Lectin-like oxidised low density lipoprotein 1 scavenger receptor regulation of signal transduction in cell function and atherosclerosis

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The candidate confirms that the work submitted is her own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that the appropriate credit has been given within the thesis where reference has been made to the work of other.

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ABSTRACT

Since the discovery of the lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1) by Sawamura and colleagues in 1997, this multi-ligand receptor has been implicated in atherosclerosis and diabetes. Oxidised LDL binding and trafficking via LOX-1 cause the activation of downstream signal transduction that cause pro-athrogenic changes such as endothelial dysfunction, apoptosis and foam cell formation. However, the molecular mechanisms have not be been fully explained. In this study, tetracycline-inducible cell lines expressing LOX-1 wild-type and trafficking-defective LOX-1-D5A were developed. The findings show different trafficking properties between LOX-1-WT and LOX-1-D5A in response to oxidised LDL. Due to these differences, LOX-1-WT and LOX-1-D5A in response to oxidised LDL exhibited differential downstream signal transduction. Moreover, 24 hour stimulation of oxidised LDL via LOX-1-WT caused decreased endothelial cell permeability; however, the underlying mechanism is not clear. The impact of deleting *LOX-1* in *ApoE* knockout mice was reduced aortic plaque coverage. This study revealed that pro-atherogenic signal transduction was reduced in aorta in LOX-1/ApoE double knockout mice compared to *ApoE* knockout mice. Furthermore, the same pro-atherogenic signal transduction was increased in the liver of *LOX-1/ApoE* knockout mice. The differential signal transduction outcomes in the aorta or liver are dependent on the status of the atherosclerosis disease. LOX-1 is reported to play a role in glucose and lipid homeostasis. Previously, deleting *LOX-1* revealed altered glucose metabolism and insulin resistance phenotype. In this study, differences in downstream insulin signalling pathways were exhibited in the skeletal muscle and adipose tissue of *LOX-1* knockout and wild-type mice. Experimental findings also revealed the influence of *LOX-1* genotype in iron metabolism in the liver. This work has provided insights on a potential role of LOX-1 clearing oxidised LDL from the circulation, and for the first time, this study potentially showed the role of LOX-1 in glucose homeostasis and iron metabolism.

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ABBREVIATIONS

AcLDL	Acetylated LDL
AGE	Advanced glycation end-products
Akt	protein kinase B
ANG II	Angiotensin-II
AP-1	Adaptor protein-1
ApoB-100	Apolipoprotein B-100
ApoE	Apolipoprotien
ATF	Activating transcription factor
BCA	Bicinchonic acid
BHT	Butylated hydroxytolunene
BSA	Bovine serum albumin
CAD	Coronary artery disease
CD	Cytoplasmic domain
CRP	C-reactive protein
CTLD	C-type lectin-like domain
CVD	Cardiovascular disease
DAPI	4’. 6-diamidino-2-phenylindole
DiI	1,1’,di-octadecyl-3,3,3’3’-tetramethylindocarbocyanine
DMEM	Dulbecco’s Modified Eagle Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ECD	Extra-cellular domain
ECL	Enhanced chemiluminescence
EDTA	Ethylene-diaminetetra acetic acid
eNOS	Endothelial nitirc oxide synthase

ERK1/2	Extracellular signal-regulated kinase 1/2 (p42/44)
FCS	Foetal calf serum
FEEL-I/II	Fascilin, EGF-like, lamin-type EGF-like and link domain-containing scavenger receptor-I/II
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
GLUT	Glucose transporter
HBS	HEPES buffered saline
HEPES	4-(2-hydroxyethyl)-1-piperazine-1-ethanesulfonic acid
HDL	High-density lipoprotein
HRP	Horseradish peroxidase
ICAM-1	Intercellular adhesion molecule-1
IL	Interleukin
LB	Luria-Bertani
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LOX-1	Lectin-like oxidized low-density lipoprotein receptor-1
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MARCO	Macrophage receptor with collagenous structure
MCP-1	Monocyte chemoattractant protein-1
MCS	Multiple cloning site
M-CSF	macrophage-colony stimulating factor
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
qRT-PCR	Quantitative real-time polymerase chain reaction
ROS	Reactive oxygen species

VEGF	Vascular endothelial growth factor
VCAM1	Vascular cell adhesion molecule-1

CHAPTER 1

Introduction

1.1 Atherosclerosis

According to the World Health Organization fact sheet, cardiovascular disease was the top cause of death in the world in 2000 and 2012 (WHO, 2014). Cardiovascular disease is the leading cause of death in the Western world and is also increasingly prevalent in developing countries. Cardiovascular disease is now responsible for more deaths worldwide than cancer, trauma and infectious diseases, leading it to be labelled a worldwide epidemic (Callow, 2006). In 2012 alone, cardiovascular diseases took more than 15 million lives worldwide, which translate to 3 in every 10 fatalities. The primary etiologic lesion of cardiovascular disease is atherosclerosis that causes vessel stenosis, embolus via thrombus formation, and ischaemia of heart and brain (WHO, 2014).

Atherosclerosis is a chronic disease of the arterial wall and is the principal cause of heart attack, stroke and gangrene of the extremities that is responsible for 50% of all mortality in the USA, Europe and Japan (Ross, 1993). Over the past 50 years, epidemiological studies have revealed a number of environmental and genetic risk factors (Lusis, 2000). The complex aetiology of atherosclerosis has hindered the advancement in describing the cellular and molecular interactions involved in the disease. Research into the disease has led to many compelling hypotheses about the pathophysiology of atherosclerotic lesion formation resulting from an excessive inflammatory fibrous-proliferative response causing various forms of insult to the endothelial cell and smooth muscle cell of the arterial wall (Glass and Witztum, 2001; Libby, 2003; Lusis, 2000). Despite the advances in understanding and preventing the

pathogenesis of atherosclerosis, there is still lack of evidence showing lipoprotein oxidation, inflammation and immunity are involved in human atherosclerosis (Libby et al., 2011).

The beginning process of atherosclerosis starts in early childhood when the first apparent deposit of fats in the intima of the large arteries canbe detected (Holman et al., 1958; McGill et al., 2000; Williams et al., 2002). With the consumption of high-fat, cholesterol diets in industrialised societies, this potentially drives the process of atherosclerosis. Thus, the abundance of plasma lipoproteins seems to be one of the primary risk factors of the disease, with the principal atherogenic lipoprotein in the blood being low-density lipoprotein (LDL) (Tall and Yvan-Charvet, 2015).

At one point, atherosclerosis was thought to be a degenerative disease in relation to aging. Studies in the past decades have shown atherosclerosis is neither a degenerative disease nor predictable (Berliner et al., 1995). On the contrary, the role of inflammation has been appreciated more linking it to atherosclerosis, either causing it directly or indirectly. Pathological studies have demonstrated distinct changes in the vessel during atherogenesis, and also showed the involvement of blood-derived inflammatory cells especially monocytes/macrophages (Lusis, 2000). A vast number of growth factors (vascular endothelial growth factor, VEGF) and cytokines (interleukin-1, IL-1; tumour necrosis factor-α, TNF-α) are also known molecules to participate in the pathogenesis of atherosclerosis (Ross, 1993). Altogether, the accumulation of atherogenic lipoproteins in the intima contributes to the recruitment of monocytes to site of the lesion, which possibly explain the underlying cellular and molecular mechanisms that lead to atherosclerosis (Libby et al., 2002).

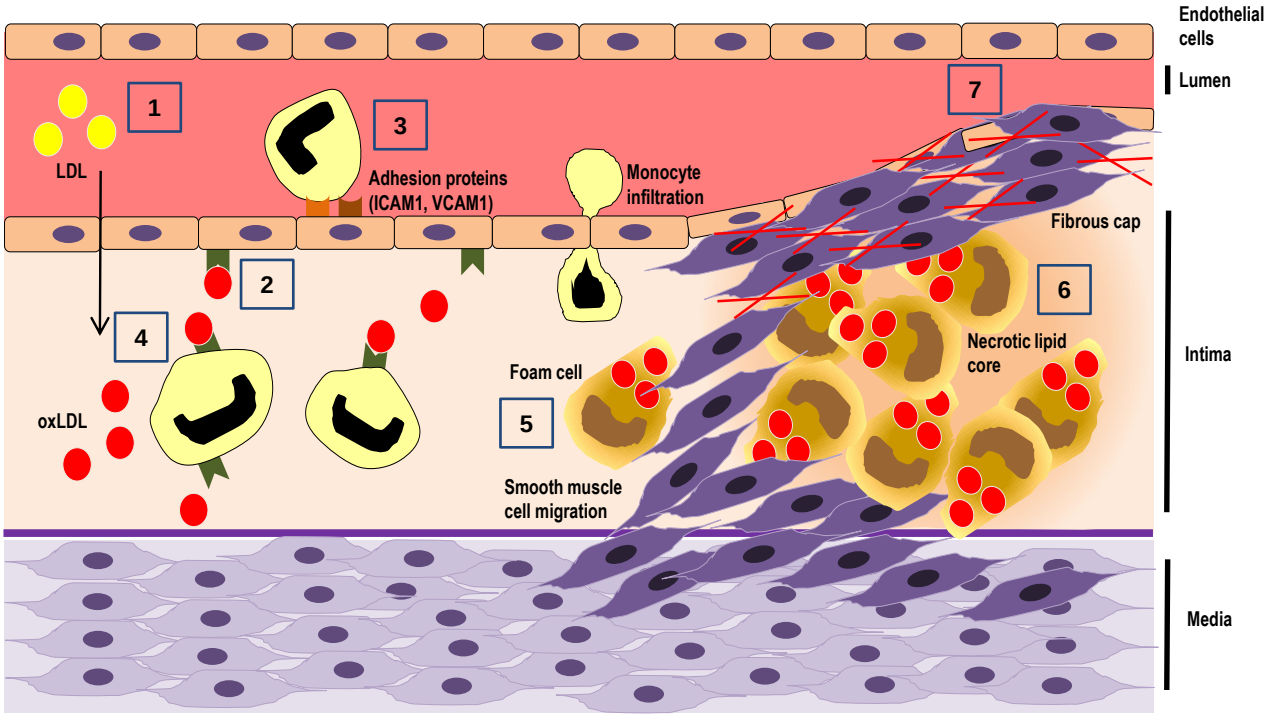

Figure 1.1. Events in atherosclerosis. (1) Low-density lipoproteins (LDL) pass into sub-endothelial layer where it gets oxidised (oxLDL). (2) OxLDL binds to scavenger receptors such as LOX-1 on endothelial cells, (3) triggering expression of adhesion molecules (ICAM1, VCAM1). (4) OxLDL bound to macrophage scavenger receptor such as CD36 accumulates in the cytoplasm, which cause (5) macrophages transform into foam cells. (6) These foam cells and migrated smooth muscle cells form a necrotic core, growing in size and cause (7) lumen narrowing. Taken from (De Siqueira et al., 2015).

1.1.1 Stages of atherosclerosis

The underlying cause of myocardial infarction, stroke, and ischaemic heart pain is due to atherosclerosis. These diseases, collectively, are known for the leading cause of death in the world, and the incidence continues to increase linked to rising epidemic of obesity and Type 2 diabetes (Braunwald, 1997; WHO, 2014). According to the theories on the pathogenesis of atherosclerosis, the possible events of atherosclerosis involve lipid deposition, vascular endothelium injury, monocyte recruitment, macrophage differentiation and vascular smooth muscle cell proliferation, as shown in figure 1.1 (Badimon et al., 1993). These factors form the characteristic of the atherosclerotic plaque.

1.1.1.1 Lesion initiation

The first detectable lesion of the disease is the so-called ‘fatty streak’, which is the retention of apolipoprotein-B (Apo-B)-containing atherogenic lipoprotein within the innermost layer of the artery wall, the intima (Williams and Tabas, 1995). The pathogenesis of atherosclerosis starts with detectable fatty streak early in life as young as 10-14 years of age, furthermore, it is attested to by the finding of fatty streak in animal models, which precede the development of intermediate lesions (McGill, 1984; Stary, 1989; Masuda and Ross, 1990a; Masuda and Ross, 1990b).

The endothelium that lines the innermost layer of artery has tight intercellular junctions that serve as a permeable barrier between the blood and tissues. Mechanical force such as fluid shear stress exerting on endothelial cells has a tremendous effect on the morphology of endothelial cells. In tubular sections of arteries, endothelial cells are polygonal in shape and aligned unidirectional to the blood flow, where blood flow is laminar and uniform. In focal areas of arteries branching or curving, the endothelial cells have no polygonal shapes and no particular co-ordination, thus the blood flow is disturbed (Gimbrone, 1999). Therefore, the regions with more arterial branching are more susceptible for lesion formation and increased permeability to molecules such as lipoproteins. Accumulation of lipoproteins, especially low-density lipoprotein (LDL), in

the sub-endothelial matrix affects the extracellular matrix components within the vascular network, which also stimulate the entry and accumulation of cholesterol-containing lipid particles in the artery wall (Tabas et al., 2007). With increased levels of circulating plasma LDL, the transport and accumulation in the sites of lesion also increase. LDL that diffuses passively through endothelial cell junction retains within the vessel wall, which involves the interaction between Apo-B of LDL and proteoglycans in the extracellular matrix (Boren et al., 1998). The most significant change during early lesion formation is oxidation of lipid when exposed to vascular oxidative products. It has been shown that LDL trapped within the walls undergoes modification, involving oxidation, glycosylation and aggregation that contribute to inflammation (Figure 1.1). The modified product gives rise to oxidised LDL, which is linked to pro-inflammatory activities and recognized by a number of scavenger receptors in macrophages. It is well-known that trapped LDL undergoes modification as native LDL is not well-recognized by scavenger receptor to generate foam cells (Goldstein et al., 1979; Cyrus et al., 1999).

1.1.1.2 Inflammation

Atherosclerosis is characterised by the recruitment of white blood cells (leukocytes) to the artery wall. Endothelial cells in the intima typically resist the adhering of leukocytes, however, when exposed to aggravating conditions such dyslipidaemia and pro-inflammatory mediators like adhesion molecules including intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), and growth factors such as macrophage colony-stimulating factor (M-CSF) are expressed by endothelial cells to attract passing leukocytes on the surface (Ross, 1993; Tabas et al., 2007). Modified LDL also inhibits synthesis of nitic oxide that is involved in initiating and maintaining vasodilation under normal physiological condition (Knowles et al., 2000). The vascular endothelium responds to high plasma levels of LDL and disturbed blood flow, and act in a paracrine and autocrine manner to maintain vascular homeostasis. Hence, alteration of endothelial cell phenotype into a dysfunctional state is a pathogenic risk factor of atherosclerosis.

The migration of leukocytes into the arterial wall is mediated by attachment to adhesion molecules under the influence of both chemotactic factors and growth-regulatory molecules released by the altered endothelium. The initial step in adhesion, which involves the capturing followed by ‘rolling’ of leukocytes along the surface of the endothelium, is mediated by selectins (cluster of differentiation 62; CD62) that are expressed on leukocytes (Tedder et al., 1995; Dong et al., 1998). Integrin very late antigen-4 (VLA-4) expressed on monocytes mediate firm adhesion to VCAM-1 on the activated endothelial cell surface (Alon et al., 1995). It is also known that monocyte chemoattractant protein-1 (MCP-1) is responsible for direct migration of monocytes at sites of lesion on the intima (Gu et al., 1998; Boring et al., 1998). In addition, the proliferation and differentiation of monocytes into macrophages is stimulated by cytokine M-CSF, which also influences the function of macrophages in lesion formation, in terms of expressing scavenger receptors (Smith et al., 1995). Anti-inflammatory small molecules such as nitric oxide regulate numerous critical cell functions during the process of atherosclerosis that involves leukocyte recruitment and migration, smooth muscle cell proliferation and control of synthesis of extracellular matrix proteins (Ross, 1993). At branch points of arteries where atheroprotective mechanisms are limited and blood flow is not uniform and laminar, the production of local endothelium-derived nitric oxide is reduced; therefore, expression of VCAM-1 and ICAM-1 is increased (De Caterina et al., 1995; Nagel et al., 1994).

1.1.1.3 Foam cell and plaque formation

Macrophages are present in all stages of atherosclerosis and function as a scavenger cell removing unwanted particles and as a source of inflammatory cytokines and growth factors molecules (Gown et al., 1986; Jonasson et al., 1986). As the process of atherosclerosis continues, monocytes transmigrate beneath the sub-endothelial layer of the intima, where monocytes differentiate into macrophages. Beneath the intima where macrophages accumulate modified lipoproteins and become foam cells (Figure 1.1). As native LDL is not being taken up by macrophages

sufficiently, presumably, modification of LDL involves reactive oxygen species produced by endothelial cells and macrophages. Several enzymes such as myeloperoxidase, sphingomyelinase and phospholipase are involved in generating highly reactive species, aggregation of lipoprotein leading to accumulation of lipids, and oxidation of LDL (Podrez et al., 2000; Marathe et al., 1999; Ivandic et al., 1999). The rapid uptake of oxidized LDL leading to foam cell formation is mediated by scavenger receptors expressed on macrophages. These scavenger receptors include SR-A1, CD36 and LOX-1 are principle receptors involved in atherosclerosis (Suzuki et al., 1997; Febbraio et al., 2000). Thus, the macrophage is an important pro-inflammatory mediator of cells in the atheromatous plaque microenvironment.

Macrophages also have a role in proliferation, as a result of scavenging oxidised LDL, they produce a number of growth factors and inflammatory cytokines such as platelet-derived growth factor (PDGF), interleukin-1 (IL-1) and tumour necrosis factor α (TNFα) (Heldin and Westermark, 1999; Merhi-Soussi et al., 2005; Branen et al., 2004). The secretion of cytokines and growth factors by macrophages are crucial for smooth muscle cell migration and proliferation and synthesis of extracellular matrix components. It was observed that smooth muscle cells in arteries respond in an autocrine way to PDGF that further stimulates self-secretion of PDGF and also releasing fibroblast growth factor (FGF) into neighbouring injured and necrotic smooth muscle cells and the overlying endothelium (Baird et al., 1990). The growing mass of extracellular atherogenic lipid, the accumulation of migrated smooth muscle cells from the medial layer of artery and smooth muscle cell-derived extracellular matrix make up the fibrous plaques (Figure 1.1). There are a number of risk factors to the development of fibrous plaques such as increased homocysteine, elevated blood pressure and diabetes. Increased homocysteine level in the blood is linked to atherosclerosis by causing endothelial cells to become dysfunctional and stimulate vascular smooth muscle cells proliferation (Gerhard and Duell, 1999). Hypertension mediated by renin-angiotensin pathway cause the

expression of angiotensin II (Ang II) in atherosclerosis, which in turn stimulates the proliferation of smooth muscle cells and synthesis of extracellular matrix components (Negoro et al., 1995). Thus, the smooth muscle cell plays the principal role in the fibro-proliferative part of this disease process.

1.2 Role of lipid and lipoproteins in atherogenesis

Increased serum lipid levels have been strongly linked to the development of atherosclerosis. Cholesterol and triglycerides are important components of the lipid fraction of the human body. Cholesterol is a hydrophobic molecule and an unsaturated alcohol of the steroid family. Cholesterol is essential for the structure of animal cell membrane and also important pre-cursor of bile acids, vitamin D and other steroids; whereas triglycerides are esters derived from glycerol and fatty acids, and they are the main constituents of body fat (Cox and Garcia-Palmieri, 1990; Oliveira et al., 2014). As cholesterol and triglycerides are hydrophobic lipid molecules, the molecules are transported between cells in the plasma by various lipoprotein particles. Plasma lipoproteins are spheroidal particles of various size and composition. The lipid core of lipoproteins are composed of triglycerides and cholesterol esters, surrounded by a monolayer of phospholipids and free cholesterol, and enclosed unevenly by apolipoproteins (Apo) (Figure 1.2). Apolipoproteins are amphipathic in nature, thus they are able to interact with both the lipids of the lipoprotein and the aqueous environment (Segrest et al., 2001). They are grouped into two classes: the non-exchangeable apolipoprotein (ApoB-100) and exchangeable apolipoprotein (ApoA, ApoC and ApoE) (Segrest et al., 1992). Apolipoproteins are important components of lipoproteins by maintaining the structure of the particle and essential for lipoprotein metabolism by binding with specific cell membrane receptors (Dominiczak and Caslake, 2011). Plasma lipoproteins are differentiated by their content of cholesterol, triglycerides and proteins, and generally classified into five major classes: chylomicrons, very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), and

high-density lipoproteins (HDL). The low-density lipoprotein is involved in the delivery of cholesterol to peripheral tissues, and enclosed by only one apolipoprotein (ApoB, predominantly B-100) (Esterbauer et al., 1992); the high-density lipoprotein is responsible in mediating the inverse process of cholesterol transport (Zhang et al., 2003), and enclosed by several apolipoproteins (ApoA, C and E) (Movva and Rader, 2008; Superko, 2009).

1.2.1 Low-density lipoproteins

The role of lipoproteins in atherosclerosis is well-established. A significant relationship between total cholesterol and coronary heart disease is known, however, the total cholesterol level is not an accurate method to predict coronary heart disease in patients. Nonetheless, total cholesterol is the sum of all cholesterol carried by both atherogenic lipoproteins (VLDL and LDL) and anti-atherogenic lipoproteins (HDL). Thus, predicting and treating the disease based on LDL-cholesterol levels is important (Carmena et al., 2004). ApoB-100 is the only protein component in a single LDL particle. LDL of about 180-250 Å in diameter is much smaller in size than the originally secreted VLDL, which ranges between 600-800 Å (Knott et al., 1986; Chen et al., 1986; Campos et al., 1996; McNamara et al., 1996).

Ground-breaking research by Brown and Goldstein on mutations of LDL receptor and accumulation of cholesterol linked to atherosclerosis have provided a very strong platform for cardiovascular research and also provide convincing evidence that increased LDL levels are sufficient for the pathogenesis of atherosclerosis (Brown and Goldstein, 1986). Indeed, a critical initial event in atherosclerosis is the retention of ApoB-containing LDL in the artery wall that mediates an inflammatory response. For any given concentration of LDL-cholesterol in the plasma, LDL retention is more crucial than LDL transport into the artery wall (Schwenke and Carew, 1989). This is supported by the findings that LDL molecules transported across the endothelium were trapped in a three-dimensional cage made of fibres and fibrils secreted by the endothelial cells

(Nievelstein et al., 1991). This explained the higher concentration of ApoB-containing LDL in the artery wall than in the plasma, as LDL associates with extracellular matrix components in the sub-endothelial space (Hoff et al., 1977).

Despite the ground-breaking findings by Brown and Goldstein, lipid-laden foam cells were formed in patients and animals lacking the LDL receptor, similar to what have been seen in patients and animals with functional LDL receptors. Due to this observation, the likely suggestion then was that atherosclerosis is not caused by the uptake of native LDL, but somewhat, native LDL has to go through a modification process. The confusing, yet interesting, enigma was resolved by *in vitro* incubation of modified LDL with a monolayer of arterial endothelial cells and showed increased cellular cholesterol content. Furthermore, *in vitro* incubation of macrophages and oxidised LDL also showed cholesterol ester accumulation (Quinn et al., 1987). The complex process of LDL oxidation involves a change in the proteins and lipids forming more intricate products. Extensive alteration in the protein composition and structure is due to non-enzymatic oxidative changes in amino acids, and proteolysis and cross-links of ApoB (Fong et al., 1987). Therefore, the oxidation of LDL induced by cells is a plausible explanation that could justify for the initial event, or even the acceleration, of the atherosclerotic process.

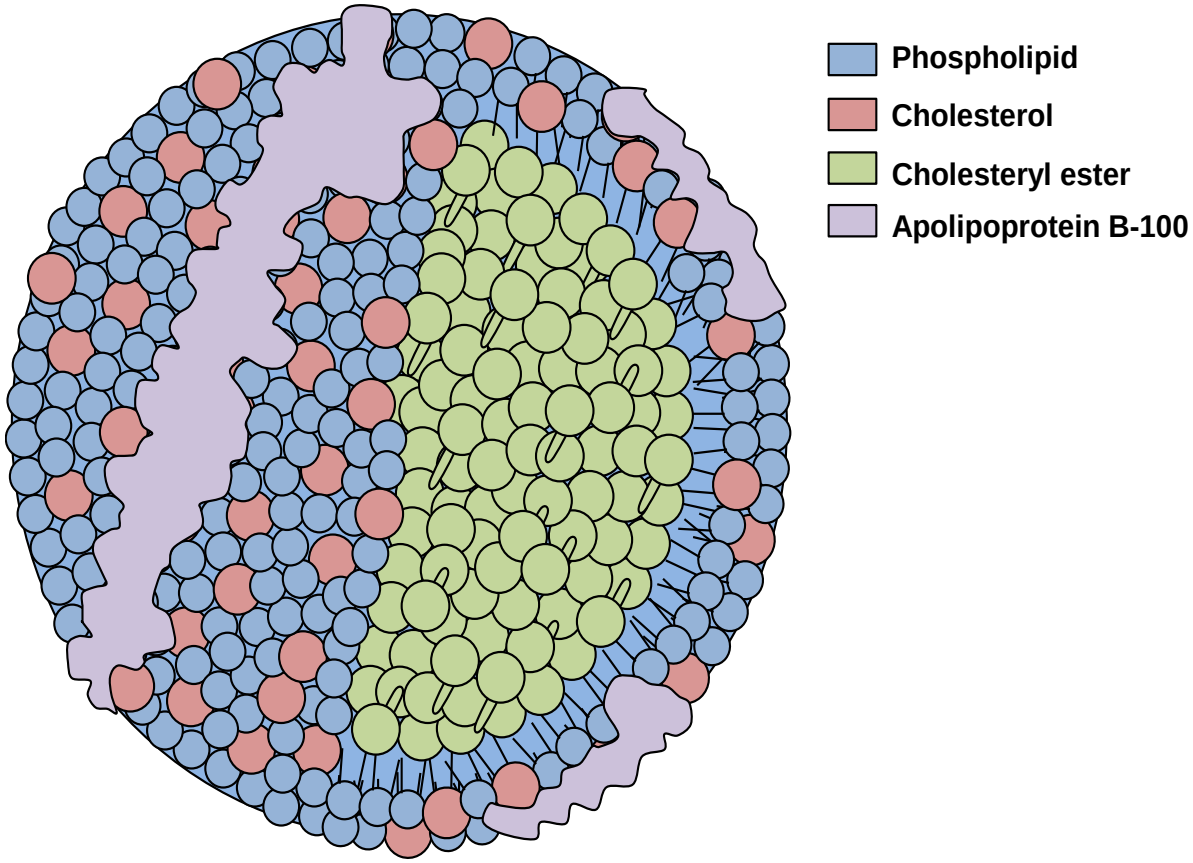

Figure 1.2. The structure of a low-density lipoprotein (LDL) particle. Schematic representation of a LDL particle consists of a hydrophobic core of predominantly cholesterol esters. This is surrounded by a monolayer made from phospholipids, cholesterol and a single apolipoprotein B-100 (ApoB-100). Adapted from (Itabe et al., 2011).

1.2.2 Oxidised low-density lipoprotein

Interest in studying oxidised LDL stemmed from observations that modification of LDL led to rapid uptake in macrophages. Evidence showing the pro-inflammatory and pro-atherogenic properties of oxidised LDL as a result of the oxidative modification process *in vivo* is rather controversial. In spite of extensive research for more than 30 years addressing the sites of LDL oxidation *in vivo*, the nature of oxidizing agents and the properties of oxidised LDL are all matters of controversy. In general, it is known that oxidation of LDL in the circulation is very limited due to the presence of anti-oxidants such as apolipoproteins, serum albumin, ascorbate, tocopherol and urate. LDL oxidation has to take place in the sub-endothelial space of artery wall, where the concentration of anti-oxidants is relatively lower than in plasma. Additionally, oxidation of LDL can be carried out intracellularly, specifically the lysosomal compartment in macrophages (Wen and Leake, 2007). Furthermore, LDL oxidation could also take place at sites of inflammation where leukocytes infiltrate due to increased vascular cell permeability, and perhaps aid into plaque formation (Memon et al., 2000). Another study showed substantial amount of lipid peroxidation products in human atherosclerotic plaques (Suarna et al., 1995).

Oxidised LDL results from exposing LDL to free radicals and non-radical oxidants generated by the vascular endothelial cells through various enzymatic mechanisms. The free radicals generated by the cells include superoxide, nitric oxide, hydroxyl radicals and carbon-centre radicals. LDL particles get oxidized by free radicals effectively oxidize the polyunsaturated fatty acids producing ApoB-derivative breakdown products and alter its receptor recognition to LDL receptor (Halliwell, 2006; Niki, 2011). As for non-radical oxidants such as hydrogen peroxide, hypochlorite and peroxynitrite, they target the proteins of LDL including cysteine, methionine and tyrosine residues. NADPH oxidase, xanthine oxidase, nitric oxide synthase, myeloperoxidase and lipoxygenase generate oxidants within the arterial wall, and these enzymes have been

shown to be present in the atherosclerotic lesions (Stocker and Keaney, 2005).

Oxidation of LDL results in a structural change of the surface lipids and the ApoB backbone becomes denatured and buried in the lipophilic environment (Parasassi et al., 2001). Binding of native LDL to its receptor occurs when positively charged lysine residues in ApoB have high affinity for negatively charged cysteine residue in the LDL receptor binding domain, whereas oxidised LDL lac this property, thus decreasing binding affinity to the LDL receptor (Blanco et al., 2010). Possibly, with the decreased number of accessible lysine residues on the surface and increased electronegativity contributes to its lowered binding capacity to LDL receptor (Benitez et al., 2004). Unlike native LDL, oxidised LDL binds to proteoglycans, the main component of the sub-endothelial extracellular matrix, mediated by the binding of basic residues of ApoB and negatively charged proteoglycans. Thus, retention of oxidised LDL within the vessel wall due to high affinity to proteoglycans contributes to development of atherosclerosis that involves apoptosis, cytokines release, inflammation and cytotoxicity (Bancells et al., 2009).

Oxidised LDL has been reported for its pro- and anti-inflammatory effect due to its nature of being a complex molecule. The extent of oxidised LDL in determining pro- or anti-inflammatory results depend on what the molecule is interacting within the cell, or what receptors being bound. The pro-inflammatory effects are primarily mediated by NF-κB, STAT 1/3, AP-1, to name a few, whereas anti-inflammatory effects are mediated by PPARs and HO-1 (Robbesyn et al., 2004). Oxidised LDL is an important factor in atherosclerotic plaque formation by producing cytotoxic effect, stimulating apoptosis, and producing leukocyte recruitment mediators in endothelial cells (Chen et al., 2003; Sanchez-Quesada et al., 2003; Chang et al., 1997). Therefore, oxidised LDL is a potent biomarker in relation to atherosclerosis.

1.3 Scavenger receptors

Scavenger receptors comprise a diverse array of integral membrane proteins and soluble-secreted extracellular domain isoforms. These proteins are termed scavenger receptor ‘supergroup’, as opposed to a superfamily, as this latter term implies primary sequence similarity shared across the whole supergroup. A key point is that scavenger receptor members within each class bear primary sequence similarity but different classes bear little or no primary sequence similarity. The common feature uniting this disparate group of proteins within the scavenger receptor supergroup is their ability to recognise common ligands such as lipoproteins, apoptotic cells, cholesterol esters, phospholipids, proteoglycans, ferritin, and carbohydrates. Scavenger receptors were initially identified on the basis of their biochemical ability to recognise and bind different modified forms of LDL e.g. oxidized LDL. Based on our current understanding of SR structure and biological function, these proteins are grouped into classes A-J (Figure 1.3) (Abdul Zani et al., 2015).

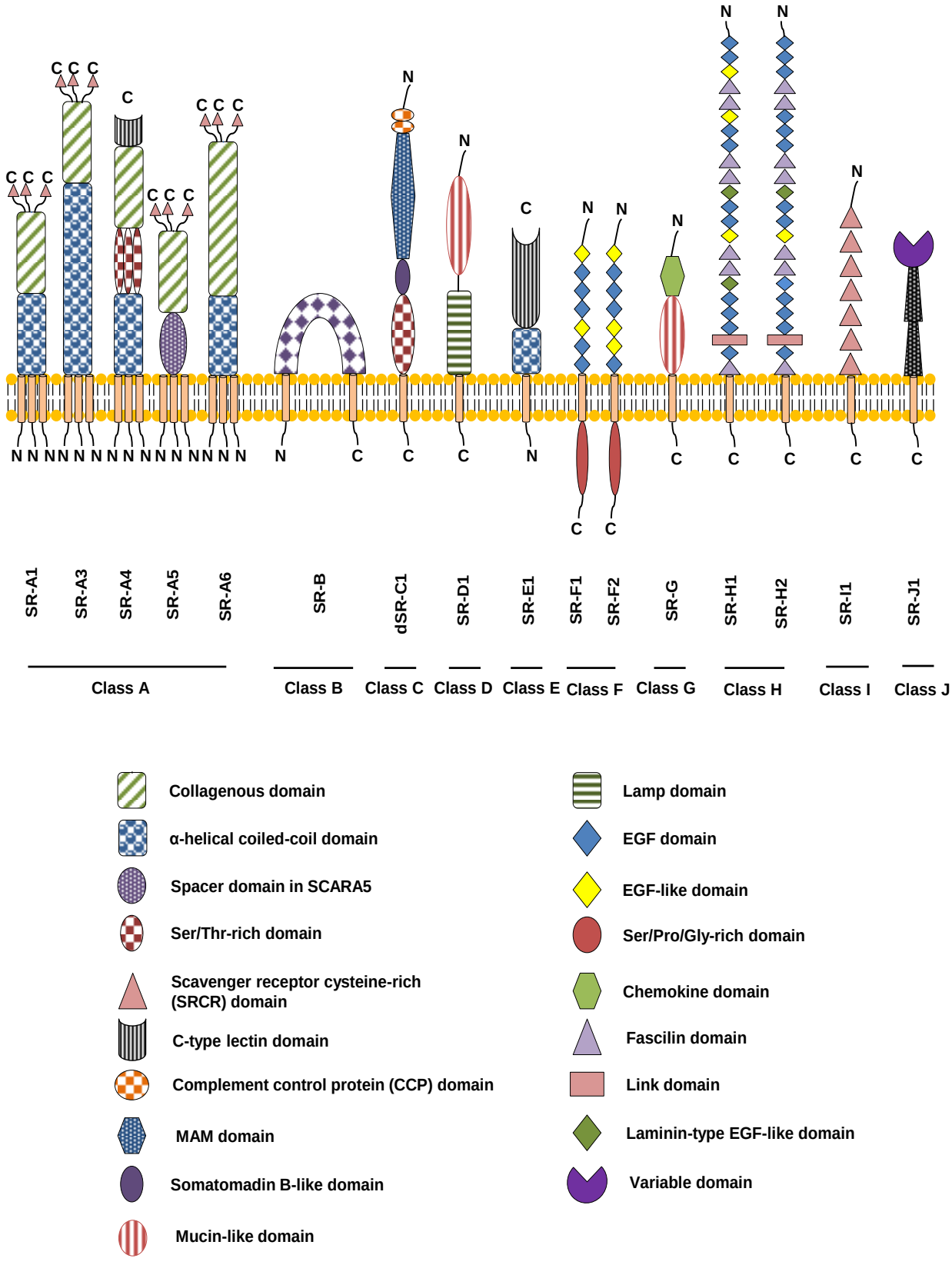

Figure 1.3. Schematic classification of scavenger receptors. The 10 different classes of scavenger receptors are denoted A-J and specific domains are denoted by the codes shown. Taken from Abdul Zani et al., 2015.

1.3.1 Class A

1.3.1.1 Genetics, protein structure and expression

These are Type II membrane proteins of ~400-500 residues with an N-terminus comprising a short cytoplasmic domain followed by a single transmembrane region and a large extracellular domain that mediates ligand recognition. Class A members include SR-A1 (SCARA1), SR-A3 (SCARA3), SR-A4 (scavenger receptor with C-type lectin; SRCL), and SR-A6 (macrophage receptor with collagenous structure; MARCO). A unique feature of Class A proteins is a collagen-like domain with collagen-binding activity with homotrimers of SR-A at the cell surface (Gowen et al., 2001).

The *SCARA1* gene is on chromosome 8 in both mice and humans. SR-A1 is relatively abundant on macrophages but also present on vascular smooth muscle and endothelial tissues, especially when endothelial cells experience oxidative stress (Mietus-Snyder et al., 1997). One common feature is exemplified by SR-A1 such as the ability to bind modified or oxidized LDL particles. The *SRCL* gene is located on human chromosome 18 and gene expression is stimulated by oxidative and hypoxic stress. SRCL contains a C-type lectin domain and is widely expressed including placenta, umbilical cord, lung, skeletal muscle, and heart. The *MARCO* gene is on human chromosome 2 (Kangas et al., 1999); the gene product lacks the α-helical coiled-coil domain present in other Class A members (Ojala et al., 2007). MARCO is expressed in tissues of the peritoneum, lymph nodes, liver and spleen macrophages. Bacteria or bacterial lipopolysaccharide (LPS) can both stimulate MARCO expression (Kraal et al., 2000), linking its function to the innate immune response to bacterial infection (Thelen et al., 2010). However, MARCO lacks the ability to bind modified LDL particles.

1.3.1.2 Signal transduction, trafficking and cell function

SR-A1 undergoes internalization from the surface plasma membrane via caveolae-mediated endocytosis routes (Figure 1.4). SR-A1 binding to

modified LDL is linked to clathrin-dependent endocytosis via recognition of a cytoplasmic dileucine motif (Chen et al., 2006). In antigen-presenting cells, SR-A1-mediated pathogen uptake involves lipid raft-dependent phagocytosis (Amiel et al., 2007). SR-A1 knockout mice display 50-70% reduction in acetylated LDL and oxidised LDL uptake with a corresponding size reduction in atherosclerotic lesions (Suzuki et al., 1997; Kamada et al., 2001). Nonetheless, there is agreement that gene knockouts cause reduced pro-inflammatory responses, macrophage apoptosis and cellular necrosis with better stabilization of atherosclerotic plaques (Manning-Tobin et al., 2009; Makinen et al., 2010). SRCL belongs to the collectin family of pattern recognition receptors, which are implicated in innate immune responses. During the pro-inflammatory response at sites of infection, SRCL can mediate recognition of complex carbohydrates and neutrophil granule glycoproteins (Graham et al., 2011; Yoshida et al., 2003). SRCL levels closely mirror cellular ability to bind, internalize and process bacterial and yeast pathogens (Jang et al., 2009; Ohtani et al., 2001).

In macrophages, the c-Jun N-terminal kinase (JNK) protein is activated in SR-A-mediated foam-cell formation (Ricci et al., 2004). Nonetheless, SR-A1-null macrophages display elevated pro-inflammatory responses including increase p42/44 (ERK1/2) mitogen-activated protein kinase (MAPK) phosphorylation, NF-κB nuclear translocation and increased secretion of cytokines (Ohnishi et al., 2011). One view is that SR-A1 and MARCO mediates rapid pro-inflammatory ligand internalization on vascular cells thus reducing interactions with toll-like receptors (TLRs) (Mukhopadhyay et al., 2011). However, SR-A1 and MARCO appear to signal through different intracellular pathways with distinct effects on immune responses (Jozefowski et al., 2005). On a more global cell phenotypic level, expression of MARCO in cultured cells stimulates development of plasma membrane-derived dendrites and lamellipodia (Pikkarainen et al., 1999), key membranous structures which mediate pathogen engulfment by eukaryote cells.

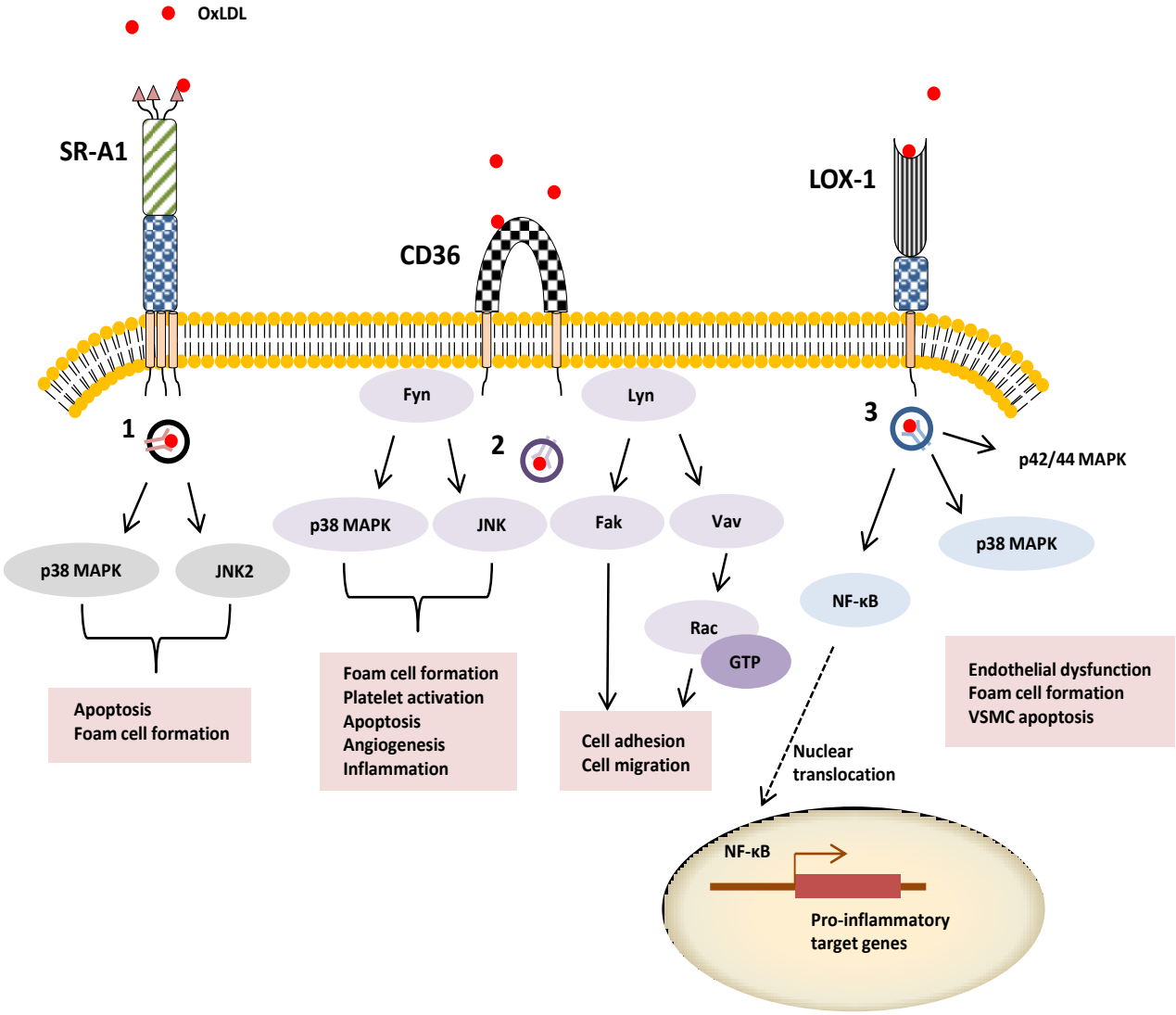

Figure 1.4. Schematic overview of ligand-stimulated scavenger receptor signal transduction. Oxidised LDL (oxLDL)-stimulated activation of intracellular signalling pathways and cellular responses is exemplified by SR-A1, SR-B2 (CD36) and SR-E1 (LOX-1). Different endocytosis pathway are denoted 1-3: (1) caveolae-mediated uptake, (2) lipid-raft-dependent uptake, (3) clathrin-independent pathway. Taken from Abdul Zani et al., 2015.

