# The pro- or anti-inflammatory effects of oxidized or chlorinated lipids and its signalling mechanisms

By

Norsyahida Binti Mohd Fauzi

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Strathclyde Institute of Pharmacy and Biomedical Sciences

University of Strathclyde, Glasgow, UK

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## ABSTRACT

It is currently well accepted that atherosclerosis is an inflammatory disease and accumulation of oxidized low density lipoprotein (OxLDL) is a principal risk factor for this disease. Since the finding that oxidized phospholipids (OxPLs) is active components of OxLDL, many studies have demonstrated their pro- and anti-inflammatory effects as well as signalling mechanisms. In comparison, less is known about chlorinated lipids, although several reports show that they are found in atherosclerotic lesions and inflammatory loci, and induce mainly pro-inflammatory effects. This study investigated the role of chlorinated lipids on pro-inflammatory cytokine production and the signalling mechanisms induced by these modified lipids, in comparison with OxPLs. Treatment of myeloid (U937) cells with 1-stearoyl-2-oleoyl-sn-3-glycerophosphocholine chlorohydrin (SOPC ClOH) but not its native lipid enhanced the effect of lipopolysaccharide (LPS)-induced interleukin-8 (IL-8) and treatment with 2-chlorohexadecanal (2-ClHDA) inhibited LPS-induced tumor necrosis-alpha (TNF-α) production. However, treatment of these compounds alone did not increase the level of IL-8 and TNF-α protein expression. Using mouse fibrosarcoma cells (L929sA) that were stably transfected with nuclear factor-kappaB (NF-κB) dependent promoter, it was demonstrated that SOPC ClOH and 2-ClHDA did not stimulate NF-κB-driven genes activity and did not inhibit TNFα-induced NF-κB driven genes activity. SOPC ClOH treatment of human embryonic kidney 293 (HEK 293) cells overexpressing PPARα induced PPRE-driven gene expression. However, treatment of these cells with native SOPC and 2-ClHDA did not induce similar effects. In addition, treatment of human umbilical vein endothelial cells (HUVECs) with SOPC ClOH and 2-ClHDA did not induce degradation of Ikappa B alpha (IκB-α) or expression of mitogen activated protein kinases (MAPKs), and pre-treatment with either SOPC ClOH or 2-ClHDA did not inhibit LPS-mediated activation of NF-κB and MAPK pathways. In conclusion, phospholipid chlorohydrin induced pro-inflammatory effects and stimulated PPRE-driven gene activity in cells overexpressing PPARα whereas 2-ClHDA induced an anti-inflammatory effect. The effects were different to those observed with short-chain OxPLs, showing that the nature of the oxidative modification is important in determining cellular response

mechanism. These modified lipids could contribute to the pathology of atherosclerosis.

# PUBLICATIONS

## Poster presentation

**Fauzi, N.M.**, Spickett, C.M. (2010). Effect of SOPC chlorohydrin on cytokine production in U937 cells. Scottish Cardiovascular Forum, University of Glasgow, UK

**Fauzi, N.M.,** Bougarne, N., DeBosscher, K., Haegeman, G. & Spickett, C.M. (2010). Pro-inflammatory and anti-inflammatory effects of oxidized and chlorinated phospholipids, and the possible role of PPARs. Lipid Oxidation, Human Diseases and Aging Meeting at -University of Turin, Italy

## Oral presentation

**Fauzi, N.M.,** Spickett, C.M. (2010). Effects of chlorinated lipids on cytokine production and activation of peroxisome proliferator-activated receptors (PPARs). Scottish Lipid Discussion Group Meeting at University of Stirling, UK

## Journals

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Breusing, N, Grune, T., Andrisic, L., Atalay, M., Bartosz, G., Biasi, F., Borovic, S., Bravo, L., Casals, I., Casillas, R., Dinischiotu, A., Drzewinska, J., Faber, H., **Fauzi, N.M.**, Gajewska, A., Gambini, J., Gradinaru, D., Kokkola, T., Lojek, A., Luczaj, W., Margina, D., Mascia, C., Mateos, R., Meinitzer, A., Mitjavila, M.T., Mrakovcic, L., Munteanu, M.C., Podborska, M., Poli, G., Sicinska, P., Skrzydlewska, E., Vina, J., Wiswedel, I., Zarkovic, N., Zelzer, S., Spickett, C.M. (2010). An inter-laboratory validation of methods of lipid peroxidation measurement in UVA-treated human plasma samples. *Free Radic Res*, 44, 1203-15.

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## ABBREVIATIONS

| | |
|---|---|
| ABCA1 | ATP-binding cassette transporter A1 |
| ACAT1 | Acetyl-coenzyme A acetyltransferase 1 |
| AP-1 | Activator protein-1 |
| APS | Ammonium persulfate |
| ApoB-100 | Apolipoprotein B-100 |
| ApoE | Apolipoprotein E |
| ARE | Antioxidant-response element |
| ATF-6 | Activating transcription factor-6 |
| BHT | Butylated hydroxytoluene |
| CBP | Cyclic AMP response element binding protein |
| CD36 | Cluster of Differentiation 36 |
| CHD | Coronary heart disease |
| CHO | Chinese hamster ovary cell |
| COX | Cyclooxygenase |
| CRE | Cyclic AMP-response element |
| CVD | Cardiovascular disease |
| DAPI | 4',6-diamidino-2-phenylindole |
| DEX | Dexamethasone |
| DMEM | Dulbecco's modified eagle medium |
| DMSO | Dimethyl sulfoxide |

| | |
|---|---|
| DTT | Dithiothreitol |
| EBM | Endothelial Basal Media |
| ECL | Enhanced chemiluminescence reagent |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| eNOS | Endothelial nitric oxide synthase |
| ER | Endoplasmic reticulum |
| ERK | Extracellular signal-regulated kinase |
| ES-MS | Electrospray mass spectrometry |
| FCS | Foetal calf serum |
| GR-α | Glucocorticoid receptor-alpha |
| GC-MS | Gas chromatography-mass spectrometry |
| GM-CSF | Granulocyte macrophage-colony stimulating factor |
| GPCR | G-protein coupled receptor |
| HBSS | Hanks buffered saline solution |
| HDL | High density lipoprotein |
| HEK 293 | Human embryonic kidney 293 |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HMG-CoA | 3-hydroxy-methylglutaryl coenzyme A |
| 4-HNE | 4-hydroxynonenal |
| HO-1 | Haem oxygenase-1 |
| HUVEC | Human Umbilical Vein Endothelial Cell |

| | |
|---|---|
| ICAM-1 | Intracellular adhesion molecule-1 |
| IDL | Intermediate density lipoprotein |
| IFN-γ | Interferon-γ |
| IκBα | IkappaB alpha |
| IKK | IκB kinase |
| IL-1β | Interleukin-1 beta |
| IL-6 | Interleukin-6 |
| IL-8 | Interleukin-8 |
| IRAK | Interleukin-1 receptor associated kinase |
| IRE1 | Inositol requiring 1 |
| JNK | c-Jun N-terminal kinase |
| KEAP-1 | Kelch-like enoyl-CoA hydratase-associated protein 1 |
| LDL | Low density lipoprotein |
| LOX | Lipoxygenase |
| LPS | Lipopolysaccharide |
| LB | Lysogeny broth |
| LBP | LPS binding protein |
| MAPK | Mitogen-activated protein kinase |
| MDA | Malonaldehyde |
| MyD88 | Myeloid differentiation primary response gene 88 |
| MCP-1 | Monocyte chemoattractant protein-1 |
| M-CSF | Macrophage colony-stimulating factor |

| | |
|---|---|
| MEM | Minimum essential medium |
| MM-LDL | Minimally modified LDL |
| MPO | Myeloperoxidase |
| MTT | 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NADPH | Nicotinamide adenine dinucleotide phosphate (reduced form) |
| NF-κB | Nuclear factor-kappa B |
| NOS | Nitric oxide synthase |
| NOX | NADPH oxidase |
| NQO1 | NADPH:quinine oxidoreductase-1 |
| OxLDL | Oxidized low density lipoprotein |
| OxPL | Oxidized phospholipid |
| PAF | Platelet activating factor |
| PAMP | Pathogen associated molecular pattern |
| PBS | Phosphate-buffered saline |
| PEI | Polyethylenimine |
| PERK | Double-stranded RNA dependent protein kinase [PKR]-like ER kinase |
| PFB | Pentafluorobenzyl |
| PPAR | Peroxisome proliferator activated receptor |
| PGC-1 | PPARgamma coactivator-1 |
| PPRE | PPAR response element |
| PUFA | Polyunsaturated fatty acid |

| | |
|---|---|
| PMA | Phorbol myristate acetate |
| RASMC | Rat aortic smooth muscle cell |
| ROS | Reactive oxygen species |
| RTK | Receptor tyrosine kinase |
| SDS | Sodium deodecyl sulfate |
| SMA | Smooth muscle-α actin |
| SR-BI | Scavenger receptor class B type I |
| SREBP | Sterol-regulatory element-binding protein |
| SRC-1 | Steroid receptor coactivator |
| SSC | Sodium chloride-sodium citrate buffer |
| TEMED | N, N, N', N-tetramethylethylenediamine |
| TNF-α | Tumor necrosis factor-α |
| TLC | Thin layer chromatography |
| TLR | Toll-like receptor |
| TNFR | Tumor necrosis factor receptor |
| TRADD | Tumor necrosis factor receptor type 1-associated death domain |
| TRAF | TNFR associated factor |
| UPR | Unfolded protein response |
| VCAM-1 | Vascular cell adhesion molecule-1 |
| VLDL | Very low density lipoprotein |
| XO | Xanthine oxidase |

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# Chapter 1

# General Introduction

## 1.1. Introduction

Atherosclerosis is an inflammatory disease with an oxidative aetiology. Oxidized low density lipoprotein (OxLDL) and oxidized lipids occur and are thought to contribute to its pathology. They have a variety of signalling effects, the mechanisms of which have been quite well studied but there are still questions remaining (Steinberg et al., 1989, Bochkov et al., 2010). Inflammation also produces chlorinated lipids (Spickett, 2007) and these are much less well studied and understood This project involved studying the pro- and anti-inflammatory effects of chlorinated lipid and phospholipid and comparing them with the known effects of oxidized phospholipids (OxPLs).

## 1.2 Cardiovascular disease

Cardiovascular disease (CVD), also called 'heart and circulatory disease', is currently the predominant cause of mortality in the UK and worldwide. This disease is expected to increase significantly during the next decades owing to the unhealthy food intake and lifestyle habits. The two main forms of CVD, coronary heart disease (CHD) and stroke, can lead to potential loss of life below age 75 (called premature death). CHD alone causes approximately one in five premature deaths in men and one in ten in women. It has also been reported that in the UK, death rates due to CHD are highest in Scotland (Scarborough et al., 2010). The British Regional Heart Study found that smoking, high blood pressure and high serum total cholesterol accounted for 90% of attributable risk of CHD (Emberson et al., 2003). Other risks factors associated with CVD include advanced age, stress, diabetes, physical inactivity, alcohol consumption and obesity (Scarborough et al., 2010).

The most common symptoms of CHD are angina and myocardial infarction. Angina, which is simply known as chest pain, occurs when blood flow to an area of the heart decreases causing imbalance between myocardial oxygen supply and demand. When this happens, the symptoms such as discomfort, heaviness, pressure, aching, burning, fullness, squeezing, or painful spasms can be felt particularly in the chest, and other area such as shoulders, arms, neck, throat, jaw and back (Gibbons et al., 1999). A myocardial infarction, or heart attack, occurs when blood flow to the heart is blocked by thrombus formation in the coronary arteries, resulting in symptoms such as acute chest pain radiating from left arm or left side of the neck, dyspnoea, severe cardiac arrhythmias, and is often fatal.

## 1.3 Atherosclerosis

### 1.3.1 Pathology of atherosclerosis.

Atherosclerosis is defined in layperson's terms as hardening of the arteries, and gives rise to cardiovascular disease and coronary heart disease, through slowly progressing lesion formation and narrowing of luminal arteries. The pathology of atherosclerosis is characterized by chronic inflammation inside the arterial wall that occurs at predilection sites with disturbed blood flow such as vascular branching points. It is thought that the initiating event of this disease is mild injury to the vascular endothelium. This injury, for instance, can result from hemodynamic stress that occurs within branch vessels (Bassiouny et al., 1994) and chronic infections by various microorganisms (Meurman et al., 2004).

Vascular injuries can result in endothelial dysfunction and alteration in endothelial structure including the absence of the elastin barrier, which serves as the major permeability barrier to LDL, and the exposure of extracellular matrix proteoglycans that are able to bind to LDL (Kwon et al., 2008), resulting in the retention of LDL in subendothelial layer of arteries. The uptake and retention of LDL within the intima subsequently initiates the formation of foam cells, leading to the first stage of atherosclerotic development, formation of lesions containing fatty streaks (Witztum and Steinberg, 2001, Libby, 2006). Progression of fatty streaks into a more fibrous lesion involves the migration of smooth muscle cells and the secretion of extracellular matrix molecules including fibrillar collagen and elastin, which together, develop a fibrous cap. Underneath the fibrous cap, a necrotic core composed of foam cells, dead macrophages, apoptotic bodies and acellular debris is formed. In more advanced stages of atherosclerosis, the fibrous cap begins to lose its stability due to reduction and degradation of the extracellular matrix. Reduction in the amount of extracellular matrix occurs due to the death of smooth muscle cells within the lesions and degradation is catalysed by proteinases produced by macrophages (Libby and Ridker, 2006).

Over many years, plaques can develop until they block the lumen and cause ischemia; however, plaque rupture and thrombosis, which occur as a result of plaque instability that is induced by calcification, ulceration at the endothelial surface and haemorrhage (Willeit and Kiechl, 2000), are usually the major clinical concern. A thrombus, if present in the coronary artery, may result in myocardial infarction, and if transferred into carotid arteries, may result in stroke (Ross, 1993, Libby, 2000).

### 1.3.2 Atherosclerosis as an inflammatory disease.

It is now well established that atherosclerosis is an inflammatory disease (Ross, 1999, Libby, 2006). However, the view of inflammation in association with atherosclerosis actually began about 100 years ago. The term atherosclerosis was first introduced by Jean Lobstein in 1989. A few years later, two different groups described the cellular inflammatory alteration in atherosclerotic arteries. Rudolf Virchow proposed that the cellular pathology was critical in atherosclerosis, although Carl von Rokitansky suggested that it had a secondary role only (Mayerl et al., 2006, Rokitansky, 1855, Virchow, 1971). In 1970s, the response-to-injury model was proposed (Ross, 1993), in which initial injury to the vessel wall by mechanical injury, toxins and oxygen radicals can result in endothelial dysfunction and further inflammation. More recently, a large number of studies have highlighted the roles of immune cells in atherosclerosis (Hansson and Libby, 2006).

As mentioned above, atherosclerosis is prone to occur at branch points of arteries that are subjected to turbulent flow. Inflammation occurs in the vasculature as a response to haemodynamic stress and other types of injury (Wick et al., 1995). In response to injury, activated intimal cells release enzymes and inflammatory components that can modify LDL particles, which in turn triggers various inflammatory signals leading to a range of pro-inflammatory effects (Pentikainen et al., 2000).

Macrophages were found to be largely present in fatty streaks lesion and the first inflammatory cells associated with atherosclerosis (Gerrity et al., 1979). This type of

immune cell also plays a role in advanced lesions (Libby, 2006). Studies in apolipoprotein E (ApoE)-decifient mice using light and electron microscopy have demonstrated that monocyte attachment to the endothelial cells occurs throughout the process of lesion development from fatty streak to advanced fibrous lesion (Nakashima et al., 1994, Dansky et al., 1999). The adherence of leukocytes occurs through the binding between adhesion molecules on endothelial cells and receptors on leukocytes (Blankenberg et al., 2003).

Various types of adhesion molecules such as selectin, integrin and immunoglobulin superfamilies, have been identified (Blankenberg et al., 2003). Selectin consists of three members; P-selectin, E-selectin and L-selectin, which are designated according to their expression site. The main site for P-selectin expression is platelets but its expression has also been found in endothelial cells. On the other hand, E-selectin is expressed exclusively in endothelial cells whereas L-selectin is expressed in leukocytes including polymorphonuclear leukocytes, eosinophils, monocytes and lymphocytes (Stocker and Keaney, 2004). In endothelial cells, P-selectin in located in Weible-Palade bodies and upon activation of variety of stimuli, it is expressed on the cell surface for a short time. Selectins recognize mucin-like glycoprotein ligands as well as fucosylated and sialylated carbohydrates such as sialyl Lewis X (Blankenberg et al., 2003). One of the main leukocyte receptors or ligand that bind to P-selectin molecules is P-selectin glycoprotein ligand-1 (PSGL-1), which also binds with lower affinity to E-selectin and L-selectin (Blankenberg et al., 2003). E-selectin is also recognized by sialyl Lewis X (Stocker and Keaney, 2004). In the event of monocyte recruitment, these adhesion molecules play a role in mediating rolling of

monocytes on the endothelial cell surface. In addition, adhesion molecules from the immunoglobulin superfamily such as vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) interact respectively with very late antigen-4 (or α4β1) and αLβ2 integrin (LFA-1) or αMβ2 integrin (Mac-1), adhesion molecules of the integrin family. This interaction provides firm adhesion of monocytes to endothelial cells (Blankenberg et al., 2003)

The expression of both P-selectin and E-selectin has been demonstrated in atherosclerotic lesions of human and hypercholesterolaemic rabbit (Huo and Ley, 2001). VCAM-1 and ICAM-1 have also been found to be highly expressed in atherosclerotic lesions of hypercholesterolaemic rabbits and mice (Cybulsky and Gimbrone, 1991, Alam et al., 2006, Cybulsky et al., 1999). In both species, it was observed that VCAM-1 expression was restricted within lesions but ICAM-1 was also found in uninvolved aorta (Cybulsky et al., 1999). In human atheromatous lesions, both VCAM-1 (O'Brien et al., 1999) and ICAM-1 (Poston et al., 1992) are also expressed. Interestingly, unlike in rabbits and mice, VCAM-1 was shown to be expressed in uninvolved inflammation area in human (See Figure 1.1) (Davies et al., 1993).

Once adhered to the endothelium, leukocytes can enter the arterial wall and this event is directed by chemoattractant cytokines (or chemokines). There are two groups of chemokines designated as CC and CXC. Generally, CC chemokines attract mononuclear cells including monocytes and T cells, and CXC chemokines attract neutrophils (Rollins, 1997). Monocyte chemoattractant protein-1 (MCP-1), a member

of CC family, is thought to play a role in monocyte recruitment. This is shown by a study in LDL-deficient mice ($LDLR^{-/-}$), where additional deletion of MCP-1 ($LDLR^{-/-}/MCP\text{-}1^{-/-}$) results in fewer intimal macrophages compared to MCP-1 wild-type mice ($LDLR^{-/-}/MCP\text{-}1^{+/+}$) (Gu et al., 1998). Interleukin-8 (IL-8) from the CXC family has also been shown to play a role in atherogenesis (Boisvert et al., 1998). IL-8 is released mainly by macrophages and is known to attract neutrophils as well as T-cells (Reape and Groot, 1999, Terkeltaub et al., 1994). This chemokine is also capable of inducing migration and proliferation of smooth muscle cells into the intima area (Yue et al., 1994), leading to formation of advanced atherosclerotic lesions (See Figure 1.1). This suggests that early recruitment of macrophages can trigger further recruitment of other inflammatory cells into the arterial wall, which together can contribute to the progression of atherosclerosis. Although it is increasingly accepted that immune cells and inflammatory mechanisms play an important role in atherosclerosis, LDL remains the most important risk factor of atherosclerosis.

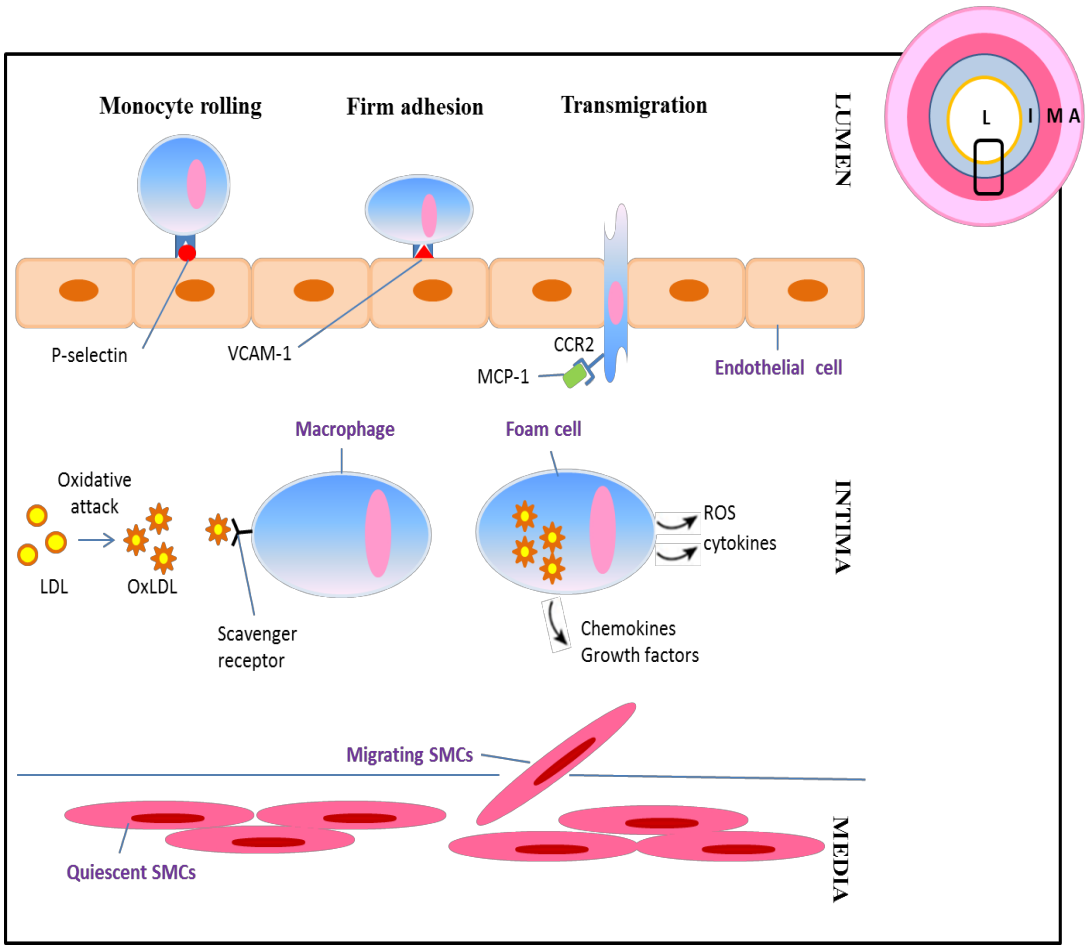


**Figure 1.1 Schematic representation of monocyte recruitment, foam cells formation and migration of smooth muscle cells that involve in progression of fatty streak to fibrous lesion.**

(L:lumen, I: tunica intima, M: tunica media, A: tunica adventitia, VCAM-1: vascular cell adhesion molecule-1, MCP-1: monocyte chemoattractant protein-1, CCR2: CC chemokine receptor type 2, LDL: low density lipoprotein, OxLDL: oxidized LDL, ROS: reactive oxygen species, SMC: smooth muscle cell). Adapted from Libby and Ridker, 2006 and Blankenberg et al., 2003.

## 1.4 Cholesterol and LDL in atherosclerosis.

### 1.4.1 Cholesterol and LDL.

It has been well established that there is a strong association between high blood cholesterol levels, hypercholesterolemia, and cardiovascular disease (Libby et al., 2000). A century ago, Russian investigators Anitchow and Chalatow successfully demonstrated that rabbits fed with a diet rich in eggs develop fatty lesions resembling to human atheroma (Anitschkow and Chalatow, 1913, Libby et al., 2000) . Further investigation by these investigators suggested that cholesterol, as opposed to ovalbumin or other non-lipid constituents of eggs, is responsible for the lesion formation (Libby, 2000, Lehr et al., 1992)) Individuals with familial hypercholesterolaemia have increased risk of cardiovascular disease (Witztum and Steinberg, 1991). Heterozygotes for this disease have a two to five fold elevation in plasma cholesterol and the level is even higher in the homozygotes. While the majority of heterozygotic individuals experience myocardic infarction by the age of 60, this event can occur much earlier in the homozygotic group. In the human population generally, it is rare to have clinically significant fatty lesions if plasma cholesterol is less than 150 mg/dl; however if plasma cholesterol exceeds this level, there is no doubt that increased risk of atherosclerosis occurs (Witztum and Steinberg, 1991). The association of cholesterol with cardiovascular disease was further supported by observations that cholesterol lowering therapy, such as 3-hydroxy-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (i.e., statins) reduce the clinical manifestation of atherosclerosis (Gotto and Grundy, 1999).

Lipids including cholesterol and triacyglycerides are transported in the plasma by large complexes called lipoproteins. These lipoproteins composed of a hydrophobic core covered by a hydrophilic shell and apoproteins. Lipoproteins are categorized according to their densities. Chylomicrons are the lipoprotein with the lowest density followed by very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoprotein (HDLs). The densities of these lipoproteins are a reflection of the amount of proteins versus lipids in the complex, in which higher protein content contributes to higher density of lipoprotein. Chylomicrons carry dietary triglycerides to peripheral tissues and dietary cholesterols to liver whilst VLDL transports triglycerides from liver to peripheral tissues. LDL and HDL are important carriers of cholesterols. LDL transports cholesterol out to peripheral tissues while HDL brings cholesterol back to the liver (depicted in Figure 1.2) (Olson, 1998). Several lines of evidence convincingly suggest that LDL promotes atherosclerosis. Cholesterol uptake through the classical LDL receptor pathway cannot result in significant accumulation of cholesterol due to negative feedback mechanism induced by intracellular cholesterol content. However, modified form of this particle can be recognized by scavenger receptor, which upon its uptake resulting in formation of foam cells (Jialal and Devaraj, 1996). Details in modification of LDL are further described in section 1.4.2.

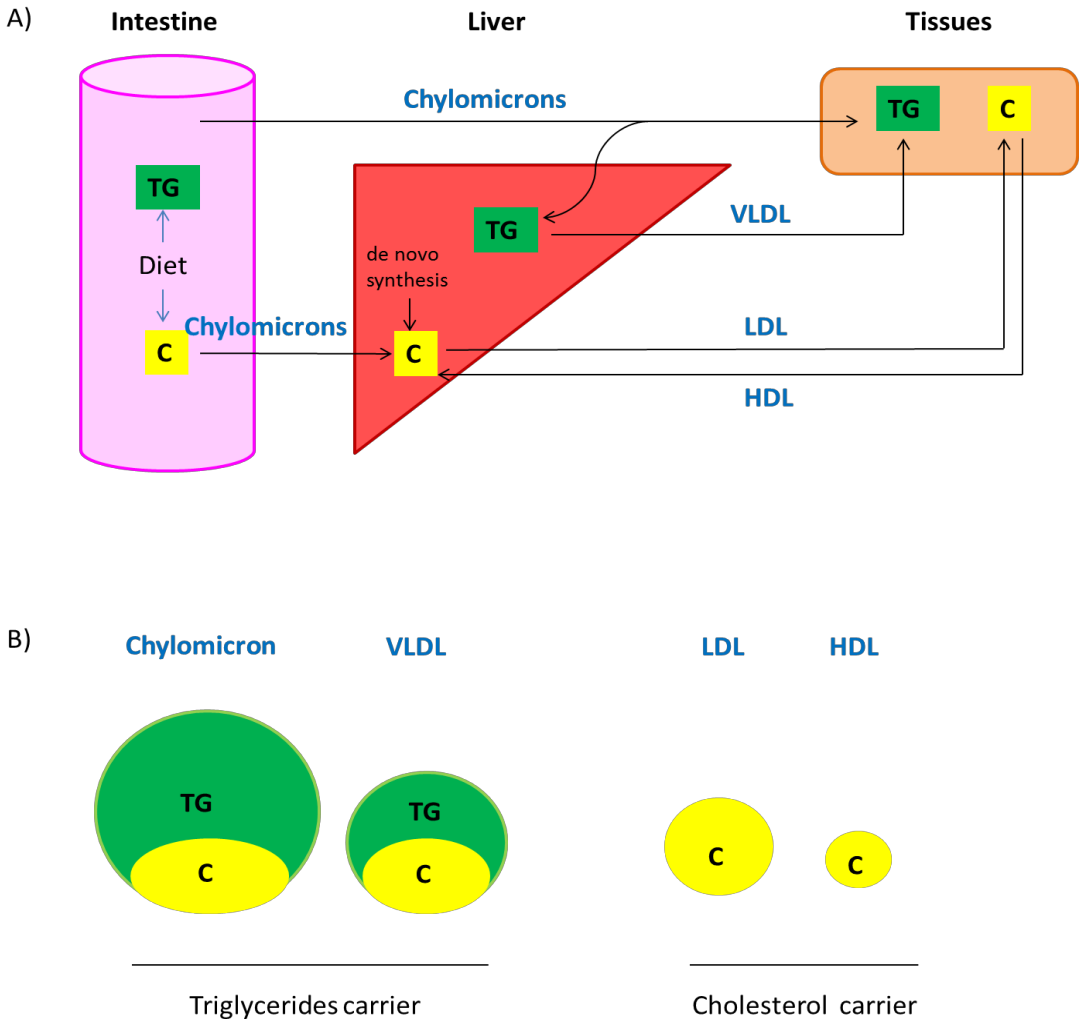


**Figure 1.2 Schematic representation of (A) lipid transport and (B) different types of lipoprotein particles.**

(TG: triglycerides, C: cholesterol, VLDL:very low density lipoproteins, LDL: low density lipoproteins, HDL: high density lipoprotein).

It is currectly well accepted that LDL is a major risk factor for atherosclerosis, therefore it is of importance to know the structure of this particle. A molecular model of LDL that encapsulates the detailed structure of the particle has been developed (Hevonoja et al., 2000) (See Fig. 1.3). Specifically, LDL is a small spherical lipoprotein with diameter size of approximately 22 nm composed of three distinct layers; outer surface layer, interfacial layer and core. The surface layer contains approximately 700 molecules of lipids, mainly phosphatidylcholine and sphingomyelin and other types of lipids including, phosphatidylethanolamine, diacylglycerol ceramide and phosphatidylinositol as well as a single apolipoprotein B (apoB)-100; the core contains about 170 triglycerides and 1600 cholesteryl esters; and the interfacial layer is composed of interpenetrating core and surface lipids (Hevonoja et al., 2000). Moreover, the LDL particle also contains lipophilic antioxidants including α-tocopherol, β-carotene and ubiquinol that can provide protection for the LDL particle from oxidative modification (Esterbauer et al., 1992).

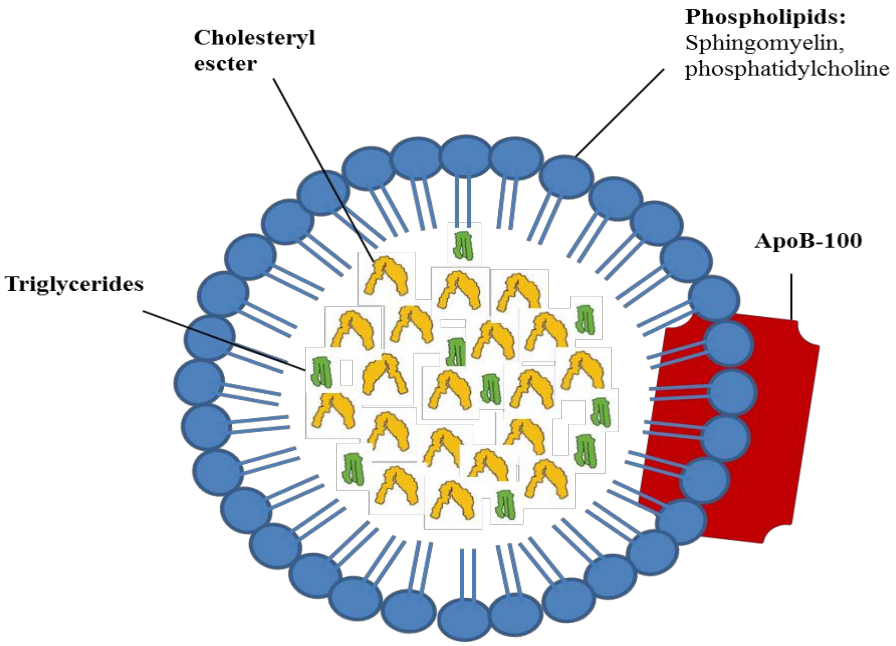


**Figure 1.3 Schematic molecular model of a LDL particle.** It contains phospholipids and an apoB-100 protein embedded in the surface layer and cholesteryl esters as well as triglycerides in the core layer. Adapted and simplified from Hevonoja et al., 2000.

### 1.4.2 Oxidative modification of LDL.

The hypothesis of oxidative stress in atherosclerosis postulates that atherosclerosis occurs due to oxidative modification of LDL in the arterial wall (Steinberg et al., 1989, Witztum, 1994, Klatt and Esterbauer, 1996). The early work that leads to this hypothesis was reported by Goldstein et al. in the early 80s (Brown and Goldstein, 1983). It started in 1977, when they noted that LDL particles did not appear to be atherogenic *in vitro* as macrophages incubated with LDL failed to internalize excess lipoprotein-cholesterol due to downregulation of the LDL receptor (Goldstein and Brown, 1977). Later on, they proposed that LDL must be somehow altered prior to its uptake by macrophages and then taken up by receptors on macrophages distinct from the LDL receptor. They observed that accumulation of macrophage-derived foam cells in homozygous hypercholesterolemia patients that have a deficiency in or absolutely no functional LDL receptor, develop macrophage-derived foam cells that was similar to those observed in normal hypercholesterolaemic patients that have normal LDL receptors. Moreover, the group demonstrated that the rate of native LDL uptake by mouse peritoneal macrophages was extremely slow, even at high concentrations of LDL and no formation of foam cells was observed (Brown and Goldstein, 1983).

Subsequently, several studies were carried out using LDL modified by physical, chemical or enzymatic methods to look for some form of LDL that could induce foam cell formation *in vitro*. Several modifications were observed; however, chemical acetylation provided the most significant effect (Brown and Goldstein, 1983). OxLDL uptake is mediated by the scavenger receptor, originally designated as

the acetyl-LDL receptor, later cloned and characterized by Kodoma et al and redesignated as scavenger receptor Type A (Kodama et al., 1988). Later on, other types of scavenger receptor for oxidized LDL such as scavenger receptor Type B [i.e., Cluster of Differentiation 36 (CD36)] (Endemann et al., 1993) and lectin-like OxLDL receptor (Sawamura et al., 1997) were discovered. These receptors are not down regulated as the cholesterol content in the macrophage increases, and are therefore responsible for the formation of foam cells. In 1981, Henriksen et al showed that native LDL incubated with cultured endothelial cell was modified into a form that is recognized by peritoneal macrophages. A later study showed that during its incubation with endothelial cells, LDL underwent an oxidative modification (Steinbrecher et al., 1984, Hessler et al., 1983), suggesting that cells can produce oxidants that can oxidatively modify native LDL components.

The role of oxidative stress in atherosclerosis is further supported by the finding that antioxidants can inhibit lesion formation (Carew et al., 1987, Chang et al., 1995, Tangirala et al., 1995). Evidence is not good in antioxidant supplementation studies in humans (Kris-Etherton et al., 2004), although in animals more positive effects have been observed.. This phenomenon has been shown in two studies where antioxidants such as probucol prevent lesion formation in the WHHL rabbit (a model for familial hypercholesterolaemia) (Carew et al., 1987, Kita et al., 1987) and butylated hydroxytoluene (BHT) inhibits lesion formation in hypercholesterolaemic rabbits (Bjorkhem et al., 1991). In 1989, it was reported that LDL extracted from atherosclerotic lesions of rabbits and humans has similar physical, immunological and biological properties to oxidative modified LDL *in vitro* (Yla-Herttuala et al.,

1989), suggesting that LDL can also undergo similar oxidative modification *in vivo* which leads to the cellular effects observed in the development of atherosclerosis.

It is believed that oxidation of LDL *in vivo* is likely to occur primarily in the intima, as it is sequestered by the proteoglycan, and the antioxidant level in this space is much lower compared to antioxidants in the plasma (Stocker and Keaney, 2004). Moreover, it has also been suggested recently that oxidation of LDL can proceed intracellularly, most probably in the lysosomal compartment of macrophages (Wen and Leake, 2007). Several factors have been reported to contribute to the *in vivo* modification of LDL (Witztum and Steinberg, 1991). Some intrinsic factors that influence oxidation of LDL are fatty acid composition, particularly polyunsaturated fatty acid (PUFA), which is prone to lipid peroxidation, antioxidant contents, phospholipase $A_2$ activity and particle size (Witztum and Steinberg, 1991).

In addition, cellular pro-oxidant activity contributes to the level of extrinsic factors required for oxidation of LDL. Vascular cells produce both free radical oxidants (i.e superoxide, hydroxyl radicals, carbon center radicals and nitric oxide) and non-radical oxidants through various enzymatic pathways including nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), xanthine oxidase (XO), NO synthase (NOS), myeloperoxidase (MPO) and lipooxygenase (LPO), all of which have been found in the atherosclerotic lesion (Stocker and Keaney, 2005). Another important factor that influences LDL modification is the concentrations of different metal ions including copper or iron. Free transition metal has been widely used to induce oxidation of LDL *in vitro*; however, their role in physiological oxidation of

LDL is controversial due to very low free metal ion levels observed *in vivo* (Halliwell and Gutteridge, 1990, Gutteridge, 1984, Dabbagh and Frei, 1995). However, it has been shown that iron containing proteins (e.g. ferritin, transferrin, haemoglobin, myoglobin) and copper containing protein (e.g. ceruloplasmin) are able to oxidize LDL *in vitro* (Fox et al., 2000), suggesting that they could also be physiologically relevant in generating OxLDL. Plasma and extracellular fluid concentrations of antioxidant components and high density lipoprotein (HDL) is also important factor for LDL oxidation (Witztum and Steinberg, 1991). HDL is a smaller lipoprotein than LDL that function as cholesterol carrier from tissue into the liver, and has been shown to partially protect LDL from oxidation by endothelial cells and copper (Parthasarathy et al., 1990).

Oxidation of LDL *in vivo* involves a complex mechanism; however, it has been demonstrated that oxidation of LDL *in vitro* follows model behaviour with lag phases followed by a propagation phase and finally a decomposition phase (See Figure 1.4) (Esterbauer et al., 1993). Free radicals initiate lipid peroxidation by abstracting electrons from a double bond within PUFA of LDL forming a carbon centered radical. The source of radicals *in vivo* will be described later, in section 1.5.2. The carbon centered PUFA radical can then react with molecular oxygen that leads to production of a lipid peroxyl radical, which then abstracts a hydrogen atom from another PUFA producing lipid hydroperoxide and another peroxyl radical. In the lag phase, antioxidant competes with chain propagation by scavenging lipid peroxyl radicals. Studies performed *in vitro* revealed that α-tocopherol is first to be depleted and β-carotene last (Esterbauer et al., 1991a, Esterbauer et al., 1991b). In

the propagation phase, where antioxidants are depleted, PUFA is quickly oxidized to lipid hydroperoxide. In the decomposition phase, fatty acids are fragmented resulting in the formation of highly reactive intermediates such as aldehydes [i.e., malonaldehyde (MDA) and 4-hydroxynonenal (4-HNE)] and ketones, which can then form an adduct with apoB-100 protein (Jurgens et al., 1987) or the head group of phospholipids, such as phosphatidylethanolamine (Horkko et al., 1999).

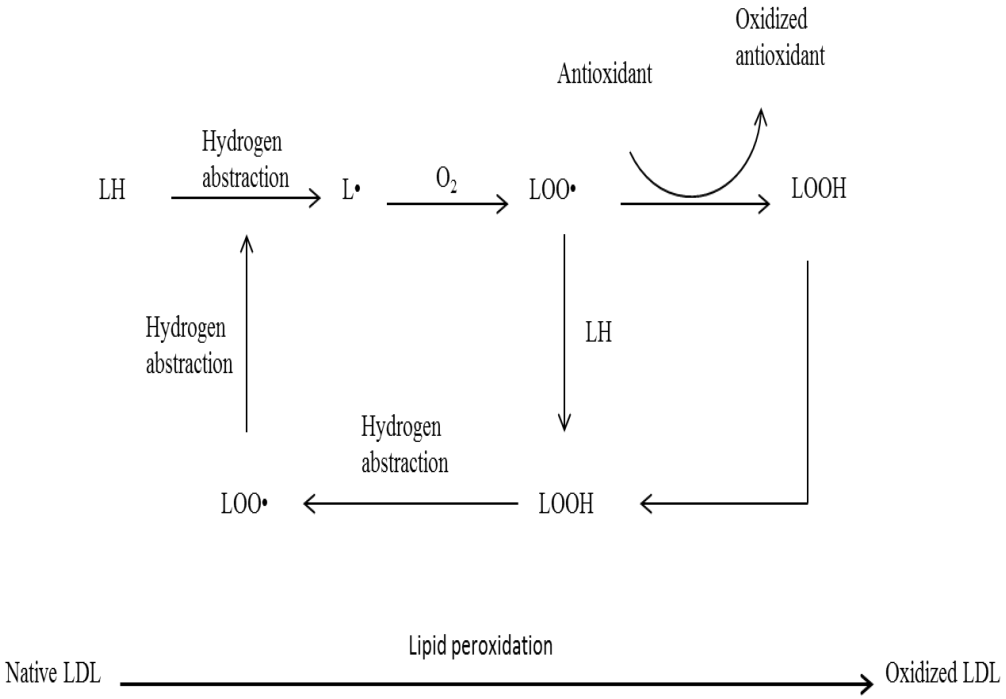


**Figure 1.4 Scheme of lipid peroxidation that involves oxidative modification of LDL.** The event has been demonstrated *in vitro* and the detail description of this event has been described within the text. (LH =a PUFA of LDL, LOO• = peroxyl radical, LOOH= hydroperoxide). Adapted from (Esterbauer et al., 1992, Esterbauer et al., 1993). The event has been demonstrated *in vitro* and the detail description of this event has been described within the text. (LH =a PUFA of LDL, LOO• = peroxyl radical, LOOH= hydroperoxide). Adapted from Esterbauer et al., 1992 and Esterbauer et al., 1993.

### 1.4.3 Biological effects of OxLDL.

Some of the biological effects induced by OxLDL that are thought to be critical in atherosclerosis include production of pro-inflammatory cytokines (i.e., TNF-α) (Jovinge et al., 1996), chemotactic factors (i.e., IL-8 and MCP-1) (Terkeltaub et al., 1994, Yla-Herttuala et al., 1991) and colony stimulating factors [i.e., macrophage colony-stimulating factor (M-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF)] (Rajavashisth et al., 1995) as well as induction of adhesion molecules on the surface of endothelial cells and macrophages (Lehr et al., 1992, Berliner et al., 1990, Carlos and Harlan, 1990). However, it is important to note that OxLDL exists in several forms that can be characterized by different degrees of oxidation and different mixtures of active components (Levitan et al., 2010).

Preparation of different forms of OxLDL as reported in the literature can be divided into two types; minimally modified LDL (MM-LDL) and extensive modified LDL (OxLDL) (Levitan et al., 2010). MM-LDL contains 2-5 nmol per mg of thiobarbituric acid reactive substance per mg of LDL cholesterol (Berliner et al., 1990) whereas OxLDL contains 10-50 nmol per mg of thiobarbituric acid reactive substance per mg of LDL cholesterol, indicating that lipid peroxidation occur much less in MM-LDL. The structure of the MM-LDL particle is slightly different from native LDL resulting in different effects produced by these particles, but MM-LDL can only be recognized by LDL receptors and not scavenger receptors, similar to native LDL. Therefore, it is thought that MM-LDL may be more physiologically relevant in the initial stage of atherosclerosis and some potential pathways of MM-LDL formation *in vivo* have been reviewed recently by Levitan et al. (Levitan et al.,

2010). Interestingly, this particle itself does not have chemotactic properties, unlike the highly OxLDL, but can induce the expression of chemotactic factors (i.e., MCP-1) by both aortic smooth muscle cells and endothelial cells (Cushing et al., 1990). As detailed previously, highly OxLDL is not recognized by the LDL receptor; instead, it has high binding affinity to scavenger receptors, suggesting they may have a role in more advance stage of atherosclerosis. These findings suggest that all degrees of oxidation are involved in atherogenesis. Many effects of OxLDL mentioned above are mediated by oxidized phospholipid components, although some effects may also be attributed to modified protein components. In the next section, the effects of oxidized phospholipids as well as the signalling mechanisms are further described.

## 1.5 Oxidized phospholipids (OxPLs) derived from OxLDL.

### 1.5.1 Overview

About seventeen years ago, OxPLs were found to be an active component of MM-LDL (Watson et al., 1995). OxPLs extracted from MM-LDL as well as autoxidation products of 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (OxPAPC) were shown to mimic the effect of MM-LDL (Watson et al., 1995). Two years later, three components of active phospholipids derived from autoxidation of PAPC: 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC), 1-palmitoyl-2-glutaroyl-sn-glycero-3 phosphocholine (PGPC) and 1-palmitoyl-2-(5,6-epoxyisoprostane E2)-sn-glycerol-3-phosphocholine (PEIPC) were discovered (See Figure 1.5). These specific components of OxPLs were also found in atherosclerotic lesions from hypercholesterolaemic rabbits (Watson et al., 1997). Following on from these key discoveries, various studies demonstrated that OxPAPC as well as its

individual components possess pro-inflammatory and pro-atherogenic affects, and therefore may have an important role in atherosclerosis. The effects induced by OxPLs will be described later in this chapter.

### 1.5.2 Initiation of phospholipid oxidation.

Oxidation of phospholipids *in vivo* can be initiated by lipid peroxidation and the detailed mechanism *in vitro* has been described above. Lipid peroxidation is initiated by free radicals, leading to the formation of peroxyl radicals. Subsequent interaction of peroxyl radicals with other lipid molecules leads to production of hydroperoxides. Free radicals are derived from exogenous and endogenous sources. One of the exogenous sources is cigarette smoke and tar. Reactive species derived from smoking include hydroxyl radicals, nitric oxide and carbon centered radicals (Church and Pryor, 1985). Another exogenous source for free radical production is UV light; which produces hydroxyl radical through water radiolysis (Takamoto and Chance, 2006).

Several enzymes in the cellular system provide the endogenous source of free radicals. Some examples of the enzymes involved are; NADPH oxidase (NOX), xanthine oxidase (XO), cyclooxygenase (COX), Nitric oxide synthase (NOS) and MPO. NOX, XO, COX and uncoupled NOS can produce superoxide anion radicals ($O_2^-$), which can immediately be dismutated by superoxide dismutase to generate hydrogen peroxide ($H_2O_2$) and oxygen ($O_2$). $H_2O_2$ can then react with $Fe^{2+}$ to form hydroxyl ions ($OH^-$) via the Fenton reaction. Alternatively, MPO can catalyse reaction between $H_2O_2$ and chloride ($Cl^-$) to form hypochlorous acid (HOCl) that can

then form a hydroxyl radical. In addition, $O_2^-$ can react with nitric oxide (NO) produced by NOS to generate a stronger oxidant, peroxynitrite ($ONOO^-$) (Margaill et al., 2005).

