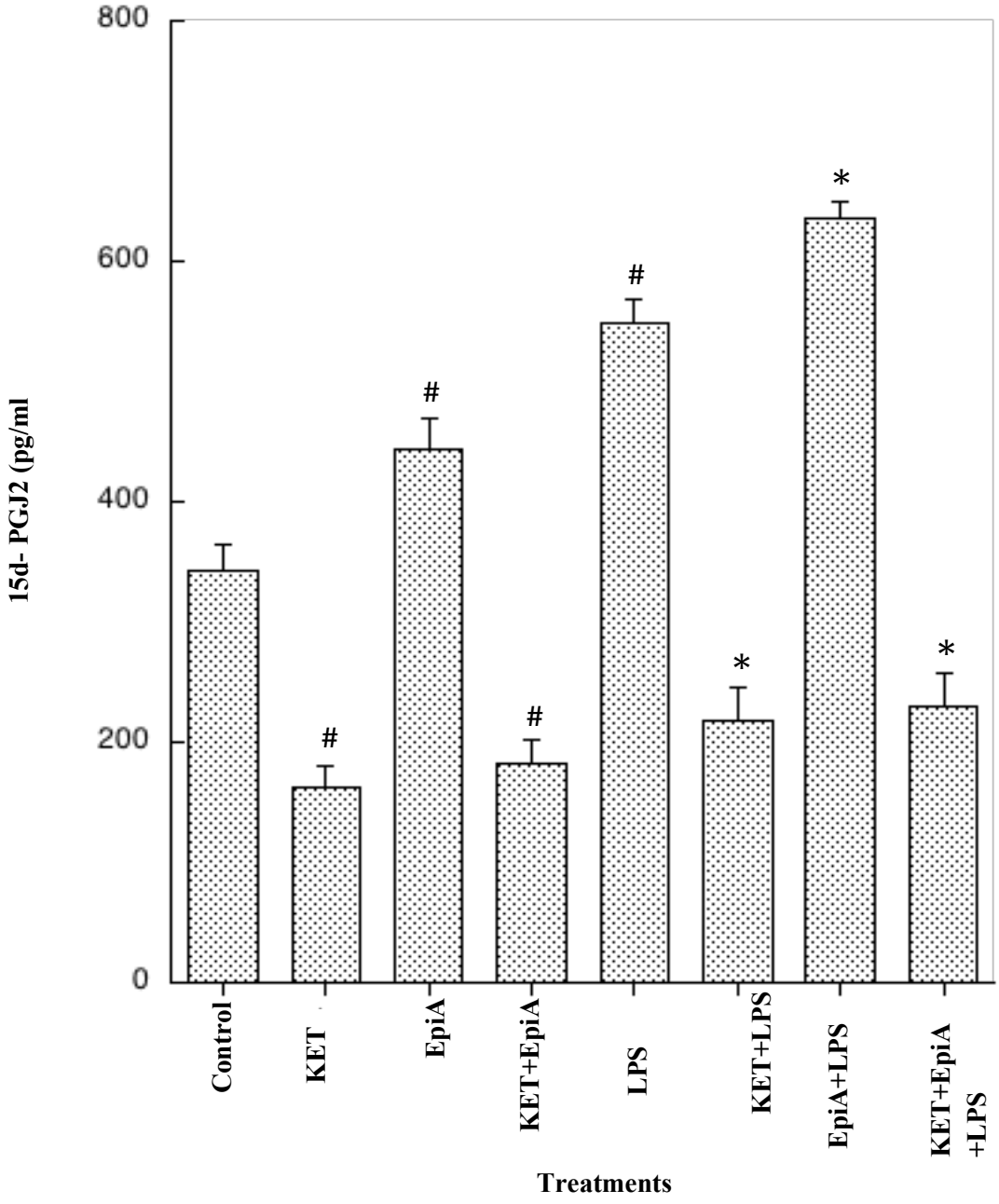

Monocytes were isolated by Ficoll/Histopaque density gradient centrifugation and maintained at 37 °C, 5% CO2 in air for 20 h. Cells were then incubated for 4 h with either 1% DMSO (v/v) in media (control), 50μM of ketoprofen or 0.001 μM 7β OH-EpiA alone or in companied with 10μg/ml LPS. Concentrations of $PGE_2$ present in plasma were measured by EIA. Values are the means of n = 3 ± s.d. *P < 0.05 versus LPS alone.

## 3.14 Effect of the NSAID Ketoprofen on 7β OH-EpiA Induced Enhancement of LPS Stimulated 15d-PGEJ$_2$ Production in Whole Human Blood

In whole human blood, 7β OH-EpiA alone enhanced 15d-PGJ$_2$ levels in plasma and also enhanced the LPS-stimulated increase in 15d-PGJ$_2$ levels (Fig. 3.50). Conversely, ketoprofen alone reduced 15d-PGJ$_2$ levels and also abolished the LPS-stimulated increase in 15d-PGJ$_2$ levels both in the absence and presence of 7β OH-EpiA (Fig. 3.50).

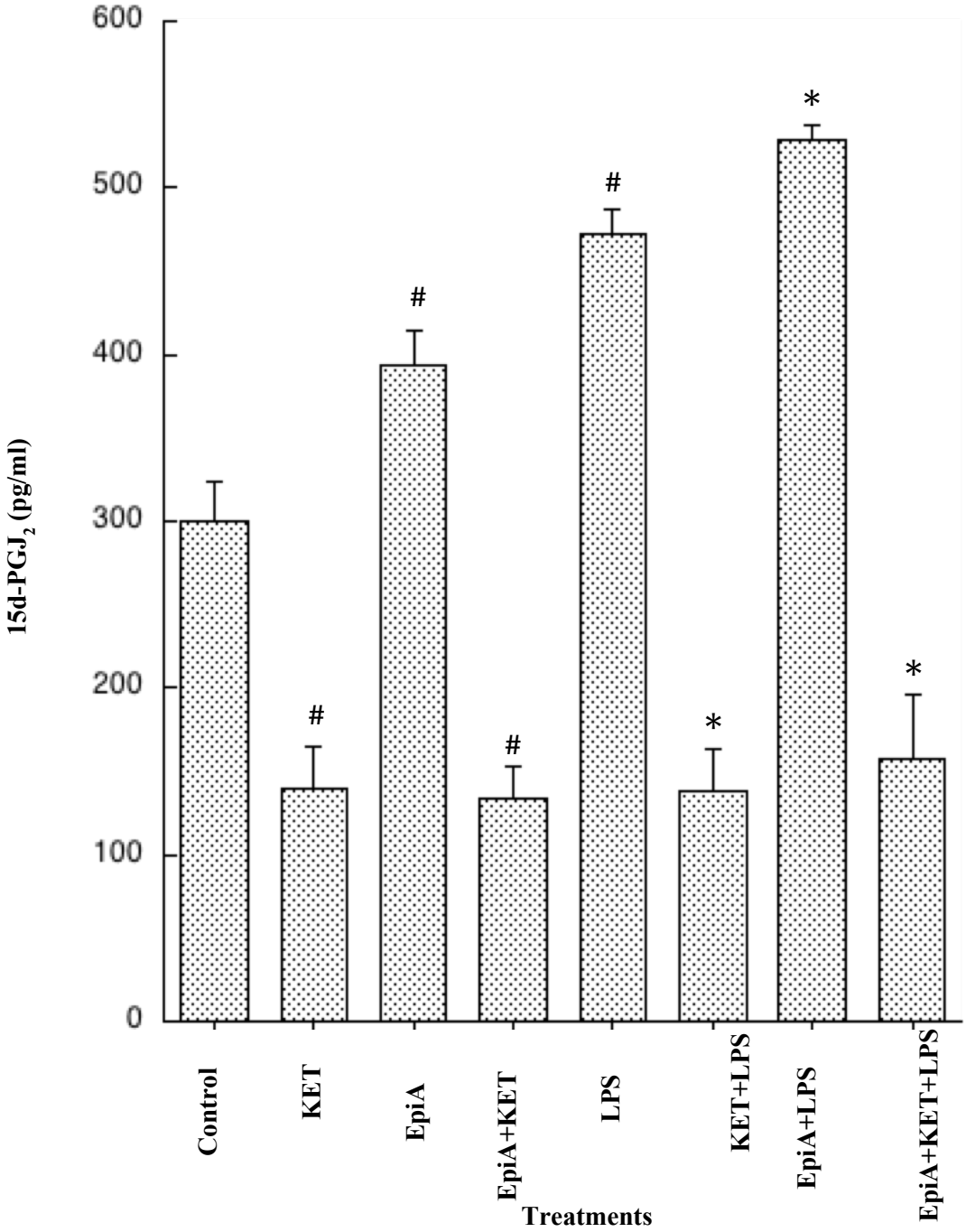
In isolated monocytes, results observed for ketoprofen were similar to those in whole blood. 7β OH-EpiA alone enhanced 15d-PGJ$_2$ levels in cell supernatants and also enhanced the LPS-stimulated increase in 15d-PGJ$_2$ levels (Fig. 3.51). Ketoprofen alone reduced 15d-PGJ$_2$ levels and also abolished the LPS-stimulated increase in 15d-PGJ$_2$ levels both in the absence and presence of 7β OH-EpiA (Fig. 3.51) indicating that 7β OH-EpiA was unable to affect the ketoprofen inhibition suggesting that the effect of 7β OH-EpiA is downstream of cyclooxygenase.

**Fig 3.50 Effect of the NSAID Ketoprofen on 7β OH-EpiA-Induced Enhancement of LPS Stimulated 15d-PGEJ$_2$ Production in Whole Human Blood**




Whole human blood was incubated at 37 °C, 5% CO2 in air for 20 h with 1% DMSO (v/v) in media (control), 50μM of ketoprofen or 0.001 μM 7β OH-EpiA alone or in companied with 10μg/ml LPS. Concentrations of 15d-PGJ$_2$ present in plasma were measured by EIA. Values are the means of n = 3 ± s.d. *P < 0.05 versus LPS alone, and #P < 0.05 versus control incubations.

**Fig 3.51 Effect of the NSAID Ketoprofen on 7β OH-EpiA-Induced Enhancement of LPS Stimulated 15d-PGJ$_2$ Production in Isolated Monocytes**




Monocytes were isolated by Ficoll/Histopaque density gradient centrifugation and maintained at 37 °C, 5% CO2 in air for 20 h. Cells were then incubated for 4 h with either 1% DMSO (v/v) in media (control), 50μM of ketoprofen or 0.001 μM 7β OH-EpiA alone or in company with 10μg/ml LPS. Concentrations of 15d-PGJ$_2$ present in plasma were measured by EIA. Values are the means of n = 3 ± s.d. *P < 0.05 versus LPS alone and #P < 0.05 versus control incubations.

## 3.15 The Effect of Arachidonic Acid on the Ability of THP-1 Cells to Produce PGs in in the Presence of LPS and 7β-OH-EpiA

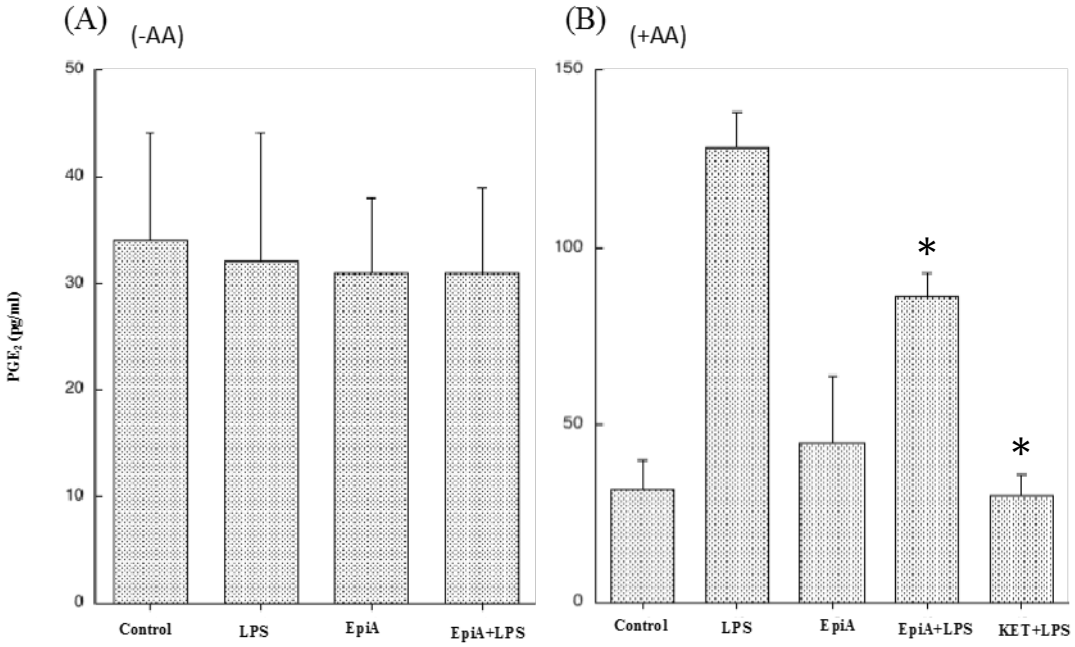
Previous experiments on the production of cytokines used the human monocytic cell line THP-1 which responded very well, and their functional profile was identical to that of normal isolated monocytes. However, in the studies measuring prostaglandin production, only very low levels of the various PGs could be detected, and the cells did not appear to respond to stimuli such as LPS, i.e. there were no changes in the low levels of the PGs. As the immediate precursor for PG production is arachidonic acid it is possible that the low levels of PG production may be due to an insufficient quantity of arachidonic acid in the THP-1 cells. In normal cells the arachidonic acid would be supplied via the blood carrying arachidonic acid from the diet. The only supply of arachidonic acid that THP-1 cells would have be would the low levels (and presumably variable) present in the foetal calf serum supplied in the medium for the cells during continuous culture. It was, therefore, decided to investigate whether supplementing culture medium with exogenous arachidonic acid would a) affect PG production in THP-1 cells and b) allow them to respond to stimuli such as LPS in future studies.

THP-1 cells were cultured in medium supplemented with arachidonic acid (10 μM). The responses of these cells were compared to the responses of cells cultured in normal medium. In THP-1 cells cultured in normal medium the low level of $PGE_2$ was not altered in the presence of either LPS or 7β OH-EpiA. In contrast, with THP-1 cells cultured in arachidonic acid-supplemented medium, an increase in $PGE_2$ levels was observed in the presence of LPS. This was approximately 4-fold higher than in cells without arachidonic acid. In addition, 7β OH-EpiA attenuated the LPS-stimulated increase in $PGE_2$ levels (Fig. 3.52). Ketoprofen also inhibited the LPS-stimulated

increase confirming that cyclooxygenase activity was responsible for the $PGE_2$ detected.