1.3.2 Class B

1.3.2.1 Genetics, protein structure and expression

The members of this class consist of SR-B1, SCARB2 and CD36. These three members have two transmembrane regions located close to the N- and C-termini, which straddle a central domain of ~400-450 residues that is glycosylated and mediates ligand recognition. The short N- and C-terminal cytosolic regions are implicated in regulatory roles in signal transduction and trafficking. *SR-B1* is on human chromosome 12. The *SR-B1* gene product binds HDL, viruses and bacteria; mutation or allelic variations in *SR-B1* are associated with an increased risk of atherosclerosis, infertility and/or an impaired innate immune response (Guo et al., 2011; Scarselli et al., 2002; Yates et al., 2011). *SCARB2* is on human chromosome 4 and predominantly expressed in liver, brain, heart and macrophages; the SCARB2 protein mediates binding to HDL particles (Ishikawa et al., 2009; Eckhardt et al., 2004). *CD36* gene is on human chromosome 7. CD36 has many functions including macrophage oxidised LDL uptake to promote foam cell formation, platelet activation/aggregation, apoptosis, angiogenesis, inflammation, its levels are elevated by a fat-rich diet, inflammation and oxidative stress (Silverstein et al., 2010; Liani et al., 2012; Nishikawa et al., 2012).

1.3.2.2 Signal transduction, trafficking and cell function

SR-B1 binds and internalizes acetylated LDL or oxidised LDL particles (Acton et al., 1994). SR-B1-mediated lipid uptake goes through lipid-raft-dependent endocytosis routes. SR-B1-mediated endocytosis causes relatively mild oxidised LDL degradation (Sun et al., 2007), suggesting key differences to other scavenger receptor-mediated ligand delivery to lysosomes. In the liver, SR-B1 may mediate HDL uptake and endocytosis; however, in peripheral tissues, SR-B1 binding to HDL may stimulate cholesterol efflux from internal stores (Peng et al., 2004). SCARB2 has a role as a viral pathogen receptor including Enterovirus 71 and Coxsackie (Yamayoshi et al., 2012a; Yamayoshi et al., 2012b) virus, and

internalization go through via clathrin-dependent endocytosis (Eckhardt et al., 2006).

CD36 binds a variety of ligands including thrombosponin-1, oxidized phospholipids/lipoproteins, long-chain fatty acids and apoptotic cells (Silverstein and Febbraio, 2009). Oxidised LDL binding to macrophage CD36 triggers intracellular signalling events (Park et al., 2012). CD36 is enriched within cholesterol-rich membrane micro-domains and interacts with other receptors such as tetraspanins and integrins. Activated CD36 signal transduction involves the tyrosine kinase Fyn, p38 MAPK and JNK (Figure 1.4). Notably, CD36-mediated intracellular signalling through Fyn is implicated in phosphorylation and activation of Vav proteins. These act as guanine nucleotide exchange factors for Rho and Rac GTPases that are implicated in actin remodelling, membrane dynamics and cell migration. Furthermore, CD36 can also bind microbial diacylglycerides to stimulate a pro-inflammatory TNFα response upon bacterial infection (Stewart et al., 2010).

1.3.3 Class C

Class C scavenger receptors are expressed only in insects such as fruit flies and mosquitoes. Members include SR-C1 and SR-C2, which are membrane-bound; SR-C3 and SR-C4 are soluble secreted proteins. Class C receptors are involved in the innate immune response against pathogens by a mechanism called pattern recognition. Pathogen-derived ligands that contain a characteristic repetitive molecular pattern bind to Class C scavenger receptor on the cell surface, thus triggering a sustained immune response. Class C proteins are either Type I membrane proteins or soluble secreted proteins where the extracellular domains contain N-proximal compliment control protein (CCP) region preceding a MAM motif (Ezekowitz et al., 2003).

1.3.4 Class D

The *CD68* gene is on human chromosome 17 and expressed on cells associated with the immune system such as monocytes, macrophages,

dendritic cells and osteoclasts. Phorbol esters, oxidised LDL and GM-CSF elevate CD68 expression, whereas bacterial LPS or TNFα inhibit CD68 levels (Li et al., 1998; Yoshida et al., 1998). This suggests a link between inflammation and CD68 function. The human CD68 gene product is a Type I membrane protein of 354 residues, which is heavily glycosylated. CD68 contains an N-proximal mucin-like domain, a proline-rich hinge region followed by a lysosome-associated membrane protein (LAMP) homology domain, a single transmembrane region and a short 12-residue cytoplasmic domain (Holness and Simmons, 1993). CD68 can bind oxidised LDL, lectins, and selectins to mediate phagocytosis and bone resorption (Ramprasad et al., 1996; da Silva and Gordon, 1999). When CD68 is expressed on monocytes, it promotes oxidised LDL binding and uptake, suggesting a role in leukocyte-mediated effects in atherosclerosis. Furthermore, soluble CD68 delivery into a mouse model reduced foam cell incidence and abdominal aortic plaque development with increased plaque stabilisation (Zeibig et al., 2011). However, CD68 depletion showed little change in oxidised LDL-mediated atherosclerosis in mice (de Beer et al., 2003), and oxidised LDL binding and accumulation in CD68-null mouse macrophages (Song et al., 2011). The role of CD68 in immune response is currently not clear. Deletion of the mouse CD68 did not impair macrophage ability to deal with innate immunity; this also did not impair cytokine production and actually enhanced adaptive immunity (Song et al., 2011).

1.3.5 Class E

Class E comprises of just one member lectin-like low-density lipoprotein receptor (LOX-1). A detailed discussion of LOX-1 can be found in the next section.

1.3.6 Class F

The class F group consists of SREC1 and SREC2 members. *SREC1* is on human chromosome 17 whereas *SREC2* is on human chromosome 22. Both protein receptors are Type I membrane proteins of 850-900 residues, an extracellular domain of ~450 residues containing multiple EGF-like

repeats, a single transmembrane region and a relatively large cytoplasmic domain of ~400 residues. SREC1 is present on neuronal and endothelial cells in heart, lung, ovary and placenta (Adachi and Tsujimoto, 2002). SREC1 binds to carbamylated LDL, acetylated LDL and oxidised LDL particles. SREC2 lacks scavenger receptor activity but preferentially forms heterodimers with SREC1 (Ishii et al., 2002). Such SREC1-SREC2 heterodimers lose the capacity to mediate lipid particle recognition suggesting that SREC2 suppresses the ligand-binding properties of SREC1. The class F members can both regulate modified LDL binding and internalization, but their effects on atherosclerosis initiation and progression are unclear. The class F protein SREC1 not only recognizes a modified lipid particles; receptor-ligand complexes can undergo clathrin-dependent endocytosis and delivery to the endosome-lysosome system (Murshid et al., 2010; Sano et al., 2012).

1.3.7 Class G

The class G member is SR-PSOX, also known as chemokine 16 (CXCL16), is a Type I membrane protein of 254 residues with an N-terminal extracellular domain, a single transmembrane region and a short cytoplasmic domain. Human *SR-PSOX* is on chromosome 17 and expressed on vascular smooth muscle cells, endothelial cells, monocytes, macrophages, and kidney podocytes (Gutwein et al., 2009). The SR-PSOX extracellular domain mediates endocytosis of phosphatidylserine or oxidised LDL, and delivery to endosome-lysosome system. The SR-PSOX extracellular domain contains a chemokine-related motif followed by a mucin-like stalk region. Cleavage within this mucin-like region by disintegrin-like metalloproteases (ADAMs) cause shedding of a soluble SR-PSOX. SR-PSOX has important innate immunity functionality through recognition of bacteria and CpG-rich DNA found in other pathogens (Gursel et al., 2006; Sheikine and Sirsjo, 2008). Manipulation of SR-PSOX levels modulates macrophage differentiation into foam cells (Zhang et al., 2008; Quan et al., 2007), suggesting a pro-atherogenic function. SR-PSOX levels can be up-regulated to recruit CD4+ T cells to affected sites during inflammatory disorders in various tissues. Mice lacking SR-PSOX

produced lower cytokines and liver natural killer cells (Yamauchi et al., 2004; Wu et al., 2007; Uza et al., 2011). This molecule may play a vital role not only in recruiting but also promoting interaction of both T and natural killer cells to dendritic cells (Shimaoka et al., 2004).

1.3.8 Class H

The class H members include Fasciclin, EGF-like, laminin-type EGF-like, and link (FEEL) domain-containing scavenger receptors 1 and 2 (FEEL1 and FEEL2), which are Type I membrane glycoproteins of up to 2570 residues. *FEEL1* and *FEEL2* are on human chromosome 3 and 12, respectively. The FEEL gene products are expressed by cells from the spleen, lymph nodes, macrophages, bone marrow, and liver. The FEEL extracellular domains have three blocks containing two Fasciclin domains interspersed with EGF-like domains and laminin EGF-like domains and a single Fascilin domain adjacent to the transmembrane domain. FEEL1 and FEEL2 bind acetylated LDL, advanced glycated end-products (AGE) and bacteria. FEEL1 expression on monocytes has been postulated to be a biomarker for increased cardiovascular disease risk (Gratchev et al., 2012).

FEEL1 also promotes bacterial recognition and stimulates lymphocyte diapedesis through lymphatic and vascular endothelial cell monolayers. FEEL1 also stimulates the recruitment of CD4+ FoxP3-positive regulatory T cells, indicating an important role in the immune response to pathogen infection (Adachi and Tsujimoto, 2002; Shetty et al., 2011). Macrophages also express FEEL2 to promote phagocytosis and clearance of aged cells, apoptotic bodies and heparin-linked proteins, showing functional similarities to other scavenger receptor members (Adachi and Tsujimoto, 2010).

1.3.9 Class I

1.3.9.1 Genetics, protein structure and expression

The members of Class I include CD163, CD5 and CD6 that are primarily restricted to the hematopoietic cell lineage. These receptors have type B

scavenger receptor cysteine-rich (SRCR) domain, which are encoded by a single exon and containing eight cysteine residues. CD163 is a 130 kDa transmembrane Type I membrane glycoprotein primarily expressed in monocytes and macrophages. *CD163* is mapped to human chromosome 12p13. The primary structure displays an extracellular domain composed of nine SRCR domains in tandem, a transmembrane region followed by a short intracellular cytoplasmic tail (Moeller et al., 2012; Nielsen et al., 2006). CD163 is also known as “haemoglobin scavenger receptor” due to its important role in mediating haemoglobin recognition and clearance in tissue macrophages (Kristiansen et al., 2001; Thomsen et al., 2013). Class I scavenger receptors are also present as soluble forms, although their functional role is unknown. It has been suggested that these soluble fragments could be potential biomarkers for inflammatory and autoimmune diseases (Alonso et al., 2010; Burdo et al., 2011; Etzerodt and Moestrup, 2013).

1.3.9.2 Signal transduction, trafficking and cell function

As suggested from its functional name as a haemoglobin scavenger receptor, CD163 helps the removal of haptoglobin-haemoglobin (Hp-Hb) complexes via the haem oxygenase-1 (HO-1) pathway to reduce pro-inflammatory haem in the circulation (Thomsen et al., 2013). This indicates the role of CD163 in anti-inflammatory response by mediating the uptake of toxic haem in macrophages (Kristiansen et al., 2001). CD163 has also been shown in animal studies to be expressed in bone marrow macrophages to initiate growth and survival of erythroblast. CD163 has also been reported to be involved in intracellular signalling such as phosphorylation of protein kinase C (PKC) (Nielsen et al., 2006)..

1.3.10 Class J

1.3.10.1 Genetics, protein structure and expression

Receptor for advanced glycation end-products (RAGE) is the sole member of class J scavenger receptor. RAGE is a 32 kDa multi-ligand transmembrane receptor that belongs to the immunoglobulin gene family.

RAGE is expressed in endothelial cells, hepatocytes, smooth muscle cells and monocytes (Ramasamy et al., 2009). Full-length RAGE is composed of an extracellular V (variable)-type domain, a single transmembrane spinning helix that connects the short C-terminal cytosolic domain and two C-type domains. RAGE is a pattern recognition receptor that has an ability to interact with and/or be activated by a number of pro-inflammatory ligands such as β-amyloid (Yan et al., 1996), S100/calgranulin (Hofmann et al., 1999), phosphatidylserine (He et al., 2011), and high-mobility group protein 1 (HMGB1) (Hori et al., 1995). Under physiological conditions, the expression of RAGE is low, but can be provoked in response to chronic conditions. These pro-inflammatory endogenous molecules are involved in inflammation and physiological stress.

1.3.10.2 Signal transduction, trafficking and cell function

The up-regulation of RAGE expression by pro-inflammatory ligands has a positive effect in a scenario where inflammation occurs; unlike most receptors their expression is down-regulated in chronic inflammatory condition (Li and Schmidt, 1997; Yao and Brownlee, 2010). AGE-bound RAGE is implicated in signal transduction mediating processes such as oxidative stress, apoptosis and inflammation (Xie et al., 2013). Stimulation of RAGE is also involved in neuronal differentiation and cell migration especially during development. Upon ligand stimulation of RAGE, the pro-inflammatory gene expression is activated by NF-κB translocation into the nucleus; RAGE itself is a target of NF-κB, thus providing a positive feedback loop to amplify the response (Li and Schmidt, 1997).

Furthermore, *in vitro* studies showed the activation of MAPK signalling cascades through RAGE-mediated activation by AGE. This triggered the oxidative stress pathway, which in turn led to activation of NF-κB (Yeh et al., 2001). Another pathway involved in the inflammatory signal transduction is the JNK MAPK pathway. From an *in vitro* study, expression of the pro-inflammatory marker protein vascular cell adhesion molecule 1 (VCAM1) was decreased by JNK inhibition (Harja et al., 2008). Additionally, activation of JNK by RAGE ligands was shown to increase

the transcriptional activity of activator protein 1 (AP1), which further increases the expression of pro-inflammatory genes.

1.4 LOX-1 scavenger receptor

Atherosclerosis is a chronic progressive disease, and its clinical manifestations include coronary artery disease, cerebrovascular disease and peripheral arterial disease. It is a multi-factorial disease with many of its risk factors being known, however, the cellular and molecular mechanisms precipitating the disease process are not well-defined. After a ground-breaking research by Steinberg and colleagues (Steinberg et al., 1989), oxidised LDL received intense interest as it promotes the development of atherosclerosis. The rapid uptake of oxidised LDL by scavenger receptors is crucial in the initial step of atherosclerosis leading to foam cells formation (Yamada et al., 1998). The interaction between lectin-like oxidised low-density lipoprotein receptor-1 (LOX-1) and oxidised LDL plays an important role in the pathogenesis of atherosclerosis (Figure 1.5).

1.4.1 Genetics, protein structure and expression

The class E scavenger receptor comprises of LOX-1, which is on human chromosome 12 within a region enriched for genes involved in the innate immune response. Sequence and structural analysis revealed LOX-1 protein is not similar to any macrophage scavenger receptors known. Nonetheless, LOX-1 shares similar identity to natural killer cell receptors (Aoyama et al., 1999; Sawamura et al., 1997) that the expression of LOX-1 might induce endothelial cell dysfunction. Human LOX-1 is a Type II membrane protein of 273 residues that structurally belongs to the C-Type lectin family. Mature form of LOX-1 protein is at 50 kDa due to glycosylation, but the precursor form is synthesised at 40 kDa (Kume and Kita, 2001). Human LOX-1 is comprised of four domains, namely, a short N-terminal cytoplasmic domain, a single transmembrane region, a coiled-coil ‘neck’ region and a C-type lectin-like domain (Ogura et al., 2009). Remarkably, the six cysteine residues of the lectin-like domain are highly

conserved, which functions as a ligand-binding domain and initiate internalization of ligands (Chen et al., 2001a).

LOX-1 tends to bind to protein moiety of oxidised LDL (Moriwaki et al., 1998); however, as LOX-1 is a scavenger receptor, it exhibits binding activity for multiple ligands such as acetylated LDL, phosphatidylserine, apoptotic cells and bacteria. Initially, LOX-1 was identified as a vascular endothelial-specific receptor for oxidised LDL, but LOX-1 is also expressed in vascular smooth muscle cells, cardiomyocytes, and immune cells (Draude et al., 1999; Iwai-Kanai et al., 2001). *In vivo*, normal physiological basal LOX-1 expression is relatively low, and enhanced by pathological conditions such as hyperlipidaemia, hypertension, diabetes and atherosclerosis (Kataoka et al., 1999; Nagase et al., 1997b). Similarly, basal expression of LOX-1 is low *in vitro*, but the expression is induced by pro-inflammatory molecules, oxidised LDL and mechanical stress (Kume and Kita, 2001; Murase et al., 1998; Nagase et al., 2001).

LOX-1 is a non-essential gene and polymorphisms within the *LOX-1* gene are linked to increased cardiovascular disease risk, but mutations within the gene are mostly non-coding that do not affect LOX-1 protein function (Mehta et al., 2007; Li et al., 2003b). Stable overexpression of LOX-1 in transgenic LDLR knockout mice has been shown to display intramyocardial vasculopathy (Inoue et al., 2005). Conversely, transient viral gene therapy in a mouse model showed liver-mediated oxidised LDL clearance and reduction in atherosclerosis (Ishigaki et al., 2008). These findings have led to much debate as to the role of LOX-1 as a protective or pro-atherogenic factor in inflammation and atherosclerosis.

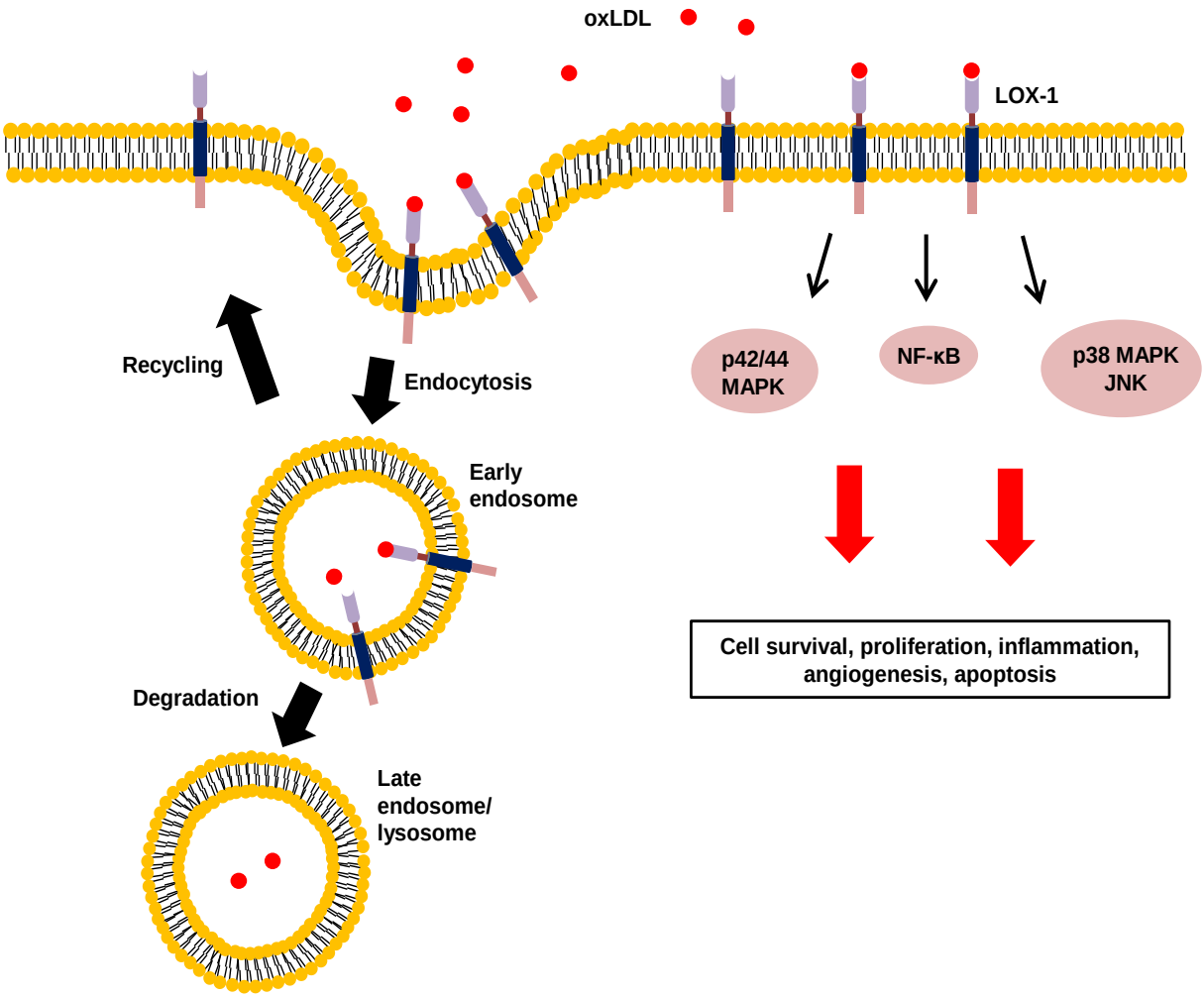

Figure 1.5. Schematic of oxidised LDL-mediated LOX-1 trafficking and signal transduction pathways. Binding and internalization of oxLDL by LOX-1 triggers the activation of inflammatory pathways: MAPKs (p38, p42/44, JNK) and NF-κB. Internalization of oxLDL-LOX-1 is mediated by clathrin-independent pathway into the early endosome, and later on LOX-1 is either recycled or degraded.

1.4.2 Signal transduction, trafficking and cell function

It is generally accepted that atherosclerosis results from interaction between oxidised LDL, leukocytes and other cellular components of the arterial wall. LOX-1 and CD36 are similar in a way that they act as cell membrane scavenger receptors mediating the binding, internalization and degradation of oxidised LDL (Figure 1.5). The class E LOX-1 protein binding with oxidised LDL stimulates a spectrum of pro-inflammatory signalling and mediating pro-atherogenic cellular responses including endothelial dysfunction, foam cell formation and vascular smooth muscle apoptosis (Kume and Kita, 2001; Kataoka et al., 2001).

Oxidised LDL provokes wide-ranging effects on a number of signalling pathways implicated in atherosclerosis. The cellular effect of oxidised LDL-dependent binding to LOX-1 stimulates the activation of numerous signal transduction pathways including MAPKs (p38, ERK1/2, JNK), Akt/eNOS, NF-κB, NADPH oxidase, Ang II Type I receptor, PKC and AP-1 transcription factor (Xu et al., 2013). LOX-1-mediated NF-κB activation by oxidised LDL is a key feature of pro-inflammatory response in immune and vascular cells. The activation causes the increased in adhesion molecules expression including intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemoattractant protein-1 (MCP-1). Collectively, these proteins are important in the early event of leukocyte adhesion to endothelial cells (Ogura et al., 2009; Clarke et al., 2006). Furthermore, binding of oxidised LDL to LOX-1 caused the generation of reactive oxygen species, which in turn, activating NF-κB signalling protein and inhibits nitric oxide production (Cominacini et al., 2001). Inflammatory cytokines such as TNF-α, lipopolysaccharide, IL-1, and TGF-β up-regulates LOX-1 mRNA (Minami et al., 2000; Draude and Lorenz, 2000; Kume et al., 1998; Nagase et al., 1998). Inflammatory mediators have profound effect on the expression of LOX-1 that includes Ang II, endothelin-1 and shear stress *in vivo* (Li et al., 1999a; Murase et al., 1998). It is believed that oxidised LDL binding to LOX-1 mediates the NADPH oxidase-ROS signalling pathways activating the redox-sensitive NF-κB, due to the so-called “NF-κB binding sites”

located in the LOX-1 gene promoter (Nagase et al., 2001). Lastly, activation of NF-κB up-regulates Ang II Type-1 receptor (AT_1R) as well as LOX-1, which in turn increase the LOX-1-mediated activity of oxidised LDL uptake.

1.4.3 LOX-1 in atherosclerosis

Research on the patho-physiological role of LOX-1 is abundant. Under physiological conditions when LOX-1 expression is normal, LOX-1 may scavenge cellular debris and clear up oxidised LDL from the circulation, and it might also be involved in host-defence system (Kakutani et al., 2000; Oka et al., 1998; Shimaoka et al., 2001; Ishigaki et al., 2008). In pathological states, LOX-1 is involved in activating endothelial cells, accumulating and partial degradation of oxidised LDL, and proliferation of smooth muscle cells, which are all linked to atherosclerosis (Kataoka et al., 1999; Nagase et al., 2000). These pathological roles of LOX-1 outweigh its physiological role causing detrimental effects.

The highly-inducible expression of the LOX-1 gene in endothelial cells by oxidised LDL has been shown to initiate early stages of atherosclerosis. Studies have shown the binding of oxidised LDL to LOX-1 cause pathological changes in the endothelial cells, such as generating superoxide anions and decreasing production of nitric oxide, resulting in cellular apoptosis (Li and Mehta, 2000b). Atherosclerosis susceptible regions such as arterial bifurcations have increased expression of LOX-1 because the endothelium is exposed to shear forces (Chen et al., 2001b; Frangos et al., 1999). Thus, fluid dynamics of blood flow may influence LOX-1 expression. Studies have shown high LOX-1 expression together with oxidised LDL in atherosclerotic lesions in endothelial cells and sub-endothelial macrophages (Kakutani et al., 2001). This further confirms the molecular basis linking oxidised LDL and LOX-1.

Adhesion of leukocytes onto the vessel wall is an important event in atherosclerosis. *In vitro* study showed increased expression of monocyte chemoattractant protein-1 (MCP-1) when human coronary artery endothelial cells (HCAEC) were incubated with oxidised LDL.

Subsequently, treatment of HCAEC with human LOX-1 anti-sense RNA inhibited the oxidised LDL-mediated expression of MCP-1 (Li and Mehta, 2000a). As LOX-1 is known as a multi-ligand scavenger receptor, an *in vivo* study showed that anti-LOX-1 antibody reduced the number of leukocytes rolling along the endothelium, signifying LOX-1 has affinity for vascular-tethering ligands (Honjo et al., 2003; Li et al., 2002b; Hayashida et al., 2002).

Proliferation and migration of vascular smooth muscle cells from the medial layer is another significant event of plaque formation in atherosclerosis. The proliferation of smooth muscle cells is mediated via the activation of NF-κB and JNK MAPK signalling pathways shown *in vitro* (Eto et al., 2006). In mouse model where genetic deletion of LOX-1 in ApoE knockout background revealed reduced smooth muscle cell proliferation (Mehta et al., 2007). Therefore, LOX-1 has a role in causing proliferation and migration of smooth muscle cells into the sub-endothelial region to become foam cells via the activation of pro-inflammatory pathways by oxidised LDL.

1.4.4 LOX-1 and other diseases

It is emphasised that activation and increased activity of LOX-1 cause a number of diseases including cardiovascular diseases such as hypertension, myocardial infarction, congestive heart failure, thrombosis and atherosclerosis. LOX-1 may also play a role in diabetes associated with cardiovascular diabetic complications. It was also reported that LOX-1 is linked to risk of cancers in breast, colon and ovaries. Furthermore, LOX-1 may also play a part in rheumatoid arthritis, obesity, kidney injury, pathogenic infection, and metabolic syndrome.

1.4.4.1 Hypertension

Hypertension is a common disease that is usually a common risk factor for cardiovascular disease. Hypertension is often associated with the increase in oxidative stress, which impairs the vascular endothelial cells by advancing the proliferation and hypertrophy of vascular smooth muscle

cells that lead to narrowing of the vascular lumen. Furthermore, oxidative stress has a negative effect on the endothelial cells by diminishing the ability of the vascular smooth muscle cells to relax and increase the contractile activity (Luo et al., 2011). The connection between hypertension and LOX-1 has been reported a decade ago in animal models (Nagase et al., 1997a). The correlation suggested that expression of LOX-1 mRNA in the aorta was up-regulated in hypertensive rats compared to normal healthy rats. An in vitro study supported this hypothesis, which detected a marked increase in LOX-1 mRNA levels in cultured human coronary artery endothelial cells upon activation of Ang II type 1 receptor, a pro-inflammatory G-protein coupled receptor (Li et al., 1999b). The interrelationship between LOX-1 and Ang II type 1 receptors and hypertension was further demonstrated in an animal model where differences between LOX-1 knockout and wild-type mice infused with Ang II were observed (Morawietz et al., 1999). The involvement of p38 and p42/44 MAPK was more pronounced in wild-type mice compared to LOX-1 knockout mice when infused with Ang II. The effect of Ang II has on LOX-1 is attenuated by angiotensin converting enzyme (ACE) inhibitor (Morawietz et al., 1999), which further supports the evidence that LOX-1 may contribute to the pathogenesis of hypertension, either directly or indirectly.

1.4.4.2 Myocardial infarction

Oxidised LDL is known to cause proliferation and migration of vascular smooth muscle cells, which can become ingested by macrophages, and over time, foam cells form. This will increase the size of the plaque, followed by plaque instability and cessation of blood flow when the vascular lumen is occluded. This sequence of events has been shown to correlate with oxidised LDL levels and the severity of acute coronary syndromes (Ehara et al., 2001). Observations on isolated rat heart perfused with oxidised LDL showed decrease in contractile activity. Subsequent study showed that LOX-1 expression was increased in the rat myocardium. Furthermore, treating the rats with an antibody specific for LOX-1, caused LOX-1 expression to markedly decrease (Li et al., 2003c).

Regulation of pre-mRNA splicing of *OLR1* and subsequent LOX-1 expression has been linked to myocardial infarction. One of the seven SNPs of LOX-1 is a functional isoform of the *OLR1* gene called LOXIN. This gene product lacks exon 5 and was suggested to protect the cells from undergoing apoptosis. Since LOXIN has no functional C-terminal domain, its functionality in cellular trafficking also diminishes (Mango et al., 2005).

1.4.4.3 Congestive heart failure

Congestive heart failure is characterized by the inability of the heart, especially the left ventricle of the heart, to pump blood to all tissues. The expression of LOX-1 in cardiac cells is relatively low, and can be induced by chemical stimuli, mechanical stimuli and oxidative stress that are activated in failing heart. In a study by Takaya and colleagues using an animal model, analysis by real-time (RT)-PCR showed LOX-1 expression was markedly increased in rats fed with high-salt diet compared to controls (Takaya et al., 2010). The expression level of LOX-1 was more pronounced in the left ventricles and the levels increased from the week the rats start high intake of salty food. It was also demonstrated that the increased expression of LOX-1 impaired the systolic function of the heart (Takaya et al., 2010).

1.4.4.4 Thrombosis

Thrombosis is a late event in atherogenesis by forming blood clots in the vessels and ceases the blood flow, which eventually leads clinical manifestation of stroke and myocardial infarction. Platelets are usually observed to initiate this event, by internalising the oxidised LDL that cause down-regulation of eNOS activity in platelets and also cause platelets to aggregate (Mehta and Li, 2002). From previous studies, the LOX-1 receptor expression has been observed in platelets, and blocking the receptor with LOX-1 antibody has been shown to diminish the formation of arterial thrombus in animal model (Mehta and Li, 2002). In another study by Puccetti and colleagues, they demonstrated the variations in LOX-1 polymorphisms affecting the platelet activation by oxidised LDL (Puccetti

et al., 2005). All this evidence supports the involvement of LOX-1 with platelets in atherothrombosis.

1.4.4.5 Diabetes mellitus

Diabetes is characterised by endothelial dysfunction, which is usually a key event in pathogenesis of atherosclerosis. Atherosclerotic cardiovascular disease, diabetic nephropathy and neuropathy are major complications of diabetes that are correlated to LOX-1. A number of factors may play a part in causing endothelial dysfunction in diabetes including oxidative stress, hyperlipidaemia, high glucose level and insulin resistance, to name a few. Other studies done using animal models demonstrated the expression of LOX-1 in vascular endothelium of diabetic rats is increased, which further suggested the association of LOX-1 and cardiovascular diabetic complications (Nowicki et al., 2012). The expression of LOX-1 in macrophages that is increased by advanced glycation end products (AGE) is also involved in the pathogenesis of diabetic atherosclerosis. In an in vitro study, LOX-1 expression was enhanced by high glucose in macrophages, which supports the role of macrophages in the formation of foam cells in vascular walls in the event of atherosclerosis (Li et al., 2004). Furthermore, the expression of adhesion molecules was increased in inflammatory cells. On the other hand, diabetes mellitus also causes diabetic nephropathy that is characterized by damage in the tubulointerstitium and development of glomerulosclerosis induced by lipid. In a study by Yamamoto and colleagues (Yamamoto et al., 2009), they demonstrated using in situ hybridization that LOX-1 mRNA levels is increased in the human renal tissue, especially in the tubulointerstitial area. The increasing level of LOX-1 expression was reported to correlate to the degree of the damage in tubulointerstitium of the kidneys. Therefore, from all the evidence reported, LOX-1 plays a crucial role in diabetic complications; however, the mechanism underlying it is still not clearly explained.

1.4.4.6 Cancer

Atherogenesis and tumorigenesis may work in parallel affecting the molecular and cellular substances in a system. The modification of native LDL to oxidised LDL is a crucial part of the pathogenesis of atherosclerosis in which some may develop certain cancers. Thus, this tells us that there is a direct or indirect connection between oxidative stress involving lipid and cancer growth. Furthermore, it was found that the cancer gene expression is found in atherosclerosis from microarray transcriptional profiling studies (Hirsch et al., 2010), which they found the commonality in atherosclerosis and cancer involves pro-inflammatory molecule NF-κB. LOX-1 is an upstream molecule of NF-κB in the inflammatory signalling pathway, and inhibiting LOX-1 with a lipid lowering drug reduces the level of NF-κB signalling activity and inhibits cellular transformation (Lu et al., 2011). This observation suggests the participation of LOX-1 in regulating the inflammatory pathway in cancer.

These studies interlinking LOX-1 and cancer brought up the possibility that LOX-1 may have pro-oncogenic function and targeting its activity may be beneficial in the treatment of cancer. Generation of reactive oxygen species in cells has been implicated in cancer development and progression by regulating the proliferation, migration and survival of the cancer cells. The family of NADPH oxidases is the main producer of ROS in epithelial and endothelial cells, and they are highly expressed in organs such as kidney and colon (Chabrashvili et al., 2002). Oxidised LDL and LOX-1 interaction increases activity of DNA oxidation and also superoxide production. It is apparent that NADPH oxidase is activated by oxidised LDL-LOX-1 interaction (Lu et al., 2011). Thus, this is one of the many possible mechanisms of LOX-1 in cancer development by relying on the presence of NADPH oxidase (Lu et al., 2011). LOX-1 can contribute to or impair tumorigenesis by regulating NF-κB activation. However, the involvement of LOX-1 in cancer is still not clearly understood.

1.4.4.7 Rheumatoid arthritis

Rheumatoid arthritis is an inflammatory disease of bone and cartilage around joints. It is reported that the lipids and lipoproteins found in synovial fluids of inflamed joints are oxidized from patients with rheumatoid arthritis in contrast with healthy patients (Ishikawa et al., 2012). It is also observed the formation of foam cells in the inflamed area, which is similar to the event of atherosclerosis. Thus, the association between rheumatoid arthritis and atherosclerosis is acknowledged involving lipid metabolism in inflamed area of the bone (Ishikawa et al., 2012). Matrix metalloproteinases (MMPs) are enzymes that involve in many arthritis diseases by destructing the articular cartilage (Ishikawa et al., 2012). The presence of oxidised LDL and LOX-1 was detected in bone chondrocytes from the rheumatoid arthritis patients (Winyard et al., 1993). In addition, synthesis of MMP-3 in the articular cartilage was observed upon activation of LOX-1 by oxidised LDL (Kakinuma et al., 2004). Blocking the production of MMP-3 by inhibiting the interaction between LOX-1 and oxidised LDL with antibody specific to LOX-1 suggested that LOX-1 can be a potential target in the treatment of rheumatoid arthritis (Ishikawa et al., 2012).

1.4.4.8 Chlamydia pneumonia

Campbell and colleagues have demonstrated the relationship between Chlamydia pneumoniae and atherosclerosis using immunohistochemistry and polymerase chain reaction (PCR) by isolating the organisms out of the atherosclerotic plaques. Chlamydia pneumoniae is distinct from other human chlamydial pathogen because of its atherogenic characteristics. In a subsequent animal study, Chlamydia pneumoniae was shown to speed up the development of atherosclerosis with the condition of the animal to be hyperlipidaemic. It was observed that the increased expression LOX-1 levels on endothelial cells caused by Chlamydia pneumoniae further stimulates the uptake of oxidised LDL (Campbell et al., 2012). Furthermore, it was shown that Chlamydia pneumoniae binds to the LOX-1 receptor; furthermore, when they inhibited the LOX-1 with specific

antibody, interaction between Chlamydia pneumoniae and LOX-1 diminished (Campbell et al., 2012).

1.5 Summary

Atherosclerosis is a leading cause of death in many parts of the world and is a systemic disease involving narrowing or occlusion of the arterial vessel wall by a plaque or lesion. The process of atherosclerosis involves aberrant lipid particle metabolism, formation of foam cells followed by the rupture of atherosclerotic plaque, leading to blood clot formation, arterial blockage resulting in heart disease and stroke. LOX-1, a member of the scavenger receptor, has been linked with each of these pathological processes. Evidence from in vitro and in vivo studies, including LOX-1 knockout and over-expression models, has implicated LOX-1 as a key modulator of the atherosclerotic pathway. This understanding could lead to novel therapeutics targeting LOX-1.

1.6 PhD project aims and hypothesis

LOX-1 is known to bind oxidised LDL mediated through the basic residues in its C-type lectin-like domain. The molecular basis, the signalling pathway *per se*, has been linked to activate a number of pro-inflammatory pathways such as MAPKs and NF-κB. However, most of the studies performed immunohistochemistry analysis from animal models. These studies did not explore the molecular and cellular mechanisms underlying LOX-1-mediated atherosclerosis. Thus, understanding the trafficking of LOX-1 and the activation of pathways relating to atherosclerosis is crucial.

Based on previous data collected in the Ponnambalam lab (University of Leeds, UK), it has been shown that deletion of LOX-1 in ApoE knockout background mice is associated with more plaques formation in the aorta compared to ApoE knockout mice alone (Supplementary figure B1) (Mughal, 2015). Furthermore, deletion of LOX-1 has also been shown to affect glucose metabolism, suggesting insulin resistance phenotype (Supplementary figure B2). These results revealed that LOX-1 potentially have a protective role in atherosclerosis and related

disease. Although it's a contentious subject to tackle, it is also a possibility that LOX-1 solely scavenges and degrades oxidised LDL down its trafficking pathway. Nonetheless, as the disease progresses, LOX-1 may not be able to degrade oxidised LDL properly, that LOX-1 may "switch" becoming more pathological and activating the pro-inflammatory pathways.

Given the contradictory evidence in this subject, I wanted to investigate LOX-1 signal transduction, trafficking and cellular outcome in cell analysis and in mouse models, thus linking both animal data and cellular study explaining mechanisms of atherosclerosis involving LOX-1. The first aim of the study is to generate a stable inducible system over-expressing LOX-1 in both vascular endothelial and non-vascular cell lines, with the purpose to assess LOX-1 and oxidised LDL. The second aim of the study involves using trafficking-defective LOX-1-D5A mutant (Murphy et al., 2008) to compare the effect of LOX-1 wild-type (WT) on trafficking and signalling pathways in response to oxidised LDL. This is followed by the third aim, which is to harvest different mouse tissue and organs, specifically aorta, liver, adipose tissue and skeletal muscle. All experimental transgenic mouse lines were fed on 0.2% cholesterol-rich (Western) diet for 12 weeks and using these tissues to elucidate the activation of signalling proteins involved in plaque formation, glucose metabolism and lipid metabolism.

CHAPTER 2

Materials and methods

2.1 Materials

2.1.1 Chemical reagents

All chemicals were of analytical grade and purchased from Sigma-Aldrich (Poole, UK) unless otherwise stated. Bicinchoninic acid assay (BCA assay) was from Thermo Fisher (Cramlington, UK) or Pierce (Rockford, USA). Enhanced chemiluminescence (ECL) reagents for Western blotting were from Thermo Fisher (Cramlington, UK). Restriction enzymes were from New England Biolabs (Hitchin, UK), Promega (Southampton, UK) or Fermentas Life Sciences (York, UK). Oligonucleotide primers were from Sigma-Aldrich (Poole, UK) or Integrated DNA Technologies (Coralville, USA). Cell culture media and reagents were from Invitrogen (Amsterdam, Netherlands). Isofluorane was purchased from Central Business Services at the University of Leeds.

2.1.2 Antibodies

The following antibodies were purchased: mouse anti-FLAG (Sigma-Aldrich, Poole, UK), goat polyclonal anti-LOX-1 (R+D Systems, Minneapolis, USA), horseradish peroxidase (HRP)-conjugated secondary antibody (Thermo Fisher, Cramlington, UK), AlexaFluor-488, -594 conjugated secondary antibodies (Invitrogen, Amsterdam, Netherlands). The following antibodies were produced by the Ponnambalam laboratory: sheep anti-LOX-1 (Diagnostics Scotland, Edinburgh, UK), rabbit anti-LOX-1 (Eurogentec, Seraing, Belgium). Antibodies were used for Western blotting and Immunofluorescence at the dilutions shown in table 2.1.

Antigen	Species	Concentration mg/ml	Dilution Factor	Source
Akt	Rabbit	0.5	WB: 1:1000	Cell Signalling Technology (Denvers, MA, USA)
Akt, phospho S473	Rabbit	0.5	WB: 1:1000	Cell Signalling Technology (Denvers, MA, USA)
eNOS	Rabbit	0.5	WB: 1:1000	Cell Signalling Technology (Denvers, MA, USA)
eNOS, phospho S1177	Rabbit	0.5	WB: 1:1000	Cell Signalling Technology (Denvers, MA, USA)
ERK1/2	Rabbit	0.5	WB: 1:1000	Cell Signalling Technology (Denvers, MA, USA)
ERK 1/2, phospho T202/Y204	Mouse	0.5	WB: 1:1000	Cell Signalling Technology (Denvers, MA, USA)
ERK 1/2, phospho T202/Y204	Rabbit	0.5	WB: 1:1000	Cell Signalling Technology (Denvers, MA, USA)
p38 MAPK	Rabbit	0.5	WB: 1:1000	Cell Signalling Technology (Denvers, MA, USA)
p38 MAPK, phospho T180/Y182	Rabbit	0.5	WB: 1:2000	Cell Signalling Technology (Denvers, MA, USA)
p53	Mouse	0.5	WB: 1:1000	Cell Signalling Technology (Denvers, MA, USA)
FLAG	Mouse	1.0	WB: 1:1000	Sigma Aldrich (Poole, UK)
FLAG	Mouse	1.0	IF: 1:300	Sigma Aldrich (Poole, UK)
Goat IgG	Donkey, HRP	0.4	WB: 1:5000	Stratech Scientific (Newmarket, UK)
Goat IgG	Donkey, AlexaFluor	2.0	IF: 1:300	Life Technologies (Grand Island, NY, USA)
Mouse IgG	Donkey, HRP	0.4	WB: 1:5000	Stratech Scientific (Newmarket, UK)
Mouse IgG	Donkey, AlexaFluor	2.0	IF: 1:300	Stratech Scientific (Newmarket, UK)
Rabbit IgG	Donkey, HRP	0.4	WB: 1:2500	Stratech Scientific (Newmarket, UK)
α-Tubulin	Mouse	2.0	WB: 1:5000	Sigma Aldrich (Poole, UK)
B-actin	Mouse	2.0	WB: 1:5000	Sigma Aldrich (Poole, UK)

Table 2.1: Primary and secondary antibodies. Details of antibody species, concentration, dilution factor and commercial source for reagents used within this study. HRP, horseradish peroxidase; WB, Western blot; IF, immunofluorescence.

2.1.3 Bacterial strains, plasmid constructs and synthetic carbohydrates

The XL10 gold *Escherichia* coli (E.coli) strain (Tet^{r}Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA1 gyrA96 relA1 lac* Hte [F' *proAB laclqZDM15* Tn*10* (Tet^r) Amy Cam^r) was from Stratagene (CA, USA). The XL10 gold strain was used for plasmid propagation and cloning. Full length wild-type and D5A mutant LOX-1 with a C-terminal FLAG tag (LOX-1-FLAG WT and LOX-1-FLAG D5A) in the mammalian expression vector pcDNA3.1(+) was kindly provided by Dr. Sam Stephens (University of Leeds, UK). pFRT/*lacZeo,* pcDNA^{TM}6/TR, pOG44, pcDNA^{TM}5/FRT/TO from Invitrogen was kindly provided by Dr. Adrian Whitehouse (University of Leeds, UK). These plasmids were used to create a stable Flp-In T-Rex inducible cell line system.