In addition, lipoxygenases (LOX), particularly 12/15- lipoxygenases can also contribute to lipid peroxidation, where LOX inserts molecular oxygen into the 1,4-pentadiene motif at C-12 or C15 of esterified arachidonic acid, resulting in production of hydroperoxides (Bochkov et al., 2010). In addition to lipid peroxidation, where oxygen is inserted into unsaturated fatty acid, oxidation of phospholipids can also occur through halogenation, by which halogen is introduced into unsaturated fatty acid; this will be discussed further in section 1.6.

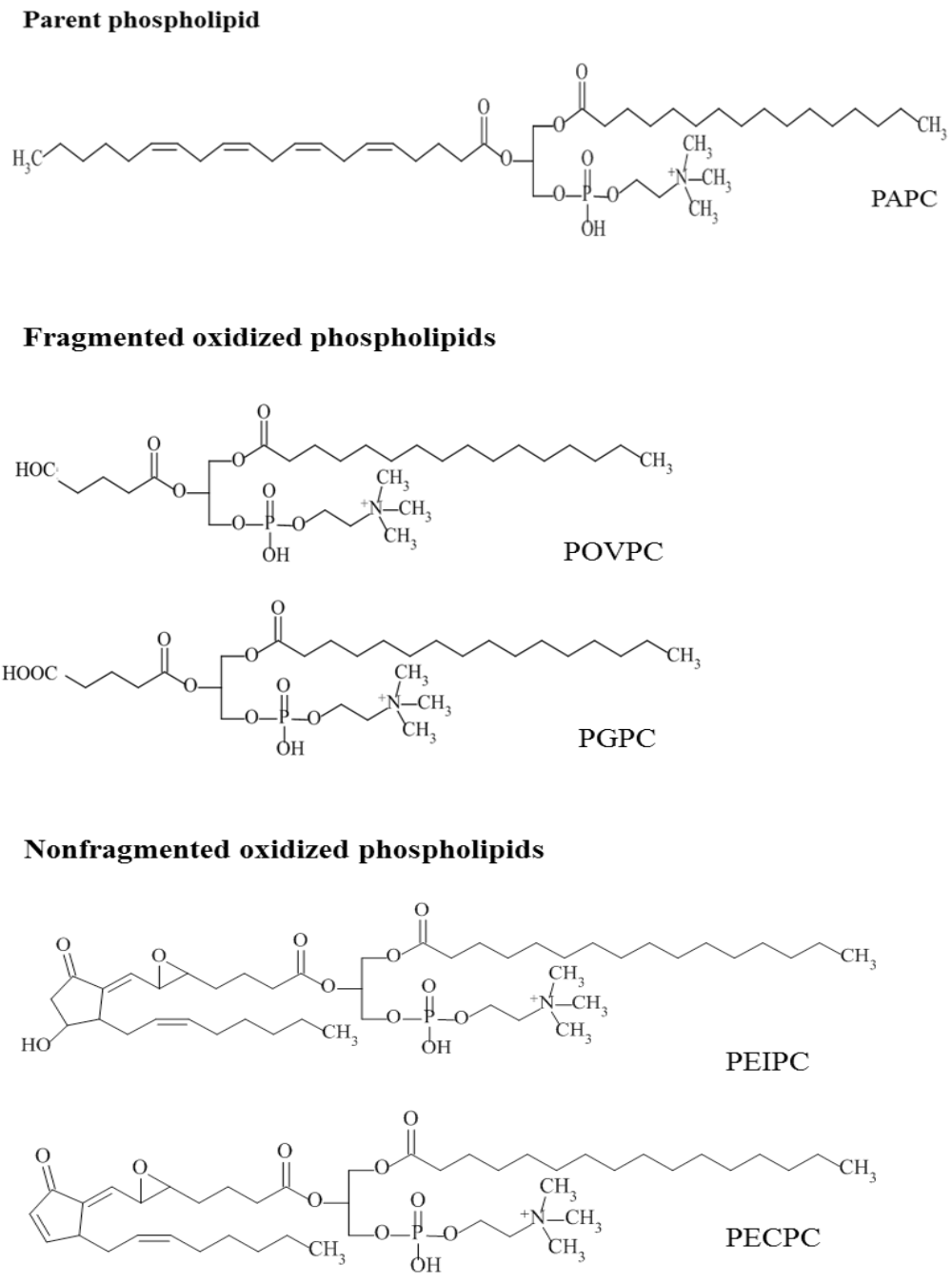


**Figure 1.5 Structures of native PAPC and its OxPLs.**

(PAPC: 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine, POVPC: 1-palmitoyl-2-(5'-oxo-valeroyL)-*sn*-glycero-3-phosphocholine, PGPC: 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine, PEIPC: 1-palmitoyl-2-(5,6-epoxyisoprostane E2)-sn-glycero-3-phosphocholine,PECPC:1-palmitoyl-2-(epoxycyclopentenone)-sn-glycero-3-phosphocholine). Adapted from Berliner, 2000.

### 1.5.3 Receptors and signalling mechanisms induced by OxPLs.

#### 1.5.3.1 Platelet activating factor (PAF) receptor

It has been suggested that OxPLs can induce their effects through interaction with the PAF receptor, based on the structure similarities observed between fragmented OxPLs and the inflammatory mediator PAF, which contains alkyl group at *sn*-1 residues and acetic acid esterified to a glycerol backbone. The PAF-like lipids shown to form during oxidation of LDL *in vitro,* mimic the effect of a synthetic PAF receptor agonist, but the affinity towards the receptor is much less than that of the PAF agonist (Marathe et al., 1999, Marathe et al., 2000). PAF-like lipids such as POVPC have been found in atherosclerotic lesions (Watson et al., 1997) and also shown to bind to the PAF receptor on human macrophages (Pegorier et al., 2006). Although some pro-inflammatory and pro-atherogenic effects of POVPC are similar to those of PAF, some of its actions are not similar, suggesting that OxPLs can also activate other receptors.

#### 1.5.3.2 Toll like receptors (TLRs)

Owing to the structural similarities between OxPAPC and its components with lipopolysaccharide (LPS), some studies have looked at the role of TLRs in mediating some effects of OxPLs (Reviewed in (Spickett et al., 2010). TLRs can recognize pathogen associated molecular patterns (PAMPs) induced by various microbial pathogens, which activate the innate immune response as well as initiating further adaptive immune responses. To date more than ten TLRs have been identified and different types of ligands may recognize different types of TLRs in combination with distinct accessory proteins. For instance, LPS is recognized by TLR4, which acts

with MD2 to mediate its response, whereas Gram positive bacterial lipoproteins are recognized by TLR2 associated with TLR1 or TLR6 (Tobias and Curtiss, 2005). LPS-induced TLR4 stimulation results in a series of protein/protein interaction resulting in activation of several inflammatory signalling pathways such as nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPKs).

NF-κB/ReI is a transcription factor that is located in cytoplasm of cell and in response to inflammatory stimuli translocates into nucleus. It regulates many genes involved in immune and inflammatory responses as well as cell survival (see Fig. 1.6) (Ahn and Aggarwal, 2005). Whereas, MAPKs are a family of serine/threonine kinases that are organized hierarchically into three-tiered modules; a MAP kinase (MAPK), a MAPK kinase (MAPKK) and a MAPKK kinase (MAPKKK) (see Fig. 1.7) (Zhang and Liu, 2002). By far the most extensively studied groups of MAPKs are extracellular signalling-regulated kinase 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK) and p-38 MAPK (Zhang and Liu, 2002) (See Fig 1.7).

High expression of TLR-4 has been identified in macrophages within human and mouse atherosclerotic lesions (Xu et al., 2001). Furthermore, in apoE knockout mice, which represents a model of human atherosclerosis, additional backcrossing with $TLR4^{-/-}$ results in reduced lesion formation compared to wild type apoE knockout mice. Moreover, in myeloid differentiation primary response gene 88 (MyD88) null mice, where the gene for MyD88, which functions as an intracellular signalling molecule for TLR, is deleted, plaque size, expression of pro-inflammatory genes and production of cytokine and chemokine were reduced (Tobias and Curtiss, 2005).

TLR4 in particular has been shown to be activated by OxPAPC, resulting in the expression of IL-8 in HeLa cells (Walton et al., 2003b). MM-LDL (of which oxPAPC is the active component) also induced activation of the TLR4 pathway in macrophages (Miller et al., 2003). In contrast, another study showed that MM-LDL and OxPAPC did not induce NF-κB activation in macrophages and HeLa cells, respectively (Yeh et al., 2001, Miller et al., 2003). Moreover, Erridge et al demonstrated that OxPAPC induced IL-8 production in human embryonic kidney 293 (HEK293) cells was not dependent on TLR4 signalling. This was demonstrated by using HEK 293 cells (TLR-4 deficient cell) transfected with TLR4/MD2 and the IL-8 promoter. OxPAPC did not increase IL-8 promoter activity, although TLR-4 was overexpressed and response to LPS (Erridge et al., 2007). The effect of OxPAPC in mediating IL-8 production is thus cell type dependent. More recently, OxPAPC was demonstrated to trigger lung injury and interleukin-6 (IL-6) production through a TLR-4/TRIF (Toll/IL-1 receptor (TIR)-domain-containing adaptor inducing IFN-beta) pathway in murine lung macrophages (Imai et al., 2008).

Paradoxically, some studies have shown that OxPAPC, POVPC and PGPC inhibit LPS-mediated NF-κB-dependent promoter activation in transfected HEK 293 cells (von Schlieffen et al., 2009, Erridge et al., 2008), suggesting that despite mediating pro-inflammatory effects through TLR4, OxPLs also mediate anti-endotoxin effects by interrupting the activation of this pathway. For example, OxPAPC has been reported to inhibit LPS-induced activation in endothelial cells and macrophages and to reduce tissue damage in response to LPS injection in mice (Bochkov et al., 2002a, Erridge et al., 2008, Walton et al., 2003a).

In addition to LPS, TNF-α can also induce activation of NF-κB and MAPK pathways. However, the activation is mediated through different receptors known TNFR1 and TNFR-2. Previous studies showed that following activation with TNF-α, the receptor is activated which leads to tumor necrosis factor receptor type 1-associated death domain (TRADD) to associate with death domain-containing adaptor protein (RIP) and TRAF2, which then leads to the phosphorylation of the IKK complex and a MAP3K that mediates NF-κB and MAPK activation, respectively. TNF-α has also been associated with apoptosis that can occur following the activation of TNFR1 and Fas death receptor. TNFR1 activates TRADD to associate with FADD and leads to the activation of caspase-3 protein which then results in apoptosis. If both NF-κB and apoptotic pathways are activated, the fate of cells depends on the strength of each pathway, a high concentration of TNF-α will lead to cell death despite cell protection by NF-κB (Dauphinee and Karsan, 2006) (Figure 1.8).

Previous research showed that OxPAPC did not inhibit the effect of TNFα-induced IκB-α degradation. With regards to apoptosis, a study demonstrated that POVPC and PGPC can activate sphingomyelinase, the upstream element that activates kinases that have been associated with the apoptotic stress response such as p-38 MAPK and JNK MAPK (Loidl et al., 2003). However, it was shown that OxPAPC did not induce expression of caspase-3 in endothelial cells, showing that OxPAPC did not activate apoptosis pathway (Gargalovic et al., 2006).

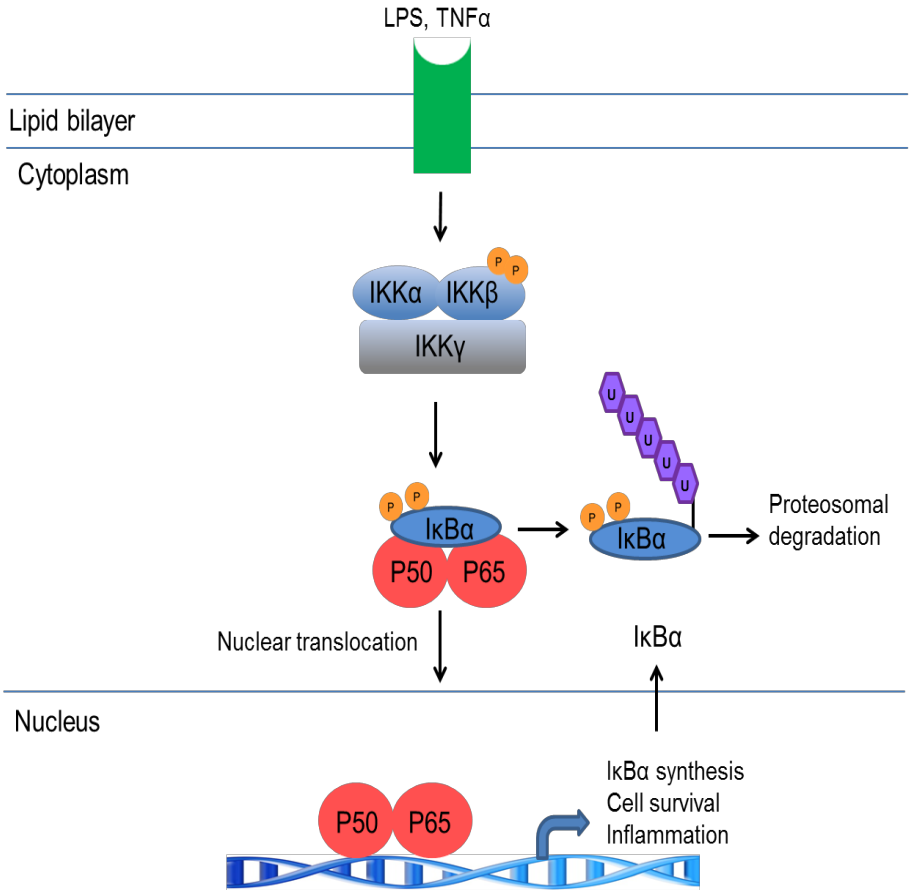


**Figure 1.6 Canonical pathway of NF-κB activation.** In an inactive state, NF-κB dimers are sequestered in the cytoplasm through interaction with an inhibitor protein, IκBα (Ghosh et al., 1998). Cell activation by various extracellular stimuli such as LPS, TNF-α and IL-1 can result in dissociation of IκBα from NF-κB, which then activates the pathway. This signalling cascade begins with the phosphorylation of IκB kinase (IKK) complexes that are composed of three subunits, the catalytical subunits IKKα and IKKβ and the regulatory subunit IKKγ (NEMO) (Stancovski and Baltimore, 1997). The activation of the IKK complex causes the phosphorylation of IκBα on its two N-terminal serine residues at position 32 and 36 (Brown et al., 1995), ubiquitinylation at position lysine 21 and 22 (Chen et al., 1995, Scherer et al., 1995) and consequently triggering the IκB-α degradation via the proteasome pathway (Delhalle et al., 2004). This results in the release of the bound NF-κB into the nucleus, which then regulates gene expression related to cell survival and inflammation. Adapted from Jost and Ruland, 2007.

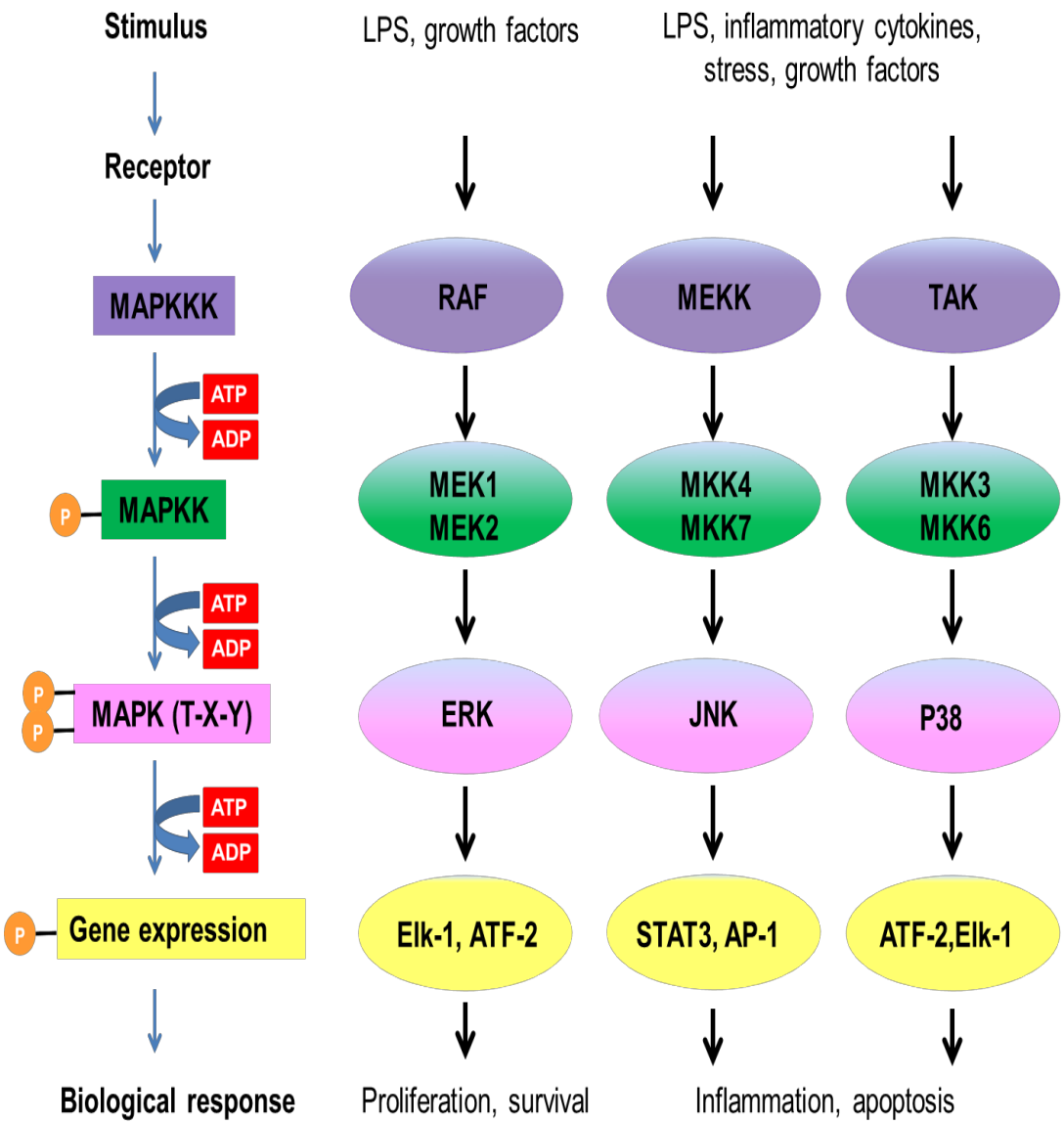


**Figure 1.7 Activation of MAPK pathways.** Upon activation of receptor tyrosine kinases (RTKs) by pro-inflammatory cytokines, bacterial products or growth factors, the MAPKKK is phosphorylated and results in activation of MAPKK. MAPKK has dual specificity enzymes that subsequently activates MAPK through dual phosphorylation on threonine and tyrosine within Thr-X-Tyr motif (where X is glutamate, proline or glycine in ERK, JNK or p-38 MAPK respectively) in their activation loop. Adapted from Liu et al., 2007 and Junttila et al., 2008.

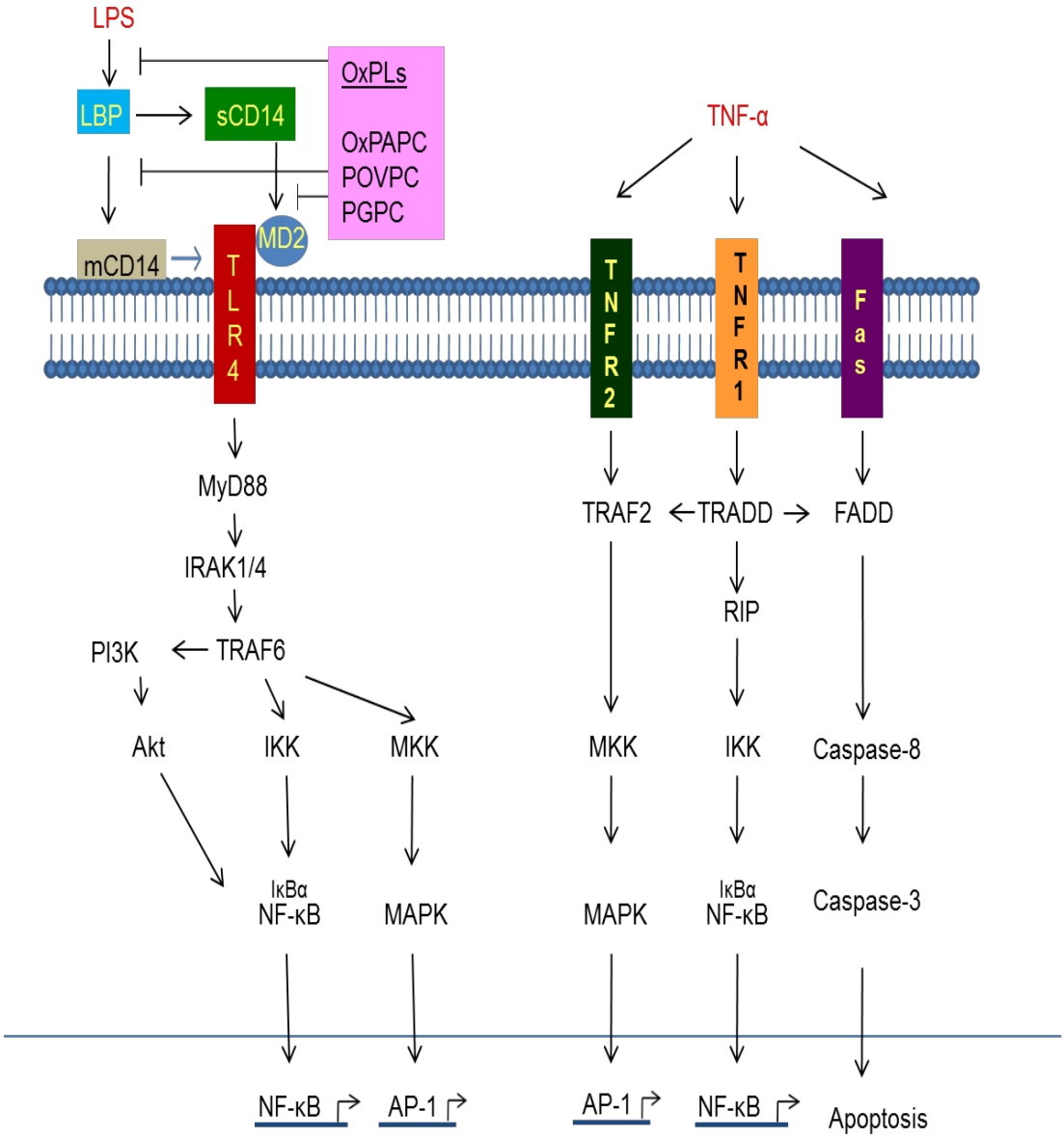


**Figure 1.8 Inhibition of LPS-mediated TLR-4 activation by OxPLs and activation of NF-κB, MAPK and apoptosis pathways by TNF-α**. Adapted from Dauphinee and Karsan, 2006 and Erridge, 2009.

#### 1.5.3.3 Peroxisome proliferator activated receptors (PPARs)

There is considerable evidence that OxPLs also play a role in the activation of PPARs. PPARs are a family of nuclear receptors consisting of three members; namely, PPAR-α, PPARβ/δ and PPARγ. Stimulation with ligands leads to formation of heterodimers with retinoid-X-receptors, which then bind to a DNA sequence of target genes via peroxisome proliferator response element (PPRE). Alternatively, PPARα can also form an inactive complex with ReIA (P65) subunit of NF-κB and interestingly, PPARα activators were shown to induce mRNA and protein expression of the endogenous NF-κB inhibitor, IκBα which leads to inactivation of the NF-κB pathway (Delerive et al., 2001). Vascular cells including monocytes/macrophages, endothelial cells and smooth muscle cells have been shown to express PPARα and γ (Delerive et al., 2001). In atherosclerosis, activation of PPARs results in pleotropic effects. For example, PPARα improves atherosclerosis through inhibition of endothelial adhesion molecules expression such as VCAM-1 and suppression of inflammatory genes including IL-6, COX-2 and endothelin-1 through formation of a negative complex with NF-κB (Duval et al., 2002). Similarly, activation of PPARγ leads to inhibition of inflammation (i.e., inhibits scavenger A and metalloproteinase gene expression) (Duval et al., 2002). Various studies have been conducted to look at the importance of PPARs in animal models of atherosclerosis and this will be discussed further in Chapter 4.

OxPAPC and individual composite lipids including POVPC, PGPC, PEIPC and PECPC have been shown to activate the PPARα pathway (Lee et al., 2000, Subbanagounder et al., 2002). However, in comparison to these lipids and the

PPARα agonists, it has been shown that PPARγ agonists did not increase level of IL-8 production by endothelial cells, suggesting that the effect of OxPAPC in mediating IL-8 production may not involve PPARγ. Other types of OxPLs have effects on activation of PPARγ; for instance, the alkyl phospholipid in OxLDL, hexadecyl azelaoyl phosphatidylcholine (azPC) was found to be able to activate PPARγ and bind to the receptor similarly to the synthetic PPARγ agonist rosiglitazone. Moreover, azPC was shown to induce COX-2 expression through activation of PPARγ (Davies et al., 2001, Pontsler et al., 2002). Further details regarding the role of OxPLs in PPAR activation are described in Chapter 4.

#### **1.5.3.4** Non-receptor mechanisms

Miscellaneous effects produced by OxPAPC can also be mediated through non-receptor mechanisms and have been reviewed recently (Spickett et al., 2010, Bochkov et al., 2010). It has been suggested that oxidation of phospholipids at sn-2 fatty acyl chain results in increased polarity; therefore if they are present within cell membranes, they are likely to alter the structure of phospholipid bilayers. Greenberg and co-workers demonstrated this possibility using a lipid whisker model, which suggests that insertion of oxygen into the unsaturated fatty acyl chains of phospholipids causes the less hydrophobic fatty acid to protrude out from the membrane, thus presenting the epitopes on oxidatively damaged cells to pattern recognition receptors (Greenberg et al., 2008). Another possible non-receptor mechanism of OxPLs is through depletion of cellular cholesterol. It has been shown that OxPAPC induced expression of IL-8 and LDL receptor in endothelial cells

through cholesterol depletion, caveolin-1 internalization and activation of sterol-regulatory element-binding protein (SREBP) (Yeh et al., 2004).

Interestingly, OxPLs are also involved in restoring cellular homeostasis by activation of stress signalling pathways such as the electrophilic stress response. This pathway is induced by electrophiles such as OxPLs containing α,β-unsaturated aldehydes, that are capable of forming Michael adducts with thiol groups on Kelch-like enoyl-CoA hydratase-associated protein 1 (KEAP-1), in turn causing the release of nuclear factor erythroid 2 p45 subunit-related factor 2 (Nrf2) into the nucleus. Accumulation of Nrf2 in turn stimulates its binding to promoters of target genes such as antioxidant-response element (ARE), leading to the transcription of antioxidant genes such as HO-1 (haem-oxygenase-1), glutamate-cysteine ligase and NADPH:quinine oxidoreductase-1 (NQO1) (Bochkov et al., 2010). Another pathway involved in the cellular adaptation is unfolded protein response (UPR). This pathway is activated by endoplasmic reticulum (ER) stress that occurs due to accumulation of misfolded proteins within the ER. The expression of the UPR target gene is mediated by three transmembrane ER stress sensors on the endoplasmic reticulum membranes; namely, activating transcription factor-6 (ATF-6), double-stranded RNA dependent protein kinase [PKR]-like ER kinase (PERK) and inositol requiring 1 (IRE1) (Bochkov et al., 2010). OxPLs are capable of stimulating these proteins, resulting in activation of transcription factors and enhanced production of inflammatory genes such as IL-8 (Gargalovic et al., 2006).

Similarly to LPS, TNF-α also induces activation of NF-κB and MAPK pathways; however, the activation is mediated through different receptors known as TNFR1 and TNFR-2. Previous studies showed that following activation with TNF-α, the receptor is activated which leads to tumor necrosis factor receptor type 1-associated death domain (TRADD) to associate with death domain-containing adaptor protein (RIP) and TRAF2, which then leads to the phosphorylation of the IKK complex and a MAP3K that mediates NF-κB and MAPK activation, respectively. Interestingly, previous research showed that OxPAPC did not inhibit the effect of TNFα-induced IκB-α degradation (Gargalovic et al., 2006), suggesting that the effect of OxPLs is specific to TLR.

## 1.6 Chlorinated lipids: Possible role in atherosclerosis and inflammation.

### 1.6.1 Myeloperoxidase and hypochlorous acid.

Phagocytic leukocyte cells play a major role in host defense and may cause oxidative damage during inflammation (Babior, 1978). Activated phagocytes produce superoxide anion via a cell membrane-bound NADPH oxidase complex (Pullar et al., 2000). Simultaneously, phagocytes also release the enzyme myeloperoxidase (MPO), which is an important component of their cytotoxic action. MPO is a tetrameric, glycosylated heme enzyme of 150kDa, consisting of heavy and light subunits of disulfide-linked promoters joined with a disulfide bridge (Nauseef and Malech, 1986), and is stored in the azurophilic granules of leukocytes. This enzyme constitutes approximately 5% of human neutrophil protein (Schultz and Kaminker, 1962), 1% of monocyte protein (Bos et al., 1978) and also found to be present in macrophages (Nagra et al., 1997).

When neutrophils are stimulated with fungi, bacteria or other activating agents, they secrete large amounts of MPO and produce superoxide in the phagolysosome and the extracellular compartment. As described above, the superoxide dismutates to hydrogen peroxide ($H_2O_2$); subsequently, MPO catalyzes the reaction of $H_2O_2$ with chloride ion ($Cl^-$) to form the powerful oxidant, hypochlorous acid (HOCl) (Kettle and Winterbourn, 1994): $H_2O_2 + Cl^- + H^+ \rightarrow HOCl + H_2O$. At plasma concentrations of halide ions, HOCl is a major oxidant generated by this heme enzyme (Harrison and Schultz, 1976, Foote et al., 1983), and MPO is the only human enzyme known to generate HOCl under these conditions (Weiss et al., 1986). In addition to their role in host defense, reactive oxidants generated by MPO including HOCl, hypobromous acid (HOBr) and hypothiocyanous acid (HOSCN) are able to produce reactive chlorinating and nitrogen species that can cause damage to host tissue at the site of inflammation (Klebanoff, 1980, Heinecke, 1997, Podrez et al., 2000).

### 1.6.2 Reaction of MPO/HOCl with biological molecules.

#### 1.6.2.1 Modification of proteins.

Reagent HOCl and enzymatically derived hypochlorite ($MPO/H_2O_2/Cl^-$) react vigorously with various biological molecules such as proteins and lipids through oxidation and chlorination. It has been suggested that HOCl works more efficiently as an oxidizing agent than a chlorinating agent; however, it is important to keep in mind that in some reactions with HOCl, chlorination occurs first, generating unstable chlorinated products that can then be oxidized to non-chlorinated products (Spickett, 2007). For instance, HOCl initially reacts with sulphur containing side chains of amino acid such as cysteine through chlorination of the thiol group, leading to the

formation of unstable sulfenyl chloride (RS-Cl) intermediates. Upon reaction with water, sulfenyl chloride is further oxidized with water resulting in the formation of sulfenic (RSOH), sulfinic ($RSO_2H$) and sulfonic acid (or cysteic acid, $RSO_3H$) (Hawkins et al., 2003). Chlorination can also generate a more stable chlorinated product; for instance, chlorination of the tyrosine side chain gives rise to 3-chlorotyrosine, which is a well-established marker for HOCl-mediated damage (Hawkins et al., 2003). Upon addition to LDL, HOCl (300 molecules of oxidant:1 particle of native LDL) has been reported to cause modification of amino acid side chains of ApoB-100, with lysine accounting for the major target, followed by tryptophan and cysteine. The pH -dependent loss of lysine (preferably in alkaline conditions, pH 12) results in the formation of unstable chloramines (Hazell and Stocker, 1993), which can be degraded by thermal and metal ion-catalyzed reaction, resulting in generation of radicals that can result in further damage of other biological molecules, particularly lipids (Malle et al., 2006). It has been reported that modification of lysine residues is responsible for HOCl-mediated formation of LDL aggregates (Hazell et al., 1994). Several studies have shown that LDL aggregates, in general, can result in accumulation of lipid in macrophages *in vitro* (Khoo et al., 1988, Suits et al., 1989, Heinecke et al., 1991) and hypochlorite modified LDL, in particular, has been shown to induce formation of foam cells (Hazell and Stocker, 1993), suggesting that HOCl reaction with proteins can result in LDL aggregation that can then induce macrophage uptake. Loss of antioxidant activity has also been reported but this occurrence is much less than protein and production of lipid peroxidation products are almost undetectable (Hazell and Stocker, 1993). This observation is in contrast to the initial sequence of reactions of OxLDL, as described

above, where lipid peroxidation and antioxidant consumption precedes modification of protein.

#### 1.6.2.2 Modification of lipids.

HOCl reacts with the double bonds of unsaturated fatty acids on phospholipids to generate chlorohydrins (hydroxy and chloride groups on adjacent carbons) (See Figure 1.9). Reaction of HOCl with mono-unsaturated fatty acids such as oleic acid generates 2 isomers, 9- and 10-monochlorohydrins (Winterbourn et al., 1992) (See Figure 1.10) whereas chlorination of polyunsaturated fatty acids such as arachidonate produces complex mixtures of products; for instance, chlorination of arachidonic acid coupled to glycerophosphocholine at the sn-2 position, results in the formation of chlorohydrins as well as lysophosphatidylcholines (Panasenko et al., 2003, Arnhold et al., 2002). In addition, Jerlich et al demonstrated that treatment of LDL with HOCl or MPO system results in the formation of phospholipid chlorohydrins (Jerlich et al., 2000).

The mechanism of chlorohydrin formation has been shown by experiments using $^{18}$O-labelled HOCl ($H^{18}OCl$) that is formed from the reaction of chlorine gas with $^{18}$O-labelled water. It was first established that reaction of HOCl ($H^{18}OCl$) with unsaturated lipids involves electrophilic attack of $Cl^+$ on the double bond to form a carbenium ion, followed by nucleophilic attack of water-derived hydroxide ($OH^-$) at the protonated carbon ($C^+$) resulting the formation of chlorohydrin (Spalteholz et al., 2004) (See Fig. 1.9). A few years later, lysophosphatidylcholine chlorohydrin was found in human atherosclerotic tissue (Messner et al., 2008a).Very recently, elevated

levels of monochlorohydrin of linoleic acid, bischlorohydrin of linoleic acid and monochlorohydrin of oleic acid have been found in adipose tissue, ascetic fluid and plasma, respectively, in an animal model of acute pancreatitis. Similarly, oleic acid chlorohydrin was found in plasma of patients with acute pancreatitis ((Franco-Pons et al., 2012).

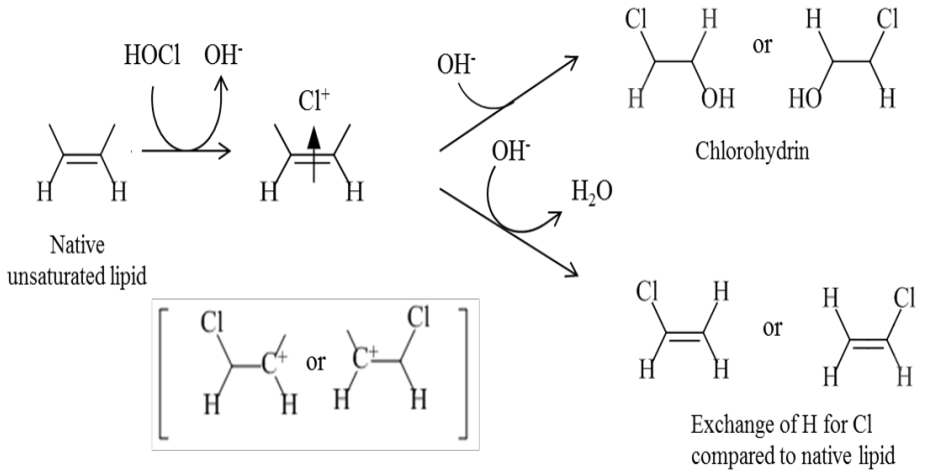


**Figure 1.9 The mechanism of chlorohydrin formation following HOCl attack on double bond of unsaturated lipids.** Adapted from Spickett, 2007.

Whilst headgroup of phosphatidylcholine is relatively inert so that the reaction with alkene bond predominates, the headgroup of phosphatidylidylethanolamine (PE) and phosphatidylserine (PS) are susceptible for HOCl attack and is relatively faster compared to the reaction of HOCl with their alkene group (Pattison et al., 2003). *N*-halogenation of headgroup of these lipids can result in formation of chloramine that can then decay to carbon-centered or nitrogen-centered radicals, such as phosphatidylglycoaldehyde and N-centered radical (Carr et al., 1998, Pattison et al., 2003, Kawai et al., 2006).

Plasmalogen, a glycerophospholipid enriched in the plasma membranes of vascular cells that is characterized by the presence of a vinyl ether bond between the glycerol backbone and the *sn-1* aliphatic group, is also susceptible to HOCl attack. The MPO/$H_2O_2$/$Cl^-$ system mediates oxidation of vinyl ether bond on plasmalogens that contain 16 carbons and 18 carbons, resulting in production of 2-chlorohexadecanal (2-ClHDA) (Albert et al., 2001) and 2-chlorooctadecanal (2-ClODA) (Thukkani et al., 2002) respectively, as well as production of lysophosphatidylcholine (LPC) (Albert et al., 2001). Furthermore, it has been shown that chlorination of plasmalogen present in LDL by MPO-derived reactive chlorinating species also generates production of 2-ClHDA, 2-ClODA and LPC (Thukkani et al., 2003). The stucture of 2-ClHDA and LPC are shown in figure 1.10. In the same year, Thukkani et al. demonstrated that 2-ClHDA and unsaturated lysophosphatidylcholine were elevated in human atherosclerotic tissues (Thukkani et al., 2003). Later on, Messner et al. demonstrated that in addition to reaction of HOCl with the sn-1 vinyl ether bond, it can also react with alkene bond plasmenylcholine at the *sn-2* position, which

leads to the formation of lysophosphatidylcholine, that upon further attack by HOCl can result in the formation of lysophosphatidylcholine chlorohydrin (Messner et al., 2006).

Reaction of HOCl and sphingomyelin has also been reported. Nusshold et al showed that HOCl can result in a concentration-dependent depletion of sphingomyelin from mouse total brain lipids. Specifically, 1µg NaOCl/100 µg sphingomyelin was demonstrated to result in approximately 40% deletion of its original content, which suggests that this types of lipid is susceptible for HOCl modification (Nusshold et al., 2010). The same group also reported that reaction of HOCl with sphingomyelin resulted in the formation monochlorohydrin, epoxide and mixed chlorohydrin/chloramide sphingomyelin species (Nusshold et al., 2010).

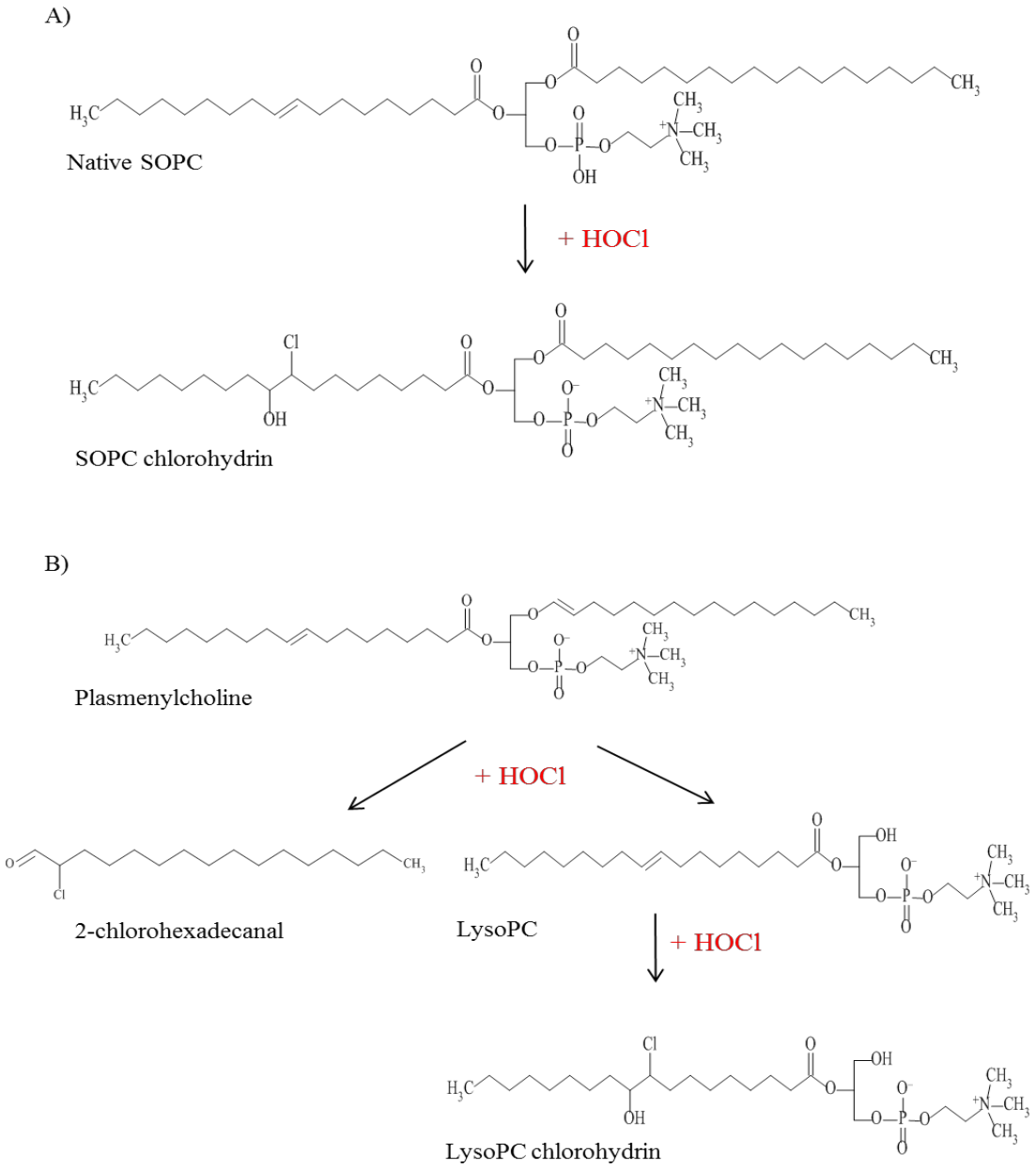


**Figure 1.10 Formation of chlorinated lipids.** (A) Formation of SOPC chlorohydrin following the reaction of native SOPC with hypochlorous acid. (B) Formation of 2-ClHDA and LysoPC following the primary reaction with HOCl as well as LysoPC chlorohydrin in the secondary reaction Adapted from Thukkani et al., 2003 and Messner et al., 2006.

### 1.6.3 HOCl-modified LDL.

#### 1.6.3.1 Occurrence *in vivo*.

Several studies have reported the involvement of MPO and HOCl in atherosclerosis. Catalytically active MPO has been found in human atherosclerotic tissue, where it colocalizes with foam cells (Daugherty et al., 1994). The specific product of MPO and HOCl activity, 3-chlorotyrosine, has been found at increased levels in LDL isolated from human atherosclerotic lesions as well as in the aortic arterial tissue lesion (Heinecke, 1998). Moreover, HOCl-modified proteins (Hazell et al., 1996) and lipids (Thukkani et al., 2003) have been found in atherosclerotic tissues.

#### 1.6.3.2 Biological effects of HOCl-modified LDL.

HOCl-LDL has been shown to induce IL-8 but not MCP-1 expression in human monocytes and increase chemotactic activities for neutrophils but not monocytes (Woenckhaus et al., 1998). HOCl-LDL has also been shown to induce production of reactive oxygen species (ROS) as well as inducing polymorphonuclear leukocyte adherence on endothelial cells (Kopprasch et al., 1998). Later on, it was established that HOCl-modified LDL reduced formation of nitric oxide (NO) via intracellular delocalization of endothelial nitric oxide synthase without altering endothelial nitric oxide synthase (eNOS) protein expression (Nuszkowski et al., 2001). Moreover, Marsche et al demonstrated that HOCl-LDL can be internalized and degraded by Class B scavenger receptors such as CD36 and scavenger receptor class B type I (SR-BI) (Marsche et al., 2003). Following this finding, it was demonstrated that treatment of macrophages with HOCl-modified LDL upregulates CD36 and PPARγ mRNA in a concentration and time-dependent manner. However, activation of SR-BI

was shown to induce a different cellular response. Stimulation with a low concentration of HOCl-LDL over a short time period upregulated SR-BI mRNA level but increased concentration and prolonged exposure resulted in the opposite effect. Based on this finding, they hypothesized that initial interaction of HOCl-LDL with SR-BI induces lipid loading, which in turn inhibits SR-BI mRNA expression. The finding by Yu et al. that the level of SR-BI mRNA was reduced with sterol loading in macrophages is thought to support this hypothesis (Westendorf et al., 2005, Yu et al., 2004).

Furthermore, MPO-modified LDL has been shown to increase level of IL-8 protein expression in endothelial cells and TNF-α protein expression in monocytes (Boudjeltia et al., 2006). Most recently, HOCl-LDL has been shown to induce apoptosis in T-cells (Resch et al., 2011). Overall, these findings suggest that HOCl-LDL may have important roles in the pathogenesis of atherosclerosis; however, it is not very well characterized whether the effect of HOCl-modified LDL caused by modification of its protein or lipid moieties. Whilst some effects of HOCl-LDL have been reported to be mediated by modification of protein components, there is increasing evidence that lipid may also have a critical role. The biological effect of HOCl-modified lipid (or chlorinated lipids) will be described further in the next section.

### 1.6.4 Biological effects of chlorinated lipids.

Since the finding that HOCl and the MPO system can react with unsaturated fatty acids resulting in lipid chlorohydrin formation in a model system (Winterbourn et al.,

1992), more studies have been done to investigate the potential effects of these products on cells and their contribution to diseases, in particular atherosclerosis. Early work showed that treatment of human red blood cells with HOCl-modified oleic acid resulted in a concentration-dependent lysis of cells. This effect was thought to be due to the incorporation of oleic acid chlorohydrin, which was bulkier and more polar compared to its parent lipid, into the hydrophobic part of the membrane (Carr et al., 1997), causing disruption to membrane structure. It was then discovered that fatty acid chlorohydrins were toxic to human endothelial cells and cell death is thought to occur due to necrosis but not apoptosis (Vissers et al., 2001).

In myeloid cells, Dever et al showed that phospholipid chlorohydrins such as chlorohydrins of stearoyl-oleoyl phosphatidylcholine, stearoyl-linoleoyl phosphatidylcholine (SLPC) and stearoyl-arachidonoyl phosphatidylcholine (SAPC), cause a dose-dependent depletion of cellular ATP. In comparison to aldehydes such as hexanal and trans-2-nonenal and OxPL such as OxPAPC, the effect of SOPC chlorohydrins on HL60 cells ATP depletion is greater; however, SOPC chlorohydrin was found to be less toxic than 4-hydroxynonenal (4-HNE) (Dever et al., 2003).