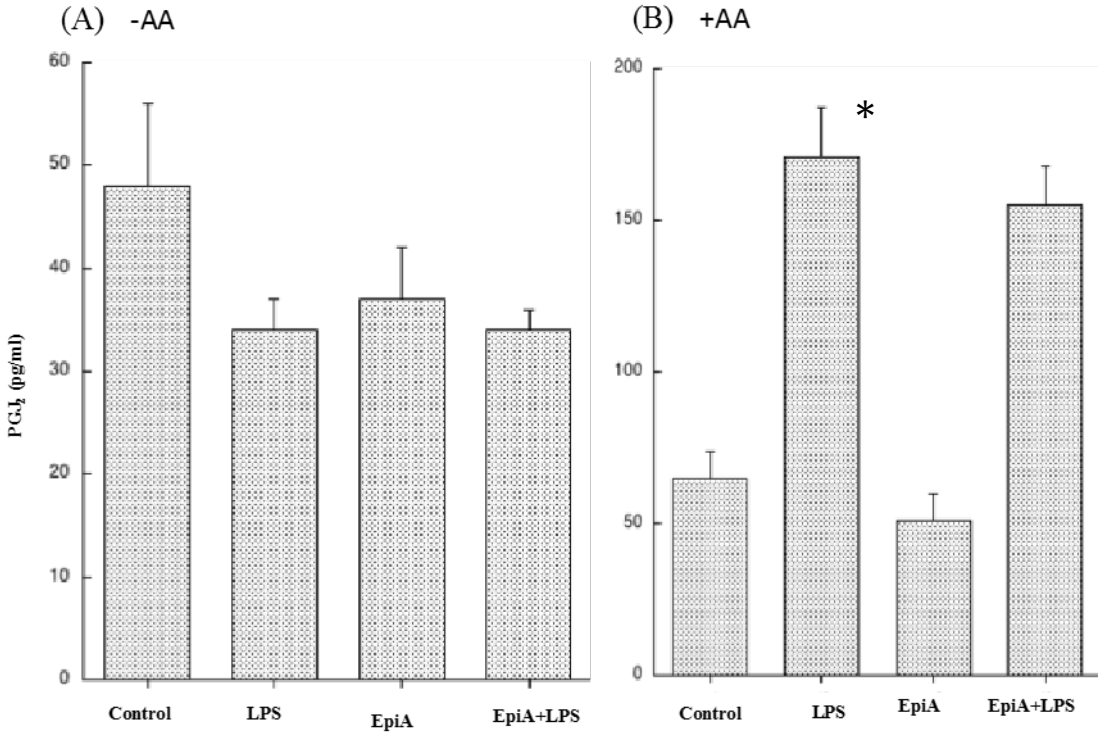
The levels of 15d-$PGJ_2$ in THP-1 cells cultured in medium supplemented with arachidonic acid (10 μM) were also measured. In THP-1 cells cultured in normal medium, the low level of 15d-$PGJ_2$ was not altered in the presence of either LPS or 7β OH-EpiA. In contrast, with THP-1 cells cultured in arachidonic acid-supplemented medium, an increase in 15d-$PGJ_2$ levels was observed in the presence of LPS. This was approximately 3.5-fold higher than in cells without arachidonic acid. 7β OH-EpiA did not affect the LPS-stimulated increase in 15d-$PGJ_2$ levels (Fig. 3.53).

**Fig 3.52 The Effect of Arachidonic acid on the Ability of THP-1 Cells to Produce $PGE_2$ in the Presence of LPS and 7β-OH-EpiA**




THP-1 cells were incubated without (A) or with (B) arachidonic acid (10μM) for 7 days at 37 °C, 5% CO2 in air. Cells were then incubated with either 1% DMSO (v/v) in media (control), 50μM of ketoprofen or 1 μM 7β OH-EpiA alone or in combination with LPS (10μg/ml) for 20 h. Concentrations of $PGE_2$ present in supernatants were measured by EIA. Values are the means of n = 3 ± s.d. *P < 0.05 versus LPS alone, and #P < 0.05 versus control incubations.

**Fig 3.53 The Effect of Arachidonic acid on the Ability of THP-1 Cells to Produce 15d-PGJ$_2$ in the Presence of LPS and 7β-OH-EpiA**




THP-1 cells were incubated without (A) or with (B) arachidonic acid (10μM) for 7 days at 37 °C, 5% CO2 in air. Cells were then incubated with either 1% DMSO (v/v) in media (control), or 1 μM 7β OH-EpiA alone or in combination with LPS (10μg/ml) for 20 h. Concentrations of 15d-PGJ$_2$ present in supernatants were measured by EIA. Values are the means of n = 3 ± s.d. *P < 0.05 versus control incubations.

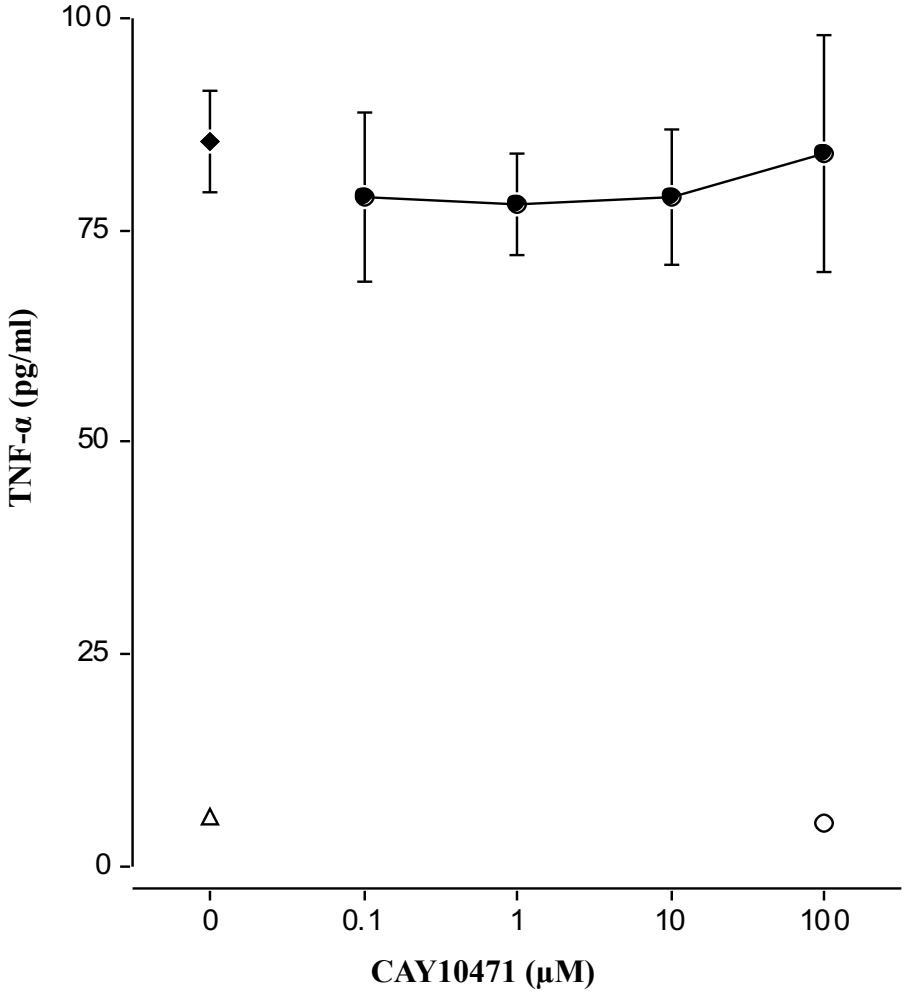
## 3.16 Effect of CAY10471 (DP2 antagonist) on LPS-Stimulated TNF-α Production in Whole Human Blood and its Effects on 7β OH-EpiA- and 15d-$PGJ_2$-induced Suppression

Previous experiments in this study indicated that the suppressive actions of 7β OH-EpiA were inhibited by ketoprofen, suggesting the involvement of a PG. 7β OH-EpiA was able to upregulate the production of both $PGD_2$ and 15d-$PGJ_2$ in whole human blood and isolated monocytes. In addition, it was confirmed that both of these PGs could also inhibit TNF- α production, suggesting that a PG which acts on DP receptors may be responsible for the end actions of 7β OH-EpiA on cytokine production.

Initial experiments were carried out to ascertain the actions of CAY10471 (a DP2/ CRTH2 receptor antagonist) alone on TNF-α production in whole blood before use in further experiments. This was carried out by using various concentrations of CAY10471 (0.1 - 100 μM) in the presence and absence of LPS (10μg/ml). CAY10471 did not alter the LPS-stimulated elevation of TNF- α nor did it have any effect on basal (control) levels at the highest concentration used (100 μM) alone (Fig. 3.54).

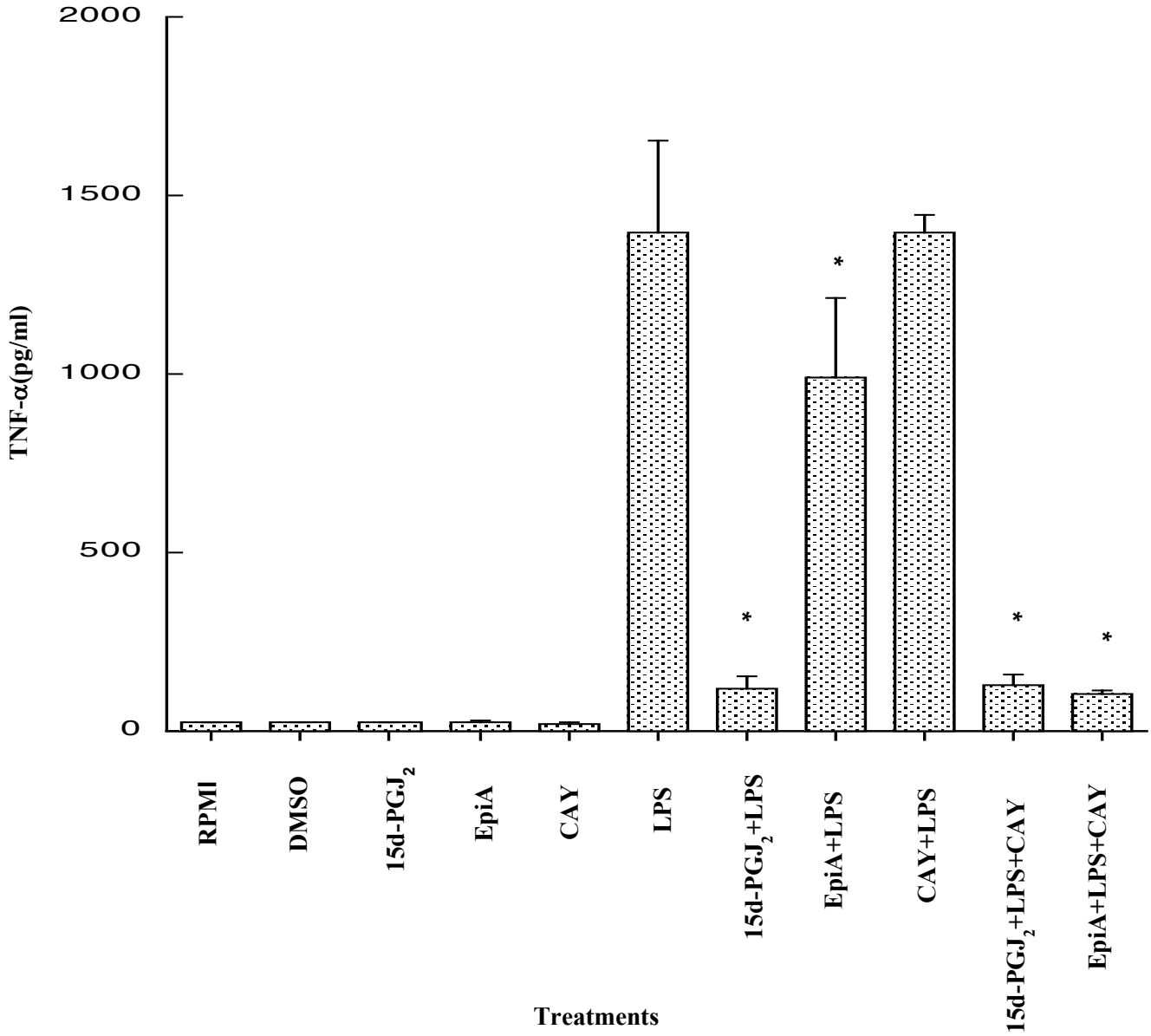
The effects of CAY10471 were ascertained on the actions of 7β OH-EpiA and 15d-$PGJ_2$ on LPS-stimulated TNF-α production in blood. The LPS-induced elevation in TNF-α levels were reduced by both 7β OH-EpiA and 15d-$PGJ_2$ as shown previously. CAY10471 did not reverse the actions of either compound nor did it affect the LPS stimulation. However, it did appear to amplify the suppression in response to 7β OH-EpiA (Fig. 3.55). This would imply that the actions of 15d-$PGJ_2$ are not mediated via a DP2 receptor and in agreement with this, any mediator of the actions of 7β OH-EpiA also do not appear to act via a DP2 receptor.

**Fig 3.54 Effect of CAY10471 (DP2 antagonist) on LPS-Stimulated TNF-α Production in Whole Human Blood**




Human blood was incubated for 20 h either with culture media alone (empty triangle), 10μg/ml LPS alone (filled diamond), 100μM CAY10471 alone (empty circle) or with increasing concentration of CAY10471 in the presence of 10μg/ml LPS (filled circle). Samples were then centrifuged and the concentration of TNF-α in plasma were determined by ELISA. All these results represent three independent experiments from single blood donor. Values are expressed as the means of n = 3 ± s.d.

**Fig 3.55 Effect of CAY10471 (DP2 antagonist) on 15d-$PGJ_2$- and 7β OH-EpiA-induced Suppression of LPS-stimulated TNF-α Production in Whole Human Blood**




Human blood was incubated for 20 h either with culture media, DMSO, 10μg/ml LPS, 10 μM $PGJ_2$, 0.001μM EpiA, 10μM CAY10471 alone or in combination with 10μg/ml LPS with or without CAY10471. Incubations were then centrifuged and the concentration of TNF-α in plasma was determined by ELISA. Values are expressed as the means of n = 3 ± s.d. *P < 0.05 versus LPS alone.

## 3.17 Cell Viability in response to Various Compounds Used

Experiments were carried out to evaluate the effect of the various compounds on cell viability. This was achieved by incubating cells in the presence of the various compounds used in the current study, after which MTT dye was added and the conversion to coloured formazan product was measured by absorbance at 540 nm. The effects of the various compounds were expressed as a percentage of the control value, cells incubated alone in culture medium.