2.1.4 Cell lines

Human embryonic kidney (HEK) 293 epithelial cell line was kindly provided by Professor Asipu Sivaprasadarao (University of Leeds, UK). HEK Flp-In^{TM} T-Rex^{TM} cell line was another kind contribution from Dr. Adrian Whitehouse (University of Leeds, UK). Immortalised porcine aortic endothelial cell line was kindly provided by Dr. Sam Stephens (University of Leeds, UK).

2.1.5 Mice

LOX-1 knockout mice on the C56BL/6 background were kindly gifted by Professor Tatsuya Sawamura (National Cerebral and Cardiovascular Centre, Osaka, Japan). *ApoE* knockout and wild-type (C57Bl/6J) mice were purchased from Charles River (Charles River Laboratories, Margate, UK). Mice were housed in a custom-built transgenic animal facility run by Central Biomedical Services at the University of Leeds. Housing conditions are described in detail in section 2.2.

2.1.6 Surgical equipment

All dissections were performed using instruments purchased from World Precision Instruments (Sarasota, USA) or InterFocus (Cambridge, UK).

2.2 Experimental Methods

2.2.1 Molecular Biology

2.2.1.1 Preparation of competent E.coli cells

E.coli XL10 gold cells were grown on Luria-Bertani (LB) agar (1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, 1% (w/v) NaCl and 1.5% (w/v) agar, pH 7.0) overnight at 37°C. A single colony was then inoculated into 50 ml of LB media (1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract and 1% (w/v) NaCl, pH 7.0) and grown for ~24 h at 37°C with shaking. The stationary phase bacteria was then diluted 1:20 in fresh LB and grown until an $OD_{550\text{-}600}$ of ~0.3-0.6 is reached. The culture is then chilled on ice for up to 15 min followed by centrifugation for 5 min at 3000 *g* at 4°C. Cells were then resuspended in a small amount of residual LB by vortexing. 20 ml of ice-cold Tfb I (30 mM potassium acetate, 100 mM $RbCl_2$, 50 mM $MnCl_2$, 10 mM $CaCl_2$ and 15% (v/v) glycerol, pH 5.8) was added per 50 ml of culture and cells resuspended in the buffer then incubated on ice for up to 45 min. Cells were centrifuged for 10 min at 3000 *g* at 4°C. The pelleted cells were then resuspended in 4 ml ice-cold Tfb II (10 mM MOPS, 75 mM $CaCl_2$, 10 mM $RbCl_2$ and 15% (v/v) glycerol, pH6.5) and incubated on ice for 30 min. Cells were aliquoted and snap frozen on dry ice before storage at -70°C.

2.2.1.2 Transformation into competent E.coli cells

10 µl of competent *E.coli* XL10 gold cells were added to the DNA and incubated on ice for 5 min. Cells were heat shocked at 42°C for 1 min, and returned to ice for 3 min. Cells were plated onto LB plates containing the appropriate antibiotic and incubated at 37°C overnight.

2.2.1.3 Plasmid DNA miniprep using TENS procedure

A rapid and cheap TENS method for plasmid DNA purification for analytical studies was as follows. 5 ml bacterial culture was set up in LB from a single colony and grown at 37°C for 16 h. 1.5 ml of the culture was centrifuged for 30 sec at 16000 *g* and the pelleted cells were resuspended in 50-100 µl of LB media. 300 µl of TENS solution (10 mM Tris pH 8, 1 mM EDTA, 0.1 NaOH, 0.5% (w/v) SDS, 0.1 mg/ml RNase) was added and vortexed briefly to lyse the cells. 150 µl of 3 M NaAc or KAc, pH 5.5, was added and tubes were inverted 4-6 times. This was centrifuged for 5 min at 16000 *g* and the supernatant transferred to a tube containing 0.9 ml ice-cold 100% ethanol, vortexed and centrifuged for 5 min at 16000 *g*. The DNA pellet was then washed twice in 70% ethanol, and then allowed to air dry and resuspended in TE (10 mM Tris, 1 mM EDTA, pH 8).

2.2.1.4 Small scale DNA purification

To obtain high quality plasmid DNA, a different procedure was carried out. 5 ml of LB plus appropriate antibiotic was inoculated with a single colony and grown at 37°C for 16 h in a shaking incubator. 1.5 ml was pelleted by centrifugation at 16000 *g.* Plasmid DNA was then purified using the Qiagen miniprep kit (Hilden, Germany) according to the manufacturer's instructions.

2.2.1.5 Large scale DNA purification

Single colonies were selected with a sterile pipette tip, inoculated into 2 ml of LB plus appropriate antibiotic, and cultured at 37°C in a shaking incubator for 6 h. This was then diluted 1:500 in 100 ml of LB plus appropriate antibiotic, and cultured at 37°C in a shaking incubator for 16 h. The DNA maxiprep was then carried out using the Qiagen maxiprep kit (Hilden, Germany) according to the manufacturer's instructions.

2.2.1.6 Restriction digests

Restriction digests contained the appropriate buffer at a final concentration of 1x for the enzymes being used. Bovine serum albumin (BSA) was

added when required, to a final concentration of 100 µg/ml. Restriction enzymes were added in accordance to the manufacturer's guidelines for the amount of DNA to be digested. Reactions were made up to a specific volume with sterile water. Typically, reactions were incubated at 37°C for 2 h before processing for analysis or sub-cloning.

2.2.1.7 Gel purification of DNA fragments

DNA fragments run on a 1-2.5% (w/v) agarose gel were visualised using a UV transilluminator and excised from the gel using a clean scalpel. DNA was purified using a QIAquick gel extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.2.1.8 Ligation of plasmid vector and DNA fragments

Ligations were set up in a total volume of 10 µl. Reactions contained 2 µl 5x T4 DNA ligase buffer, 0.5 µl T4 DNA ligase (Invitrogen, Amsterdam, Netherlands), a 1:3 molar ratio of vector to insert is usually used with 100 ng of vector being used. Insert was replaced with sterile water in negative controls. Ligations were incubated in 16°C water bath overnight.

2.2.1.9 Agarose gel electrophoresis

Electrophoresis of DNA samples was carried out using 1-2.5% (w/v) agarose gels containing 1 µg/ml ethidium bromide in 0.5X TAE buffer (2 mM Tris, 1 mM acetic acid, 0.5 mM EDTA, pH 8) or 0.5X TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8). Gels were run in 0.5X TAE or 0.5X TBE buffer at 100 V for ~1 h. DNA was visualised in a G:BOX XT4 Chemi imaging workstation (Syngene, Cambridge, UK).

2.2.1.10 Plasmid DNA sequencing

600 ng of plasmid DNA in 30 µl deionised water was sent for sequencing carried out by the DNA Sequencing Service (Dundee University, UK). Sequencing of pcDNA5/FRT/TO plasmids were carried out using the CMV forward primer (5'-CGCAAATGGGCGGTAGGCGTG-3') and BGH reverse primer (5'-TAGAAGGCACAGTCGAGG-3').

2.2.1.11 RNA extraction and purification

RNA extraction was performed using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, cells grown in 6-well plate were lysed in 350 µl of buffer RLT and homogenized using QIAshredder (Qiagen, Hilden, Germany). An equal volume of 70% (v/v) ethanol was added to the lysate and the RNA bound to resin in a spin column by centrifugation for 15 sec at 8000 *g*. RNA was washed in buffer RW1 for 15 sec and then incubated with DNase I for 15 min to digest any deoxyribonucleases. A further 15 sec wash with RW1 preceded 2 washes in buffer RPE, firstly for 15 sec, then for 2 min. All washes were spun down at 8000 *g.* RNA was then eluted in 80 µl RNase-free water. Quality and quantity of RNA was verified after each extraction. RNA content of samples was quantified using OD_{260} measurements in a nanophotometer (Implen, Munich, Germany). Quality was then assessed by calculating the OD_{260}/OD_{280} ratio.

2.2.1.12 Quantitative real-time reverse-transcriptase polymerase chain reaction (qPCR)

RNA was reverse transcribed into cDNA using High Capacity Reverse Transcription kit (Applied Biosystems, California, US) according to the manufacturer's instructions. Briefly, a master mix of 2 µl 10X Reverse Transcription buffer, 0.8 µl 25X dNTP, 2 µl 10X random primers, 1 µl Multiscribe Reverse Transcriptase and 4.2 µl nuclease-free water was made up and added to 10 µl RNA sample. Reverse transcription was carried out using a Biometra TProfessional thermocycler (Göttingen, Germany). Incubation times were 25°C for 10 min, followed by 37°C for 120 min and 85°C for 5 min. The resultant cDNA was diluted 1 in 4 with DEPC-treated water. 5 µl of diluted cDNA was combined with 300 nM (final concentration) of the relevant primers and 12.5 µl Power SYBR Green PCR master mix (Applied Biosystems, California, US). The reaction mix was made up to 25 µl with DEPC-treated water. PCR reactions were performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, California, UK) with ROX reference dye used for

normalisation. The reaction initiated at 50°C for 2 min prior denaturing for 10 min at 95°C. Samples then underwent 45 cycles of denaturing at 95°C for 15 sec, and annealing and extension at 60°C for 1 min. PCR products were quantified relative to housekeeping genes encoding GAPDH. The primers used are summarised in table 2.2.

2.2.1.13 DNA extraction for genotyping

Ear notches were taken from mice in accordance with Home Office regulations. DNA from ear notches were extracted using MyTaq Extract PCR kit (Bioline, London, UK) according to the manufacturer's instructions. Briefly, 20 µl of buffer A, 10 µl of buffer B and 20 µl deionised water were added and vortexed. The mixture was incubated at 75°C for 5 min followed by further incubation at 95°C for 10 min. The mixture then spun down for 1 min at top speed. The extracted DNA in the supernatant was diluted 1 in 9 with deionised water.

2.2.1.14 DNA amplification using polymerase chain reaction

PCR amplification of DNA was carried out using MyTaq HS Red mix (Bioline, London, UK) according to manufacturer's guidelines. For LOX-1 and ApoE PCR, 1 µl of DNA was mixed on ice with 12.5 µl MyTaq HS Red mix, 0.5 µl of 20mM primers (table 2.3) and top it up to 25 µl with deionised water. PCR was carried out using a Biometra TProfessional thermocycler (Göttingen, Germany). The PCR cycles were as follows:

LOX-1

95°C	10 min	
95°C	30 sec	x 35 cycles
60°C	30 sec	
72°C	30 sec	
72°C	7 min	
4°C	Hold	

ApoE

94°C	3 min	
94°C	30 sec	x 35 cycles
68°C	40 sec	
72°C	1 min	
72°C	2 min	
10°C	Hold	

Mouse LOX-1	
Forward	5’- GTC ATC CTC TGC CTG GTG TTG T -3’
Reverse	5’- TGC CTT CTG CTG GGC TAA CAT C -3’
Human LOX-1	
Forward	5’- GAA ACC CTT GCT CGG AAG CTG A -3’
Reverse	5’- CAG ATC CAG TCT TGC GGA CAA -3’
Porcine LOX-1	
Forward	5’- GAG TCT TTC CAC TCT GCG GT -3’
Reverse	5’- CGG TCA CCA GTA ATC CCA GG -3’
Mouse GAPDH	
Forward	5’- GGG TGT GAA CCA CGA GAA AT -3’
Reverse	5’- CCT TCC ACA ATG CCG AAG TT -3’
Human GAPDH	
Forward	5’- GTC TCC TCT GAC TTC AAC AGC G -3’
Reverse	5’- ACC ACC CTG TTG CTG TAG CCA A -3’
Porcine GAPDH	
Forward	5’- CCA TGT TTG TGA TGG GCG TG -3’
Reverse	5’- CCA GGG GCT CTT ACT CCT TG -3’

Table 2.2: Primer sequences for real-time quantitative PCR.

LOX-1	
Forward	5’- CGC CAA CCA TGG CTA TGG GAG AAT GG -3’
Reverse	5’- CAG CGA ACA CAG CTCCGT CTT GAA GG -3’
ApoE	
Common	5’- GCC TAG CCG AGG GAG AGC CG -3’
Wild-type reverse	5’- TGT GAC TTG GGA GCT CTG CAG C -3’
Mutant reverse	5’- GCC GCC CCG ACT GCA TCT -3’

Table 2.3: Primer sequences for DNA genotyping.

2.2.2 Protein analysis by SDS-PAGE

2.2.2.1 Preparation of cell lysates

Media was aspirated from flasks and cells were washed twice in cold PBS. Cells were then lysed in 2% (w/v) SDS in PBS containing 1 mM PMSF and protease inhibitor cocktail (Roche) and scraped into microcentrifuge tubes. This was followed by incubating lysates at 95°C for 5 min and sonicated for 5 sec.

2.2.2.2 Preparation of tissue lysates

Frozen mouse tissue was cut into appropriate size using a scalpel. The tissue was then lysed and homogenized in 1X cell lysis buffer (Cell signalling Technology, Massachusetts, US) using a hand-held TissueRuptor (Qiagen, Hilden, Germany). This was followed by sonication of tissue lysates on ice for 20 sec prior to centrifuging at 15,000 rpm for 20 min at 4°C, with resultant supernatant transferred to a fresh tube.

2.2.2.3 BCA assay

After preparing lysates, total protein concentration can be quantified by bicinchoninic acid (BCA) assay. On a 96-well plate, 10 µl of standard bovine serum albumin (BSA) controls at different concentration of 0, 0.2, 0.4, 0.6, 0.8, 1.0 mg/ml and 5 µl of protein samples were added in duplicates. Reagents A and B of Pierce BCA protein assay (Thermo Fisher Scientific, Massachusetts, US) were mixed together in a 50:1 ratio, and 200 µl of the mixture was added into each well. The plate was then incubated at 37°C for 20 min, followed by reading at 562 nm using a Tecan plate reader connected to a PC running on Magellan version 6.0 software (Tecan, Reading, UK). Values were obtained from it to calculate the maximal amount of sample to be loaded for SDS-PAGE gel and immunoblot analysis.

2.2.2.4 SDS-PAGE

Protein samples were added to an equal volume of 2X SDS-PAGE sample buffer (100 mM Tris pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 0.1% (w/v)

bromophenol blue, 4% (w/V) β-mercaptoethanol) and boiled at 95°C for 10 min. Samples were briefly centrifuged (maximum speed, 10 sec) to collect all droplets. To separate proteins, SDS-PAGE gels were made up with the solutions listed in table 2.4, and the gels were allowed to set for >15 min. Samples were loaded in a 5% stacking gel (30% (w/v) acrylamide, 1 M Tris pH 6.8, 10% (w/v) SDS, deionised water, 10% (w/v) APS, TEMED) and subjected to electrophoresis in a discontinuous running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS) at 130V for ~2 h.

2.2.2.5 Western blotting

Separated proteins were transferred onto a nitrocellulose membrane (Whatman Protran 0.2 µm pore size, Schleicher & Schuell Bioscience, Dassel, Germany) in transfer buffer (25 mM Tris-HCl pH 7.5, 106 mM glycine, 20% (v/v) methanol) at 4°C for 3 h at 300 mA or overnight at 30 mA. The membrane was briefly stained with 0.1% Ponceau S in 5% (w/v) acetic acid to confirm successful transfer and equal protein loading, before being rinsed with TBS-T (20 mM Tris-HCl pH 7.6, 137 mM NaCl, 0.1% (v/v) Tween-20) and blocked in 5% (w/v) skimmed milk in TBS-T for 1 h. Membranes were then probed with primary antibodies dissolved in 1% (w/v) BSA in TBS-T with 1 mM sodium azide at 4°C overnight. Membranes were washed 3 times for 10 min in TBS-T and incubated for 1 h in species-specific horseradish peroxide (HRP)-conjugated secondary antibodies at room temperature. Membranes were washed 3 times for 10 min in TBS-T and incubated briefly with EZ-ECL combined enhanced chemiluminescence substrate detection kit (Geneflow Ltd, Staffordshire, UK). Bound antibodies were then visualised by enhanced chemiluminescence in a G:BOX XT4 Chemi imaging workstation (Syngene, Cambridge, UK). Band intensity was determined using 2-D densitometry running on dedicated image analysis software (Syngene).

Solutions	Volume (ml) required for different percentage gels		
	10%	**12%**	**15%**
30% acryl	6.7	8	10
3 M Tris (pH 8.8)	2.5	2.5	2.5
ddH_2O	10.2	8.9	6.9
10% SDS	0.2	0.2	0.2
10% APS	0.4	0.4	0.4
TEMED	16 µl	16 µl	16 µl

Table 2.4: Volumes of solutions for one resolving gel for different percentage gels.

2.2.3 Cell culture

2.2.3.1 Cell passage

Epithelial human embryonic kidney 293 (HEK293) cell line and Flp-In™ T-Rex™ -293 cell line were cultured in Dulbecco's modified eagle medium (DMEM; Gibco, Cramlington UK) containing 10 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-Glutamine, 1X non-essential amino acids and 10% (v/v) foetal calf serum (FCS; Life Technologies, Paisley, UK). Immortalised porcine aortic endothelial cell (PAEC) was cultured in Roswell Park Memorial Institute 640 (RPMI640; Gibco, Cramlington, UK) containing 10 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-Glutamine, 1X non-essential amino acids and 10% (v/v) foetal calf serum (FCS; Life Technologies, Paisley, UK). All three cell lines were incubated at 37°C in a hydrated 5% CO_2 atmosphere. Cells were passaged every 2-3 days by trypsinisation with 1 ml of TrypLE Express (Invitrogen, Amsterdam, Netherlands), followed by incubation at 37°C for 3 min, until cells had detached from plate. Complete DMEM or RPMI was used to quench the trypsin and remove the cells from the plate, and then plated out. For HEK293 and Flp-In™ T-Rex™ -293 cells, flasks or plates were pre-coated with poly-l-lysine (Sigma-Aldrich, Poole, UK) for 30 min at room temperature. Poly-l-lysine was removed and washed once with PBS, and air-dried for at least 2 h.

2.2.3.2 Calcium phosphate-based gene transfection of mammalian cells

Cells were plated the day before transfection at ~20% confluency. Pre-warmed Opti-MEM reduced serum (Gibco, Cramlington, UK) media was added to cells before transfection in the volumes shown in table 2.5 plus HBS, plasmid DNA and 2.5 M $CaCl_2$. The mixture was incubated for 20 min at room temperature prior to adding to cells in drop-wise manner. The transfection reagent was left on cells overnight and media was changed the next day with full serum media. Cells were ready for further experiment 24-48 h post-transfection. This method was used for transient cell transfection.

2.2.3.3 Lipid-based gene transfection of mammalian cells

Cells were plated the day before transfection at the appropriate number for the plate being used. The Lipofectamine 2000 (Invitrogen, Amsterdam, Netherlands) transfection reagent was used and carried out according to the manufacturer's instructions. This was carried out in Opti-MEM media. Media was replaced with full serum media 4-6 h post-transfection. This method was used for generating stable cell line.

2.2.3.4 Analysis of intracellular signalling pathways

Cells were seeded into 6-well plates and cultured for at least 24 h in full serum media until ~80% confluent. At the same time, 1 μg/ml of tetracycline was added to induce LOX-1 expression. Media was then aspirated and cells were washed twice with PBS. Cells were then starved in Opti-MEM reduced serum media for 2 h prior to stimulation with 10 μg/ml oxidised LDL (oxLDL) as specified in each experiment. After the specified time course, plates were put on ice and media aspirated; cells were then washed twice with ice-cold PBS. Plates were then removed from ice and cells lysed using an appropriate buffer.

Plate size	Medium volume	HBS	$CaCl_2$	DNA
24 well	300 µl/well	18 µl	1.1 µl	1 µg
6 well	1.5 ml/well	86 µl	5.1 µl	5 µg
10 cm dish	8 ml/well	500 µl	30 µl	30 µg

Table 2.5: Transfection volumes for calcium phosphate-based gene transfection.

2.2.3.5 Cell surface biotinylation

Cells were stimulated (oxLDL in opti-MEM) for a specified time course before washing twice with ice-cold PBS and incubated with 0.25 mg/ml EZ-Link Sulfo-NHS-LC-Biotin (Thermofisher) in PBS containing 2 mM $MgCl_2$ and 2 mM $CaCl_2$ for 40 min at 4°C. Biotinylation was quenched by washing twice with ice-cold 1X TBS (20 mM Tris-HCl pH 7.6, 137 mM NaCl) followed by washing twice with ice-cold PBS. Cells were lysed in 500 µl NP-40 buffer (50 mM Tris pH7.5, 150 mM NaCl, 1% NP-40) for 5 min on ice before scraping the cells. Lysates were cleared by centrifugation at 15000 rpm for 30 min at 4°C. Equivalent protein amounts were incubated with 35 µl neutravidin-agarose beads (ThermoFisher) overnight at 4°C. Beads were pelleted by brief centrifugation, supernatant removed and beads washed 4 times with 500 µl ice-cold NP-40 buffer. 40 µl of 2X SDS-PAGE sample buffer was added and proteins eluted by heating at 95°C for 10 min before analysis by SDS-PAGE and immunoblotting.

2.2.3.6 Assessment of endothelial monolayer permeability using trans-endothelial electrical resistance (TEER)

Human endothelial cells were seeded in 450 µl at 1.5 x 10^5 cells/well (sufficient to give a monolayer) into a 0.4 µm pore size Transwell filter inserted into a 24-well plate (BD Biosciences, Oxford, UK) containing 500 µl culture media and left to adhere overnight. At t=0 h the trans-endothelial electrical resistance (TEER) across each monolayer was measured using a MILLICELL-ERS TEER machine (Merck Millipore). Following this, 50 µl of culture media + 10 µg/mL oxidised LDL was added to the upper chamber. After a further 1, 2, 4, 6 or 8 h, TEER across each monolayer was measured again and the relative increase in permeability (corresponding to a decrease in electrical resistance across the endothelial monolayer) was calculated as follows: Relative TEER (%) = (TEER of sample of interest– TEER of blank (TEER across insert with no cells) / TEER of control cells at 0 h – blank)*100.

2.2.4 LDL and oxidised LDL preparation

2.2.4.1 Purification of LDL particles from blood

20 ml of blood was collected by a medical doctor from consenting healthy adult volunteers (under University of Leeds of Faculty of Biological Sciences local ethical approval and license). Blood was added to 3.8% (w/v) trisodium citrate in a 9:1 ratio to prevent blood coagulation. Human plasma was separated from blood cells by centrifugation at 4000 rpm for 20 min at room temperature. The clear, yellowish plasma was transferred to a fresh tube containing OptiPrep Density Gradient medium (Sigma) in 4:1 ratio to give a final concentration of 12% (v/v) iodaxinol. 1 ml of HBS (0.85% (w/v) NaCl, 10 mM HEPES pH 7.4) was added to a 4.7 ml Beckman Opti-Seal centrifuge tube. The plasma-OptiPrep mix was layered under the HBS solution and centrifuged at 100,000 rpm at 16°C for 3 h. The different lipoprotein fractions formed discrete bands in the tube. LDL forms a deep orange band towards the top of the tube. The LDL band was removed with a 25 gauge needle attached to a 1 ml syringe. The extracted LDL was dialysed into PBS at 4°C for 24 h. The concentration of LDL was measured using a BCA assay.

2.2.4.2 Oxidation of LDL

Following dialysis into PBS, native LDL was stored in fresh tube containing 100 µM EDTA and 20 µl butylated hydroxytoluene (BHT) at 4°. LDL particles were oxidized by incubation with 5 µM $CuSO_4$ at 37°C for 24 h. The oxidation reaction was terminated by adding 100 µM EDTA and 20 µM BHT. The concentration of oxLDL was measured using a BCA assay.

2.2.4.3 Agarose gel electrophoresis of lipid particles

The relative electrophoretic mobility of oxLDL and native LDL was analysed by agarose gel electrophoresis followed by Sudan black staining. 4 µg of lipid particles were loaded to 0.5% (w/v) agarose gel in borate buffer (80 mM boric acid, 90 mM Tris-HCl pH 8.3, 3 mM EDTA) and run at 100 V for 1 h. The gel was then fixed in 75% (v/v) ethanol plus 5% (v/v) acetic acid for 15 min. The gel was then stained with a saturated solution

of the lipid stain Sudan black in 60% (v/v) ethanol and 0.05% (w/v) NaOH for 2 h. This was followed by de-staining of gel in 50% (v/v) ethanol.

2.2.4.4 Fluorescent DiI-labelling of lipid particles

OxLDL particles were labelled with the fluorescent compound 1,1 dioctadecyl-3,3,3',3'-tetramethyllindocarbocyanine perchlorate (DiI; Sigma) in DMSO. 300 µg of DiI was added to each milligram of lipoprotein and incubated in the dark at 37°C for 18 h. The labelled oxLDL was then centrifuged at 13,000 rpm for 10 min before dialysing against PBS in the dark for 24 h at 4°C. The concentration of DiI-oxLDL was measured by BCA assay.

2.2.5 Immunofluorescence analysis

Media was aspirated from cells seeded on poly-l-lysine (Sigma-Aldrich, Poole, UK) coated coverslips in 24-well plates and cells were rinsed twice in PBS. Cells were fixed in 500 µl 10% (v/v) formalin (Sigma-Aldrich, Poole, UK) for 5 min at 37°C. Fixative was aspirated and coverslips rinsed twice in PBS. Coverslips were then incubated in 5% (w/v) BSA in PBS to block non-specific antibody binding to cells, followed by washing twice in PBS. Coverslips were inverted onto a 25 µl drop of primary antibody solution diluted in 1% (w/v) BSA in PBS (table 2.1) in a moist staining chamber and incubated overnight at room temperature. Coverslips were washed 3 times with PBS and inverted onto a 25 µl secondary antibody solution containing 4 µg/ml donkey Alexa Fluor-conjugated secondary antibody (Invitrogen, Amsterdam, Netherlands), 2 µg/ml 4,6-diamidino-2-phenylidole (DAPI) in 1% (w/v) BSA in PBS and incubated for 2 h at room temperature. Coverslips were washed 3 times with PBS and mounted onto slides using Fluoromount G (Southern Biotech, Alabama, US). Images were acquired either using a wide-field deconvolution microscope DeltaVision (Applied Precision Inc., Issaquah, US) or an EVOS-fl inverted digital microscope (Life technologies, Paisley, UK). Relative protein levels or co-distribution were analysed and quantified using Image J (NIH, Bethesda, US).

2.2.6 Animal housing and husbandry

All mice were housed in individually ventilated cages at no more than 5 per unit. Animals only shared a cage with siblings of the same sex. The complex lighting was on a ‘fade up-fade down’, 12 h light and 12 h dark cycle from 0630 to 1830. Room temperature was 21°C +/- 2°C. Standard chow feed (Rat and Mouse No.1 Maintenance; Special Diet Services, Essex, UK) and water were available *ad libitum*. Mice were checked upon daily. Breeding cages were set up with 1 male and 2 female over the age of 8 weeks. Pups were weaned at 18 days and ear notched for identification thereafter. Stringent records were kept of husbandry and experimental procedures. Any mice displaying stunted development or overly aggressive behaviour were excluded from experiments.

2.2.6.1 Pro-atherogenic diet

Mice were fed with 0.2% cholesterol Western diet (Special Diet Services, Essex, UK) for 12 weeks from 8 weeks of age. Before 8 weeks old, they were fed standard chow (Special Diet Services, Essex, UK). Feed was available *ad libitum* and animals were weighed weekly.

2.2.6.2 Organ harvesting

Isofluorane (CBS, University of Leeds) anaesthesia was induced and a cardiac puncture technique was performed to withdraw blood. Mice were laid on their back, and using a 1 ml syringe and a 22 gauge needle, needle was inserted perpendicular to chest wall, straight to the apex of the heart in the left ventricle. Blood was slowly withdrawn by gently pulling back on the plunger to obtain the maximum amount of blood available. Blood was transferred to heparin-coated tubes and immediately placed on ice. This was followed by performing a midline laparotomy. The abdominal contents were displaced to the right. 5 ml of PBS was slowly perfused into the left ventricle to flush out any remaining blood. Organs such as the heart, liver, adipose tissue, and aorta were dissected, and then placed in a tube before snap freezing the tissues in liquid nitrogen.

2.2.7 Statistical analysis

This was performed using the unpaired two-tailed Student's *t*-test for 2 groups, one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test or two-way ANOVA followed by Bonferroni multiple comparison test using GraphPad Prism software (La Jolla, CA, US). Significant differences between control and test groups were evaluated with *p* values less than 0.05 (*), 0.01 (**), 0.001 (***) and 0.0001 (****) indicated on the graphs. Error bars in graphs denote ± SEM (Standard error of mean).

CHAPTER 3

Development of a tetracycline-inducible LOX-1 expression system

3.1 Introduction

LOX-1 is a scavenger receptor for oxidised LDL that is associated with pathological states such as atherosclerosis and diabetes (Dunn et al., 2008; Mitra et al., 2011; Chen et al., 2001c). LOX-1 is expressed in vascularised tissues such as heart, lung and liver (Sawamura et al., 1997). LOX-1 is expressed in different vascular and immune systems including the endothelium, monocytes, macrophages and smooth muscle. Expression of LOX-1 in resting or basal state is relatively low in primary endothelial cells *in vitro* (Mehta and Li, 1998). However, LOX-1 expression can be elevated by pro-inflammatory stimuli such as oxidised LDL, Ang II, TNFα and glucose (Aoyama et al., 1999; Li et al., 1999a; Li et al., 2003d).

One problem in studying the function of LOX-1 is that the expression itself is associated with increased programmed cell death i.e. apoptosis (Li and Mehta, 2009). An inducible expression system in mammalian cells would make it easier to study the functional role of this scavenger receptor. One solution is the use of a system that relies on the integration of a gene of interest using a FLP recombinase-mediated integration to enable tetracycline-inducible expression of the GOI at this locus (O'Gorman et al., 1991; Yao et al., 1998). The Flp-In™ T-Rex™ system is designed to create mammalian cell lines which stably express proteins of interest and protein expression is constant across a population of cells. The system uses the FRT site for integration of the LOX-1 cDNA

by FLP recombinase. The tightly controlled expression of the LOX-1 transgene is regulated by the tetracycline repressor (TetR), which binds to two tetracycline operator sequences upstream of the gene of interest and thus blocks gene transcription at the level of new RNA synthesis. By using this system, it also removes variations in transgene expression levels caused by genome specific variability, and also enables control of the timing of gene expression (Thomas et al., 2004).

The oxidised LDL binding to LOX-1 causes an increase in apoptosis in a variety of cell types. This LOX-1-mediated apoptosis is caused by activation of caspase-9 and caspase-3, which down-regulate anti-apoptotic proteins (Li and Mehta, 2009). It is likely that culture media contains lipid particles and related substances that become oxidised and become ligands that activate LOX-1 signalling and promote apoptosis. The aim of this chapter was thus to generate a stable cell platform for studying LOX-1 signalling and functionality *in vitro***.**

3.2 Results

3.2.1 Generation of the expression construct with LOX-1 gene

The specific objectives were to generate an inducible LOX-1 expression system and test whether such a system could mediate LOX-1-specific oxidised LDL binding and uptake. Initially, the LOX-1 sub-cloned into pcDNA3.1 vector has a five amino acid linker peptide (GPGPG) and 5' *BamHI* and 3' *EcoRI* sites to the cDNA. Polymerase chain reaction (PCR) was used to generate an amino acid FLAG peptide sequence (DYKDDDDK) flanked by 5' *EcoRI* and 3' *XhoI* restriction sites (Murphy et al., 2006). Both digested LOX-1-WT and LOX-1-D5A extracted and purified from pCDNA3.1 plasmids were detected at ~3 kbp by 1.5% (w/v) agarose gel electrophoresis (Figure 3.1A). Appropriate control with uncut plasmid was detected at ~8 kbp (Figure 3.1A).

The LOX-1 cDNAs needed to be sub-cloned into the multiple cloning sites (MCS) of the pcDNA5/FRT/TO expression plasmid. pcDNA5/FRT/TO plasmid was digested by restriction enzymes, *BamHI* and *XhoI,* to produce 'sticky' ends. This linearized plasmid was detected at ~5 kbp along with the uncut plasmid control, which migrated faster than the digested product (Figure 3.1B). Finally, the digested and purified LOX-1-FLAG was sub-cloned into the pcDNA5/FRT/TO vector. Analysis of recombinant clones showed that both pcDNA5/FRT/TO LOX-1-WT and LOX-1-D5A inserts were detected at ~8 kbp; as the 'vector only' control was detected at ~5 kbp (Figure 3.2). Therefore, this confirms the directional insertion of LOX-1-WT and LOX-1-D5A into the pcDNA5/FRT/TO plasmid. DNA sequencing confirmed the sequence and orientation of the LOX-1-FLAG cDNA inserts within the pcDNA5/FRT/TO plasmid.

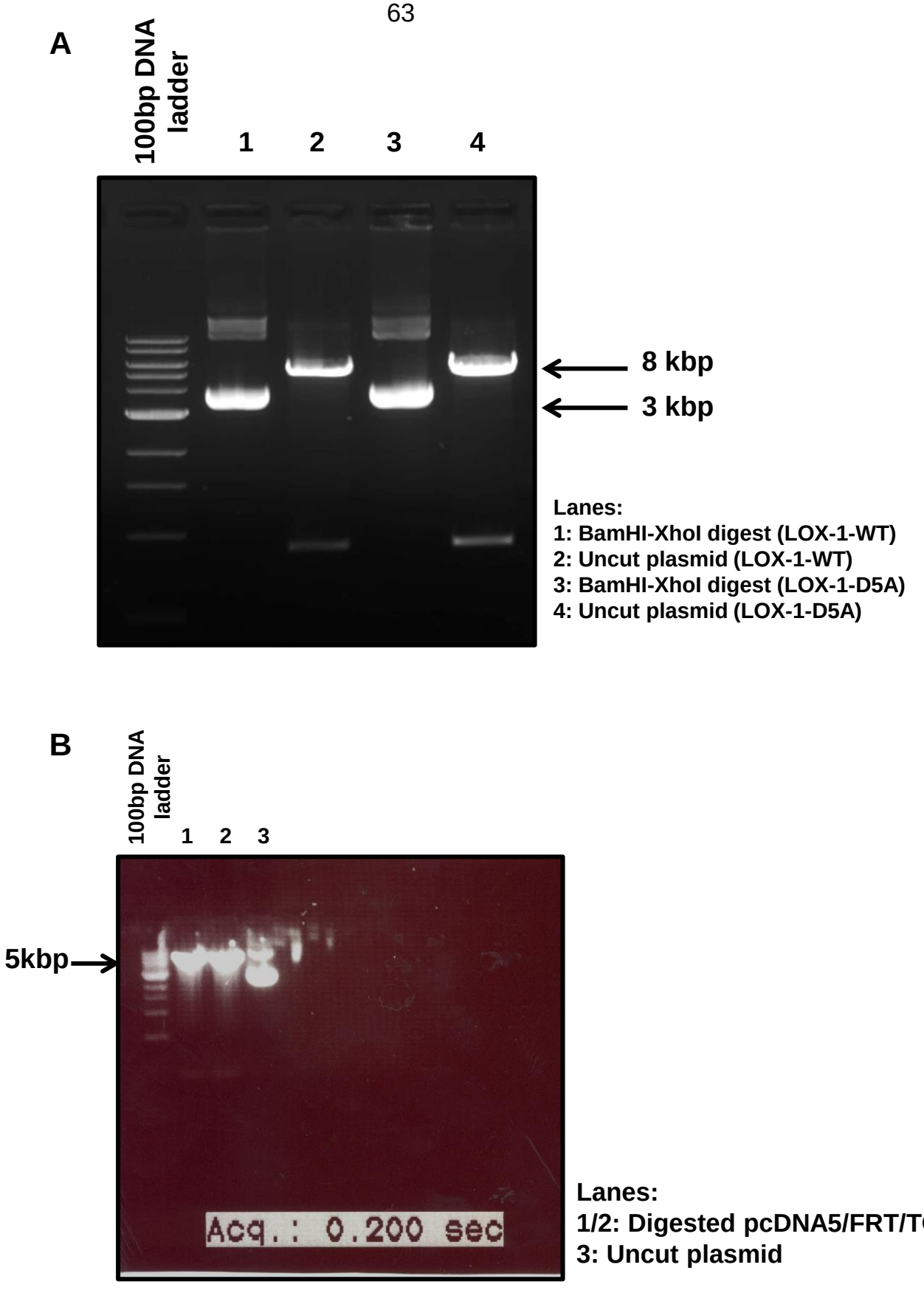

Fig. 3.1. Restriction digestion and analysis of LOX-1-bearing plasmids. Agarose gel electrophoresis of (A) digested products of pCDNA3.1 LOX-1-WT and LOX-1-D5A constructs, and (B) pcDNA5/FRT/TO (5 kbp) plasmid digested using *BamHI* and *XhoI.*

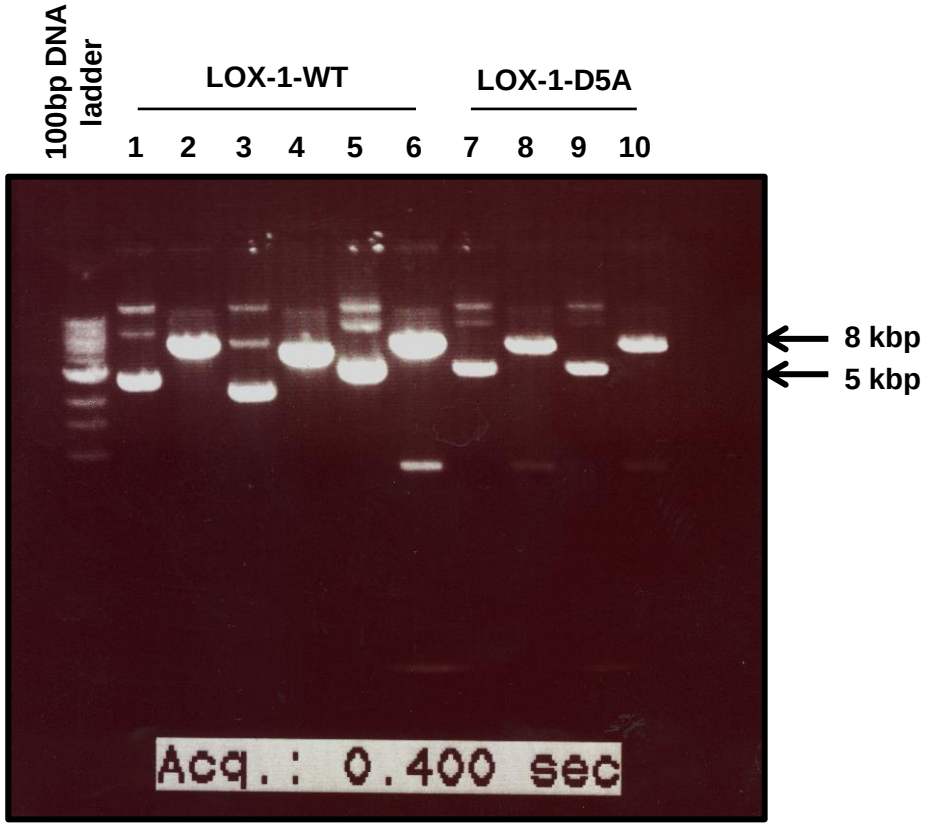

Fig. 3.2. Recombinant plasmid analysis of LOX-1 clones. Analysis of pcDNA5/FRT/TO-LOX-1 recombinant plasmids using 1.5% (w/v) agarose gel electrophoresis of clones with size of 6 kbp (even number lanes 2, 4, 6, 8, 10) and vector only as negative control with size of 5 kbp (odd number lanes 1, 3, 5, 7, 9).

3.2.2 Characterisation of Flp-In™ T-Rex™ cell lines

The generation of Flp-In™ T-Rex™ cell line, in which is the parent cell lines can receive and express gene of interest requires the stable integration of 2 plasmids, pFRT/*lacZeo* and pcDNA6/TR. The HEK293 Flp-In™ T-Rex™ cell line is available commercially, but the porcine aortic endothelial cell (PAEC) line needed to be constructed. The T-Rex cells maintain stable integration of both plasmids within the genome by conferring resistance to markers on these plasmids for resistance to zeocin and blasticidin antibiotics, respectively. This requires transfection and integration of the FRT site plasmid (pFRT/*lacZeo*), the selection of PAEC colonies by zeocin resistance and the verification by PCR assay. After successful transfection of pFRT/*lacZeo,* colonies formed were selected with 150 µg/ml zeocin and the PAEC clones were allowed to grow to confluency prior to expansion and PCR analysis. After the extraction of genomic DNA from each clone (1-6), PCR assay was carried out to confirm the successful integration of *lacZeo* gene. In figure 3.3A, the expected 450 bp PCR product was observed. This was confirmed by the positive control (pFRT/*lacZeo*) at 450 bp and the negative control (un-transfected parent cell line) showing absence of this PCR product.

Subsequently, the second plasmid pcDNA6/TR, which expresses the tetracycline repressor protein, is then expressed by transfection and integration of pFRT/*lacZeo*, where integrants were selected by blasticidin resistance. Like previously, colonies formed were selected with 15 µg/ml blasticidin where the cells then were propagated to confluency. After DNA extraction, successful PAEC clones (1-7) with the expected 1.5 kbp PCR product were seen (Figure 3.3B), as this was confirmed by the positive control PCR (pcDNA6/TR). Thus, the PAEC line containing the Flp-In™ T-Rex™ expression system has now been generated.

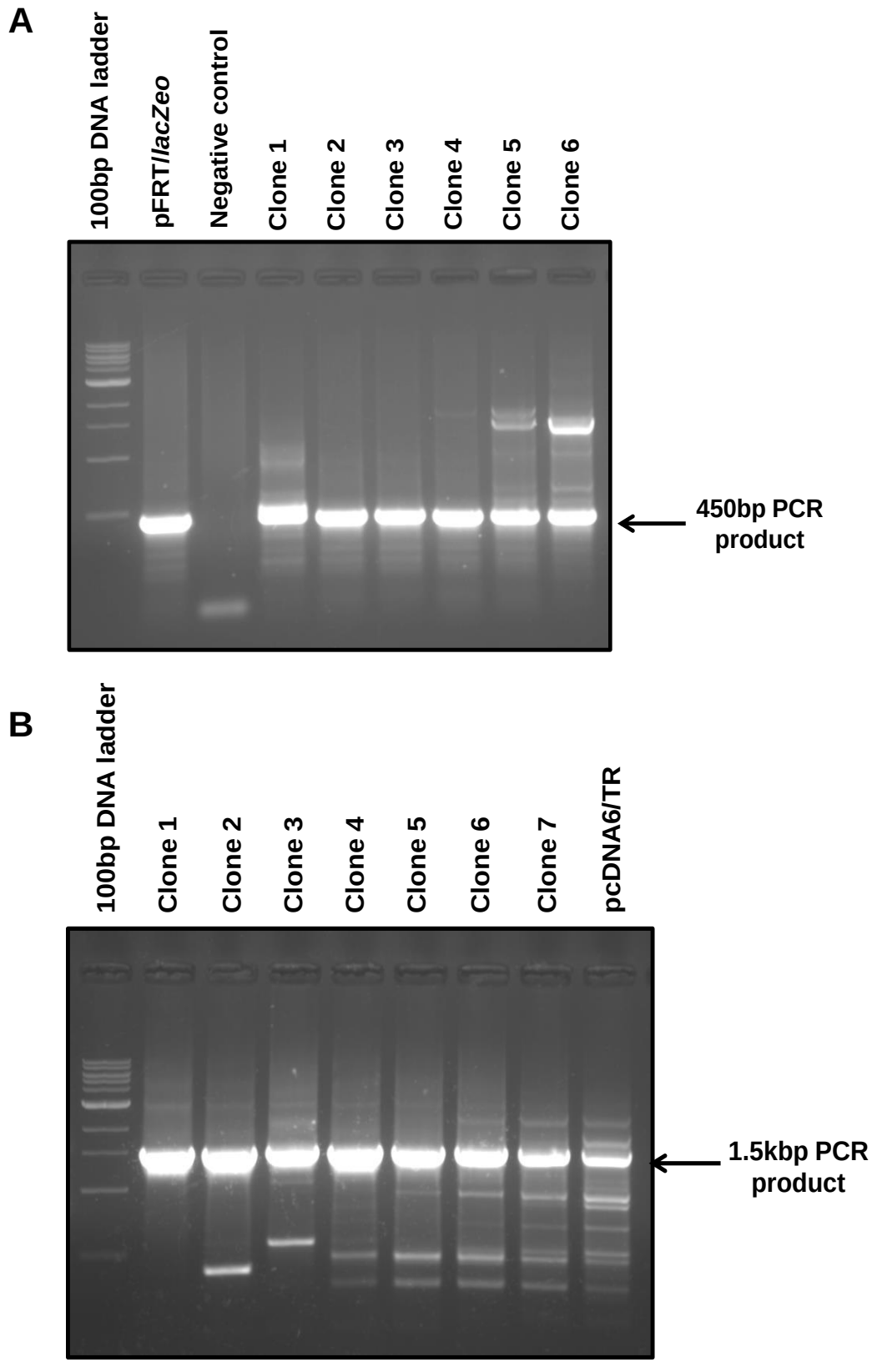

Figure 3.3. PCR analysis of stable integration of the tetracycline-inducible expression system. Analysis of PAEC clones were carried out using PCR assays for the confirmation of integration of (A) pFRT/*lacZeo* and (B) pcDNA6/TR. Samples were subjected to agarose gel electrophoresis and staining with ethidium bromide.