Moreover, the same group has reported that chlorohydrin at concentration of 25 μM increased caspase-3-activity, suggesting that lipid chlorohydrins may be able to induce apoptosis (Dever et al., 2006). SOPC chlorohydrin has also been shown to induce leukocyte adherence to endothelial cells in artery of apoE knockout through P-selectin and ICAM-1 as well as increased production of ROS (Dever et al., 2008). Recently, fatty acid chlorohydrins have been reported to induce expression of TNF-α

and interleukin-1β (IL-1β) mRNAs in peritoneal macrophages following intraperitoneal injection of both control and acute pancreatitis mice with fatty acid chlorohydrins (Revised manuscript (Franco-Pons et al., 2010)

Several studies have also reported the effects of chlorinated lipids derived from modification of plasmenylcholine, which includes α-chloro fatty aldehydes (e.g., 2-ClHDA) and LysoPC. One of the earliest findings was that 2-ClHDA induces neutrophil chemotaxis (Thukkani et al., 2002) and LysoPC induces cell surface expression of P-selectin in human coronary artery endothelial cells (Thukkani et al., 2003). Additionally, similar to the observation in HOCl-modified LDL described above, 2-ClHDA has been shown to inhibit production of NO via delocalization of eNOS (Marsche et al., 2004). 2-ClHDA has also been implicated in myocardial damage as analysed by the release of lactate dehydrogenase (LDH) from isolated perfused rat hearts (Thukkani et al., 2005).

Additionally, 2-ClHDA has been demonstrated to form Schiff base adducts with primary amine-containing molecules such as ethanolamine glycerophospholipids and lysine, which could result in disruption of cell membrane and alteration of protein function (Wildsmith et al., 2006b). Moreover, Wildsmith et al. reported that human coronary artery endothelial cells and human neutrophils induce oxidation of 2-ClHDA, generating chlorinated fatty acid (α-ClFA), and reduction of 2-ClHDA, generating chlorinated fatty alcohols (α-ClFOH). These metabolites have been shown to be able to incorporate into cellular lipids including monoglycerides, trigelycerides, phosphatidylcholine and phosphatidylethanolamine (Wildsmith et al., 2006a). Both

2-ClHDA and its metabolite 2-chlorohexadecanoic acid (2-ClHA) have been shown to upregulate COX-2 mRNA and protein expression in endothelial cells (Messner et al., 2008a).

Taken together, these studies underline the potential significance of chlorinated lipids to induce both pro- and anti-inflammatory effects comparable to the well-studied OxPAPC. However, the knowledge of the biological effects of chlorinated lipids and their mechanism of action is still very limited.

## 1.7 Aims

To reiterate, atherosclerosis is currently viewed as an inflammatory disease and oxidative modification of LDL plays a key role in mediating the pathology of this disease. Whilst mounting evidence has shown that OxLDL can induce various biological effects, recent studies have focussed on the specific components of modification of LDL that contribute to its effect. Various effects of OxLDL have been reported to be mediated by modification of lipid components of LDL. OxPLs such as OxPAPC have been found to be active agents in OxLDL and have been shown to induce both pro- and anti-inflammatory effects in vascular cells, such as myeloid cells, smooth muscle cells and endothelial cells. Various receptors and signalling mechanisms involved in mediating its biological effects have been extensively studied. In addition to OxPLs, HOCl-modification of lipid moieties of OxLDL has been shown to generate a variety of chlorinated lipid products. However, there are limited studies demonstrating the effect and mechanism of action of this type of lipid. Some studies demonstrate that chlorinated lipids have a comparable

effect to OxPLs; for instance phospholipid chlorohydrin induces expression of P-selectin similar to OxPAPC, suggesting that chlorinated lipids could also be involved in mediating inflammatory effects. However, it is not known whether chlorinated lipids can similarly induce other effects caused by OxPAPC and mediate such effects through similar mechanisms.

The overall aim of this study was to address these gaps in knowledge and specifically to address the following research questions:

- Do chlorinated lipids alone induce IL-8 and TNF-α production in myeloid cells or inhibit these cytokine production when treated in combination with LPS?
- Do chlorinated lipids activate the PPARα, β and γ pathway?
- Do chlorinated lipids activate NFκB and MAPK or inhibits their stimulation by LPS in smooth muscle cells and endothelial cells ?

To address these questions, the project will utilize a number of cell types; leukocyte cells (U937), endothelial cells (HUVECs), smooth muscle cells (RASMCs), HEK 293 cells and fibrosarcoma cells (L929sA). The role of oxidized and chlorinated lipids in inflammatory signalling will be investigated initially by examining the ability of these compounds to activate cells to induce inflammatory cytokine production, which will be measured by enzyme-linked immunosorbent assay (ELISA). Then the role of PPARs pathway will be determined using luciferase assay and the role of MAPK or NF-κB signalling pathways will be tested using Western blotting.

# Chapter 2

# Materials and Methodology

## 2.1 Materials

All materials and reagents used were obtained from Sigma Aldrich Company Ltd. (Poole, Dorset, UK) unless otherwise stated. Phospholipids including 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC), 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PAPC), 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine (PGPC) and 1-palmitoyl-2-(5'-oxo-valeroyL)-*sn*-glycero-3-phosphocholine (POVPC) were obtained from Avanti Polar Lipids, Inc, (USA) and the α-chloro fatty aldehyde, 2-chlorohexadecanal (2-ClHDA), was a generous gift from Professor Andrew Pitt (Aston University, UK). The synthesis of 2-ClHDA will be described briefly in the next section.

Plasmids; PSG5hPPARα, PSG5hPPARβ, PSG5hPPARγ, PSG5 and PPRE-containing reporter plasmid $J_3$-TK-luc were a kind gift from Professor G. Haegeman from University of Ghent, with a kind permission from Professor B. Stael, Institute Pasteur, Lille, France (the original source for plasmids). The plasmids PSG5hPPARα, PSG5hPPARγ and PPREJ3-tk-luc were described previously (Chinetti et al., 1998, Staels et al., 1998, Vu-Dac et al., 1995). The vector pPGKβGeobpA that contained a neomycin-resistant/β-galactosidase fusion gene was a kind gift of Professor G. Haegeman (originally obtained from Dr. P. Soriano Fred Hutchinson Cancer Research Center, Seattle, WA). All antibodies including mouse monoclonal anti p-ERK (E-4), rabbit polyclonal anti IκBα (C-21), rabbit polyclonal anti-p38 (N-20), rabbit polyclonal anti-ERK-1 (K23, rabbit polyclonal NF-κB T-p65 (C-20) and rabbit polyclonal GAPDH (FL-335) were obtained from Santa Cruz Biotechnology Inc. (CA, USA).

## 2.2 Oxidation and analysis of phospholipids.

### 2.2.1 Preparation of oxidized and chlorinated lipids.

Stearoyl-oleoyl phosphatidylcholine (SOPC) in chloroform (40 mg/ml) was aliquoted and dried under nitrogen. Aliquots of SOPC (0.5 mg) were reconstituted in 50 µl of Hanks buffered saline solution (HBSS) and sonicated at $35^0$C for 15 min, to form multilamellar lipid vesicles. The vesicles were treated with a 100 µM sodium hypochlorite (NaOCl) at pH 6.0 for 20 min to achieve maximum conversion of native SOPC to chlorohydrin. The concentration of NaOCl in the stock solution was determined by spectrophotometer at 291 nm ($\varepsilon_{292}$ = 350 $M^{-1}cm^{-1}$). Excess hypochlorite was removed by passing the mixture through a reverse phase Sep-pak cartridge (Waters, UK) as described previously (Dever et al., 2003). The columns were washed with water and methanol, and lipids were extracted with 100% methanol and 1:1 methanol/chloroform. The organic solvent was dried under nitrogen and lipid chlorohydrin was reconstituted in RPMI medium. Native SOPC was prepared using the same method, but sodium hypochlorite was replaced with HBSS.

Chlorinated form of SOPC used in this study was partially purified and will be referred to in an abbreviated form as SOPC ClOH. The concentrations of SOPC ClOH used in this study are concentrations of native lipid treated with NaOCl. The actual concentrations of lipid are presumed to be slightly lower from the filtration through Sep-Pak columns. However, the lipid loss is expected to be minimal due to the strong solvents used for elution. In previous method developed by Stewart, Spickett and Pitt (unpublished data), subsequent to removal of hypochlorite, lipids

were sterile filtered. However, in this study lipids were not sterile filtered but reconstituted in serum free media and directly used on cells. It should be noted that lipid was reconstituted in serum free media to avoid partial binding of lipids protein constituents of the serum supplemented medium.

In addition, oxidation of PAPC was prepared by autoxidation. Aliquots of PAPC in chloroform (1 mg/ml, 100 µl) were dried under nitrogen stream and were autoxidized for 7 days at $40^0$C in the dark. Prior to addition to cells, OxPAPC was reconstituted by vortexing in warm media. The concentrations of OxPAPC used in this study are concentrations of native PAPC. The correct formation of OxPAPC was confirmed by mass spectrometry as described below.

The synthesis of 2-ClHDA was undertaken entirely by Professor Andrew Pitt based on the protocol adapted from Thukkani et al (Thukkani et al., 2002). In brief, hexadecaal was synthesized by partial oxidation of hexadecanol at -70ºC using Swern oxidation with oxalyl chloride-activated dimethyl sulfoxide (DMSO) as a catalyst (Mancuso et al., 1978). Dimethy acetal hexadecanal was then synthesized by acid methanolysis of hexadecanal. Subsequently, using an acetal chlorination system utilizing $MnO_2$-trimethylchlorosilane, dimethyl acetal of 2-ClHDA was produced (Bellesia et al., 1992). Finally, 2-ClHDA was synthesized by refluxing in 1:1 trifluoroacetic acid/dichloromethane and purified by flash chromatography using 7:3 hexane/dichloromethane. The purity was confirmed by thin layer chromatography (TLC) and by gas chromatography-mass spectrometry (GC-MS) of its pentafluorobenzyl (PFB) oxime derivative.

### 2.2.2 Detection of chlorohydrin formation and oxidation products: Electrospray Mass Spectrometry.

The conversion of native lipid to SOPC ClOH was monitored by electrospray mass spectrometry (ES-MS). Aliquots (1 mg/ml) were diluted 200-fold in 0.1% formic acid in methanol. ES-MS was carried out in a LCQ-Duo (Finnigan) mass spectrometer in positive ion mode by direct infusion (Sheath Gas Flow Rate = 30 arb; Spray voltage = 4.5 kV; Capillary temperature = 230ºC). For detection of SOPC ClOH, spectra in the range 500–1050 *m/z* were obtained and loss of native lipids and an increase of mass-to-charge-ratio (m/z) of 52 (monochlorohydrin) was monitored. Spectra between 400 and 1100 m/z were collected to obtain a profile of the autoxidation products of PAPC (Dever et al., 2006). Individual components of OxPAPC, 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine (PGPC) and 1-palmitoyl-2-(5'-oxo-valeroyL)-*sn*-glycero-3-phosphocholine (POVPC) were also monitored between 400-1100 m/z.

## 2.3 Determining the effects of chlorinated lipids on production of IL-8 and TNF-α in U937 cells.

### 2.3.1 Growth of U937 cells.

U937 myeloid cell lines were obtained from European Collection of Animal Cell Cultures (UK). The cells were cultured in RPMI-1640 containing 2 mM L-glutamine and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), with addition of 10% foetal calf serum (FCS) and 100 units/ml penicillin and 0.1 mg/ml streptomycin (Invitrogen, Paisley, UK). Cells were cultured at $37^0$C in a humidified atmosphere of 5% $CO_2$. Upon reaching 90% confluence, U937 cells were diluted (1

in 5) with fresh media in a new flask or plated out for experiments or frozen in 12% DMSO in FCS and stored in liquid nitrogen for later use.

### 2.3.2 Treatment of U937 cells with native, chlorinated and oxidized lipids in the presence or absence of LPS.

To test the effect of chlorinated and oxidized lipids on production of pro-inflammatory cytokines by activated myeloid cells, U937 cells ($5\times10^5$ cells/well, 1000 µl) seeded in triplicates in 24 well plate, were first differentiated with 0.1 µg/ml phorbol myristate acetate (PMA) for 72 hours. The change in cell morphology; cells becoming irregular in shape and change in cell characteristics such as adherence to the plate (Hida et al., 2000), were observed under microscope. Half of the old media (500 µl) was removed, and cells were stimulated at different time points (0- 24 hours) with serum free medium (500µl) or 100 µM SOPC ClOH (500 µL of doubled concentration). Activated cells were also stimulated with different concentrations of native SOPC, SOPC ClOH and PGPC. In addition, cells were treated with native and modified lipids together with *Salmonella abortus equi* lipopolysaccharide (*S. abortus equi* LPS) to determine their effect in the presence of pro- inflammatory mediators. Initially, cells were stimulated with 100 ng/ml of *S. abortus equi* LPS at different time intervals (0-24 hours) and with various concentrations of *S. abortus equi* LPS (0, 0.1, 1, 10 and 100 ng/ml) for 24 hours to find the best concentration and time for co-treatment with chlorinated and oxidized lipids. Cells were then treated for 24 hours with various concentrations of native SOPC, SOPC ClOH and PGPC, together with *S. abortus equi* LPS at concentration of 100 ng/ml.

Additionally, another set of experiments was carried out with a lower concentration of *Escherichia coli* LPS (*E.Coli* LPS, 10 ng/ml) over a different time course (4 hours for TNFα and 18 hours for IL-8 analysis). Similar to described above, prior to stimulation, cells were differentiated with 0.1 μg/ml PMA (in 96 well plate containing $5x10^4$ cells/well, 200 μl). Medium was removed and cells were treated either with 200 μl of serum free medium and different concentrations of native SOPC, SOPC ClOH and 2-ClHDA (1, 2.5, 5, 10, 25 μM) alone or pre-treated for 30 minutes before addition of *E.Coli* LPS. OxPAPC (25 μM) was used as a positive control. At the end of treatment, cells were centrifuged and supernatants were collected.

### 2.3.3 Measurement of IL-8 and TNF-α production by ELISA.

The concentration of TNF-α and IL-8 present in supernatants obtained from treatment above were measured by ELISA assay (R&D, UK) and assays were performed according to the manufacturer's instructions. Briefly, capture antibody, a mouse anti-human IL-8 or TNF-α (100 μl), was incubated in 96 well plates overnight at room temperature. On the next day, contents were discarded and washed three times with phosphate-buffered saline 1 (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4$ and 1.5 mM $KH_2PO_4$; pH 7.4) containing 0.05% Tween 20. The same number of washes was applied for the whole assay and after every last wash; plates were blotted on paper towels to remove excess liquid. Remaining antibodies were then blocked for one hour with 1% BSA in PBS containing 0.05% sodium azide ($NaN_3$) (300μl). Plates were washed before incubation with samples or standards (100 μl) for approximately 2 hours. The contents were decanted and plates were

washed before adding biotinylated goat anti-human IL-8 or anti-human TNF-α antibody (100 µl). Following two hours incubation, plates were washed again and incubated with Streptavidin-horse radish peroxidase (100 µl) for 20 minutes. After the final wash, TMB-substrate (100 µl) was added and the reaction was stopped after 5-10 minutes by adding 50 µl of sulphuric acid ($H_2SO_4$) (Fison, Ipswich, UK). The absorbance was read immediately at 450 nm in a plate reader.

### 2.4 Cell Viability Assay.

U937 cells ($5x10^4$ cells/well), seeded in triplicates in 96 well plate, were either differentiated or undifferentiated with 0.1 µg/ml PMA before stimulated with 100 µl of SOPC ClOH, *S. abortus equi* LPS, and acrolein (concentrations are varied) for 24 hours. Moreover, PMA-activated cells were treated with the highest concentrations of SOPC ClOH (100 µM), LPS (100 ng/ml) and acrolein (75 µM) for different periods of time (0-24 hours). Additionally, activated cells were treated with different concentrations of SOPC ClOH (5 and100 µM), 2-ClHDA (5 and 100µM), OxPAPC (10, 25, 50 µM), PGPC (8 and 32 µM) and *E. coli* LPS (5 and 100 ng/ml), and a positive control, hypochlorous acid (0.1, 0.5, 1, 5,10 mM) for 24 hours. At the end of stimulation, cells were incubated with 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (0.5 mg/ml) for 90 minutes before centrifuging at 200 g for 10 minutes. Supernatants were carefully removed from the plates without removing blue crystals at the bottom of the wells. Blue crystals were dissolved by mixing with DMSO and the absorbance was read at 540 nm in a plate reader.

## 2.5 Determining the effects of chlorinated lipids on activation of PPARs.

### 2.5.1 Growth of HEK 293 cells and stably transfected L929sA cells.

Mouse fibrosarcoma (L929sA) cells were stably transfected with the recombinant plasmid p(IL-6κB)$_3$50hu.IL6P-luc+ by Prof Haegeman's laboratory members using calcium phosphate ($CaPO_4$) precipitation (Vanden Berghe et al., 1998). The recombinant plasmid was as described previously (Plaisance et al., 1997, Vanden Berghe et al., 1998) (Figure 2.1). Stably transfected L929sA and human embryonic kidney 293 (HEK 293) cells (1) were maintained in Dulbecco's modified eagle medium (DMEM) (Gibco, Invitrogen, CA, USA) that contained 5% newborn calf serum and 5% FCS, 100 units/ml penicillin and 0.1 mg/ml streptomycin. Upon reaching confluence, the medium was aspirated and a solution of trypsin [0.05% trypsin, 0.53 mM ethylenediaminetetraacetic acid (EDTA) in PBS 1, 10 ml] was added into the flask. Cells were incubated for 3-5 minutes at 37°C before cells were diluted into half by fresh growth media. Cells were further subcultured into a new flask or seeded into plates for experiments.

After preliminary experiments in Belgium, further investigation on PPARs pathway was carried out in our laboratory in Glasgow. HEK 293 cells (2) were maintained in minimum essential medium (MEM) media (Gibco Life Technologies Ltd, Renfrewshire, UK) supplemented with penicillin (250 units/ml), streptomycin (100 µg/ml), L-glutamin (27 mg/ml), 5% (v/v) sodium bicarbonate, 1% (v/v) non-essential amino acids and 10% (v/v) FCS (all obtained from Invitrogen, Paisley, UK). Medium was changed every two days thereafter. Cells were passaged using 1 x sodium

chloride-sodium citrate (SSC) [150 mM NaCl and 17mM sodium citrate ($Na_3C_6H_5O_7$); pH 7.4].

### 2.5.2 Measuring the effects of oxidized and chlorinated lipids on TNFα-induced activation of NF-κB promoter.

L929sA cells (10,000 cells/well, 200 µl) stably transfected with an NF-κB dependent recombinant promoter construct were plated in 96 wells plate and were allowed to proliferate to 70 to 80 percent confluent. The medium was aspirated and cells were either treated with serum free media, oxPAPC (1-100 µM), SOPC ClOH (1-100 µM), 2-ClHDA (1-100 µM), a positive control dexamethasone (1µM) and ethanol (0.1%) or pre-treated for 30 minutes prior to stimulation with TNF-α (2000 units/ml) for 6 hours. At the end of treatment, medium was aspirated and cells washed once with ice cold PBS 1. Lysis buffer (50 µl) containing 0.2 M $K_2HPO_4$, 0.2 M $KH_2PO_4$ and 26 % (v/v) Triton X100 (pH 7.6) was added into each wells. Cells were lysed by freeze-thaw and used for luciferase assay (Section 2.5.6).

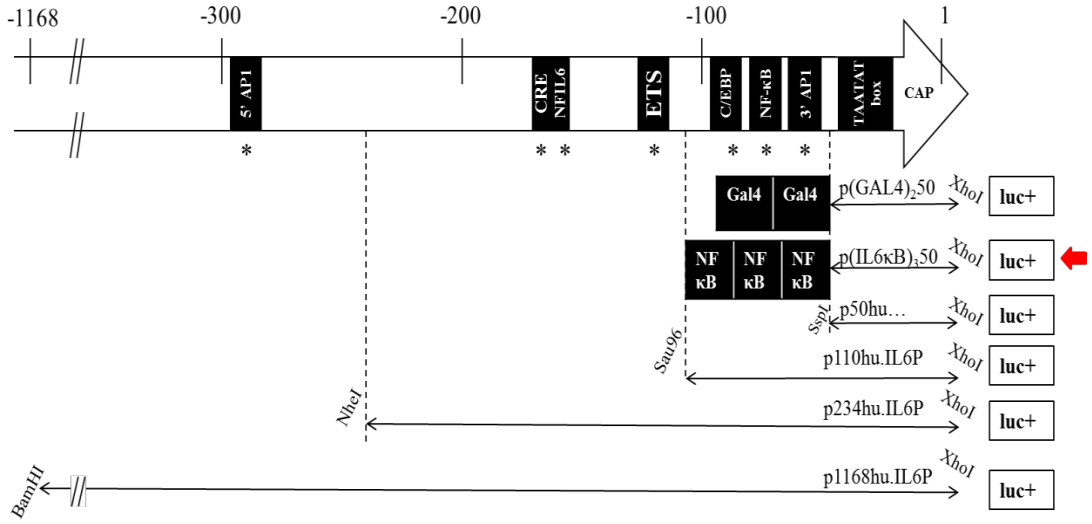


**Figure 2.1 Schematic representation of full length IL-6 promoter and its recombinant reporter gene constructs.** Adapted from Vanden Berghe et al., 1998).. Arrow shows the recombinant construct used for stable transfection in L929sA cells.

### 2.5.3 Transfection of HEK 293 cells using $CaPO_4$ and treatment of transiently transfected cells with oxidized and chlorinated lipids.

To determine whether OxPAPC, SOPC ClOH and 2-ClHDA can activate the PPAR pathway, different concentrations of these compounds were tested in HEK cells transfected with or without PPARα, β, or γ using a standard procedure for calcium phosphate ($CaPO_4$) coprecipitation (Graham and van der Eb, 1973). HEK 293 cells (90,000 cells/well, 400 µl) were seeded in 24 wells plate to achieve 70-80% confluent. On the next day, old medium was replaced with fresh medium (350 µl/well) and cells were transfected by adding DNA-$CaPO_4$ precipitate (50 µl/well). DNA-$CaPO_4$ precipitates were prepared by adding 3.75 ml of DNA-calcium chloride ($CaCl_2$) mixture composed of 5 µl/well $CaCl_2$/HEPES (1.25 M $CaCl_2$, 0.125 M HEPES, pH 7.05), 400 ng DNA/well (200 ng $PPREJ_3TKluc$, 100 ng PSG5hPPAR-α, -β, and –γ, 50 ng β-galactosidase or 100 ng empty vector [PSG5]) and 20 µl/well of 0.1 x Tris-EDTA (1 mM Tris.HCl pH7.2, 0.1 mM EDTA) into 3.75 ml of HEPES-buffer saline (2x buffer saline/1x HEPES; 0.596% (w/v) HEPES, 1.6% (w/v) NaCl, 0.074% (w/v) KCl, 0.05% $Na_2HPO_4$ and 0.2% dextrose; pH 7.05; 25 µl/well) in dropwise fashion. In another tube of DNA-$CaPO_4$ precipitates, 100 ng of PPAR construct was replaced with 100 ng empty vector. The mixture was placed in a $37^0$C incubator for approximately 7 minutes to allow the formation of fine precipitate and as mentioned above, 50µl of DNA precipitate was added into each well. After 6 hours of treatment with DNA-$CaPO_4$ precipitate, the medium was replaced with fresh medium. Following 48 hours after transfection, the media was removed and cells were stimulated with serum free medium, OxPAPC (1-100 µM), SOPC chlorohydrin (1-100 µM), 2-ClHDA (1-100 µM) and ligands for PPARα (500

nM GW647), β (10 μM L165,041) or γ (10 μM rosiglitazone). Cells were incubated for 6 hours or 16 hours at 37C in 5% $CO_2$ incubator. At the end of stimulation, medium was discarded carefully from each well. Lysis buffer (0.174 M $K_2HPO_4$, 0.026 M $KH_2PO_4$, 0.2% Triton X100; pH 7.6; 150 μl) was added and cells were freeze-thawed and were used for luciferase assay (Section 2.5.6.1).

### 2.5.4 Propagation of plasmid DNA.

Experiments in section 2.5.2 and 2.5.3 were carried out in Professor Haegeman's laboratory as part of a collaborative visit. To repeat some experiments, plasmids were sent to our laboratory. Before plasmids were transfected into cells, the amount of plasmids (PSG5hPPAR-α, PSG5hPPAR-β, PSG5hPPARγ, PSG5 and PPRE-containing reporter plasmid $J_3$-tk-luc) were propagated using the following techniques; Transformation of TOP10 chemically competent *E. coli* (Invitrogen, Paisley, UK) and DNA purification by endotoxin-free plasmid purification kit (Qiagen) and were carried out based on the manufacturer's instructions

In brief, five vials containing One Shot® TOP10 chemically competent *E. coli* were thawed on ice and 100 ng of plasmids were then added into each vials and incubated for 30 minutes on ice. Cells were then heat shocked for 30 second in the 42ºC water bath to allow insertion of plasmids into cells. Vials were then removed and placed on ice. Prewarmed super optimal broth with catabolite repression (S.O.C.) medium (250 μl) were added to each vials and were shaken at $37^0$C for 1 hour at 225 rpm. Cells (20μl) from each transformation vials were spread on separate antibiotic-selective agar plates composed of lysogeny broth (LB) medium (10g Bacto-tryptone, 5g yeast

extract and 10g NaCl) and carbenicillin (100 µg/ml). Plates were inverted and incubated overnight at $37^0$C.

To continue with DNA purification; firstly, a single colony was picked from a plate and inoculates placed in 5 ml of LB medium that contained100 µg/ml carbenicillin. Culture was incubated for 6 hours at $37^0$C with vigorous shaking and then was diluted into 1/100 dilution before further incubated and shaken for 16 hours at $37^0$C. Bacterial cells were harvested by centrifugation at 4500 x g for 10 minutes at room temperature. Subsequently, bacterial pellet was resuspended in 10 ml of resuspension buffer that contain RNase A (100µg/ml) and LyseBlue reagent (1:1000). Lysis buffer was then added and the solution was incubated for 5 minutes at room temperature. Consequently, chilled neutralization buffer was added into the lysate and mixed thoroughly. After addition of this buffer, white precipitated material composed of DNA, proteins, cell debris and detergent was formed, and the cell lysate became less viscous. Lysate was poured into the barrel of the QIAfilter Cartridge and incubated for 10 minutes to ensure separation of floating materials and lysate. The cap from the outlet nozel was removed and cell lysate was filtered through the cartridge into a 50 ml tube. Endotoxin removal buffer was added to the filtered lysate and the lysate was mixed and incubated on ice for 30 minutes. A QIAGEN-tip 500 was equilibrated by adding 10 ml of equilibration buffer and emptied by gravity flow before lysate was applied to the QIAGEN-tip. Lysate was allowed to enter resin by gravity flow and column was then washed twice with wash buffer. Subsequent to this, DNA was eluted with 15 ml of elution buffer and then precipitated by adding iso-propanol. After centrifugation at 4500 g for one hour at $4^0$C, supernatant was discarded. DNA

pellet was washed with 5 ml of endotoxin-free 70% ethanol and centrifuged at 4500 g for another one hour. Supernatant was removed and DNA pellet was air-dried before DNA was dissolved in 0.3 ml of endotoxin-free Tris-EDTA buffer. DNA concentration was determined using a NanoDrop 2000C spectrophotometer (ThermoScientific, UK).

**Table 2.1** Concentrations of purified DNA samples measured by the NanoDrop 2000/2000c.

| Sample | Nucleic acid concentration (ng/ul) | A260 | A280 | 260/280 |
|---|---|---|---|---|
| PPRE | 1784.4 | 35.687 | 18.960 | 1.88 |
| PPARα | 1287.5 | 25.749 | 13.726 | 1.88 |
| PPARβ | 782.9 | 15.659 | 8.310 | 1.88 |
| PPARγ | 1585.7 | 31.715 | 16.934 | 1.87 |

Concentration was calculated using nucleic acid calculation present in the software based on absorbance at 260 nm. A260 and A280 represents the absorbance at 260 nm or 280, respectively, normalized to 10 mm pathlength. The ratio 260/280 was used to determine the purity of DNA. The DNA was accepted as pure if the ratio was more than 1.8. If the ratio is lower, this indicates the presence of protein or other contaminants that absorb at or close to 280 nm (ThermoScientific, UK).

### 2.5.5 Transfection of HEK 293 cells using PEI and treatment of transiently transfected cells with native, SOPC ClOH, 2-ClHDA and PGPC.

HEK293 cells were seeded in 12 well plates to achieve 50-60% confluent and cells were transfected using polyethylenimine (PEI) method. The PEI-DNA solution was then prepared in 1:5.7 ratio; so for 1 µg DNA, 5.7 µl of PEI (1 mg/ml) was used (Cunningham et al., 2012). PEI (Polyscience Inc,) was added into DNA and was then mixed with medium (200µl/well) and was incubated for 15 minutes at room temperature. To determine the amount of DNA required for desired transfection condition, different combination of DNA was used [combination of 200, 100, 500 or 25 ng of $PPREJ_3$-tk-luc with various amount of PSG5hPPAR-α constructs (10-1000 ng)]. During this incubation period, old medium of HEK cells was replaced with fresh medium (800 µl/well). Subsequently, PEI-DNA mixture was added into each well in dropwise fashion and cells were transfected overnight. On the next day, old medium was replaced with fresh medium and after six hours, cells were quiesced overnight. Cells were treated with ethanol (did not exceed 0.1%) and various concentration of PPARα agonist, WY14643 at concentration ranging from 500 nM to 50 µM or without agonist. At the end of stimulation, medium was aspirated from each well and 200 µl of lysis buffer [25 mM Tris, 8 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1% Triton X100 and 15% Glycerol; pH 7.8] was added. Cells were harvested and were used for luciferase assay (Section 2.5.6.2). Only results with the optimized conditions used for further treatment with chlorinated and oxidized lipids were shown in Chapter 4.

To determine the effect of native SOPC, SOPC ClOH, 2-ClHDA, POVPC and WY14643, cells were transfected with PEI-DNA mixture containing 50 ng $PPREJ_3Tluc$ and 50 ng of PSG5hPPAR-α, as this combination was shown to give the desired transfection efficiency. After quiescence, cells were treated with serum free media, native SOPC, SOPC ClOH, 2-ClHDA (10 and 50 μM), PGPC (5 μg/ml), WY14643 (50 μM) and ethanol. At the end of stimulation, cells were lysed and were used for luciferase assay, similar to that described above (Section 2.5.6.2).

### 2.5.6 Luciferase Reporter Activity.

Two different methods of luciferase assay were undertaken, as described below.

#### 2.5.6.1 Luciferase assay I.

Luciferase and β-galactosidase assays were carried out according to the manufacturer's instructions (Promega, Madison, WI, USA). Luciferase activity, expressed in arbitrary light units, was corrected for the protein concentration in the sample by normalization to constitutive β-galactosidase levels. Cell lysate (25 μl) was added to two black 96-well plates in order to measure luciferase and galactosidase activity. β-galactosidase levels were quantified with a chemiluminescent reporter assay Galacto-Light kit (Tropix, Bedford, MA, USA). 25 μl of galactosidase buffer (2% (v/v) of galactosidase in reaction buffer diluents (Analytical Bio) was added to the samples and incubated at room temperature for 40 minutes. In another plate, luciferase buffer containing luciferase reagent [270 μM Coenzyme A, 470 μM luciferin (both from Sigma St Louis, MO, USA) , 530 μM ATP (Boehringer Mannheim, Germany)] in a reaction buffer composed of 20 mM

Trycine, 1 mM $Mg(CO_3)Mg(OH)_2.5H_2O$, 0.1mM EDTA, 33.3 mM DTT and 2.7 mM $MgSO_4$ (all from Sigma St Louis, MO, USA) were added into the sample. Because luciferase buffer is not stable due to the presence of ATP and luciferin, the measurement was carried out immediately after their addition into samples. The relative light units of both plates were measured by a luminometer (Perkin Elmer, Victor X3).

#### 2.5.6.2 Luciferase assay II.

Transfected HEK cells were washed twice with ice cold PBS 2 at the end of stimulation. 200 µl of lysis buffer [25 mM Tris phosphate, 8 mM $MgCl_2$, 1 mM DTT (Roche Diagnostics GmbH), 1% Triton X100 and 15% (v/v) glycerol; pH 7.8] were added into each well. Cells were scraped from the plate and transferred into a 1.5 ml tube and spin at 13000 rpm for 2 min in microcentrifuge. Supernatant (100 µl) was collected into a tube and 100µl of luciferase buffer [1mM ATP, 1% (v/v), bovine serum albumin (BSA) (Bochringer Mannheim, UK) and 0.2 mM luciferin substrate (Lonza, Slough, UK) in lysis buffer] added to it. The relative light units were measured immediately using luminometer (F12 luminometer, Berthold detection system)

## 2.6 Determining the effects of chlorinated lipids on activation of NF-κB and MAPK pathway.

### 2.6.1 Isolation and growth of RASMCs.

Dissection of Sprague-Dawley rats and isolation of aortas have been done under sterile condition. Rats were a kind gift from Dr. Paul Coats and Dr. Satirah. Isolated

aortas were transferred into universal tubes containing 0.9% normal saline to avoid dryness. Then, the aorta were minced into small pieces and transferred into 6 wells plate containing 5 ml of Waymouth MB/Hams F12 (1:1) medium (from Invitrogen) supplemented with 10% FCS and 100 units/ml penicillin and 0.1 mg/ml streptomycin (Invitrogen, Paisley, UK) and incubated at $37^0$C with 5% $CO_2$. Medium were removed after 24 hours for the first time, and then cells were fed every two days. Rat aortic smooth muscle cells (RASMCs) achieved approximately 90% confluence after 10 to 15 days. Aorta tissues were removed from the plate and cells were detached using TrypLE and transferred into a T-25 flask, plated in 6 well plate or kept in liquid nitrogen.

### 2.6.2 Identification of RASMCs by immunohistochemistry staining.

RASMC at an initial count of 2.0 x $10^4$ cells/well (200µl/well) were incubated in chamber slides overnight to get a suitable confluency. Medium were removed and cells were fixed with 2% (v/v) paraformaldehyde for 20 minutes and washed three times with 200 µl PBS1. Cells were then permeabilised by adding 200 µl/well of 0.5% (v/v) Triton X100 in PBS1 for 15 minutes at room temperature and cells were washed three times with PBS1. For blocking, 200 µl of 10% (v/v) heat inactivated serum from goat) was added followed by incubation at $37^0$C for 30 minutes. 200 µl of rabbit anti-smooth muscle actin was added. In parallel, normal rabbit IgG were prepared as a control. Cells were incubated at $4^0$C overnight and washed again thrice with PBS. Secondary antibody, goat anti-rabbit fluorescein-labeled antibodies (FITC) in 0.1% (v/v) Triton X100 (200 µl) was added and incubated at $37^0$C for one

hour. This was followed by washing before 4',6-diamidino-2-phenylindole (DAPI) was added and incubated overnight in the dark at room temperature.

### 2.6.3 Growth of HUVECs.

Primary Human Umbilical Vein Endothelial Cells (HUVECs: ≥ 500,000 cells/vial) were purchased from Lonza (Slough, UK). HUVECs were maintained in Endothelial Basal Media (EBM-2) containing 2% (v/v) of fetal bovine serum and supplemented with EGM-2 Single Quots containing 2 ml hydrocortisone, 0.5 ml epidermal growth factor human recombinant (rhEGF), 0.5 ml gentamycin sulfate, amphotericin-B (GA-1000), 0.5 ml recombinant long R insulin-like growth factor-1 in aqueous solution ($R^3$-IGF-1), 2 ml recombinant human fibroblast growth factor-B (rhFGF-B), 0.5 ml heparin, 0.5 ml ascorbic acid and 0.5 ml endothelial growth factor human recombinant (VEGF) (Medium and all growth factors are included in endothelial cell growth media kits from Lonza, Slough, UK).

Medium was changed every two days until cells were confluent. Stimulation of cells was carried out in HUVECs from passage 2 to 6 only. To subculture the cells, medium was removed and cells were washed twice with a sterile solution of 0.5% (w/v) trypsin (Invitrogen, Paisley, UK), 0.2% (w/v) EDTA in phosphate buffered saline 2 (PBS; 154 mM NaCl, 5.36 mM KCl, 8 mM $Na_2HPO_4$, 1.46 mM $KH_2PO_4$, pH 7.4). The solution was removed and the flasks were incubated at $37^0$C, 5% $CO_2$ for 2-3 minutes until cells rounded. The flasks were gently tapped to ensure the cells were completely detached and then washed in 10 ml DMEM or EBM-2. Cells were further passaged (1 in 10 dilution) into a new flask or seeded into plates. HUVECs

were frozen in a freezing media containing DMSO, media and FCS (1:9:2) (Invitrogen, Paisley, UK) and stored in liquid nitrogen for later use. Cells were cultured at $37^0$C in humidified atmosphere of 5% $CO_2$.

### 2.6.4 Investigation of effects of chlorinated and oxidized lipids on stimulation of NF-κB and MAPK pathways.

To confirm the responsiveness of primary RASMCs, cells within passage 0 to 4 were seeded in 6 well plates and were grown to 90% confluent and stimulated with 100 μg/ml *E.coli* LPS and 20 ng/ml TNF-α for different time intervals or with different concentrations of LPS and TNF-α. Different concentrations of these mediators were also tested at different time intervals.

Because primary RASMCs were not responsive towards LPS and TNF-α, HUVECs were used as a model to investigate the effect of chlorinated and oxidized lipids. HUVECs were seeded on 12 well plates and were grown to 90% confluent before stimulation with the following treatment. Half medium of HUVECs was removed and cells were treated with serum free M199 and 2X concentrations of OxPAPC, SOPC ClOH, PGPC, ethanol and 2-ClHDA at different time points. Subsequently, cells were treated with LPS (100μg/ml) at different time (15-120 min) and concentration (1-100 μg/ml). Combination treatment were then carried out, in which HUVECs were pre-treated with oxidized or chlorinated lipids for one hour followed by treatment of LPS at concentration 30 μg/ml for another 15 minutes or one hour. Cells were also treated alone with chlorinated lipids or were pretreated for 60

minutes before stimulation with TNF-α for a further 15 minutes to investigate the effect of chlorinated lipids on the NF-κB pathway.

### 2.6.5 Western Blot Assays.

#### 2.6.5.1 Preparation of Whole Cell Extracts.

At the end of the stimulation, cells were placed on ice to stop the reaction. Cells were immediately washed twice with ice cold PBS 2 before the addition of 180 µl of pre-heated Laemmli's sample buffer [63 mM Tris-HCl (pH6.7), 2 mM $Na_4P_2O_7$, 5 mM EDTA, 10% (v/v) glycerol, 2% (w/v) SDS, 50 mM DTT (Roche diagnostics GmbH), 0.007% (w/v) bromophenol blue]. Cells were scraped using a syringe rubber plunger, collected into 1.5 ml tubes, and were repeatedly pushed through a 21 gauge needle to lyse them. The samples were then boiled for 5 minutes to denature the cellular proteins. Subsequently, cells were used for Western blotting or kept in -20$^0$C until needed (Cadalbert et al., 2010).

#### 2.6.5.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

A resolving gel solution was prepared by adding N, N'-methylenebis-acrylamide (30:0.8), 0.375M Tris (pH8.8), 0.1% (w/v) sodium deodecyl sulfate (SDS) and 10% (w/v) ammonium persulfate (APS). N, N, N', N-tetramethylethylenediamine (TEMED) was then added to initiate polymerisation. 15% (v/v) acrylamide was used for detecting low molecular weight of protein (<30 kDa) and 10% (v/v) acrylamide was prepared for higher molecular weight of protein (30-90 kDa). The resolving gel was poured between two plates and overlaid with 180 µl 0.1 % (w/v) SDS. After gel polymerisation, the SDS was removed and a stacking gel containing 3% (v/v)

acrylamide (N, N'-methylenebis-acrylamide (30:0.8) in 125 mM Tris (pH 6.8), 0.1% (w/v) SDS and 0.05% (w/v) APS and TEMED was added. The comb was removed when the gel polymerized and the gel was assembled in a Bio-Rad electrophoresis tank. Electrophoresis buffer containing 25 mM Tris, 129 mM glycine and 0.1% (w/v) SDS was then poured into the tank. Aliquots of sample (20-30 µl) were loaded into each well using a microsyringe. The protein concentration was not determined prior to loading, but correct protein loading was confirmed by checking the expression of a housekeeping gene, which gives information on the total amount of protein loaded in the gel. A prestained SDS-PAGE molecular weight marker solution was run in parallel in order to identify the protein of interest. Samples were electrophoresed at a constant voltage of 130 V until the bromophenol dye reached the bottom of the gel.

#### 2.6.5.3 Electrophoretic Transfer of Proteins to nitrocellulose Membrane.

The proteins on SDS-PAGE were transferred to nitrocellulose membrane (Whatman, Kent, UK) by electrophoretic blotting (Cadalbert et al., 2010). Two pieces of Whatman 3 MM paper, two pieces sponge pads and 1 nitrocellulose membrane were immersed in transfer buffer [25 M Tris, 19 mM glycine, 20% (v/v) methanol] and arranged on the cassette. The gel was pressed firmly against the nitrocellulose membrane to remove air bubbles and sandwiched between papers and sponges. Because SDS in the gel provides negative charge on the proteins, the cassette was oriented in a Bio-Rad Mini Trans-Blot$^{TM}$ tank with the gel facing the cathode. An ice reservoir was located next to it to cool the tank during transfer of protein and a constant current of 300 mA was applied for 105 minutes.

### 2.6.5.4 Immunological Detection of Protein.

The nitrocellulose membrane was removed from the cassette after electrophoretic transfer. Remaining sites on the nitrocellulose were blocked using 2% (w/v) BSA in NaTT buffer (150 mM NaCl, 20 mM Tris (pH7.4), 0.2% (v/v) Tween 20) for 2 hours. Subsequently, blocking buffer was removed and membrane was incubated overnight with antiserum specific to the target protein appropriately diluted in 0.2% BSA in NaTT buffer. The membrane was then washed 6 times with NaTT at15 minute intervals. The membrane was then incubated for 2 hours with secondary horseradish peroxidase-conjugated IgG directed against the first immunoglobulin diluted to approximately 1:10000 in 0.2% BSA in NaTT. Another six washes of the membrane were then carried out. To detect protein, the membrane was incubated in enhanced chemiluminescence reagent (ECL) for 2 min (Cadalbert et al., 2010). The membrane was blotted onto a paper towel to remove excess liquid, mounted on exposure cassette and covered with cling film. The membrane was exposed to X-ray film (Kodak ls X-OMAT) for the required time in a darkroom and developed using X-OMAT machine (KODAK M35-M X-OMAT processor).

### 2.6.5.5 Nitrocellulose membrane stripping and reprobing.

For detection of other cellulose bound proteins, the nitrocellulose membrane blotted with previous antibody was reprobed. The antibody used on the membrane was removed using stripping buffer (0.05 M Tris-HCl, 2% SDS and 0.1 M of β-mercaptoethanol). The membrane was incubated in 15 ml of stripping buffer for one hour at $60^0$C in an incubator/shaker. The stripping buffer was removed and the membrane was given 3 times 15 min washes with NaTT buffer. At this stage, another

primary antibody was added onto the membrane and incubated overnight. Subsequently, membrane was washed, primary antibody was added and finally membrane was exposed to the film following the same step as described in section 2.4.4.

### 2.6.6 Scanning Densitometry

Images obtained from western blot were scanned using Epson perfection 1640SU scanner using Adobe photoshop 5.0.2 software. The bands were normalized to control and quantified using Scion image (Scion Corp., Maryland, USA)

## 2.7 Investigate the effect of chlorinated lipids on cell morphology

A preliminary study was carried out to investigate whether chlorinated lipids could induce cell death in endothelial cells. HUVECs were seeded in 12 well plates and allow to reach 50% confluence. Half of the media was removed and replaced with 2X concentrations of SOPC ClOH and 2-ClHDA (various final concentrations) alone, or pre-treated with chlorinated lipids for 30 minutes prior to stimulation with TNF-α (10 ng/ EDTA ml TNF-α). After 24 hours stimulation, cells were observed under microscope.

## 2.8 Data Analysis

The data generated were expressed as mean +/- S.E.M for two or three separate observations or as a representative of at least three separate experiments, where replicates within experiment varied from four to six replicates. The statistical significance of differences between mean values from control and treated groups

were determined either by one-tailed Students Unpaired t-test or a two-tailed one way analysis of variance (ANOVA) followed by a Dunnets test. Data were considered significant with $P<0.05$.

# Chapter 3

# Effect of Oxidized and Chlorinated Lipids on Myeloid Cell Cytokine Production

## 3.1. Introduction

It is generally well accepted that atherosclerosis is an inflammatory disease and oxidation of LDL has an important role in the initiation and progression of this disease. OxLDL has been shown to produce various effects including proliferation of smooth muscle cells and macrophages, cell injury, cytotoxicity and inflammation (Chisolm and Chai, 2000). Since the discoveries that OxPLs such as OxPAPC, PGPC, POVPC and PEIPC are the active agents of minimally modified LDL, various studies have been conducted to demonstrate their effects *in vitro* and *in vivo* (Bochkov et al., 2010). With regards to pro-inflammatory effects, OxPAPC has been shown to induce IL-8 production by activated macrophages (Erridge et al., 2007). Interestingly, OxPAPC has also been implicated in some potentially therapeutic effects such as inhibition of LPS-mediated TNF-α production in macrophages (Erridge et al., 2008) and inhibition of LPS-induced IL-8 production in human aortic endothelial cells and myeloid cells (Walton et al., 2003a, Erridge et al., 2007).

Latterly, there is increasing evidence suggesting that HOCl-LDL can also induce various biological effects, as described earlier in Chapter 1. Whilst various studies reported that the effects of HOCl-LDL are mediated by modification of its protein components; for instance, HOCl- LDL protein induces mRNA and protein level of IL-8 in human monocytes (Woenckhaus et al., 1998), there is also increasing evidence suggesting that modification of lipids by HOCl, which results in the formation of chlorinated lipids, may also contribute to the effect induced by HOCl-LDL.

With regards to pro-inflammatory effects, SOPC chlorohydrin (SOPC ClOH), a product formed by chlorination at double bond of oleic acid attached to glycerol backbone at sn-2 position of phospholipid SOPC, has been shown to induce inflammation by enhancing production of reactive oxygen species in PMA-activated leukocyte (Dever et al., 2006). Moreover, α-chloro fatty aldehyde, 2-ClHDA, has been shown to induce delocalization of eNOS that results in reduction of anti-atherogenic nitrogen oxide production (Marsche et al., 2004) and up-regulation of COX-2 expression and subsequent production of prostacyclin that provide anti-atherogenic effects (Messner et al., 2008a). These findings suggest that it is likely that these chlorinated lipids have pleiotropic effects and may have an important role in atherosclerosis. However, no study has been done to investigate the effect of chlorinated lipids on pro-inflammatory cytokines production. Therefore, the aim of this study was to investigate the hypothesis that, similarly to OxPAPC, SOPC ClOH and 2-ClHDA may induce IL-8 production when incubated alone with myeloid cells, and in the presence of LPS, may inhibit LPS-induced IL-8 and TNF-α production.