Almost all of the agents used in the current study did not have an effect on cell viability except for dexamethasone and 15d-$PGJ_2$ (Table 3.1).

**Table 3.1 The Effect of Various Agents Used in the Present studies on the Viability of THP-1 Cells Estimated by MTT Assay**

| Treatment | Cell viability % ± s.d. |
|---|---|
| Control (RPMI 1640 alone) | 100.0 ± 1.09 |
| DMSO | 97.9 ± 1.30 |
| LPS | 100.9 ± 0.21 |
| 7β EpiA 10μM | 100.8 ± 1.10 |
| 7β EpiA 0.01μM | 101.6 ± 1.21 |
| 7β EpiA 0.01μM+LPS | 101.2 ± 0.21 |
| Dexamethasone | 110.4 ± 1.14 * |
| $PGE_2$ | 104.9 ± 1.23 |
| $PGD_2$ | 102.7 ± 1.32 |
| 15d-$PGJ_2$ | 105.3 ± 0.93 * |
| Ketoprofen | 100.1± 1.35 |
| Dead Cells | 0 ± 0.12 |

THP-1 cells (1×106 cells/ml) of RPMI 1640) were placed into 96-well plates and different treatments were added as follows; 0.1 % DMSO, LPS (10 μg/ml), 7β OH-EpiA, dexamethasone (100 μM), $PGE_2$ (10 μM), $PGD_2$ (10 μM), 15d-$PGJ_2$ (10 μM), ketoprofen (50 μM) and dead cells (obtained by boiling cells for 2 min and allowing to cool before adding to incubation plates). The plates were then incubated at 37°C, 5% CO2, 100% humidity for 24 h. MTT solution was then added to cells and incubations continued for a further 4h. DMSO (100%) was then added and plates incubated for 4h prior to the measurement of absorbance at 540 nm (see methods section). All values are the mean ± s.d. of n = 5, *P < 0.05.

## 3.18 Gene Expression Studies

Various experiments were carried out to ascertain the effects of 7β OH-EpiA on the expression of genes relevant to the processes which 7β OH-EpiA has been previously shown to modulate at the functional level in the present study. This was achieved by incubating cells with the various agents already shown to have effects followed by extraction of mRNA and quantitation of specific gene products by quantitative RT-PCR (qRT-PCR). These experiments were carried out in THP-1 cells in order to have sufficient amounts of sample and reduce variability. A few preliminary experiments showed that isolated monocytes were at times low in numbers and variable in mRNA extraction.

### 3.18.1 Reference Gene Stability in THP-1 cells

The stability of reference genes, which were used in this study (PPIB, RPL37A, HPRT1 ACTB and GAPDH) were validated using a comprehensive ranking of the stability of candidate reference genes via the free available program RefFinder (http://leonxie.esy.es/RefFinder/) (De Spiegelaere *et al*., 2015). The results were collected from the three common analysis gene stability programmes, which are geNorm, BestKeeper and NormFinder. The results showed that PPIB was the most stable control gene ranked by all three programs. Since genes, which have a lower mean (M value) with low standard deviation (SD) are considered to be the most stable reference gene calculated by geNorm and BestKeeper programs whereas a high value represented less stable expression (Andersen *et al*., 2004; Pfaffl *et al*., 2004; Vandesompele *et al.*, 2002b). PPIB was deemed to be the most invariant or stably expressed out of the candidate genes tested because its M value was below 1.5 with SD of 0.09. The candidate reference genes tested had higher values as listed in table (3.2).

**Table 3.2 Stability of Reference Genes in THP-1 cells by NormFinder, geNorm and BestKeeper.**

Most Stable ↑

Least Stable ↓

| Rank | GeNorm | BestKeeper | NormFinder |
|---|---|---|---|
| **1** | PPIB | PPIB | PPIB |
| **2** | β-ACTIN | RPL37A | β-ACTIN |
| **3** | RPL37A | β-ACTIN | HPRT1 |
| **4** | HPRT1 | HPRT1 | RPL37A |
| **5** | GAPDH | GAPDH | GAPDH |

The stability of various gene expression, A low rank number indicates the most stable and a high rank shows the least stable genes. The results show that PPIB was the most stable reference gene according to those tested and included in this list. This summarised three different validation programs: NormFinder, geNorm and BestKeeper.

**Table 3.3 Stability Analysis of Selective Reference Genes by geNorm (M value) and BestKeeper (SD value).**

| Reference gene | M value (geNorm) | SD value (BestKeeper) |
|---|---|---|
| PPIB | 1.3 | 0.09 |
| β ACTIN | 1.58 | 1.93 |
| RPL37A | 1.90 | 1.20 |
| HPRT1 | 2.0 | 0.90 |
| GAPDH | 2.1 | 1.4 |

The stability expression of reference genes as illustrated in many studies have an M value lower than 1 and are preferred to be at least lower than 1.5 (Cao *et al*., 2012). In this list, PPIB appears to be the most stable reliable control gene.

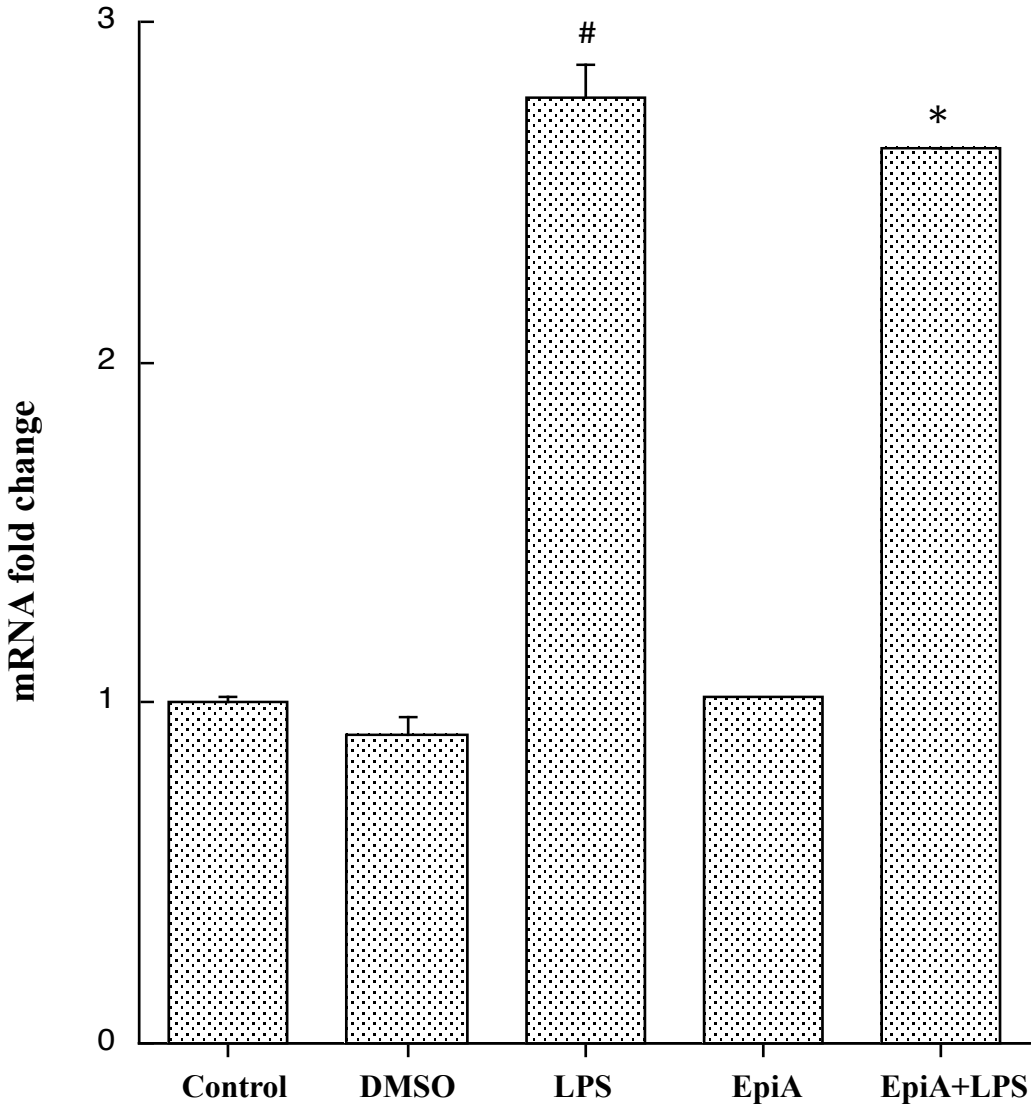
### 3.18.2 Effect of 7β OH-EpiA on Expression of Genes Involved in LPS Responses

In this series of experiments, the effects of 7β OH-EpiA on the levels of mRNA for genes involved in the responses to LPS were studied; including its receptor (TLR 4), a target cytokine (TNF-α), an enzyme involved in the biosynthesis of a mediator suppressing LPS actions (PGDS) and a receptor which mediates suppression of LPS responses (DP2 receptor).

#### 3.18.2.1 Effect of 7β OH-EpiA on Expression of the Toll Like Receptor 4 (TLR- 4) in THP-1 Cells

It was previously shown that 7β OH-EpiA suppresses both TNF-α and IL-1β production in response to LPS. The first component in the chain of processes that occur in responding to LPS, the receptor, is an obvious potential target resulting in suppression. Thus, measurements of TLR 4 mRNA were carried out to evaluate the effects of 7β OH-EpiA. THP-1 cells were incubated with (0.001 μM) EpiA with or without (10 μg/ml) LPS followed by extraction of total mRNA and qRT-PCR with primers for the TLR 4 gene as described in the methodology section. Incubation with LPS resulted in an increase (2.78 ± 0.1) in the fold-induction of mRNA levels for the TLR 4 gene (Fig. 3.56). 7β OH-EpiA alone did not have any effect on TLR 4 expression and remained close to the control levels of expression but showed a very small reduction (2.63 ± 0.002) in the LPS-induced expression (Fig. 3.56).

**Fig 3.56 The effect of 7β OH-EpiA on TLR-4 mRNA Expression in THP-1 Cells**

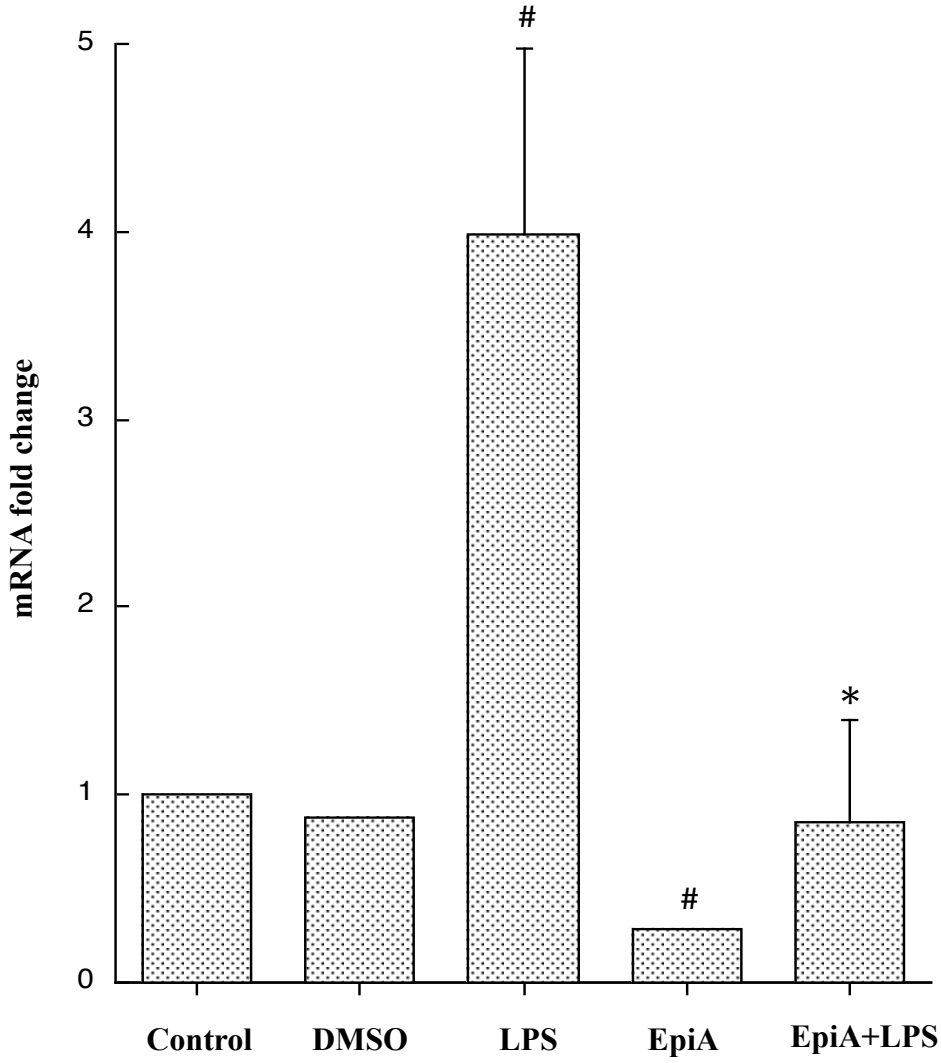



Cells ($2x10^6$ cells/ml) were placed into 6-well plates after which LPS (1μg/ml), EpiA (0.01μM) or a combination of EpiA with LPS, DMSO alone and culture medium (control) were added and cells incubated for 24 h. Total RNA was prepared from the cells. After qPCR, analysis was performed on the cDNA using selected primers for TLR 4 as described in the methods section. Relative expression concentrations of TLR4 mRNA transcripts were normalised to the reference gene PPIB using the delta-delta Ct method (Livak & Schmittgen, 2001). Values are the means of n = 3 ± s.d. *P <0.05 versus LPS alone, #P < 0.05 versus control incubations.

### 3.18.2.2 Effect of 7β OH-EpiA on mRNA Expression of the Tumour Necrosis Factor-α (TNF-α) Gene in THP-1 Cells

As it was previously shown that 7β OH-EpiA reduced the production of TNF-α in response to LPS, it was decided to ascertain if this was an action at the level of the expression of the TNF-α gene and not on the release processes. THP-1 cells were incubated with (0.001 μM) EpiA with or without (10 μg/ml) LPS followed by extraction of total mRNA and qRT-PCR with primers for the TNF-α gene as described in the methodology section. Incubation with LPS resulted in an increase (4.15 ± 2.58) in the fold-induction of mRNA levels for the TNF-α gene (Fig. 3.57). 7β OH-EpiA alone reduced TNF-α expression below the control levels of fold expression (0.48 ± 0.06) and also greatly reduced the LPS-stimulated fold expression (0.84 ± 1.9) of the TNF-α gene (Fig. 3.57).