3.2.3 Generation of Flp-In™ T-Rex™ expression cell lines

Both the LOX-1-WT and LOX-1-D5A mutant FLAG-tagged cDNAs were sub-cloned into pcDNA5/FRT/TO expression vector (Fig. 3.2). These plasmids were then co-transfected into the HEK293 or PAEC Flp-In™ T-Rex™ cell lines along with a pOG44 plasmid expressing Flp recombinase. A negative control used in the experiment was the transfection of 'empty' plasmid pcDNA5/FRT/TO alone. Upon stable integration of the pcDNA5/FRT/TO containing the LOX-1 cDNA into the FRT site, the cells are rendered hygromycin resistant and zeocin sensitive. Thus, the successful stable integrants are maintained in culture media containing hygromycin and blasticidin antibiotics (but lacking zeocin).

After successful transfection of LOX-1-FLAG into Flp-In™ T-Rex™ HEK293 and PAEC cell lines, colonies were selected with hygromycin and blasticidin antibiotics to allow propagation. To screen for successful expression of LOX-1-FLAG in the cells from various numbers of colonies picked, the cells were induced with tetracycline in the culture media for 16-24 h. Subsequently, cells were lysed and subjected to immunoblotting analysis. Figure 3.4 shows analysis of different HEK293T and PAEC Flp-In™ T-Rex™ clones with negative control, LOX-1-WT (Figure 3.4A) and LOX-1-D5A (Figure 3.4B) with the absence or presence of tetracycline induction (16-20 h). In this context, LOX-1-WT and LOX-1-D5A mentioned are FLAG-tagged, which can be detected as bands of 37-45 kDa in the presence of tetracycline. As for the negative control (empty vector), in the absence and presence of tetracycline, no bands were detected, thus confirming that the expression of tagged LOX-1 proteins is encoded by the integrated transgene within each clone. The clone with the highest LOX-1 expression was chosen to be used in all the experiments onwards.

To further validate the successful expression of LOX-1-WT and LOX-1-D5A in these cell lines, quantitative real-time PCR (qRT-PCR) were carried out. As shown in figure 3.5A, mRNA levels of both LOX-1-WT and LOX-1-D5A were significantly increased by overnight incubation with tetracycline in HEK293 cells. As for PAEC cells, two different primer pairs,

namely human LOX-1 and porcine LOX-1 primer pairs were tested for qRT-PCR, as shown in Fig. 3.5B and Fig. 3.5C, respectively. Using the human-specific LOX-1 primers in PAEC (Figure 3.5B), both mRNA expression of wild-type and mutant LOX-1 were significantly expressed after tetracycline treatment. To check for native porcine LOX-1 mRNA expressed in PAEC cells, porcine LOX-1 primers sequence was used. Figure 3.5C shows significant levels of endogenous porcine LOX-1 mRNA upon tetracycline addition. The housekeeping gene GAPDH was used as an internal control for this qRT-PCR analysis of relative mRNA levels.

One issue was asking how the addition tetracycline over a time period regulates expression of LOX-1 protein. To assess the dynamics of tetracycline-induced LOX-1 protein expression, cells were treated with tetracycline for 0, 2, 4, 8, 16 and 24 h, then cells were lysed and subjected to immunoblotting. Fig. 3.6A shows tetracycline-induced expression of both LOX-1-WT and LOX-1-D5A. The expression profiles are similar with maximal tetracycline-induced expression of both proteins evident 16-24 h after tetracycline addition. The bands corresponding to these LOX-1 proteins were quantified and relative levels analysed for LOX-1-WT (Fig. 3.6B) and LOX-1-D5A (Fig. 3.6C). From these experiments, the optimal time period for maximal LOX-1 expression occurs 16-24 h after tetracycline addition.

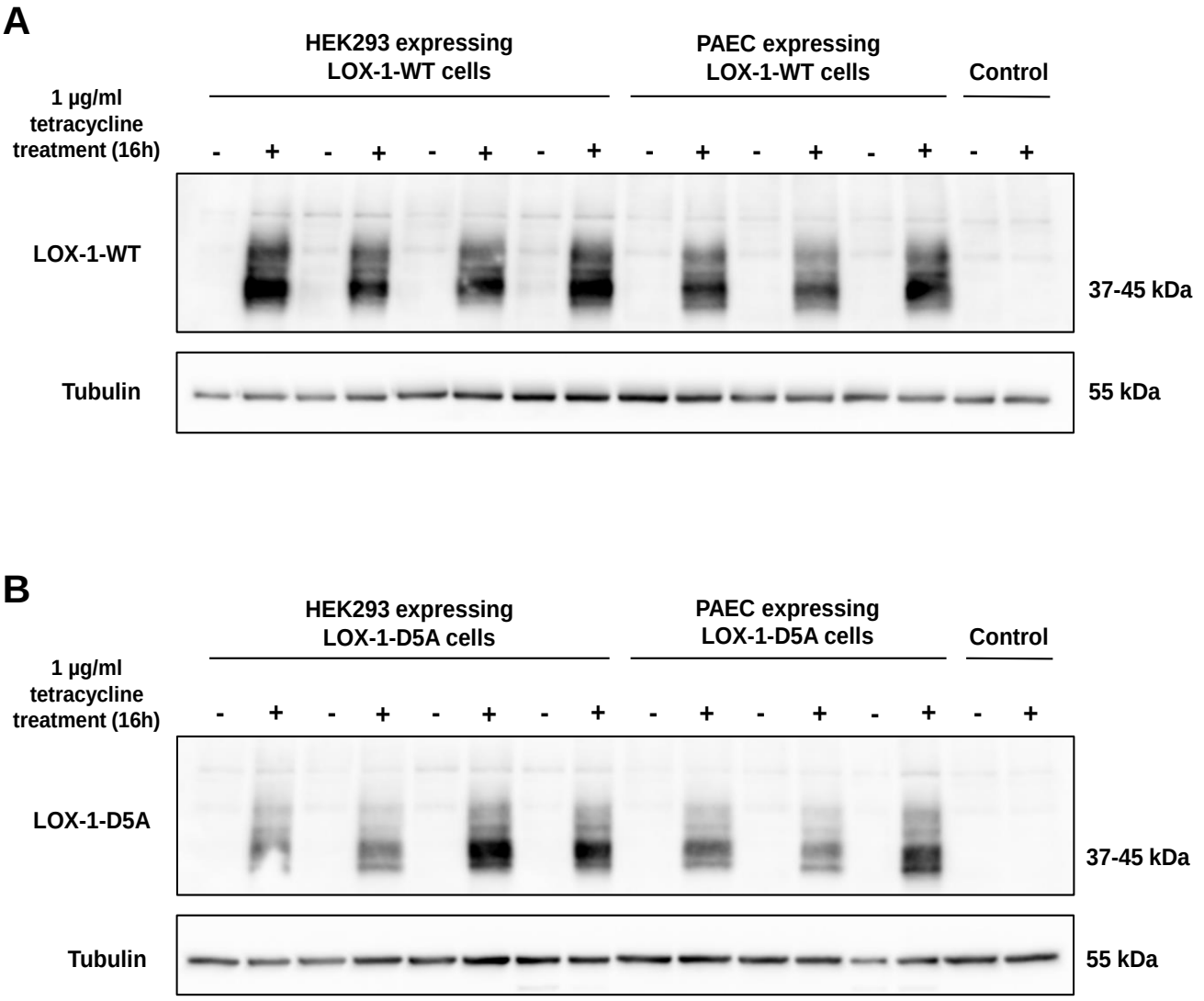

Fig. 3.4. Tetracycline-induced expression of LOX-1-WT and LOX-1-D5A. HEK293 and PAEC clones expressing (A) LOX-1-WT and (B) LOX-1-D5A were compared to clones carrying empty vector (negative control). Cells were induced with 1 µg/ml tetracycline for >16 h. Cells were then lysed and processed for immunoblot analysis using mouse anti-FLAG antibodies to detected the FLAG-tagged LOX-1 proteins.

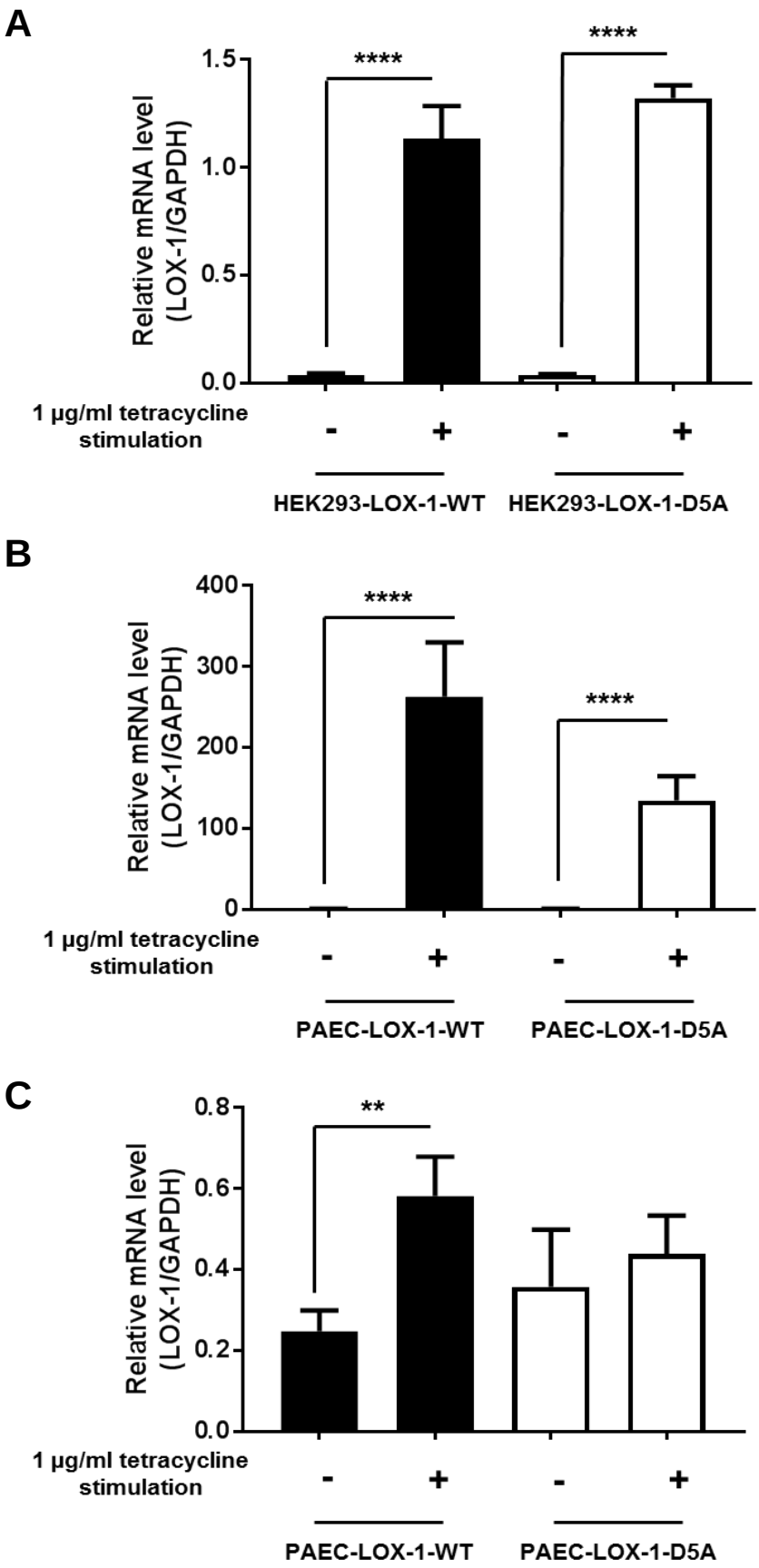

Fig. 3.5. Quantification of LOX-1-WT and LOX-1-D5A mRNA levels using qRT-PCR. The quantification of mRNAs in (A) HEK293 and (B, C) PAEC clones were performed using qRT-PCR encoding hLOX-1-WT and hLOX-1-D5A (A and B), and porcine LOX-1-WT (C). The mRNA levels were normalized to GAPDH as a housekeeping gene and internal control. Error bars indicate ±SEM (n≥3). $p<0.05$ (*), $p<0.01$ (**), $p<0.0001$ (****).

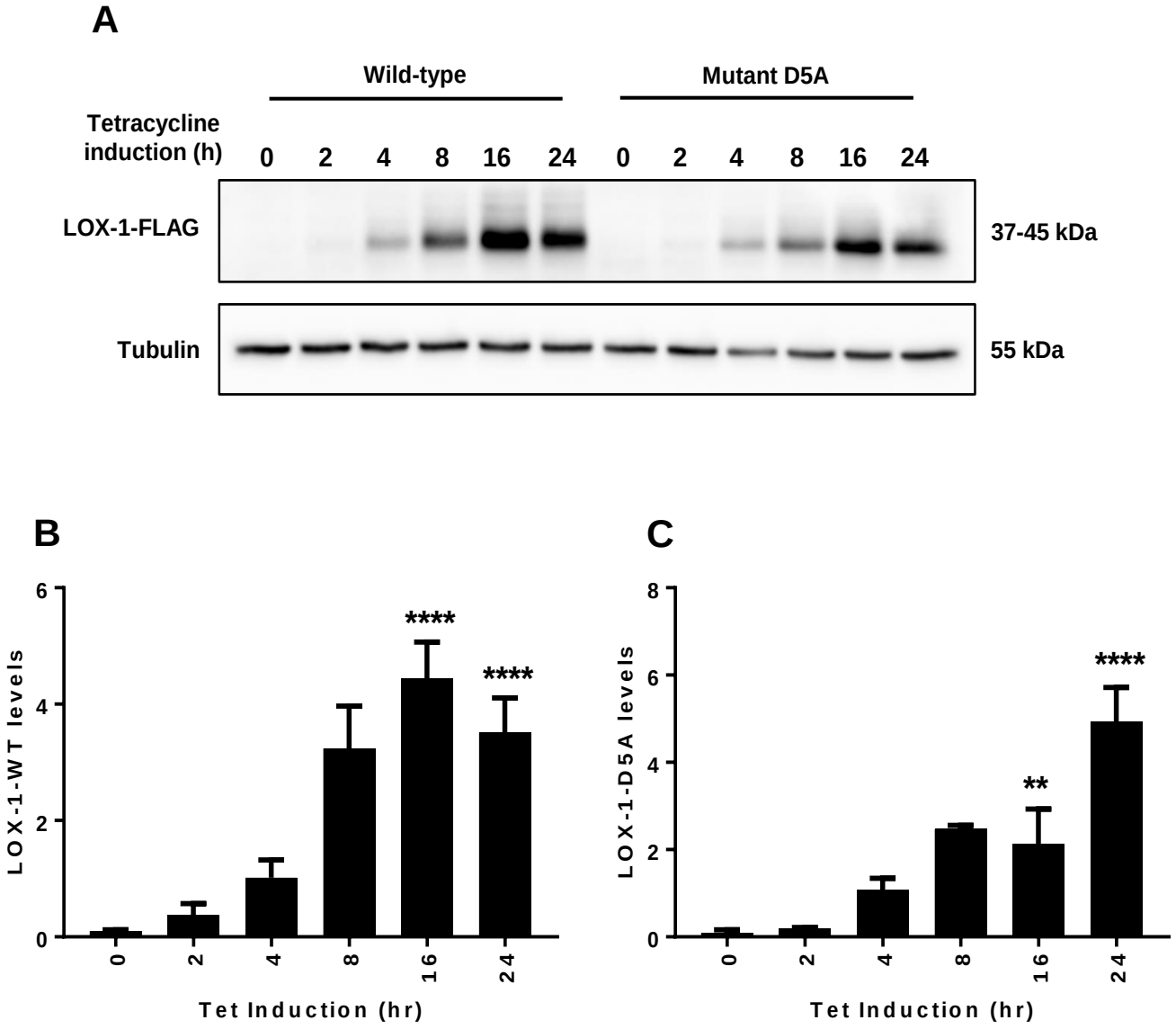

Fig. 3.6. Induction of the LOX-1 expression by tetracycline within 24 h. (A) HEK293 cell expressing LOX-1-WT and LOX-1-D5A were induced with 1 µg/ml tetracycline or 0, 2, 4, 8, 16 or 24 h. Cells were then lysed and processed for Western blot analysis using antibodies against FLAG-tag and quantification of (B) LOX-1-WT and (C) LOX-1-D5A. Error bars indicate ±SEM (n≥3). $p<0.05$ (*), $p<0.01$ (**), $p<0.0001$ (****).

3.2.4 Analysis of oxidised LDL binding to cells expressing LOX-1

3.2.4.1 Extraction and oxidation of low-density lipoprotein

In order to study the interaction of LOX-1 with oxidised LDL, LDL needed to first be isolated from human blood. Human LDL particles were purified from human plasma by ultracentrifugation using self-generating gradients of iodixanol (Graham et al., 1996). This method was used due to shorter centrifugation times than traditional sodium or potassium bromide gradients (Chapman et al., 1981; Kelley and Kruski, 1986) and because the high salt concentrations can modify lipoprotein structure and therefore require removal. Copper sulphate was used to oxidise the LDL particles. There are different methods that can be utilised for oxidation, such as transition metals or incubation with cultured cells, but LDL incubation with copper sulphate is one of the most widely used methods and produces extensively oxidised LDL (Levitan et al., 2010). The oxidation is halted with the anti-oxidants EDTA and butylated hydroxytoluene (BHT). Oxidation increases the negative charge on the particle due to reactive aldehyde conjugation to lysine residues.

Native and oxidised LDL particles treated with the anti-oxidants EDTA and BHT were run on 0.5% (w/v) agarose gels followed by Sudan black staining. Oxidised LDL has an increased electrophoretic mobility in comparison to native LDL as shown in figure 3.7. Oxidation of the LDL particle results in fragmentation of ApoB-100 and aggregation of particles, which leads to non-homogeneity in oxidised LDL particles. Therefore, this method confirms that the LDL particle has undergone oxidation by the copper ions.

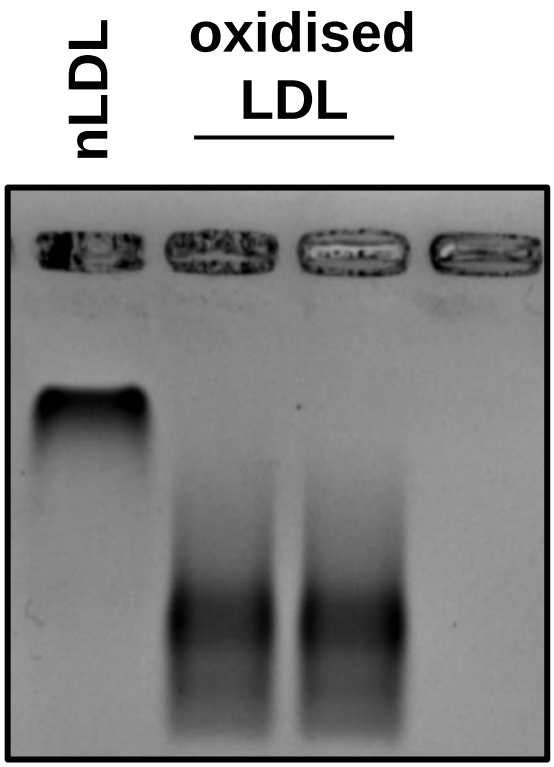

Fig. 3.7. Purification and oxidation of low-density lipoprotein particles. Low-density lipoprotein (LDL) was extracted from human plasma using iodaxonal gradient centrifugation and oxidised using 5 µM copper sulphate ($CuSO_4$) for 24 h at 37ºC (Oxidised LDL; lanes 2 and 3). Control was incubated with 100 µM EDTA and 20 µM BHT at room temperature (nLDL; lane 1). 4 µg of each sample was analysed on 0.5% (w/v) agarose gel and stained with the lipid stain Sudan black.

3.2.4.2 LOX-1 binding to oxidised LDL particles

To further validate the binding of oxidised LDL to LOX-1, a cell-based assay was used. The oxidised LDL particles were incubated with a fluorescent lipophilic dye (DiI), which enables tracking of the oxidised LDL particles using fluorescence microscopy (Murphy et al., 2006; Murphy et al., 2008). HEK293 and PAEC clones expressing these LOX-1 cDNAs were induced with tetracycline overnight. After serum-starvation for 2 h, cells were incubated with DiI-oxidised LDL for 15 min on ice. Fixation and staining cells with anti-FLAG antibodies allows detection of LOX-1-WT or LOX-1-D5A proteins in these clonal lines. Analysis of a negative control PAEC clone revealed no staining of FLAG-tagged LOX-1 as expected (Figure 3.8B). Nonetheless, the negative control PAEC clone exhibited significant amounts of DiI-oxidised LDL binding (Figure 3.8C). Furthermore, negative control PAEC showed significant DiI-oxidised LDL accumulation within cells in large punctate structures (Figure 3.8D, arrows).

The different LOX-1-expressing clones also showed staining for LOX-1 proteins using the anti-FLAG antibody (Figure 3.9, D and E); no such staining was visible in the negative control (Figure 3.9F). In contrast to PAEC, HEK293 negative control showed little or no binding of DiI-oxidised LDL to LOX-1 (Figure 3.9I). Upon tetracycline-induced expression of LOX-1-WT or LOX-1-D5A, there was notable increase in staining for DiI-oxidised LDL (Figure 3.9, G and H). There was DiI-oxidised LDL co-distribution with LOX-1-WT (Figure 3.9J, arrows). There was notably less staining of DiI-oxidised LDL for cells expressing LOX-1-D5A compared to LOX-1-WT expression (Fig. 3.9K). These findings suggest that LOX-1 expression in the HEK293 cells enables specific detection of oxidised LDL binding and uptake.

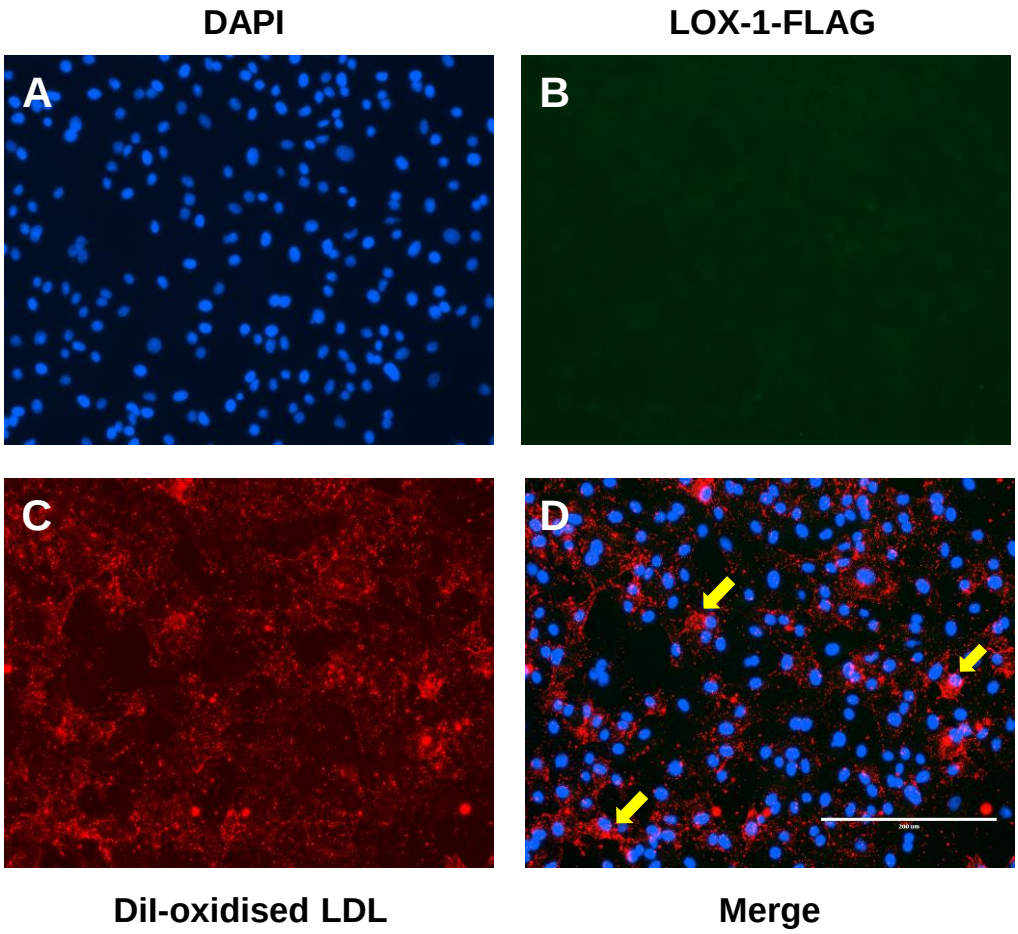

Fig. 3.8. Staining of DiI-oxidised low-density lipoprotein in control PAEC cells. Control porcine aortic endothelial cells (PAECs) were induced with 1 µg/ml tetracycline overnight prior to incubation with DiI-oxidised LDL (red) for 15 min on ice. Endothelial cells were fixed and processed for immunofluorescence microscopy using mouse anti-FLAG and secondary anti-mouse AlexaFluor-488 conjugate (green); nuclei were stained using DAPI (blue). Arrows denote cells exhibiting high DiI-oxidised LDL uptake. Scale bar, 200 µm.

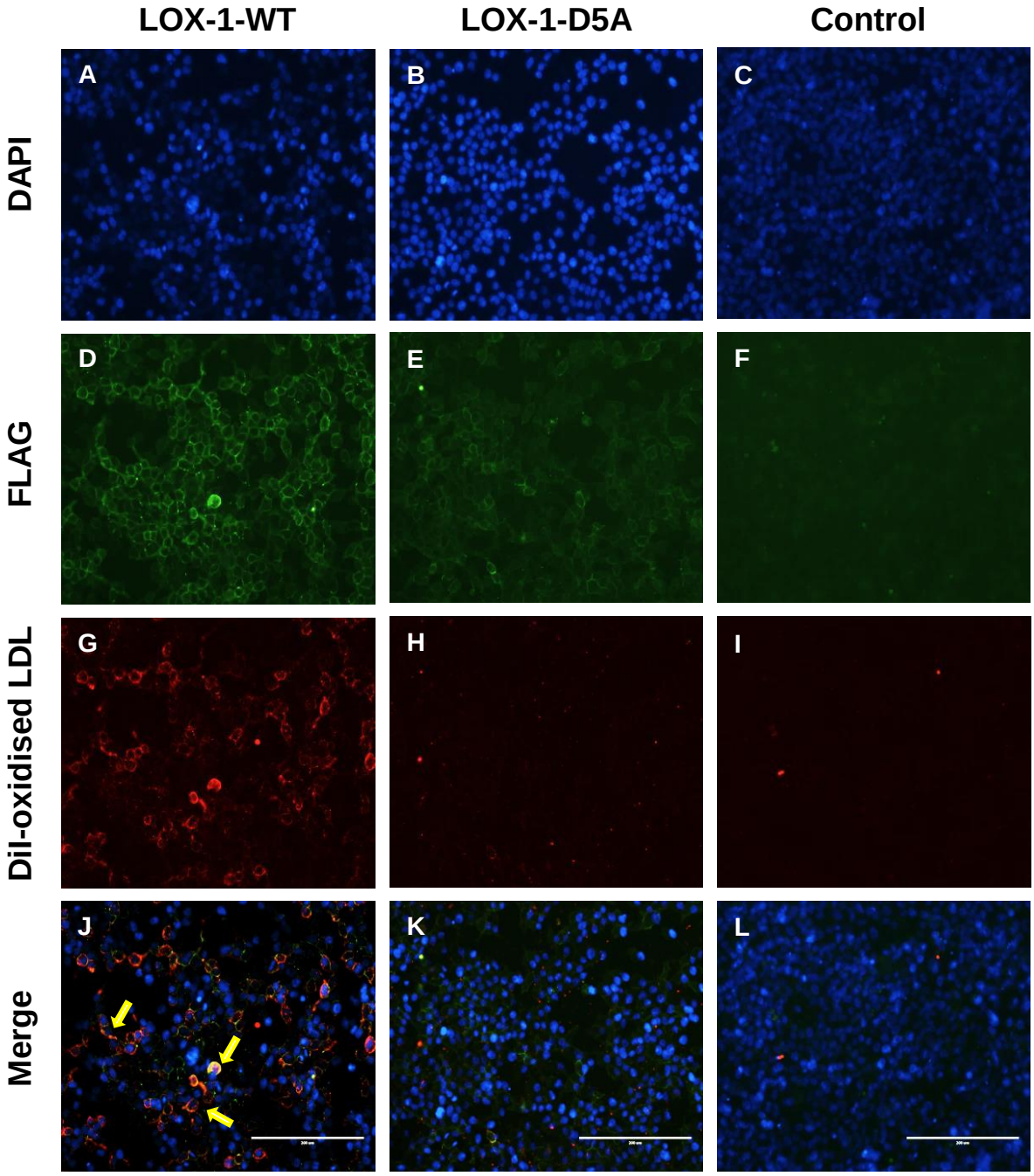

Fig. 3.9. Visualisation of Oxidised LDL uptake in HEK293 clones expressing LOX-1 proteins. Human embryonic kidney (HEK) 293T cell transfected with LOX-1-WT (A), LOX-1-D5A (B) and control (C) were induced with 1 µg/mL overnight prior to incubation with DiI-oxidised (red) LDL for 15 min on ice. Epithelial cells were fixed and processed for immunofluorescence microscopy using anti-FLAG (green); nuclei stained using DAPI (blue). Scale bar, 200 µm.

3.3 Discussion

In this chapter, I constructed an inducible system that expressed either LOX-1-wild-type-FLAG (LOX-1-WT) or a trafficking-defective mutant, LOX-1-D5A. The idea behind generating Flp-In^{TM} T-Rex^{TM} cell line, firstly, it requires the integration of two plasmids (Figure 3.10A), where one contains the *Flp* Recombination Target (FRT) sites, and the other expresses the tetracycline (tet) repressor (Andrews et al., 1985; Hillen and Berens, 1994). To date, only HEK293 cells have both plasmids integrated into the genome. Subsequently, full-length human LOX-1 cloned into pcDNA5/FRT/TO vector was co-transfected with pOG44 plasmid into HEK293 and PAEC Flp-In^{TM} T-Rex^{TM} cell lines (Figure 3.10B). Expression of LOX-1 is repressed by the tet repressor protein that binds to the tet operator O_2 sequence upstream of the LOX-1 to prevent transcription. Tetracycline must be added into the culture media so then it binds to the tet repressor protein that distorts the structure and releasing it from the tet operator sequence, which in turn allows the transcription and translation of LOX-1 (Figure 3.10C).

One potential problem with LOX-1 expression in cell lines is that activation of this receptor triggers apoptosis (Li and Mehta, 2009). To solve this problem, using controlled and inducible expression by LOX-1 is one way of studying the functional role of this protein in cells. Here, human embryonic kidney 293 cells (HEK293) and immortalised porcine aortic endothelial cells (PAEC) were used to stably express LOX-1-WT and mutant LOX-1-D5A proteins. As the HEK293 cell line does not bind or take up significant levels of oxidised LDL due to little or no scavenger receptor being expressed, thus effects of LOX-1 can be studied in detail. In contrast, the PAEC cell line does bind and take up significant levels of oxidised LDL. This could be due to the possible expression of other scavenger receptors including SR-A1, CD36 and LOX-1. Although LOX-1 expression in the PAEC background is more physiological, it is difficult to study the effects of LOX-1 in isolation considering the background expression and levels of other scavenger receptors.

The addition of the FLAG peptide tag in the full-length human LOX-1 at the C-terminus upstream of the C-type lectin-like extracellular domain (LOX-1-FLAG; Fig. 3.11) enabled monitoring the protein. Both HEK293 and PAEC cells expressed a ~40 kDa LOX-1-FLAG protein as detected by anti-FLAG antibodies, which corresponds to the molecular mass of human LOX-1 in vascular cells and tissues (Xie et al., 2004). It is noteworthy to point out that there was no 'leaky' expression of LOX-1 in the absence of tetracycline. This controlled and tetracycline-inducible expression system was further validated using qRT-PCR to monitor the mRNA levels of LOX-1 in the absence and presence of tetracycline.

Analysis of HEK293 cells expressing LOX-1 or LOX-1-D5A suggested substantial differences in oxidised LDL binding and uptake. LOX-1-D5A-expressing cells showed dramatically reduced oxidised LDL binding and uptake (compared to LOX-1-WT). One explanation could be that the D5A mutation perturbs LOX-1 trafficking and availability for oxidised LDL binding at the plasma membrane. This mutation does not significantly affect the mRNA levels of LOX-1-D5A suggesting that any effects are occurring due to altered function of the protein and not due to overall expression levels. The following chapter will detail the use of these Flp-In^{TM} T-Rex^{TM} cell lines to study the functional role of the LOX-1 protein.

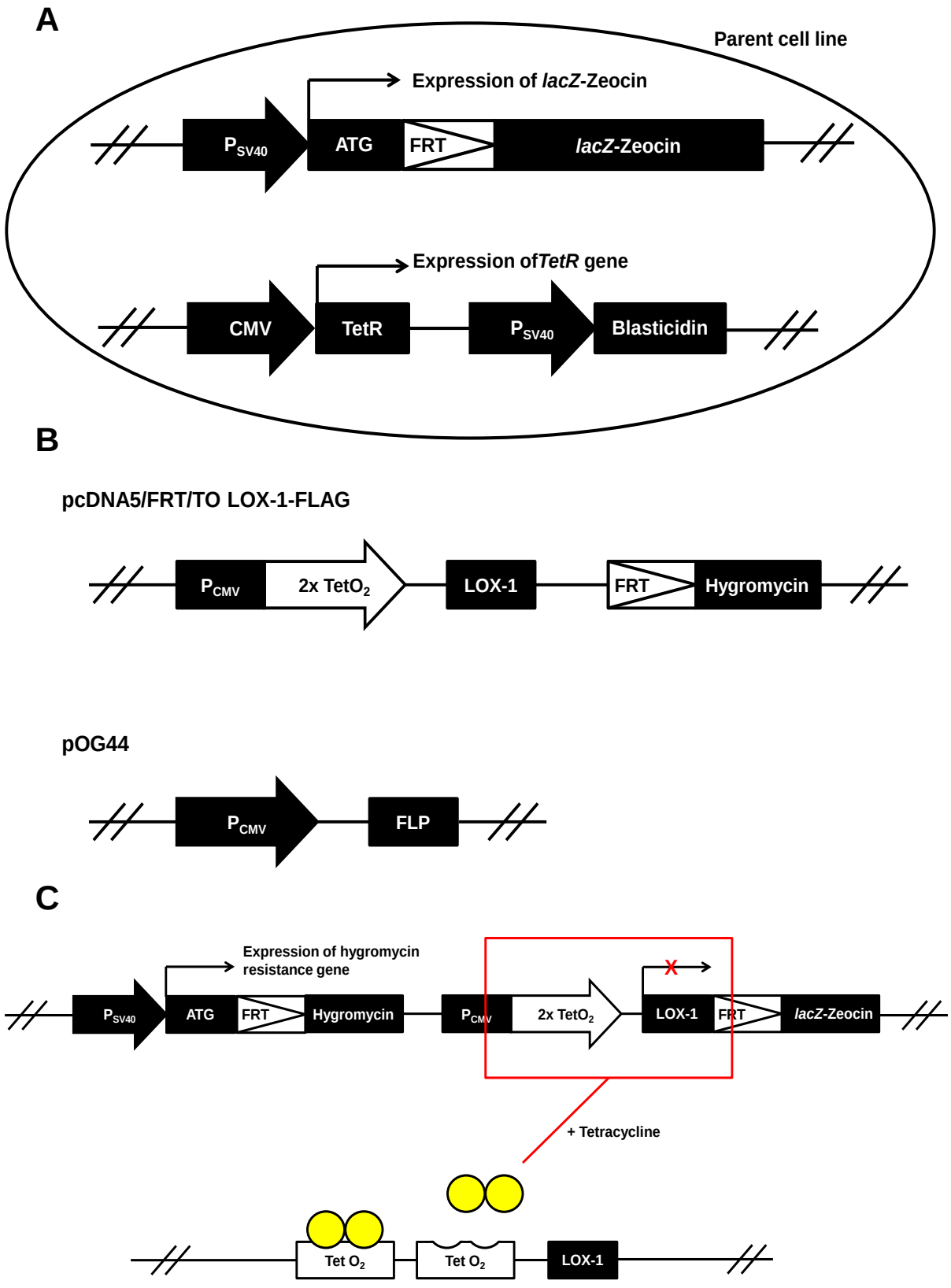

Fig. 3.10. Schematic diagram of the Flp-In™ T-Rex™ system. (A) Integration of pFRT/*lacZeo* and pcDNA6/TR into the genome of the parent cell line. (B) Co-transfection of LOX-1 in pcDNA5/FRT/TO vector and pOG44 in Flp-In™ T-Rex™ cell line. (C) Expression of LOX-1 is repressed by tet repressor protein binding with Tet operator O_2 sequence. Addition of tetracycline distorts the shape of the tet repressor unable to bind to the Tet operator, which initiates transcription and translation of LOX-1.

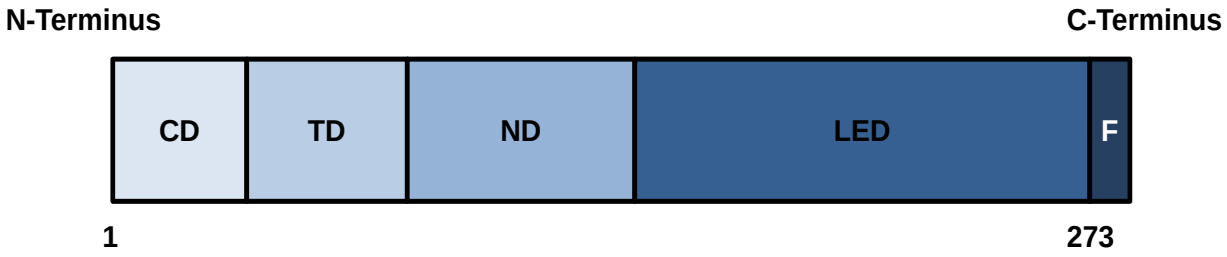

Fig. 3.11. Domain structure of the human LOX-1-FLAG construct. CD, cytoplasmic domain; TD, transmembrane domain; ND, neck domain; LED, lectin-like extracellular domain; F, FLAG-tag.

CHAPTER 4

LOX-1 binding to oxidised LDL regulates signal transduction and cellular responses

4.1 Introduction

A keynote study by Brown and Goldstein discovered that the LDL receptor was a key molecule in the uptake and metabolism of cholesterol and this was linked to the formation of lipid and cholesterol-rich foam cells which contribute to atherosclerosis (Brown et al., 1976). Human patients with familial hypercholesterolemia inherit impaired LDL receptor alleles and display increased cholesterol accumulation within atherosclerotic lesions. This led to the idea that another membrane-bound receptor must be involved in recognising the modified or oxidised form of LDL (Basu et al., 1976). The first scavenger receptor to be identified, namely SR-A1, is known for the binding to modified forms of LDL particles (Kodama et al., 1990), and other 9 classes of scavenger receptors have been identified (Murphy et al., 2005).

About 40 years ago, the oxidatively modified form of LDL was suggested to be the key mediator of foam cell formation in macrophages; LDL modification by endothelial cells was one contributory factor to this process (Steinbrecher et al., 1984). It was shown in immunohistochemical analysis of human vascular tissues and animal studies that oxidised LDL accumulated in atherosclerotic lesions (Itabe et al., 1994; Ehara et al., 2001; Nishi et al., 2002; Palinski et al., 1996). This oxidised LDL activates a number of pro-atherosclerotic signal transduction pathways, which effectively activates endothelial cells and cause the proliferation of smooth

muscle cells (Berliner et al., 1990; Shih et al., 1999; Kusuhara et al., 1997; Huang et al., 1995). The macrophages, smooth muscle cells and fibroblasts are known to internalize oxidised LDL through receptor-mediated pathways involving a number of scavenger receptors such as SR-A1, SR-B1, CD36 and LOX-1 (Dhaliwal and Steinbrecher, 1999). The early stage of atherosclerosis involves endothelial dysfunction due to the binding of oxidised LDL to scavenger receptors on endothelial cells, although the scavenger receptor basal levels is usually low (Bickel and Freeman, 1992).

The endothelial receptor for oxidised LDL, namely LOX-1, is a membrane protein structurally belonging to the C-type lectin family and is expressed *in vivo* in vascular endothelial cells (Sawamura et al., 1997). LOX-1 has a role in binding and internalisation of oxidised LDL in endothelial cells. There is a large body of evidence that oxidised LDL results in an increase in pro-inflammatory and pro-atherogenic aspects of vascular function (Li and Mehta, 2000a; Li and Mehta, 2000b). Additionally, LOX-1 is dynamically up-regulated by disease conditions such as diabetes, hypertension and dyslipidaemia and mediators such as Ang II, cytokines and AGE (Kume et al., 1998; Murase et al., 1998; Kataoka et al., 1999; Chen et al., 2001c).

Almost a decade ago, it was shown that a dynamin-2-dependent pathway regulates the internalisation and accumulation of oxidised LDL via LOX-1 (Murphy et al., 2008). However, the dynamics and fate of both oxidised LDL and LOX-1 receptor within the endosome-lysosome system was unknown. One could predict after the dissociation of oxidised LDL and LOX-1 in the endosome (Murphy et al., 2008), LOX-1 receptor is probably recycled back to the surface membrane. It has been reported that oxidised LDL is proteolytically degraded in endothelial cells and macrophages (Mehta et al., 2006; Lougheed et al., 1999). If this was the plausible scenario, LOX-1 would not be involved in atherogenesis; in fact, LOX-1 scavenges and degrades oxidised LDL in the vascular system. In the study by Murphy and colleagues, mutations within an acidic motif (DDL) in the LOX-1 cytoplasmic domain is responsible for endocytosis,

and mutation in this motif (also known as mutant LOX-1-D5A) affects such plasma membrane-to-endosome trafficking (Murphy et al., 2008). However, the underlying mechanism of this differential trafficking of LOX-1-WT and LOX-1-D5A is still unclear. It is therefore important to address whether oxidised LDL binding and uptake would affect trafficking, which would have consequences on signal activation.

To address this question, the work carried out in this chapter evaluated oxidised LDL-mediated trafficking and regulation of downstream signal transduction linking to cellular function. Here, I show that oxidised LDL activation of LOX-1 triggers differential signal transduction and trafficking outcomes. Differences in the functional roles of wild-type and mutant LOX-1 could potentially explain how signal transduction pathways regulate atherosclerosis.