## 3.2. Methodology

Native, oxidized lipids and chlorinated lipids were obtained from Avanti Polar Lipid (USA) or obtained from Professor Andrew Pitt (Aston University) as described in section 2.1. Chlorinated lipids were prepared and analysed as describe in section 2.2.1 and 2.2.2. Growth and subculturing of mammalian cells were described in section 2.3.1. U937 cells were differentiated using 0.1μg/ml PMA before cell stimulation with various treatments as described in section 2.3.2. Production of IL-8 and TNF-α was measured by ELISA as described in section 2.3.3 and cell viability assay carried out as described in section 2.4.

## 3.3. Results

### 3.3.1. Detection of lipid chlorohydrin and oxidized phospholipids.

Prior to treatment of cells, chlorinated and oxidized lipids were analysed by mass spectrometry to ensure the native lipids had been converted into the required products. The conversion of native SOPC to SOPC ClOH was observed by electrospray mass spectrometry in the positive ion mode. Figure 3.1 represents the spectra of native SOPC and SOPC ClOH. The formation of mono-chlorohydrin was detected as an increase in mass-to charge ratio of 52. Treatment of SOPC [788 *m/z* ($M+H^+$) or 810 *m/z* ($M+Na^+$)] with hypochlorous acid (HOCl) resulted in almost complete conversion to the monochlorohydrins, observed at 862 *m/z* ($M+Na^+$). Sodiated molecules ($M+Na^+$) were detected as increase in mass-to charge ratio of 22 due to exchange of hydrogen with sodium ion. Sodium was derived from the glass tubes during preparation of chlorination and oxidation of phospholipids. Peaks present in the spectrum corresponding to M+1, M+2 and M+3 due to the presence of

$^{13}C$, $^{37}Cl$ or both. Figure 3.2 represents the spectra of oxPAPC. Upon oxidation of PAPC [782 *m/z* ($M+H^+$) or 804 *m/z* ($M+Na^+$)], a mixture of oxPAPC products was detected. Phospholipid oxidation products were observed at 616 *m/z* ($M+Na^+$) known as POVPC and at 632 *m/z* ($M+Na^+$) known as PGPC. Other peaks observed at 868 *m/z* were due to addition of 2 molecules of oxygen and the peak observed at 900 *m/z* was due to addition of 3 molecules of oxygen to the sodiated form of PAPC. Another peak at 882 *m/z* ($M+Na^+$) was a dehydration product of oxidised PAPC that has 3 molecules of oxygen added to it.

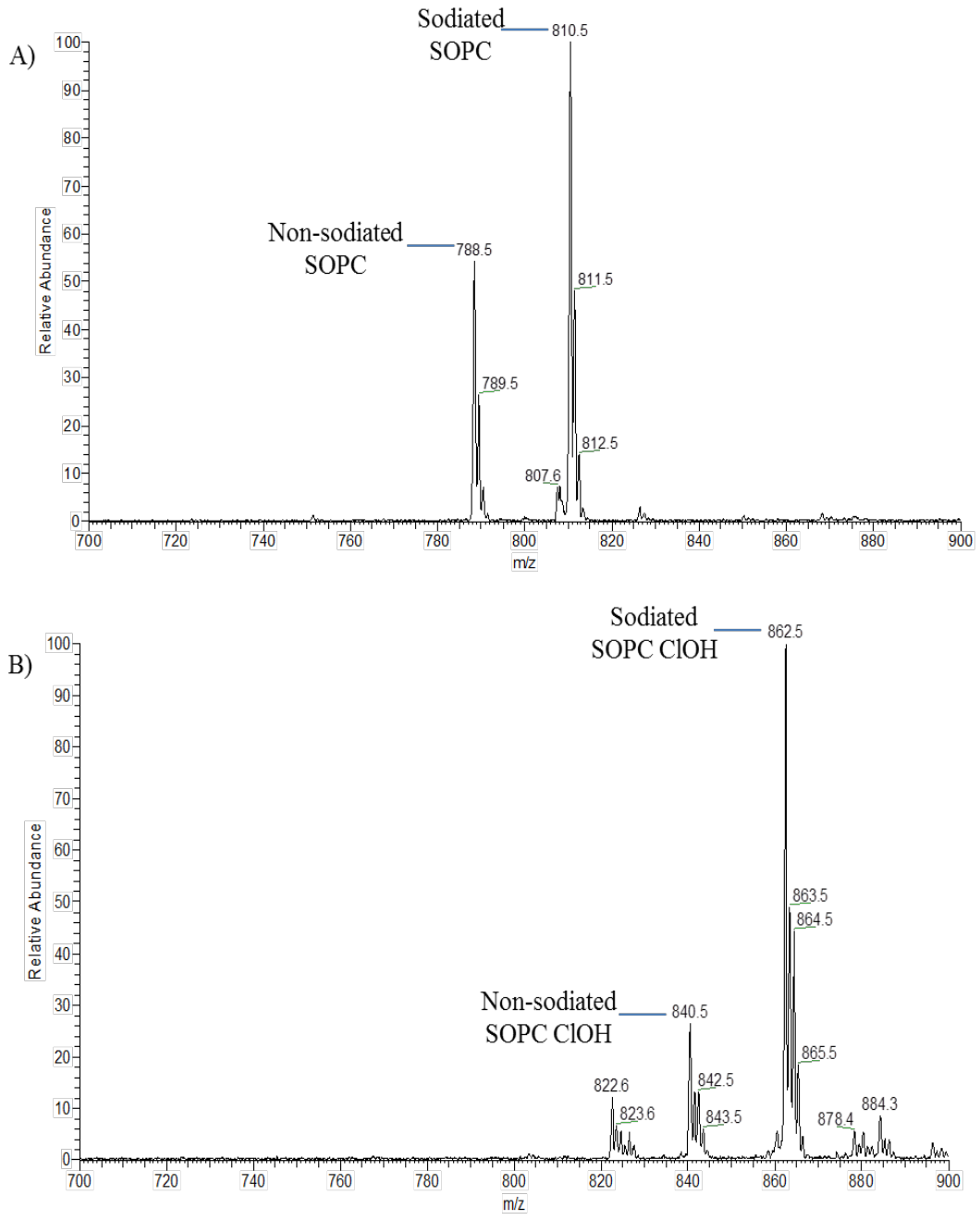


**Figure 3.1 Detection of HOCl-oxidized SOPC by positive-ionization electrospray mass spectrometry.** (A) Native SOPC was introduced into the mass spectrometer ion source dissolved in flow injection solvent composed of 0.1% formic acid in methanol. The signal of the lipid was observed at 788 *m/z* ($M+H^+$ ion) and 810 *m/z* ($M+Na^+$ ion) and (B) SOPC ClOH was produced by treatment with NaOCl as described in section 2.2.1, and was observed at 840 m/z ($M+H^+$ ion) and 862 ($M+Na^+$ ion).

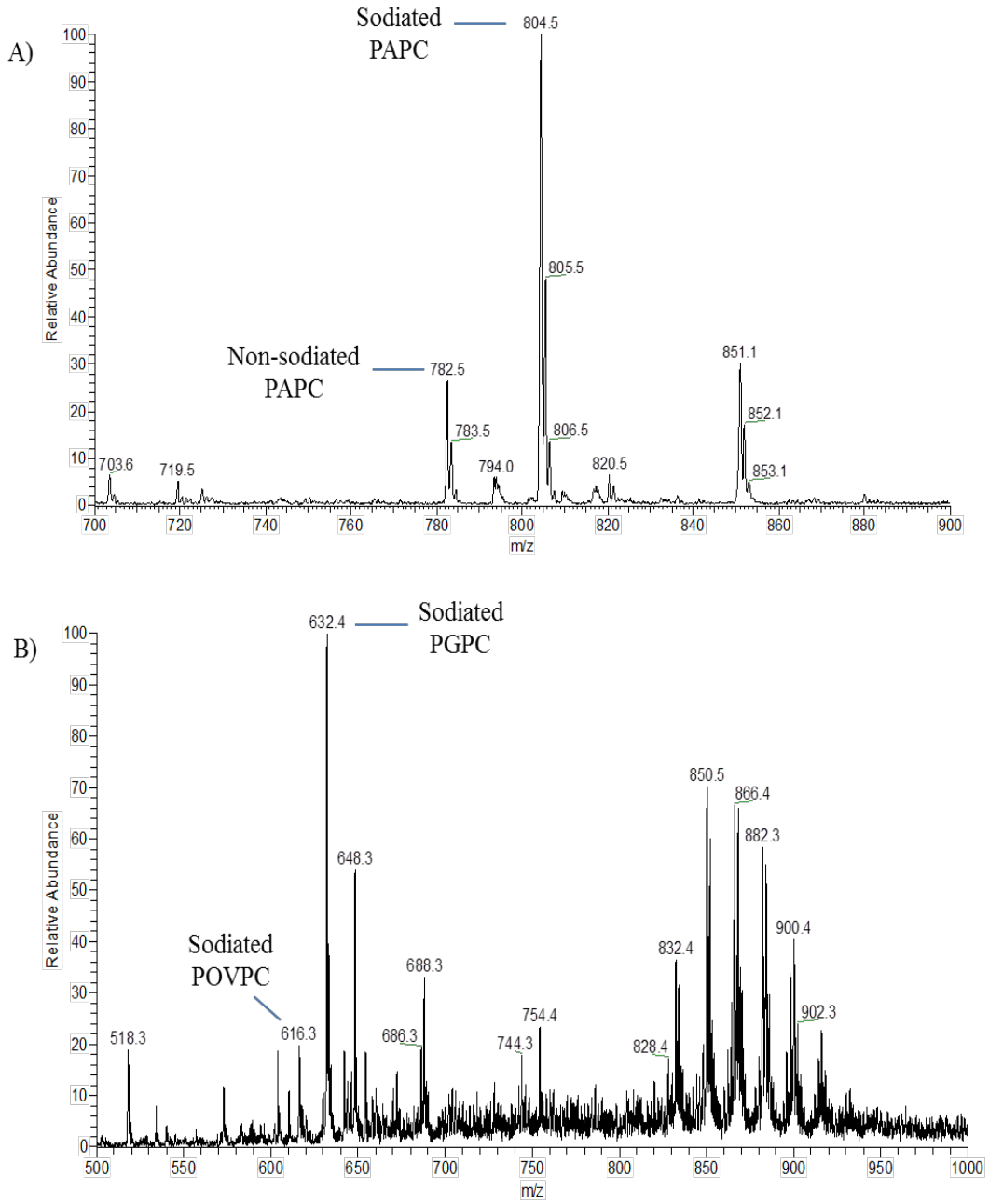


**Figure 3.2 Detection of autoxidation of PAPC by positive-ionization electrospray mass spectrometry.** (A) Native PAPC was introduced into the mass spectrometer ion source dissolved in flow injection solvent composed of 0.1% formic acid in methanol. The spectrum of the lipid was observed at 782 *m/z* ($M+H^+$ ion) and 804 m/z ($M+Na^+$ ion). (B) Oxidized PAPC mixture was produced by autoxidation in air for 7 days (2.2.1), was resuspended in flow injection solvent (1 mg/ml) and 20 µl (20µg) was analysed (2.2.2): PGPC and POVPC were observed at 632 m/z ($M+Na^+$ ion) and 610 ($M+Na^+$ ion), respectively.

### 3.3.2. Effect of chlorinated and oxidized lipids on U937 cell production of IL-8 and TNF-α.

Figure 3.3 shows that treatment of U937 cells with SOPC ClOH (100μM) at different time intervals did not cause significant increase in TNF-α and IL-8 production. It can also be seen that the cell response to control (media alone) and chlorohydrin were very similar. The basal level production of IL-8 by U937 cells was approximately 1000 times higher than production of TNF-α protein. Treatment of U937 cells with native lipid and chlorohydrin derivative of SOPC at different concentrations resulted in higher TNF-α release (Figure 3.4). At the highest concentrations of treatment (100 μM), the average chlorohydrin stimulated TNF-α levels appeared to be higher than the control treated cells, but possibly due to high variability, statistical analysis did not show any significant difference. There was clearly no induction of IL-8 by SOPC chlorohydrin over the concentration range investigated.

OxPAPC has been shown to induce IL-8 production but not TNF-α (Erridge et al., 2007). Similarly, PGPC, an individual component of OxPAPC mixture has been shown to induce IL-8 production (Yeh et al., 2004). Therefore, the effect of PGPC on U937 cells was determined and compared with that of chlorohydrin. The results are shown in Figure 3.5. With TNF-α production, there appeared to be a trend towards decreasing cytokine levels with cells treated with 8 and 16 μM PGPC but at the highest concentration of PGPC (32 μM), the level of TNF-α increased to the same level as control, suggesting that overall PGPC did not induce production of TNF-α. On the other hand, the average IL-8 levels measured were higher in cells treated with 16 and 32 μM PGPC; however, owing to the relatively large variability in the

analysis, none of these effects was statistically significant. Thus although the trend observed was generally in agreement with previous findings, it cannot be concluded that PGPC caused significant alterations in IL-8 and TNF-α production under the conditions used in this experiment.

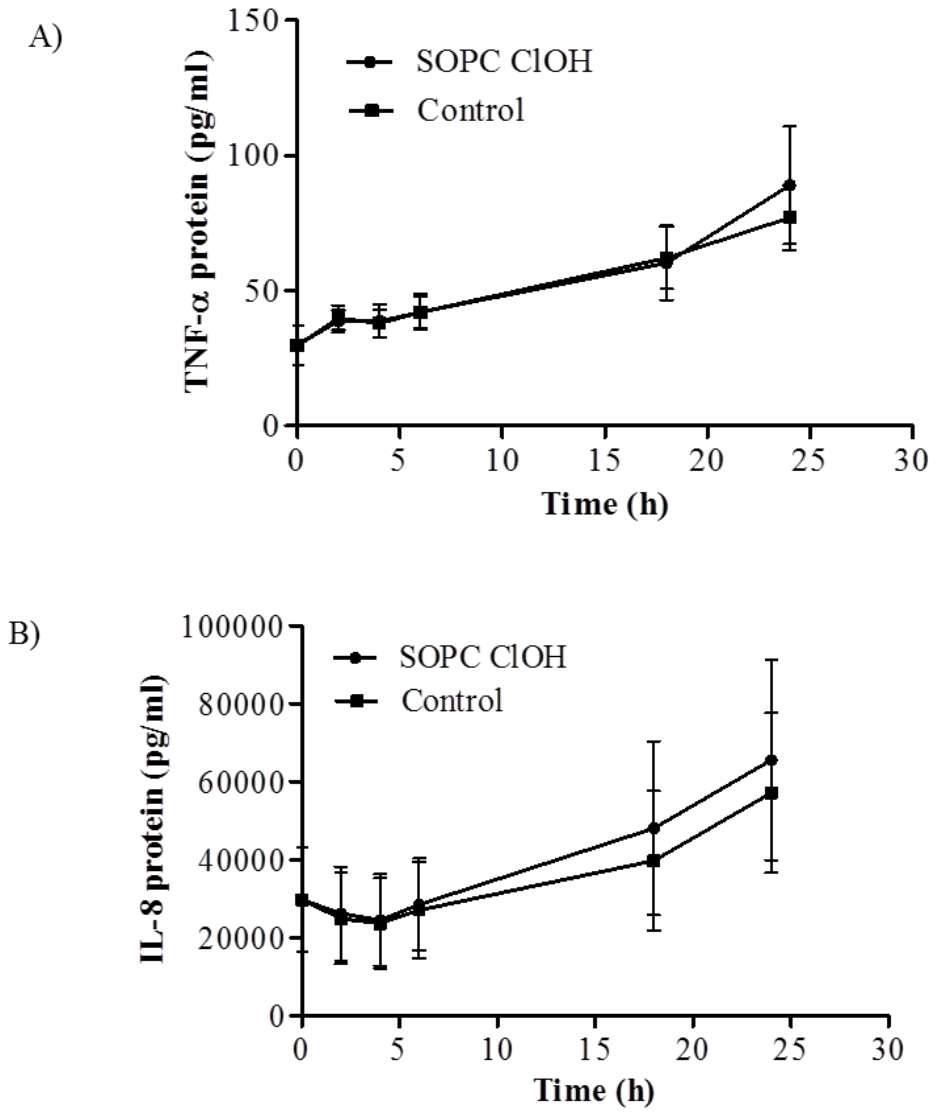


**Figure 3.3 Chlorohydrin derivative of SOPC did not induce production of TNF-α and IL-8 in U937 cells.** Cells were treated with medium (control) or SOPC ClOH (100 μM) for a period of 24 hours. Culture medium was assessed for (A) TNF-α and (B) IL-8 production by ELISA as described in section 2.3.3. Lipid concentrations are based on the native lipid treated with HOCl. Data are expressed as mean ± S.E.M of three separate experiments.

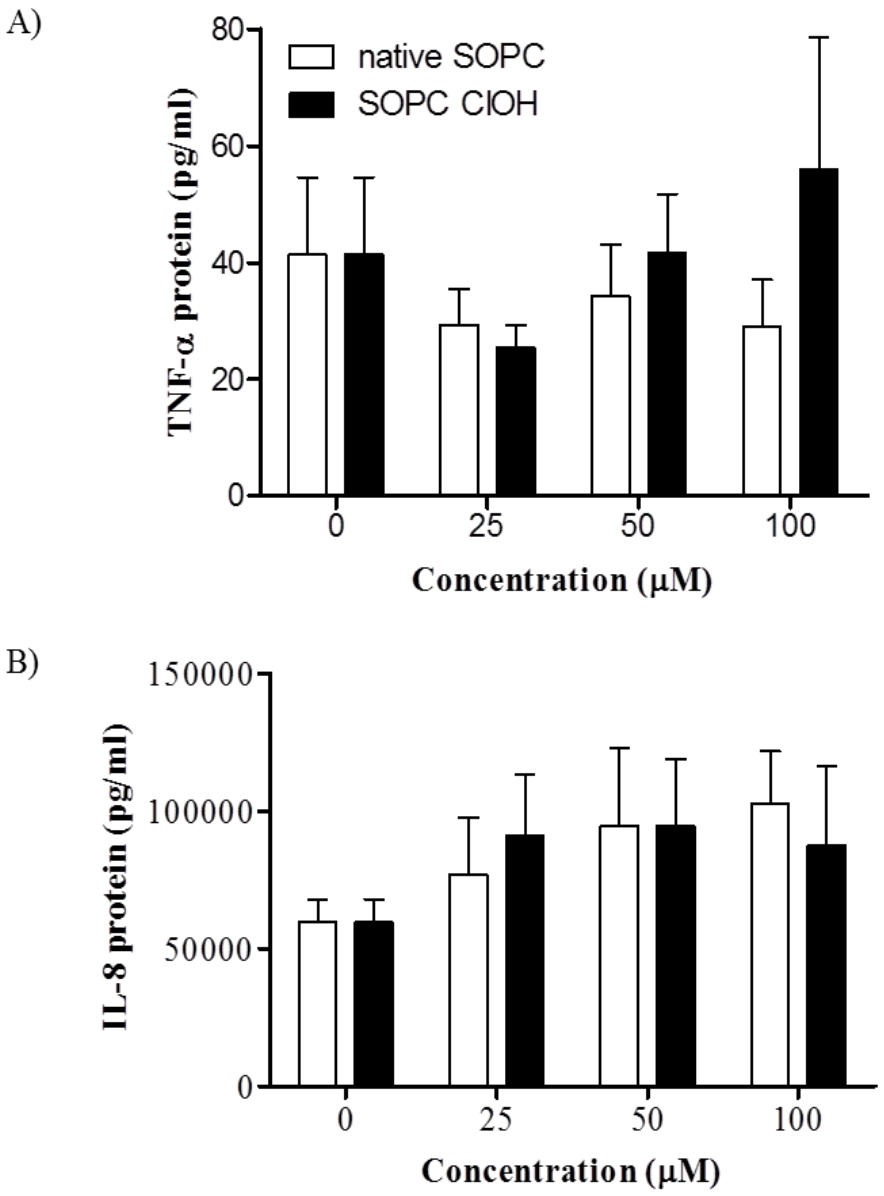


**Figure 3.4 Native SOPC or chlorohydrin derivative of SOPC did not induce production of TNF-α and IL-8 in U937 cells.** Cells were treated with indicated concentrations of native or chlorohydrin of SOPC for 24 hours. Culture medium was assessed for (A) TNF-α and (B) IL-8 production by ELISA as described in section 2.3.3. Lipid concentrations are based on the native lipid treated with HOCl. Data are expressed as mean ± S.E.M of at least three separate experiments.

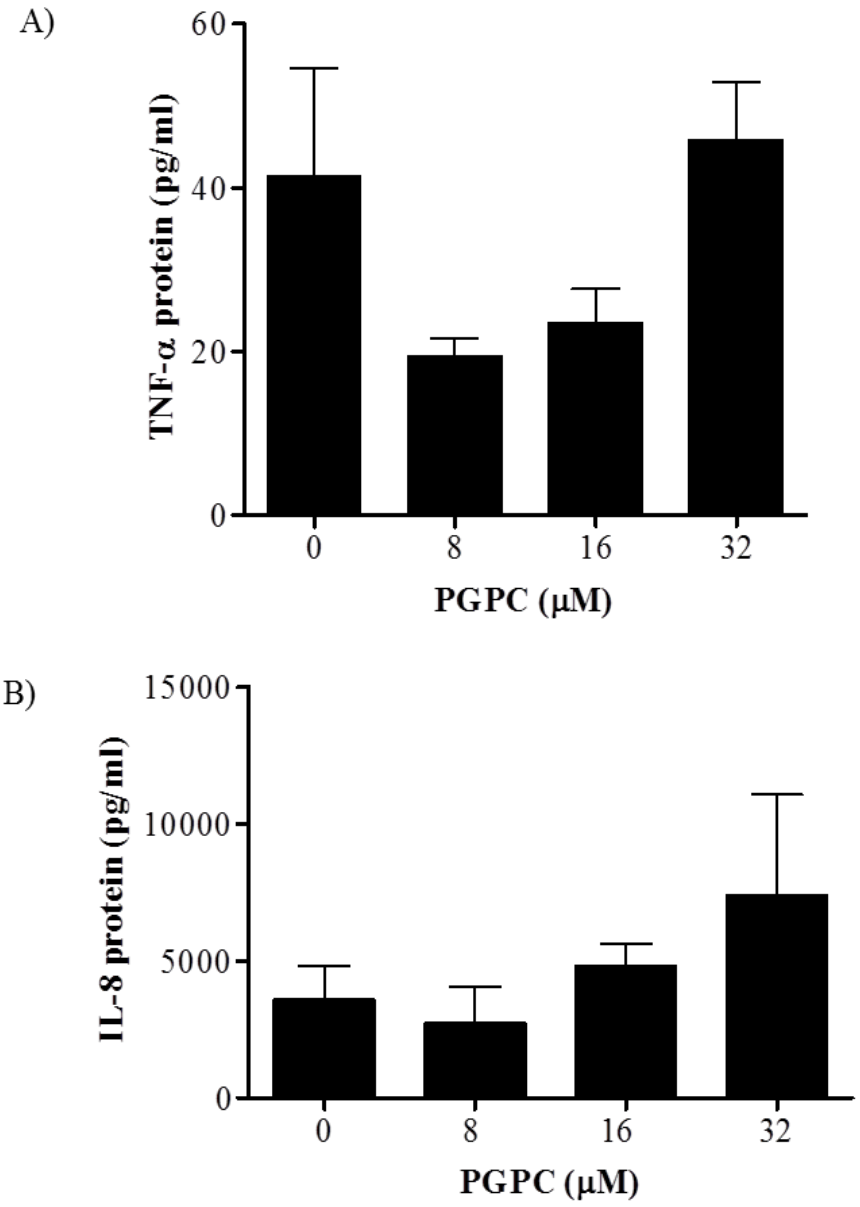


**Figure 3.5 PGPC did not induce production of TNF-α and IL-8 in U937 cells.** Cells were treated with indicated concentrations of PGPC for 24 hours. At the end of stimulation, supernatant was collected and was assessed for (A) TNF-α and (B) IL-8 production by ELISA as described in section 2.3.3. Data are expressed as mean ± S.E.M of three separate experiments.

### 3.3.3. Effect of *S. abortus equi* LPS on production of IL-8 and TNF-α in U937 cells.

Lipopolysaccharide (LPS), the component of the outer membrane of gram negative bacteria, is a well-established mediator that induce pro-inflammatory cytokine production (Mackman, 2003), particularly IL-8 and TNF-α. OxPAPC has been previously shown to inhibit production of LPS-induced IL-8 and TNF-α production (Erridge et al., 2007, Erridge et al., 2008). Because some effects of OxPAPC have been shown to be comparable to lipid chlorohydrin, for instance OxPAPC induces expression of P-selectin, similar to that observed in SOPC chlorohydrin, it is of interest to investigate whether phospholipid chlorohydrins also have inhibitory effects on LPS-induced proinflammatory cytokine production in U937 cells. First of all, the time course and concentration dependence of LPS-induced cytokine production was tested. Figure 3.6 illustrates the effect of LPS on U937-mediated IL-8 and TNF-α production at different time points. LPS (100 ng/ml) induced IL-8 production in a time-dependent manner and a significant increase was observed from 6 hours onwards ($P<0.001$ vs control). However, the trend was different for TNF-α production, where a significant increase was observed at 4, 6 and 24 hours only.

Moreover, LPS was shown to induce a concentration-dependent increase in TNF-α and IL-8 production (Figure 3.7). The level of both TNF-α and IL-8 production was increased by more than half in cells treated with 10 ng/ml LPS compared to control; however, probably due to high variability in the analysis, the effect was not significant. In cells treated with the highest concentration of LPS (100 ng/ml), significant increase of TNF-α and IL-8 level were observed. At this concentration,

the average TNF-α level measured was approximately 15 fold higher than control, while average IL-8 level was approximately 4.5 fold greater than control.

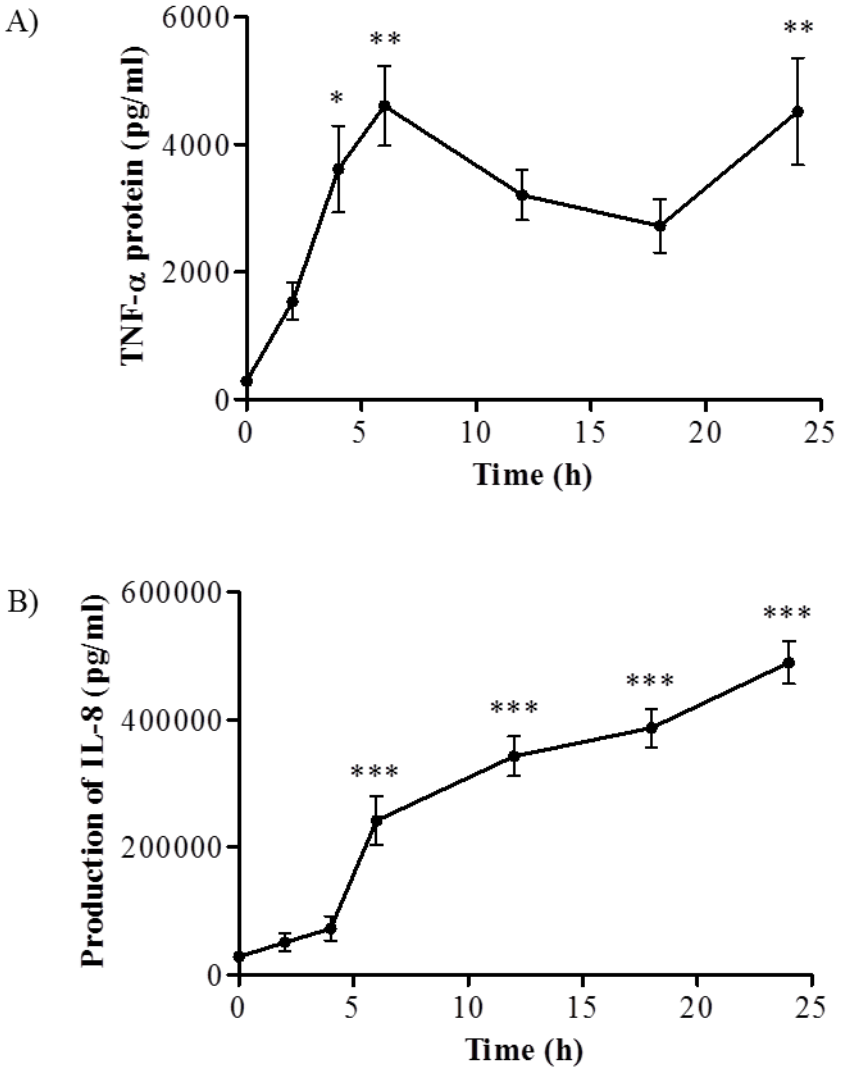


**Figure 3.6 Differential effects of TNF-α and IL-8 production in U937 cells following treatment with *S. abortus equi* LPS.** Cells were treated with LPS (100 ng/ml) for a period of 24 hours. Culture medium was assessed for (A) TNF-α and (B) IL-8 production by ELISA as described in section 2.3.3. Data are expressed as mean ± S.E.M of at least three separate experiments. Significant differences compared to control is shown by *, 1 symbol corresponds to $P<0.05$, 2 symbols correspond to $P<0.01$ and 3 symbols correspond to $P<0.001$.

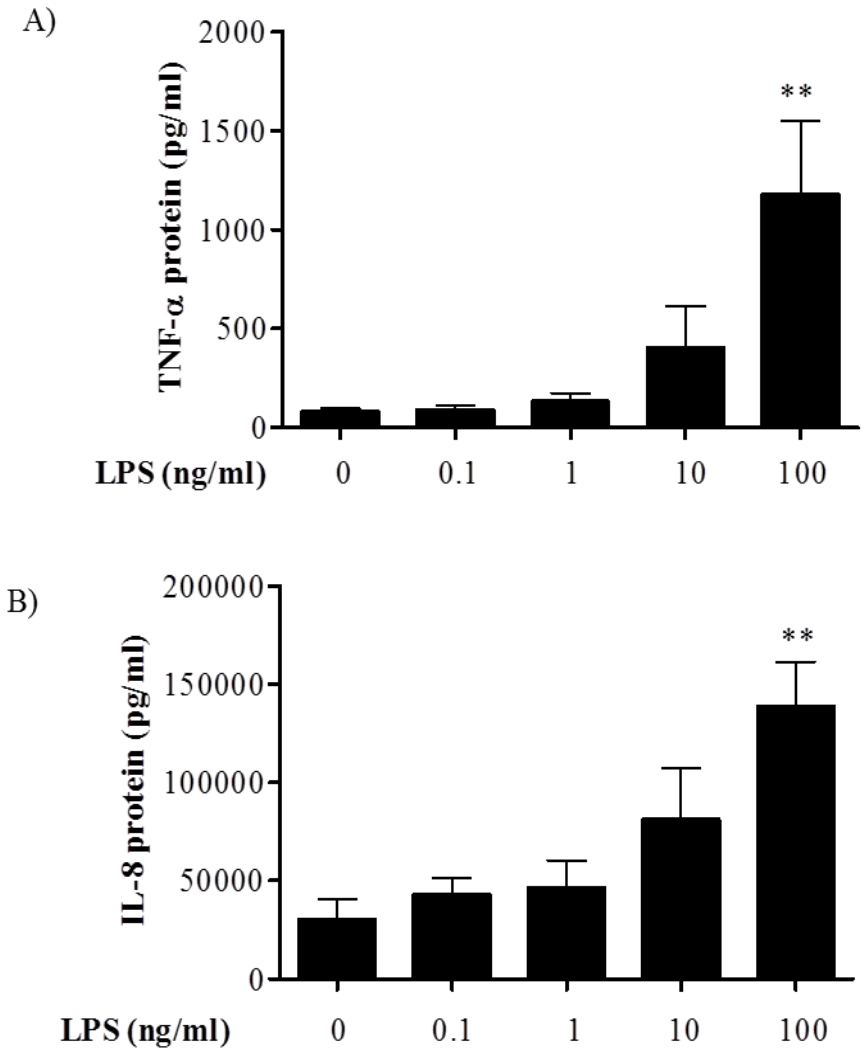


**Figure 3.7 Concentration-dependent effects of *S. abortus equi* LPS on production of TNF-α and IL-8 in U937 cells.** Cells were treated with different concentrations of LPS for 24 hours. Culture medium was assessed for (A) TNF-α and (B) IL-8 production by ELISA as described in section 2.3.3. Data are expressed as mean ± S.E.M of at least three separate experiments. **, $P<0.01$ vs control.

### 3.3.4. Effect of chlorinated and oxidized lipids on production of IL-8 and TNF-α in U937 cells stimulated with *S. abortus equi* LPS.

U937 cells were treated with different concentrations of native and SOPC chlorohydrin together with 100 ng/ml LPS for 24 hours. The results show that while the presence of LPS significantly increased the TNF-α production, SOPC ClOH did not cause a significant effect on LPS-induced cytokine production (Figure 3.8). With regards to IL-8 production, SOPC ClOH was shown to enhance the effect of LPS. The level of IL-8 was slightly increased in cells treated with 25 μM SOPC ClOH and was statistically significant at concentration 50 μM and 100 μM compared to the control with zero lipid plus LPS. With regards native SOPC, there appeared to be a similar trend to SOPC ClOH; however, none of the concentrations of native SOPC induced significant effects on LPS-stimulated cells. There was also no significant effect observed between SOPC chlorohydrin and native SOPC.

PGPC the individual component of OxPAPC was tested in this system to compare with SOPC ClOH. Surprisingly, Figure 3.9 shows that PGPC, which was expected to inhibit LPS-induced cytokine production, did not inhibit LPS-induced IL-8 or TNF-α production.

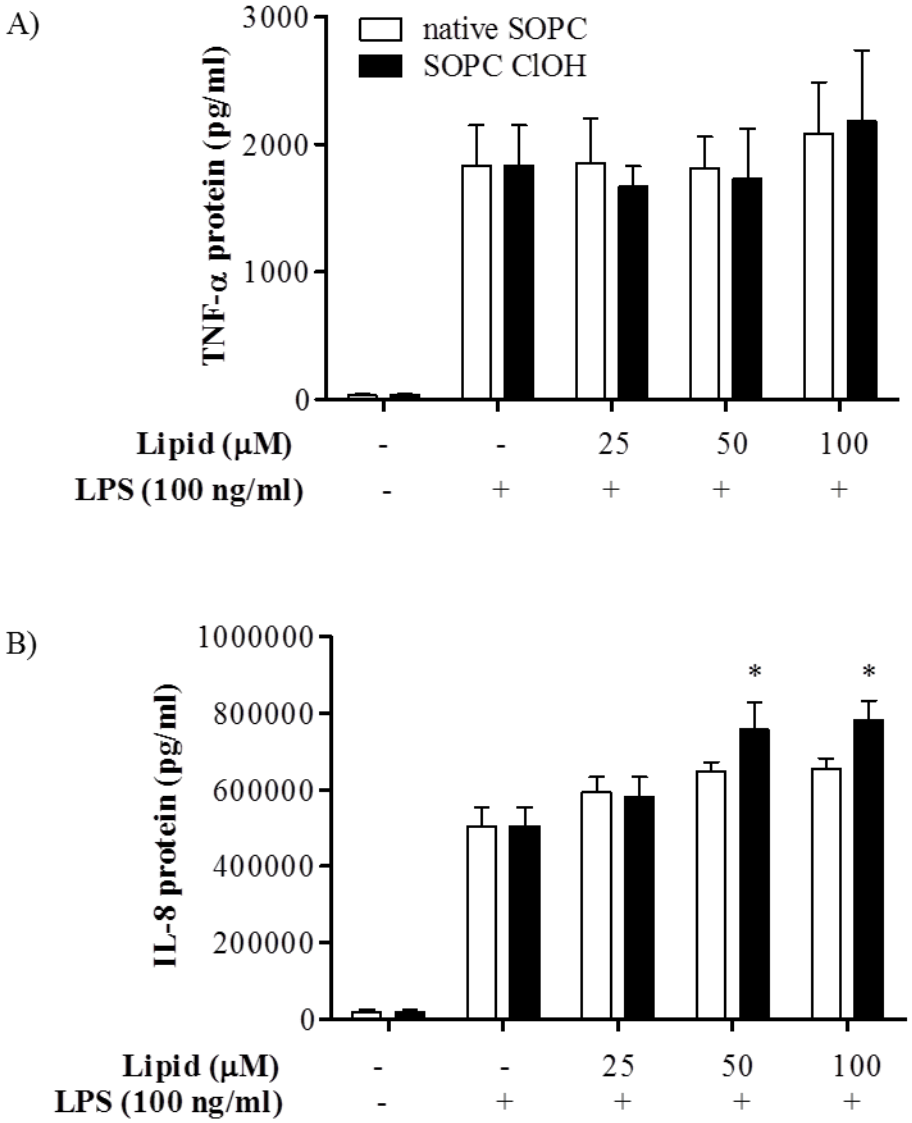


**Figure 3.8 Differential effects of native and chlorohydrin of SOPC on *S. abortus equi* LPS-induced production of TNF-α and IL-8 in U937 cells.** Cells were treated with the indicated concentrations of native SOPC or SOPC chlorohydrin together with LPS (100 ng/ml) for 24 hours. Culture medium was assessed for (A) TNF-α and (B) IL-8 production by ELISA as described in section 2.3.3. Data are expressed as mean ± S.E.M of at least three separate experiments. Significant differences compared to positive control (LPS) is shown by *, 1 symbol correspond to $p<0.05$. There was no significant different between native lipid and chlorohydrin.

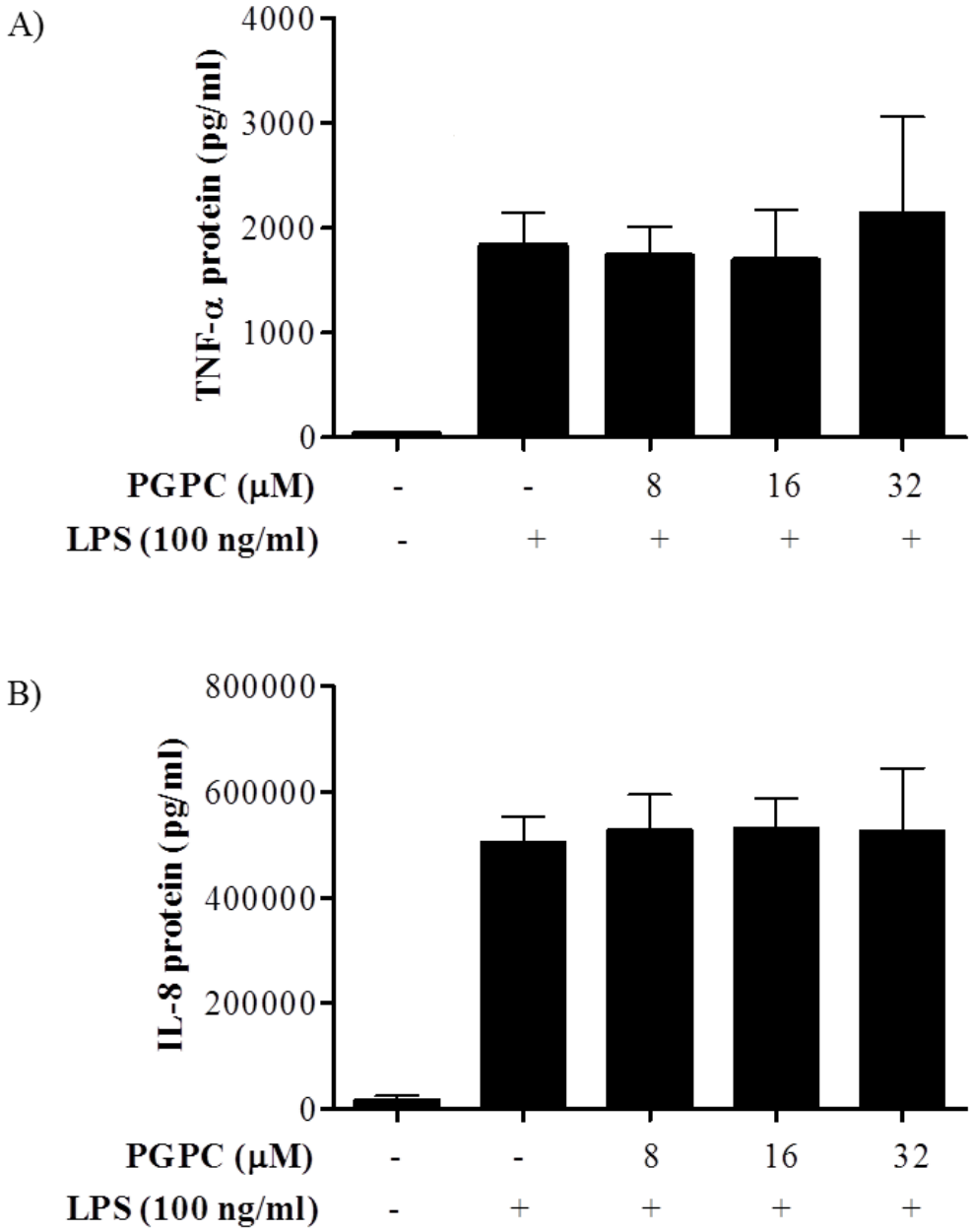


**Figure 3.9 PGPC had no effect on *S. abortus equi* LPS-induced TNF-α and IL-8 production in U937 cells.** Cells were treated with indicated concentrations of PGPC with LPS (100 ng/ml) for 24 hours. Culture medium was assessed for (A) TNF-α and (B) IL-8 production by ELISA as described in section 2.3.3. Data are expressed as mean ± S.E.M of three separate experiments.

### 3.3.5. Effect of native, chlorinated and oxidized lipids on *E.coli* LPS-mediated TNF-α and IL-8 production in U937 cells.

In addition to the experimental design above, another approach was applied to investigate to what extent the effect of SOPC ClOH on U937 cells is dependent on the exact treatment conditions. Previous work in our group (Erridge et al., 2007) has shown that when cells are treated in the absence of LPS, oxPAPC (20-30 μg/ml) increases the production of IL-8 (18 hours) but not TNF-α (4 hours). In addition, pre-treatment of cells with OxPAPC prior to stimulation with *E.coli* LPS for 18 and 4 hours inhibited LPS-mediated IL-8 and TNF-α production respectively in THP-1 cells (Erridge et al., 2007, Erridge et al., 2008). Using an experimental design adapted from this work, *E.coli* LPS was used instead of *S.abortus equi* LPS and a lower concentration of *E.coli* LPS (10 ng/ml) was applied. In the earlier experimental design, following treatment with PMA, a 2X concentration of lipid dissolved in medium without serum was mixed with an equal volume of cells in medium containing 10% (v/v) serum (therefore the final concentration of serum was 5% (v/v) ). To exclude interference of serum components on the result of the assay, in this experiment, medium containing 10% (v/v) serum was removed before cells were stimulated with serum free media supplemented with chlorinated and oxidized lipids.

The results for the effects of native and chlorohydrin of SOPC on TNF-α production in the presence or absence of LPS are shown in Figure 3.10. It is clear that treatment of U937 cells with native or chlorohydrin of SOPC for 4 hours did not induce production of TNF-α. Whilst treatment of cells with LPS (10 ng/ml) increased approximately 20 fold the induction of TNF-α, treatment with native or SOPC ClOH

did not have significant effect on although the average level of TNF-α in cells treated with the highest concentration of SOPC ClOH (25 μM) was higher than cells treated with LPS alone. Similarly, 18 hours stimulation of cells with native or chlorohydrin of SOPC alone did not induce production of IL-8, although the average level of IL-8 was higher in cells treated with 25 μM SOPC ClOH compared to control. Treatment of cells with LPS induced a 4 fold increase in IL-8 level and when pretreated with SOPC ClOH (1-25 μM), the average level of IL-8 production appeared to be higher than cells treated with LPS alone. However, the effect was not statistically significant (Figure 3.11). Higher concentrations of SOPC ClOH (50 and 100 μM) were also tested, but still no significant effects were observed (data not shown). With regards to native SOPC, it is clear that it has no effect on LPS-induced IL-8 production.

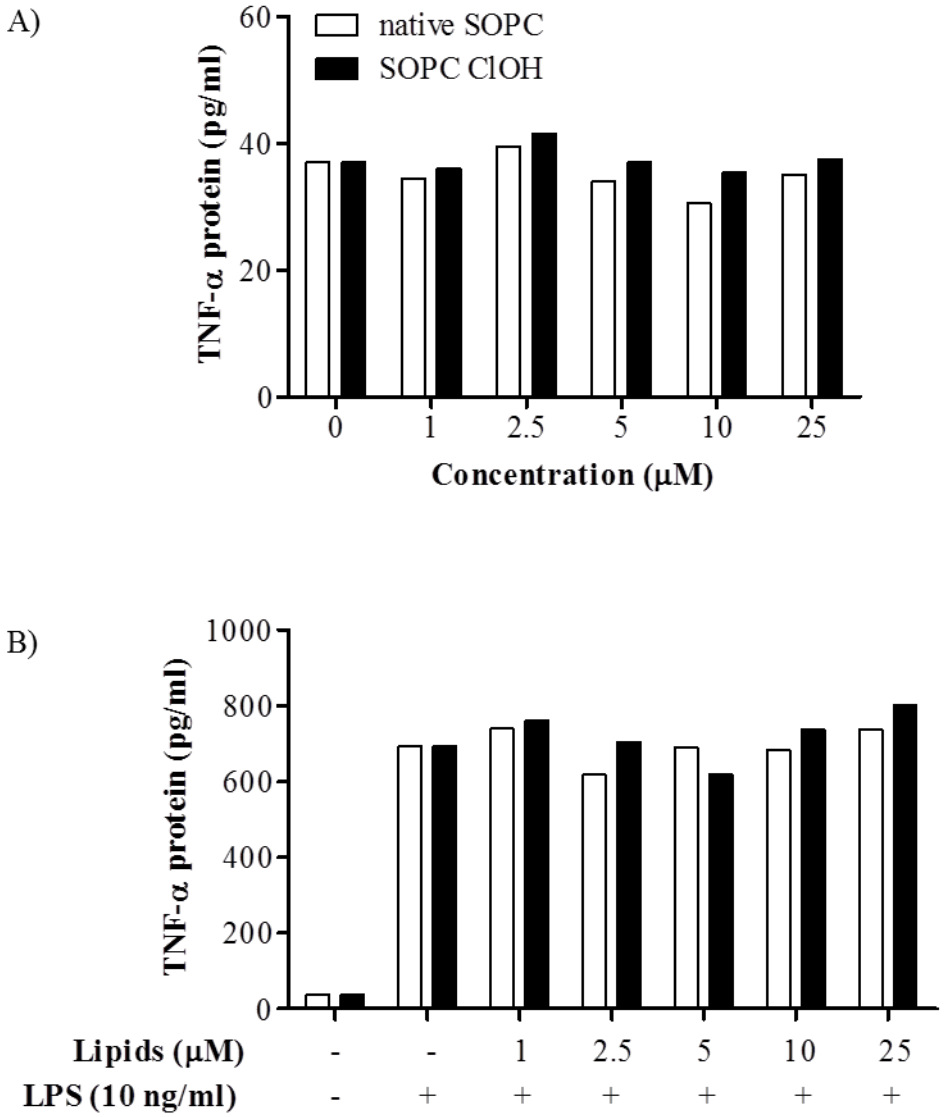


**Figure 3.10 Native SOPC or SOPC ClOH neither induced basal nor *E.coli* LPS-induced TNF-α production in U937 cells.** Cells were (A) treated with indicated concentrations of native or chlorohydrin of SOPC alone for 4 hours or (B) pretreated for 30 minutes before further stimulated with LPS (10 ng/ml) for 4 hours. Supernatant was assessed for TNF-α production by ELISA as described in section 2.3.3. Data are expressed as mean ± S.E.M of two separate experiments.