**Fig 3.57 The Effect of 7β OH-EpiA on TNF-α Gene Expression (mRNA) in THP-1 Cells**



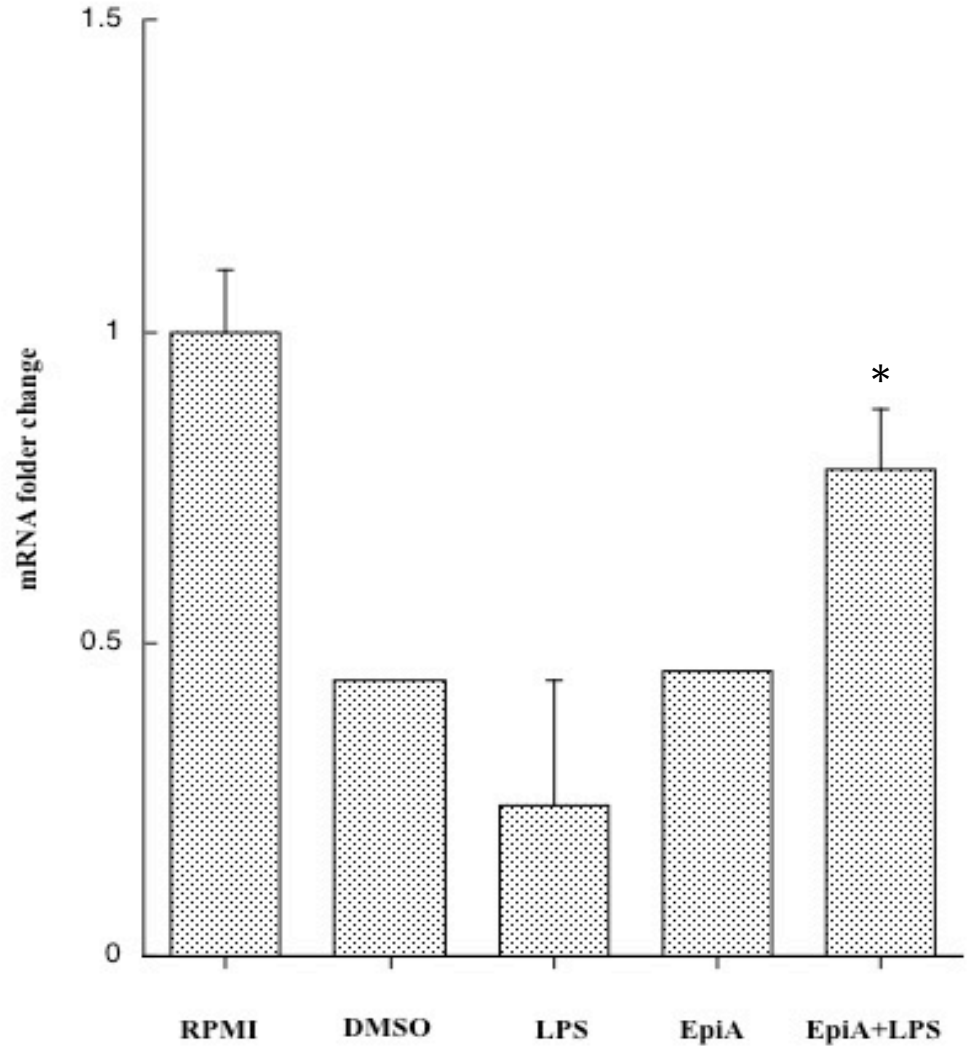

Cells ($2x10^6$ cells/ml) were placed into 6-well plates after which LPS (1μg/ml), EpiA (0.01μM), combination of EpiA with LPS, DMSO alone and culture medium (control) were added and cells incubated for 24 h. Total RNA was prepared from the cells. After reverse transcription, quantitative real-time PCR (qPCR) analysis was performed on the cDNA using selected primers for TNF-α as described in the method section. Relative expression concentrations of TNF-α mRNA transcripts were normalised to the reference gene PPIB using the delta-delta Ct method (Livak & Schmittgen, 2001). Values are the means of n = 3 ± s.d. *P <0.05 versus LPS alone, #P < 0.05 versus control incubations.

### 3.18.2.3 Effect of 7β OH-EpiA on mRNA Expression of the Prostaglandin $D_2$ Synthase (PTGDS) Gene in THP-1 Cells

7β OH-EpiA in the present study has been shown to reduce both TNF-α and IL-1β production in response to LPS. This effect appeared to involve a PG-mediator, as the effect was inhibited by the cyclooxygenase inhibitor, ketoprofen. Taken with the observation that 7β OH-EpiA also selectively enhanced the production of $PGD_2$ and 15d-$PGJ_2$ this implies that a post-cyclooxygenase process is involved. 15d-$PGJ_2$ is produced as a spontaneous conversion of $PGD_2$, thus, a potential candidate is the enzyme which produces $PGD_2$, prostaglandin $D_2$ synthase. Therefore, the effect of 7β OH-EpiA on the expression of the prostaglandin $D_2$ synthase gene (PTGDS) was evaluated. THP-1 cells were incubated with (0.001 μM) EpiA with or without (10 μg/ml) LPS followed by extraction of total mRNA and qRT-PCR with primers for the PTGDS gene as described in the methodology section.

Incubation with LPS resulted in a decrease (0.2 ± 0.1) ($P = <0.01$) in the fold-induction of mRNA levels for the PTGDS gene compared to control (Fig. 3.58). However, there was no effect compared to the solvent control (DMSO). 7β OH-EpiA alone reduced PTGDS fold expression below the control levels of expression (0.5 ± 0.3) but was similar to the DMSO control. Compared to its DMSO solvent, 7β OH-EpiA in the presence of LPS, increased the expression fold (0.83±0.002) of PTGDS mRNA (Fig. 3.58).

**Fig 3.58 The Effect of 7β OH-EpiA on PTGDS mRNA Expression in THP-1 Cells**



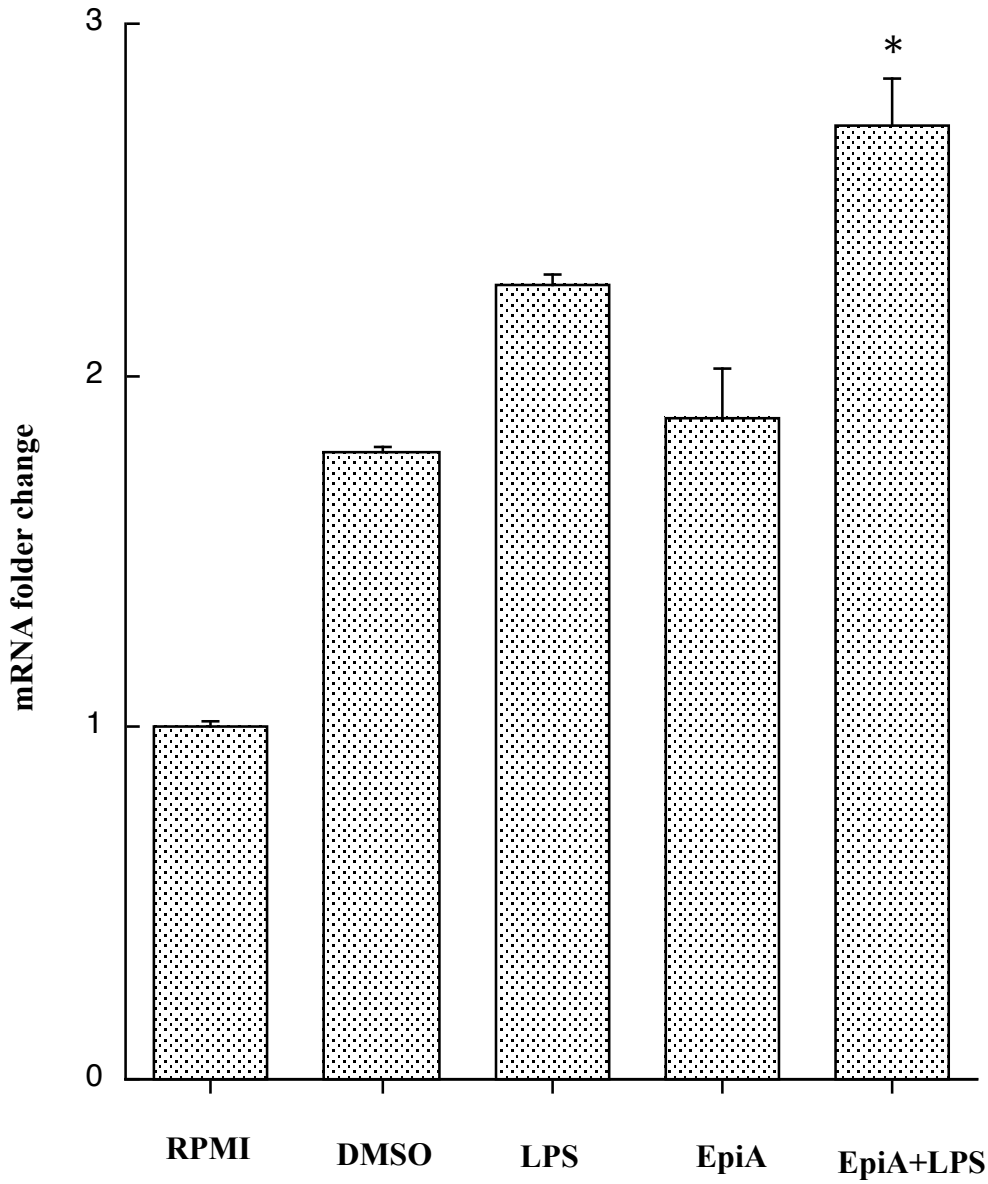

Cells ($2x10^6$ cells/ml) were placed into 6-well plates after which LPS (1μg/ml), EpiA (0.01μM), combination of EpiA with LPS, DMSO alone and culture medium (control) were added and cells incubated for 24 h. Total RNA was prepared from the cells. After reverse transcription, quantitative real-time PCR (qPCR) analysis was performed on the cDNA using selected primers for TNF-α as described in the method section. Relative expression concentrations of PGDS mRNA transcripts were normalized to the reference gene PPIB using the delta-delta Ct method (Livak & Schmittgen, 2001). Values are the means of n = 3 ± s.d. *P <0.01 versus DMSO solvent control.

#### 3.18.2.4 Effect of 7β OH-EpiA on Expression of the Prostaglandin $D_2$ DP2 Receptor (PTGDR2) in THP-1 Cells

Previous observations raised the possibility that either $PGD_2$ or its metabolite 15d-PGJ2 may be a mediator of the actions of 7β OH-EpiA. The more recently described receptor for $PGD_2$, the DP2 receptor, was chosen as a target in expression experiments, as the functional properties of this receptor had been studied using the actions of 7β OH-EpiA and 15d-$PGJ_2$ on the LPS-stimulated production of using a selective receptor antagonist.

Incubation of THP-1 cells with LPS resulted in an increase (2.28 ± 0.01) in the fold-induction of mRNA levels for the PTGDR2 gene compared to both culture medium control and the DMSO solvent control (Fig. 3.59). 7β OH-EpiA increased PTGDR2 expression above the control level of expression (1.81 ± 0.1) but was similar to the DMSO control. Compared to its DMSO solvent, 7β OH-EpiA in the presence of LPS showed a small increase in the expression (2.8 ± 0.3) of PTGDR2 mRNA (Fig. 3.59).

**Fig 3.59 The Effect of 7β OH-EpiA on PTGDR2 Expression in THP-1 Cells**




Cells ($2x10^6$ cells/ml) were placed into 6-well plates after which LPS (1μg/ml), EpiA (0.01μM), combination of EpiA with LPS, DMSO alone and culture medium (control) were added and cells incubated for 24 h. Total RNA was prepared from the cells. After reverse transcription, quantitative real-time PCR (qPCR) analysis was performed on the cDNA using selected primers for TNF-α as described in the method section. Relative expression concentrations of PTGDR2 mRNA transcripts were normalized to the reference gene PPIB using the delta-delta Ct method (Livak & Schmittgen, 2001). Values are the means of n = 3 ± s.d. *P <0.05 versus LPS alone and DMSO control.

# Chapter Four: Discussion

# 4. Discussion

## 4.1 Overall Discussion

There are many studies in vivo and in vitro focusing on the cytoprotective effects of the dehydroepiandrosterone metabolite 7β OH-epiandrosterone (Niro *et al*., 2012). It can suppress pathologies in a variety of tissues including brain, heart and the colon. It has been shown to exert neuroprotective effects in vivo at low doses in animal models by preventing neuronal cell death following cerebral ischemia (Pringle et al., 2003) as well as in Alzheimer's disease (Dudas *et al*., 2004). 7β OH-EpiA has been also shown to reduce dextran sodium sulphate induced colitis (Hennebert *et al*., 2008) and it also increases the production of cytoprotective prostaglandins such as $PGD_2$ and its direct metabolite 15d-$PGJ_2$ (Davidson *et al.*, 2008; Hennebert *et al*., 2008). As inflammatory responses which produce pro-inflammatory cytokines lead to pathologies in many tissues and these responses also result in the production of PGs, the aim of this study was to investigate whether 7β OH-EpiA had beneficial effects on pro-inflammatory cytokines (TNF-α and IL-1β) and anti-inflammatory cytokines (IL-4 and IL-10) in response to LPS as an important pathogenic stimulus. In addition, a further aim was to evaluate the effects 7β OH-EpiA on downstream PG production. LPS is a potent bacterial endotoxin that is able to potently stimulate a generalised, acute inflammatory reaction (Rietschel *et al*., 1994). This was studied in whole human blood, human isolated monocytes and also to an extent in the human monocytic cell line THP-1. It was also intended to evaluate the direct role of prostaglandins in the actions of 7β OH-EpiA. This was achieved pharmacologically by using the NSAID ketoprofen to ascertain whether there was any modification of the actions of 7β OH-EpiA on LPS-stimulated TNF-α and the production of several important PGs ($PGE_2$, $PGD_2$, 15d-$PGJ_2$). The potential involvement of steroid receptors,

particularly the glucocorticoid receptor, was also evaluated using the dual glucocorticoid / progesterone receptor antagonist, mifepristone. This was achieved by comparing the actions of 7β OH-EpiA with dexamethasone in the inhibition of TNF-α production. This was followed by mRNA expression studies for selective related genes to find out the effect of 7β OH-EpiA on the genes related to their functions as investigated in the present study.

This discussion section will consist, in the first instance, of an evaluation of the methodologies used throughout the current research and will be followed by a discussion of the experimental data obtained in this study and compared to those from previous studies.