4.2 Results

4.2.1 Analysis of LOX-1 signal transduction pathways in non-vascular HEK293 cells

4.2.1.1 Oxidised LDL regulates LOX-1 plasma membrane-to-endosome endocytosis and recycling

Cell surface LOX-1 is constitutively internalised from the plasma membrane by a clathrin-independent and dynamin-2-dependent pathway (Murphy et al., 2008). A molecular chaperone is also thought to interact with the N-terminus cytoplasmic domain of LOX-1 to mediate internalisation and trafficking (Bakthavatsalam et al., 2014). Moreover, the Lys to Asn amino acid substitution at position 167 (K167N) within the extracellular C-type lectin-like domain of LOX-1 has been shown to reduce oxidised LDL binding affinity and uptake (Biocca et al., 2009). Murphy and colleagues previously identified cytoplasmic acidic motif within LOX-1 (Murphy et al., 2008), thus I hypothesised that LOX-1-WT and LOX-1-D5A

could differentially regulate oxidised LDL internalisation in order to modulate downstream responses.

To test this idea, HEK293 Flp-In™ T-Rex™ cells expressing LOX-1-WT or LOX-1-D5A were stimulated for 0, 15, 30, 60 or 120 min with oxidised LDL prior to monitoring LOX-1-WT and LOX-1-D5A dynamics using cell surface biotinylation. In order to measure plasma membrane-associated LOX-1 levels and LOX-1 activity, both biotinylated cell surface (affinity isolation) and total cellular protein (total lysates) pools were subjected to immunoblotting for FLAG-tagged LOX-1-WT and LOX-1-D5A (Figure 4.1A). Quantification revealed that both LOX-1-WT and LOX-1-D5A were present at the cell surface at 0 min (Figure 4.1, C and D). Oxidised LDL promoted a significant reduction in cell surface LOX-1-WT levels at 15, 30 or 60 mins (Figure 4.1C). In comparison, oxidised LDL stimulation revealed greater stability and longevity in cell surface LOX-1-D5A levels (Figure 4.1D). Oxidised LDL stimulation did not seem to affect the total cellular LOX-1-WT and LOX-1-D5A levels.

To further analyse LOX-1-dependent oxidised LDL internalisation, HEK293 Flp-In™ T-Rex™ cells were subjected to an oxidised LDL stimulation time course of 0, 15, 30 and 60 min, prior to analysis of LOX-1-WT (Figure 4.2) and LOX-1-D5A (Figure 4.3) trafficking using fluorescence-based microscopy. As previously mentioned, LOX-1-WT was present on the cell surface at 0 min (Figure 4.2, A and B). Interestingly, LOX-1-D5A was mostly seen in large punctate structures near the nucleus within the cytoplasm at 0 min (Figure 4.3, A and B). Following oxidised LDL stimulation for 15 min, LOX-1-WT was observed to localise in the cytoplasm and after 30 and 60 min, LOX-1 was seen in the cytoplasm and the cell surface (Figure 4.2, B-H). In comparison, LOX-1-D5A failed to reveal localisation at the cell surface (Figure 4.3, A and B), but in the cytoplasm, even after 15 min (Figure 4.2, C and D). Eventually, LOX-1-D5A was seen to localise at the cell surface at either 30 or 60 min (Figure 4.3, E-H). Taken together, these data suggest the differential localisation of LOX-1-WT and LOX-1-D5A can cause unique oxidised LDL-stimulated signal transduction events.

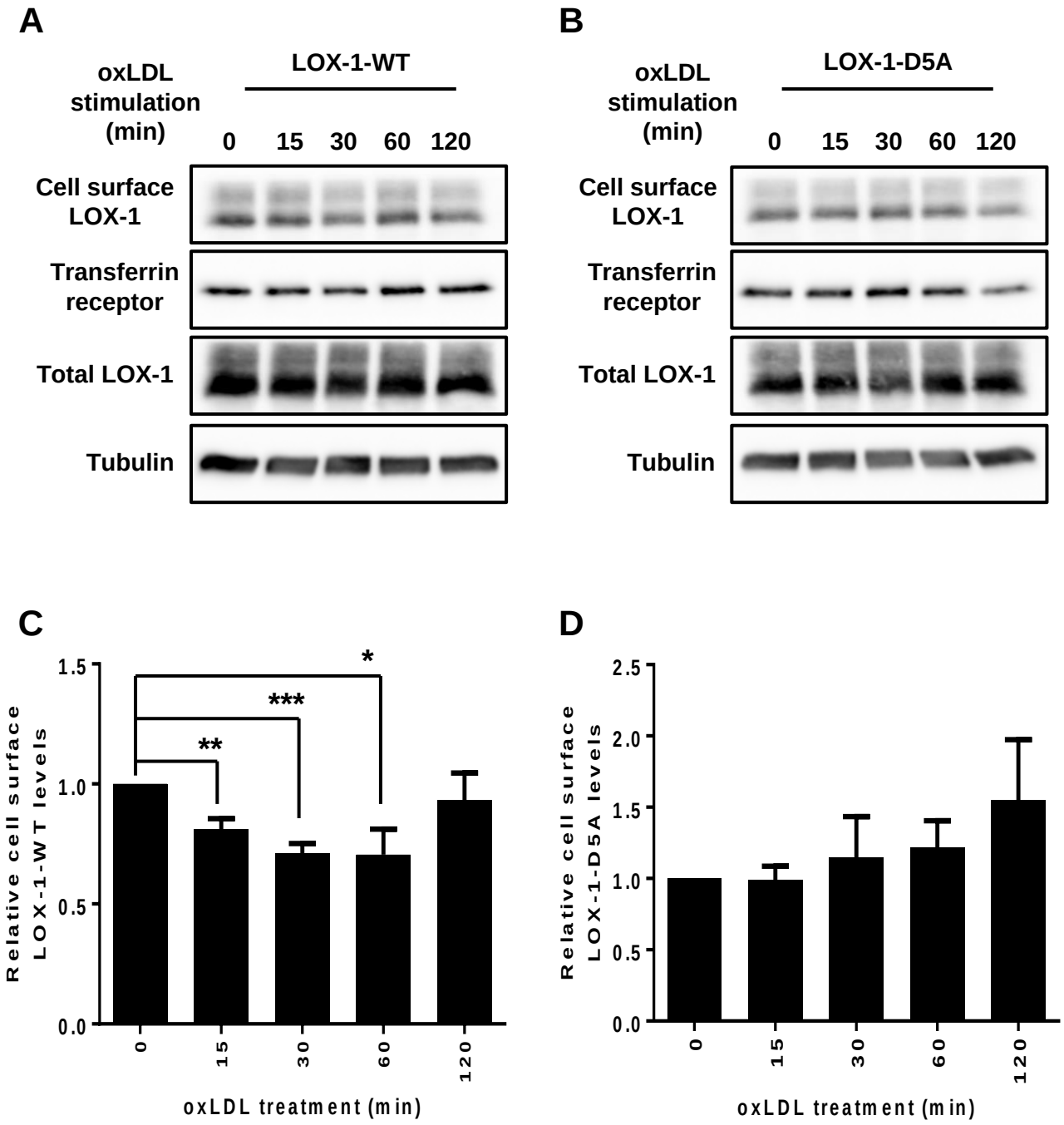

Figure 4.1. Oxidised LDL-mediated LOX-1 internalization. HEK293 cells expressing (A) LOX-1-WT and (B) LOX-1-D5A were stimulated with oxLDL (10 µg/mL) for 15, 30, 60 or 120 min before cell surface biotinylation and immunoblot analysis of cell surface and biotinylated LOX-1 proteins. Transferrin receptor was used as positive control. (C and D) Quantification of cell surface LOX-1-WT and LOX-1-D5A, respectively, relative to cell surface protein levels at time 0. Error bars indicate ±SEM (n≥3). p<0.05 (*), p<0.01 (**), p<0.001 (***), p<0.0001 (****).

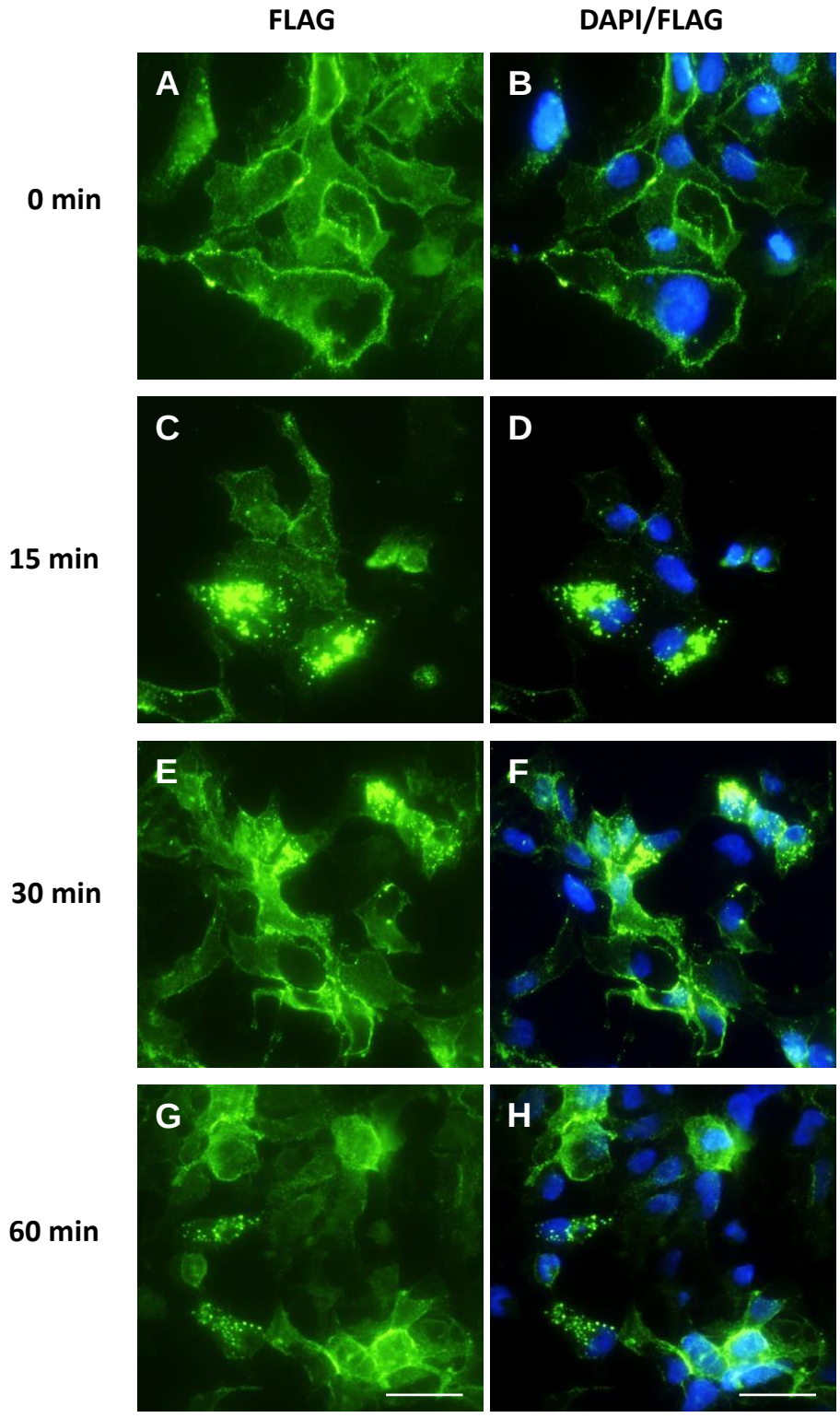

Figure 4.2. Oxidised LDL-mediated LOX-1-WT trafficking. HEK293 expressing LOX-1-WT was stimulated with oxLDL (10 µg/mL) for 15, 30 or 60 min. Cells were fixed and processed for immunofluorescence microscopy using mouse anti-FLAG (green); nuclei stained using DAPI (blue). Scale bar, 100 µm.

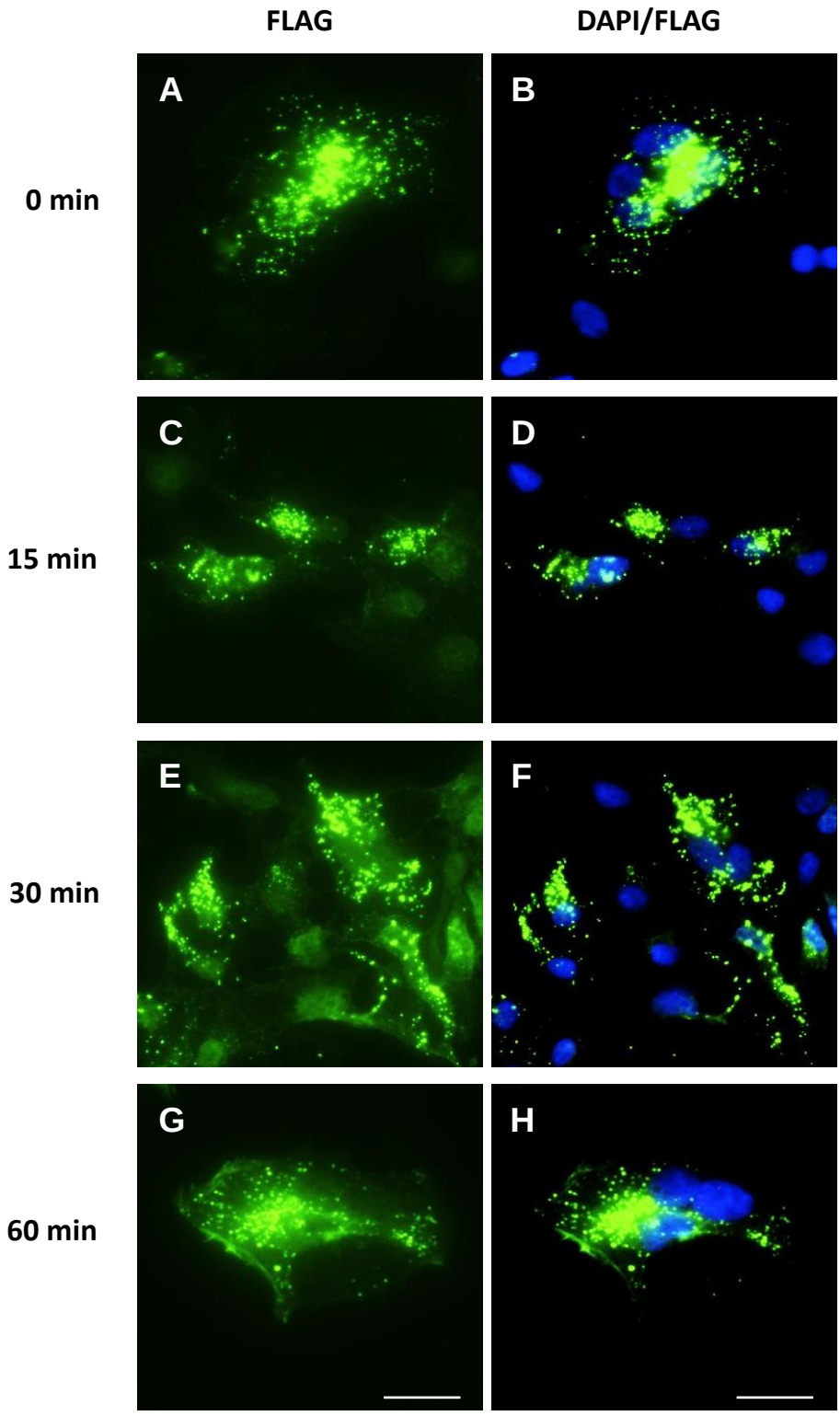

Figure 4.3. Oxidised LDL-mediated LOX-1-D5A trafficking. HEK293 expressing LOX-1-D5A was stimulated with oxLDL (10 µg/mL) for 15, 30 or 60 min. Cells were fixed and processed for immunofluorescence microscopy using mouse anti-FLAG (green); nuclei stained using DAPI (blue). Scale bar, 100 µm.

4.2.1.2 Oxidised LDL triggers differential LOX-1-mediated signal transduction

Upon binding to oxidised LDL, LOX-1 can activate multiple signalling events (Twigg et al., 2012), including activation of NF-κB (Cominacini et al., 2000), increased expression of adhesion molecules (Li et al, 2002) and increased apoptosis (Schneiderman et al., 1998). Although there are many mechanisms linking LOX-1 signalling with cardiovascular disease, little is known about the membrane trafficking regulation of oxidised LDL-mediated LOX-1 signalling. As LOX-1-WT and LOX-1-D5A exhibit different trafficking, I hypothesised that such membrane trafficking affects downstream signal transduction.

To investigate this, LOX-1-expressing HEK293 cells were induced overnight with tetracycline prior to stimulation with oxidised LDL for 0, 5, 15, 30 or 60 min before immunoblot analyses (Figure 4.4) of phospho-Akt, phospho-NF-κB, phospho-p38 MAPK and phospho-ERK1/2 levels. Quantification of phospho-Akt (Figure 4.4C) revealed oxidised LDL stimulation significantly decreased the activation of phosphorylated Akt in LOX-1-D5A cells at 0, 15, 60 and 120 min compared to LOX-1-WT cells. Similarly, quantification of phospho-p38 MAPK (Figure 4.4G) was significantly lower in LOX-1-D5A cells than in wild-type LOX-1 cells at 5 min. Although it was not significant, but the trend could be seen that the activation of phospho-p38 MAPK started to increase at 15, 60 and 120 min. Interestingly, for LOX-1-D5A, phosphorylation of ERK1/2 peaked significantly at 60 min (Figure 4.4D). Phosphorylated NF-κB levels were not significantly affected by oxidised LDL for either LOX-1-WT or LOX-1-D5A (Figure 4.4H). These data show LOX-1-WT expression causes decreased Akt phosphorylation linked to a gradual increase in phospho-p38 MAPK levels caused by oxidised LDL.

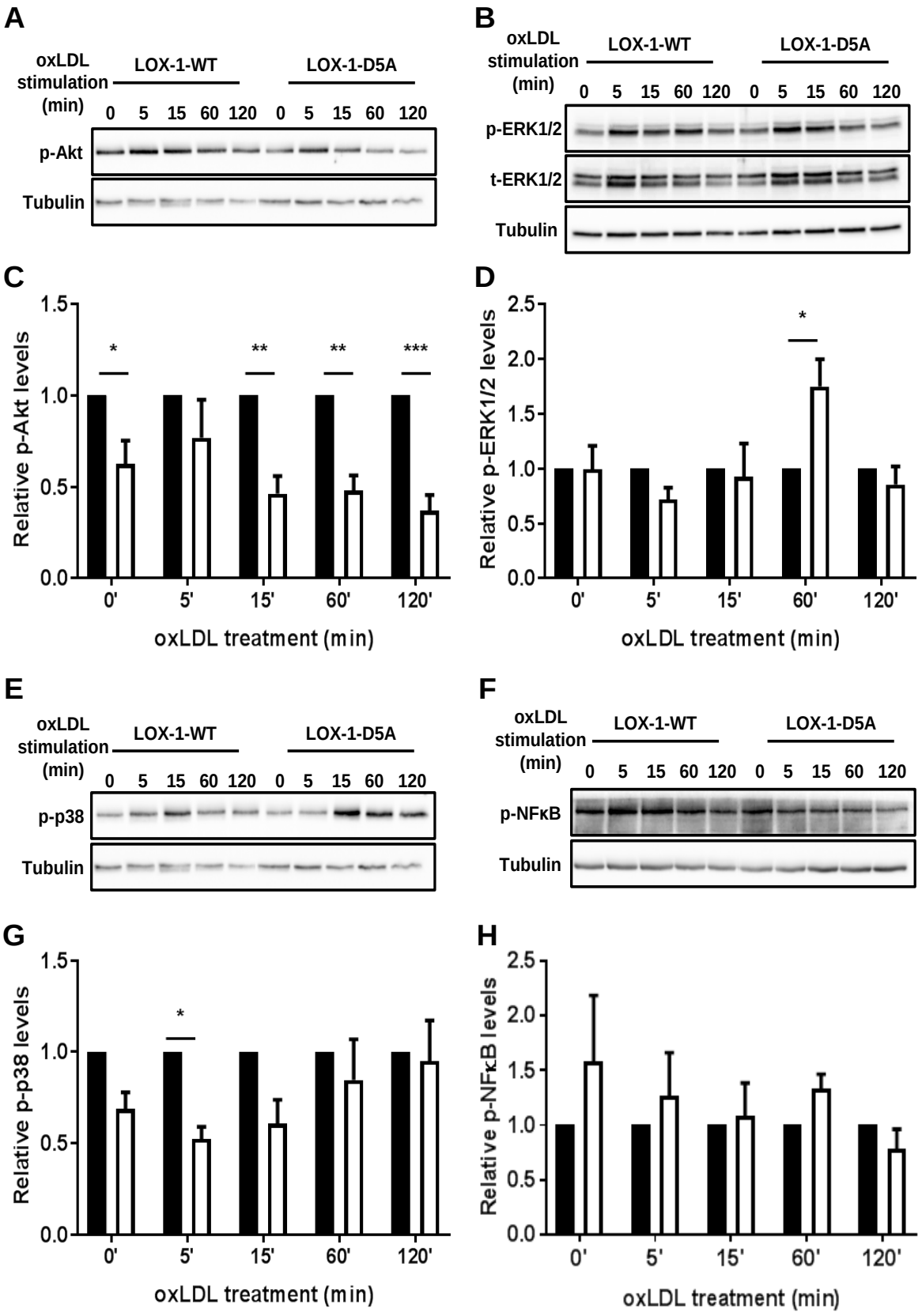

Figure 4.4. Oxidised LDL activation of signal transduction. HEK293 cells expressing either LOX-1-WT or LOX-1-D5A were treated with oxLDL (10 µg/mL) for 5, 15, 60 or 120 min prior to cell lysis and immunoblot analysis against (A) p-Akt, (B) pERK1/2. (E) p-p38 and (F) p-NF-κB. (C, D, G and H) Quantifications of each activated proteins relative to LOX-1-WT; black bars indicate LOX-1-WT; white bars indicate LOX-1-D5A. Error bars indicate ±SEM (n≥3). p<0.05 (*), p<0.01 (**), p<0.001 (***), p<0.0001 (****).

4.2.1.3 Inhibition of oxidised LDL binding to LOX-1 affects downstream intracellular signalling

Pharmacological inhibitors that target the extracellular domain of LOX-1 also attenuate oxidised LDL-dependent expression of pro-atherogenic molecules, but the mechanisms underlying such regulation is unclear (Li et al., 2002a; Li et al., 2002b). One such small molecule and LOX-1 inhibitor is a humanised antibody termed JTX92 which blocks LOX-1-mediated responses (Murphy et al., 2006). Since LOX-1 undergoes constitutive endocytosis (Murphy et al., 2008), one possibility is that JTX92 modulates LOX-1 signalling to downstream pathways or events. The addition of JTX92 caused a reduction in levels of LOX-1-WT (Figure 4.5A). Surprisingly, JTX92 addition stimulated LOX-1-D5A levels over a similar time course (Figure 4.5A). JTX92 was added prior to stimulation of oxidised LDL for 0, 15, 30, 60, or 120 min, followed by cell lysis and immunoblot analysis for phospho-Akt, p53, phospho-p38 MAPK and phospho-ERK1/2 (Figure 4.5A). Quantification showed that JTX92 blocked oxidised LDL-stimulated activation of Akt, p38, ERK1/2 and p53 in cells expressing LOX-1-WT (Figure 4.5, B-E, black bar). However, phosphorylation of Akt, p38 MAPK and ERK1/2 were significantly increased in cells expressing LOX-1-D5A after 30 min in response to oxidised LDL stimulation (Figure 4.5, B-E, white bar). These findings suggest that JTX92-mediated binding differentially regulates LOX-1-WT versus LOX-1-D5A signal transduction in HEK293 cells.

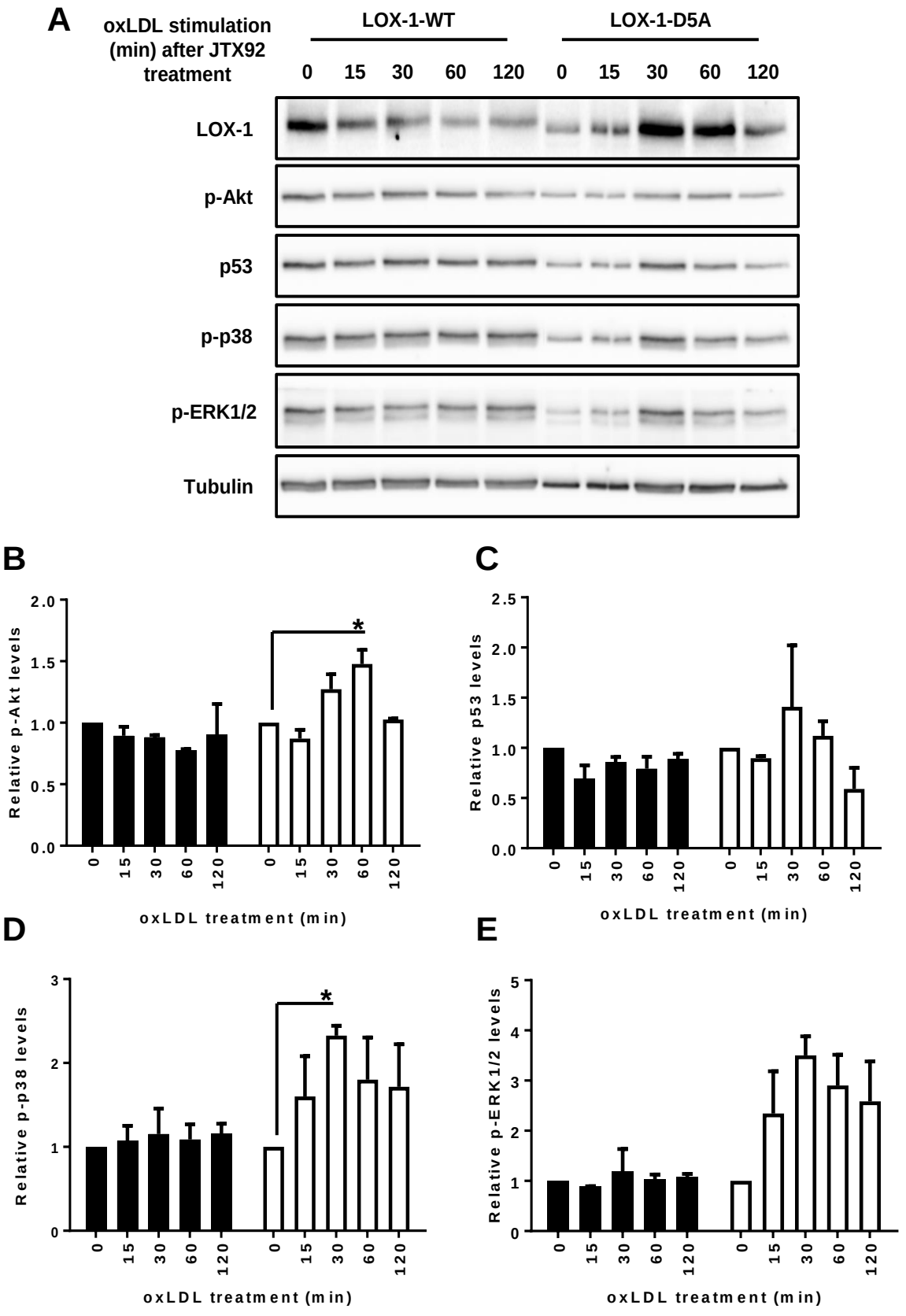

Figure 4.5. Inhibition of oxidised LDL binding to LOX-1 affects signal transduction. (A) Immunoblotting of phosphorylated Akt, p53, p38, ERK1/2 in HEK293 cells following 1 h pre-incubation with LOX-1 blocking antibody (JTX92, 10 µg/mL) prior to oxLDL stimulation (10 µg/mL) for 15, 30, 60 or 120 min. (B-E) Quantification of activated proteins upon oxidised LDL activation in the presence of JTX92 relative to time 0; black bars indicate LOX-1-WT; white bars indicate LOX-1-D5A. Error bars indicate ±SEM (n≥3). p<0.05 (*), p<0.01 (**), p<0.001 (***), p<0.0001 (****).

4.2.2 Analysis of signal transduction pathways in vascular endothelial PAEC cells

4.2.2.1 Oxidised LDL causes differential LOX-1-mediated signal transduction

Oxidised LDL stimulates NF-κB, Akt, p38 MAPK and ERK1/2 MAPK signalling pathways in endothelial and smooth muscle cells (Robbesyn et al., 2004; Jing et al., 1999; Chien et al., 2003) including apoptosis (Wang et al., 2016). In this context, I asked whether overexpression of LOX-1-WT or LOX-1-D5A caused by oxidised LDL modulated events in signal transduction pathways (Figure 4.6 and Figure 4.7). Induction of LOX-1-WT (Figure 4.6A) or mutant LOX-1-D5A (Figure 4.7A), was detected after without (Tet OFF) or with (Tet ON) induction. Immunoblot quantification showed significantly reduced levels of phosphorylated Akt, eNOS and NF-κB proteins at 0 min in LOX-1-WT cells compared to control (Tet OFF) (Figure 4.6, B-E). Stimulation with oxidised LDL in cells expressing LOX-1-WT did not show significant changes in Akt, eNOS and NF-κB phosphorylation. Activated p38 MAPK was slightly increased in response to oxidised LDL at 5 min in LOX-1-WT expressing cells but eventually it returned to baseline levels (Figure 4.6D). Conversely, for LOX-1-D5A expressing cells, phospho-Akt, phospho-eNOS, phospho-p38 MAPK and phospho-NF-κB levels were up-regulated at 0 min compared to control (Tet OFF) (Figure 4.7, B-E). Stimulation with oxidised LDL caused decreased phosphorylation of Akt and eNOS, whilst activity levels of p38 and NF-κB were increased compared to the control. These data suggest that wild-type and mutant LOX-1 cause differential activation of downstream signal transduction pathways.

Next, I compared the differential capabilities of LOX-1-WT and LOX-1-D5A in stimulating signal transduction pathways, immunoblotting of phospho-eNOS, phospho-Akt, phospho-NF-κB, phospho-p38 MAPK and phospho-ERK1/2 were analysed and quantified (Figure 4.8A). Quantified levels of phosphorylated Akt were significantly increased at 0 min in LOX-1-D5A cells, and there was not much change of phospho-Akt levels in

response to oxidised LDL (Figure 4.8B). Interestingly, levels of activated NF-κB were not altered in response to oxidised LDL, though expression levels of phospho- NF-κB was significantly less in LOX-1-D5A (Figure 4.8C). There were no differences of activated p38 MAPK and ERK1/2 levels in LOX-1-WT and LOX-1-D5A, apart from a slight increase in response to oxidised LDL at 5 min, and then the levels returned to baseline (Figure 4.8, D and E). Taken together, these data suggest short-term stimulation (0-1 h) of oxidised LDL through LOX-1 did not have significant effects on downstream signalling events.

In vascular endothelial cells, although LOX-1 has been linked to atherosclerosis-related events such as endothelial dysfunction and increased expression of adhesion molecules through the activation of several signalling pathways including MAPK proteins and NF-κB, how such events are integrated are unclear (Thakkar et al., 2015; Mehta et al., 2006; Li and Mehta, 2009). Therefore, I asked whether a longer time-frame oxidised LDL stimulation caused differential signalling expression levels in PAEC Flp-In^{TM} T-Rex^{TM} cells (Figure 4.9A). Endothelial cells were stimulated with oxidised LDL for 0, 1, 4, 8 or 24 h prior to cell lysis, followed by immunoblot analysis for phospho-eNOS, phospho-Akt, phospho-NF-κB, phospho-p38 and phospho-ERK1/2. Quantification of oxidised LDL-stimulated phospho-Akt levels in both LOX-1-WT and LOX-1-D5A expressing cells shows a significant reduction at 4, 8 or 24 h post-stimulation (Figure 4.9B). On analysing eNOS and ERK1/2 under similar conditions, there was a gradual reduction in phospho-eNOS and phospho-ERK1/2 after 8 or 24 h of oxidised LDL stimulation in both LOX-1-WT and LOX-1-D5A cells (Figure 4.9, C and D). Interestingly, levels of stress-activated phospho-p38 MAPK was elevated more rapidly within 1 h of oxidised LDL stimulation and this returned back to baseline at 4, 8 or 24 h post-stimulation in both LOX-1-WT and LOX-1-D5A cells (Figure 4.9E). These data suggest that oxidised LDL presence over a longer time period causes down-regulation of different signal transduction pathways and these profound signalling differences at 24 h may represent the activation of apoptotic signalling pathways.

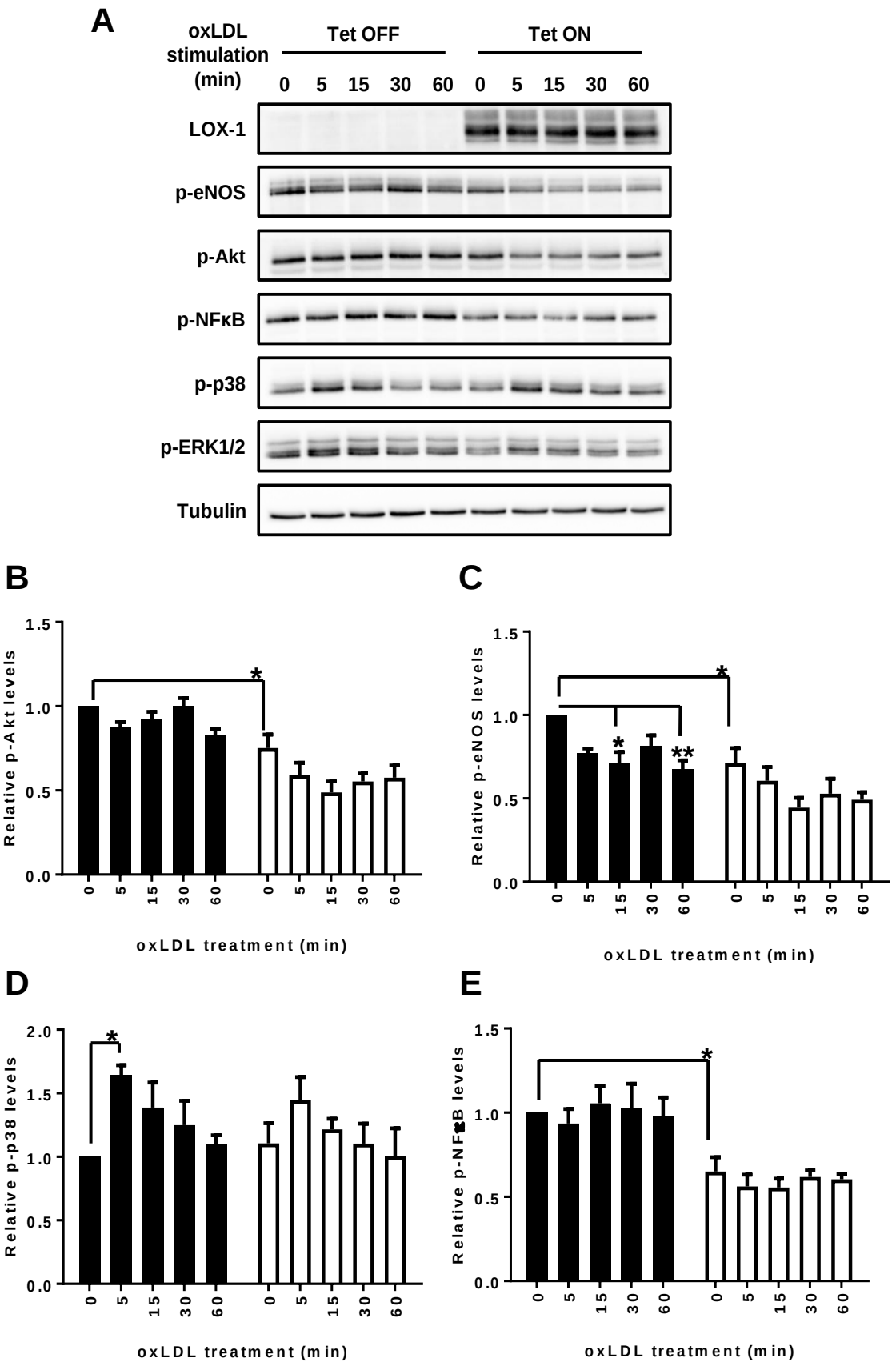

Figure 4.6. Oxidised LDL activation of LOX-1-WT signal transduction. (A) PAEC cells expressing LOX-1-WT were induced overnight with 1 µg/mL tet prior to stimulation with oxLDL (10 µg/mL) for 5, 15, 30 or 60 min followed by cell lysis and immunoblot analysis. (B-E) Quantifications of each activated proteins relative to Tet OFF at time 0; black bars indicate Tet OFF; white bars indicate Tet ON. Error bars indicate ±SEM (n≥3). p<0.05 (*), p<0.01 (**), p<0.001 (***), p<0.0001 (****).

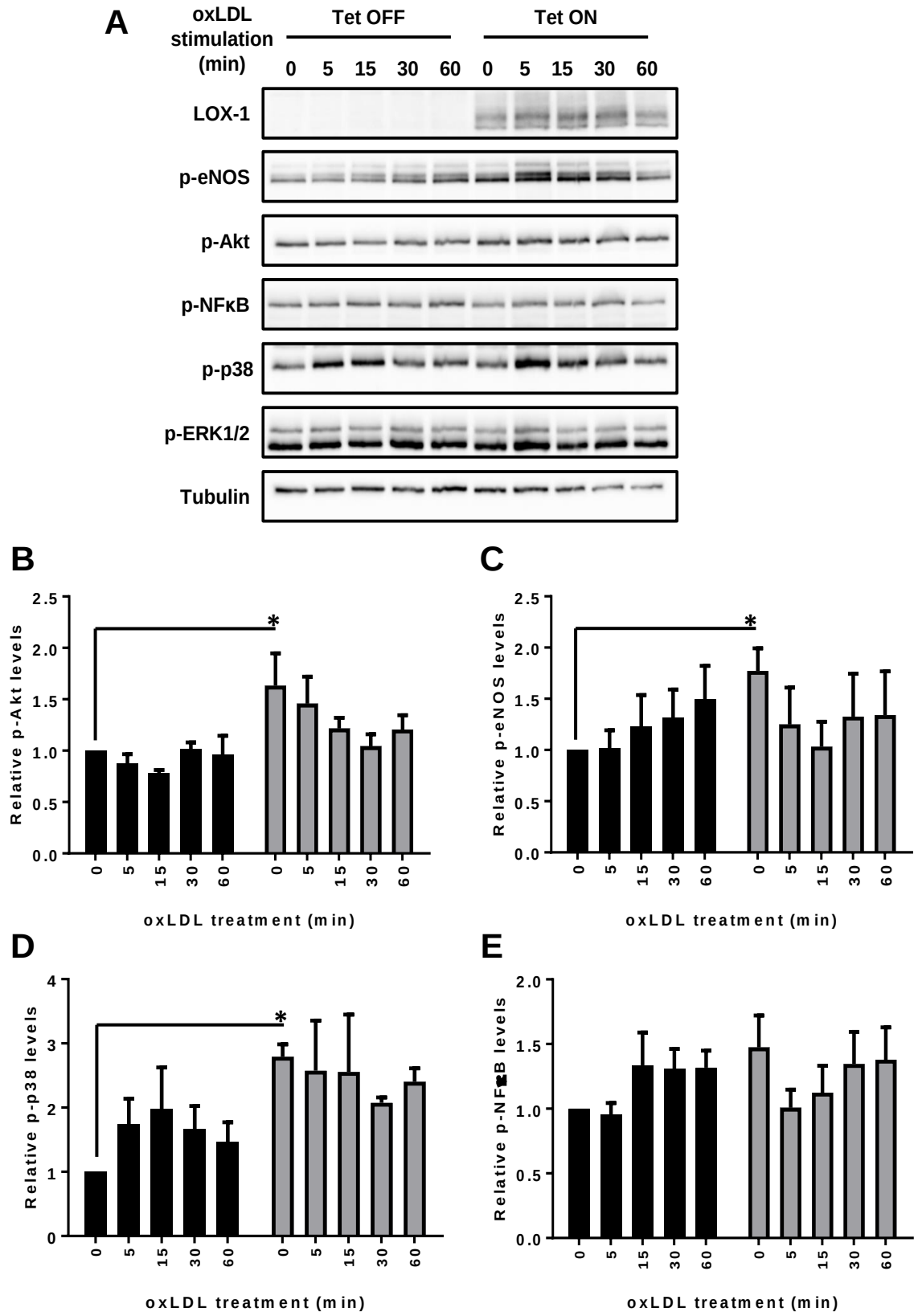

Figure 4.7. Oxidised LDL activation of LOX-1-D5A signal transduction. (A) PAEC cells expressing LOX-1-D5A were induced overnight with 1 µg/mL tet prior to stimulation with oxLDL (10 µg/mL) for 5, 15, 30 or 60 min followed by cell lysis and immunoblot analysis. (B-E) Quantifications of each activated proteins relative to Tet OFF at time 0; black bars indicate Tet OFF; white bars indicate Tet ON. Error bars indicate ±SEM (n≥3). p<0.05 (*), p<0.01 (**), p<0.001 (***), p<0.0001 (****).

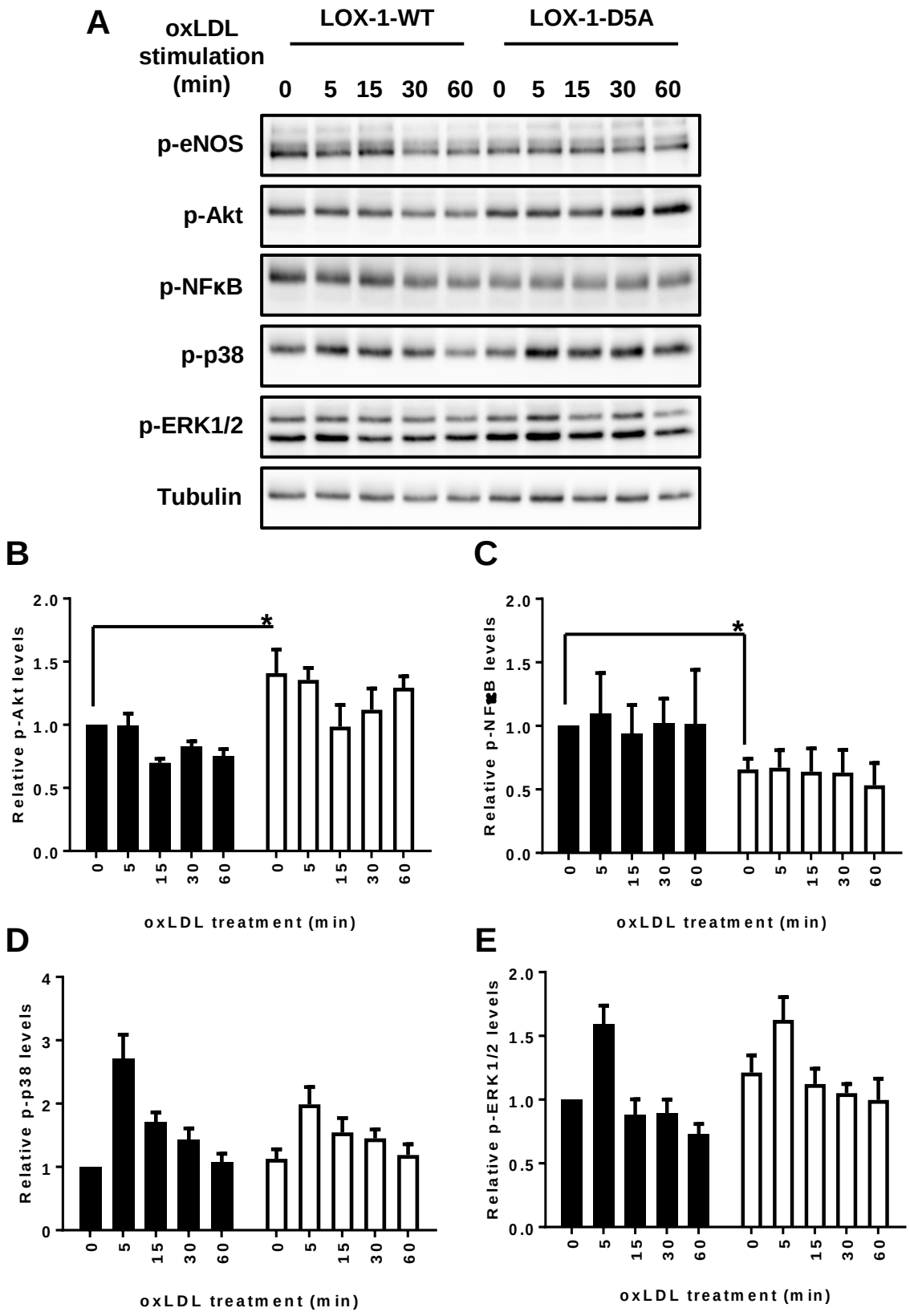

Figure 4.8. Differential oxidised LDL activation of signal transduction in LOX-1-WT and LOX-1-D5A. (A) PAEC cells expressing either lOX-1-WT or LOX-1-D5A were stimulated with oxLDL (10 µg/mL) for 5, 15, 30 or 60 min followed by cell lysis and immunoblot analysis. (B-E) Quantifications of each activated proteins relative to LOX-1-WT at time 0; black bars indicate LOX-1-WT; white bars indicate LOX-1-D5A. Error bars indicate ±SEM (n≥3). p<0.05 (*), p<0.01 (**), p<0.001 (***), p<0.0001 (****).