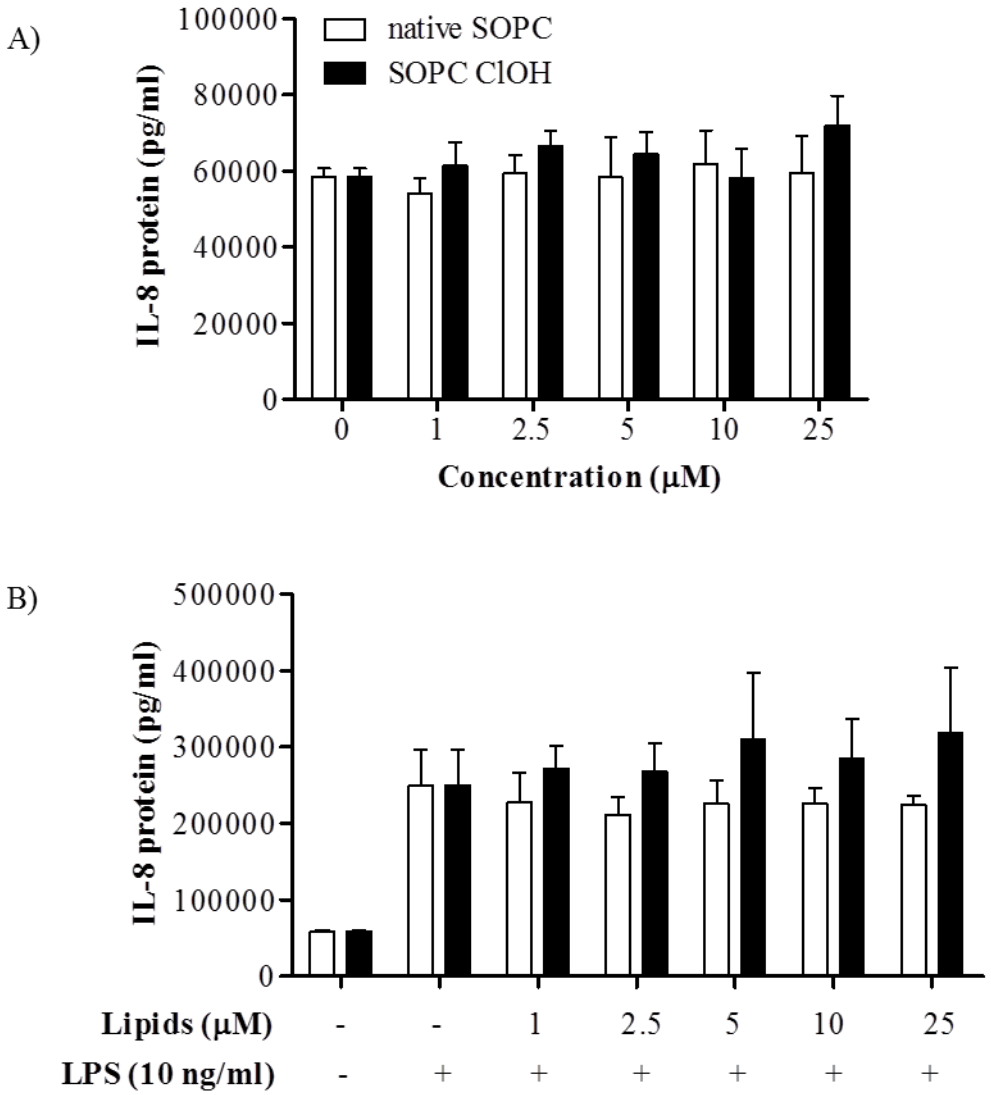


**Figure 3.11 Native SOPC or SOPC ClOH neither induced basal nor *E.coli* LPS-induced IL-8 production in U937 cells.** Cells were (A) treated with indicated concentrations of native or chlorohydrin of SOPC for 18 hours or (B) pre-treated for 30 minutes with lipids before further stimulated with LPS (10 ng/ml) for 18 hours. Supernatant was assessed for IL-8 production by ELISA as described in section 2.3.3. Data are expressed as mean ± S.E.M of three separate experiments.

The effect of the α-chloro fatty aldehyde, 2-ClHDA, a chlorinated lipid derived from reactive chlorinating attack on a vinyl ether bond of plasmenylcholine was also tested. In cells treated with 2-ClHDA (1 to 5 μM) for 4 hours, the average level of TNF-α was slightly reduced compared to control, however the level of TNF-α was increased again to a level similar to control in cells treated with 10 and 50 μM 2-ClHDA. Although the average level was slightly decreased in cells treated with the lower concentration, it was clear that 2-ClHDA did not affect basal level of TNF-α (Figure 3.12A). In cells treated with LPS, TNF-α production was markedly increased. Pre-treatment of cells with 1 μM of 2-ClHDA was already able to decrease LPS-induced TNF-α production; however the level of TNF-α was increased again at a concentration of 2.5 μM. Interestingly, pre-treatments with higher concentrations of 2-ClHDA (5, 10 and 25 μM) were shown to concentration-dependently inhibit LPS-induced TNF-α production and significant decreased was observed in cells pre-treated with 10 ($P<0.05$) and 25μM ($P<0.001$) 2-ClHDA (Figure 3.12B). A single experiment was conducted using a higher concentration of 2-ClHDA (100μM) to investigate whether it can further inhibit the effect of LPS-induced cytokine production. It was found that pretreatment with 100 μM 2-ClHDA induced only 1-fold further inhibition of LPS-induced TNF-α production compared to 25 μM 2-ClHDA (data not shown). In Figure 3.13, the results demonstrate that treatment of cells with 2-ClHDA alone did not induce production of IL-8. In the presence of LPS, the average level of cells pre-treated with 2-ClHDA was observed to be slightly higher than control; however, it is clear that 2-ClHDA did not affect LPS-induced IL-8 production.

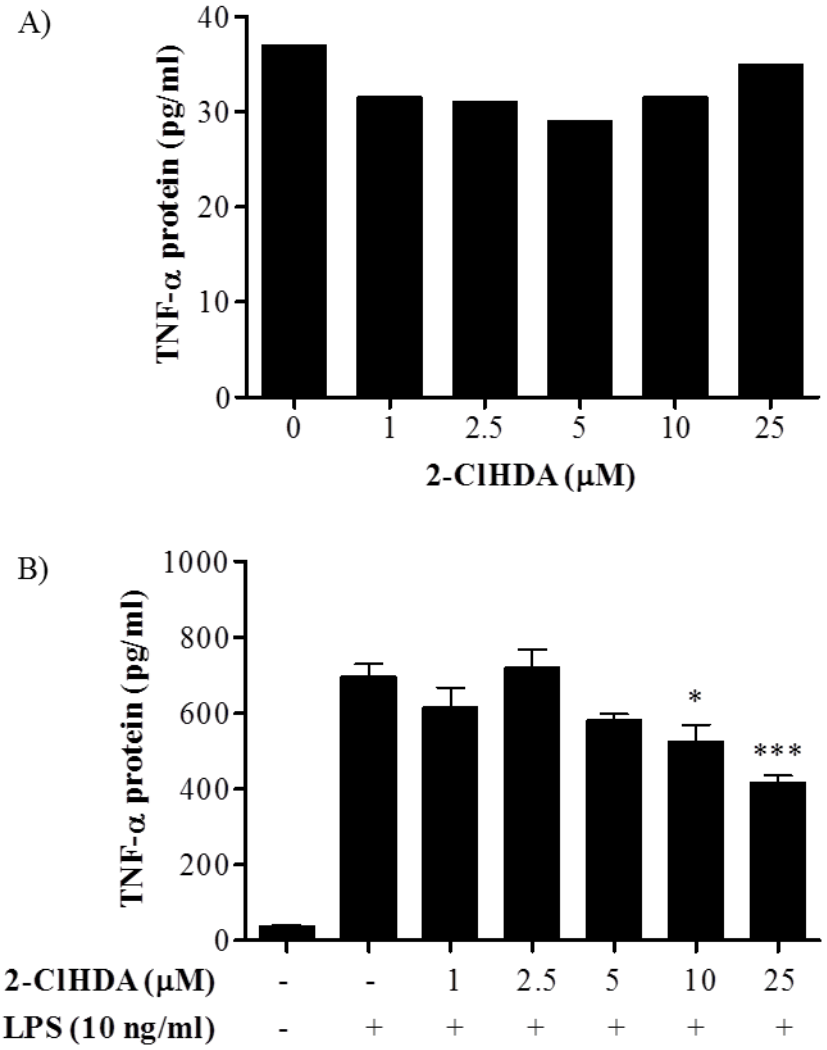


**Figure 3.12 Differential effects of 2-ClHDA on basal and *E.coli* LPS-induced TNF-α production in U937 cells.** Cells were (A) treated with indicated concentrations of 2-ClHDA for 4 hours or (B) pre-treated for 30 minutes before stimulated with LPS (10 ng/ml) for 4 hours. Culture medium was assessed for TNF-α production by ELISA as described in section 2.3.3. Data for treatment with chlorinated lipid alone are expressed as mean ± S.E.M of two separate experiments and data for treatment of compound in presence of LPS are expressed as mean ± S.E.M. of three separate experiments. Significant differences compared to positive control (LPS) is shown by *, 1 symbol correspond to $p<0.05$ and 3 symbols correspond to $p<0.001$.

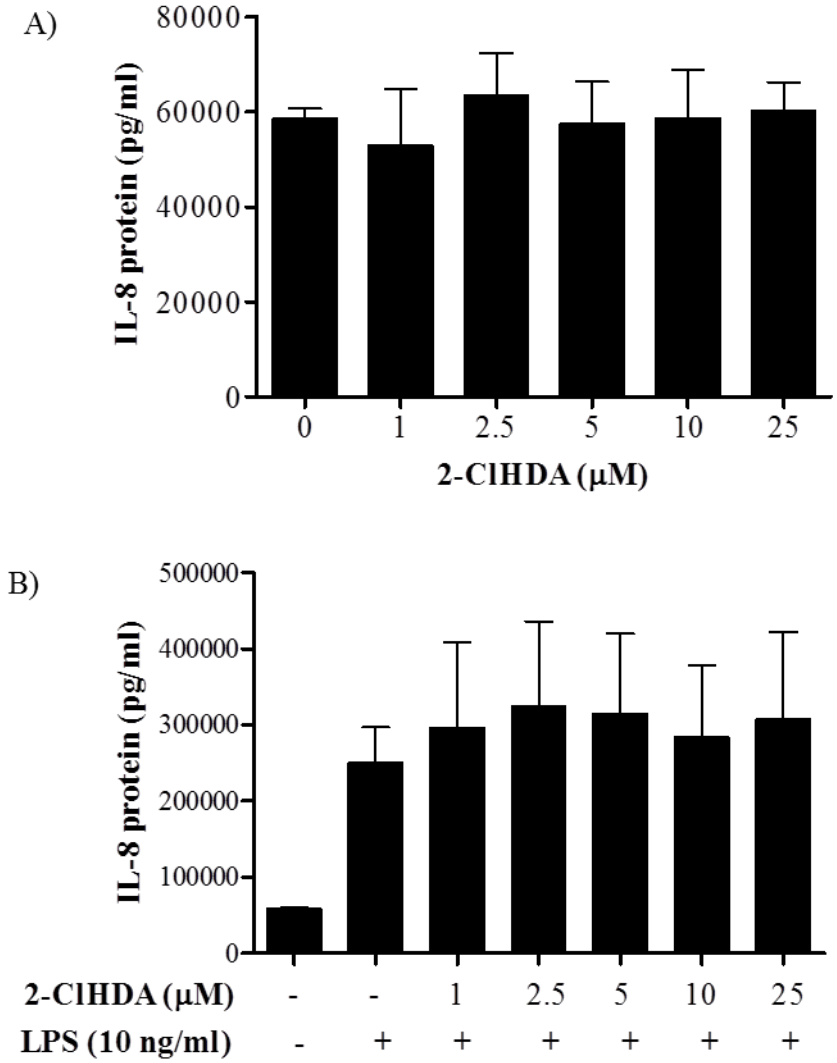


**Figure 3.13 2-ClHDA neither induced basal nor *E.coli* LPS-induced IL-8 production in U937 cells.** Cells were (A) treated with indicated concentrations of 2-ClHDA for 18 hours or (B) pre-treated for 30 minutes with this chlorinated lipid prior to further stimulation with LPS (10 ng/ml) for 18 hours. Supernatant was assessed for IL-8 production by ELISA as described in section 2.3.3. Data are expressed as mean ± S.E.M of three separate experiments.

As mentioned above, OxPAPC has been previously shown to induce IL-8 production but not TNF-α and in the presence of LPS, OxPAPC inhibit LPS-induced TNF-α and IL-8 production. Because in the earlier experiment , the effect of PGPC was not consistent with OxPAPC, therefore, in this experiment, 25 μM OxPAPC was used to compare with the effect of SOPC ClOH and 2-ClHDA. Figure 3.14A illustrates the effect of OxPAPC on production of TNF-α production by U937 cells in the presence or absence of LPS. Treatment of cells with OxPAPC alone clearly showed that OxPAPC did not induce TNF-α production. When cells were treated with LPS for 4 hours, the level of TNF-α was significantly increased. However, pre-treatment for 30 minutes with OxPAPC drastically reduced LPS-induced TNF-α level to the same level as the control.

In Figure 3.14B, the effect of OxPAPC on induction of IL-8 production is shown. 18 hours stimulation with OxPAPC for 18 hours appeared to result in a higher level of IL-8 release. However, the effect observed was not statistically significant. When compared to previous work in our group (Erridge et al., 2007), the concentration used to induce a significant increase in IL-8 production was 50 μM; therefore it is possible that with a higher concentration of OxPAPC, a significant effect could be observed. Nevertheless, the trend observed in U937 cells treated with OxPAPC in the present study was apparently similar to that observed in previous study (Erridge et al., 2007). Treatment of cells with LPS induced a significant increase in IL-8 and pretreatment with OxPAPC inhibit LPS-induced IL-8 production. Compared to OxPAPC inhibition of TNF-α, the extent of inhibition of LPS-induced IL-8 was lower.

To investigate whether individual components of OxPAPC, PGPC, may induce a similar effect to OxPAPC, the effect of PGPC was tested. Results show that basal level of TNF-α formation was reduced by PGPC and the reduction was statistically significant in cells treated with the highest concentration of PGPC (32 μM) (Figure 3.15A). In the presence of LPS, production of TNF-α was markedly increased but pre-treatment with PGPC inhibited LPS-induced TNF-α production in a concentration dependent manner (3.15B). With regards to IL-8, treatment of cells with PGPC induced a concentration dependent increase in cytokine production which was statistically significant in cells treated with the highest concentration of PGPC (32 μM) (Figure 3.16A). Paradoxically, in the presence of LPS, PGPC was found to inhibit LPS-induced TNF-α production (3.16B). The effect observed for PGPC in this experimental set up is in contrast to the earlier experiments in this chapter where no significant effect of PGPC on cytokine production was observed.

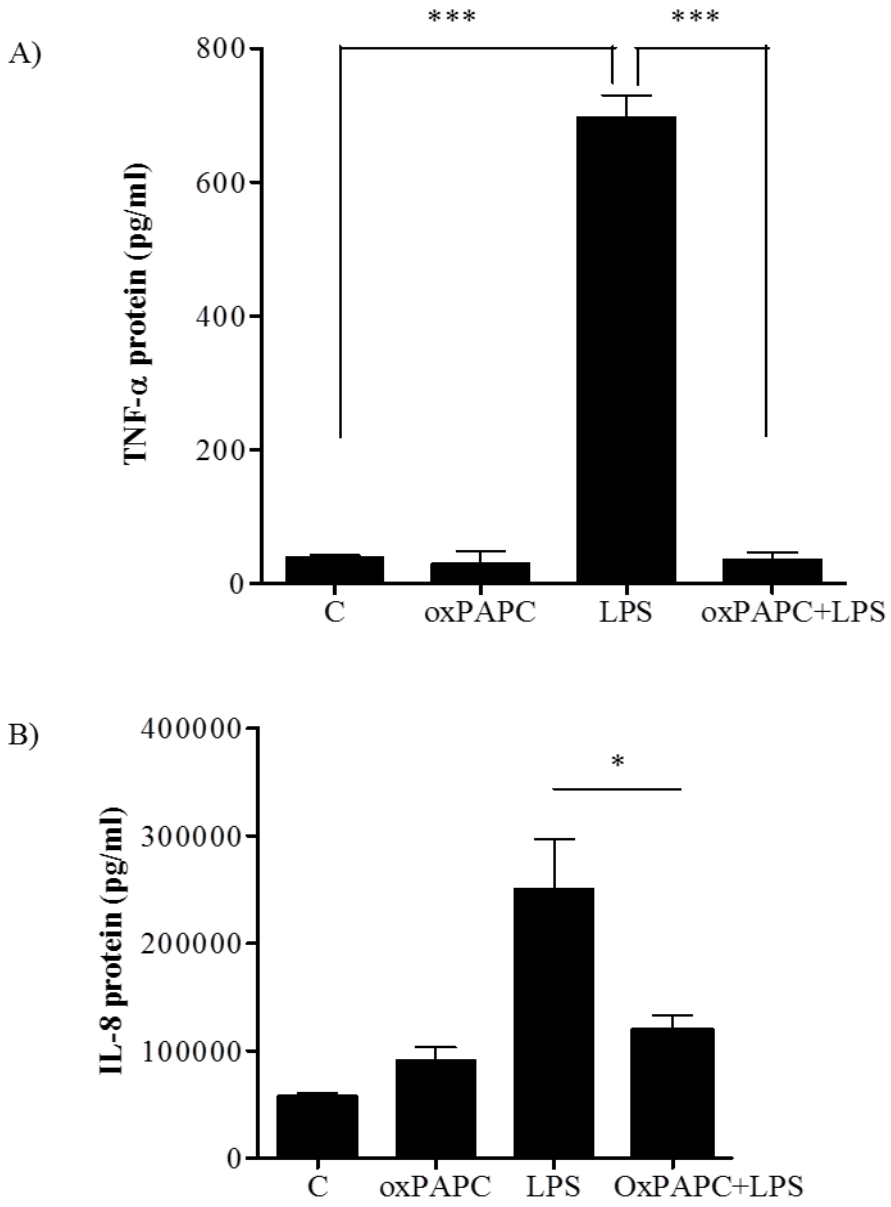


**Figure 3.14 Differential effects of OxPAPC on basal and *E.coli* LPS-induced TNF-α and IL-8 production in U937 cells.** (A) Cells were treated with oxPAPC (25μM) alone for 4 hours or pretreated with OxPAPC (25 μM) for 30 minutes and followed by stimulation with LPS (10 ng/ml) for 4 hour to assess the production of TNF-α. (B) Cells were treated with OxPAPC for 18 hours or pretreated with oxPAPC for 30 minutes before stimulation with LPS for 18 hours to assess the production of IL-8. Data are expressed as mean ± S.E.M of three separate experiments. Significant differences compared to positive control (LPS) is shown by *, 1 symbol correspond to $p<0.05$ and 3 symbols correspond to $p<0.001$.

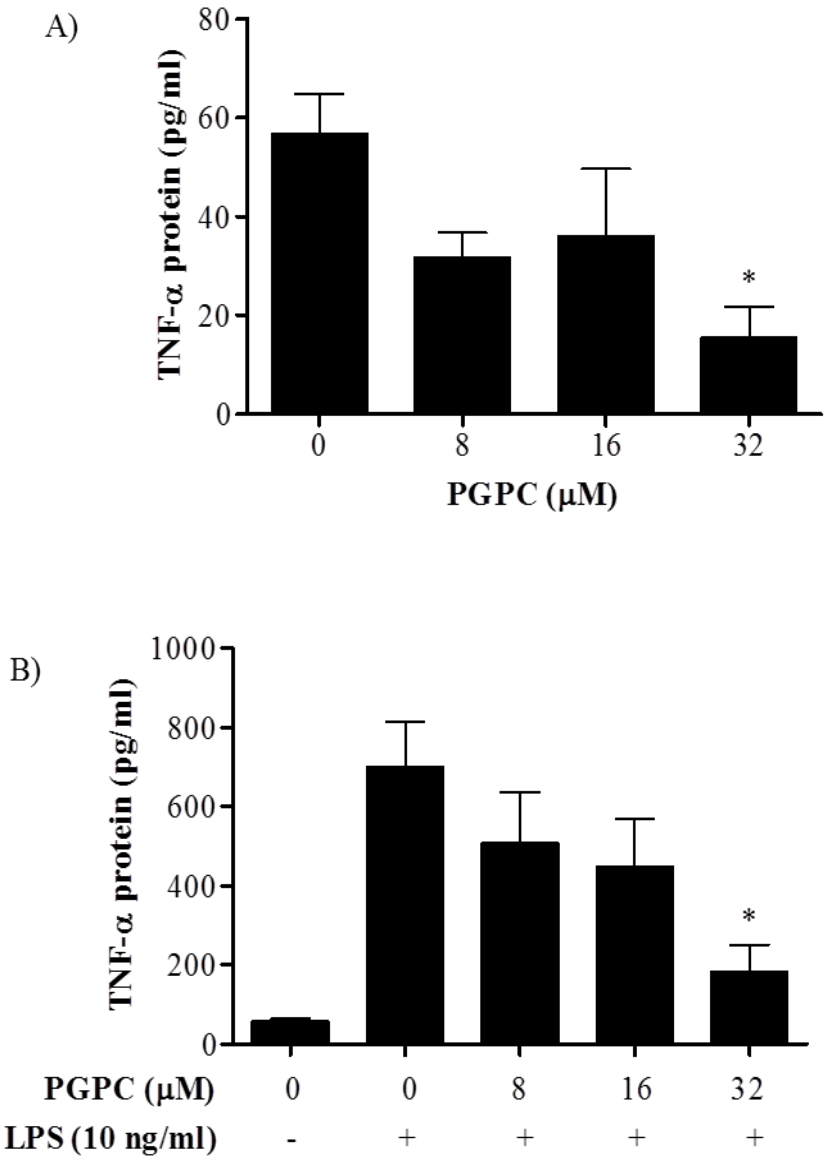


**Figure 3.15 Differential effects of PGPC on basal and *E.coli* LPS-induced TNF-α production in U937 cells.** Cells were (A) treated with indicated concentrations of PGPC for 4 hours or (B) pre-treated for 30 minutes with indicated concentrations of PGPC before further stimulated with LPS (10 ng/ml) for further 4 hours. Supernatant was assessed for TNF-α production by ELISA as described in section 2.3.3. Data are expressed as mean ± S.E.M of at least three separate experiments. Significant differences compared to positive control is shown by *, 1 symbol correspond to $p<0.05$.

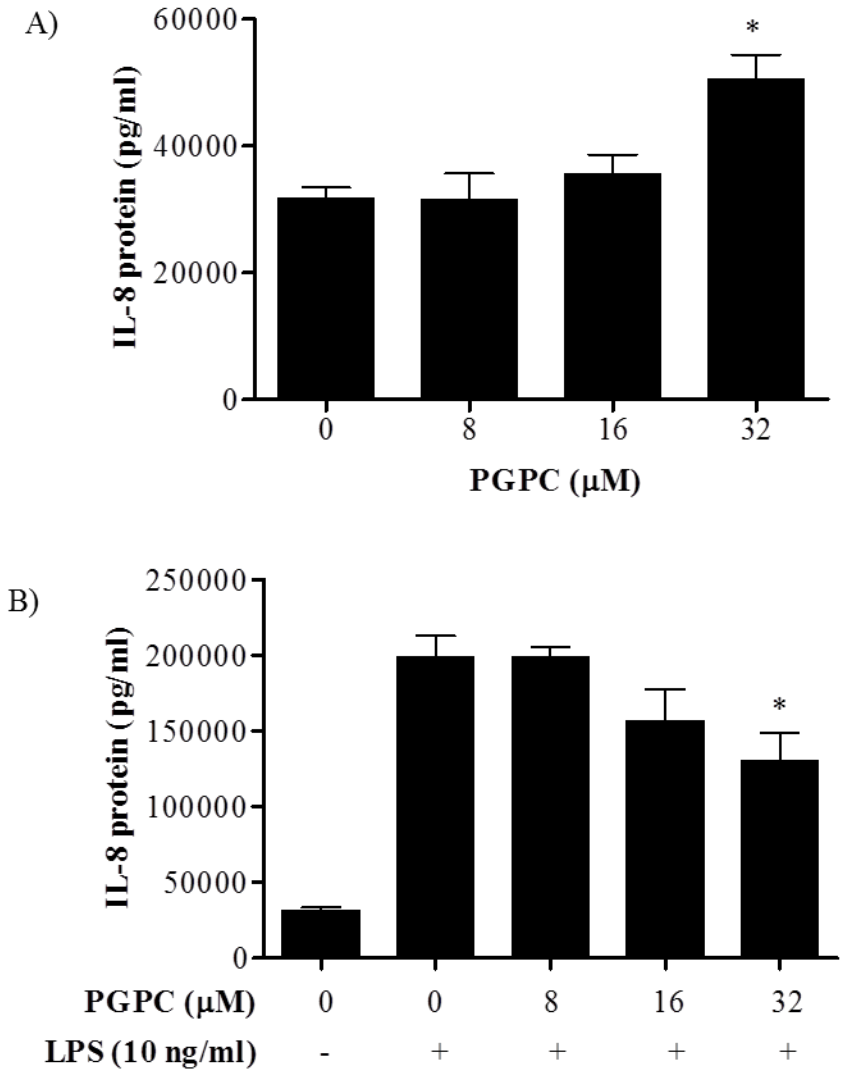


**Figure 3.16 Differential effects of PGPC on basal and *E. coli* LPS-induced IL-8 production in U937 cells.** Cells were (A) treated alone with indicated concentration of PGPC for 18 hours or (B) pretreated for 30 minutes with indicated concentrations of PGPC and followed by stimulation with LPS (10 ng/ml) for 4 hour to assess the production of IL-8 production by ELISA as described in section 2.3.3. Data are expressed as mean ± S.E.M of three separate experiments. Significant differences compared to positive control (LPS) is shown by *, 1 symbol correspond to $p<0.05$and 3 symbols correspond to $p<0.001$.

### 3.3.6. Effect of oxidized and chlorinated lipids on myeloid cell viability.

In view of the fact that some lipid treatments caused a decrease in cytokine production, it was important to investigate whether this was simply due to toxicity of the compounds to the cells. Consequently, the effect of chlorinated lipids including phospholipid chlorohydrin and α-chlorofatty aldehyde as well as oxidized phospholipids including oxPAPC and PGPC on myeloid cell viability was tested using the well-established MTT assay (Figure 3.17).

Initially, cells were differentiated with 0.1 μg/ml phorbol myristate acetate (PMA) and treated with the low and high concentration of oxidized and chlorinated lipids. Following a 24 hour treatment of U937 cells, SOPC chlorohydrin (5, 100 μM), 2-chlorohexadecanal (5, 100 μM), OxPAPC (5, 50 μM) and PGPC (8, 32 μM) did not cause any significant reduction of cell viability. There was also no reduction of cell viability in U937 cells treated with *S. abortus equi* LPS (5, 100 ng/ml). The effect of *E.coli* LPS was not tested in this assay, but it was not expected to be substantially different to *S. abortus equi* LPS.

HOCl was used as a positive control in the experiment. The results demonstrated that HOCl reduced cell viability in a concentration dependent manner and was significant at 10 mM with 80% reduction ($p<0.001$ vs control). Another positive control that can be used for loss of cell viability assay is acrolein. Interestingly, acrolein (75 μM) caused a significant decrease in viability of cells that were not treated with PMA but had no significant effect in PMA-treated U937 cells (Figure 3.18) However, with regards to SOPC ClOH, no significant effect was observed even in undifferentiated

U937 cells (data not shown). This finding suggests that PMA-activated U937 cells were more resistant than non-activated U937 cells, and also shows that the decreased cytokine levels observed with certain treatment were not simply due to loss of viability.

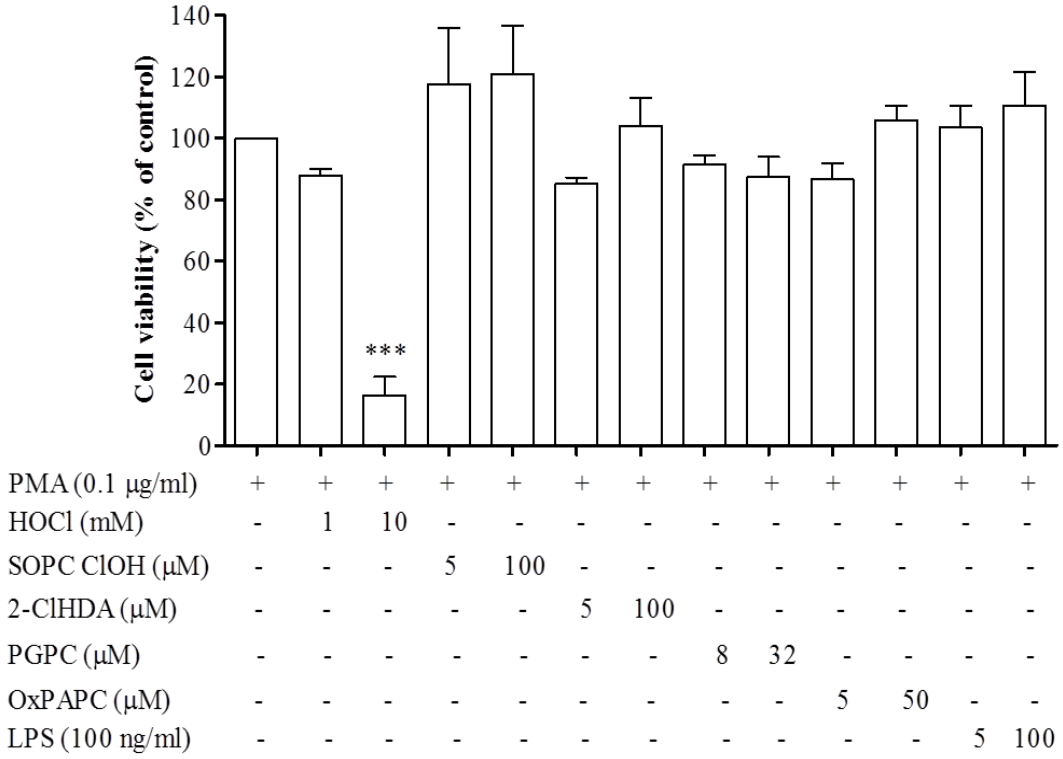


**Figure 3.17 Effects of HOCl, SOPC ClOH, 2-ClHDA, PGPC, OxPAPC and *S. abortus equi* LPS on cell viability determined by MTT assay.** U937 cells were treated with two different concentrations of hypochlorous acid, SOPC chlorohydrin, 2-chlorohexadecanal, PGPC, oxPAPC and S. abortus equi LPS for 24 hours. Cells were incubated with MTT reagent (0.5 mg/ml) for 90 minutes before centrifuging and resuspending in DMSO as outlined in section 2.4. The data was read at 540 nm and data are presented as a comparison with control cell viability. The data are average of three separate experiments ± S.E.M. Significant differences compared to control is shown by *, where 3 symbols correspond to $p<0.001$.

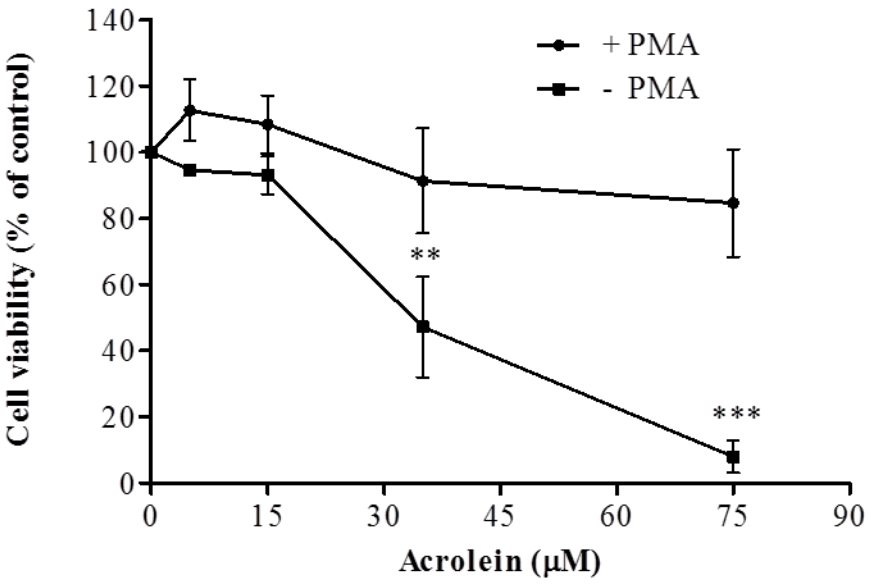


**Figure 3.18 The effect of acrolein on cell viability determined by MTT assay.** Non stimulated and PMA stimulated U937 cells were treated with different concentrations of acrolein. Cells were incubated with MTT reagent (0.5 mg/ml) for 90 minutes before centrifuging and resuspending in DMSO as outlined in section 2.4. The data was read at 540 nm and data are presented as a comparison with control cell viability. The data are average of three separate experiments ± S.E.M. Significant differences compared to control is shown by *, where 2 symbols correspond to $p<0.01$, 3 symbols correspond to $p<0.001$.

### 3.4. Discussion

It is well established that oxidative modification of LDL plays a key role in atherosclerosis (Ross, 1999). OxPLs were identified as a major active component of MM-LDL and various pro-inflammatory effects of the well-established model of OxPL, OxPAPC, have been reported. For example, OxPAPC has been shown to induce cell adhesion molecule expression (i.e, P-selectin), stimulate chemokine release (i.e., MCP-1 and IL-8) as well as induce mRNA and protein level of COX-2 (Bochkov et al., 2010). More recently, OxPAPC and its component lipids have also been implicated with the ability to induce anti-inflammatory effects, one of which is OxPAPC can inhibit the pro-inflammatory cytokine production (i.e., TNF-α ) induced by bacterial products such as LPS (Erridge et al., 2008). In comparison to OxPLs, less is known about chlorinated lipids such as SOPC ClOH and 2-ClHDA. Because some effects induced by chlorinated lipids have been found to be comparable to OxPAPC; for instance SOPC ClOH was reported to induced P-selectin expression (Dever et al., 2008) and 2-ClHDA was demonstrated to induce expression of COX-2 (Messner et al., 2008a), this study was set out to determine whether these chlorinated lipids can also regulate pro-inflammatory cytokines production similar to OxPAPC.

Prior to stimulation with chlorinated and oxidized lipids, human macrophage-like U937 cell lines were activated with PMA to model the effect of activated macrophages, which have been shown to mimic the effect of differentiated tissue macrophages present in atherosclerotic lesions, particularly in term of their ability to induce pro-inflammatory cytokine production (Hida et al., 2000). The immortal

monocytic U937 cell line was used in this study as these cells are readily available and can be grown to produce high numbers of cells, in contrast to primary myeloid cells which require frequent blood donation. A previous study has shown that in standard medium without PMA, U937 cells tend to have a regular spherical shape, however, following activation with PMA, U937 cells became adherent by forming cell clusters, extended pseudopodia and produced cytokines including IL-8 and TNF-α (Hida et al., 2000). In agreement with this previous finding, it was observed in the present study that the shape of U937 cells was more irregular and cells were adhered to the plastic surface of plates. Additionally, it has been demonstrated in the present study that in the absence of PMA, production of IL-8 and TNF-α by U937 cells was undetectable and even cells were treated with the highest concentration of LPS (100 ng/ml), the level of IL-8 and TNF-α still could not be measured (data not shown), showing that activation of macrophages is a key step for the production of cytokines. Although primary human leukocytes closely mimic the *in vivo* state and generate more physiologically relevant data, we did not use primary cells in this study due to some limitations. For instance, blood donation or tissue biopsy are required to isolate primary cells and primary cells cannot be readily expanded *ex vivo* (Daigneault et al., 2010).

Concentrations of oxidized and chlorinated lipids used in this study were not toxic to cells and this was assessed by MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay. MTT is a colorimetric assay that measures cell proliferation or when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. The principle of this assay is that when MTT enters mitochondria of

metabolically active cells (viable cells), it is reduced to insoluble purple formazan dye crystals by mitochondrial succinate dehydrogenase. Acrolein, an unsaturated aldehyde that is known to cause cell toxicity, was used as a comparison to oxidized and chlorinated lipids. Interestingly, acrolein did not cause toxic effects in PMA-activated U937 cells but caused cell death in non-activated cells. A previous study suggests that PMA induced an anti-apoptotic effect in U937 cells through activation of Cdk9 protein, which is a catalytic subunit of cellular protein kinase TAK (tat-associated kinase) that mediates viral transactivator protein Tat. Using dominant negative Cdk9 cells, they showed that percentage of viable cells was lower compared to parental U937 cells after treatment with PMA (Foskett et al., 2001). In PMA-activated cells, HOCl has been shown to induce cell toxicity and was therefore used as a positive control.

The effect of *S. abortus equi* LPS on induction of IL-8 and TNF-α protein was characterized in the present study. Stimulation of cells with LPS induced a time-dependent increase of IL-8 production. A similar trend was shown previously in different types of cells including polymorphonuclear neutrophils (Ethuin et al., 2001) and endometriotic stromal cells (Iba et al., 2004). However, the pattern of TNF-α production induced by LPS was different from IL-8, as the level of TNF-α production was maximal at 6 hours, which is consistent with the previous finding by Garrelds et al. (Garrelds et al., 1999) but decreased after 12 hours before increasing again after 24 hours stimulation. One possible explanation for this trend could be a secondary effect, in which after a certain time cells were dying, leading to decrease in TNF-α level and following cell death, various inflammatory proteins release from

the cells that can initiate more TNF-α from living cells. Although different pattern of cytokines expression observed in cells treated with LPS at different time points, treatment with different concentration of cells has been found to dose-dependently increase the production of cytokines.

The first finding in this study indicates that both SOPC ClOH and 2-ClHDA did not increase production of IL-8 when treated alone in U937 cells. Although in a reverse time course experiment SOPC ClOH has been shown to induce an increased pattern of IL-8 or TNF-α production, the effect was found to be due to the serum-free media as opposed to the chlorohydrin itself. Cells were not quiesced before treatment, as half of the media containing serum was removed and replaced with doubled concentration of treatment dissolved in serum free media to obtain the required concentration. Because of this, one possible explanation is that nutrient limitation of cells may induce autophagy that can then stimulate cytokine production (Harris, 2011); therefore, it was observed that the longer treatment of cells with serum-free media, the higher level of cytokines were released by the cells. If the cells had been quiecesed, it would be expected that there would be no increase in cytokine production.

Furthermore this study demonstrated for the first time that SOPC ClOH induced a synergistic effect when treated together with LPS, where it has been found to enhance the production of IL-8 induced by LPS. The mechanism involved in mediating this effect has not been investigated in this study. Previously, it has been suggested that SOPC ClOH may enhance the effect of PMA-mediated production of

reactive oxygen species in leukocytes through CD36 dependent mechanism (Dever et al., 2008). Another study showed that in HEK cells transfected with CD36, LPS was shown to induce production of IL-8. Because no expression of TLR4 was observed in HEK cells, this suggests that induction of IL-8 can also be mediated through CD36 pathway. Therefore, it is possible that the SOPC chlorohydrin induced further increased in LPS-induced IL-8 production through CD36 pathway. In contrast to SOPC ClOH, 2-ClHDA was found to inhibit production of LPS-induced TNF-α but not IL-8 through as yet unknown mechanism. The effect of chlorinated lipids was compared with the effect of OxPLs, OxPAPC and PGPC. The effect of SOPC ClOH and 2-ClHDA was apparently not consistent with these OxPLs where treatment of OxPLs alone induced IL-8 production, whereas treatment in cells stimulated with LPS resulted in inhibition of LPS-induced TNF-α and IL-8 production.

Some discrepancies have been observed in two different experimental approaches used in this study. With regards to the effect of SOPC ClOH, while treatment of cells together with LPS for 24 hours may induce production of LPS-mediated IL-8 production, pre-treatment of cells for 30 minutes prior to stimulation by LPS for 18 hours has been found to have no significant effect. Whether pre-treatment of cells with the chlorohydrin before stimulation with LPS versus co-treatment of cells with the chlorohydrin plus LPS might contribute to this inconsistency is not known, as the effect cannot be directly compared due to different incubation times where the latter had a longer incubation period. In contrast, PGPC was found to have no clear effect when cells were treated in medium supplemented with serum, but without serum PGPC was found to induce IL-8 production and inhibit LPS-induced IL-8 and TNF-

α. This suggests that although serum may facilitate compound delivery, as mentioned above, it is possible that serum might also block it by sequestering OxPLs.

Overall, SOPC ClOH and 2-ClHDA alone did not induce IL-8 production. In the presence of LPS, SOPC ClOH appeared to enhance LPS-induced IL-8 production, whereas 2-ClHDA may inhibit LPS-induced TNF-α production. Similarly to OxPLs, chlorinated lipids may also induce pleotropic effects; however, with regards to pro-inflammatory cytokine production investigated in this study, chlorinated lipids appear to regulate production of cytokine differently from OxPLs.

# Chapter 4

# The Role of Oxidized and Chlorinated lipids in Regulating PPAR pathway

## 4.1 Introduction

Peroxisome proliferator activated receptors (PPARs) are ligand activated transcription factors that belong to a nuclear receptor superfamily. Three isoforms termed PPARα (or NRIC1), PPARβ/δ (or NRIC2), and PPARγ (or NRIC3), have been identified. PPARα is present at high level in tissues having a high metabolic rate such as kidney, heart, muscle and liver. PPARγ is mainly expressed in adipose tissue and intestine and PPARβ/δ is predominantly expressed in brain, heart, liver, adipose tissue and small intestine. A broad spectrum of compounds can serve as PPAR ligands, including endogenous ligands such as fatty acids and oxidized fatty acids as well as synthetic ligands such as fibrates and antidiabetic glitazones (Michalik and Wahli, 2008).

PPARs play important roles in the regulation of lipid homeostasis and inflammation (Michalik et al., 2006). They are encoded by distinct single copy genes located on different chromosomes. Human PPARα is located on chromosome 22, PPARβ/δ on chromosome 6 and PPARγ on chromosome 3. Two mechanisms of action of PPARs have been described; the transactivation or PPRE-dependent mechanism and the transrepression or PPRE-independent mechanism. In the first mechanism, PPARs bind, upon heterodimerization with retinoid X receptor (RXR), to DNA sequence elements termed PPAR response elements (PPRE) that are present in the promoter region of the target gene. This process is followed by recruitment of coactivators such as histone acetyltransferase p300, cyclic AMP response element binding protein binding protein (CBP) and steroid receptor coactivator (SRC-1) that bind to the PPARγ coactivator (PGC)-1, leading to gene transcription and gene expression

(Moreno et al., 2010). A second mechanism involves interference with other signalling pathways. For example, PPARα has been shown to interfere with the NF-κB signalling pathway by binding directly to p65 NF-κB to form an inactive complex. In addition, PPARα activators have been demonstrated to induce IκBα mRNA and protein expression (Delerive et al., 2001), resulting in the retention of NF-κB in the cytosol.

OxLDL and OxPLs have been demonstrated to mediate their effects through activation of PPARα and γ (Lee et al., 2000, Taketa et al., 2008). The activation of PPARs by PPAR activators, OxLDL and OxPLs was shown to mediate a number of anti-inflammatory effects studied in either cells in culture or in mice knockout models. For example, Babaev et al. demonstrated that $LDL^{-/-}$ mice reconstituted with bone marrow from $PPAR\alpha^{-/-}$ mice and fed on a high fat diet, developed larger atherosclerotic lesions compared to $LDL^{-/-}$ mice reconstituted with bone marrow from $PPAR\alpha^{+/+}$ animals, (Babaev et al., 2007). The same study demonstrated that macrophages from PPARα-deficient mice had; enhanced OxLDL uptake, reduced scavenger receptor class B1 (SR-B1) mRNA expression and protein level, reduced acetyl-coenzyme A acetyltransferase 1 (ACAT1), an enzyme involved in cholesterol efflux, and increased LPS-mediated inflammatory gene activation (Babaev et al., 2007).

In addition, Taketa and coworkers reported that OxLDL can activate PPARα and PPARγ pathways mediated induction of ATP-binding cassette transporter A1 (ABCA1) mRNA expression and inhibition of monocyte chemoattractant protein-1

(MCP-1) mRNA expression, by increase in intracellular level of 15-deoxy-delta-12,14-prostaglandin J2 (15d-$PGJ_2$) that is known as a ligand for PPARγ, through ERK1/2 MAPK-dependent COX2 expression (Taketa et al., 2008). Firstly, this group showed that OxLDL induced overexpression of COX-2 (Taketa et al., 2008), an enzyme that is known to catalyse the conversion of intracellular fatty acids to prostanoids including prostaglandin (PGD2, PGE2, PGF2α), prostacyclin (PGI2) and thromboxane A2 (O'Banion M.K.). Treatment of macrophage cells with ERK1/2-specific inhibitor was shown to decrease the level of COX-2 mRNA expression. This group then showed that treatment with OxLDL resulted in production of several prostaglandins such as prostaglandin E2 ($PGE_2$) and prostaglandin D2 ($PGD_2$) as well as 15-deoxy-delta-12,14-prostaglandin J2 (15d-$PGJ_2$). When cells were overexpressed with dormant negative-ERK or transfected with COX-2 siRNA, the level of OxLDL-induced 15d-$PGJ_2$ was reduced (Taketa et al., 2008). Finally, it was shown that PPARα and PPARγ siRNA inhibit OxLDL-induced ABCA1 mRNA expression and stimulate OxLDL-induced MCP-1 mRNA expression, suggesting that PPARs have anti-inflammatory properties. In another study, Jiang et al reported that activation of PPARγ by 15d-$PGJ_2$ was shown to inhibit PMA-induced TNF-α, interleukin-1β ( IL-1β) and IL-6 production in human monocytes (Jiang et al., 1998). Taken together, these findings suggest that activation of PPARα and γ has the potential to reduce the risk of atherosclerosis.

Nevertheless a number of studies have shown pro-inflammatory effects. OxPLs including OxPAPC, PGPC, POVPC and the synthetic ligand for PPARα, WY14643, were demonstrated to induce IL-8 and MCP-1 protein synthesis in endothelial cells

(Lee et al., 2000). The same group also showed, by using endothelial cells derived from PPARα knockout mice, that PPARα is crucial for induction of MCP-1/JE by MM-LDL and OxPAPC (Lee et al., 2000). Furthermore, OxLDL was found to induce CD36 expression on macrophages through activation of PPARγ (Nagy et al., 1998). Moreover, an oxidized alkyl phosphatidylcholine present in OxLDL, hexadecyl azelaoyl phosphatidylcholinne (azPC), was found to be capable of binding to PPARγ and resulting in increased CD36 expression (Davies et al., 2001). Figure 4.1 depicts the above-described mechanisms.