## 4.2 Evaluation of Methodology

### 4.2.1 Evaluation of the Use of Whole Human Blood, Mononuclear Cells and THP-1 Cells

Whole human blood and isolated mononuclear cells are commonly used in immunological and pharmacological studies on inflammation since they are the major source of the main inflammatory cytokines particularly TNF-α and IL-1β. Pro-inflammatory cytokines produced in acute and chronic inflammatory responses result in end-response pathology (Thurm & Halsey, 2005). This was also the case in this study where LPS was shown to stimulate these cytokines. The pro-inflammatory cytokines can in turn enhance the production of other cytokines and downstream secondary mediators such as prostaglandins, mainly $PGE_2$. All these events have been shown to occur in vitro and in vivo (Ertel *et al.*, 1995; Strieter *et al.*, 1990). Therefore, whole human blood and isolated monocytic cells were used in this study to characterize the actions of 7β OH-EpiA on inflammatory responses.

Due to the limitation in the number of isolated monocytes, monocytic cell lines have also been used in research studies. Monocytic cell lines such as THP-1 cells have been used widely in order to provide a useful and effective model system for studying the response of human monocytes in immunological studies (Auwerx, 1991). The THP-1 cell line was originally established and characterised from the peripheral blood of a one-year-old patient with acute monocytic leukaemia (Tsuchiya *et al*., 1980). These cells are mainly used in studying biological functions and regulation of human monocytes because they share phenotypic characteristics with mature monocytes (Qin, 2012). LPS can stimulate THP-1 cells and initiate them to release cytokines such as TNF-α in the same manner as mature monocyte do in identical experimental conditions (Schildberger *et al.*, 2013). Based on the sensitivity of these cells to LPS, THP-1 was chosen for this study as a model of human monocyte function. Isolated monocytes from peripheral human blood do not yield 100% pure monocytes but will also contain other cells. Coupled with the long isolation process and inability to be stored but required to be used directly after isolation makes human monocytes very difficult to use for all experiments (Chanput *et al*., 2014). Another severe drawback facing studies using isolated monocytes is the low concentration of isolated RNA even during overexpression studies while the level obtained from using THP-1 cells was consistently high.

In many studies, THP-1 cells have been reported to have disadvantages in vitro, all these disadvantages appear to be associated with differentiation such as an increased sensitivity to LPS during differentiation. (Chanput *et al*., 2014). This was not observed in the present study and LPS was able to stimulate THP-1 cells with an increase in the concentrations of both TNF-α Fig (3.1) and IL-1β Fig (3.2) in the same manner as isolated monocytes. Thus, these cells were considered to be useful for further studies in characterising the actions of 7β OH-EPIA on cytokine production in blood cells.

### 4.2.2 Isolation of Monocytes by Ficoll Density Centrifugation

Isolation of monocytes using adherence after ficoll density gradient centrifugation from whole human blood was used in this study because it is cheaper compared to isolation using magnetic bead technology which can be prohibitively expensive. Separation of monocytes using magnetic beads is usually conducted by positive selection, normally this would be to target the main monocyte marker CD14. However, it has been reported that there are many physiological changes in monocytes and therefore changes in their function using positive selection. It has been reported that phagocytosis was affected in CD14+ monocytes isolated by comparing to isolation methods using adherence techniques which was measured by flow cytometry (Delirezh *et al*., 2013). Another major drawback using positive isolation via CD14 is that this activates monocytes before any test compounds can be incubated with the cells (Neu *et al*., 2013) (Landmann *et al*., 1998). Indeed, in a study comparing positive isolation with negative isolated monocytes it was reported that the response to LPS was very weak from positive isolated monocytes. This was attributed to microbeads used in isolations which were still attached to cells and which did not degrade even after 6 days of culture (Bhattacharjee *et al*., 2017). Cells were obtained by the adherence method in the present study and isolated cells did not appear stimulated prior to incubation with LPS. Thus, this methodology was used in subsequent experiments using monocytes.

The adherence method yielded around $1.6\times 10^6$ cells/ml and this number of cells was adequate to carry incubations with LPS in cytokine production experiments such as TNF-α measurements. However, the RNA concentration was very low 2ng/µl compared to the yield from THP-1 cells which gave around 200ng/µl for an equivalent

cell number. Isolation of monocytes by different methods (negative or positive isolation) have been shown to face many problems such as cost and the prolonged exposure to antibodies used as markers to isolate cells which can cause transcriptional changes resulting in altered gene expression profile (Beliakova-Bethell *et al*., 2014; Lyons *et al*., 2007). For these reasons, isolated monocytes were used for cytokine stimulation with THP-1 cells used in parallel, but THP-1 cells alone were used for gene expression studies.

## 4.3 Ability of Lipopolysaccharide (LPS) to Stimulate Cytokine Production

LPS is one of the most potent (pathological / physiological) activators of innate immune cells. LPS is a component that forms the outer membrane of gram-negative bacteria, which has been well studied in vivo and in vitro with respect to inducing the inflammatory cascade ,by using LPS, and therefore stimulate/ enhance production of pro-inflammatory cytokines (Raetz & Whitfield, 2002; Rietschel *et al.*, 1994). Innate immune cells such as monocytes can bind LPS through their TLR4, which is one of the pattern recognition receptors (PRRs)(Hoshino *et al*., 1999). LPS binding to the TLR4 and MD2 complex activates the NF-κB pathway via the adaptor molecular MyD88 which in turn activates production of pro-inflammatory cytokines such as TNF-α and IL-1β (Forster-Waldl *et al*., 2005). TNF-α and IL-1β are the major most abundant pro-inflammatory cytokines, which are involved in local and systemic inflammation during infection, depending on the level produced and can at high levels, in the worst conditions, lead to septic shock (Zhang & An, 2007). Among innate immune cells, monocytes play important roles in response to bacterial infection therefore, isolated monocytes and THP-1 cells were deemed to be appropriate models to study the mechanisms involved in the LPS-stimulated production of cytokines and

how this may be affected by 7β OH-EpiA.

In a previous study, LPS was shown to induce cytokine production (TNF-α) from monocytes at concentrations as low as 5 ng/ml indicating the high sensitivity of monocytes to small amounts of LPS (Kreutz *et al*., 1997). As well as LPS reported in another study to induce significant amounts of IL-1β by using (1μg/ml) in both monocyte and whole blood (Davidson *et al*., 1998). In a study by Schytte Blix and his co-workers in 1999, it was reported that LPS isolated from two different types of gram-negative bacteria (*E. coli* and *A. Actinomycetemcomitans*) both stimulate the production of pro-inflammatory cytokines, mainly TNF-α and IL-1β, from whole human blood. The production of these cytokines occurred with concentrations as low as 0.1 μg/ml of LPS (Schytte Blix et al., 1999) indicating that diverse sources of LPS have similar potency. Also, studies, using whole human blood incubated with LPS concentrations in the same range as shown in the present study (1μg/ml-100μg/ml) elicited an increase in TNF-α levels in a time and concentration-dependent manner (Foster *et al.*, 1993). The use of whole human blood in this way could be an important model system which could provide insights into the control of LPS-stimulated overproduction of TNF-α and IL1β. This is important as in vivo high concentrations of LPS result in septic shock leading to death due to the high level of pro-inflammatory cytokines particularly TNF-α and IL-1β being produced (Zweigner *et al*., 2001).

In the present study, LPS was able to stimulate a concentration-dependent increase in the quantity of TNF-α and IL-1β in human whole blood, isolated monocytes and in THP-1 cells. This occurred with LPS concentrations between (0.1-100 μg/ml) and the maximum concentration of TNF-α and IL-1β (measured using ELISA) was obtain with concentrations of LPS between 10-100 μg/ml (Figs. 3.2 and 3.7 respectively). This was

similar to results reported by Foster and his group in 1993. They stimulated human and rat blood with different concentrations of LPS (*E.coli*) (1-100 μg/ml) and showed that TNF-α production occurred in a concentration and time dependent manner for both human and rat blood. (Foster *et al*., 1993). In the present study an LPS concentration of 10 μg/ml was used throughout as an optimal concentration for stimulating cytokines production in human blood. In THP-1 cells there was a reliable increase in TNF-α at three different concentrations of LPS (1, 10 and 100 μg/ml) therefore 1 μg/ml of LPS was used due to the higher sensitivity of these cells to lower concentrations of stimuli as reported in the study by Kreutz in 1997 (Kreutz *et al*., 1997). This concentration of LPS (1μg/ml) has also been used in a study that examined the inhibitory effect of chloroquine on LPS stimulation of TNF-α production from THP-1 cells (Jang *et al*., 2006).

## 4.4 Modulation of Cytokine Production by 7β OH-EpiA

### 4.4.1 Inhibition of Pro-Inflammatory Cytokines in Response to LPS Stimulation

Inflammatory responses occur in a cascade that is characterized by the production of pro-inflammatory cytokines, mainly TNF-α and IL-1β. These cytokines have the ability to stimulate further downstream inflammatory mediators such as prostaglandins mainly PGE2, which also exerts a negative feedback action on the production of these cytokines Fig (1.3). Therefore, it was decided in the current study, to investigate effects of 7β OH-EpiA on the production of pro-inflammatory cytokines directly. In particular, TNF-α and IL-1β in response to LPS stimulation in whole human blood, THP-1 cells as well as isolated monocytes. This was compared to the actions of the glucocorticoid analogue, dexamethasone as a positive "landmark" steroid. In whole human blood Fig (3.3), very low concentrations of 7β OH-EpiA in the nanomolar

range, were able to reduce TNF-α production. This was also shown in isolated monocytes and THP-1 cells. This was similar to the actions of dexamethasone, but low concentrations of dexamethasone were not effective in reducing TNF-α production and this only occurred with much higher concentrations in the > 10μM range which has also been reported in other studies (Joyce *et al*., 1997).

This influence of steroids on the level of cytokines has been reported in human studies. It was observed that a decrease in steroid hormone levels increases the level of some cytokines such as TNF-α in cerebral spinal fluid (CSF) of Alzheimer's disease patients (Tarkowski *et al.*, 2003). Also, in women with an age-related reduction of oestrogen it has been observed that there is an increased level of TNF-α, IL-1 and IL-6 in plasma. This has been attributed directly to the steroid's action in vitro, the supernatants of isolated monocytes from postmenopausal women have been shown to decrease the level of these cytokines in response to exogenously added 17β-estradiol (Bernard-Poenaru *et al*., 2001).

The actions of steroids generally occur via interaction with their specific receptors forming a complex that has been reported to inhibit binding of the NF-κB complex to regulatory areas of target genes, or to prevent nuclear translocation and a transcriptional activation of the TNF-α gene by a component of NF-κB (p65) (Fig 1.7) (Ghisletti *et al*., 2005).

In the current study, low concentrations of 7β OH- EpiA suppressed production of TNF-α and also showed a decreased expression of TNF-α mRNA in response to LPS stimulation. 7β OH-EpiA was also able to reduce production of IL-1β in whole human blood and monocytic cells (Figs 3.8, 3.10, 3.12 respectively). Dexamethasone,

synthetic steroid, also reduced production of TNF-α (Figs 3.3, 3.4, 3.5) and IL-1β (Figs 3.9, 3.11, 3.13).

Dexamethasone has been shown to decrease quantities of TNF-α in monocytic cells as well as levels of anti-inflammatory cytokines such as IL-10 (Franchimont *et al*., 1999) and the data obtained in this study is consistent with these observations. There is a possibility that 7β OH- EpiA may be involved in the suppression of inflammatory responses by other lipids such as fatty acids. There is substantial evidence which indicates that the dietary ω-3 polyunsaturated fatty acid (ω-3 PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) can suppress inflammatory responses. For example, in vitro, both EPA and DHA in human kidney-2 HK-2 cells can significantly inhibit LPS-induced NF-κB activation in a dose-dependent manner but this study did not measure the levels of pro-inflammatory cytokines (Li *et al*., 2005). Another study showed that in response to EPA there was a clear reduction of LPS-stimulated TNF-α at the transcriptional and protein level via a switch-off of NF-κB in macrophages (Lo *et al*., 1999). The mechanisms by which ω-3 PUFAs act as anti-inflammatory mediators leading to inhibition of production of TNF-α and IL-1β in monocytes is still unclear (Endres *et al*., 1989) In vivo, $PGE_2$ and IL-1β were significantly decreased in response to the TLR3 ligand poly I:C in rabbits previously administered eicosapentaenoic acid (EPA) for 42 days compared to untreated animals. In addition, 15d-$PGJ_2$ was shown to increase in response to stimulation by IL-1β and TNF-α in the plasma of rabbits pre-treated with EPA (Davidson *et al*., 2013). This is interesting as in turn 15d-$PGJ_2$ has inhibitory effects on the production of these two cytokines. Although in the study of Davidson et al. (2013) EPA produced this profile of effects on cytokines and PGs it is possible that these actions of EPA in vivo may be indirectly mediated and may involve a circulating mediator such as a steroid. This

would unlikely be a glucocorticoid as they would produce a global suppression of cytokines and PGs whereas an increase in 15d-$PGJ_2$

was observed, raising the possibility that a compound such as 7β OH-EpiA could be involved. This is possible because in the present study 7β OH-EpiA selectively increased 15d-$PGJ_2$ production. Thus, it would be interesting to see what effect EPA has on the production of 7β OH-EpiA in vivo in order to ascertain if it is indeed a mediator of EPA actions. The suppressive action of 7β OH-EpiA on TNF-α and IL-1β could be mediated by the stimulation of other modulatory factors such as anti-inflammatory cytokines (IL-4, IL-10) or PGs ($PGD_2$, 15d-$PGJ_2$). There are two studies that have clearly demonstrated the ability of 7β OH-EpiA to increase biosynthesis of $PGD_2$ and its metabolite 15d-$PGJ_2$ (Davidson *et al*., 2008; Le Mee *et al*., 2008). Thus, it was decided in the current study, to investigate effects of 7β OH-EpiA on the production of anti-inflammatory cytokines directly. In particular, IL-10, IL-4 and PGs ($PGE_2$, $PGD_2$ and 15d-$PGJ_2$) in response to LPS stimulation in whole human blood, THP-1 cells as well as isolated monocytes. This was compared to the actions of the glucocorticoid analogue, dexamethasone as a positive steroid control which will be discussed in the next section.

### 4.4.2 Effect of 7β OH-EpiA on the Production of Anti-inflammatory Cytokines

New approaches in treating autoimmune diseases such as RA have targeted anti-inflammatory cytokines such as IL-4 and IL-10 by stimulating their production in order to reduce the production of TNF-α, IL-1β and IL-6 and this has been successfully used in rat models (Katsikis *et al.*, 1994). For example, IL-10, has been

demonstrated as a potential cytokine for cell protection since intra-ventricular administration of IL-10 can reduce infarct size following reperfusion ischaemia damage. Since during infarction many infiltrating cells such as monocyte/macrophages which increase in the zone of the myocardium are thought to be responsible for the high expression of mRNA for inflammatory cytokines including TNF-α, IL-1β and IL-6, IL-10. Treatment has shown an inhibition of the infiltration of macrophages which correlates with a reduction in TNF-α, IL-1β and IL-6 production (Krishnamurthy *et al*., 2009). Its production during injury suggested that increasing local concentrations of IL-10 may be useful in the treatment of inflammation. Release of IL-10 from monocytes/ macrophages in response to LPS or IL-1β has been reported to be similar (Garcia *et al.*, 2017).