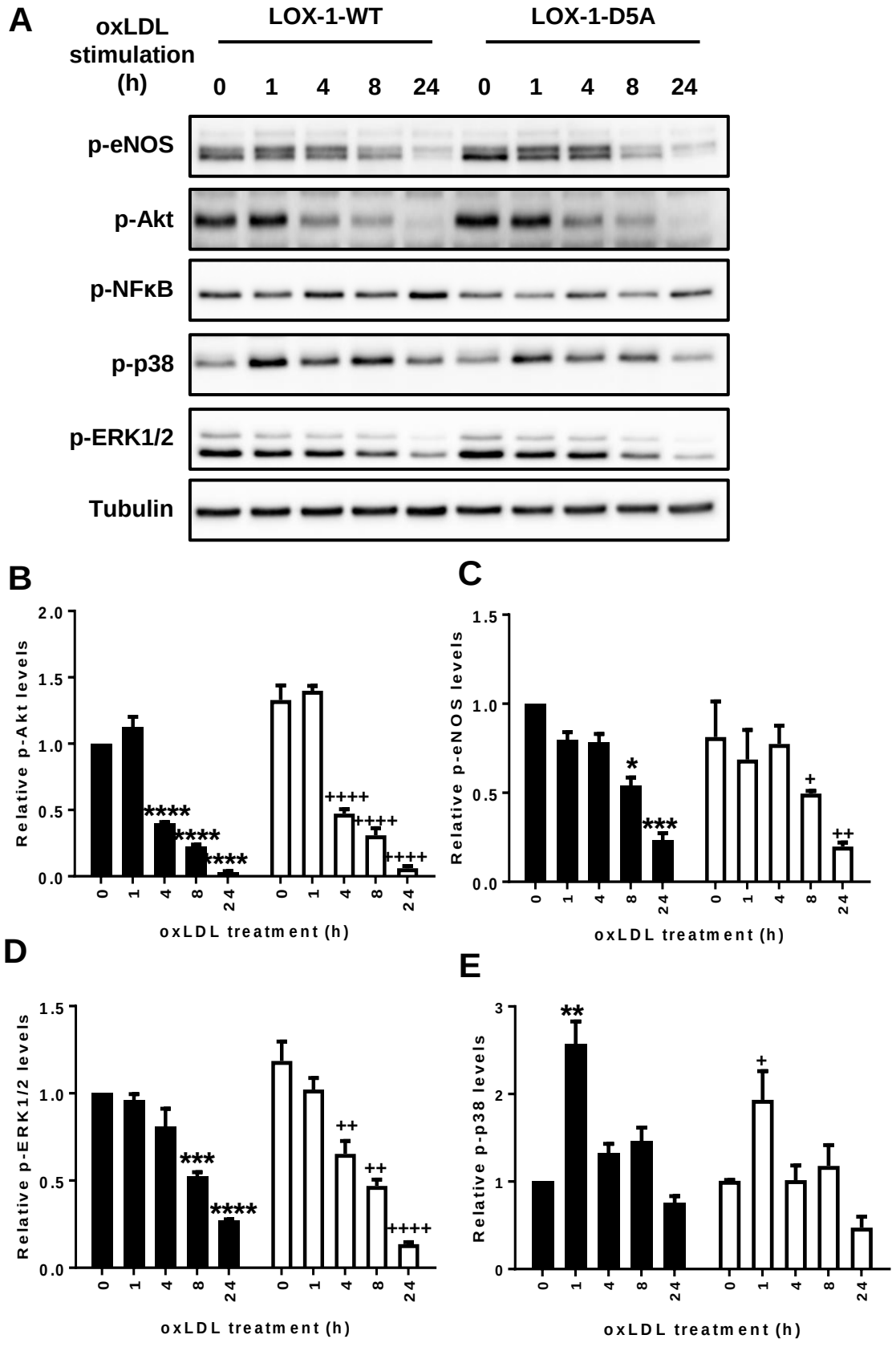

Figure 4.9. Longer time-frame stimulation of oxidised LDL. (A) PAEC cells expressing either lOX-1-WT or LOX-1-D5A were stimulated with oxLDL (10 µg/mL) for 1, 4, 8 or 24 h followed by cell lysis and immunoblot analysis against phosphorylated eNOS, Akt, NF-κB, p38 and ERK1/2. (B-E) Quantifications of each activated proteins relative to LOX-1-WT at time 0; black bars indicate LOX-1-WT; white bars indicate LOX-1-D5A. Error bars indicate ±SEM (n≥3). p<0.05 (*), p<0.01 (**), p<0.001 (***), p<0.0001 (****).

4.2.2.2 Oxidised LDL through LOX-1 decreases endothelial barrier permeability

One of the hallmarks of early endothelial dysfunction caused by oxidised LDL is increased endothelial barrier permeability to serum proteins (Liao et al., 1995; Rangaswamy et al., 1997; Essler et al., 1999; Orr et al., 2007). In this study, I wanted to know whether endothelial cell permeability decreases upon oxidised LDL stimulation via LOX-1-WT. To test this idea, I used a non-invasive technique namely, trans-endothelial electrical resistance (TEER) to assess the permeability of the PAEC monolayer. PAEC Flp-In^{TM} T-Rex^{TM} cells with empty vector pcDNA5/FRT/TO (control) or LOX-1-WT were stimulated with oxidised LDL (Figure 4.10A). Readings of the TEER measurement were taken at 0, 1, 2, 4, 6 and 8 h. Quantified measurements showed significant increase of TEER reading in LOX-1-WT (red line) cells at time 0 compared to control (blue line) cells. After 1 h stimulation of oxidised LDL, TEER reading of PAEC-expressing LOX-1-WT cells were significantly higher than that observed for control cells; this value gradually decreased after 2 h and returned back to baseline after 4 h. Endothelial barrier permeability is not stimulated by oxidised LDL; in fact, there is decreased permeability (increased TEER) upon oxidised LDL addition.

To further assess whether there was monolayer leakage caused by oxidised LDL, an alternative approach monitoring the movement of a florescent marker such as fluorescein isothiocyanate (FITC)-labelled dextran was used. In this assay, PAEC Flp-In^{TM} T-Rex^{TM} control or LOX-1-WT expressing cells were grown to a confluent monolayer on a Transwell filter and stimulated with oxidised LDL for 24 h; then cells were incubated with FITC-dextran for 30 min and followed by fluorescence measurement (Figure 4.10B). Quantification of FITC-dextran movement across the PAEC monolayer showed significant lower fluorescence intensity in the LOX-1-WT expressing cells compared to negative control. Both TEER and FITC-dextran permeability measurements indicate reduced PAEC permeability caused by LOX-1-WT expression.

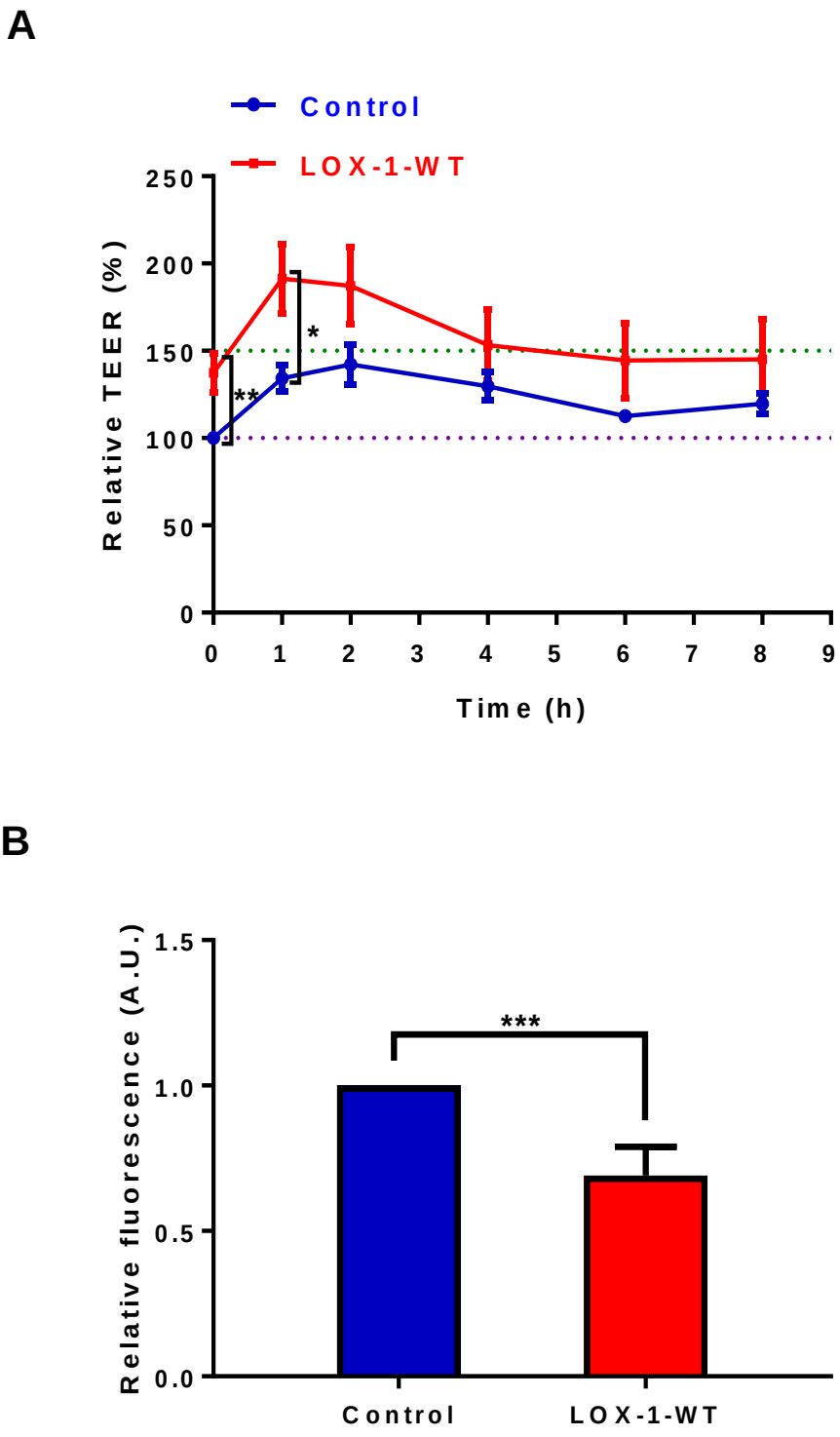

Figure 4.10. Oxidised LDL-induced via LOX-1 decreases cell permeability. (A) Trans-endothelial electrical resistance (TEER) reading was measured from PAEC cells expressing LOX-1-WT (red) and control cells at 0, 1, 2, 4, 6, and 8 h following stimulation with oxLDL (10 µg/mL). (B) Quantifications of FITC-dextran movement across the PAEC layer grown in Transwell filter by measuring fluorescence intensity. Prior to measurements, PAEC cells were treated with oxLDL (10 µg/mL) for 24 h, followed by incubating cells with FITC-dextran for 30 min. Error bars indicate ±SEM (n≥3). $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).

4.3 Discussion

In this study, I show that the presence of oxidised LDL reveals different trafficking properties of LOX-1-WT compared to mutant LOX-1-D5A proteins. These differences in oxidised LDL-stimulated LOX-1 trafficking have impacts on downstream signal transduction pathway(s), which influences endothelial cell barrier permeability (Fig. 4.11). In this model, LOX-1 wild-type and mutant LOX-1-D5A bind with similar affinity for oxidised LDL, but differentially programme ligand-dependent receptor internalisation and recycling (Fig. 4.11). Our findings reveal that oxidised LDL-mediated LOX-1 signalling activates Akt and p38 MAPK signal transduction. This is linked to oxidised LDL-stimulated LOX-1 endocytosis and delivery to endosomes linked to increased activation of the canonical MAPK pathway resulting in phosphorylation of the ERK1/2 master regulator (Fig. 4.11).

A key feature of oxidised LDL binding to cell surface LOX-1-WT is receptor endocytosis, linked to receptor recycling and possible degradation. In contrast, LOX-1-D5A expression results in a significantly lower pool of LOX-1 on the cell surface and addition of oxidised LDL causes a significantly lower fraction of LOX-1-D5A that undergoes endocytosis. Oxidised LDL-dependent LOX-1-WT internalisation was markedly higher in comparison to oxidised LDL-stimulated LOX-1-D5A expressing cells. This effect correlates with increased distribution of activated LOX-1-WT in endosomes and recycling back to cell surface. Previously it was shown that LOX-1-D5A lacks the ability to undergo oxidised LDL-stimulated internalisation (Murphy et al., 2008). In the work presented in this chapter, the D5A mutation within the LOX-1 cytoplasmic domain also affects trafficking and recycling of this membrane protein from endosomes. It was noteworthy that oxidised LDL stimulation did not affect the overall levels of either LOX-1-WT or D5A mutant, suggesting that oxidised LDL activation did not stimulate proteolysis or clearance.

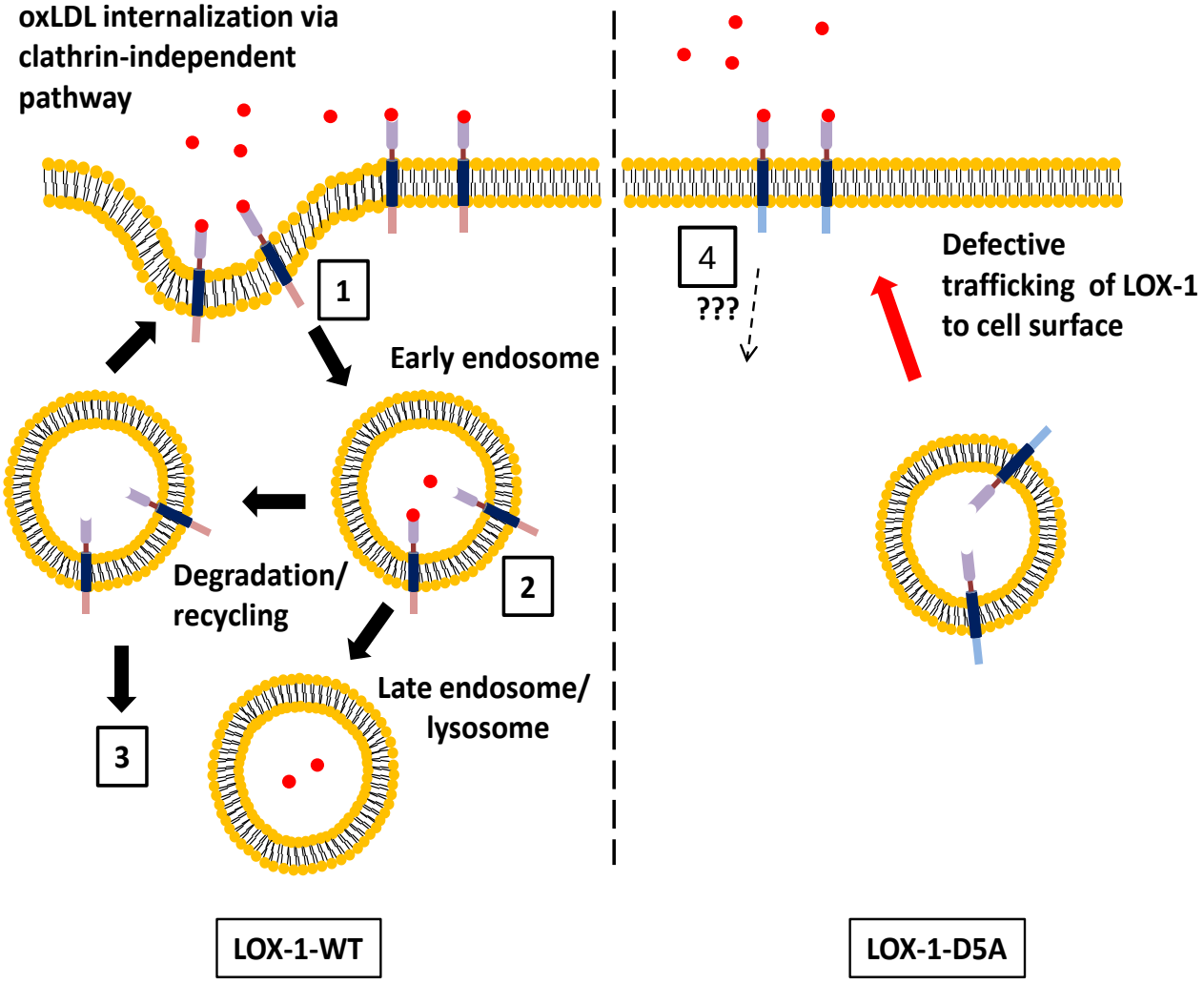

Figure 4.11. Schematic of oxidised LDL-stimulated LOX-1 trafficking. (1) Binding and uptake of oxLDL through LOX-1-WT. (2) OxLDL-LOX-1 in the early endosomes. (3) Un-coupling of oxLDL and LOX-1; LOX-1 is possibly recycled or degraded. (4) Trafficking-defect LOX-1-D5A unable to internalize oxidised LDL

One explanation for how LOX-1-WT and LOX-1-D5A differentially promote differential downstream signal transduction is through the binding and internalisation of LOX-1 and oxidised LDL. One study suggests that the constitutive endocytosis of LOX-1 is independent of oxidised LDL binding, and thus the downstream signalling events are linked to oxidised LDL activation of LOX-1 (Murphy et al., 2008). Oxidised LDL binding to membrane receptors is linked to activation of multiple signal transduction pathways (Robbesyn et al., 2004; Jing et al., 1999; Chien et al., 2003). The work in this chapter shows that the expression of LOX-1-WT stimulates Akt and p38 MAPK activation in response to oxidised LDL. Surprisingly, activation of either ERK1/2 or NF-κB was not evident under these conditions. Furthermore, despite the inability of LOX-1-D5A to internalise oxidised LDL, ERK1/2 activation and signalling was increased. As p38 MAPK is usually activated by cellular stress, it is likely that oxidised LDL would trigger this stress-activated kinase (Zhang and Liu, 2002), which plays fundamental roles in survival, proliferation and apoptosis (Widmann et al., 1999). In contrast, the Akt signal transduction pathway is best known for its involvement in cell survival (Dudek et al., 1997; Kauffmann-Zeh et al., 1997). Therefore, these findings suggest that oxidised LDL binding to LOX-1 cause cross-communication between the Akt and p38 MAPK signal transduction pathways, with likely consequences for cell survival. Additionally, targeting the LOX-1 extracellular domain using the monoclonal antibody JTX92 inhibited oxidised LDL binding, and prevented the downstream activation of MAPK and Akt signalling. Furthermore, the presence of JTX92 caused increased loss in LOX-1-WT levels, suggesting that this caused increased endocytosis, trafficking and proteolysis in this system. Notably, LOX-1-D5A expressing cells were capable of oxidised LDL-stimulated MAPK and Akt signalling even in the presence of JTX92 monoclonal antibody. Such findings suggest that oxidised LDL activation of LOX-1 can trigger differential signalling outcomes depending on membrane trafficking, plasma membrane and endosome localisation.

I have used a PAEC Flp-In™ T-Rex™ system to also stably express LOX-1-Wt and LOX-1-D5A constructs. I have showed low mRNA levels of native porcine LOX-1 (mentioned previously in Chapter 3) with the possibility that other scavenger receptors such as SR-A1 and CD36 might also contribute to oxidised LDL binding on PAEC cells. Overexpression of human LOX-1-WT in PAEC cells caused decreased activation of Akt, eNOS and NF-κB in response to oxidised LDL, whereas overexpressing the mutant LOX-1-D5A enhanced such signalling events. The endothelial monolayer within blood vessels produces nitric oxide generated by endothelial nitric oxide synthase (eNOS), which is critical for regulating blood pressure and vessel integrity; eNOS phosphorylation and activation is dependent upon Akt (Dimmeler et al., 1999). One possible explanation is that binding and activation of LOX-1 in response to oxidised LDL inhibits activation of the Akt/eNOS pathway, which exerts an effect on redox-sensitive transcription factor NF-κB.

These observed differences in signal transduction events between LOX-1-WT and LOX-1-D5A impact on the levels of phospho-eNOS, and thus directly on the rate of NO synthesis. One possibility is that the presence of native porcine LOX-1 or other scavenger receptors in PAECs could modulate signalling outcomes and make the effects of LOX-1-WT or LOX-1-D5A less clear-cut. Additionally, exposure of either LOX-1-WT or LOX-1-D5A to oxidised LDL for a longer time-frame (0-24 h) revealed that reduced activation of signal transduction pathways involving Akt/eNOS and ERK1/2. Surprisingly, under the same conditions, p38 MAPK was not affected by long-term oxidised LDL rapidly returning back to baseline within 24 h.

The role of oxidised LDL in causing endothelial dysfunction in atherogenesis is well-established (Liao et al., 1995; Rangaswamy et al., 1997; Essler et al., 1999; Orr et al., 2007). Surprisingly, in this study I showed that oxidised LDL via LOX-1-WT causes decreased endothelial cell barrier permeability. This suggests that the expression of LOX-1-WT causes fewer and smaller gaps within the PAEC monolayer cells. Under such conditions, there would be less ‘leakage’ or movement of cells and

molecules across the layer. Under these conditions, LOX-1-WT expression could inhibit trans-endothelial migration (TEM) of monocytes and lipoproteins into in the sub-endothelial space or intima. The mechanism underlying this phenomenon is unclear, as activation of the Akt/eNOS and ERK1/2 MAPK signal transduction pathways was reduced after 24 h of oxidised LDL stimulation. Another aspect of this work is that in PAEC Flp-In^{TM} T-Rex^{TM} cells, both native porcine LOX-1 and other porcine scavenger receptors could contribute to cellular responses to oxidised LDL and thus complicate the analysis.

CHAPTER 5

LOX-1-dependent signal transduction pathways in atherosclerosis

5.1 Introduction

Atherosclerosis-related cardiovascular disease is a major cause of morbidity and mortality in the world. Atherosclerosis is a well-established phenomenon but the underlying molecular and cellular mechanisms remain ill-defined. Arterial plaque initiation, development and progression is a dynamic process encompassing endothelial dysfunction, retention and modification of lipids, and recruitment of cells of the immune system such as monocytes, macrophages, T-cells and B-cells.

Atherosclerosis begins with the binding and accumulation of lipids and lipid particles followed by diverse inflammatory events (Ross, 1999). Oxidised or modified LDL particles can promote key steps involved in arterial plaque or lesion formation (Steinberg et al., 1989). The binding and rapid uptake of oxidised LDL by scavenger receptors, namely SR-A1, CD36 and LOX-1, present in macrophages and endothelial cells is important in foam cell development within atherosclerotic lesions (Yamada et al., 1998). The LOX-1 trafficking pathway oxidised LDL involves constitutive internalisation or endocytosis via clathrin-independent pathway for delivery to the endosome-lysosome system (Murphy et al., 2008). LOX-1 mediates multiple pro-atherogenic cellular responses such as endothelial dysfunction, apoptosis, vascular inflammation, foam cell formation and cholesterol metabolism in fat cells called adipocytes (Chui et al., 2005; Xu et al., 2012).

LOX-1 is implicated in promoting atherosclerosis from studies using different human and animal models (Morawietz, 2007; Hu et al., 2008a; Hu et al., 2008b; Khaidakov et al., 2012). The translation of animal models, however, has been less conclusive. A seminal study by Mehta and colleagues showed a significant reduction of aortic plaque incidence in transgenic mice carrying both low-density lipoprotein receptor (*LDLR*) and *LOX-1* null alleles (Mehta et al., 2007). Pro-atherogenic and pro-inflammatory signalling, such as NF-κB and p38 MAPK phosphorylation, were also reduced in *LDLR/LOX-1* double knockout/null mice compared to *LDLR*-null mice (Mehta et al., 2007). Furthermore, the overexpression of a *LOX-1* transgene in *ApoE* knockout mice on a high-fat diet showed increased uptake and infiltration of oxidised LDL in the heart and in the common carotid artery (Inoue et al., 2005; White et al., 2011). Nonetheless, ectopic expression of LOX-1 in the liver of the *ApoE* knockout mice showed reduction in plasma LDL levels, thus showing reduced incidence of atherosclerotic plaques (Ishigaki et al., 2008). Nonetheless, these findings suggest that LOX-1 can promote beneficial (anti-atherogenic) or harmful (pro-atherogenic) effects in atherosclerosis.

LOX-1 could play a role in the immune system by recognising oxidised LDL as a foreign molecule or antigen. Presumably, monocytes which infiltrate into the sub-endothelial layer of the intima cause increased expression of adhesion molecules such as VCAM-1 and ICAM-1. Subsequently, differentiated macrophages and endothelial cells in the intima could internalise oxidised LDL to process or degrade this substance. Such findings raise the question whether LOX-1 promotes or inhibits inflammation and atherosclerosis. The aims of the work presented in this chapter therefore were to evaluate the effect of loss of functional *LOX-1* genotype on signalling transduction pathways in transgenic mice to evaluate effects on atherosclerosis.

5.2 Results

5.2.1 Influence of the *LOX-1* genotype on animal physiology

5.2.1.1 Genotyping of transgenic mice

Recent work from the Ponnambalam laboratory (University of Leeds, UK) has shown that *LOX-1/ApoE* double knockout mice showed increased plaque formation compared to *ApoE* knockout mice (Supplementary figure B1) (Mughal, 2015). I investigated this further by assessing the link between genotype, diet and signal transduction in these transgenic mouse lines. I first assessed the different transgenic mouse lines by subjecting ear notch tissues to PCR. The C57Bl/6 mouse line carrying the *ApoE* knockout showed a 245 bp sequence whilst those containing the gene showed the presence of a 155 bp sequence (Figure 5.1A). *LOX-1* knockout mice displayed loss of 405 bp sequence within exon 7 of the mouse *LOX-1* (*OLR1*) gene (Figure 5.1B). However, future experiments will need to confirm the deletion of LOX-1 exon 7 is associated with loss of LOX-1 mRNA and protein.

5.2.1.2 Diet-induced changes in mouse weight

Six week old male mice fed on a standard chow diet were weighed weekly starting from 6 weeks of age (Figure 5.2, A and B). Quantification of the weight gain showed a significant ~20-25% increase in both wild-type and LOX-1 knockout mice after 6 weeks on standard chow diet (Figure 5.2A). Conversely, in *ApoE* knockout and double *LOX-1/ApoE* knockout mice, there was a significant ~40% increase after 6 weeks of standard diet feeding (Figure 5.2B). There was no difference in their body weights at 12 weeks of age between these groups. After standard chow diet, mouse organs such as liver (Figure 5.2, C and D) and heart (Figure 5.2, E and F) were weighed and compared within each group. Quantification of organ weights, for both heart and liver, showed no differences between wild-type and *LOX-1* knockout mice and between *ApoE* knockout and *ApoE/LOX-1* double knockout mice at 12 weeks of age.

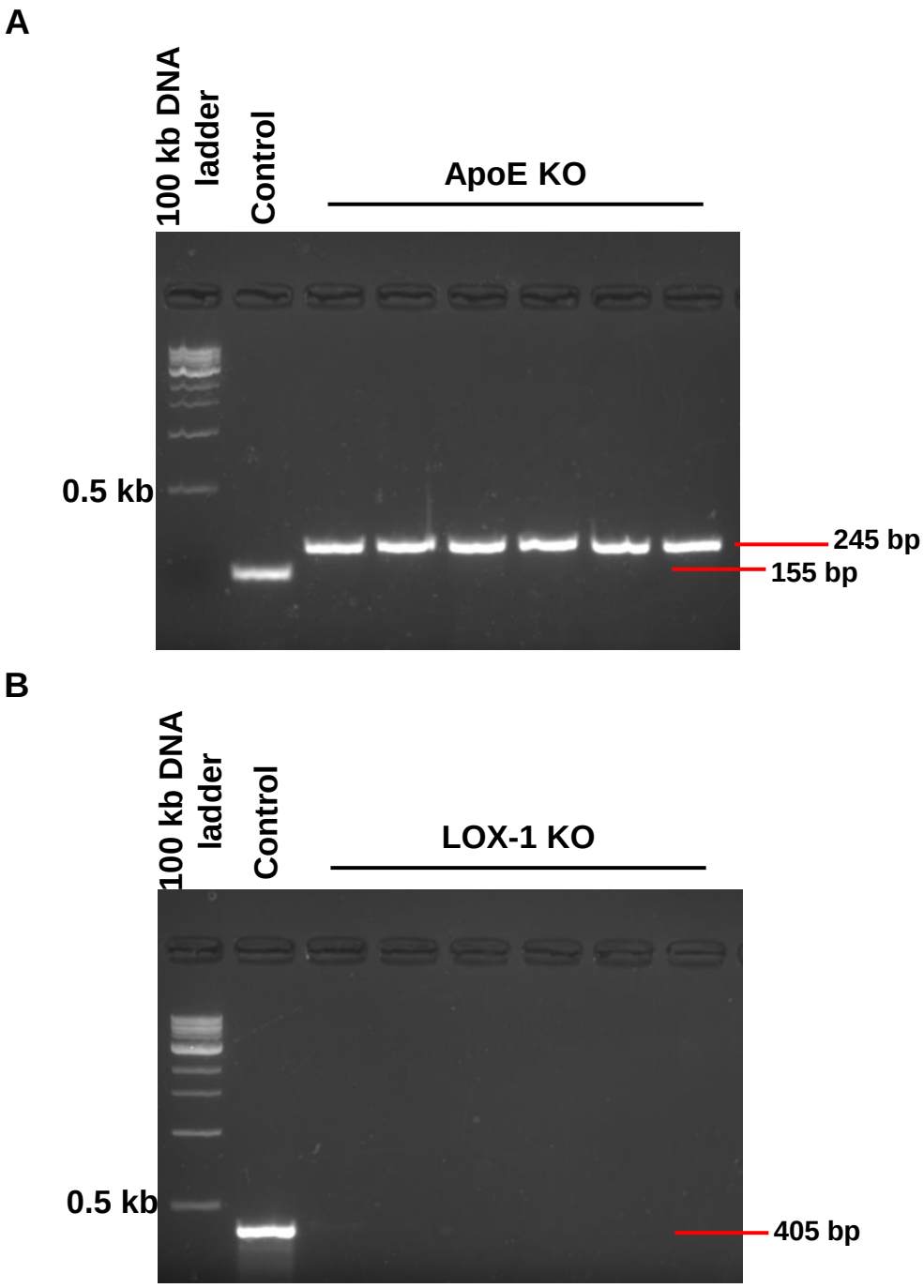

Figure 5.1. Profiling of transgenic mouse lines. *ApoE*-null, *LOX-1*-null, and wild-type mice were genotyped for *LOX-1* and *ApoE* alleles using polymerase chain reaction (PCR). (A) With ApoE primers, a low band (155 bp) indicates presence of the gene whilst a band (245 bp) indicates its absence. (B) The presence of a DNA band (405 bp) with LOX-1 primers indicates a functional gene whilst its loss signifies knockout.

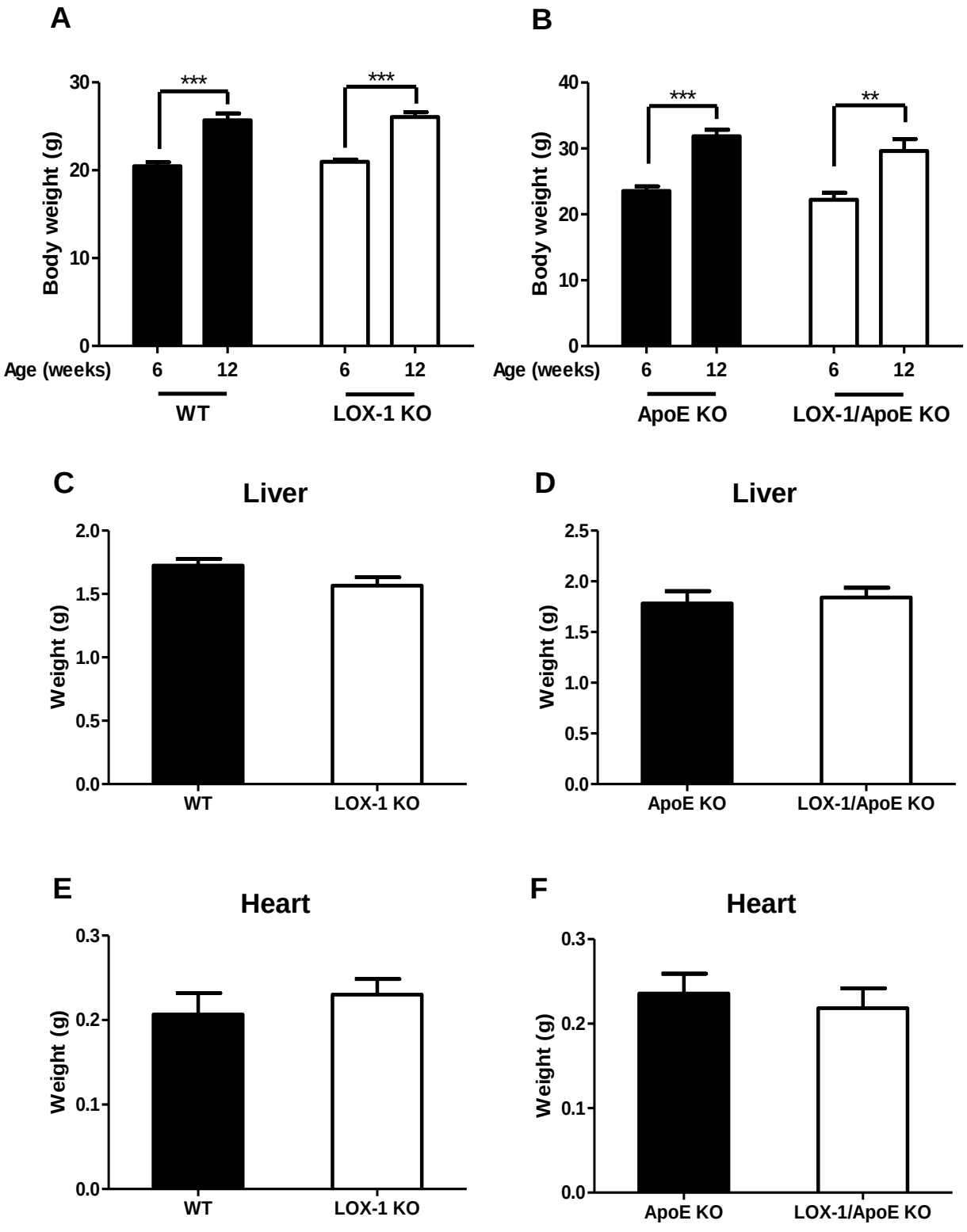

Figure 5.2. Weight and organ weight of transgenic mouse lines on Standard chow diet. Weight measurements of (A) wild-type (WT) and LOX-1 knockout (KO) and (B) ApoE KO and LOX-1/ApoE KO taken at age 6 weeks and age 12 weeks. (C) Liver weight of WT and LOX-1 KO and (D) liver weight of ApoE KO and LOX-1/ApoE KO at 12 weeks. (E) Heart weight of WT and LOX-1 KO and (D) heart weight of ApoE KO and LOX-1/ApoE KO at 12 weeks. Error bars indicate ±SEM (n≥8). p<0.05 (*), p<0.01 (**), p<0.001 (***), p<0.0001 (****).

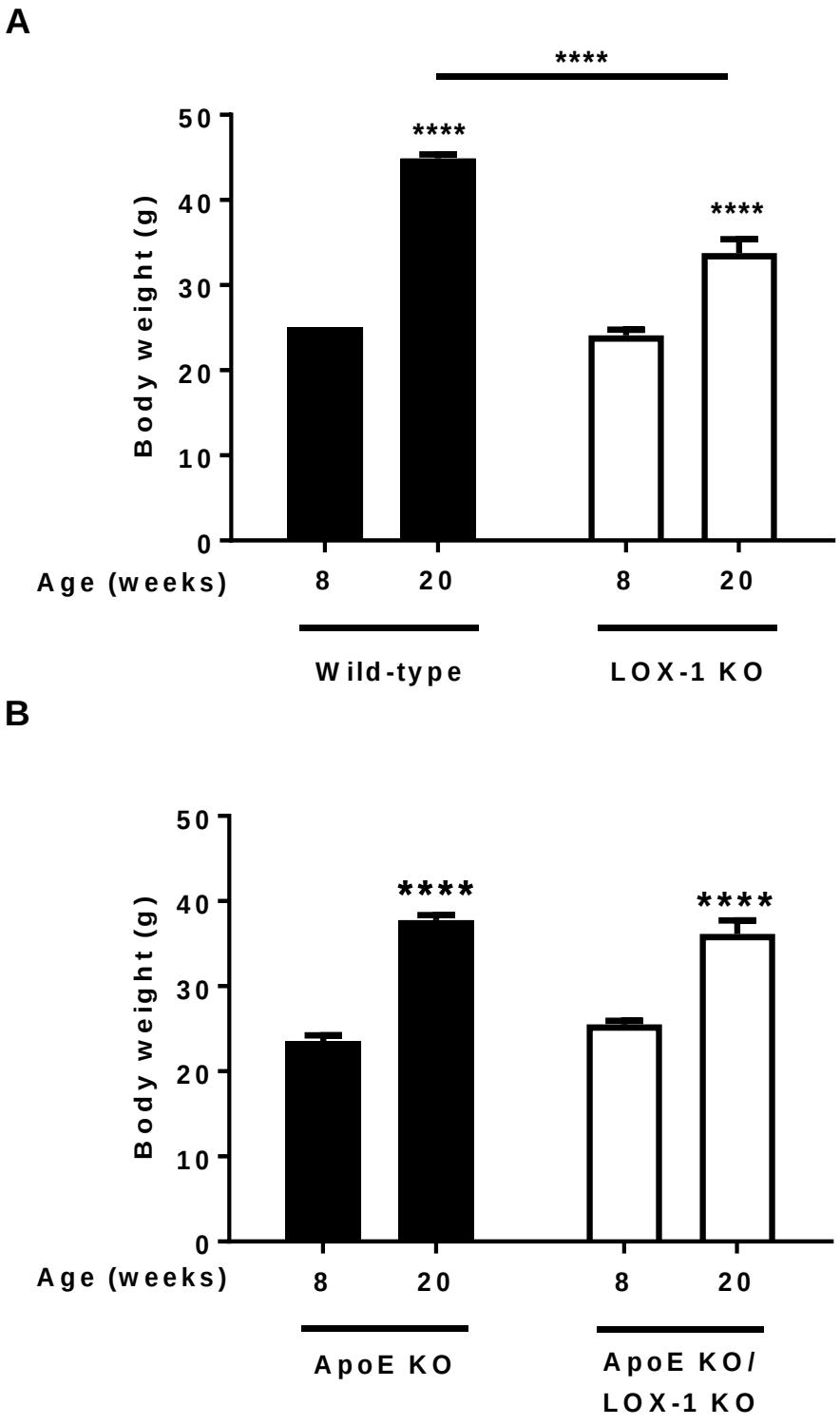

Figure 5.3. Weight of transgenic mouse lines on Western diet. Weight measurements of (A) wild-type (WT) and LOX-1 knockout (KO) and (B) ApoE KO and LOX-1/ApoE KO taken at age 8 weeks and age 20 weeks. Error bars indicate ±SEM (n≥8). $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).

To better understand the basis for plaque development in the mouse aorta, I fed the mice with fat and cholesterol-rich Western diet for 12 week period and evaluated different parameters (Figure 5.3). Quantification of mouse body weight revealed a significant ~65-70% increase for wild-type mice and a significant ~40-45% increase for *LOX-1* knockout mice after 12 weeks on Western diet (Figure 5.3A). Additionally, there was a significant ~45% difference in body weight between wild-type and *LOX-1* knockout mice at 20 weeks of age (Figure 5.3A). Quantification of change in body weight during Western diet showed a significant ~65-70% increase in *ApoE* knockout mice, with a significant ~50% increase in *LOX-1/ApoE* double knockout mice (Figure 5.3B). These findings suggest that presence of functional *LOX-1* has impacts on change in body weight.

5.2.2 Pro-atherogenic signal transduction pathways in the mouse aorta

The concept of oxidative stress and inflammation as central regulators of in atherosclerosis and vascular disease raises interesting questions on the role of LOX-1 in these processes. A pro-oxidative state implies that endothelial cells in the intima could be injured, generate reduced nitric oxide levels, promote inflammation, modify or oxidise LDL, all hallmarks of atherosclerosis (Mehta, 2006). The effect of knocking out *LOX-1* in a wild-type or *ApoE* knockout mouse background was investigated and found that *LOX-1/ApoE* double knockout mice have a higher aortic plaque coverage compared to control *ApoE* knockout mice; whilst there were little or no plaques in the aortas of wild-type or *LOX-1* knockout mice on a Western diet (Supplementary figure B1) (Mughal, 2015).

Based on these previous findings, I hypothesised that pro-inflammatory and pro-atherogenic signalling events are elevated in *LOX-1/ApoE* double knockout mice compared to controls. To test this idea, I subjected 8 week old male mice to a 12 week Western diet and harvested the aorta from all four mouse lines, namely, wild-type mice, *LOX-1* knockout mice, *ApoE* knockout mice and *LOX-1/ApoE* double knockout mice. Immunoblotting was used to probe for changes in status of NF-κB,

Akt, ERK1/2 and p38 MAPK using antibodies directed to either the native protein or phosphorylation epitopes (Figure 5.4A and Figure 5.5A). Even though there were no plaques evident in wild-type and *LOX-1* knockout mice, I wanted to evaluate the impact of loss of *LOX-1* alone on signal transduction events in the mouse aorta. Quantification of band intensities revealed significant increases in phosphorylation of NF-κB, Akt, p38 MAPK and ERK1/2 in comparison to control wild-type mice (Figure 5.4, B-E). Such data suggest that the loss of functional *LOX-1* causes activation of signal transduction events in the mouse aorta. Surprisingly, quantification of immunoblotting of mouse aortas showed significant reduction in phosphorylation of NF-κB, Akt, ERK1/2 and p38 MAPK in *LOX-1/ApoE* double knockout mice compared to control *ApoE* knockout alone (Figure 5.5, B-E). Such findings clearly show a trend of opposing signalling effects caused by the presence or absence of *LOX-1* in the aorta.