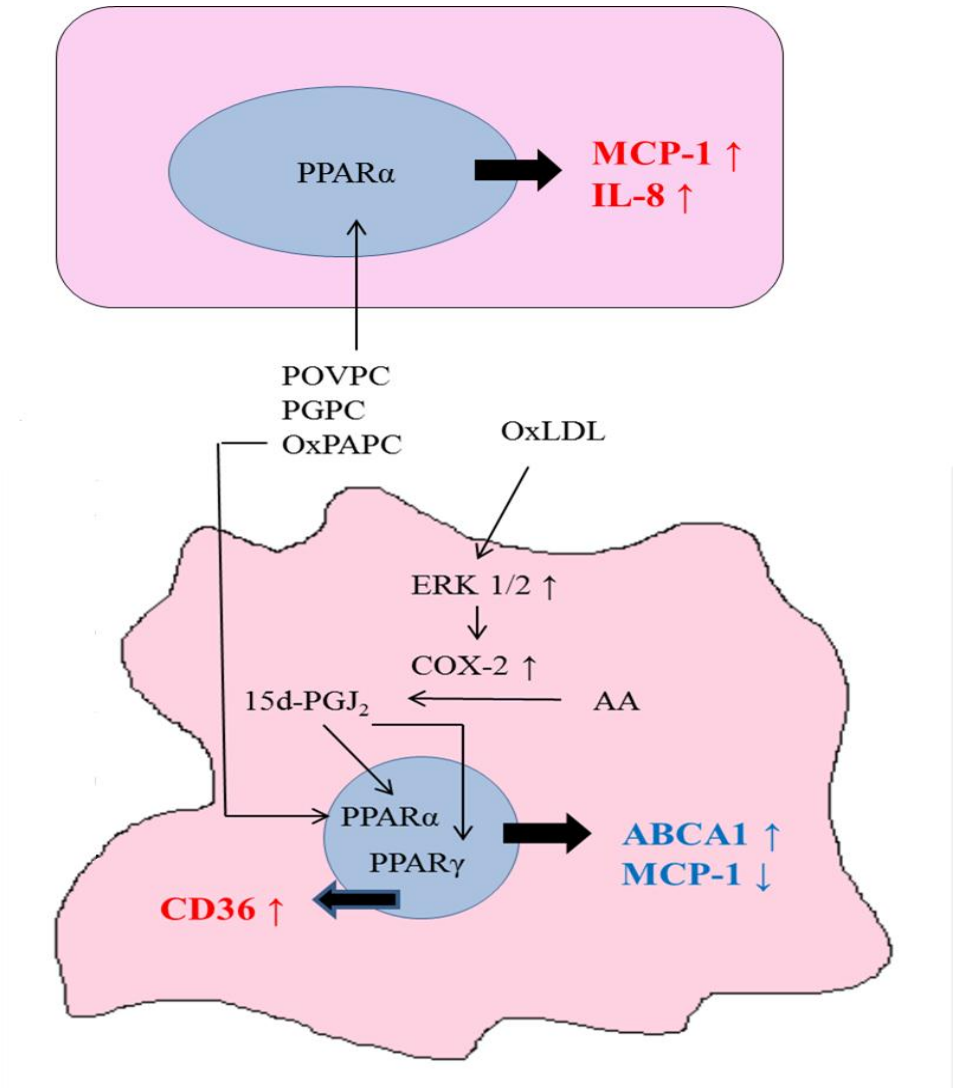


**Figure 4.1 Schematic diagram depicting the role for PPARα and PPARγ in OxLDL-induced pro- and anti-inflammatory effects by endothelial cells and macrophages.** In endothelial cells, PPARα can be activated by OxPAPC, PGPC and POVPC, leading to production of chemokines. OxLDL treated in macrophages, can activate PPARγ, leading to induced CD36 expression and a component of OxLDL such as OxPAPC can activate PPARα. OxLDL can also activate PPARα and γ through ERK-dependent COX-2 expression in macrophage. COX-2 catalyses the formation of intracellular 15d-$PGJ_2$ from arachidonic acid (AA), activating PPARs and ultimately leading to increased expression of ABCA1 and decreased expression of MCP-1 mRNA. Responses regarded as pro-inflammatory are shown in red and anti-inflammatory ones are in blue. Adapted from Lee et al., 2000 and Taketa et al., 2008.

The role of PPARβ in inflammation is currently unclear. However, PPARβ activators were shown to induce some therapeutic effects. A study carried out *in vivo* by Barish et al., demonstrated that treatment of apoE knockout mice with an orally active PPARβ agonist reduced atherosclerotic lesion size. Moreover, they showed that in macrophages derived from wild type mice, but not PPARβ-deficiency mice, PMA, IL-1β and interferon-γ (IFN-γ)- induced MCP-1 expression was inhibited by a PPARβ agonist (Barish et al., 2008), suggesting a receptor-dependent mechanism.

Oxidation of LDL and phospholipids by the enzyme MPO and HOCl can generate HOCl-modified LDL and various chlorinated lipid products. These chlorinated molecules can induce a variety of pro-inflammatory effects, comparable to those of OxPLs; however, the knowledge of signalling mechanisms involved in mediating their effects is still limited. Due to the lipid nature of chlorinated lipids and comparable effects to OxPLs, it is possible that chlorinated lipids could also induce activation of PPARs. Specifically, HOCl-LDL was shown to mediate its uptake by macrophages through CD36 and SR-B1. These effects were shown, by a binding study of HOCl-LDL protein versus lipid moiety in chinese hamster ovary cell (CHO), to be mediated by the protein moiety of HOCl-LDL (Marsche et al., 2003). However, later on chlorinated phospholipid was shown to enhance PMA-induced ROS through a CD36-dependent mechanism (Dever et al., 2008), suggesting that lipid components could also be the active component of HOCl-LDL.

The above data highlights the potential for the family of PPARs to mediate the effects of OxPLs. Far fewer studies have examined a similar possibility for

chlorinated lipids. Therefore, the aim of this study was to investigate whether oxidized and chlorinated lipids can induce activation of PPARα, β and γ resulting in transactivation of PPRE and transrepression of NF-κB-dependent IL-6 promoter activity. This was performed using reporter assays in cells transfected with different PPAR constructs.

## 4.2 Methodology

Native, oxidized lipids and chlorinated lipids were obtained from Avanti Polar Lipid (USA) or obtained from Professor Andrew Pitt (Aston University) as described in section 2.1. Chlorinated lipids were prepared and analysed as describe in section 2.2.1 and 2.2.2. DNA constructs including PPRE, empty vector, an expression vector for β-galactosidase and PPARα, β and γ were obtained from Professor G Haegeman (University of Ghent, Belgium) as described in section 2.1. Growth and subculturing of mammalian cells was carried out as described in section 2.5.1. DNA (1 μg/μl; 10 μl) was expanded using chemically competent cells and plasmid DNA purification was done using an endotoxin free plasmid maxi kit, as described in 2.5.4. Transfection with $CaPO_4$ or PEI was described in section 2.5.3 and 2.5.5. Luciferase assays for NF-κB-driven and PPRE-driven luciferase activity were carried out as described in section 2.5.6.

## 4.3 Results

### 4.3.1 Effects of dexamethasone on basal and TNFα-stimulated NF-κB dependent promoter activity in L929sA cells.

Dexamethasone (DEX) is a synthetic ligand for the glucocorticoid receptors-α (GR-α). Activated GR has previously been shown to interfere with the NF-κB pathway (Beck et al., 2009). In this study, DEX was used as a positive control and tested in parallel with chlorinated and oxidized lipids treatment. Figure 4.2 shows that TNF-α alone stimulated an appproximate 30 fold increased in NF-κB reporter activity in L929 sA cells. Whilst DEX had no effect on basal levels, it substantially reduced the TNF-α response by greater than 90%. This results suggest that L929sA cells, stably transfected with this reporter gene, represent an appropriate cell model to test the effect of chlorinated and oxidized lipids on the activity of NF-κB.

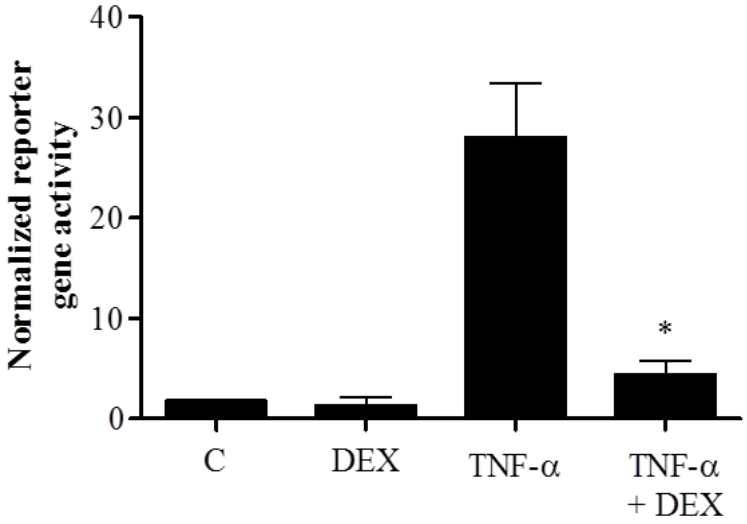


**Figure 4.2 Dexamethasone inhibited TNFα-induced NF-κB-driven gene expression in L929sA cells.** L929sA cells, stably transfected with p(IL6κB)$_3$50hu.IL6P-luc+, were incubated with DEX (1 μM) alone or in combination with TNF-α (2000 units/ml) for 7 hours. Supernatants were removed and cells were harvested and assayed for luciferase activity. Data for luciferase was normalized to galactosidase and presented as percentage of luciferase/galactosidase. Data are representative of three independent experiments and presented as mean ± S.D of 6 replicates within one plate. Significant differences compared to control is shown by *, 1 symbol corresponds to $p<0.05$.

### 4.3.2 Effects of chlorinated lipids on basal and TNFα-stimulated NF-κB dependent promoter activity in L929sA cells.

This study was conducted to investigate whether chlorinated and oxidized lipids induce anti-inflammatory effects through interference with the activity of NF-κB. In figure 4.3, native SOPC was shown to inhibit the basal activity by almost 50% at 100 μM. However, the result was inconclusive due to the fact that it had to be based on a single experiment. Whilst stimulation with TNF-α resulted in a 2.5 fold increased in NF-κB-Luc activity, pre-treatment with native SOPC seems to have no significant effect.

In figure 4.4, the effect of SOPC ClOH on NF-κB reporter activity was also examined. Treatment of cells with SOPC ClOH cause a biphasic effect, 50 μM of this chlorinated lipid resulted in increased level of luciferase activity whereas treatment with a higher concentration (100 μM) resulted in reduced basal activity. Statistical analysis cannot be carried out because the result was an average of two independent experiments. In addition, it was found that SOPC ClOH did not affect NF-κB-Luc reporter activity in response to TNF-α. In figure 4.5, 2-ClHDA appeared to reduce the basal NF-κB activity in a concentration dependent manner; however, due to large variability in the analysis, the effect was not significant. There was also no significant effect of 2-ClHDA on TNFα-induced NF-κB-driven gene expression.

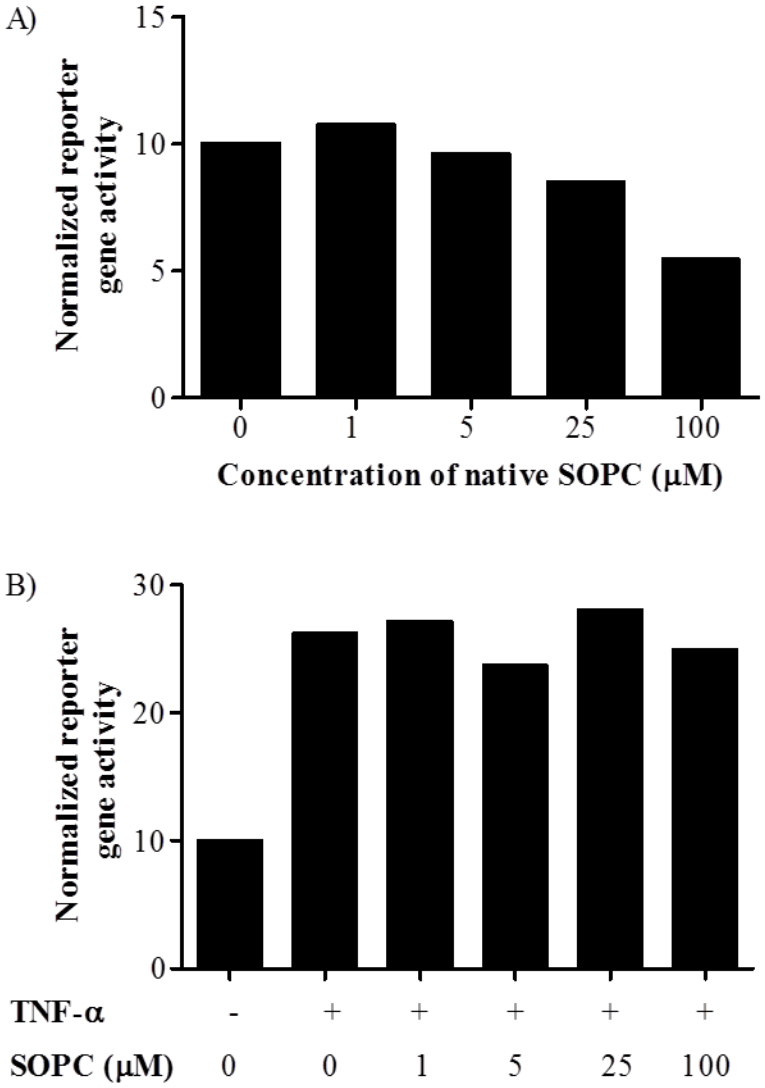


**Figure 4.3 Effects of native SOPC on basal and TNFα-induced activation of NF-κB driven gene expression in L929sA cells.** L929sA cells, stably transfected with p(IL6κB)$_3$50hu.IL6P-luc+, were (A) incubated with medium or various concentrations of native SOPC for 7 hours or (B) preincubated for an hour with native SOPC, before stimulated with TNF-α (2000 units/ml) for further 6 hours Supernatants were removed and cells were harvested and analysed by luciferase assay. Data for luciferase was normalized to galactosidase and presented as percentage of luciferase/galactosidase. Data were obtained from a single experiment.

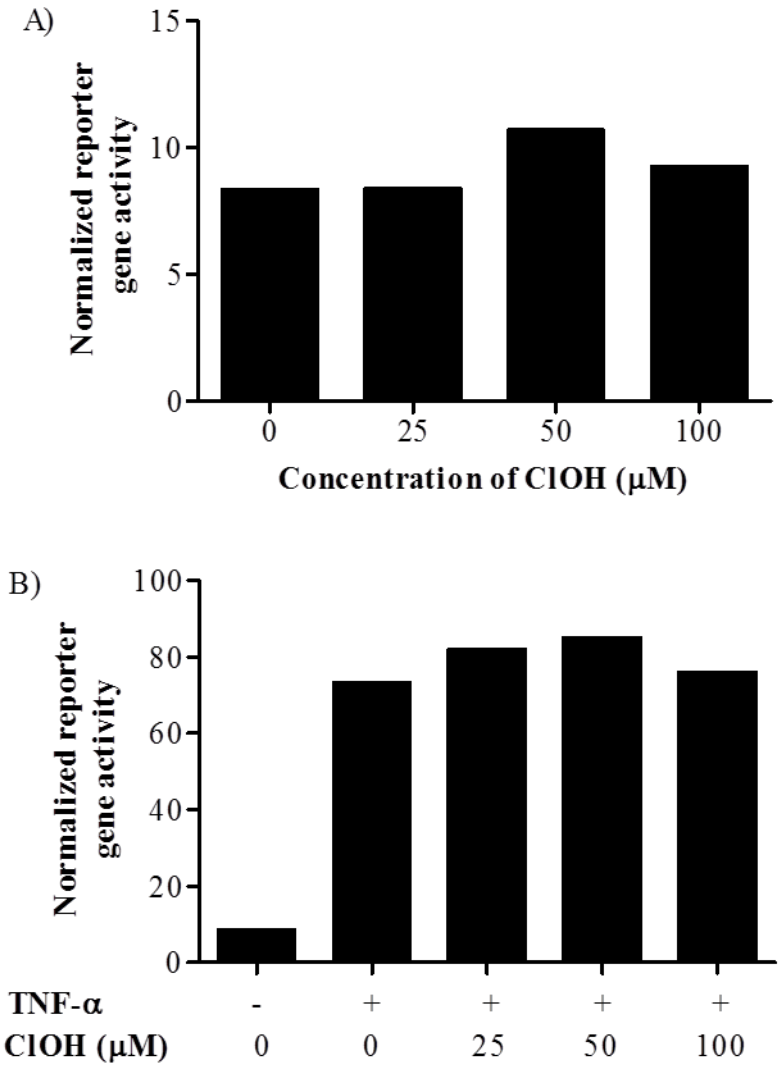


**Figure 4.4 Effects of SOPC ClOH on basal and TNFα-induced NF-κB-driven gene expression in L929sA cells.** L929sA cells that were stably transfected with p(IL6κB)$_3$50hu.IL6P-luc+, were (A) treated with medium or various concentrations of SOPC ClOH for 7 hous or (B) pre-treated for an hour with SOPC ClOH before addition of TNF-α (2000 units/ml) for 6 hours. Supernatants were discarded and cells were harvested and the luciferase assay was performed. Data for luciferase was normalized to galactosidase and presented as percentage of luciferase/galactosidase. The results are mean of two independent experiments.

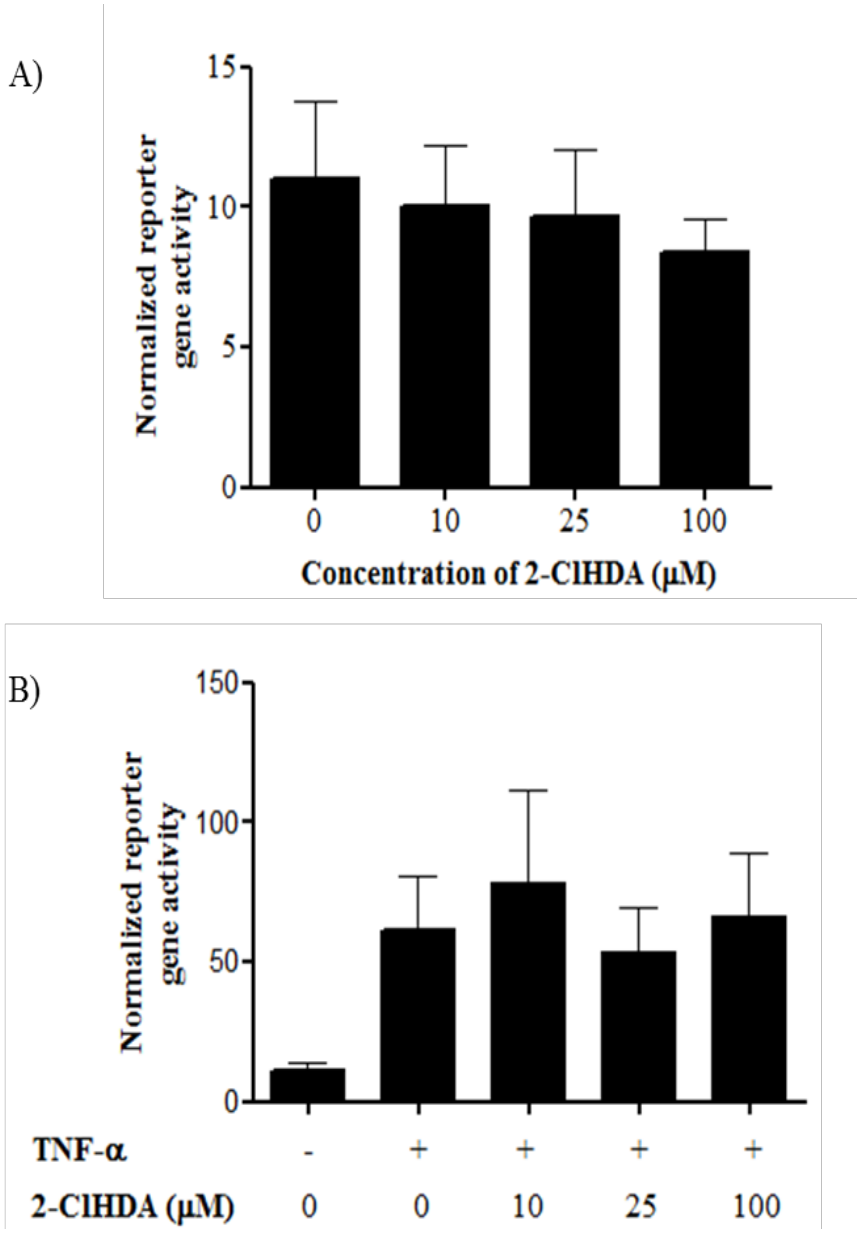


**Figure 4.5 Effect of 2-ClHDA on basal and TNFα-induced NF-κB-driven gene expression in L929sA cells.** L929sA cells with stably integrated p(IL6κB)$_3$50hu.IL6P-luc+ were (A) treated for 7 hours with medium or various concentrations of 2-ClHDA or (B) pre-treated for one hour with 2-ClHDA prior to TNF-α (2000 units/ml) stimulation for 6 hours. Samples were collected and assayed for luciferase activity. Data for luciferase is normalized to galactosidase and presented as percentage of luciferase/galactosidase. The results are mean ± S.E.M of at least three independent experiments

### 4.3.3 Effects of OxPLs on basal and TNFα-stimulated NF-κB dependent promoter activity in L929sA cells.

It has also been demonstrated that PPARα ligand such as WY14643 can inhibit TNFα-induced NF-κB-luc activity (Bougarne et al., 2009). Therefore in this study, the effects of OxPLs on the basal and TNFα-induced NF-κB-driven gene expression were investigated as a comparison to the effects of chlorinated lipids. This is shown in Figure 4.6. Surprisingly, whilst TNF-α stimulated NF-κB reporter activity, pre-treatment of L929sA cells with OxPAPC over a micromolar concentration range did not significantly affect either basal or TNFα-stimulated NF-κB driven luciferase activity. Only at the highest concetration used in this experiment was reporter activity slightly increased compared to control (Figure 4.6A). This observation was in agreement with previous finding that OxPAPC did not inhibit TNFα-mediated signalling at intracellular level, instead the inhibitory effect is exclusive for LPS-mediated signalling (Bochkov et al., 2002a).

In contrast to a OxPAPC mixture, its individual component PGPC (Figure 4.7A) and POVPC (Figure 4.8A) were demonstrated to increase basal luciferase activity at concentration 25 μM but this effect was reduced at a concentration of 100 μM. It has also been demonstrated that while PGPC (Figure 4.7B) could slighly inhibit the effect of TNF-α, POVPC at concentration 5 and 25 μM has been shown to enhance the effect of TNF-α (Figure 4.8B). Unfortunately, data generated with these short chain oxidized phosphatidylcholines are inconclusive, due to the fact that analyses had to be based on single experiments. More work is needed and repetitions are

essential to be able to draw solid conclusions about the effect of short chain oxidized phosphatidylcholines on the NF-κB pathway.

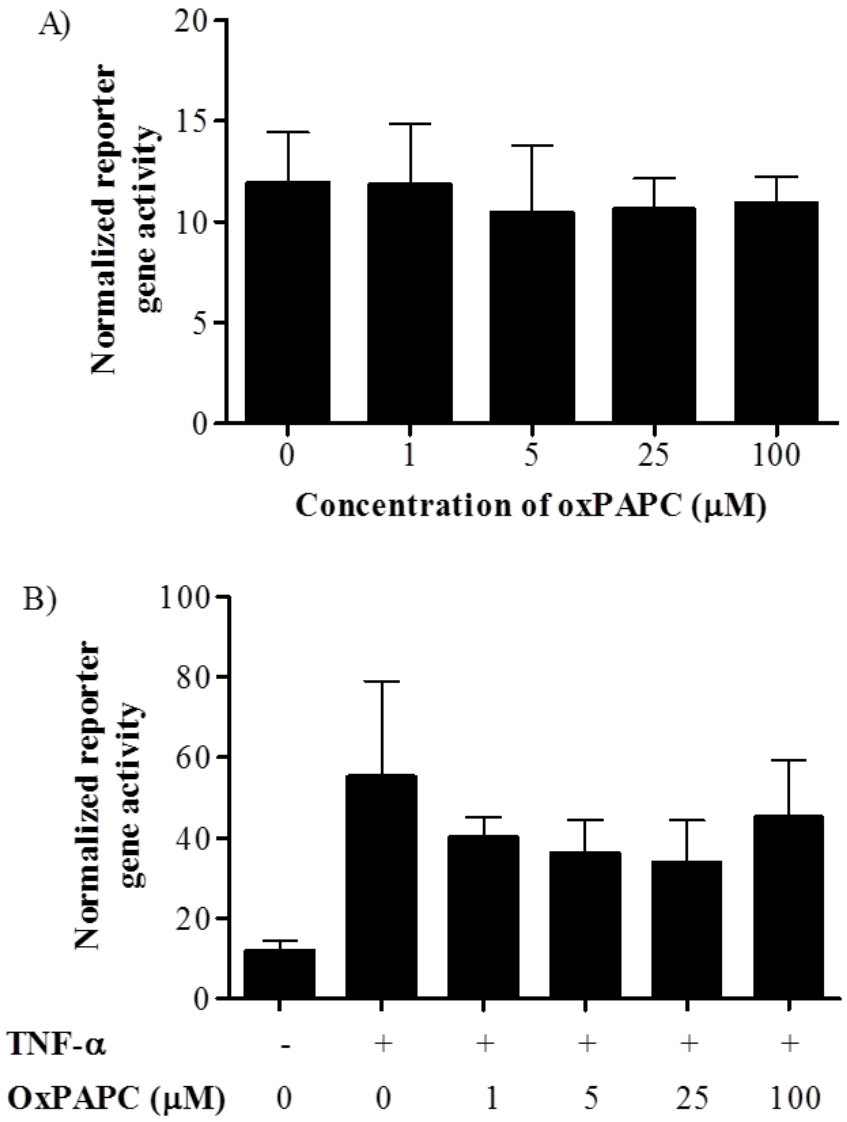


**Figure 4.6 Effect of OxPAPC on basal level and TNF-α-induced NF-κB-driven gene expression in L929sA cells.** L929sA cells transfected with p(IL6κB)$_3$50hu.IL6P-luc+ were (A) stimulated with various concentrations of OxPAPC for 7 hours or B) pre-treated for an hour prior to stimulation with TNF-α (2000 units/ml) for 6 hours. Cells were collected and used for luciferase assay. Data for luciferase is normalized to galactosidase and presented as percentage of luciferase /galactosidase. The results are mean ± S.E.M of at least three independent experiments.

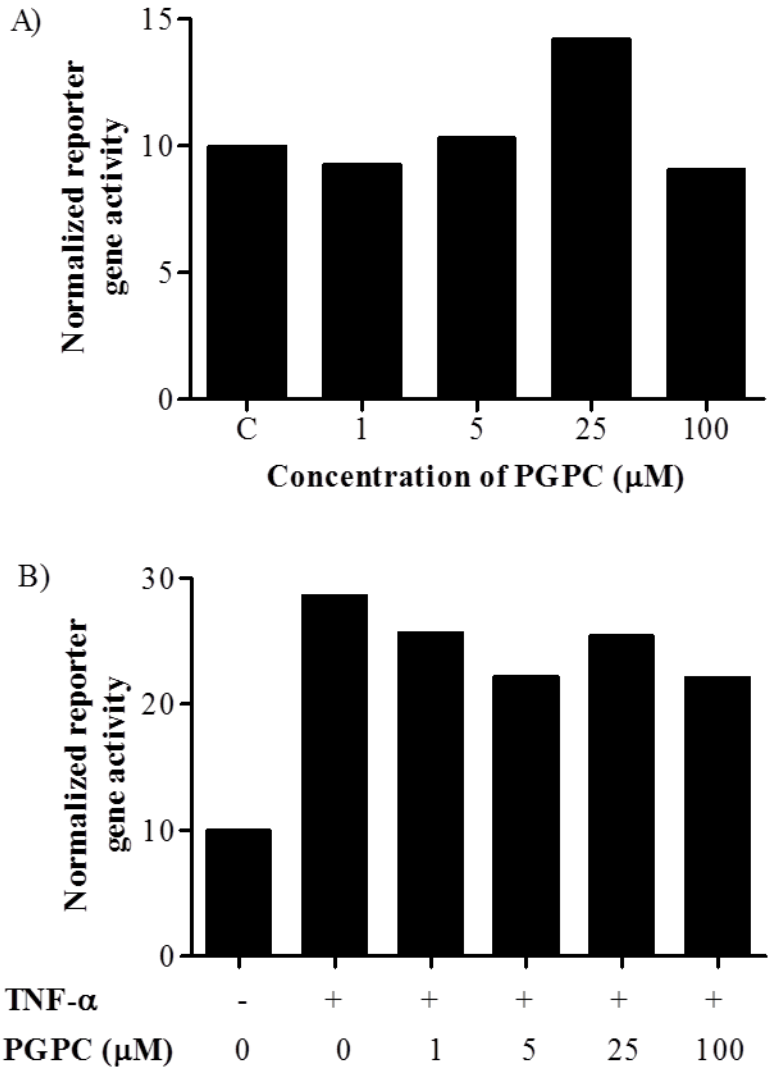


**Figure 4.7 Effect of PGPC on basal and TNFα-induced NF-κB-driven gene expression in L929sA cells.** L929sA cells with stably integrated p(IL6κB)$_3$50hu.IL6P-luc+ were (A) stimulated for 7 hours with various concentrations of PGPC or (B) pretreated for an hour with PGPC before further stimulated with TNF-α (2000 units/ml) for 6 hours. Cell lysates were prepared and were used for luciferase assay. Data for luciferase is normalized to galactosidase and presented as percentage of luciferase /galactosidase. Data was collected from a single experiment.

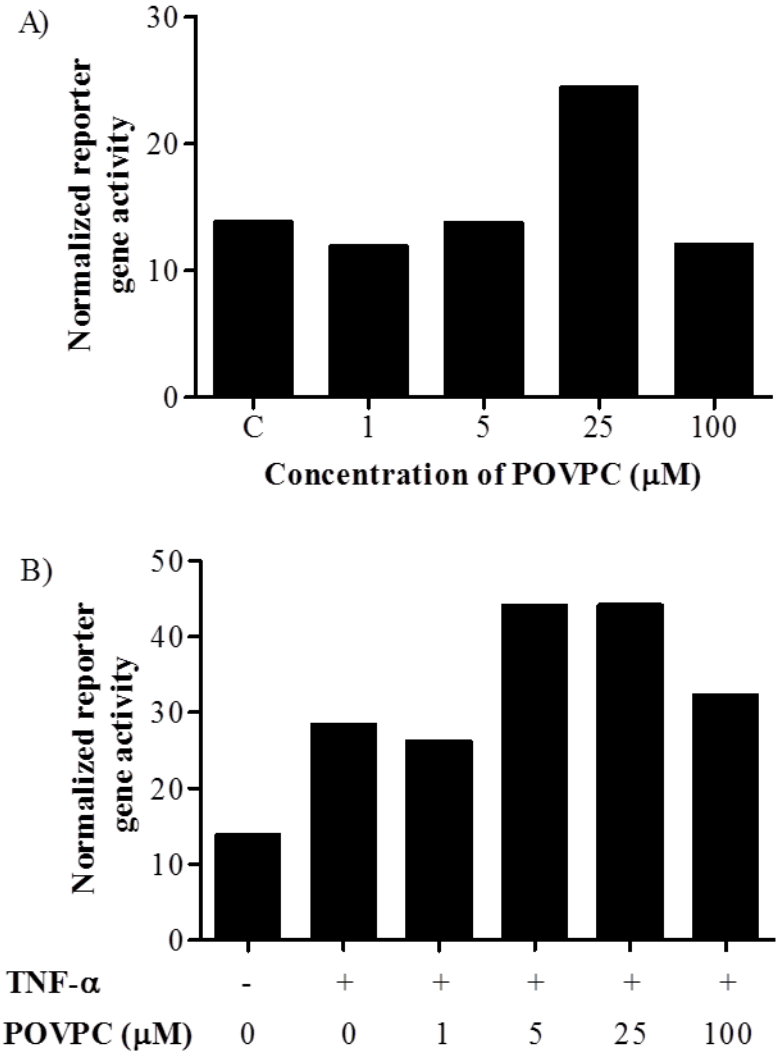


**Figure 4.8 Effect of POVPC on basal and TNFα-induced NF-κB-driven gene expression in L929sA cells.** L929sA cells that were stably transfected with p(IL6κB)$_3$50hu.IL6P-luc+ were incubated with (A) various concentrations of POVPC for 7 hours or pre-incubated for an hour with POVPC before TNF-α (2000 units/ml) was added for 6 hours stimulation. Samples were collected and analysed by luciferase assay. Data for luciferase is normalized to galactosidase and presented as percentage of luciferase/galactosidase. Results presented were obtained from a single experiment.

### 4.3.4 Optimization of transient transfection condition in HEK 293 cells.

To determine whether chlorinated and oxidized lipids can activate PPAR-dependent signalling, HEK 293 cells were transiently transfected with a multiple PPRE-driven promoter construct (J3TK-luc) in the absence or presence of PPARs [α (section 4.3.3), β (section 4.4.4), and γ (section 4.3.5)], and treated with native SOPC, SOPC ClOH, 2-ClHDA, OxPAPC and POVPC. Optimization of transfection efficiency (ratio of transfection reagent $CaPO_4$ to amount of DNA and ratio of DNA constructs in 400ng of total DNA) was performed by our collaborators (Dr. N. Bougarne, Professor G. Haegeman and Professor K. De Bosscher) at the University of Ghent, Belgium and was applied in this study (Section 4.3.3 to 4.3.5) .

Before further investigation was carried out in our laboratory, a series of experiments were carried out to obtain the optimized conditions for transfection using PEI as the transfection agent. In order to achieve the desired transfection efficiencies, the ratio of transfection reagent to DNA, amount of DNA and length of transfection were taken into consideration.

HEK 293 cells were transiently transfected with various combinations of PPARα and PPRE DNA constructs ranging from 25 to 200 ng. Combination of 50 ng of PPRE and 50 ng of PPARα (figure 4.9B) has been shown to induce low basal level of PPRE-Luc expression and was used to investigate the effect of chlorinated and oxidized lipids on HEK 293 cells. Further experiment showed that combination of 25 ng PPRE and 25 ng PPARα also induce a comparable basal PPRE-Luc level. Treatment with PPARα activator was shown to significantly induce PPRE-driven

gene expression in HEK 293 cells (Figure 4.9A). Induction with ethanol, which was used as a solvent to dilute WY14643, was shown to induce reporter gene expression levels compared to control and treatment with agonist increased the luciferase activity at 1.4 times higher than ethanol. This suggests that the presence of solvent can affect luciferase gene expression. However, the result was inconclusive as it was obtained from a single experiment.

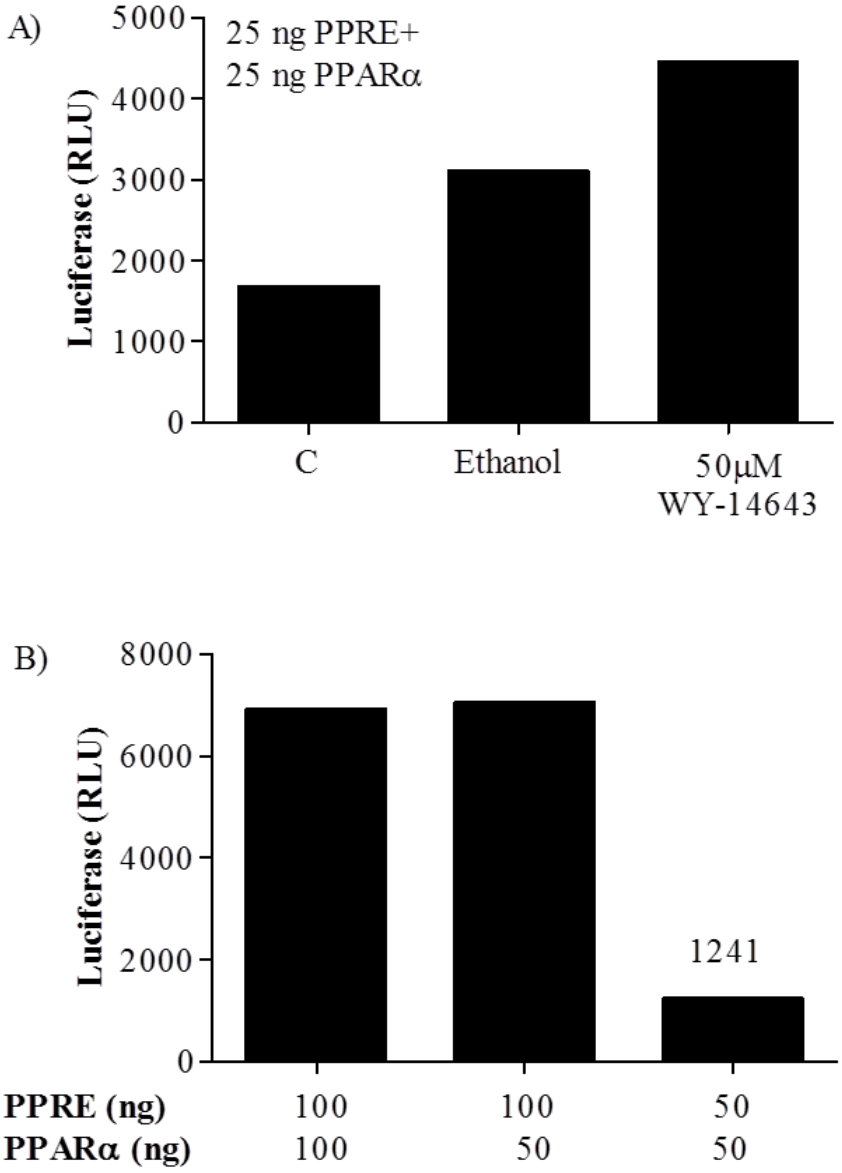


**Figure 4.9 Optimization of transfection in HEK 293 cells.** HEK 293 cells were transiently transfected for 48 hours, using transfection agent polyethyleneimine, (A) with 25 ng of PPRE and 25 ng PPARα DNA constructs and treated with 0.1% (v/v) vehicle or PPARα gonist for 8 hours or (B) transiently transfected with 50 ng of PPRE and 50 ng PPARα DNA constructs.

### 4.3.5 Effect of native, chlorinated and oxidized lipids on transactivation of PPARα in HEK 293 cells.

Results in Figure 4.10A shows that in the absence of PPARα construct, native SOPC slightly but significantly reduced basal levels of PPRE-Luc activity in HEK293 even at low concentration (5 μM), which were further reduced when cells were treated with a higher concentration of native lipid (25 μM). Because it is known that PPARα is present in HEK cell, this suggests that native SOPC could affect the activity of endogenous PPARα. When HEK293 cells were overexpressed with PPARα, the level of PPRE-Luc activity in control was increased 3.5 fold compared to the control in cells without overexpression of PPARα. Interestingly, treatment with native SOPC reduced the level of PPRE-Luc activity at all concentrations tested compared to control (Figure 4.10A). The PPAR activator was without effect and thus it is unclear whether the effect of SOPC is upon agonist stimulated or basal levels.

With regards to SOPC ClOH, the inhibitory effect was more prominent than native SOPC where the reduction of basal level of PPRE-luc in cells transfected without PPARα was observed even at 1μM of SOPC ClOH. Treatment with higher concentration (2.5 μM) resulted in approximately 50% inhibition compared to control and and the level remained the same even when higher concentrations (5 and 25 μM) were used. In cells overexpressing PPARα, SOPC ClOH was found to concentration-dependently reduced the PPRE-luc activity (Figure 4.10B).

Whilst native and chlorohydrin of SOPC was shown to reduce basal level of PPRE-luc in cells without PPARα, 2-ClHDA was found to have an opposite effect (Figure

4.10C), 2-ClHDA stimulated 3 fold increased in reporter activity. This indicates the presence of endogenous PPARα in HEK cells which can be activated by GW647 (Figure 4.10A). Following overexpression of PPARα, 2-ClHDA increased PPRE-driven gene expression but only at a concentration of 100 μM (Figure 4.10C); however, it is of importance to note that there was a large variability within this assay.

The effect of OxPLs was also tested. It was that the basal level of luciferase expression was reduced by PGPC after 6 hours stimulation (Figure 4.11A). In cells transfected with PPARα, the level of PPRE-luc activity was reduced by PGPC. The effect of a mixture OxPAPC was then tested to compare with the effect of PGPC. In contrast to PGPC, OxPAPC did not inhibit basal level of PPRE-luc. The effect observed was in contrast to previous study where OxPAPC was found to increase endogenous PPRE-luciferase activity in endothelial cells (Lee et al., 2000). In cells overexpressed with PPARα, OxPAPC was found to reduce the level of luciferase expression (Figure 4.11B). Subsequently, the treatment protocol was adjusted and HEK cells were treated for longer times with the inducing agents. Results show that after 16 hours stimulation, the highest concentration of OxPAPC (100 μM) induced endogenous level of PPRE driven gene (Figure 4.11C). It is well known that PPARs support ligand-independent transactivation upon overexpression (Delerive et al., 2001). Moreover, the validated PPARα agonist GW647 used in this experiment showed an inconsistent effect, where in some experiments, the agonist had either no effect, or inhibited or enhanced basal actity of PPRE-Luc. In PPARα-overexpressed cells the agonist had either no effect or enhanced PPRE-Luc activity. Due to time

constraints, the results demonstrated in this section were based on a single experiment; therefore, further experiments are necessary to draw a conclusive result.

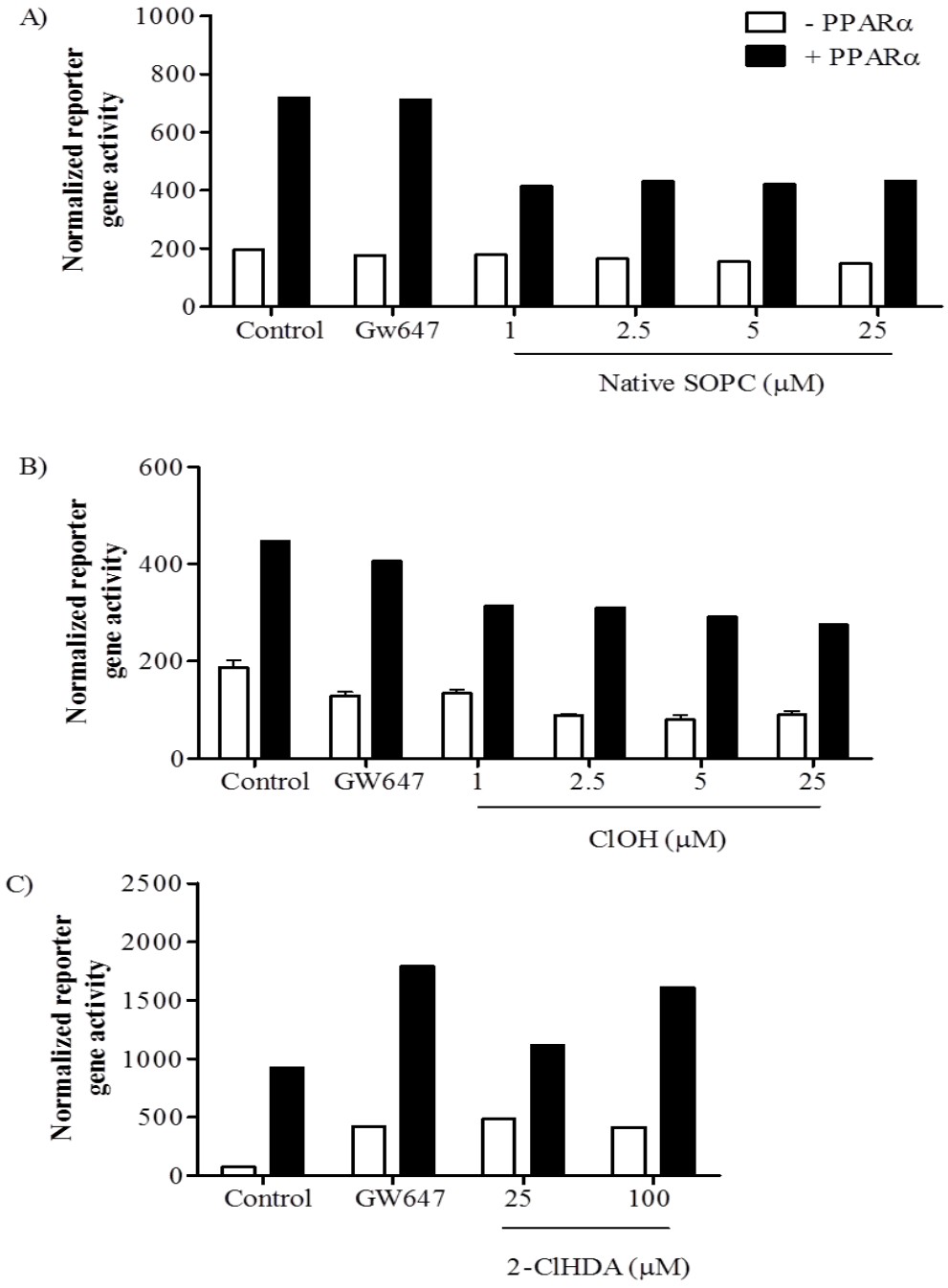


**Figure 4.10 Effect of native SOPC, SOPC ClOH, 2-ClHDA and GW647 on transactivation of PPARα in HEK 293 cells.** HEK cells were transfected with J3-TK-LUC (a PPRE driven gene promoter), β-galactosidase, empty vector and with (solid bar) or without (open bar) an expression vector for PPARα. The cells were treated for 6 hours with A) native SOPC, B) SOPC ClOH, C) 2-ClHDA or GW647. Data for luciferase is normalized to galactosidase and presented as percentage of luciferase/galactosidase and results presented are from a single experiment.