Macrophages from asthmatic patients show a decreased level of IL-10 mRNA and IL-10 protein compared to those isolated from healthy donors (John *et al*., 1998). This reduction in IL-10 in asthmatic patients appears to be associated with an increased level of TNF-α, and IL-1β production from alveolar macrophages and circulating monocytes. This is thought to be due to the reduced IL-10 production as many studies have shown that recombinant human IL-10 inhibits TNF-α production. Additional evidence for this view is provided in studies where, adding anti-IL-10 antibodies to isolated human monocyte cultured with LPS show a marked increase in the production of TNF-α, IL-1β and IL-6 (Donnelly *et al*., 1995). There are several proposed mechanisms whereby IL-10 may do this, such as inhibitory effects on the activity of NF-κB in monocytes (Wang *et al.*, 1995). also, IL-10 has been reported to down-regulate surface TNF-R expression in monocytes (Joyce *et al*., 1994). Corticosteroid therapy in asthmatic patients has shown an increase in the level of mRNA of IL-10

and production of IL-10 which subsequently results in a decrease in the level of TNF-α, IL-1β and IL-6 in alveolar macrophages and isolated monocytes (John *et al*., 1998). Most glucocorticoids, such as the analogue dexamethasone, have been reported to have immunosuppressive and anti-inflammatory activity (Coutinho & Chapman, 2011). Dexamethasone can increase levels of anti-inflammatory cytokines such as IL-10 in some situations, however, other studies have shown that it decreases IL-10. Thus, data from previous studies are contradictory. The current study shows dexamethasone decreased levels of IL-10 in all models albeit at relatively high concentrations(10-100 μM) (Fig 3.18), (Fig3.20) and (Fig 3.22). This is in agreement with other work that has shown dexamethasone to suppress gene expression and level of IL-10 following LPS stimulation in isolated monocyte (Fushimi *et al*., 1997). 7β OH-EpiA has been studied with respect to its actions on IL-10 by Davidson et al., in 2008, which had shown no effects of 7β OH-EpiA on the level of IL-10 in isolated monocytes (Davidson *et al*., 2008). Similarly, in the current study, the effects of 7β OH- EpiA on the level of IL-10 also showed the same lack of results in whole human blood (Fig 3.17), in monocyte cells. (Fig 3.19) as well as THP-1 cells (Fig 3.21). Meanwhile, dexamethasone in this study decreased levels of IL-10 in all tissues (Fig 3.18), (Fig3.20) and (Fig 3.22).

Many studies have focussed on another anti-inflammatory cytokine i.e. IL-4 which has been reported to reduce the production of pro-inflammatory cytokines such as TNF-α (Finnegan *et al*., 2002) and IL-1β in macrophage and monocyte supernatant culture media after 4 and 20 hours of incubation following LPS stimulation (Finnegan *et al*., 2002; te Velde *et al*., 1990). Thus, it is possible that 7β OH-EpiA may be acting via increasing the production of IL-4; however, this is deemed unlikely as the data obtained in this study show that 7β OH-EpiA does not alter IL-4 production in blood or in monocytes. On the other hand, the data also showed that dexamethasone

increased production of IL-4 in response to LPS stimulation.

## 4.5 Evaluation of the Effects of the Non-Steroidal Anti-inflammatory Drug (NSAID) Ketoprofen on 7β OH-EpiA-Induced Suppression of TNF-α and PGs Production

The principal targets of NSAIDs action are cyclooxygenases, the enzymes responsible for the conversion of arachidonic acid and EPA into inflammatory mediators, such as prostaglandins (Brown *et al*., 2013). Initially, NSAIDs were thought to act via inhibition of a single COX enzyme (COX1) activity but after the discovery of the second isoform of COX (COX2) this was also found to be inhibited by NSAIDs (Zarghi & Arfaei, 2011). COX2 was found to be expressed in response to stimuli such as pro-inflammatory cytokines including TNF- α (Medeiros *et al*., 2010), IL-1β (Molina-Holgado *et al*., 2000)) or LPS. Most NSAIDs are non-selective with respect to the COX isoforms and are able to effectively inhibit PG synthesis.

It has also been suggested that NSAIDs can act to regulate gene expression directly via their interaction with a class of nuclear receptor superfamily members, termed peroxisome proliferator-activated receptors (PPAR) (Puhl *et al*., 2015). The natural ligand for PPARγ receptor that is expressed in monocytes / macrophages was thought to be 15d-PGJ2 (Bell-Parikh *et al*., 2003). The principle action after 15d-$PGJ_2$ binding to PPARγ was initially thought to be a suppressive effect on pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 (Jiang *et al*., 1998). However, the concentrations of 15d-$PGJ_2$ that inhibit cytokine production are several orders of magnitude lower than those required for binding to PPAR-γ. In addition, PPAR-γ activators do not affect LPS-stimulated TNF-α production (Chawla *et al*., 2001). Thus, it is highly unlikely that 15d-$PGJ_2$ acts via PPAR-γ which implies that 15d-$PGJ_2$ exert

its anti-inflammatory effects via interaction with a yet unknown, possibly another prostaglandin, receptor (Thieringer *et al*., 2000).

Because previous and the current studies have shown that 7β OH-EpiA can enhance the biosynthesis of selective prostaglandins. This study examined the effect of ketoprofen on the ability of 7β OH-EpiA to modulate TNF-α, $PGE_2$ and 15d-$PGJ_2$ synthesis. The data showed that ketoprofen effectively reversed the 7β OH-EpiA-induced reduction in TNF-α production. This indicated that a cyclooxygenase product is most likely involved in the mechanism by which7β OH-EpiA suppresses TNF-α production. Ketoprofen was also shown to directly attenuate the 7β OH-EpiA-induced biosynthesis of 15d-$PGJ_2$ strengthening the possibility that its actions on cytokine production may be mediated via the biosynthesis of 15d-$PGJ_2$ (see Fig 1.4). The inhibitory effect of ketoprofen on COX was confirmed by its suppressive effect on $PGE_2$ production. As ketoprofen can inhibit both COX1 and COX2 it is not possible to indicate whether COX1 or COX2 is important in the 7β OH-EpiA-induced biosynthesis of 15d-$PGJ_2$. However, this is not so critical to total PGs production as both COX1 and COX2 can produce the $PGH_2$ precursor for the downstream production of 15d-$PGJ_2$. This provides information suggesting that the control point at which 7β OH-EpiA acts to upregulate 15d-$PGJ_2$ production is downstream of COX.

## 4.6 Evaluation of the Effects of Mifepristone (Glucocorticoid Receptor Antagonist) on the Ability of 7β OH-EpiA and Dexamethasone in Reducing TNF- α Production in Response to LPS Stimulation

Mifepristone, also known as RU486, is a progesterone receptor antagonist which has glucocorticoid (GR) and progesterone receptors (PR) antagonist activity with high binding to the GR compared to dexamethasone and PR (Peeters *et al*., 2008). Both

glucocorticoids and progesterone exert their anti-inflammatory action via binding to GR and PR respectively. Dexamethasone (a synthetic glucocorticoid) has potent anti-inflammatory effects by decreasing pro-inflammatory cytokines such as TNF-α and IL-1β and acts through binding to the GR. In vitro, activation of NF-κB leads to increases in inflammatory cytokines, this activation can be suppressed by glucocorticoids via the GR. In vivo, the production and release of glucocorticoids act to prevent or reduce the pathology of septic shock and this can be reversed by mifepristone since mifepristone can block glucocorticoid receptors. (Shukla *et al*., 2014). In addition, mifepristone displays high affinity binding to progesterone receptors which is five times greater than progesterone itself (Shukla *et al.*, 2014).

Progesterone has been shown to be anti-inflammatory and can suppress LPS-induced expression of TNF-α and COX2 in mouse microglia. The action of this hormone was reported to occur as a result of increased phosphorylation of IκB and P65 which in turn inactivates NF-κB. An important aspect of this inhibitory effect of progesterone is that it can be blocked by mifepristone and results in increasing degradation of IκB and P65 which in turn results in activation NF-κB (Lei *et al*., 2014). Another study also described that progesterone supressed production of cytokines such as TNF- α and IL-1β in response to LPS in macrophages and also showed this action was clearly reversed in response to RU486 (mifepristone) (Butts *et al.*, 2008). An early study on mifepristone, suggested that mifepristone could be responsible for bacterial contamination of tissue causing infection that might progress to cause septic shock (Brouckaert *et al.*, 1992). Another study showed that the cortisol and dexamethasone attenuation of their ability to produce TNF-α and IL-1β in LPS-stimulated macrophages is reversed in the presence of mifepristone. This occurred as a result of blocking the GR (Carr *et al.*, 1991).

Therefore, this current study examined whether 7β OH-EpiA could utilise glucocorticoid or progesterone receptors for its effects by using mifepristone as an antagonist and comparing this with dexamethasone as positive control. Dexamethasone clearly shows an ability to decrease TNF-α and prostaglandin production in response to LPS stimulation and exhibits competition in mixtures of dexamethasone and mifepristone. This was similar to study by (Joyce *et al*., 1997) using mifepristone (100 μM) did not show any effect on TNF-α in response to LPS-stimulation. Similarly, mifepristone did not have any effect on 7β OH EpiA reduction of TNF-α production in the presence of LPS. This could have occurred as a result of several possibilities. One possibility could be that 7β OH EpiA acts via GR or PR but mifepristone was unable to affect the response. This raises the more likely possibility that 7β OH-EpiA does not act through either a GR or a PR and may utilise another, possibly unreported, receptor.

## 4.7 Effect of 7β OH-EPIA on the Production of Prostaglandins

### 4.7.1 Evaluation of the Mechanisms by which 7β OH-EpiA Modulates Prostaglandin Production

Prostaglandins have been shown in other studies as well this current study to reduce the production of pro-inflammatory cytokines mainly TNF-α. PGs have also been shown in this study to effectively reduce, and in some cases prevent, the production of pro-inflammatory cytokines especially TNF-α. Thus, it is possible that the actions of 7β OH-EpiA in suppressing TNF-α production could be mediated via inducing the production of prostaglandins.

#### 4.7.1.1 Effect of the Ability of 7β OH-EpiA to Reduce Production of Prostaglandins in Response to LPS Stimulation in Whole Blood Cells

Activation of human blood cells using LPS has been used as model of inflammation to enhance PG production in many published experiments. In the current study, LPS was shown to be an effective stimulus for the production of three PGs ($PGE_2$, $PGD_2$ and 5d-$PGJ_2$) in whole human blood and isolated monocytes. This action of LPS has been reported to occur as result of inducing the expression of COX2 (mRNA and protein) with the involvement of MyDD88 and NF-κB because it has been shown that silencing the P65 subunit of NF-κB with siRNA decreased COX2 mRNA induction and $PGE_2$ levels in microglia (the monocytic lineage of microglia) (Font-Nieves *et al*., 2012) as well as in human isolated monocyte cells that have been shown to express COX2 mRNA $PGE_2$ after exposure to LPS for 24hrs compared to rested cells (Hempel *et al*., 1994). Exogenous 15d-$PGJ_2$ has been reported to be cytoprotective in cerebral ischaemia by preventing ischaemia–reperfusion-induced brain damage (Ou *et al*., 2006).

In animal models, treatment with 15d-$PGJ_2$ prior to inducing ischaemia suppressed infarct volume. Intracerebral infusion of an adenoviral construct of cyclooxygenase-1 (Adv-COX-1) gene transfer has been associated with an increased level of 15d-$PGJ_2$ in ischemic brain of rate; this was accompanied by a reduced infarct volume (Lin *et al*., 2006). The accumulation of the endogenously produced 15d-$PGJ_2$ in the brain has been reported to be protective during pathogenic insults, indicating that 15d-$PGJ_2$ is a possible mediator of the protective actions of 7β OH-EpiA. High levels of 15d-$PGJ_2$ in the brain raises the question of where the source of increased 15d-$PGJ_2$ production from within or outside the brain tissues/ cells and in response to which stimuli? Up to now, it is well documented that circulating mononuclear cells of the innate immune

system, in response to inflammatory stimuli, produce increased level of prostaglandins and that these cells are a major source of both cytokines and cytokine-induced prostaglandins following pathogenic stimulation. Early studies using animal models, indicated that inflammatory stimuli (LPS and poly I:C) and cytokines (IL-1 and TNF-α) increase production of $PGE_2$ in blood and CSF and also increase the entry, from the peripheral circulation, of radiolabelled $PGE_2$ into the third cerebral ventricle which showed that $PGE_2$ crossed the blood brain barrier (BBB) (Davidson *et al*., 2001). Therefore, the neuroprotective actions of 7β OH-EpiA could occur inside the CNS but coming from the systemic circulation which would be straightforward due to the lipid solubility of 7β OH-EpiA which would allow it to cross the blood brain barrier and induce PG production inside the CNS. This was shown in a study by Pringle *et al*. 2003, where they administered 7β OH-EpiA intravenously into animals and demonstrated a neuroprotective efficacy against ischaemia-induced neuronal damage (Pringle *et al.*, 2003).

The current study examined the effect of 7β OH-EpiA on PGs production in blood cells. The results show that 7β OH-EpiA reduced the level of LPS-stimulated $PGE_2$ in both plasma and monocyte supernatants. This is similar to studies be Le Mee *et al*., 2008 and Davidson *et al*., 2008 which showed that incubation of isolated human monocytes with TNF-α and 7β OH-EpiA for 24hr decreased levels of $PGE_2$ (Davidson *et al*., 2008; Le Mee *et al*., 2008). These studies had a similar time frame and concentrations of 7β OH-EpiA as those used in the current study. This would rule out $PGE_2$ as a mediator of the cytokine-suppressive action of 7β OH-EpiA. However, 7β OH-EpiA increased the production of both $PGD_2$ and 15d-$PGJ_2$. Thus, it appears that these PG derivatives are potential mediators of 7β OH-EpiA in its ability to suppress cytokine production. Both PGs were also effective in suppressing LPS-stimulated

TNF-α production. This action of 7β OH-EpiA on enhancing $PGD_2$ and 15d-$PGJ_2$ production would not be expected predictable for steroids. Normally, conventional steroids (cortisol, dexamethasone) have been reported to reduce production of prostaglandins as also shown in this study.