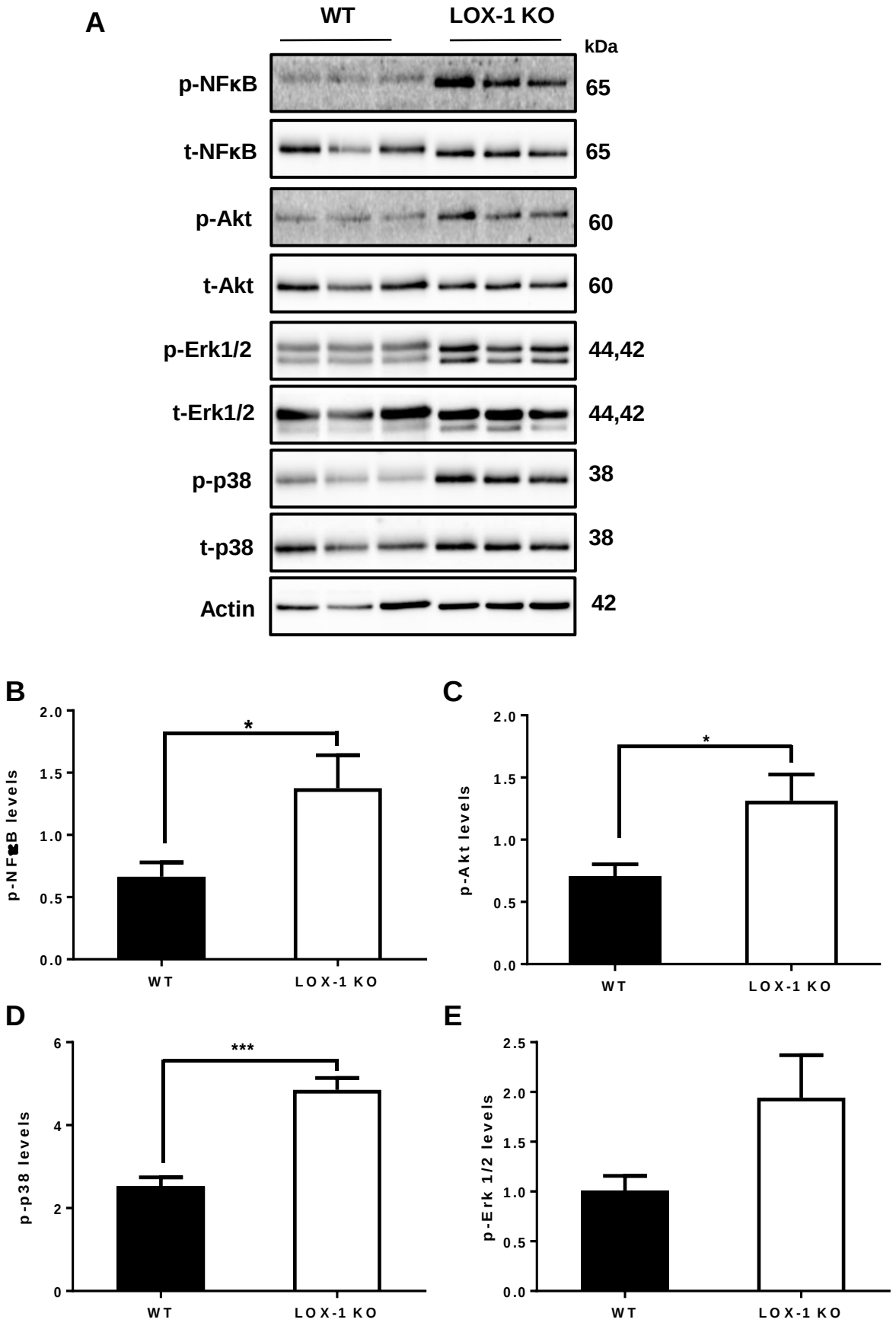

Figure 5.4. LOX-1 modulates aorta signal transduction pathways in wild-type and LOX-1 knockout mice. (A) Wild-type (WT) and LOX-1 knockout (KO) fed on a Western diet for 12 weeks were analyzed for pro-inflammatory signalling in aorta. Tissues were compared for phosphorylation of (B) NF-κB, (C) Akt, (D) p38 and (D) ERK1/2 relative to total proteins. Error bars indicate ±SEM (n≥8). $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).

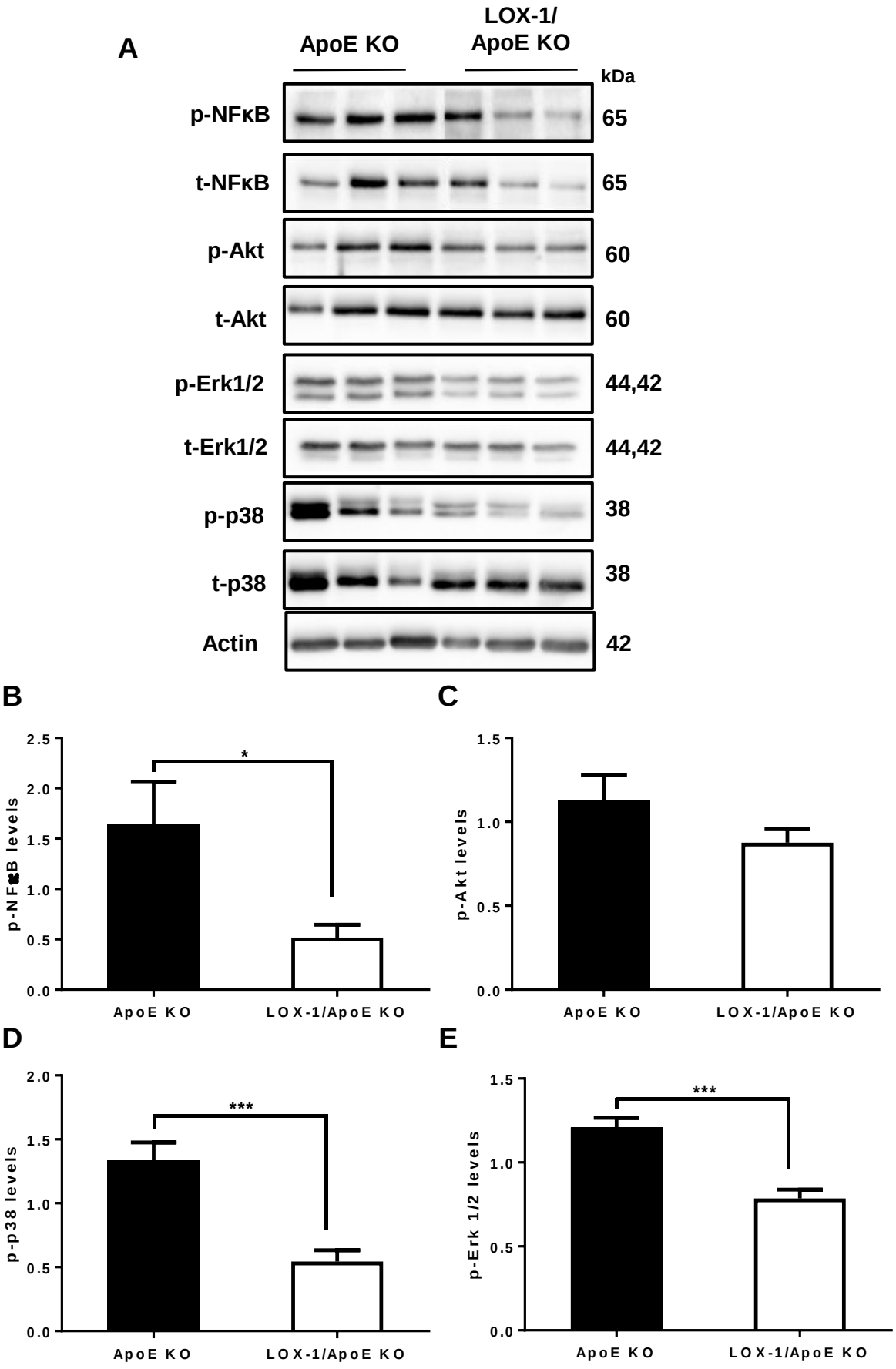

Figure 5.5. LOX-1 modulates aorta signal transduction pathways in ApoE knockout and LOX-1/ApoE knockout mice. (A) ApoE knockout (KO) and LOX-1/ApoE (KO) fed on a Western diet for 12 weeks were analyzed for pro-inflammatory signalling in aorta. Tissues were compared for phosphorylation of (B) NF-κB, (C) Akt, (D) p38 and (D) ERK1/2 relative to total proteins. Error bars indicate ±SEM (n≥8). $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).

5.2.3 Pro-atherogenic signal transduction pathways in mouse liver

The liver is an important organ for clearing oxidised LDL (Itabe et al., 2011). One possibility is that LOX-1 expressed in the sinusoidal endothelial cells of the liver plays a major role in binding oxidised LDL for endocytosis and clearance. However, the pro-inflammatory signalling pathways influenced by LOX-1 in the liver are unknown. I evaluated such signalling events involving LOX-1 after 12 weeks of Western diet. Liver tissues from wild-type mice, *LOX-1* knockout mice, *ApoE* knockout mice and *LOX-1/ApoE* double knockout mice were probed by immunoblotting to monitor the phosphorylation status of NF-κB, Akt, ERK1/2 and p38 MAPK (Figure 5.6A and Figure 5.7A). The signalling differences in liver tissues between wild-type and *LOX-1* knockout mice were quantified (Figure 5.6, B-E). Quantification revealed decreased phosphorylation of NF-κB (Figure 5.6B), Akt (Figure 5.6C) and p38 (Figure 5.6D) in *LOX-1* knockout mice in comparison to wild-type mice. There were no significant effects on ERK1/2 phosphorylation (Figure 5.6E).

Next, I quantified signalling differences in the liver between *ApoE* knockout mice and *ApoE/LOX-1* double knockout mice using immunoblotting (Figure 5.7A). Quantification of band intensities revealed significant increased phosphorylation of NF-κB (Figure 5.7B), p38 (Figure 5.7D) and ERK1/2 (Figure 5.7E) in *ApoE/LOX-1* double knockout mice compared to *ApoE* knockout mice. However, I could not detect changes in phospho-Akt levels in either mouse strain (Figure 5.7C). Such findings clearly show a trend of opposing signalling effects caused by the presence or absence of *LOX-1* in the liver.

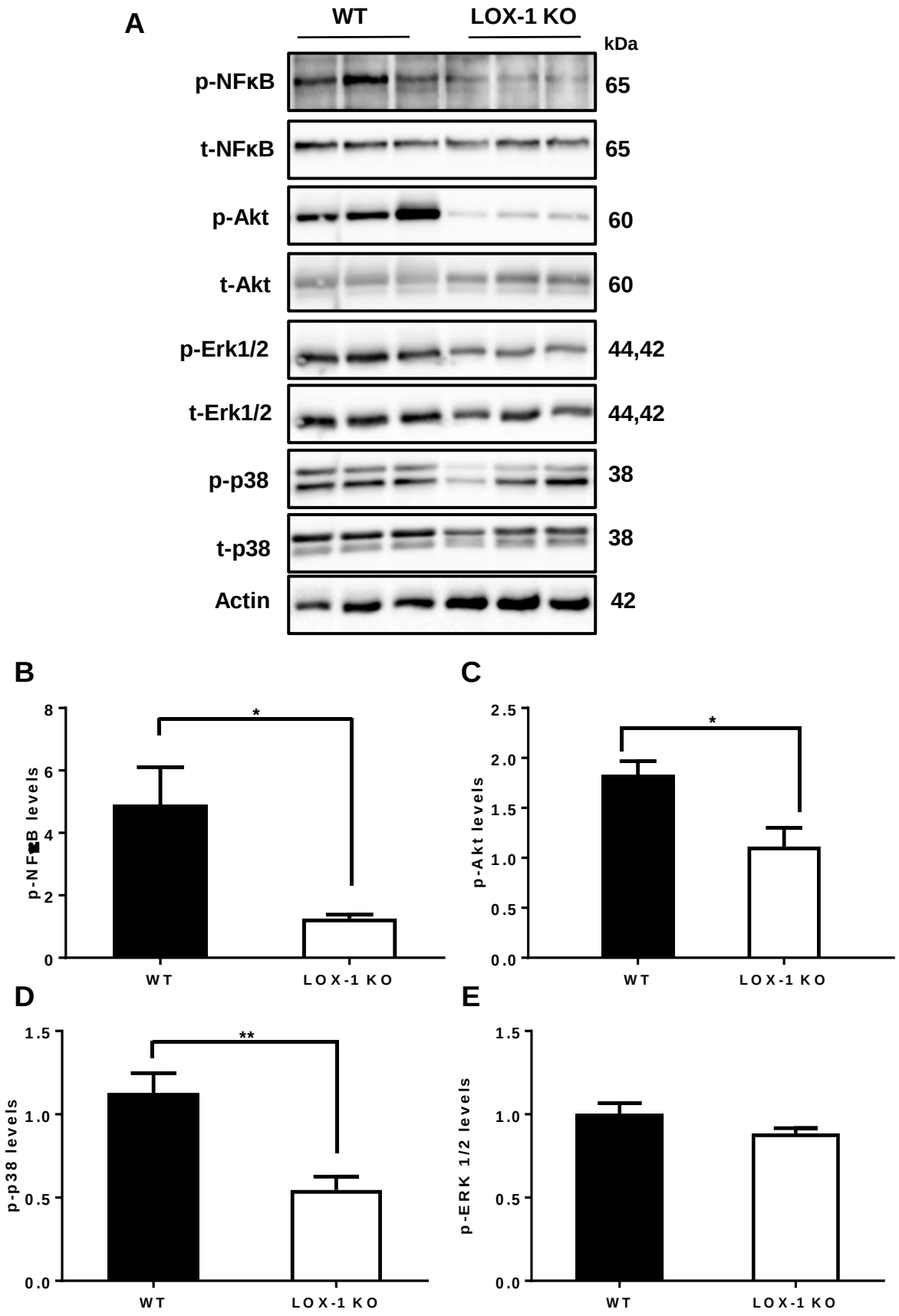

Figure 5.6. LOX-1 modulates liver signal transduction pathways in wild-type and LOX-1 knockout mice. (A) Wild-type (WT) and LOX-1 knockout (KO) fed on a Western diet for 12 weeks were analyzed for pro-inflammatory signalling in liver. Tissues were compared for phosphorylation of (B) NF-κB, (C) Akt, (D) p38 and (D) ERK1/2 relative to total proteins. Error bars indicate ±SEM (n≥8). p<0.05 (*), p<0.01 (**), p<0.001 (***), p<0.0001 (****).

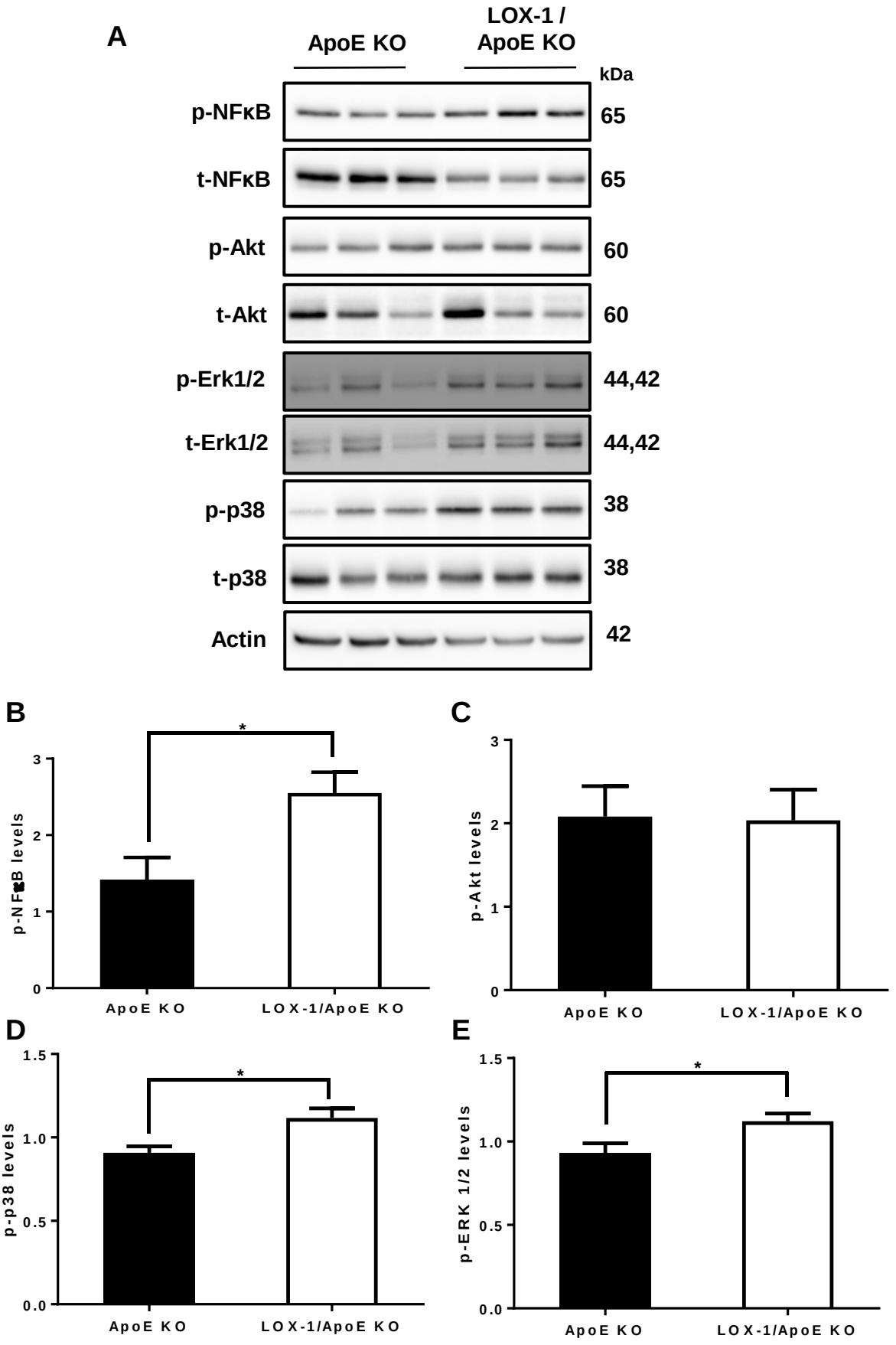

Figure 5.7. LOX-1 modulates liver signal transduction pathways in ApoE knockout and LOX-1/ApoE knockout mice. (A) ApoE knockout (KO) and LOX-1/ApoE (KO) fed on a Western diet for 12 weeks were analyzed for pro-inflammatory signalling in aorta. Tissues were compared for phosphorylation of (B) NF-κB, (C) Akt, (D) p38 and (D) ERK1/2 relative to total proteins. Error bars indicate ±SEM (n≥8). $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).

5.3 Discussion

The work carried out in this chapter showed the impact of deleting *LOX-1* on different signal transduction pathways such as Akt, NF-κB, ERK1/2 and p38 MAPK. However, the differential signal transduction outcomes in the aorta or liver could be dependent on the status of atherosclerosis disease and progression. In our proposed model (Figure 5.8), under conditions where atherosclerosis is relatively advanced, loss of functional *LOX-1* has wide impact on the signal transduction, plaque development and plasma lipids. The addition of the Western diet may increase abnormality in plasma lipids, with increasing plasma LDL and decreasing plasma HDL levels (Figure 5.8).

In the mouse aorta, although plaque incidence is increased by loss of functional *LOX-1* within the *ApoE* knockout background (Supplementary figure B1) (Mughal, 2015), the multiple signalling and activation events are reduced (Figure 5.8). One possible explanation for this phenomenon is that oxidised LDL is trapped within the arterial plaques and cannot activate its pro-inflammatory events (Tsimikas et al., 2007). On the other hand, *LOX-1* deletion causes increased signalling in liver, namely NF-κB, ERK1/2 and p38 MAPK activation (Figure 5.8). This is due to high levels of oxidised LDL in the blood circulation (Ishigaki et al., 2008), that in the absence of LOX-1, oxidised LDL is not being efficiently removed, excreted and/or degraded. Such abnormalities could lead to endothelial dysfunction, impaired glucose metabolism, abnormal lipid levels and plaque formation.

This is not the first study to examine the link between atherosclerosis, plaque formation, presence of oxidised LDL and pro-inflammatory signalling pathways. In fact, a seminal study used overexpression of LOX-1 in endothelial cells of *ApoE* knockout mice to find that increased aortic oxidised LDL levels led to endothelial dysfunction and increased production of reactive oxygen species (Akhmedov et al., 2014). This observation agrees with the work presented in this chapter as *LOX-1/ApoE* double knockout mice showed reduction in pro-inflammatory

signalling in the aorta (Figure 5.5). However, what was lacking in the work by Akhmedov et al. (2014) is that they have not shown the effect of overexpressing LOX-1 in endothelial cells of the liver. One conclusion is that in advanced atherosclerosis, LOX-1 is unable to compensate for the increased levels of oxidised LDL in the aorta. Thus, LOX-1 may play a role in regulating the status of pro-inflammatory gene transcription by activating the p65 RelA subunit of NF-κB.

Moreover, Ishigaki and colleagues showed enhanced clearance of oxidised LDL by hepatic LOX-1 after adenoviral administration of LOX-1 in the liver of *ApoE* knockout mice (Ishigaki et al., 2008). Yet again, this result coincides with our current study when *LOX-1/ApoE* double knockout mice showing increased pro-inflammatory signalling in the liver. This further proves the importance of liver-specific LOX-1 being expressed. Scavenger receptors have been reported to be expressed in Kuppfer cells and sinusoidal endothelial cells in the liver (Poli, 2000; Schneiderhan et al., 2001; Ling et al., 1997). The liver has been shown as an important organ in effectively clearing pro-atherogenic oxidised or modified LDL particles from the circulation when administered exogenously (Ling et al., 1997). Additionally, another study showed fluorescent labelled oxidised LDL was found to accumulate in rat Kupffer cells (Pieters et al., 1994). A contradictory study by Zhang and colleagues revealed the oxidised LDL-mediated increase in phosphorylation of p65 RelA (NF-κB subunit) and increased production of reactive oxygen species via LOX-1 in liver endothelial cells (Zhang et al., 2014). However, the use of relatively high concentrations of oxidised LDL may have activated other liver scavenger receptors and these high concentrations of oxidised LDL may not be physiologically relevant. Taken together, I have now shown the important role of LOX-1 in the liver, although the fate of oxidised LDL and long-term accumulation of oxidised LDL in the liver are unknown.

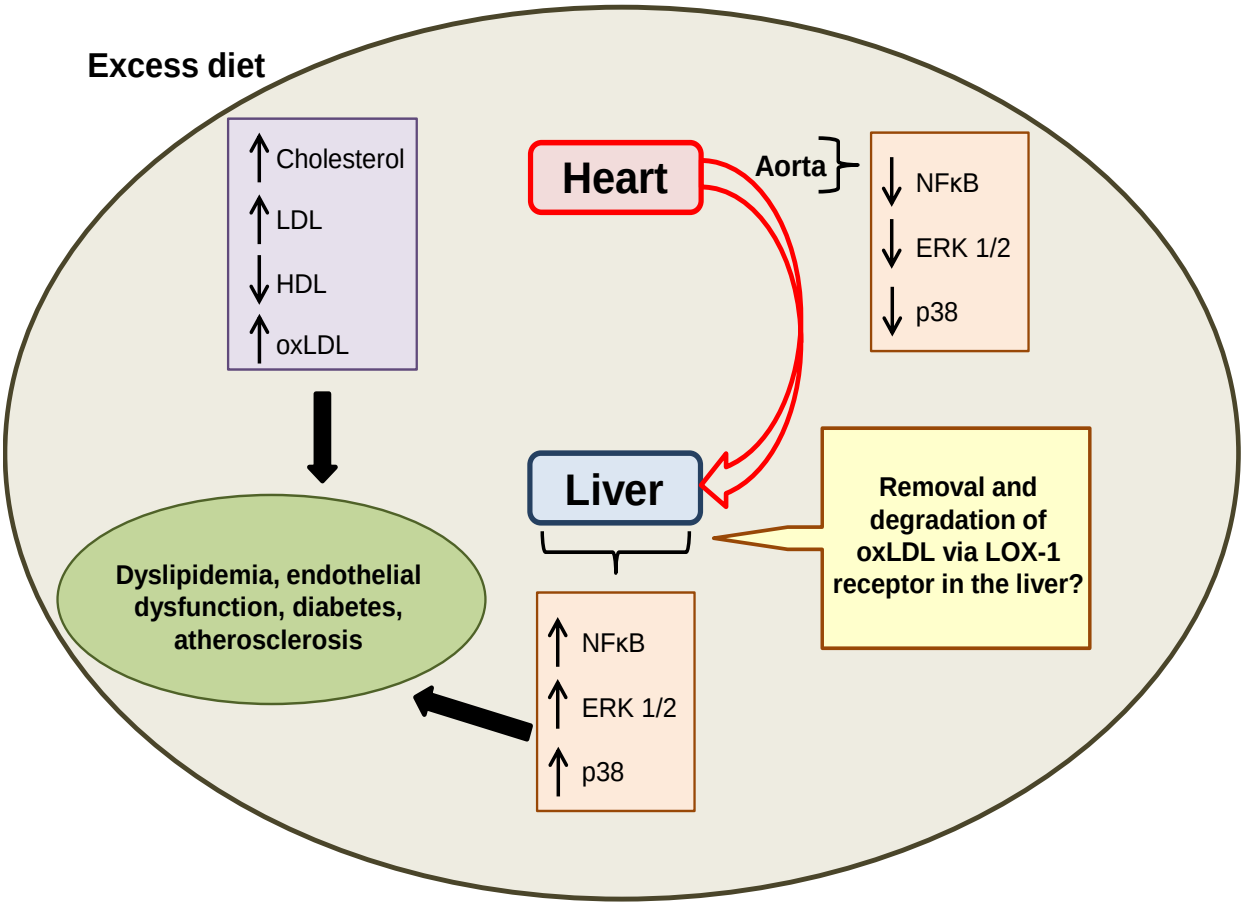

Figure 5.8. Consequences of knocking out *LOX-1* gene. Knocking out LOX-1 in an advanced atherosclerotic state alters the plasma cholesterol and lipid levels, with a significant increase of plasma oxLDL levels. Subsequently, increased oxLDL levels activates pro-inflammatory signalling pathway, especially in the liver. This causes hyperlipidemia and diabetes leading to atherosclerosis.

One of the early events in atherosclerosis is endothelial dysfunction. As shown in this chapter and in our proposed model in figure 5.9, loss of functional *LOX-1* showed increased phosphorylation of NF-κB and MAPKs. As there was no evidence of plaques in the aorta of wild-type and *LOX-1* knockout mice (Supplementary figure B1) (Mughal, 2015), altered signal transduction could explain how atherosclerotic lesions are initiated. It is possible in a pre-lesion state of the aorta, LOX-1 expressed on the endothelium enables scavenging for oxidised LDL for endocytosis and clearance. As the disease progresses, LOX-1 receptor potentially modulate pro-atherogenic signalling. One view is that LOX-1 thus switches roles depending on how is the relative seriousness of the disease state.

The work in this chapter thus demonstrates that in the early stage of atherosclerosis, LOX-1 may be involved in clearing oxidised LDL present in blood plasma to potentially target this substance for clearance and/or degradation. I have shown the deletion of functional *LOX-1* caused increased pro-inflammatory signalling, in spite of scarcity of plaques detected in the mouse aorta. Such increased in pro-inflammatory signalling in the *LOX-1* knockout model could mean that the endothelium is being primed to move into a pro-atherogenic state. For the first time, I showed loss of functional *LOX-1* in a pro-atherosclerotic *ApoE* knockout mouse model did not cause plaque reduction, despite the reduced pro-inflammatory signalling.

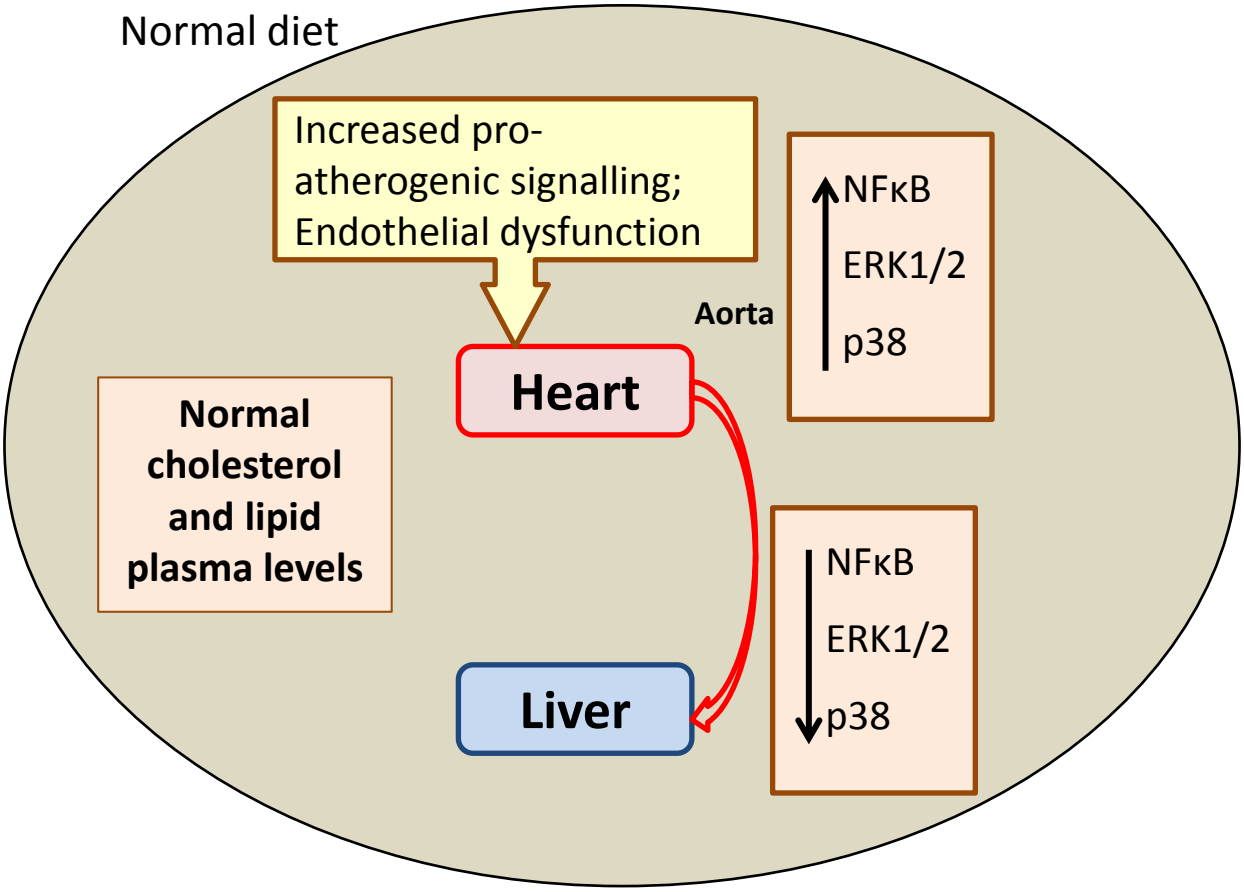

Figure 5.9. Consequences of knocking out *LOX-1* gene. Knocking out LOX-1 in normal physiological state does not alffect the plasma cholesterol and lipid levels. However, pro-atherogenic signalling are increased in the aorta that might mediate endothelial dysfunction, and therefore, potentially explains pre-lesion state in the aorta.

CHAPTER 6

LOX-1 functional regulation of lipid and glucose metabolism

6.1 Introduction

Patients with diabetes mellitus display an increased risk of atherosclerosis with 80% of diabetic mortality due to heart attacks and strokes (Nathan et al., 2005; Faxon et al., 2004). Type 1 (insulin-dependent) and type 2 (non-insulin-dependent) diabetes increases the risk major arterial dysfunction such as coronary artery disease and peripheral artery disease. The effects of diabetes on vasculature are complicated; however, hyperglycaemia, insulin resistance, dyslipidaemia and elevated free fatty acids levels are major factors in atherosclerosis in diabetes (Beckman et al., 2002). Many *in vitro* studies showed that increased glucose levels cause a plethora of pro-atherogenic responses such as increased production of reactive oxygen species and increased NF-κB activation (Brownlee, 2005; Mazzone et al., 2008; Piga et al., 2007; Yan et al., 1994). Consequently, this increases the expression of adhesion molecules in endothelial cells and attracts circulating monocytes, which depicts the early stage of atherosclerosis (Piga et al., 2007).

Metabolic syndrome is a complex disorder linking dyslipidaemia, obesity, insulin resistance and hypertension (Stern et al., 2005). Interestingly, increased plasma oxidised LDL levels are associated with metabolic syndrome (Sigurdardottir et al., 2002; Holvoet et al., 2008). A number of studies have drawn associations between LOX-1 and the pathophysiological findings of diabetes and the metabolic syndrome. Chen

and colleagues showed increased LOX-1 expression in vascular endothelial cells of diabetic rats suggests the role of LOX-1 in diabetes-related endothelial dysfunction leading to atherosclerosis (Chen et al., 2001c). Another study also showed the increased expression of LOX-1 in response to high levels of glucose, in which such expression depended upon the activation of NF-κB and MAPK signalling pathways (Li et al., 2003d).

The impact of *LOX-1* deletion on glucose metabolism in animal models was examined recently (Mughal, 2015). Glucose tolerance testing was performed on mice after overnight fasting and showed that blood glucose levels were increased in *LOX-1* knockout mice compared to wild-type mice (Mughal, 2015). Given this, I wanted to assess the signalling mechanisms linked to glucose sensing and metabolism linked to the *LOX-1* genotype. The aims of the work presented in this chapter are to elucidate the link between membrane receptors and oxidised LDL and insulin signal transduction pathways in cells and animals.

6.2 Results

6.2.1 Insulin activates LOX-1-mediated Akt and ERK1/2 MAPK signalling

Insulin is a potent anabolic hormone which is important for glucose homeostasis. Insulin-mediated activation of the insulin receptor activates downstream signalling events such as the PI3K and MAPK pathways (Saltiel and Kahn, 2001). In this study, I hypothesised that LOX-1 regulates insulin signalling pathway by affecting the activation of downstream kinases and/or enzymes that control the cell function. To test this idea, I used the previously described HEK293 cells which can display inducible wild-type human LOX-1-FLAG expression compared to cells carrying the mock-transfected empty vector, which acted as a negative control. Cells were serum-starved in insulin-free cell culture media for 2 h, prior to stimulation with insulin for 15 min. Immunoblotting was used to assess the biochemical status of insulin receptor, Akt, ERK1/2 and p38 MAPK proteins (Figure 6.1A). Quantification of these revealed that there was no significant change in the levels of insulin receptor when LOX-1-expressing cells were compared to controls (Figure 6.1B). Stimulation with insulin caused an increased activation of Akt with little or no difference in between control and LOX-1-expressing cells (Figure 6.1C). In contrast, the basal levels of phosphorylated ERK1/2 (Figure 6.1D) and p38 MAPK (Figure 6.1E) were decreased and were not significantly modulated by insulin stimulation. These data suggest a role for LOX-1 in modulating basal MAPK signalling in this model system.

I then tested our hypothesis using PAEC cells with inducible wild-type LOX-1-FLAG expression compared to mock-transfected empty vector. After serum starvation for 2h, cells were stimulated with insulin and subjected to immunoblotting to monitor insulin receptor, Akt, ERK1/2, p38 MAPK and eNOS (Figure 6.2A). Quantification of these data showed that basal phosphorylated eNOS levels were substantially reduced in LOX-1-exressing cells compared to control (Figure 6.2B). After stimulation with insulin, there was an increased trend in phosphorylated eNOS levels in

LOX-1 cells compared to control, although it did not reach statistical significance (Figure 6.2B). Similarly, non-stimulated basal phosphorylated Akt levels in LOX-1 cells were reduced compared to control (Figure 6.2C). The levels of phosphorylated Akt were significantly increased after insulin stimulation in both LOX-1-expressing cells and controls (Figure 6.2C). Phospho-ERK1/2 levels were significantly elevated after insulin stimulation in both LOX-1-expressing cells and control (Figure 6.2D). However, phospho-ERK1/2 levels were noticeably higher in LOX-1-expressing cells compared to control (Figure 6.2D). There were no significant differences in basal phospho-ERK1/2 in the presence of LOX-1 vs control (Figure 6D). Basal or insulin-stimulated phospho-p38 MAPK was not significantly affected by LOX-1 expression (Figure 6.2E).

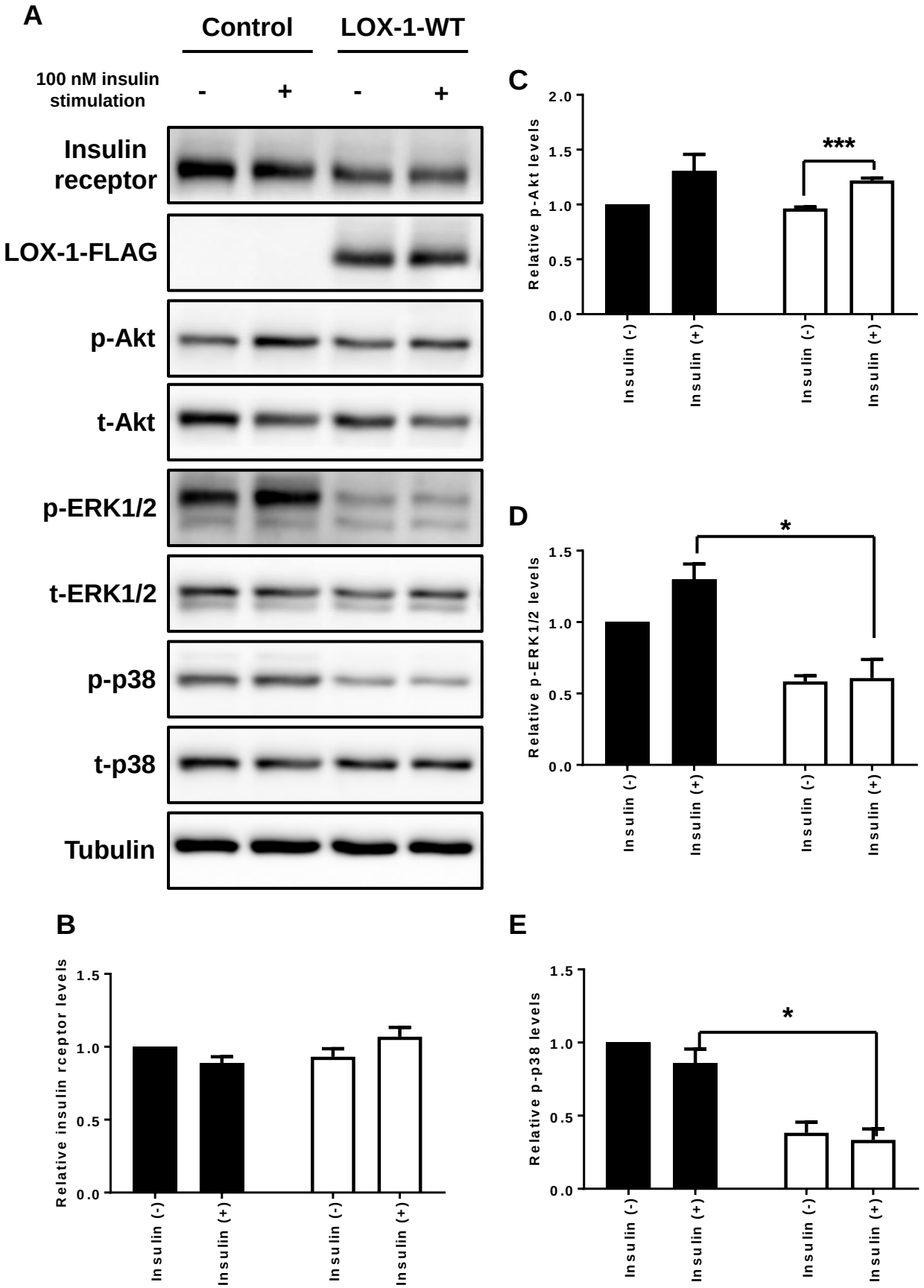

Figure 6.1. LOX-1 modulates insulin-induced activation of signal transduction. (A) HEK293 cells expressing LOX-1-WT and control cells were stimulated with 100 nM insulin for 15 min prior to cell lysis and immunoblot analysis. (B-E) Quantifications of each activated proteins relative to control cells; black bars indicate control; white bars indicate LOX-1-WT. Error bars indicate ±SEM (n≥3). p<0.05 (*), p<0.01 (**), p<0.001 (***), p<0.0001 (****).

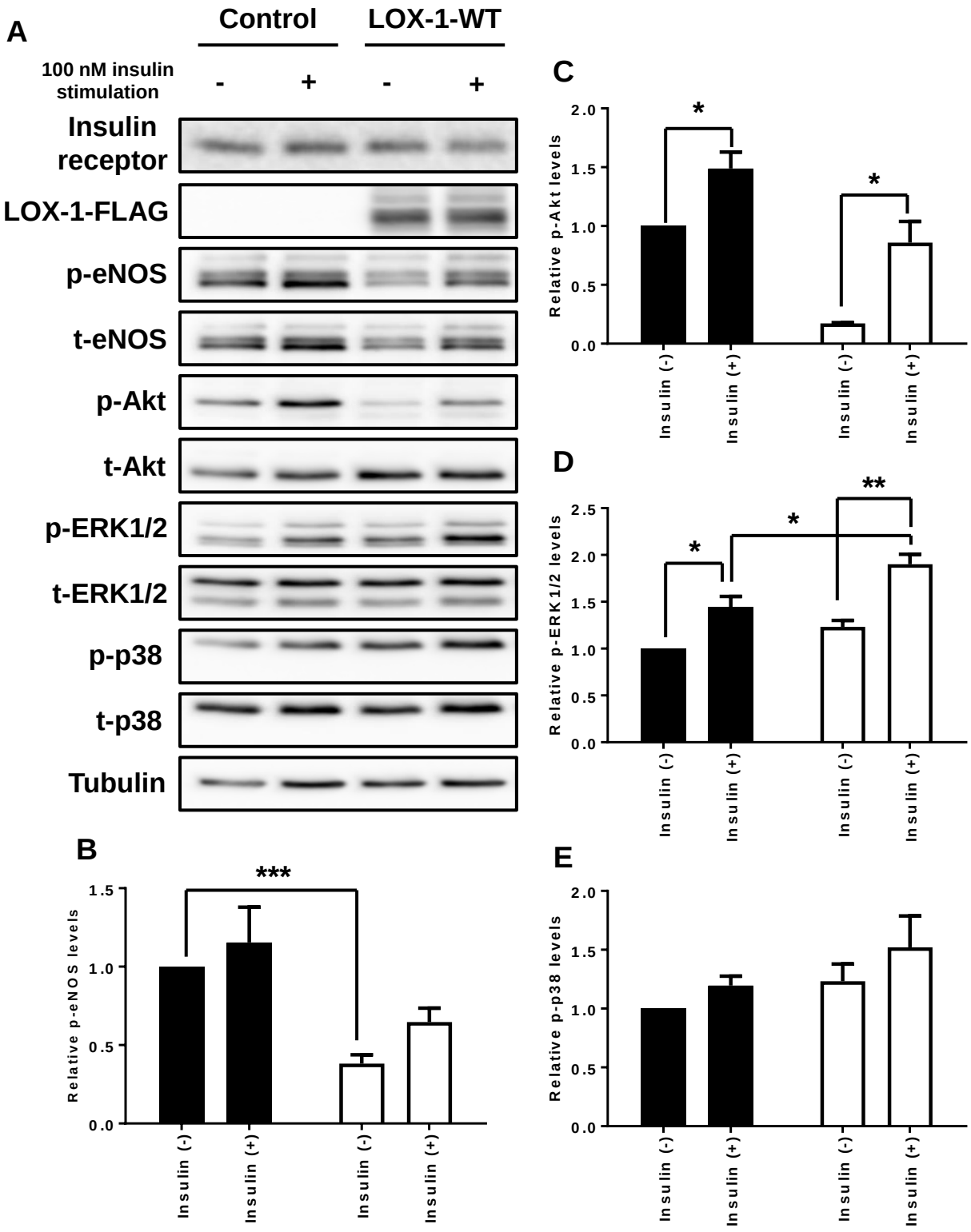

Figure 6.2. LOX-1 modulates insulin-induced activation of signal transduction. (A) PAEC cells expressing LOX-1-WT and control cells were stimulated with 100 nM insulin for 15 min prior to cell lysis and immunoblot analysis. (B-E) Quantifications of each activated proteins relative to control cells; black bars indicate control; white bars indicate LOX-1-WT. Error bars indicate ±SEM (n≥3). $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).