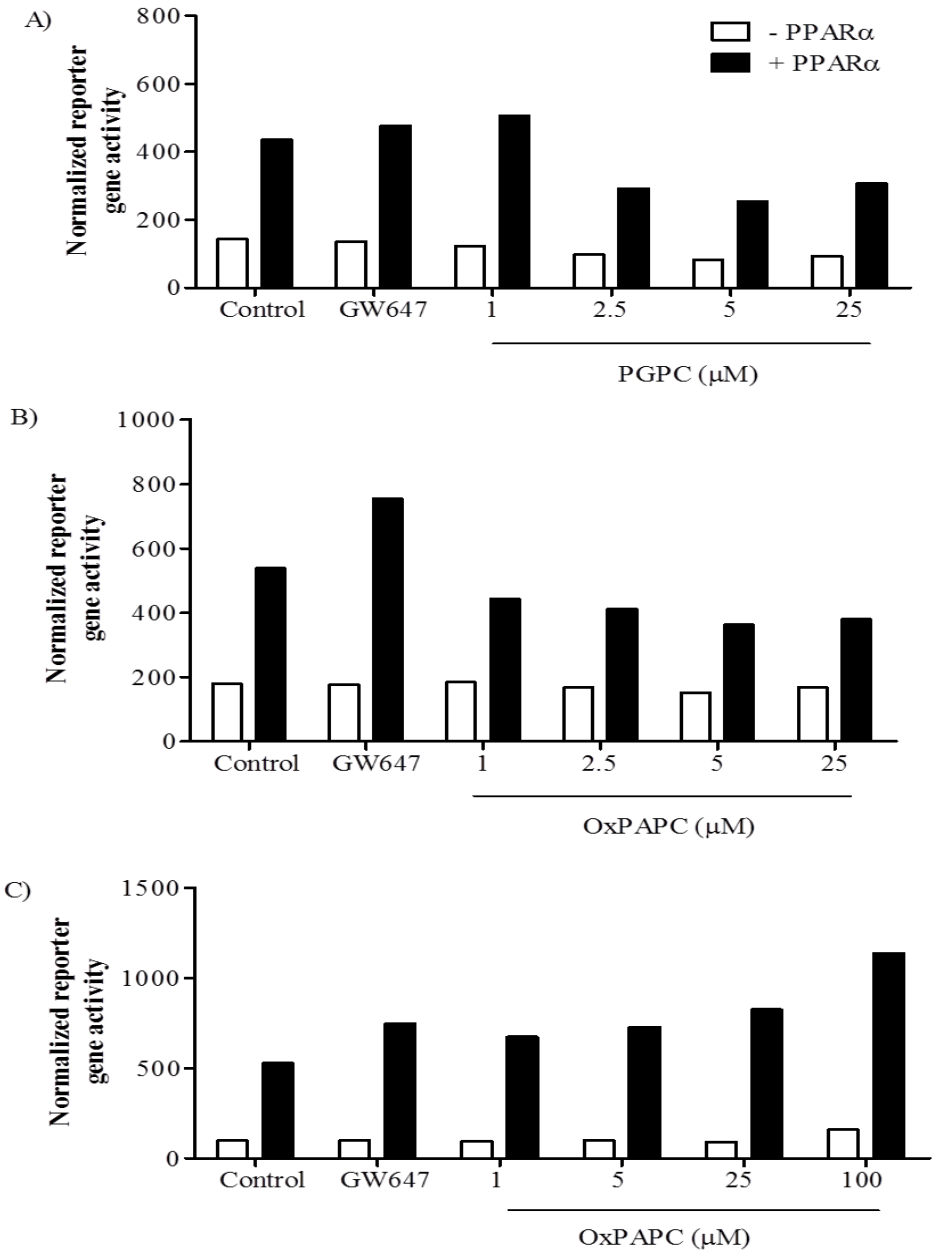


**Figure 4.11 Effect of PGPC, OxPAPC and GW647 on tranactivation of PPARα in HEK 293 cells.** HEK cells were transfected with J3-TK-LUC (a PPRE driven gene promoter), β-galactosidase, empty vector and with (solid bar) or without PPARα (open bar). The cells were treated for 6 hours with A) PGPC, B) OxPAPC or GW647 and for 16 hours for C) OxPAPC or GW647. Data for luciferase is normalized to galactosidase and presented as percentage of luciferase/galactosidase. Values were obtained from a single experiment.

### 4.3.6 Effect of chlorinated and oxidized lipids on transactivation of PPARβ in HEK 293 cells.

To investigate whether SOPC ClOH, 2-ClHDA and OxPAPC induce PPRE-driven gene expression through activation of PPARβ, these compounds were administered to HEK cells that either transiently transfected with PPRE or co-transfected with PPRE and PPARβ construct. Figure 4.12A illustrates that SOPC ClOH enhanced basal PPRE-Luc activity at 25 and 100 μM of the lipid. However this effect was in contrast to the effect of SOPC ClOH observed in Figure 4.10B where SOPC ClOH did not induce PPRE-luc activity; in fact, the PPRE-Luc activity was reduced at a concentration of 25 μM. The PPRE-luc activity in cells overexpressed with PPARβ also increased following treatment with 25 μM SOPC ClOH but dramatically reduced in cells treated with 100 μM SOPC ClOH. On the other hand, data show that 2-ClHDA had negligible effect on expression of PPRE-driven gene expression and no effect following overexpression with PPARβ (Figure 4.12B). This lack of effect was in contrast to the previous experiment illustrated in Figure 4.10C where 2-ClHDA increased the basal activity of PPRE-Luc. Finally, treatment with OxPAPC resulted in a minor increase in basal luciferase activity at a low concentration (3 μM) but had no effect in cells additionally transfected with PPARβ (Figure 4.12C). Moreover, the effect of L165 041, an agonist for PPARβ was found to be not consistent between different plates although the same passage of cells was used. However, it is important to note that the the data was collected from a single experiment only, more repetitions are essential to draw firm conclusions.

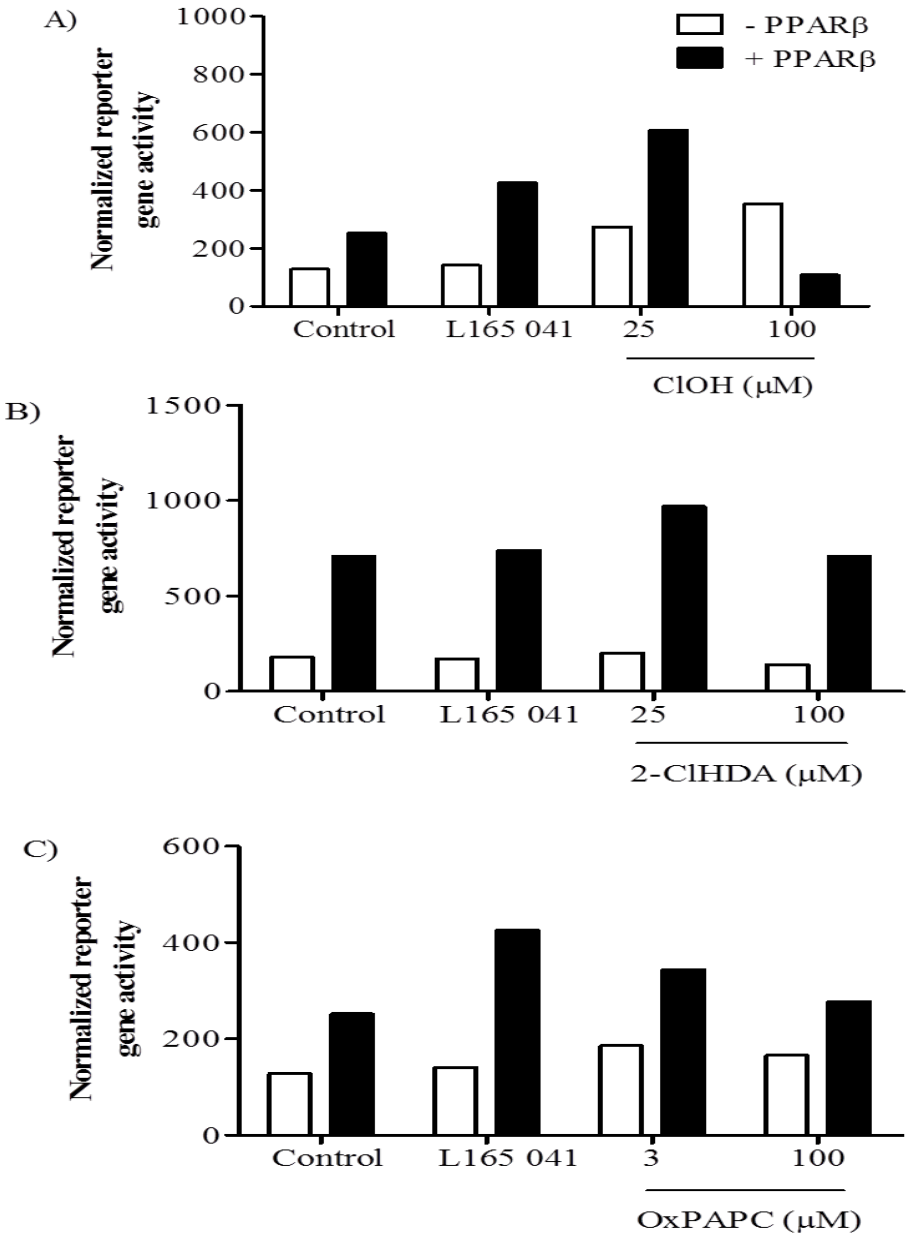


**Figure 4.12 Effect of SOPC ClOH, 2-ClHDA, OxPAPC and L165041 on transactivation of PPARβ in HEK 293 cells.** Cells were transfected with J3-TK-LUC (a PPRE driven gene promoter), β-galactosidase, empty vector and with (solid bar) or without an expression vector PPARβ (open bar). The cells were treated for 6 hours with (A) SOPC ClOH, (B) 2-ClHDA, (C) OxPAPC or L165041. Data for luciferase is normalized to galactosidase and presented as percentage of luciferase/galactosidase. Data was obtained from a single experiment.

### 4.3.7 Effect of chlorinated and oxidized lipids on transactivation of PPARγ in HEK 293 cells.

To determine whether SOPC ClOH, 2-ClHDA and OxPAPC modulate PPAR-γ dependent transcription, a combination of PPRE-Luc with or without an expression vector for PPARγ were introduced into HEK cells by transient transfection. The results show that treatment with SOPC ClOH reduced basal activity of PPRE-Luc (Figure 4.13A) and this observation was consistent with results presented in figure 4.10B and in contrast to the result presented in figure 4.12A. In cells overexpressing PPARγ, treatment with 25 μM SOPC ClOH increased luciferase expression to the same level as the PPARγ agonist, rosiglitazone. However at a higher concentration, SOPC ClOH reduced the level of luciferase expression (Figure 4.13A). With regards to 2-ClHDA, this compound was apparently without effect on the basal activity of PPRE-Luc (Figure 4.13B), which is in contrast to the result illustrated in figure 4.10C but consistent with the result observed in figure 4.12B. Upon overexpression, due to high variability within assay, it was not clear whether 2-ClHDA could increase PPRE-Luc activity; a low concentration of 2-ClHDA caused a slight increase whilst the highest concentration caused a minor decrease (Figure 4.13B). In cells treated with OxPAPC, results demonstrated that this compound did not increase basal level of PPRE-driven gene expression, similar to that observed in an earlier experiment (Figure 4.11B). However, in PPARγ-overexpressed cells, OxPAPC enhanced luciferase expression (Figure 4.13C). With regards to rosiglitazone, this agonist was found to has no effect on basal luciferase expression compared to control and in cells overexpressing PPARγ, this agonist appeared to increased PPRE-Luc activity. Again, this results were based on a single experiment and the effects

observed are therefore inconclusive. However, if time permits, it is interesting to further investigate whether chlorinated and oxidized lipids can further increased PPRE-Luc activity in cells overexpressed in PPARγ as there is a trend where low concentration of chlorinated lipids increased PPRE-luc activity and the range of concentrations of OxPAPC used in this assay resulted in increased the luciferease expression compared to control.

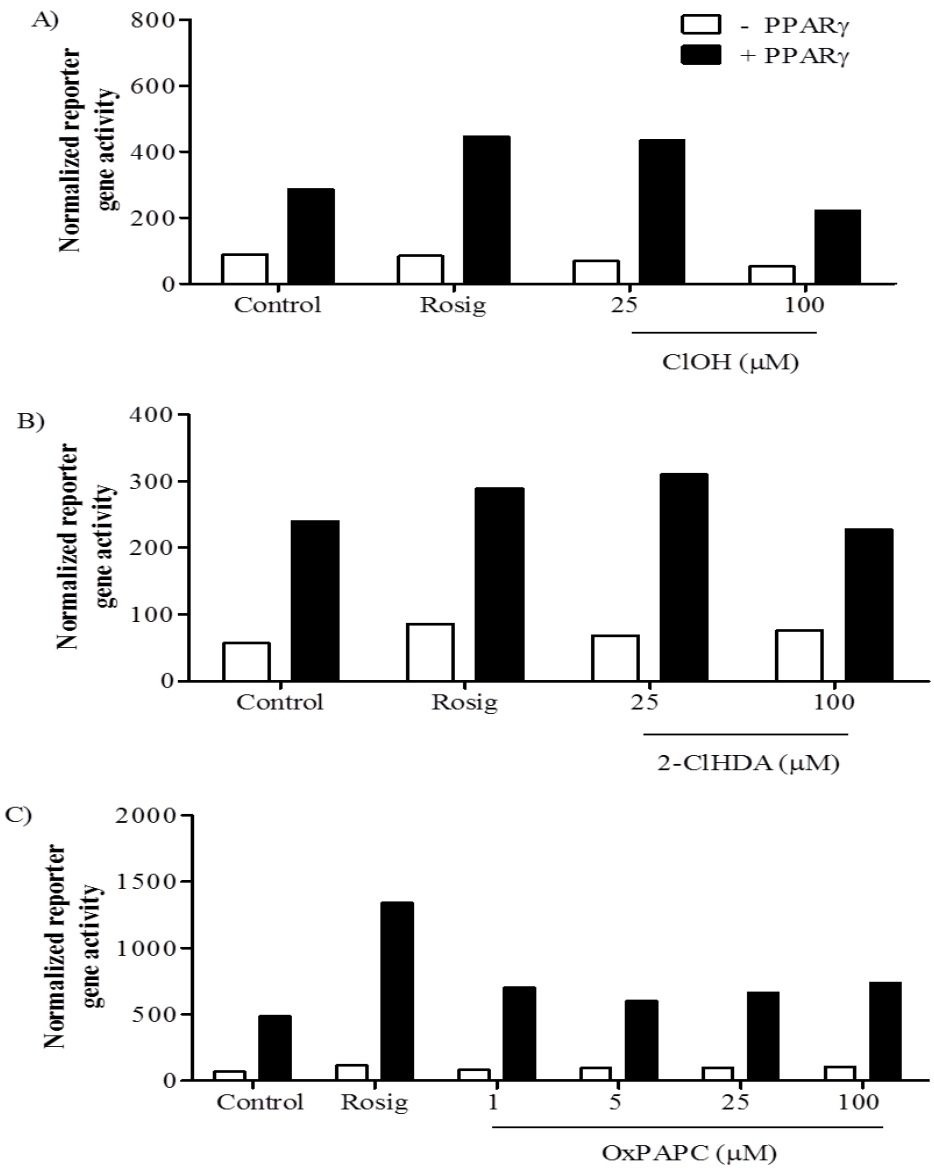


**Figure 4.13 Effect of SOPC ClOH, 2-ClHDA, OxPAPC and rosiglitazone on transactivation of PPARγ in HEK 293 cells.** Cells were transfected with J3-TK-LUC (a PPRE driven gene promoter), β-galactosidase, empty vector and with (solid bar) or without PPARγ (open bar). The cells were treated for 6 hours with (A) SOPC ClOH, (B) 2-ClHDA, (C) OxPAPC or rosiglitazone (rosig). Luciferase/galactosidase ratios were determined. Presented results were obtained from a single experiment.

### 4.3.8 SOPC ClOH but not 2-ClHDA and PGPC induced transcription of PPRE-luciferase reporter gene in HEK 293 cells.

To further investigate the effect of chlorinated lipids on PPRE-Luc activity in cells overexpressing PPARα, transiently transfected HEK cells were stimulated with the low and high concentrations of SOPC ClOH and 2-ClHDA. Native SOPC was also tested to verify that the effect was mediated by chlorohydrin but not its native lipid. Results in Figure 4.14A show that SOPC ClOH but not native SOPC induced PPRE-driven gene expression. Treatment of HEK cells with oxidized phospholipid, POVPC at concentration 5 μg/ml was selected based on a previous study that showed a significant increase in PPRE-luciferase expression in endothelial cells. However, in this study, although the compound appeared to increase the PPRE-dependent luciferase activity compared to control, the effect observed was not statistically significant. A higher concentration of POVPC was also applied (10 μg/ml); however, the compound induced cell death in HEK cells. Similarly, 2-ClHDA was found to have no effect on PPRE-Luc activity. Surprisingly, treatment of HEK cells with a validated PPARα agonist, WY14643, also did not significantly induce PPRE-driven gene expression although a slight increase of PPRE-luc activity was observed.

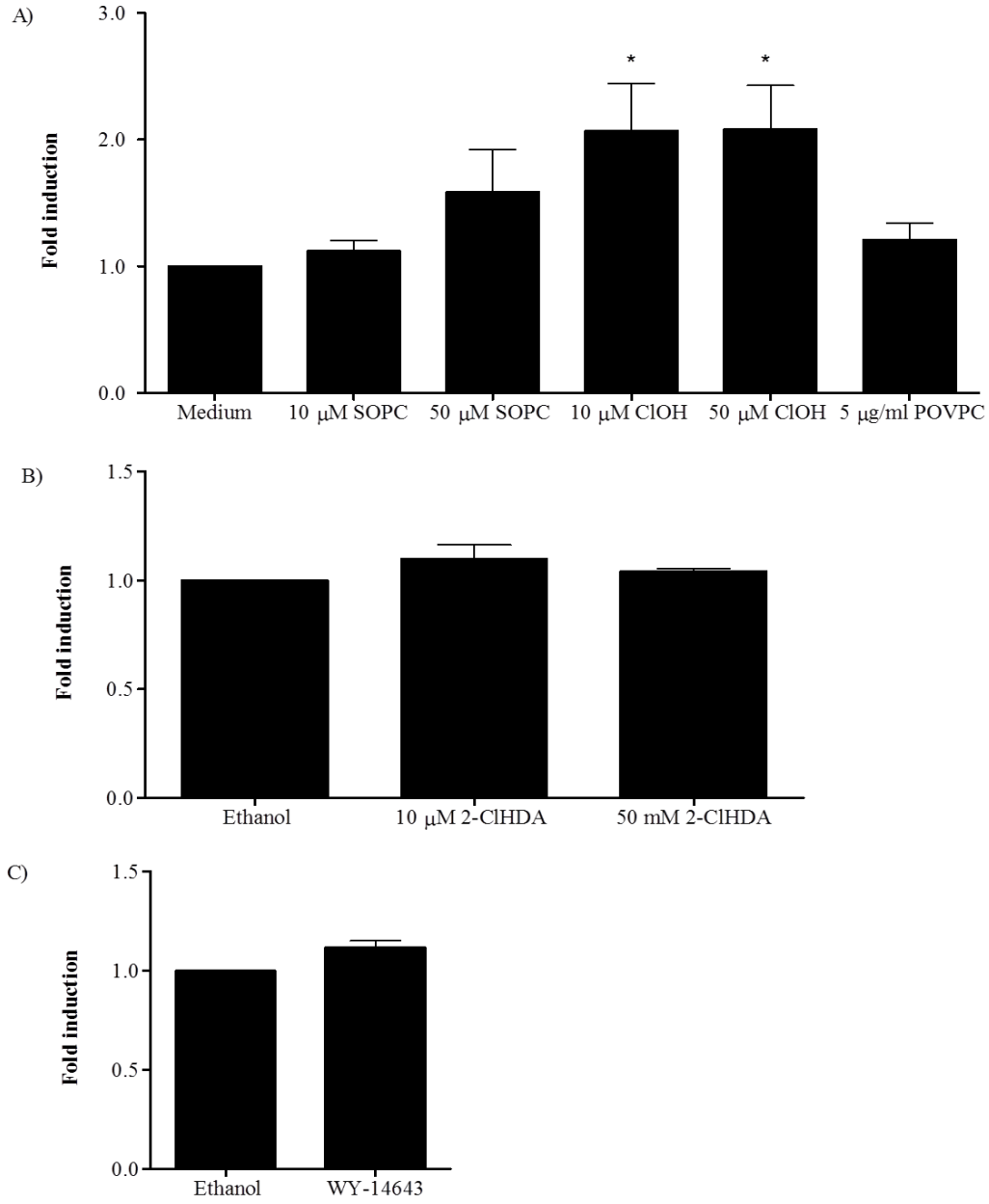


**Figure 4.14 Effect of native SOPC, SOPC ClOH, 2-ClHDA PGPC and WY-14643 on transactivation of PPARα in HEK 293 cells.** HEK cells were transfected with J3-TK-LUC (a PPRE driven gene promoter and PPARα. The cells were treated for 8 hours with A) medium, native SOPC, SOPC ClOH and POVPC, B) ethanol (with final concentration less than 0.1 %) and 2-ClHDA or C) ethanol and WY-14643. The data represent the mean ± S.E.M of the fold induction, relative to medium or ethanol-treated response, from three separate experiments.*, $P<0.05$.

## 4.4 Discussion

Several studies have shown that OxLDL induces activation of PPARα and PPARγ, and that 9-HODE, 13-HODE and OxPLs including OxPAPC, POVPC, PGPC, which are components of OxLDL are involved in OxLDL-mediated PPARα and PPARγ activation (Nagy et al., 1998, Lee et al., 2000, Delerive et al., 2000). Activation of PPARs by these components can lead to both pro- and anti-inflammatory effects. A number of reports show that OxLDL can induce CD36 expression on macrophages through transactivation of PPARγ, which in turn induces the OxLDL uptake leading to formation of foam cells, the hallmark of atherosclerosis (Collot-Teixeira et al., 2007). Similarly, 15d-$PGJ_2$ and the synthetic ligand of PPARγ, troglitazone, were shown to induce expression of CD36 in human monocytes (Nagy et al., 1998). This led to the idea that some components of OxLDL that can activate CD36 expression could also activate PPARs pathway.

In addition, OxLDL as well as the cholesterol-lowering drug (statin) were shown to induce anti-inflammatory effects by activating PPARα and PPARγ through ERK1/2-dependent COX-2 expression. This suggests that several mechanisms that can activate COX-2 could also activate PPARs. With regards to chlorinated lipid products, there is no study demonstrating their effect on PPAR activation. However, there are some reports showing that the phospholipid chlorohydrin can induce their effects through a CD36-dependent mechanism (Dever et al., 2008) and α-chloro fatty aldehyde can induce expression of COX-2 (Messner et al., 2008a), suggesting that chlorinated lipids could also mediate some of their effects through activation of PPARs.

Previously, a study showed that synthetic PPARα agonists, WY14643 and GW647 can inhibit TNF-induced NF-κB-driven gene expression in L929sA cells transfected stably with an NF-κB-dependent promoter. It was also shown that inhibition of NF-κB-driven genes by both agonists reduced production of pro-inflammatory cytokine IL-6 production (Bougarne et al., 2009), suggesting that activation of PPARα could induce anti-inflammatory effects through interference with NF-κB. To date, no study has reported the anti-inflammatory effects of chlorinated lipids. However, because of the lipid nature of chlorinated lipids and the role of PPARs in lipid signalling, it was hypothesized that chlorinated lipids could also induce similar effects as the known PPARα agonist-mediated anti-inflammatory effects. In contrast to the hypothesis, SOPC ClOH and 2-ClHDA did not regulate TNFα-induced NF-κB driven gene expression. This result was in agreement with result presented in chapter 5, whereby chlorinated lipids did not reverse IκBα degradation induced by TNF-α in endothelial cells, suggesting that chlorinated lipids did not inhibit the effect of TNF-α stimulation. However, native SOPC appeared to reduce NF-κB-driven gene expression in L929sA cells.

With regards to oxidized phospholipids, OxPAPC, the results observed that oxPAPC did not inhibit TNFα-induced NF-κB-driven gene expression. Studies from different groups also showed that OxPAPC did not inhibit TNFα-induced IκBα degradation in THP-1 cells (Erridge et al., 2008) and endothelial cells (Bochkov et al., 2002a), suggesting that TNFα-induced NF-κB activity could not be inhibited by OxPAPC. Moreover, it has been proposed that the protective effect of OxPAPC, PGPC and POVPC are restricted to inhibition of TLR2 and 4 signalling pathway. For instance,

OxPAPC was shown to inhibit binding of LPS to its accessory protein LBP, CD14 and MD2, leading to inhibition of TLR4 activation which then inhibits LPS-induced pro-inflammatory effects (Erridge et al., 2008). Therefore, it was concluded that OxPAPC probably did not regulate TNFα-induced NF-κB-driven gene expression. In contrast, it has been demonstrated in this study that POVPC at concentration 5 and 25 μM enhanced TNFα-induced NF-κB driven gene expression; however, more repetitions are needed to validate the effect. Moreover, whilst OxPAPC alone did not affect basal level of NF-κB-Luc activity, PGPC and POVPC were found to induce a bell-shaped effect on NF-κB-driven gene expression, suggesting that the individual component of OxPAPC may have a dual effect, in which at moderate concentrations, the lipids increase basal activity and at higher concentrations no effect is observed.

The preliminary study conducted in collaborators laboratory showed that native SOPC and SOPC chlorohydrin reduced PPRE-Luc activity in HEK cells overexpressed with PPARα. However, as mentioned earlier, this was based on a single experiment. Further investigation carried out in our laboratory showed for the first time that in cells overexpressed with PPARα, SOPC ClOH, but not native SOPC, induces PPRE-driven gene expression. Activation of PPARα can lead to both pro- and anti-inflammatory effects; although, possible effects related to SOPC ClOH activation of PPARα is not addressed in this study. Interestingly, some effects of chlorinated phospholipids are comparable to the effect induced by OxLDL as well as PPARα and PPARγ activators. Specifically, OxLDL and synthetic PPARα agonist, WY14643, were shown to induce CD36 expression in macrophages and SOPC ClOH was demonstrated to increase generation of PMA-induced ROS in CD36-dependent

manner. Because induction of CD36 expression can be mediated by PPARα and γ activators (Duval et al., 2002), it is tempting to speculate that the activation of PPARα by this chlorinated phospholipid could contribute to some of its pro-inflammatory effects.

On the other hand, Taketa et al. proposed that oxLDL may induce some anti-inflammatory effects through activation of PPARα and γ by intracellular 15d-$PGJ_2$, the formation of which is catalysed by COX-2 (Taketa et al., 2008). In endothelial cells, Messner et al showed that 2-ClHDA induces COX-2 expression, suggesting that it could also play the same role as OxLDL (Messner et al., 2008a). They also showed that 2-ClHDA may induce production of prostacyclin (Messner et al., 2008a), which is an endogenous prostanoid produced by the action of COX-2. Prostacyclins are expressed predominantly in endothelial cells and play important roles as vasodilators and anti-thrombotic agents, and were reported to mediate apoptosis through PPARβ (Hatae et al., 2001). In the preliminary study, 2-ClHDA was found to slightly induce luciferase activity in cells overexpressed with PPARα, β and γ in the preliminary study. However, it appears that 2-ClHDA has no significant effect in cells overexpressed with PPARα after a few repetitions were conducted in the later part of this chapter. Due to time limitation, the effect of 2-ClHDA on cells overexpressed with other PPARs including PPARβ and γ could not be carried out; but due to some interesting effect observed in the preliminary study, it is worthwhile to further investigate the effect of this compound. The present study also showed that incubation of OxPAPC for 6 hours in HEK 293 cells transiently transfected with PPRE and PPARα did not induce PPRE-driven gene expression but the effect was

observed after 16 hours stimulation, suggesting that the effect of OxPAPC is time dependent. Moreover, it is of importance to note that the effect of a validated PPARα agonist, WY14643 was poor where it has been shown in this study that this agonist did not even induce a significant effect. With regards to other types of PPAR agonist used in the preliminary study such as GW647 (PPARα agonist), L165041 (PPARβ agonist) and rosiglitazone (PPARγ agonist), the effect observed was not consistent, with some assay, they just induced a slight increase of PPRE-Luc or even induced a slight decrease, while in some other assays, they clearly increased PPRE-Luc expression.

Together these findings suggest that SOPC ClOH but not native SOPC, 2-ClHDA and POVPC, can mediate activation of PPARα through transactivation mechanism, but not trans-repression mechanism. The effect of SOPC ClOH and 2-ClHDA on PPARβ and γ in this study was not clear, although it is possible that this compound can also induce activation of these PPARs. Previous works suggest that OxPAPC may induce both pro- and anti-inflammatory effects through activation of PPARα, and chlorohydrin could also induce both effects through this pathway.

# Chapter 5

# The Role of Oxidized and Chlorinated Lipids in Regulating NF-κB and MAPK Pathways

## 5.1. Introduction

Recruitment of OxLDL in atherosclerotic lesion is one of the main characteristics of atherosclerosis. Previous studies reported that some effects of OxLDL are mediated through NF-κB and MAPK signaling pathways. For instance, it has been shown that OxLDL modulates COX-2 expression in endothelial cells through a p38 MAPK, NF-κB and cAMP-response element (CRE) dependent pathway (Norata et al., 2004). Consistent with the effect observed in OxLDL, its active agent OxPAPC also induces tissue MAPK pathway and previous study showed that induction of tissue factor by OxPAPC involved activation of ERK; although, NF-κB is not activated by oxPLs (Bochkov et al., 2002a).

Interestingly, OxLDL as well as OxPAPC was shown to be able to inhibit NF-κB and MAPK signaling induced by LPS (Robbesyn et al., 2004, Erridge et al., 2008) and it was hypothesized that the inhibition is mediated through blocking the binding of LPS to its accessory proteins, LBP and CD14 (Erridge, 2009). Activation of NF-κB and MAPK pathways can also be activated by TNF-α; however, previous study showed that OxPAPC did not inhibit the effect of TNFα-induced IκB-α degradation (Bochkov et al., 2002a).

TNF-α has also been associated with apoptosis that can occur following the activation of TNFR1 and Fas death receptor. A study demonstrated that OxLDL alone induced apoptosis signaling cascades through activation of Fas receptor (Sata and Walsh, 1998). Interestingly, the apoptotic effect of OxLDL was not prevented by treatment with TNF-α at a concentration that was shown to induce anti-apoptotic

A20 gene expression in endothelial cells (Heermeier et al., 2001). In vascular smooth muscle cells, POVPC and PGPC were shown to induce caspase-3 expression and activate sphingomyelinase that can activate p-38 MAPK and JNK MAPK signaling pathways (Loidl et al., 2003). However, OxPAPC did not induce expression of caspase-3 in endothelial cells (Gargalovic et al., 2006), suggesting that OxPLs may have different effect in different types of cells.

Compared to OxLDL and OxPAPC, less is known about chlorinated lipids. Various studies showed that chlorinated lipids such as stearoyl oleoyl phoscphatidylcholine chlorohydrin (SOPC ClOH) and 2-chlorohexadecanal (2-ClHDA) induce pro-inflammatory and pro-atherogenic effects. Nevertheless, the mechanism of action, particularly the regulatory role of MAPK and NFκB in relation to the effect produced by chlorinated lipids, is unknown. Previously, one form of OxLDL (HOCl-modified LDL) was demonstrated to have an apoptotic role in Jurkat T-cell lines (Resch et al., 2011). However, whether chlorinated lipids can induce apoptosis in endothelial cells has not been investigated. Therefore, in this study, OxPAPC was examined, in view of repeating the study in human aortic endothelial cells and as a positive control to investigate the role of chlorinated lipids in mediating the activation of NF-κB, MAPK and apoptotic pathways.

## 5.2. Methodology

Native, oxidized lipids and chlorinated lipids were obtained from Avanti Polar Lipid (USA) or obtained from Professor Andrew Pitt (Aston University) as described in section 2.1. All antibodies used in this experiments were obtained from Santa Cruz Santa Cruz Biotechnology Inc. (CA, USA) as described in section 2.1. Chlorinated lipids were prepared and analysed as describe in section 2.2.1 and 2.2.2. Growth and subculturing of rat aortic smooth muscle cells (RASMCs) and human umbilical vein endothelial cells (HUVECs) were described in section 2.6.1 and 2.6.3, respectively. Treatment of RASMCs with LPS and TNF-α and treatment of HUVECs with chlorinated and oxidized lipids was described in section 2.6.4. IκBα loss and phosphorylation of ERK, p38 MAPK and JNK was determined by Western blotting as described in section 2.6.5. A preliminary study on cell morphology to investigate the potential effect on cell death was carried out as described in section 2.7.

## 5.3. Results

### 5.3.1. Detection of smooth muscle-α actin by electron microscopy.

To investigate the mechanism of actions induced by chlorinated lipids, primary smooth muscle cells derived from rat aortas were used as a model. In order to confirm that cells isolated from aorta are only smooth muscle cells, immunohistochemistry staining was utilized. Presence of smooth muscle-α actin (SMA), which is one of a few genes whose expression is relatively restricted to vascular smooth muscle cells and localized only in microfilament bundles (Skalli et al., 1989), was observed under electron microscopy (Figure 5.4). This suggests that

cells isolated are smooth muscle cells and therefore cells were used to investigate the effect of chlorinated lipids.

### 5.3.2. Effects of LPS and TNF-α on NF-κB and p38 MAPK pathways in RASMC.

Before stimulation with oxidized or chlorinated lipids, RASMC (within passage 0 to 4) were treated with classical inflammatory mediators, LPS (100μg/ml) and TNF-α (20 ng/ml) for different time intervals to confirm the responsiveness of these cells. Multiple experiments with different concentrations were carried out, but the results obtained showed that neither LPS nor TNF-α induce IκB-α degradation or p38 MAPK expression (Figure 5.5), suggesting that the cells were not responsive to the treatment. Therefore, HUVECs, another cell type relevant to vascular biology, were then used as a model to understand the mechanism of chlorinated lipids.

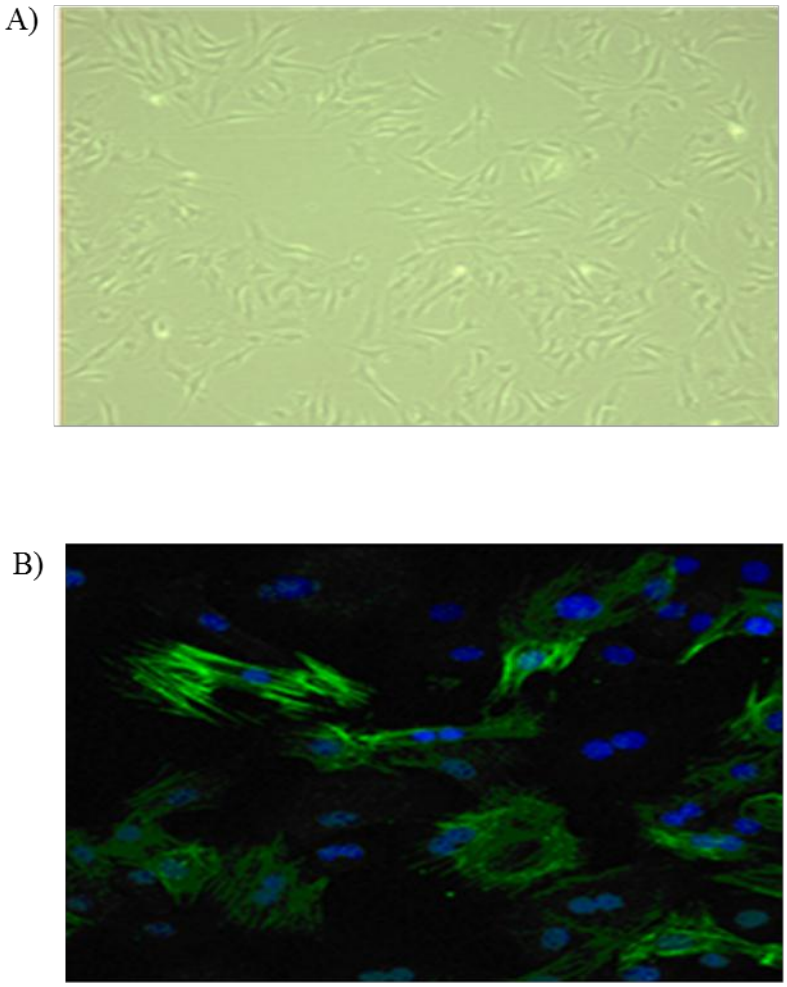


**Figure 5.1 Morphology of RASMCs and immunohistochemical detection of smooth muscle-αctin.** A) Morphology of RASMCs was observed by light microscopy. B) Immunohistochemistry staining has been conducted as described in section 2.6.1.2 and presence of smooth muscle-α actin in microfilament (fluorescence) was observed by electron microscopy (blue: nuclei, green: SMA).

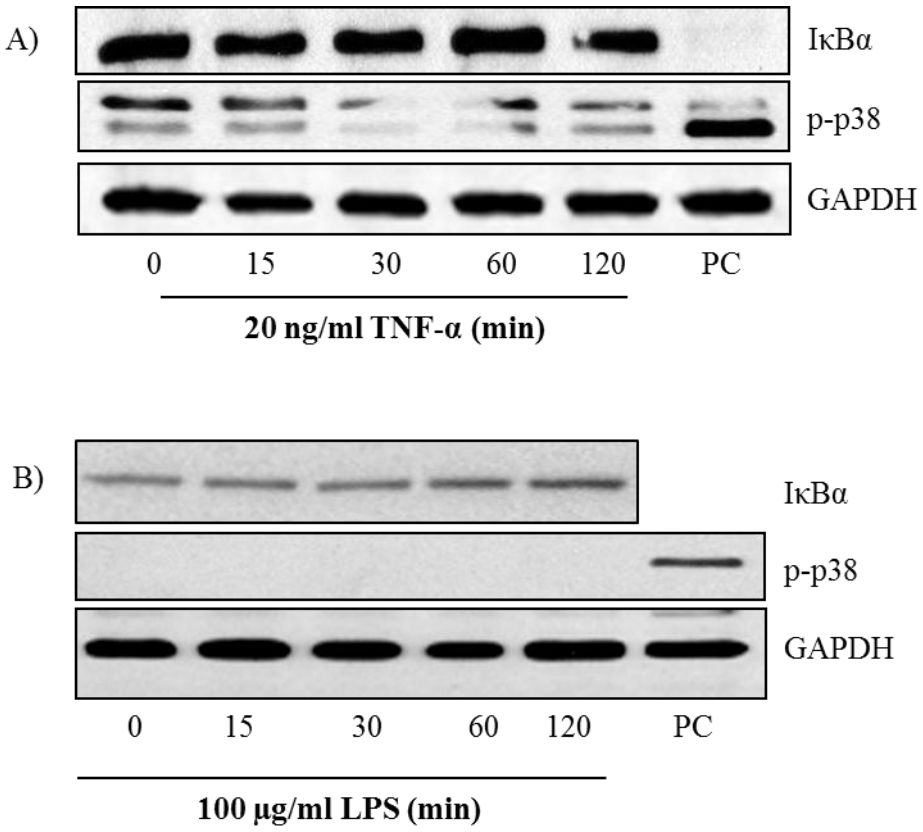


**Figure 5.2 Effect of TNF-α and LPS in rat RASMCs.** RASMCs were stimulated with A) TNF-α (20 ng/ml) and B) LPS (100 µg/ml) for the time indicated. Whole cell lysates were prepared, separated by SDS PAGE and assessed for IκBα loss (38 kDa) and expression of p38 (43 kDa) as described in section. The results are representative of 3 independent experiments. PC, positive control.

### 5.3.3. Effects of chlorinated and oxidized lipids on NF-κB and MAPK pathways in HUVECs.

Because RASMCs did not give a response following treatment with LPS or TNF-α, which are well known mediators for activation of NF-κB and MAPK pathway, the study was changed to examine the effects of chlorinated and oxidized lipids in HUVECs. Before HUVECs were treated with chlorinated and oxidized lipids, a control experiment was conducted to demonstrate that treatment of HUVECs with TNF-α resulted in IκBα degradation and phosphorylation of p38 MAPK (results not shown), confirming that HUVECs were responsive to TNF-α and both signalling pathways could be measured by Western blotting.

Cells were then treated with SOPC ClOH (25 μM) over a period of 4 hours (Figure 5.6). The results clearly show that SOPC ClOH neither induced IκBα degradation nor phosphorylation of JNK (Figure 5.6A and B). In Figure 5.6C, SOPC ClOH was shown to induce phosphorylation of p38 MAPK after 240 minutes stimulation, however the same effect was also observed in cells stimulated with vehicle. Similarly, in figure 5.6D, phosphorylation of ERK was observed after 15 minutes stimulation with SOPC ClOH; however, it was subsequently discovered that the protein expression was not due to the modified lipid but instead due to the effect of the serum-free M199 media that was used to dilute the lipids.

With regards to 2-ClHDA, this compound was found to neither induce IκBα degradation (Figure 5.7) nor phosphorylation of ERK (data not shown). Whilst it was also found that cells treated with 2-ClHDA for 20 and 24 hours resulted in a decrease

in the total amount of protein, this effect was apparently not due to 2-ClHDA but the effect of serum free M199 medium. With regards to OxPAPC, it is clear that OXPAPC did not induce IκBα degradation. However, it was observed that after 30 minutes stimulation, OxPAPC induced phosphorylation of ERK and this stimulation remained constant until 4 hours, which is the longest incubation time used in this study (Figure 5.8). The effect of OxPAPC was not an artefact as treatment with medium alone for 30 and 240 minutes did not induce phosphorylation of ERK. This result was in agreement with previous finding by Bochkov and coworkers (Bochkov et al., 2002b).

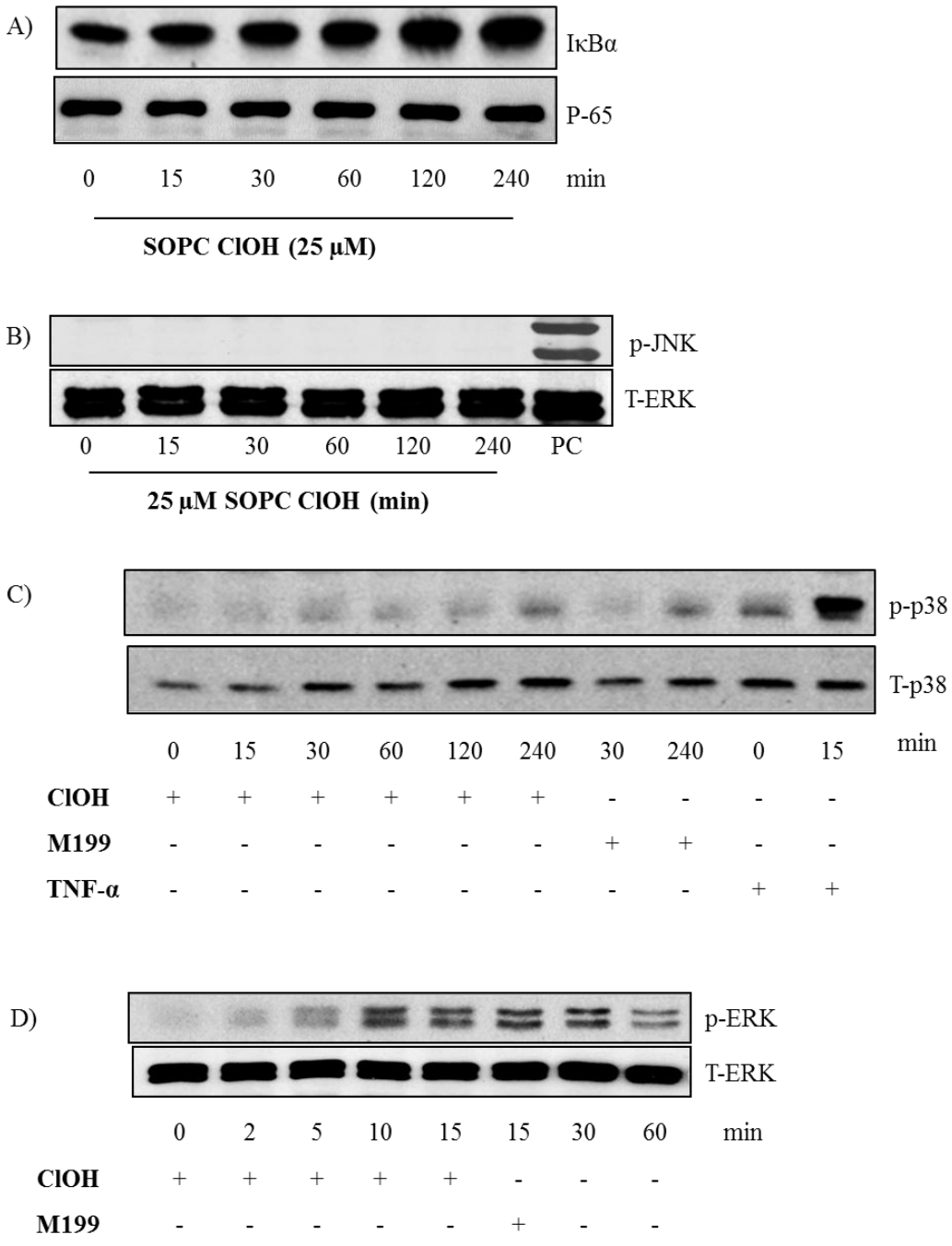


**Figure 5.3 Effect of SOPC ClOH on NF-κB and MAPKs in HUVECs.** Cells were stimulated with SOPC ClOH (25 μM), or a positive control, TNF-α (20 ng/ml) for the time indicated. Whole cell lysates were prepared, separated by SDS PAGE and assessed for (A) IκBα (B) p-JNK (C) p-p38 (D) p-ERK as described in section 2.6.5. Results presented were obtained from a single experiment.

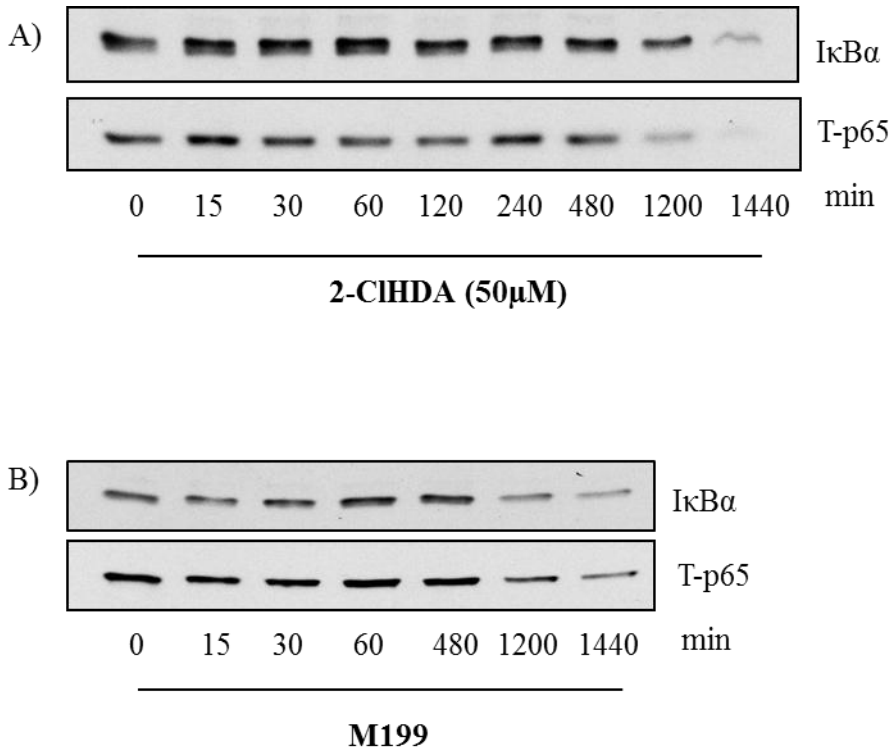


**Figure 5.4 Effect of 2-ClHDA on degradation of IκBα and phosphorylation of ERK in HUVECs.** Cells were stimulated with (A) 2-ClHDA (50 μM), which was dissolved in ethanol and reconstituted in M199 medium (final concentration of ethanol = 0.1%) or negative control, serum free medium M199, at different time intervals as indicated above. Whole cell lysates were prepared, separated by SDS PAGE and assessed for IκBα as described in section 2.6.5. Results were obtained from a single experiment.