In the present study $PGD_2$ and 15d-$PGJ_2$ levels increased in response to 7β OH-EpiA. This action of 7β OH-EpiA would not normally be expected of other steroids, especially glucocorticoids, which are generally suppressive agents with respect to PG production. This finding was similar to two other published studies. Those studies showed that the level of $PGD_2$ and of 15d-$PGJ_2$ increased following exposure of monocytes to 7β OH-EpiA after an incubation period of around 18 hrs (Davidson *et al*., 2008; Le Mee *et al*., 2008). $PGD_2$ can be transformed into its metabolite 15d-$PGJ_2$; this occurs spontaneously and makes the quantitation of actual $PGD_2$ levels difficult. In the present study $PGD_2$ was quickly reacted with methoxime as $PGD_2$ is an unstable prostaglandin, its half-life is 0.9 mins in blood (Suzuki *et al*., 1986) and in plasma it is 30 min (Schuligoi *et al.*, 2007). It can be further metabolized to 11β-PGF, a stereoisomer of $PGF_{2\alpha}$ and is also readily dehydrated to produce prostaglandins of the J series, such as $PGJ_2$, Δ12-$PGJ_2$, and 15-deoxy-$\Delta^{12,14}$-$PGJ_2$ (Fitzpatrick & Wynalda., 1983).

Measurement of the metabolites of $PGD_2$ has been reported to be suitable in the supernatants of cell cultures, where $PGD_2$ levels may reach up to ng/ml levels. Most studies that focus on biosynthesis of $PGD_2$ face the problem that it is readily degraded when the incubation conditions contain serum proteins such as albumin or in acidic and basic media. This has been derived from data on the development of anti-$PGD_2$

antibodies for analysis. Antigenic protein conjugates of $PGD_2$, synthesized for the production of antisera, show considerable amounts of decomposition. Thus, the

resulting antibody response is heterogeneous with poor specificity (Ito *et al*., 1988). This made $PGD_2$ assay systems based on the metabolite compounds unreliable and difficult to interpret. A specific and high-affinity antiserum has been obtained by immunizing rabbits with the stable $PGD_2$ analogue PGD2-11-methoxime (Ito *et al*., 1988). Therefore, $PGD_2$-MOX ELISA, with conjugates of this derivative is very specific for $PGD_2$ MOX, which was used in this study to prevent further $PGD_2$ degradation and thus, give a more accurate quantitation of $PGD_2$ levels. A divergence in the production of PGs can occur in other situations. This is supported by other studies which show that inflammation resolution is associated with a reduced synthesis of $PGE_2$ but an increase in the levels of $PGD_2$ and its dehydration metabolite 15d-$PGJ_2$ which has also been described in carrageenan-induced pleurisy in rats. The level of $PGD_2$ and 15d-$PGJ_2$ in cell-free inflammatory exudates in a rat model increased during the resolution stage of inflammation (Gilroy *et al*., 1999). The effect of 7β OH-EpiA on PGs appears to be highly specific for the 7β isomer of OH-EpiA since the 7α isomer, namely 7α OH-EpiA was not able to alter either PG or cytokine production in any of the preparations currently studied. This would suggest a very specific receptor interaction with the 7β isomer, however, no receptors for the action of 7β OH-EpiA have been suggested or identified. It's possible that with respect to suppression of both PG and cytokine production, the actions of 7β OH-EpiA may be acting via a glucocorticoid receptor (GR).

In the present study, a dual GR and progesterone receptor (PR) antagonist, mifepristone, was not able to modulate the actions of 7β OH-EpiA. Coupled with the observation that the comparative actions of dexamethasone on PG and cytokine

production were reversed by mifepristone, this would strongly suggest that neither a GR nor PR is the receptor by which 7β OH-EpiA mediates its actions. Dexamethasone was able to reduce the production of all of the prostaglandins measured ($PGE_2$, $PGD_2$ and 15d-$PGJ_2$). The ability of glucocorticoids to inhibit PG biosynthesis may be through the indirect inhibition of phospholipase $A_2$ ($PLA_2$). This action appears to be mediated indirectly by Annexin A1 in a GR-dependent manner (Kol *et al*., 1998). Also, dexamethasone has been reported to repress IL-1β-stimulated COX2 at a post-transcriptional level in a GR-dependent manner. This suppressive action of dexamethasone on COX2 has been demonstrated to be antagonized by mifepristone which confirms that dexamethasone was acting in a GR-dependent manner to suppress COX2 (Newton *et al.*, 1998).

#### 4.7.1.2 Effect of Mifepristone (GR/ PR Antagonist) on the Ability of 7β OH-EpiA to Modulate Production of Prostaglandins

As indicated earlier, mifepristone is an anti-progestin and acts via its high affinity binding to the PR but can also bind to the GR. In this part of the study mifepristone was used to find out its effect on the biosynthesis of PGs in response to 7β OH-EpiA. Many early studies indicated that steroids inhibit production of prostaglandins without affecting the conversion of AA by COX (Gryglewski, 1976) i.e. they do not act by inhibiting COX activity. In this study, mifepristone had no effects on the 7β OH-EpiA-stimulated increase in biosynthesis of 15d-$PGJ_2$ or the decrease in levels of $PGE_2$. That also confirmed the previous findings in this study which showed that 7β OH-EpiA does not act via binding to GR. In contrast, mifepristone was able to reverse the dexamethasone inhibition on PG production following LPS stimulation confirming that mifepristone was active through GR. Therefore, the results obtained from this

study suggest that 7β OH-EpiA is unlikely to act via a glucocorticoid receptor in modulation PG synthesis action.

It's possible that this steroid acts in two ways a) by high affinity binding resulting in reduced TNF-α production and b) a lower affinity binding which results in an increased biosynthesis of $PGD_2$ and 15d-$PGJ_2$ and lowered $PGE_2$. This raises the possibility that 2 different mechanisms and thus, 2 different receptors could be involved in 7β OH-EpiA action on PG production. It is also possible that TNF-α may regulate either of these putative receptors.

#### 4.7.1.3 Effect of Arachidonic Acid (AA) on the Ability of 7β OH-EpiA to Reduce or Increase Production of Prostaglandins in THP-1

In the current study, prostaglandin production from THP-1 cells in response to LPS was very low compared to that produced by isolated monocytes. Since the polyunsaturated fatty acid arachidonic acid (AA) is a primary precursor for the biosynthesis of prostaglandins it is possible that the low levels of PG production may be due to a deficient quantity of arachidonic acid. Because the long-term maintenance of cells in culture medium does not overtly include a variety of polyunsaturated fatty acids this would lead to a deficiency in those fatty acids necessary for PG production, namely arachidonic acid and also other essential fatty acids. Since mammalian cells in vitro cannot synthesize AA it could only be obtained from the FCS supplement in culture medium. This would be very low and reliable in the continuous culture of cells. THP-1 cells would unlikely be able to synthesise arachidonic acid as they do not contain the requisite desaturase enzymes (Lee *et al*., 2016). There may be small levels of arachidonic acid contained within the FCS used to culture the cells but this is not a major source of AA which essentially renders the cells deficient in this fatty acid (Stoll & Spector, 1984). Thus, the arachidonic acid would have to be delivered to the cells

preformed. Moreover, THP-1 cells were originally derived from an acute monocytic leukaemia patient, which would possibly exhibit fundamentally different lipid metabolism. Exogenous arachidonic acid would be taken up into the cell membrane from the incubation medium and released in response to stimuli such as LPS or 7β OH-EpiA via $PLA_2$ activation in this study.

THP-1 cells can express $PLA_2$ and COX (Leyva-Illades *et al*., 2010) so should be able to synthesis PGs from the arachidonic acid supplied. The cells should then be able to produce $PGH_2$ which is the immediate precursor of the various prostanoids such as $PGD_2$ and $PGE_2$. Therefore, it was decided to investigate whether adding exogenous AA to culture medium could affect the LPS-stimulated production of $PGE_2$ and 15d-$PGJ_2$ in THP-1 cells.

In the current study, LPS did not have any effect on the biosynthesis of $PGE_2$ from THP-1 cells which had been cultured in arachidonic-free medium (containing only the variable amount of arachidonic acid in the FCS). However, in a parallel series of measurements where THP-1 cells were cultured in medium supplemented with arachidonic acid, LPS stimulated an increase in the production of $PGE_2$. This increased level of $PGE_2$ was suppressed by ketoprofen confirming active COX metabolism in the biosynthetic pathway. 7β OH-EpiA also decreased levels of LPS-stimulated $PGE_2$ which was in agreement with the effects in isolated normal monocytes. The effect of arachidonic acid-supplemented medium was also studied on the levels of 15d-$PGJ_2$ produced by THP-1 cells. As with the effect of arachidonic acid supplementation on $PGE_2$ production, LPS was similarly able to stimulate an increase in levels of 15d-$PGJ_2$ but showed no effect in un-supplemented medium. 7β OH-EpiA did not appear to alter 15d-$PGJ_2$ levels from THP-1 cells cultured in arachidonic acid supplemented

medium which is different from its effects on normal monocytes where 15d-$PGJ_2$ levels were increased. This is possibly because of a deficiency in $PGD_2$ synthase but this is unlikely as LPS was able to stimulate an increase in 15d-$PGJ_2$ levels and also the cells were shown to express the $PGD_2$ synthase gene which was upregulated by 7β OH-EpiA in these cells. THP-1 cells grown in media without supplementation with AA may have produced the various PGs but may have been below the limit of detection of the EIA assays used.

## 4.8 Gene expression

### 4.8.1 Validation of reference genes

RT-qPCR is a powerful tool for analysing gene expression. It depends on measuring the increase in fluorescence emitted by a DNA-specific dye during the PCR reaction. For relative quantification, the expression of a target gene is measured in relation to one or multiple reference genes. Reference genes, which are also normally known as "housekeeping" genes, are likely to be stably or invariantly expressed in all cells under normal and experimental treatment conditions. The reference genes are largely utilised as internal controls and instrumental tools for calibration and normalisation of gene expression data in molecular biological studies. Therefore, a group of candidate reference genes were identified by studying their expression (Eisenberg & Levanon, 2013). Quantitative real-time reverse transcription PCR (qRT-PCR) can quantify and validate the level of RNA expression of reference genes (Cao *et al*., 2012; Dheda *et al*., 2004).

Glyceraldehyde-phosphate-dehydrogenase (GAPDH) is a common reference gene used in peripheral blood mononuclear cell culture, whole blood and tissue studies (Dheda *et al*., 2004). Many studies normalise the qRT-PCR results to either GAPDH

or beta actin (ACTB) in LPS-stimulated cells. However, these two reference genes (GAPDH and ACTB) have been shown to differ across samples and they are not stably expressed in many experimental settings ((Maeß, 2010; Eisenberg & Levanon, 2013). A previous study evaluated some candidate reference genes for LPS-stimulated THP-1 cells including: Ribosomal Protein L37A (RPL37A), ACTB, GAPDH, Peptidylprolyl Isomerase B (PPIB), and Hypoxanthine Phosphoribosyltransferase1 (HPRT1) using qRT-PCR (Cao *et al*., 2012). It indicated that PPIB was the most stable reference gene (Cao *et al*., 2012). Another study involving LPS-stimulated monocytes emphasised the importance of the validation of control genes where it was identified that PPIB and B2M are the most stably expressed genes in monocytes but ACTB is considered to be an unstable gene (Piehler *et al*., 2010). In contrast, Maess's study (2010) which was also conducted using THP-1 cells, found that ACTB is stably expressed whereas PPIB is a less stable gene (Maess *et al*., 2010). In summary, two out of three separate studies strongly agree that PPIB is a stable reference gene in THP-1 cells (Piehler *et al*., 2010; Cao *et al*., 2012). The variation between these three publications highlights the need for precise validation of reference genes used in the current study. Validation of reference genes is critically important because the RNA expression levels of these reference genes may vary within cell types and with different experimental conditions i.e. stimulation (Cao *et al*., 2012). Therefore, candidate internal reference genes in the present project were carefully evaluated before their use in gene expression studies with qRT-PCR. In order to correctly evaluate the results, a number of reference genes were chosen to be assessed. These were: PPIB, GAPDH, RPL37A, ACTB, and HPRT1 as listed in Table 2.4.

Normalising the values obtained from different experiments was a challenge because improper analysis can lead to misinterpretation of entire results. However, all issues were fully addressed following an examination of their expression stability under

experimental test conditions relevant to this particular study. RNA expression of these genes should not therefore be significantly changed with the inflammatory stimulus, LPS, which was used here. The gene stability can be assessed using validation software programs such as the geNorm, BestKeeper, and NormFinder which are recommended by many researchers and are based on Microsoft Excel (Maess *et al*., 2010; Cao *et al*., 2012). The mRNA expression levels of all seven candidate reference

genes used in this thesis (PPIB, GAPDH, RPL37A, ACTB and HPRT1) were evaluated by qRT-PCR in THP-1 cells based on the above suggested conditions. It was found that PPIB was the most reliable reference gene compared to the rest of the genes measured because mRNA expression of PPIB was the most consistent among different samples even in the presence of LPS.

The stability of potential control genes can be determined by Normfinder, geNorm and BestKeeper using RefFinder web tool (http://leonxie.esy.es/RefFinder/) (De Spiegelaere *et al.*, 2015). All reference genes used in the present study were also assessed through these validation programs. The first program was Normfinder which uses a qRT-PCR data normalisation tool which calculates the stability of each gene and ranks its expression according to the corresponding stability value. A higher value shows lower stability and vice versa (Cao *et al*., 2012). The second program is geNorm which forms the expression stability of control genes by measuring the M value of each gene. The M value is a mean of the variation between a particular gene and all other reference genes used. A lower M value shows the most stable expression. It is generally agreed that genes with $M < 1.5$ should be considered as a stable, reliable reference gene (Cao *et al*., 2012). The third validation program is BestKeeper which defines the expression variability between a group of reference genes based on quantification cycle (Van Herreweghe *et al*. 2010) values, coefficient of variance and

the standard deviation (SD). It is suggested that genes with a SD > 1 are considered unstable control genes and should be rejected from any list (Cao *et al*., 2012). Taking

Normfinder, geNorm and BestKeeper-data analysis into account, PPIB was ranked as the most stable control gene tested in this present project because its M value was below 1.3 (M value of PPIB = 1) and had a low SD (SD of PPIB = 0.09) and all other genes were excluded from consideration this is listed in Table 3.1 and Table 3.2 (Results Section).

It should be noted that reference genes for THP-1 cells are not restricted to these genes only (PPIB, GAPDH, RPL37A, ACTB and HPRT1), other stable, expressed control genes could have been examined and assessed as to their suitability if time had permitted.

### 4.8.2 Effect of 7β OH-EpiA on TLR-4 and TNF- α genes expression in THP-1 cells

Toll-like receptors (TLR) play a pivotal role in the innate immune response, and the expression levels of these receptors may reflect the sensitivity of immune cells to infections. Transcription of several pro-inflammatory cytokines, including IL-1, IL-6, and TNF-α, depends on NF-κB translocation to the nucleus (O'Neill, 2006). The binding of lipopolysaccharide (LPS) to TLR4 triggers human monocytes to produce cytokines such as TNF-α and IL-1β which play a dominant role in the inflammatory response, as can be observed during sepsis. Steroids have the ability to inhibit pro-inflammatory cytokine production as shown extensively in the present study and others. They highlight the importance of TLR4 and cytokine release during the activation of innate immune systems in THP-1 cells (Sligl *et al*., 2009) (Sherwin *et al*., 2012). In order to determine the intracellular targets of 7β OH-EpiA action, part of the

present project focused on mRNA expression for both TLR4 and TNF-α in LPS-stimulated THP-1 cells. Extensive studies showed that LPS can directly activate

NF-κB signalling through TLR4 and dexamethasone can block NF-κB translocation resulting in reduced expression of pro-inflammatory cytokines (Gomes *et al*., 2015; O'Neill, 2006). This finding was similar to a study that showed LPS was able to upregulate TLR4 expression in THP-1 cells (Wan *et al*., 2016). This was in agreement with the current study where LPS upregulated TLR4 expression. Thus, the expression of TLR4 is an important control target which can regulate cytokine production.

The regulation of TLR4 expression by steroids appears to be complex in that the steroids alone do not appear to have an effect but exhibit full efficacy following stimulation e.g. with LPS. Two studies that demonstrate this have investigated the efficacy of low doses of corticosteroids in the reversal of septic shock in human patients, suggesting that they might reverse mortality of septic shock faster (Sligl *et al*., 2009; Sherwin *et al*., 2012). Another study focused on genes that are up- and down-regulated by NF κB, and the effects of corticosteroids over the LPS-induced response. Their results suggested that administration of corticosteroids in parallel with LPS modifies the initial transcriptional profile and is sufficient to balance the resulting inflammatory response towards a beneficial outcome (Foteinou *et al*., 2009). Also in another study using dexamethasone it was shown that dexamethasone decreased levels of expression of TLR4 in peripheral blood monocytes compared to untreated cells but dexamethasone alone had no effects on TLR4 (Zhou *et al*., 2017). The current study also shows that 7β OH-EpiA alone has no effects on TLR4 expression but 7β OH-EpiA administered concomitantly with LPS non-significantly decrease TLR4 expression which could be due to using very low concentration of 7β OH-EpiA (0.001μM). It has been shown that the decrease in TLR4 expression caused by $PGE_2$

was adequate to reduce TNF-α in response to LPS in macrophages (Degraaf *et al*., 2014). On the basis of this strong association between TLR4 and TNF-α, it was decided that it would be interesting to evaluate the effect of 7β OH-EpiA on expression of mRNA for TNF-α. Since this study shows that a low concentration of 7β OH-EpiA was able to reduce the protein level of TNF-α in both serum and plasma. It would thus be useful to treat LPS-stimulated THP-1 cells with the same concentration of 7β OH-EpiA and measure the expression of TNF-α. It was found that LPS was able to increase the expression level of TNF-α mRNA in response to LPS. These findings indicate that 7β OH-EpiA can inhibit TNF-α produced in response to LPS possibly through decreasing the expression of both TLR4 and TNF α genes. These expression data for TNF-α were in parallel with results obtained from the measurement of TNF-α activity levels. Evidence of the effects of 7β OH-EpiA in increasing the levels of $PGD_2$ and its metabolite 15d-$PGJ_2$ in whole blood cells, was demonstrated earlier in this thesis, which seem to be the key components involved in this suppression of TNF-α and TLR-4. Therefore, it would be interesting to evaluate the effect of 7β OH-EpiA on expression of mRNA for Prostaglandin endoperoxide synthase (PGDS) and its receptor (PTGDR2) in this present study.

### 4.8.3 Effect of 7β OH-EpiA on Prostaglandin Endoperoxide Synthase (PTGDS) and PTGDR2 Expression in THP-1 Cells

Cyclooxygenases 1 and 2 (COX-1 and -2) are responsible for converting arachidonic acid (AA) (from position 2 of phospholipids of cell membrane) to prostaglandin endoperoxide $H_2$, the precursor of the prostaglandins ($PGD_2$, $PGE_2$, $PGF_{2\alpha}$ and $PGI_2$) (Smith *et al*., 2000; Rouzer & Marnett, 2003). Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used to treat inflammation, pain and fever, and these actions are generally attributed to inhibition of COX-2 (Masferrer *et al*., 1995). Ketoprofen is

an NSAID which, in this study, suppressed the ability of 7β OH-EpiA to increase 15d-$PGJ_2$ in plasma and supernatant. Because the suppressive effects of 7β OH-EpiA have been linked to the increased production of $PGD_2$ and its metabolite 15d-$PGJ_2$, the effect of 7β OH-EpiA on mRNA expression of the prostaglandin endoperoxide synthase (PTGDS) gene was evaluated.

Prostaglandin $D_2$ ($PGD_2$) which is a major cyclooxygenase product of arachidonic acid. $PGD_2$ is generated from $PGH_2$ via the activity of prostaglandin $D_2$ synthase (PGDS). $PGD_2$ is produced by two distinct $PGD_2$ synthases, the lipocaline-type (LPGDS) and hematopoietic (HPGDS) enzyme. Both differ in their amino acid moieties, tissue distribution and immunological consequence. While LPGDS is abundantly found in the central nervous system, retina, melanocytes, heart, and reproductive organs, HPGDS has mostly been implicated in an inflammatory context and is mainly located in blood cells (immune cells) (Trivedi *et al*., 2006). HPGDS is therefore be expressed by a variety of immune cells, including mast cells, dendritic cells, Langerhans cells and Th2 cells. Up to now, HPGDS has mostly been implicated in allergic responses contributing to a pro-inflammatory phenotype(Joo & Sadikot, 2012).

The pro-inflammatory effects of $PGD_2$ seem to be mediated by binding two different G-protein-coupled receptors (GPCRs): DP1 receptor and DP2 receptor which is also called chemoattractant receptor expressed on Th2 cells (CRTH2 receptor). $PGD_2$ can bind to two receptors with high affinity (Hata & Breyer, 2004; Xue *et al*., 2005; Arima & Fukuda, 2011). Although the CRTH2 receptor and the DP1 receptor have similar affinity for $PGD_2$, the CRTH2 receptor can bind to more diverse ligands compared to the DP1 receptor. For instance, the CRTH2 receptor has the ability to bind the $PGD_2$ metabolite (15-deoxy-$\Delta^{12, 14}$-$PGJ_2$) but not the DP1 receptor (Hata & Breyer, 2004).

At sites of allergic inflammation, $PGD_2$ is produced by mast cells to activate consequent inflammatory responses by interaction with the DP1 receptor and CRTH2 receptor (Xue *et al*., 2005). It has been reported that $PGD_2$ can also be produced by other immune cells such as antigen-presenting dendritic cells and type 2 T-helper lymphocytes (Th2 cells), indicating that $PGD_2$ may have a role in the antigen-specific

immune system (Hata & Breyer, 2004). The present study focuses on the expression of DP2 receptor gene (PTGDR2) in THP-1 cells after LPS activation to compare their expression in the presence and absence of 7β OH-EpiA and to see if 15-deoxy-$\Delta^{12, 14}$-$PGJ_2$, as ligand for DP2 receptors, is involved in controlling the suppressive action on pro-inflammatory cytokine production. However, there was no gene expression detected for either the DP1 or DP2 receptors in THP-1 cells and monocytes. Also this could be due to lack of AA in incubation medium of THP-1 cells.

In the last part of this study, results show that LPS alone failed to stimulate expression of (H-PGDS) PTGDS in THP-1 cells, but in presence of both LPS and 7β OH-EpiA PTGDS expression was higher than LPS alone. Although, a number of investigations demonstrated expression of H-PGDS in peripheral tissues and localized in the antigen-presenting cells, mast cells, megakaryocytes, and Th2 lymphocytes but not Th1 (Tanaka *et al*., 2000). There are very few investigations directed to the potential expression of H-PGDS in monocytes. The present data provides an indication of the expression of PTGDR2 in response to LPS in THP-1 cells. This study also shows that 7β OH-EpiA can induce the expression of the PTGDR2 gene. This indicates a potential downstream mechanism by which it upregulates the biosynthesis of $PGD_2$ and consequentially its metabolite 15-deoxy-$\Delta^{12,14}$-$PGJ_2$ which would in turn suppress LPS-stimulated TNF-α production.

## 4.9 Conclusion

In conclusion, this study demonstrated that 7β OH-EpiA is a potent inhibitor of pro-inflammatory cytokine production from monocytic cells. The effects of 7β OH-EpiA are not mediated by increasing the production of either IL-4 or IL-10 indicating that the mechanism does not involve an upregulation of anti-inflammatory cytokines. 7β OH-EpiA also decreases $PGE_2$ but on the other hand, it induces biosynthesis of $PGD_2$ and 15d-$PGJ_2$. A failure of mifepristone to reverse the actions of 7β OH-EpiA, when it has been confirmed that mifepristone can reverse dexamethasone actions under the same conditions, clearly indicates that 7β OH-EpiA is highly unlikely to be acting via a GR or PR. As both $PGD_2$ and 15d-$PGJ_2$ were shown to suppress TNF-α production and that 7β OH-EpiA increased the production of these PGs, it is possible that they mediate the inhibitory actions of 7β OH-EpiA on inflammatory cytokine production in blood cells. This suggestion was strengthened by the observation that ketoprofen, which was confirmed to inhibit the production of all 3 PGs, was able to reverse the actions of 7β OH-EpiA on cytokine production. However, this still raises questions of the mechanisms by which 7β OH-EpiA can selectively enhance the production of both $PGD_2$ and 15d-$PGJ_2$ whilst suppressing the production of $PGE_2$. This would indicate a very specific upregulation of the enzymes which control $PGD_2$ biosynthesis such as $PGD_2$ synthase. This raises another question over whether this would be a direct effect on the enzymes or whether it occurs via a receptor-mediated signal system. A putative

receptor for 7β OH-EpiA could have an effect on the expression of the gene for $PGD_2$ synthase in an analogous manner to GRs and indeed the expression of mRNA for $PGD_2$ synthase was shown to be upregulated in the present studies. This could also be used to identify the receptors through which 7β OH-EpiA transduces its actions.

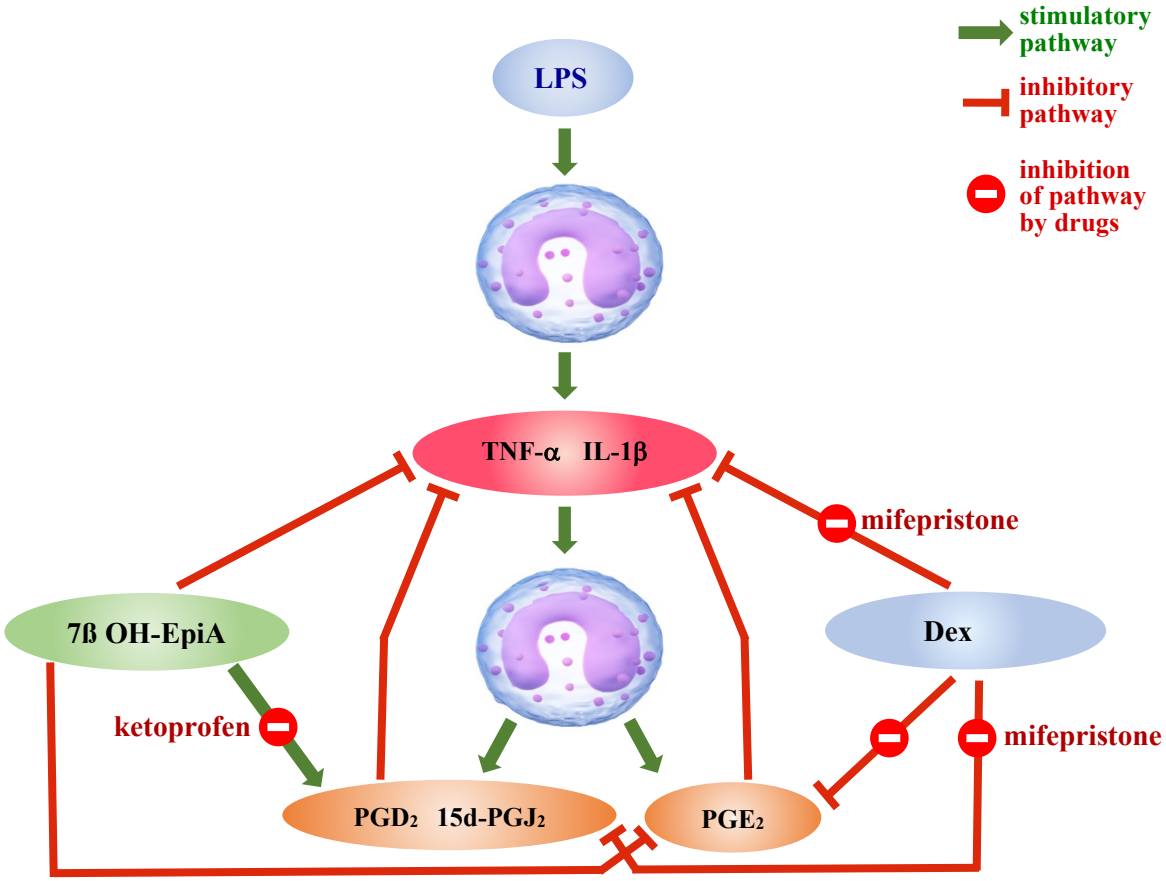



**Fig 4.1 Summary overview of the main contributions of the current thesis.**

The figure illustrates the effects of 7β OH-EpiA (compared to dexamethasone – Dex) on LPS-stimulated production of cytokines and prostaglandins (inhibition of $PGE_2$ and enhancement of $PGD_2$ and 15d-$PGJ_2$) from monocytic cells. It also shows the effects of either the NSAID, ketoprofen (inhibition of PG production) or the GR/ PR receptor antagonist mifepristone, which blocked the inhibitory actions of Dex but did not have any effects on either of the 7β OH-EpiA pathways (stimulation of $PGD_2$ and 15d-$PGJ_2$ or suppression of cytokine production).

## 4.10 Future Work

Clearly, much work has still to be carried out on 7β OH-EpiA. Further work to confirm its mechanism of action and identify its receptor are required as the comparison between the inactive 7α OH-EpiA isomer and active 7β OH-EpiA isomer clearly indicate a selective receptor action. Also, it is important to assess the effects of 7β OH-EpiA on the activity of $PGD_2$ synthase as time did not permit this in the present study.

# Chapter Five: References

# 5. References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