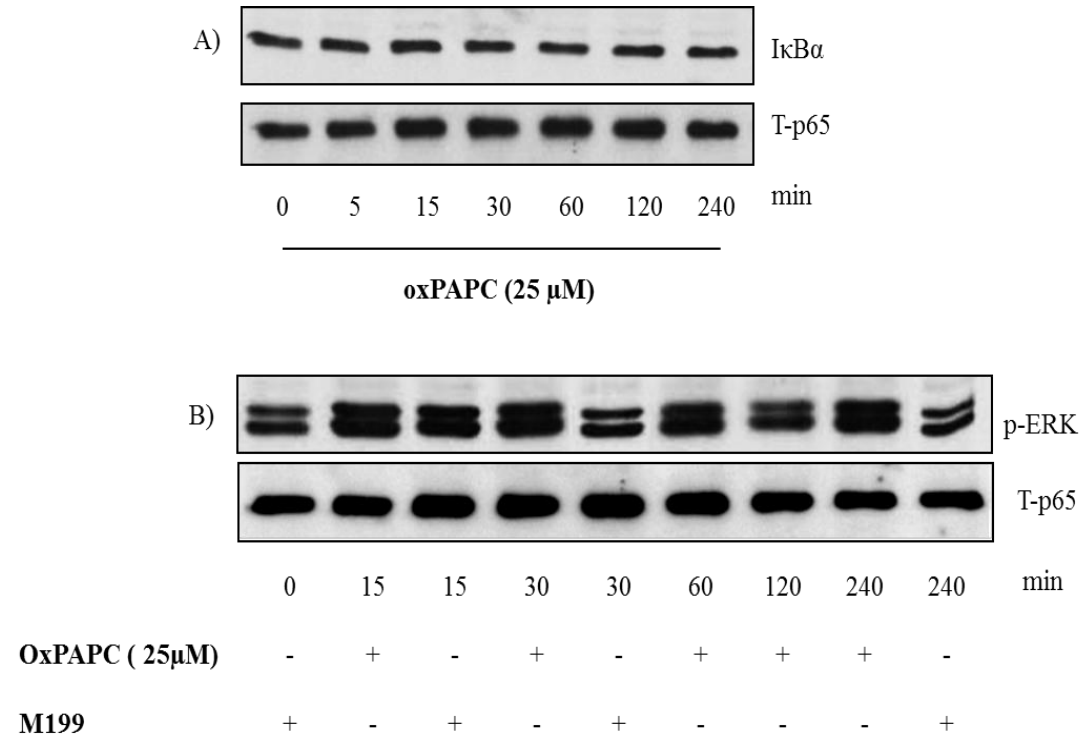


**Figure 5.5 Effect of oxPAPC on degradation of IκBα and phosphorylation of ERK.** Cells were stimulated with 25 µM OxPAPC or negative control, serum free medium M199 at different time points. Whole cell lysates were prepared, separated by SDS PAGE and assessed for (A) IκBα (B) p-ERK as described in section 2.6.5. Data are obtained from a single experiment.

### 5.3.4. LPS stimulated IκBα degradation and p-ERK expression in HUVECs.

Previous studies demonstrated that OxPAPC inhibited LPS-mediated cellular signalling, in particular NF-κB activation. To investigate whether chlorinated lipids also have the same role, an initial experiment using different concentrations of LPS over a time course of stimulation was undertaken and the appropriate conditions selected for stimulation with chlorinated lipids. Figure 5.9 (A) illustrates that LPS (100 μg/ml) produced a substantial IκBα loss after 60 minutes incubation. LPS also increased the expression of p-ERK after 5 minutes stimulation and reached a maximum level after 15 minutes before expression gradually declined thereafter. HUVECs were then treated with different concentrations of LPS at 60 minutes and it was found that the lowest concentration that caused degradation of IκB-α was 30 μg/ml (figure 5.9 B). In addition, increase ERK phosphorylation was observed at 1μg/ml LPS after 15 minutes stimulation and the level was the same in cells treated with higher concentrations of LPS (Figure 5.9 C).

LPS at a concentration of 30 μg/ml was selected and tested for both IκBα loss and expression of p-ERK at different time intervals (Figure 5.10). The result shows that IκBα degradation started after 30 minutes with maximum degradation (0.308 fold of basal value) after 60 minutes. Phosphorylation of ERK began after 5 minutes and a maximum level was obtained after 15 minutes (1.797 fold of basal value). For treatment with oxidized and chlorinated lipids, 30 μg/ml of LPS was used at 60 and 15 minutes for NF-κB and ERK activation, respectively.

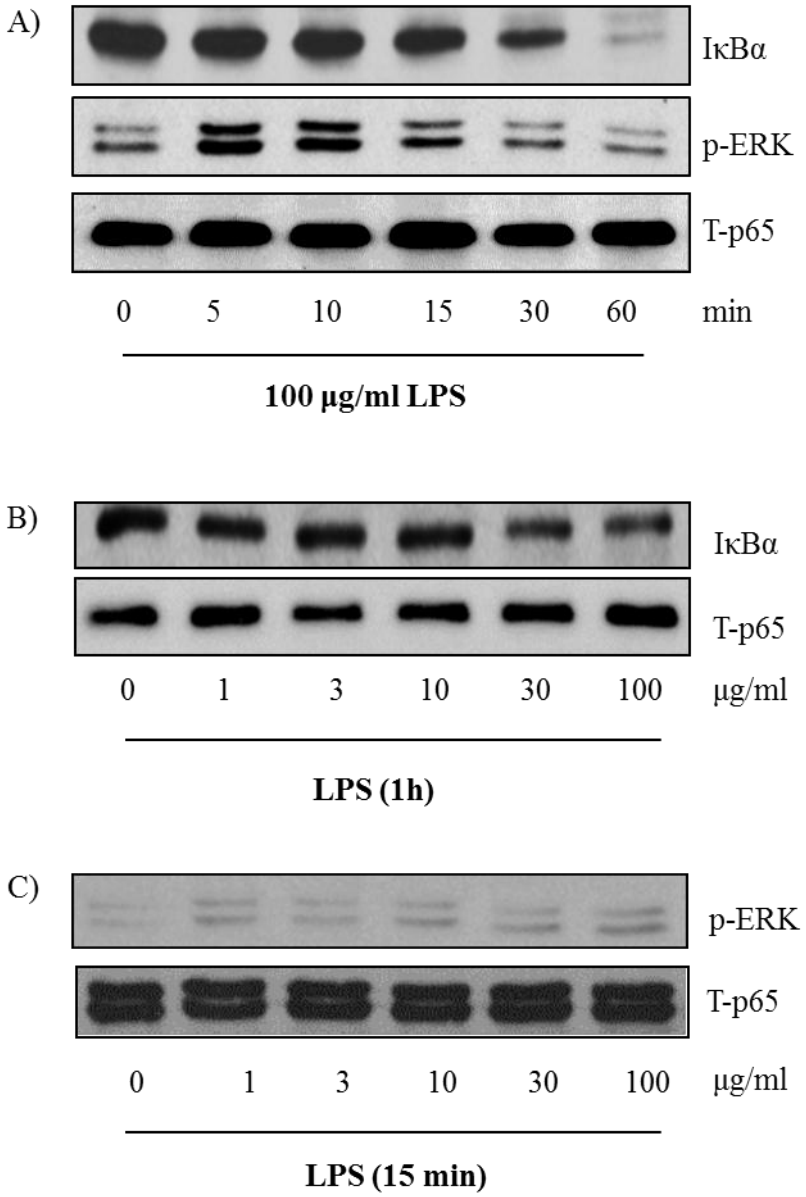


**Figure 5.6 LPS mediated IκBα loss and phosphorylation of ERK.** Cells were stimulated with LPS at concentration. (A) 100 µg/ml for a period of 120 minutes and (B) with different concentrations of LPS as indicated for (B) 60 minutes and (C) 15 minutes. Whole cell lysates were prepared, separated by SDS PAGE and assessed for IκBα loss and phosphorylation of ERK. Data was collected from single experiment.

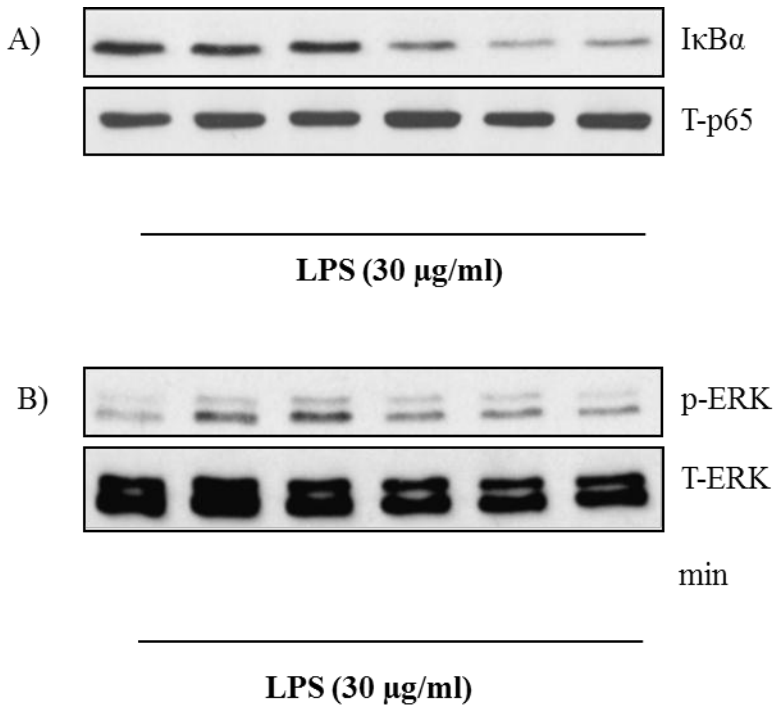


**Figure 5.7 LPS mediated IκBα loss and phosphorylation of ERK.** Cells were stimulated with LPS at concentration 30 µg/ml for a period of 120 minutes. Whole cell lysates were prepared, separated by SDS PAGE and assessed for (A) IκBα loss and (B) phosphorylation of ERK. Data represent two independent experiments.

### 5.3.5. Effect of chlorinated and oxidized lipids on LPS-induced IκBα degradation.

HUVECs were pre-treated with SOPC ClOH or oxPAPC for 60 minutes, followed by stimulation with LPS (30 μg/ml) for another 60 minutes. LPS alone induced degradation of IκBα with a magnitude of 0.312 fold of basal control (Figure 5.11A). However, pre-treatment with SOPC ClOH (1-50 μM) did not reverse IκBα degradation mediated by LPS. Similarly, treatment with 2-ClHDA did not affect LPS-induced IκBα degradation (data not shown). The effect of the oxidized phospholipid complex, OxPAPC, was compared to chlorinated lipids tested in parallel. In contrast to chlorinated lipids, OxPAPC (25 μM) was shown to induce a reversal of LPS-induced IκBα degradation. In addition, results in figure 5.12A showed that at OxPAPC concentration-dependently inhibited LPS mediated IκBα degradation, the highest concentration used in this experiment induced approximately 99% inhibition of the LPS-mediated effect.

### 5.3.6. Effect of chlorinated and oxidized lipids on LPS-induced p-ERK phosphorylation.

The effect of SOPC ClOH and OxPAPC pre-treatment on LPS-stimulated induction of phosphorylation of ERK was also examined (Figure 5.11B). LPS (30 μg/ml) markedly increased ERK phosphorylation after 15 minutes stimulation. Pre-treatment of cells with the lowest (1 μM) the highest (50 μM) concentrations of SOPC ClOH used in this study slightly reduced the LPS-mediated ERK phosphorylation; however, there was apparently no effect observed in cells treated with 3-25 μM SOPC ClOH. The effect of OxPAPC (25 μM) tested in parallel with SOPC ClOH

has been shown to slightly inhibit LPS-induced ERK phosphorylation. However further experiments showed that OxPAPC clearly inhibits LPS-induced phosphorylation of ERK after treatment with 10 μM OxPAPC (Figure 5.12B).

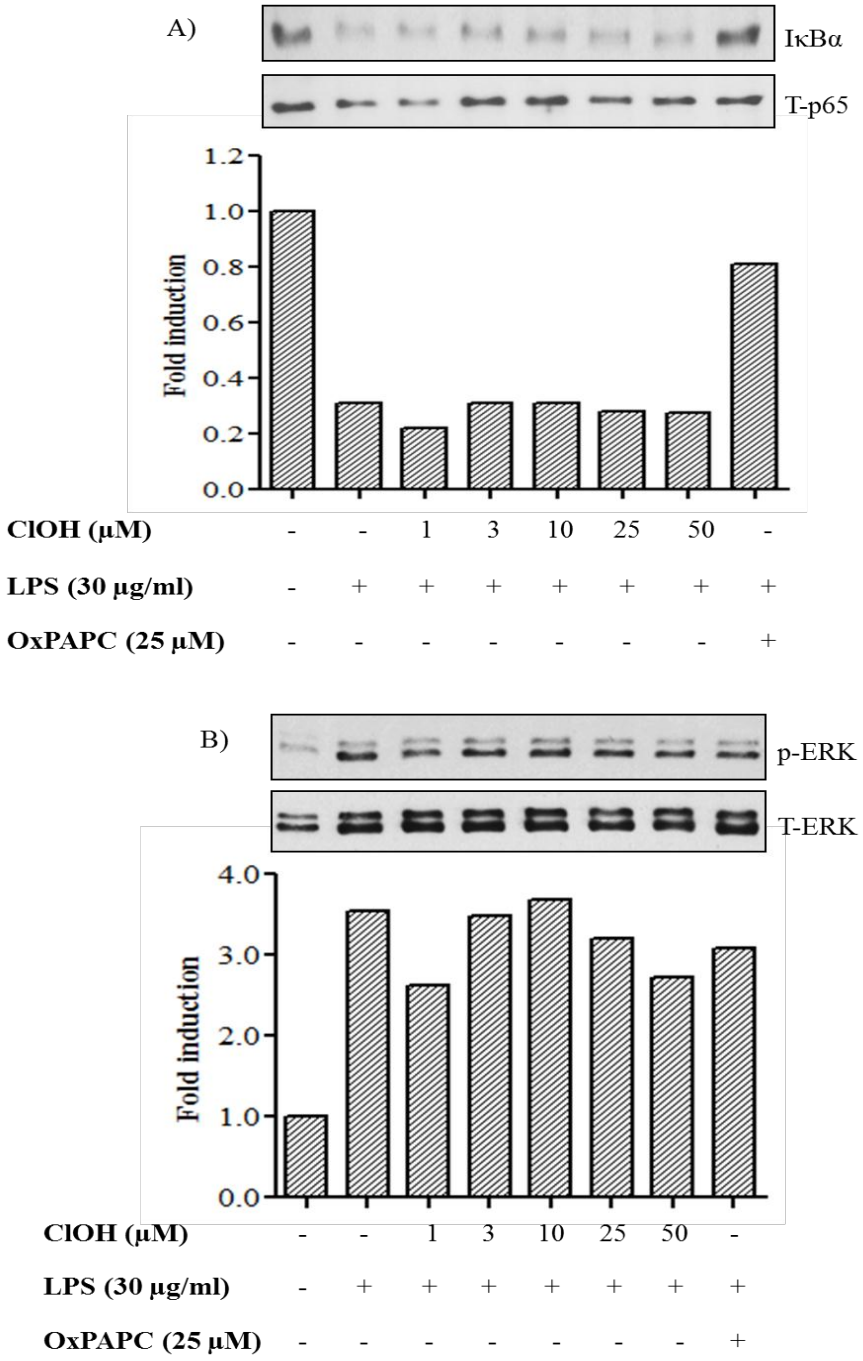


**Figure 5.8 Effects of SOPC ClOH on IκBα degradation and phosphorylation of ERK mediated by LPS in HUVECs.** Cells were pre-treated with different concentrations of SOPC ClOH and OxPAPC (25μM) for 30 minutes before stimulation with LPS (30 μg/ml) for 1 hour or 15 minutes. Whole cell lysates were prepared, separated by SDS PAGE and assessed for (A) IκBα loss and (B) phosphorylation of ERK as outlined in section 2.6.5. Blots were quantified for fold induction by scanning densitometry.

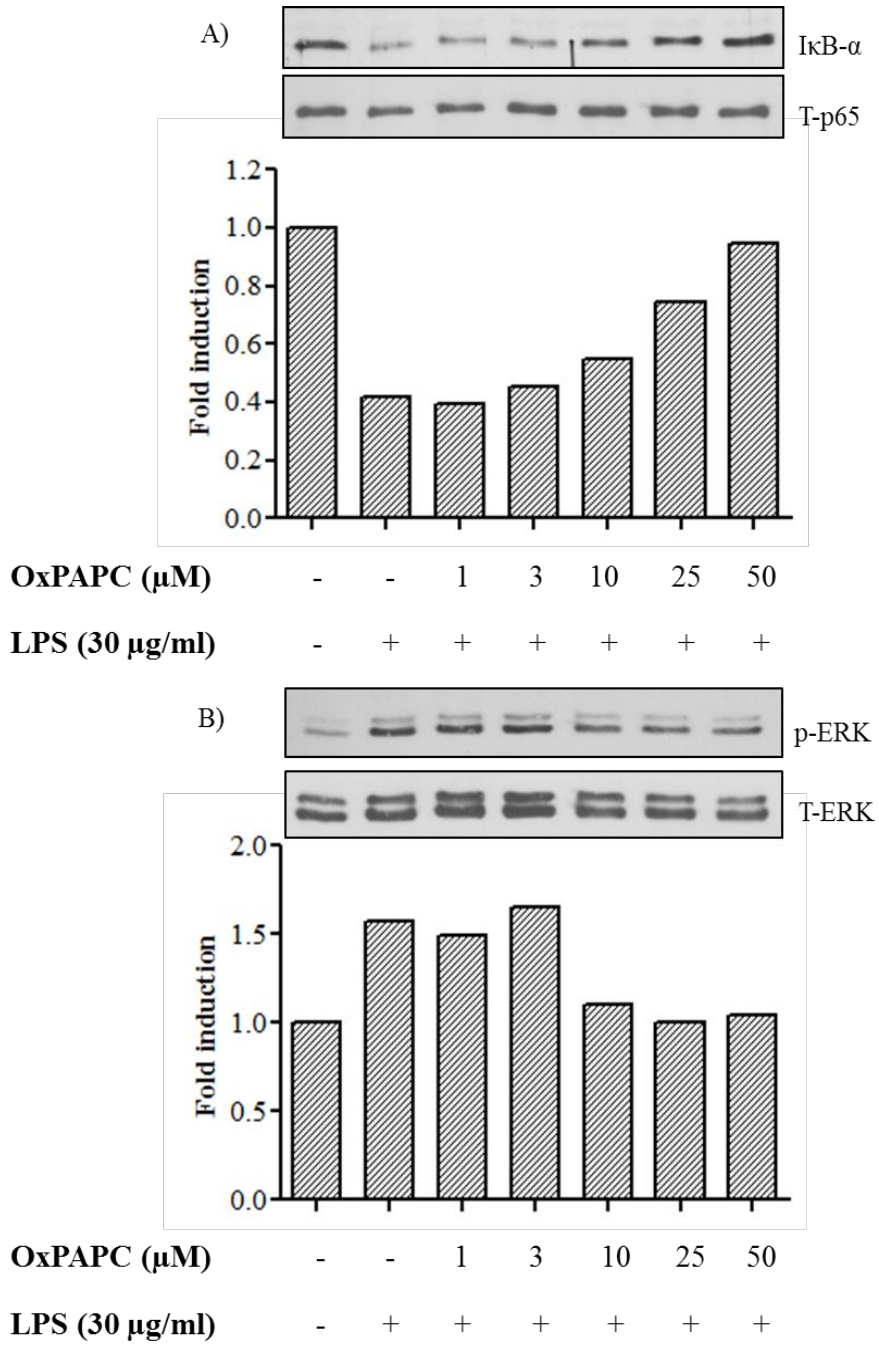


**Figure 5.9 Effects of OxPAPC on IκBα degradation and phosphorylation of ERK mediated by LPS in HUVECs.** Cells were pre-treated with different concentrations of OxPAPC for 30 minutes before stimulation with LPS (30 μg/ml) for 1 hour or 15 minutes. Whole cell lysates were prepared, separated by SDS PAGE and assessed for (A) IκBα loss and (B) phosphorylation of ERK as described in section 2.6.5. Blots were quantified for fold induction by scanning densitometry.

### 5.3.7. Effect of chlorinated lipids on TNFα-induced IκBα degradation.

It is known that LPS and TNF-α can induce activation of NF-κB and MAPK pathways. OxPAPC was reported to be capable of inhibiting this signalling however, the effect is specific for LPS induction only (Bochkov et al., 2002a). Based on an earlier result shown in figure 5.11A, chlorinated lipids did not inhibit LPS-mediated IκBα degradation however, whether the effect of TNF-α can be inhibited by these lipids is not known. In figure 5,13A, results show that treatment of cells with SOPC ClOH alone did not affect degradation of IκBα, suggesting that the compound alone did not activate NF-κB pathway. This is consistent with the above experiments where treatment of chlorinated alone did not induce NF-κB activity. Upon treatment with TNF-α, IκBα was degraded; however, pre-treatment with SOPC ClOH appeared to have no effect of TNF-α mediated IκBα degradation. A similar effect was observed in cells treated with 2-ClHDA, where 2-ClHDA neither induced basal NF-κB activity nor inhibited TNF-α mediated NF-κB activation (Figure 5.13B).

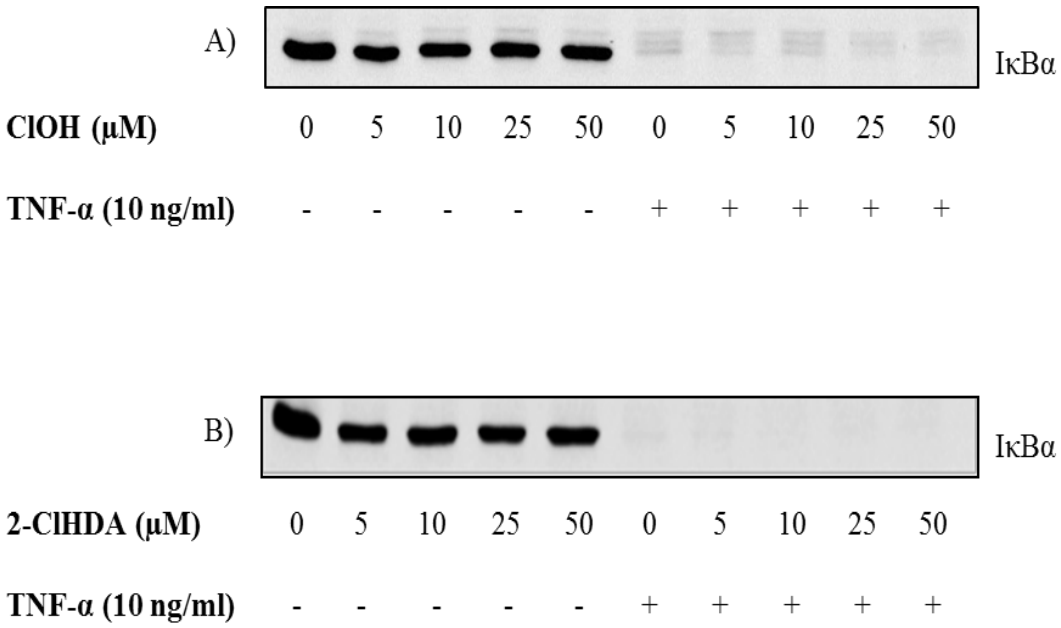


**Figure 5.10 Effect of SOPC ClOH and 2-ClHDA on IκBα degradation mediated by TNF-α in HUVECs.** Cells were either treated with different concentrations of SOPC ClOH alone for 75 minutes or pre-treated with and for 60 minutes before stimulation with TNFα (10 ng/ml) for 15 minutes. (B) 2-ClHDA Whole cell lysates were prepared, separated by SDS PAGE and assessed for IκBα loss as described in section 2.6.5.

### 5.3.8. Effect of chlorinated lipids on HUVECs morphology.

SOPC ClOH has been shown previously to induce cell death in myeloid cells (Dever et al., 2006), however the effect in endothelial cells is not known. Thus, a preliminary study was conducted to investigate whether chlorinated lipids have the potential to induce cell death in HUVECs. Figure 5.14 shows that treatment with M199 alone and different concentrations of SOPC ClOH over 24 hours did not change the morphology of HUVECs. Upon stimulation with TNFα, a few of cells were found to round up. However the confluency was comparable to control. When cells were pre-treated with SOPC ClOH, even the lowest concentration of lipid (5 μM) resulted in cell death following TNFα-stimulation and similar effects were observed with increasing concentrations of lipid. The confluency was reduced by approximately 10% compared to cells treated with 10 ng/ml TNF-α alone.

Similarly, treatment with 0.1% ethanol (vehicle) and 2-ClHDA alone did not have any effect on cell morphology; but, treatment with TNF-α caused some cells to round up. Pre-treatment with the lowest concentration of 2-ClHDA was found to cause even more cell rounding; however, cell confluency was comparable to the one treated with TNF-α alone (approximately 90%). Pre-treatment with 10 μM 2-ClHDA resulted in an increase in the number of cells rounded up and confluency was found to be slightly reduced by approximately 10%. Pre-treatment with the highest concentration of 2-ClHDA caused more cell death and the confluency of cells were found to be reduced to 70% compared to cells treated with TNF-α. At the lowest concentration of lipids, SOPC ClOH was found to have an effect in inducing cell

death in TNF-α stimulated cells. However, at the highest concentration, 2-ClHDA was found to have more prominent effect.

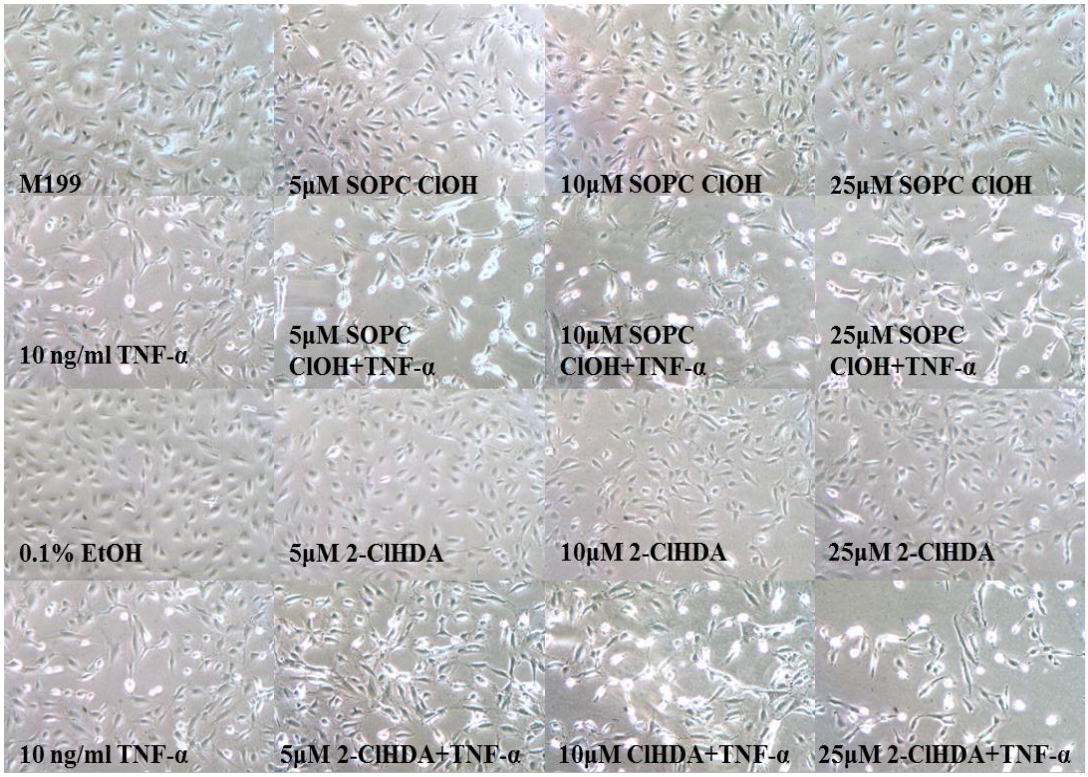


**Figure 5.11 Observation of HUVECs morphology by light microscopy.** Cells were treated with different concentrations of chlorinated lipids alone or pretreated for 30 minutes with chlorinated lipids and followed by stimulation with TNF-α (10 ng/ml) for 24 hours. Result was obtained from a single experiment.

## 5.4. Discussion

It is well established that OxLDL plays an important role in atherosclerosis and the mechanisms involved in mediating its effects have been described extensively. Since OxPLs were found to be the active agents of OxLDL, various studies were then undertaken to investigate their effects as well as signalling mechanisms.OxPAPC products has been reported to affect nuclear factor of activated cell T-cells (NFAT), MAPK, and JAK pathways, and also to inhibit the NF-κB pathway induced by bacterial products such as LPS. More recently, several reports showed that chlorinated lipids may also have similar effects to OxPAPC; however, knowledge of their signalling mechanisms is still lacking.

In the present study, HUVECs were utilized to investigate two inflammatory signalling pathways, MAPK and NF-κB, which may possibly be involved in mediating the effects of chlorinated lipids. Moreover, these cells were used to determine whether chlorinated lipids can attenuate the effects induced by LPS and TNF-α. HUVECs were used as a model because these cells have been widely used to study the role of endothelial cells in atherosclerosis and it is well established that LPS and TNF-α induce the expression of adhesion molecules and cytokine production in HUVECs (Yan et al., 2001), through activation of NF-κB and MAPK signalling. Our findings are unfortunately inconclusive due to limited repetitions, although the preliminary data suggests that effects of SOPC ClOH and 2-ClHDA were apparently not mediated through MAPK or NF-κB pathways, but potentially through another mechanism as yet unidentified.

NF-κB is an oxidative stress transcription factor that controls various genes active in inflammation, including cytokines such as TNFα and IL-8, enzymes such as inducible nitric oxide synthase (iNOS) and adhesion molecules. Whether OxLDL induces activation of NF-κB pathway remains a subject of controversy. Hamilton and coworkers demonstrated that OxLDL induced the NF-κB pathway in monocytes (Hamilton et al., 1998), whereas another group found that OxLDL down-regulated NF-κB-mediated transcription induced by pro-inflammatory cytokines. However, Bochkov et al demonstrated that OxPAPC, the active component of oxLDL, did not induce classical NF-κB pathway (Bochkov et al., 2002b).

Previous studies showed that α-chloro fatty aldehyde (2-ClHDA) can induce the expression of COX-2 by increasing the transcription of the COX-2 gene, and that 2-ClHDA induced degradation of IκB-α, that leads to activation of NF-κB pathway (Messner et al., 2008a). Because the promoter for the human COX-2 gene can be regulated by p65 NF-κB, it is possible that increased levels of COX-2 may be mediated through this pathway. In addition, it was shown that the expression of P-selectin induced by OxLDL is dependent on the NF-κB pathway (Wang et al., 2011). As SOPC ClOH also induced the expression of induction of P-selectin, it was thought that this effect could be mediated through NF-κB as well. However, results obtained from this study showed that both 2-ClHDA and SOPC ClOH did not induce degradation of IκBα in HUVECs. The effect of 2-ClHDA obtained in this study was inconsistent to the results shown by Messner and colleagues (Messner et al., 2008a). This group showed that compared to control, 2-ClHDA (50μM) induced IκBα degradation in HAEC after 20 hours stimulation and the effect. However, because

the total protein was not shown, it is arguable whether this effect is real. In the present study HUVECs were also treated with 50 μM 2-ClHDA or media alone for up to 24 hour. Both treatments reduced IκBα protein. Together, these findings suggest that SOPC ClOH did not induce activation of NF-κB pathway but 2-ClHDA was apparently may have different effect in different type of cells.

The MAPK pathways are also activated during inflammation. Previous studies reported that some effects of OxLDL, including cell proliferation and cytotoxicity on macrophages, endothelial cells and smooth muscle cells, were mediated through the p38 MAPK and ERK pathways (Senokuchi et al., 2004, Li et al., 2003, Chien et al., 2003). Similarly, OxPAPC was demonstrated to induce expression of tissue factor through the ERK pathway (Bochkov et al., 2002). In agreement with results obtained by Bochkov et al., we demonstrated that OxPAPC induced the expression of ERK. In contrast, SOPC ClOH and 2-ClHDA did not induce the activation of this pathway, suggesting that the effects of both lipids may be mediated through other pathways.

Activation of NF-κB and MAPK pathways were shown to induce various pro-inflammatory and pro-atherogenic genes, suggesting that these pathways are suitable targets to prevent inflammation. Previously, OxPAPC products were shown to inhibit LPS-mediated IκBα degradation in endothelial cells, this effect was suggested to be mediated through inhibition of TLR-4 activation (Chow et al., 1999, Beutler, 2000). Initial experiments with LPS demonstrated degradation of IκBα and activation of MAPK, particularly the ERK pathway. In combination with OxPAPC, we demonstrated that degradation of IκBα was attenuated and expression of ERK was

inhibited. This result was consistent with the previous study by Bochkov et al., which suggests that OxPLs inhibit LPS induced TLR-4 pathway by preventing the interaction of LPS with CD14 and LBP (Bochkov et al., 2002a).

With regards to chlorinated lipids, no studies have previously been carried out to investigate whether these lipids can inhibit LPS-mediated activation of NF-κB and MAPK. However, the results in chapter 3 showed that 2-ClHDA inhibited TNF-α production induced by LPS in macrophage-like cells, U937. Surprisingly, the results obtained demonstrated that 2-ClHDA did not inhibit LPS-mediated IκBα degradation (data not shown). It is possible that the effect of 2-ClHDA is cell specific, therefore further investigation could be carried out in macrophages to understand the anti-inflammatory effects of 2-ClHDA on cells stimulated with LPS.

Stimulation of cells with the potent proinflammatoy cytokine, TNF-α, resulted in activation of TNFR1, which can initiate programmed cell death and activation of a transcription factor NF-κB and a kinase, JNK (Shen and Pervaiz, 2006). Persistent activation of TNFα-induced JNK activation can result in apoptosis. In addition, ROS is known to play an important role in TNFα-mediated JNK activation and ROS itself can induce activation of JNK. Prolonged JNK activation by TNF-α and ROS can be suppressed by NF-κB, which provides protection from TNFα-induced apoptosis (Shen and Pervaiz, 2006). Moreover, antioxidant enzymes such as manganese superoxide dismutase (MnSOD), the expression of which is under transcriptional control of NF-κB, limits JNK activity through inhibition of ROS formation (Shen and Pervaiz, 2006). With regards to HUVECs, TNF-α can mediate cell apoptosis but

only if NF-κB is directly inhibited (Zen et al., 1999). In the present study, it was found that SOPC ClOH and 2-ClHDA did not inhibit TNFα-induced IκBα; however, these lipids were able to induce cell death in TNFα-stimulated endothelial cell. Therefore, it is tempting to speculate that these lipids could interfere with NF-κB activity at the transcriptional level

In conclusion, chlorinated lipids did not induce activation of ERK, JNK and p38 MAPK and NF-κB pathways in endothelial cells. In addition, these modified lipids did not inhibit activation of either pathway in LPS- or TNFα-stimulated cells. Moreover, although it has been reported previously that chlorohydrin may induce apoptosis and toxicity, observation by microscopy showed that chlorohydrin did not induce cell death in endothelial cells, although, it is possible that it can activate cell death signal in TNFα-activated cells.

# Chapter 6

# General Discussion

## 6.1 Importance of oxidized and chlorinated lipids in inflammation.

It is becoming increasingly accepted that HOCl-modified LDL is important in atherosclerosis, but, whether the effects are mediated through protein modifications or lipid modifications are not completely understood; although, more recently chlorinated lipids including chlorohydrins and α-chlorofatty aldehydes were shown to have comparable effects to OxPLs of MM-LDL. The overall aims in the present study, therefore, were to investigate the biological effects and the signalling mechanisms of phospholipid chlorohydrin (SOPC ClOH) and α-chloro fatty aldehyde (2-ClHDA) in comparison to oxidized phospholipid (OxPAPC, POVPC and PGPC). Firstly, human myeloid cell lines were used to investigate effects of SOPC chlorohydrin and 2-ClHDA alone or in presence of microbial product (LPS) on pro-inflammatory cytokines production. Subsequently, transfected L929sA cells and HEK cells were used to study the nuclear signalling pathways (NF-κB dependent promoter and PPRE driven gene, respectively) and HUVECs were used to determine the effect of these chlorinated lipids on intracellular signalling pathways (MAPK and NF-κB pathways).

This study demonstrates for the first time that co-treatment of SOPC ClOH but not native SOPC with LPS (lipids and LPS were treated at the same time), induced production of IL-8 but not TNF-α by myeloid cells. However, treatment with chlorohydrin alone was not sufficient to induce IL-8 production. Thus, it appears that SOPC ClOH enhances the effect of LPS, rather than inducing direct stimulation on the signalling pathways leading to IL-8 production. However, the mechanism of

which chlorohydrin enhanced LPS-induced IL-8 production has not been investigated in the present study.

Interestingly, it has been reported that SOPC ClOH enhanced PMA-induced ROS production through activation of CD36 dependent mechanism (Dever et al., 2008), which suggest that binding of SOPC ClOH to scavenger receptor CD36 is an important step in mediating cell signalling pathways. In the present study, cells were activated by PMA prior to stimulation with chlorohydrin or LPS or combination of both treatments. It is well established that PMA, the PKC agonists, can induce CD36 expression (Yesner et al., 1996) and production of IL-8 can be mediated through CD36 pathway; therefore, it is possible to speculate that in presence of LPS, SOPC ClOH could enhance IL-8 production through activation of CD36 pathway. However, it is of importance to keep in mind that the present study showed that SOPC ClOH did not enhance TNF-α production, which supposedly could also be mediated through CD36 pathway. Therefore, further investigation is needed to determine whether CD36 pathway could contribute to the effect of SOPC ClOH with regards to increase IL-8 production.

Moreover, the present study shows that SOPC ClOH did not increase or inhibit LPS-induced IκBα degradation or ERK phosphorylation. This is in contrast to the finding in myeloid cells, where SOPC ClOH enhanced the effect of IL-8 production. However, unlike myeloid cells, it has been shown that CD36 was not expressed in HUVECs (Swerlick et al., 1992). Based on the hypothesis that IL-8 production can be mediated by SOPC ClOH through CD36 pathway, this finding adds to the

possibilities that CD36 could involve in mediating the effects of SOPC ClOH. On the other hand, the present study has also shown that pre-treatment of SOPC ClOH did not affect LPS-mediated IL-8 production; however the incubation time was shorter, therefore at this point, it is not known whether pre-treatment of cells with chlorohydrin could induce different effect compare to when cells were treated with SOPC ClOH and LPS at the same time.

Some effects of SOPC ClOH are comparable to OxPLs; however, the effect of SOPC ClOH with regards to IL-8 and TNF-α production appears to be in contrast to those reported for OxPAPC. Several studies, including the present one, have consistently shown that OxPAPC inhibited LPS-induced IL-8 and TNF-α production in THP-1 cells and reversed LPS-induced IκBα degradation in endothelial cells, and OxPAPC alone mediated phosphorylation of ERK (Erridge et al., 2008, Bochkov et al., 2002b). Moreover, it has been shown that HOCl-LDL may induce IL-8 production in human monocytes; however, the effect observed is mediated by HOCl-modified proteins (Woenckhaus et al., 1998). Overall this suggest that phospholipids chlorohydrin may have different role from chlorinated protein and OxPLs; but, this would have implications for their potential effects *in vivo*, where they induce effects when other pro-inflammatory mediators were exist.

With regards to a-chloro fatty aldehyde, it was shown for the first time that 2-ClHDA reduced LPS-induced TNF-α level. This effect is comparable to that induced by oxPAPC (Erridge et al., 2008, Ma et al., 2004); however, it is important to note that, unlike OxPAPC, 2-ClHDA did not inhibit LPS-induced IL-8 production. The

signalling mechanism of 2-ClHDA mediated inhibitory effect is not investigated in the present study; however, it could possibly regulate different signalling mechanisms compare to OxPLs. Previously, OxPAPC was shown to inhibit TNF-α production by preventing LPS activation of TLR-4 receptor, which in turn results in deactivation of NF-κB and MAPK pathway. However, in the present study, 2-ClHDA did not inhibit LPS-induced degradation of IκBα and phosphorylation of ERK, suggesting that the effect of 2-ClHDA could be mediated through as yet unknown mechanism. Moreover, the present study demonstrates that 2-ClHDA alone did not induce TNF-α in myeloid cell and did not induce IκB-α degradation or phosphorylation of ERK in HUVECs, suggesting that 2-ClHDA did not activate NF-κB pathway. However, this was in contrast to previous finding, where 2-ClHDA was shown to induce activation of NF-κB pathway in human coronary artery endothelial cells (Messner et al., 2008a).

The concentration of chlorinated lipids used in present study was pathophysiological relevant; therefore, the effect observed in this in vitro studies could reflect the same effect *in vivo*. Earlier, evidence suggest that concentration of free HOCl in inflammatory area can reach more than 300μM (Katrantzis et al., 1991), however, in the *in vivo* situation, it is possible that HOCl can interact with other biological molecules in the environment, particularly proteins, indicating that lower concentration of lipid chlorohydrin and α-chloro fatty aldehyde might present in physiological level. Later on, it has been identified that concentration of 2-ClHDA in human neutrophils was ranging from 25 to 100 μM (Thukkani et al., 2002). With regards to OxPLs, it has been identified that level of PGPC in human atherosclerotic

lesion was 20-40 μg/g tissue and SOPVPC, PAzPC and SAzPC were 1-8 μg/g tissue (Ravandi et al., 2004), whereas in atherosclerotic lesion in rabbit aorta, level of POVPC, PGPC and PEIPC were 40-100 μg/g tissue (Watson et al., 1997, Subbanagounder et al., 2000). By estimating 1 g tissue is corresponding to 1 ml volume, the concentration POVPC in 1 μg/g tissue is 1.7 μM. This suggests that concentration of oxidized phospholipids in atherosclerotic lesions could be ranging from 1 to 100 μM (Greig et al., 2012).

Another important finding of the present study was that SOPC ClOH can cause activation of PPARα through PPRE-dependent mechanism in transfected HEK 293 cells. Similarly, the present study also demonstrated that OxPAPC induced PPRE-driven gene through PPARα, which is in agreement with previous finding by Lee et al, where they have shown that OxPLs induce PPRE luciferase promoter activity in endothelial cells and activate PPARα in CV-1 cells (Lee et al., 2000). Similar group also reported that PPARα agonist WY14643 but not PPARγ agonist induces IL-8 production in endothelial cells (Lee et al., 2000). In contrast, different studies have reported that in myeloid cells, PPARγ agonist, but not WY14643, induces IL-8 mRNA level (Fu et al., 2002, Zhang et al., 2001). This suggests that the effect of PPAR agonists may be cell specific; possibly depending on the level of PPAR expression, as it has been shown that higher level of PPARα is expressed in endothelial cells while higher level of PPARγ is expressed in myeloid cell (Jackson et al., 1999). With regards to 2-ClHDA, it has been shown previously that 2-ClHDA induced activation of COX-2, which catalyses the production of prostacyclin (Messner et al., 2008a). Prostacyclin was shown to be able to induce activation of

PPARβ (Barish et al., 2008). However, in the present study, because the experiment was carried out once and the variability within assay was high, it was not clear whether 2-ClHDA could induce PPARβ activation.

## 6.2 Future Work

The present study has demonstrated that SOPC ClOH enhanced LPS-induced IL-8 production and 2-ClHDA prevented TNF-α production induced by microbial products. Moreover, SOPC ClOH has been shown to induce PPRE-driven gene activation through activation of PPARα. Concurrently, however, the study has inevitably raised more questions regarding these emerging myeloperoxidase-derived chlorinated lipid products. For example, what is the signalling mechanisms involved in mediating synergy effect of SOPC ClOH and inhibitory effect of 2-ClHDA in myeloid cell? Further investigation into this matter would perhaps involve the study of signalling mechanisms in myeloid cells, to determine whether the effect of SOPC ClOH and 2-ClHDA is cell type specific. In addition, although SOPC ClOH was shown to activate PPARα, further studies are needed to determine whether activation of PPARα is mediated through receptor-dependent or receptor-independent mechanisms. Radioligand binding assay is one possible indirect method to determine whether chlorohydrin could bind to the receptor. In that assay, cells are treated with radioligand and subtype specific agonist and the affinity to bind to receptor is measured by calculating the amount of receptor-bond tracer (Thibault and Schiffrin, 2000). Moreover, the present study has shown that SOPC ClOH may have similar effect to PPARα agonist (Lee et al., 2000), where SOPC ClOH can activate PPARα but it did not induce IL-8 production in myeloid cells. Because PPARα agonist can

induce IL-8 level in endothelial cell, it is of interest to further investigate whether SOPC ClOH could induce IL-8 production in this type of cells. As SOPC ClOH can activate PPARα that is known to induce both pro- and anti-inflammatory effects; more detailed studies are required to investigate what type of biological effects could be induced by SOPC ClOH through activation of this pathway. With regards to 2-ClHDA, it may be of interest to repeat some experiments with PPARβ; possibly treatment of cells with longer period of time, to verify whether 2-ClHDA can induce activation of PPARβ. SOPC ClOH was used as a model to demonstrate the effect of chlorohydrin but chlorohydrin found in human atherosclerotic lesion is lysophosphatidylcholine chlorohydrin (LysoPC ClOH) (Messner et al., 2008b); therefore, it is of interest to investigate whether the effect of this compound is comparable to SOPC ClOH. In addition, preliminary study has shown that SOPC ClOH in presence of TNF-α can induce apoptosis in HUVECs; suggesting that further studies are needed to understand the effects of these chlorinated lipids with regards to apoptotic signalling pathways.

## 6.3. Conclusions

It is becoming increasingly accepted that HOCl-LDL involve in pathophysiology of atherosclerosis. As modification of lipid components can generate production of phospholipid chlorohydrins and α-chloro fatty aldehydes, these could contribute to adverse effect of HOCl-LDL. As describe in Chapter 1, various studies have shown that chlorohydrins and 2-ClHDA may produce mainly pro-inflammatory effects. The present study has generated novel data demonstrating biological effects of SOPC ClOH with regards to pro-inflammatory indications and 2-ClHDA with regards to

anti-inflammatory indications. Moreover, this study has shown that SOPC ClOH can induce activation of PPARα, which is known to be able to induce both pro- or anti-inflammatory effects. In conclusion, these findings suggest that chlorinated lipids may induce pleotropic effects, comparable to OxPLs, suggesting that chlorinated lipids may regulate the pathogenesis of atherosclerosis.

# Chapter 7

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