6.2.2 Insulin-stimulated signal transduction is independent of oxidised LDL-stimulated signalling

Oxidative stress is involved in a number of pathological conditions, including diabetes and atherosclerosis. Maziere and colleagues have previously showed the inhibitory effect of oxidised LDL on insulin signalling (Maziere et al., 2004). In this study, I hypothesised that oxidised LDL downregulates insulin signalling pathway. To test this idea, I pre-treated both LOX-1 and control cells with oxidised LDL for 24 h, then stimulated with insulin followed by immunoblotting analysis to monitor the status of insulin receptor, ERK1/2, p38 MAPK, Akt and eNOS proteins (Figure 6.3A). Quantification of these data showed that reduction in basal phospho-eNOS levels in LOX-1-expressing cells was independent of oxidised LDL or insulin stimulation (Figure 6.3B). Treatment of both cells with oxidised LDL showed increased levels of phospho-Akt; even though it was not statistically significant, insulin stimulation caused an increased trend of elevated phospho-Akt in both LOX-1-expressing cells and controls (Figure 6.3C).

Quantification of phospho-ERK 1/2 revealed an increase in both control and LOX-1-expressing cells (Figure 6.3D). However, addition of insulin did not significantly affect the oxidised LDL-stimulated increase in phospho-ERK1/2 levels (Figure 6.3D). Although there were ligand-stimulated increases in phospho-p38 levels in both LOX-1-expressing cells and controls, this was not significant (Figure 6.3E). These data suggest that oxidised LDL and LOX-1 act independently in modulating events linked to insulin signalling.

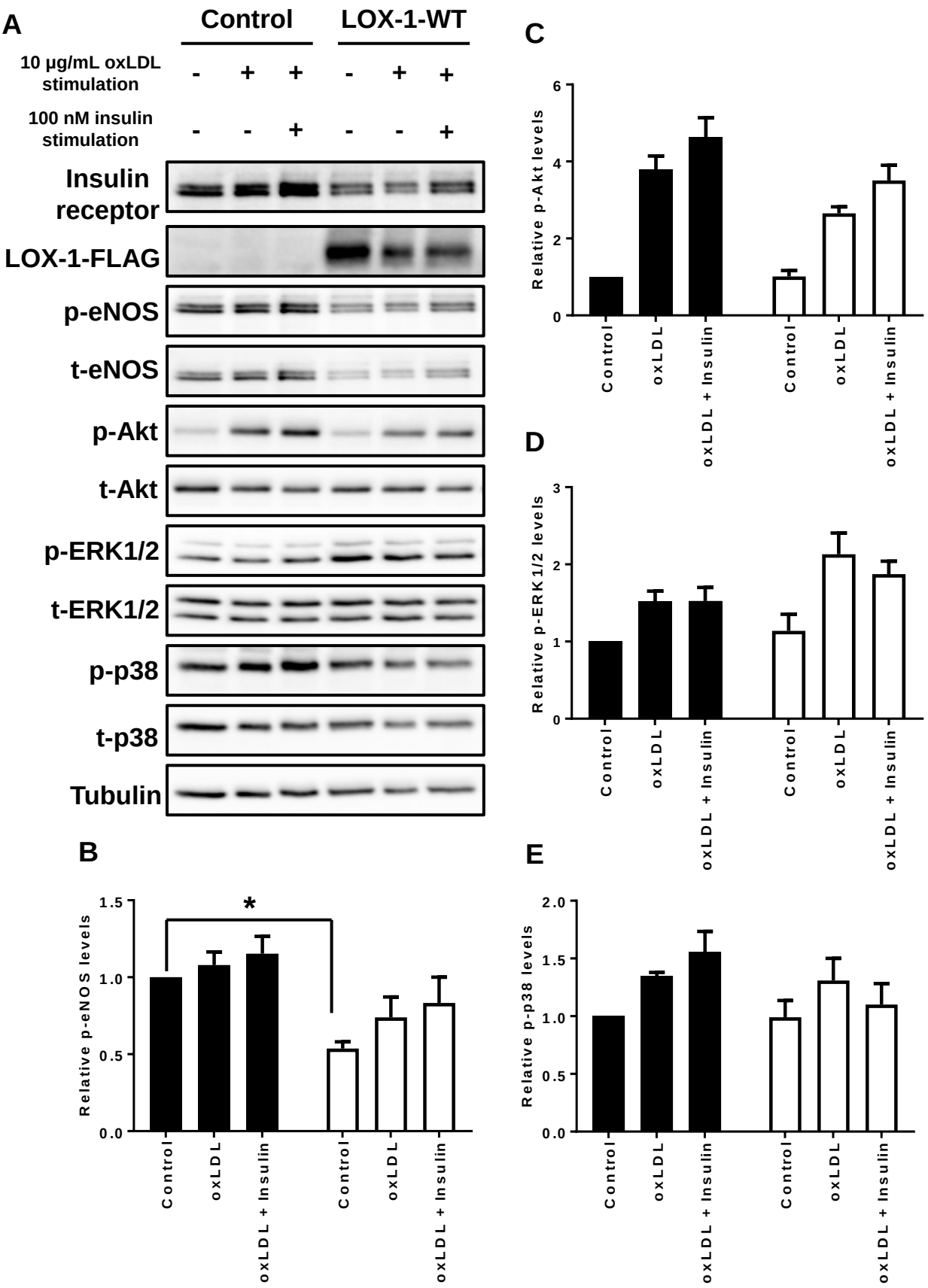

Figure 6.3. Effect of oxidised LDL on insulin-induced signal transduction. (A) PAEC cells expressing LOX-1-WT and control cells were pre-treated with 10 µg/mL oxLDL, followed by stimulation with 100 nM insulin for 15 min prior to cell lysis and immunoblot analysis. (B-E) Quantifications of each activated proteins relative to control cells; black bars indicate control; white bars indicate LOX-1-WT. Error bars indicate ±SEM (n≥3). p<0.05 (*), p<0.01 (**), p<0.001 (***), p<0.0001 (****).

6.2.3 Role of LOX-1 signalling pathways in glucose metabolism

Skeletal muscle is one of the major organs that is targeted by insulin and has an important role in insulin-induced glucose uptake (Bergman, 1989).. Insulin that is secreted by pancreatic β cells binds insulin receptors on cells of the skeletal muscle and activates downstream signal transduction and metabolic pathways (Petersen et al., 2004; Long and Zierath, 2008). It was reported that the rate of insulin-induced glucose uptake is slower in obese patients with type 2 diabetes compared to normal healthy patients (Sjostrand et al., 2002; Barrett et al., 2009). Up to now, there has been no association between LOX-1 signalling pathway(s) and glucose metabolism in skeletal muscle. I hypothesised that *LOX-1* genotype modulates Akt and MAPKs signal transduction pathways. To address this idea, muscle tissues from wild-type and LOX-1 knockout mice fed on Western diet were analysed by immunoblotting to monitor the status of Akt, p38 MAPK and SAPK/JNK protein kinases (Figure 6.4A). Quantification of these data revealed significant ~30-40% increase in phospho-Akt levels in *LOX-1* knockout mice compared to wild-type mice (Figure 6.4B).

Insulin has also been shown to concurrently activate p38 MAPK and SAPK/JNK protein kinases, which are implicated in regulation of glucose uptake and glycogen synthase activities (Antonescu et al., 2005; Moxham et al., 1996). Quantification of immunoblotting data showed significant ~35% reduction in phospho-p38 levels in *LOX-1* knockout mice compared to wild-type mice (Figure 6.4C). Lastly, levels of phospho-SAPK/JNK was significantly ~2-fold higher in *LOX-1* knockout mice in comparison to wild-type mice (Figure 6.4D). The presence of a functional *LOX-1* genotype has complex effects on signalling in skeletal muscle.

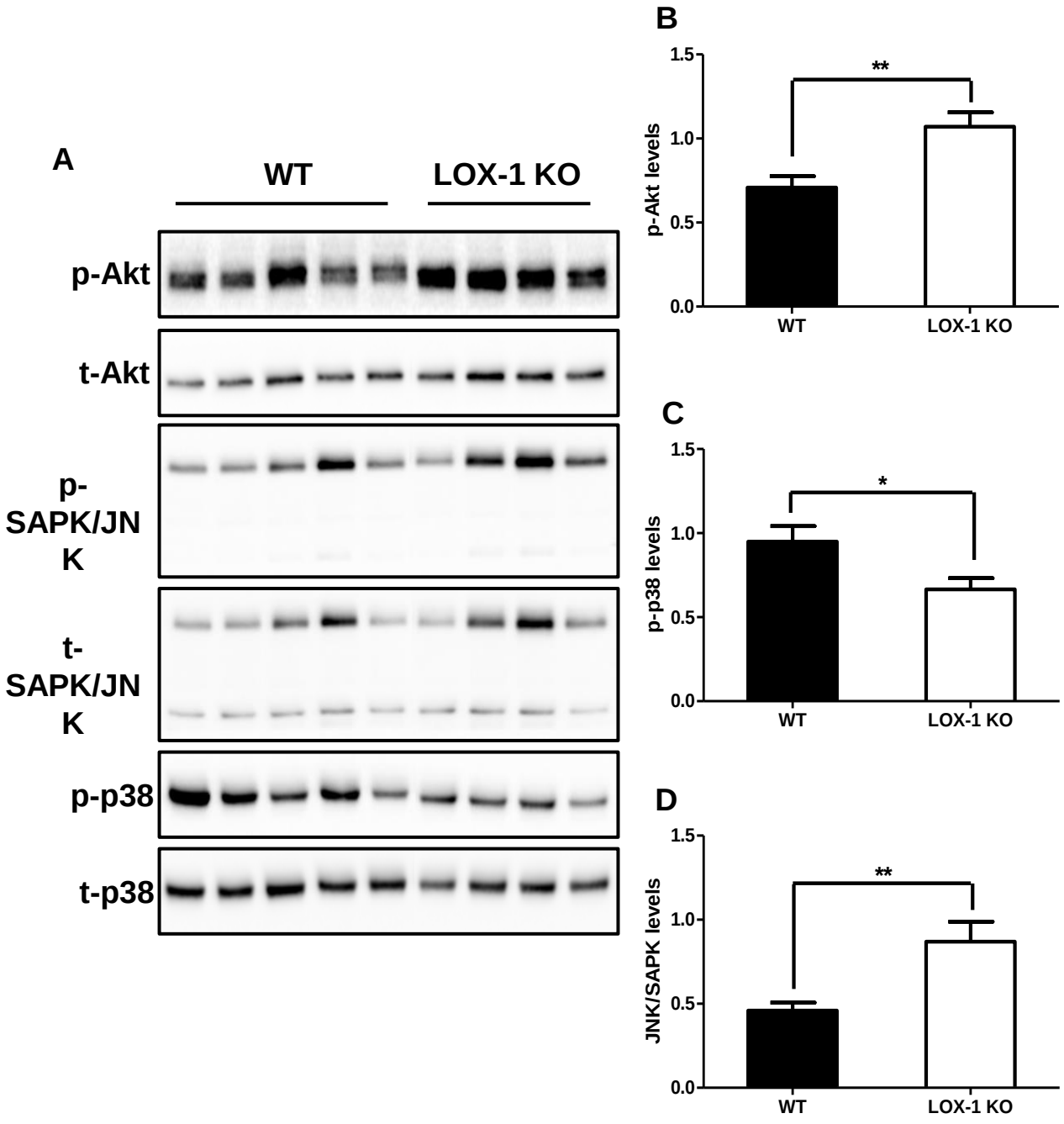

Figure 6.4. LOX-1 signal transduction pathways in skeletal muscle of wild-type and LOX-1 knockout mice. (A) Wild-type (WT) and LOX-1 knockout (KO) fed on a Western diet for 12 weeks were analyzed for signal transduction in skeletal muscle. Tissues were compared for phosphorylation of (B) NF-κB, (C) Akt, (D) p38 and (D) ERK1/2 relative to WT. Error bars indicate ±SEM (n≥8). $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).

6.2.4 Analysis of LOX-1 function in signal transduction in adipose tissues

Atherosclerosis-related hepatic inflammation has been implicated on adipose tissue signal transduction which is important in obesity; this can cause systemic inflammation and altered metabolism of lipids and glucose (Fain et al., 2004). Treating cultured adipocytes with oxidised LDL showed effects on Akt and MAPK signalling pathways which affected glucose uptake (Scazzocchio et al., 2009). Recently, LOX-1 was linked to diet-induced expression of pro-inflammatory cytokines (Takanabe-Mori et al., 2010). Therefore, in this study, I used immunoblotting of epididymal adipose tissues to assess the status of Akt, ERK1/2 and p38 MAPK kinases in wild-type and *LOX-1* knockout mice on a Western diet (Figure 6.5A). Quantification of these data revealed significant >2-fold increase in phospho-p38 levels in *LOX-1* knockout mice compared to wild-type control (Figure 6.5C), but there was no significant change on either phospho-Akt (Figure 6.5C) or phospho-ERK1/2 levels (Figure 6.5D).

Adipose tissue stores fat, and the accumulation of adipose tissue causes predisposition to obesity, which is linked to insulin resistance. Thus, I next evaluated the same signalling pathways in the more advanced atherosclerosis *ApoE* knockout model (see Chapter 5). *LOX-1/ApoE* double knockout mice and *ApoE* knockout mice were fed on Western diet, and epididymal adipose tissues subjected to immunoblotting analysis for Akt, p38 MAPK and ERK1/2 (Figure 6.6A). Quantification showed that the loss of a functional *LOX-1* genotype caused ~2-fold decrease in basal phospho-Akt levels (Figure 6.6B). However, neither phospho-p38 nor phospho-ERK1/2 levels were significantly affected under these conditions in these different transgenic mouse lines (Figure 6.6, C and D). There is thus a clear link between atherosclerosis status as a function of LOX-1 and signalling events in adipose tissues.

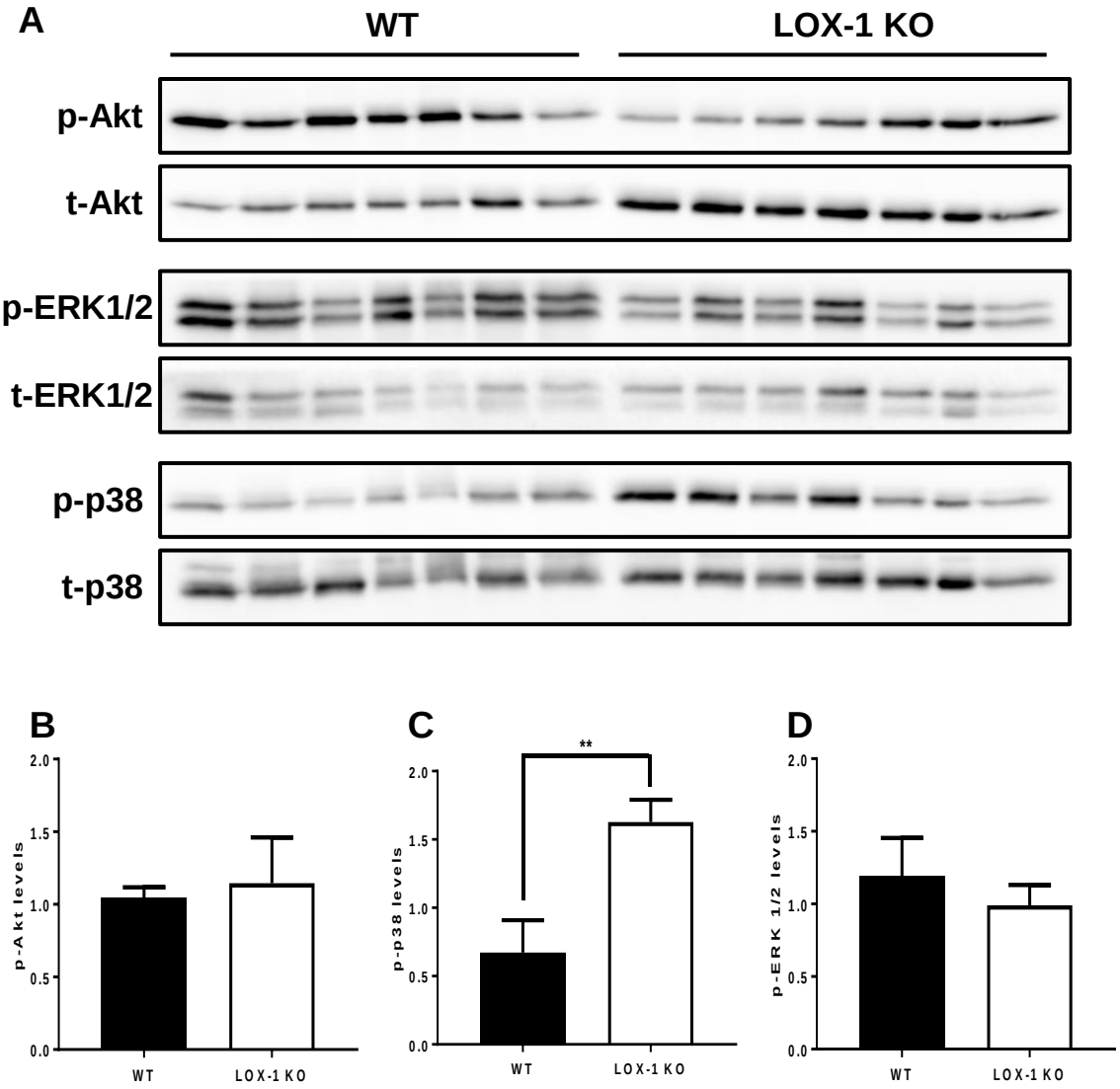

Figure 6.5. LOX-1 signal transduction pathways in epididymal adipose tissue of wild-type and LOX-1 knockout mice. (A) Wild-type (WT) and LOX-1 knockout (KO) fed on a Western diet for 12 weeks were analyzed for signal transduction in adipose tissue. Tissues were compared for phosphorylation of (B) Akt, (C) p38 and (D) ERK1/2 relative to WT. Error bars indicate ±SEM (n≥8). Error bars indicate ±SEM (n≥3). $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).

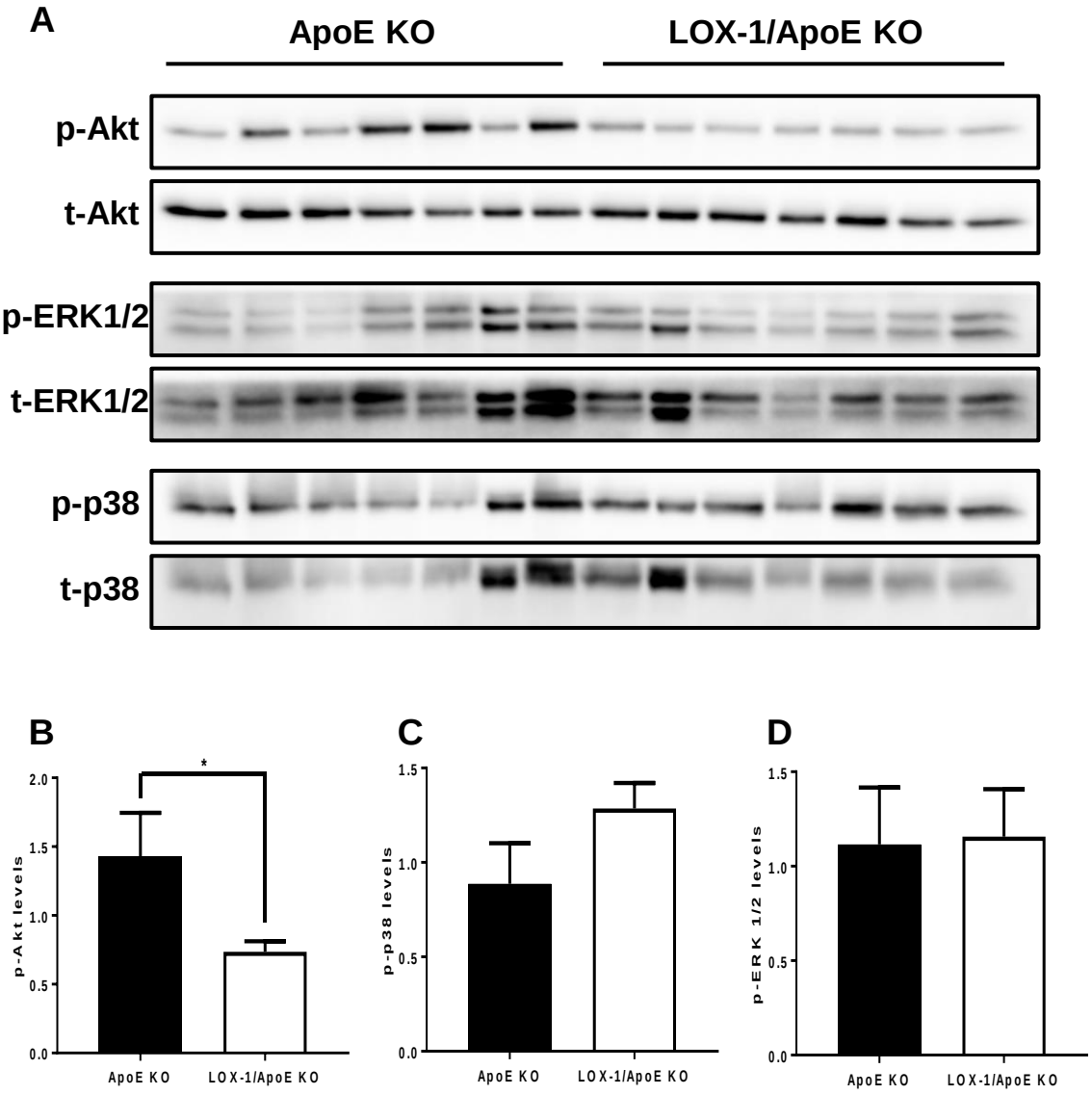

Figure 6.6. LOX-1 signal transduction pathways in epididymal adipose tissue of ApoE knockout and LOX-1/ApoE knockout mice. (A) ApoE knockout (KO) and LOX-1/ApoE KO fed on a Western diet for 12 weeks were analyzed for signal transduction in adipose tissue. Tissues were compared for phosphorylation of (B) Akt, (C) p38 and (D) ERK1/2 relative to ApoE KO. Error bars indicate ±SEM (n≥8). $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).

6.2.5 Role of LOX-1 signalling pathways in iron metabolism

It has been previously reported that hepatic iron-overload and insulin resistance are linked (Ferrannini, 2000; Moirand et al., 1997). Iron regulates the expression of ferritin and the transferrin receptor which are central regulators of iron metabolism in mammals. I hypothesised that the insulin-resistance phenotype of *LOX-1* knockout mice (Supplementary figure B2) might affect regulatory proteins that regulate iron metabolism. To test this idea, I examined liver from wild-type and *LOX-1* knockout mice after Western diet using immunoblotting to monitor transferrin receptor, ferritin and p53 tumour suppressor (Figure 6.7A). Quantification of these data revealed that~3-fold increase in ferritin levels in the *LOX-1* knockout mice compared to wild-type mice (Figure 6.7B). In contrast, there was ~2-fold decrease in transferrin receptor levels in *LOX-1* knockout mice compared to wild-type (Figure 6.4C). I further assessed the p53 tumour suppressor which is an indicator of programmed cell death (apoptosis); quantification revealed ~2-fold increase in p53 levels in the *LOX-1* knockout mice compared to wild-type (Figure 6.7D). These findings alone do not elucidate iron load in the liver, however, such findings support a functional link between the *LOX-1* genotype, fat and lipid metabolism, and require corroboration with more direct assays of iron content.

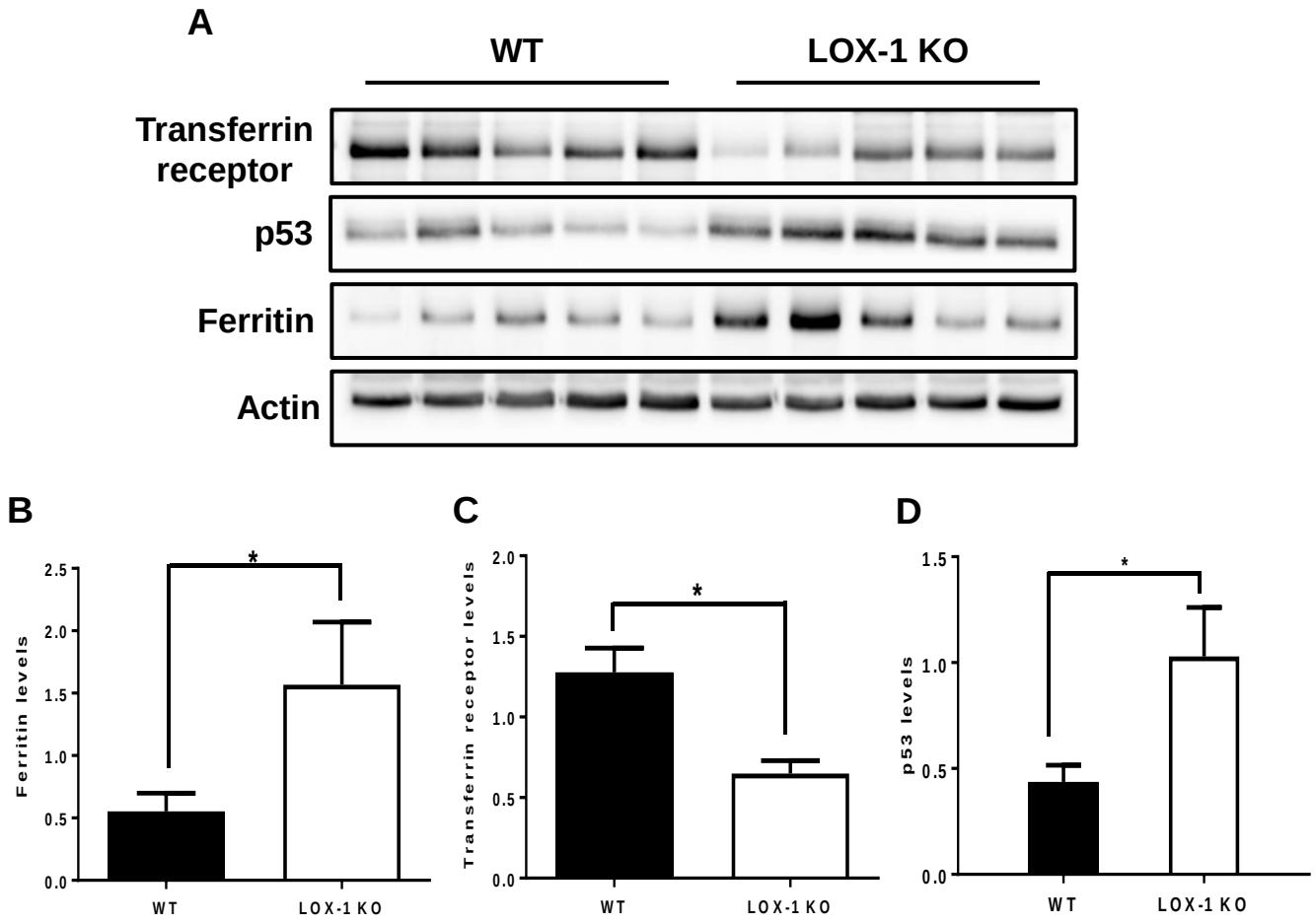

Figure 6.7. LOX-1 regulates iron metabolism in liver. (A) Wild-type (WT) and LOX-1 knockout (KO) fed on a Western diet for 12 weeks were analyzed for ferritin, transferrin receptor and p53 in liver. Tissues were compared for (B) ferritin levels, (C) transferrin receptor levels and (D) p53 levels relative to WT. Error bars indicate ±SEM (n≥8). $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).

6.3 Discussion

In this chapter, I have assessed a functional role for LOX-1 in metabolic syndrome, which is implicated in obesity and insulin resistance, in major insulin-targeted organs, namely skeletal muscle, adipose tissue and liver. The use of inducible expression system has allowed the analysis of LOX-1 modulation of signalling events in non-vascular and vascular endothelial cells. A key feature of this work is that stimulating vascular PAEC cells showed that the presence of LOX-1 caused increased insulin-stimulated ERK1/2 activation. Such signalling has been postulated to increase glycogen synthase activity, thus promoting glucose storage via glycogen synthesis (Dufresne et al., 2001). The master protein kinase Akt showed an increased trend of insulin-stimulated activation in the presence of LOX-1, but this lacked statistical significance. The importance of Akt in insulin signalling is that it is required for GLUT4 translocation to the cell surface, which is a major pathway for glucose uptake in skeletal muscle (Mackenzie and Elliott, 2014).

Furthermore, I assessed the effects of oxidised LDL on insulin-stimulated signalling events. Interestingly, oxidised LDL-mediated activation of LOX-1 did not affect insulin-stimulated signal transduction pathways involving Akt or ERK1/2. One conclusion is that LOX-1 acts in an oxidised LDL-independent manner to modulate insulin receptor activation and signal transduction. Such regulation could occur through a direct interaction between LOX-1 and insulin receptor: thus LOX-1 could be a functional co-receptor for insulin by binding and modulating insulin receptor signalling and activity. Previously, it was reported that the existence of LOX-1 and Ang II receptors promote production of reactive oxygen species and increased activation of MAPKs (Yamamoto et al., 2015; Wang et al., 2011). More recently, it has been shown that LOX-1 directly binds the Ang II type 1 receptor (AT1) via its extracellular domain (Yamamoto et al., 2015). Taken together, LOX-1 could interact with insulin receptor to modulate tyrosine kinase activity and/or signalling to downstream Akt and ERK1/2 pathways.

Previously, the phenotype of both *LOX-1* knockout and wild-type mice on glucose tolerance has been assessed (Mughal, 2015), showing that loss of functional *LOX-1* caused abnormal glucose metabolism with insulin-resistant features (Supplementary figure B2). This suggested that LOX-1 is required for insulin-stimulated glucose metabolism. Insulin regulates glucose metabolism by decreasing glucose output from the liver and increasing uptake of glucose into muscle and adipose tissues (Pessin and Saltiel, 2000). For effective maintenance of glucose levels from the circulation the translocation of glucose transporter to the cell surface in muscle and fat cells is a critical aspect of insulin-stimulated activity. Furthermore, insulin also plays a role in lipid metabolism by increasing lipid synthesis in liver and fat cells, and reducing fatty acid release from triglycerides in adipose tissue. Thus, targeting skeletal muscle and adipose tissues is important for evaluating the role of LOX-1 on glucose and lipid metabolism.

I have shown the increased activation of Akt levels in skeletal muscle when *LOX-1* is deleted. As activation of Akt is well-known to cause the translocation of the GLUT4 glucose transporter to the plasma membrane, the work in this chapter shows that the presence of functional LOX-1 suppresses the activation Akt and JNK/SAPK in skeletal muscle. The suppression of Akt activity in skeletal muscle of wild-type mice could thus inhibit effective glucose uptake. On the other hand, loss of LOX-1 function caused decreased p38 activation: this is important as increased p38 MAPK activity is also implicated in insulin-stimulated glucose uptake (Gehart et al., 2010). Thus in skeletal muscle, LOX-1 could have opposing actions in suppressing Akt but promoting p38 MAPK activation. The exact mechanism of insulin-stimulated regulation of glucose metabolism is conflicting as glucose transporter activity may be independent of p38 MAPK activation (Antonescu et al., 2005; Ribe et al., 2005).

I found that SAPK/JNK activation in skeletal muscle was enhanced when *LOX-1* function was compromised. Activation of SAPK/JNK is implicated in glycogen synthase expression (Moxham et al., 1996). Insulin stimulation also causes glycogen synthase dephosphorylation (Jensen

and Lai, 2009). Taken together, this suggests that a functional *LOX-1* genotype influences the ability of skeletal muscle tissues to correctly respond to insulin. These findings support that previously reported where blood glucose levels in *LOX-1* knockout mice displayed an abnormal response to insulin (Mughal, 2015). One plausible explanation is that LOX-1 expression in skeletal muscle modulates multiple insulin-stimulated signalling events and significantly affects signal transduction pathways leading to GLUT4-mediated glucose uptake. Thus, targeting LOX-1 could be a future priority in new therapies for diabetes.

Adipose or fat tissues regulate lipid homeostasis by storing and utilising energy from triglycerides which is also responsive to insulin. Accumulation of adipose tissues is a key factor in obesity, and is associated with type 2 diabetes and a pre-atherosclerosis state (Despres et al., 2001). Studies on insulin-resistance showed metabolic improvement after removal of fat or adipose tissues (Gabriely and Barzilai, 2003; Gabriely et al., 2002; Thorne et al., 2002). Insulin activates the tyrosine kinase activity of the insulin receptor, which then activates a multiple downstream signalling pathways such as Akt and ERK1/2 master regulators (Cheatham and Kahn, 1995). Deletion of LOX-1 did not have any influence on either Akt or ERK1/2 activation; however, phospho-p38 levels were elevated suggesting increased protein kinase activity. LOX-1 expression in adipose tissues is reported to regulate pro-inflammatory cytokine production (Takanabe-Mori et al., 2010). In this case, such LOX-1-mediated signalling and regulation of p38 MAPK status might impact on pro-inflammatory cytokine expression and/or secretion.

The link between iron and lipid metabolism has always been a controversial subject as the mechanisms are not fully understood. The liver is a major organ for storing iron and maintaining lipid homeostasis (Fargion et al., 2001). Iron association with dyslipidaemia and atherosclerosis is likely to induce oxidative stress and inflammation in the liver. In the work in this chapter indicates a functional link between LOX-1, iron and lipid metabolism linked to atherosclerosis. Iron loading in liver can be monitored by increased ferritin levels and decreased transferrin

receptor levels (Andrews and Schmidt, 2007). I observed high ferritin levels and low transferrin receptor levels displayed by *LOX-1* knockout mice. Studies have suggested that increased iron storage in liver is associated with insulin resistance and dyslipidaemia (Dongiovanni et al., 2011; Gabrielsen et al., 2012; Wlazlo et al., 2013). This further supports our conclusion of the insulin resistance phenotype exhibited by *LOX-1* knockout mice. I further showed that increased p53 levels paralleled the increase in ferritin levels in the *LOX-1* knockout background. Not only does activation of the p53 tumour suppressor cause apoptosis, it also promotes insulin resistance (Derdak et al., 2011).

LOX-1 thus has a major influence on metabolic syndrome that includes insulin resistance, dyslipidaemia and iron homeostasis. The exact mechanisms of lipid and glucose metabolism in skeletal muscle, adipose tissue and liver influenced by *LOX-1* genotype are not fully understood. Nonetheless, I have linked the impact of LOX-1 on insulin resistance and dyslipidaemia by demonstrating the insulin-induced downstream insulin signalling pathways.

CHAPTER 7

General discussion

The studies presented in this thesis provide novel insights into how oxidised LDL-mediated LOX-1 trafficking and downstream signal transduction modulate expression of pro-atherogenic and pro-inflammatory proteins. This chapter will now provide an overview of the subject matter in the context understanding within the cardiovascular field.

7.1 Oxidised LDL-mediated LOX-1 trafficking and downstream signal transduction

In this current study, it has been demonstrated that LOX-1 membrane trafficking is mediated by oxidised LDL (Figure 7.1). Although there was no indication of whether LOX-1 gets recycled or degraded, I show that LOX-1 is present on the cell plasma membrane in the absence of oxidised LDL. In fact, LOX-1 is also being constitutively internalized independent of oxidised LDL, which has a similar trafficking activity to transferrin receptor (Murphy et al., 2008). Furthermore, mutations in the intracellular domain have caused LOX-1 to lose its ability to internalize oxidised LDL (Murphy et al., 2008). It was astounding to find that the mutation has caused a trafficking-defect, in which I have demonstrated that LOX-1-D5A mostly localised in the endosome and not on the cell surface. I also confirmed that the mutation did not have an effect on the mRNA expression, thus it could just be that the mutation causes a slow transport of LOX-1-D5A to the cell membrane, but the mechanism is unknown.

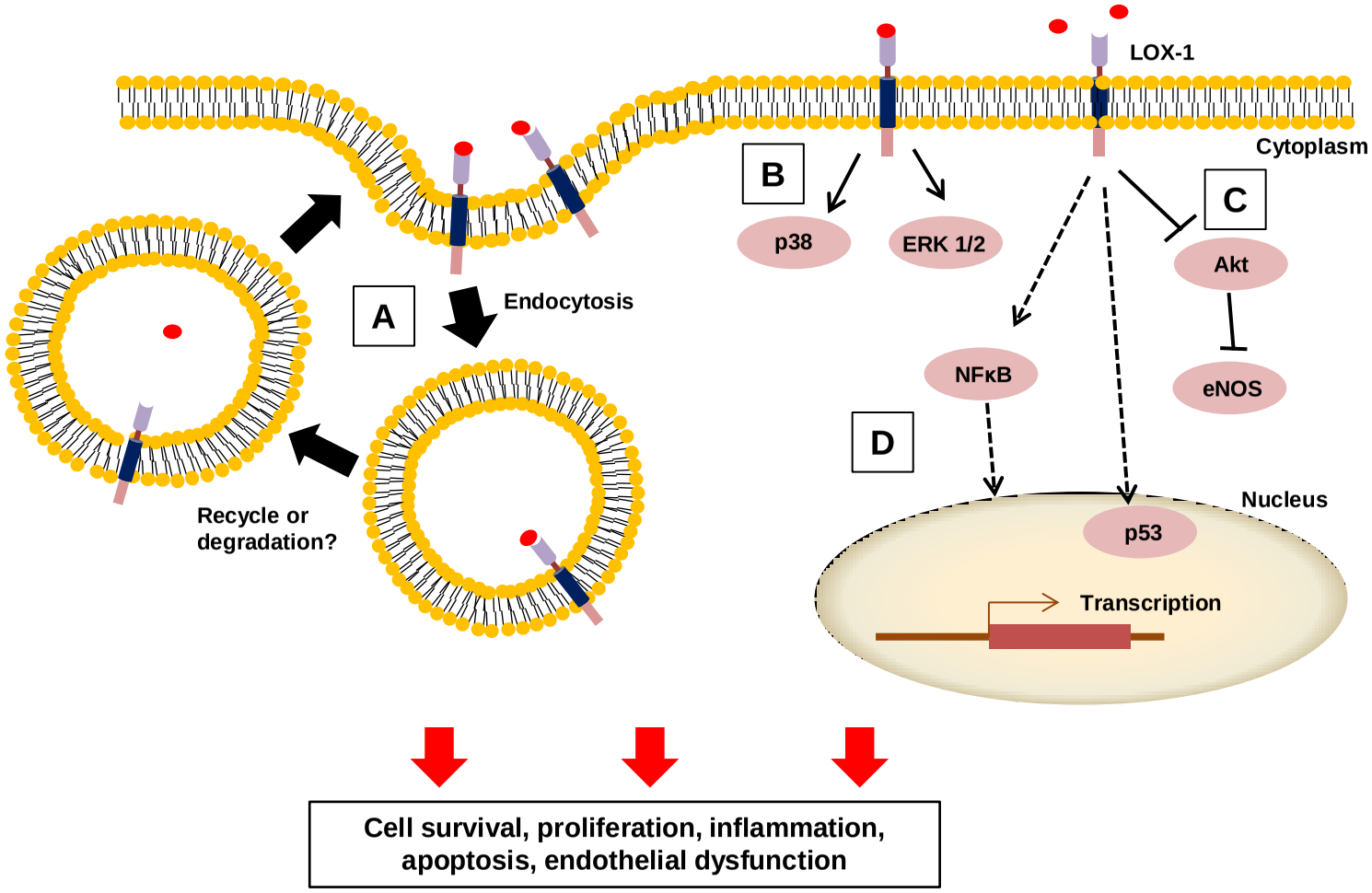

Figure 7.1. LOX-1 trafficking and signalling pathways regulating cell function. (A) Oxidised LDL (OxLDL) stimulate LOX-1 internalization by clathrin-independent pathway. Consequently, LOX-1 is either degraded or recycled, whereas OxLDL could be partially degraded in the cell. **(B)** OxLDL binding to LOX-1 impacts on downstream p38 and ERK1/2 phosphorylation. **(C)** LOX-1 activation supresses the Akt/eNOS activity, in response to the presence of OxLDL. **(D)** LOX-1 activates p53 causing apoptosis; and activates NFκB regulating the transcription of pro-inflammatory target genes. Differential activation of signalling pathways by LOX-1promotes inflammation, apoptosis, and endothelial dysfunction. Dashed arrows denote undefined mechanisms.

In vascular endothelial cells, LOX-1 activation in response to oxidised LDL has been suggested to affect activity of MAPKs, Akt, eNOS and NF-κB signalling pathways (Mehta et al., 2004; Li et al., 2003b; Li et al., 2001; Lu et al., 2009). Oxidised LDL stimulation causes reduction in phosphorylated Akt and eNOS levels, which is in agreement with most studies (Figure 7.1). Binding oxidised LDL by LOX-1 causes an activation of p38 and ERK1/2 MAPKs signalling proteins. One possibility is that the activation of these MAPKs is required to regulate internalization. Additionally, oxidised LDL up-regulates LOX-1 expression through phosphorylation of ERK1/2 (Hsieh et al., 2001). Activation of ERK1/2 has also been implicated on LOX-1-mediated gene expression (Li et al., 2003a). NF-κB, which is activated by pro-inflammatory stimuli, modulates the expression of pro-inflammatory cytokines and adhesion molecules (Xu et al., 2012; Lawrence, 2009). It was previously reported that binding of oxidised LDL to LOX-1 activates NF-κB (Cominacini et al., 2001). However, in this study, oxidised LDL-stimulated LOX-1 activation did not increase the activity levels of NF-κB.

The aim of the study was to understand differential signal transduction event between LOX-1-WT and LOX-1-D5A, in which there was not much difference in response to oxidised LDL. The variations between different studies could be that the cell lines used in the current study may not have similar mechanisms that are present in primary vascular endothelial cell lines. Thus, it may be difficult to interpret the results and compare what signalling pathways are actually activated to modulate plaque formation. Another limitation of this *in vitro* study was the lack of commercial LOX-1 antibodies for immunoblotting and the difficulty of finding antibodies that cross-react with the porcine cell line. Thus, for future experiments, exploring the LOX-1 downstream activation and nuclear localization of NF-κB could reveal the transcription of pro-inflammatory target genes involved.

7.2 LOX-1 signalling pathways regulate plaque formation and glucose metabolism

A seminal study by Mehta and colleagues showed deletion of LOX-1 in ApoE knockout background mice showed reduced plaque formation (Mehta et al., 2007), and more studies confirmed this phenotype (Inoue et al., 2005; Kataoka et al., 1999). Previous study has shown deletion of LOX-1 in ApoE knockout background mice exhibited more plaques forming in the aorta (Mughal, 2015). Based on this data, the aim of the study was to evaluate LOX-1 signal transduction mechanisms in the aorta and the liver. I have shown LOX-1 caused increased pro-atherogenic signalling pathway in advanced atherosclerotic state, suggesting in a stressed condition, LOX-1 may be activating pro-atherogenic signalling pathway. On the contrary, LOX-1 expressed in the aorta pre-lesion state is required to clear oxidised LDL from the circulation. Thus, LOX-1 has a dual role in modulating plaque formation depending on the progress of the disease. To further support these findings, staining of plaques in the aortic sinus and detecting pro-inflammatory cytokines in plasma could explain the activation of these signalling pathways.

Aortic atherosclerosis has also been associated with lipid metabolism in the liver (Simpson and Harms, 1983). Thus, I evaluated pro-atherogenic signalling mechanism in the liver. Deletion of LOX-1 in ApoE knockout background mice increased pro-atherogenic signalling pathway. Similar observations were seen in which oxidised LDL uptake in the liver was increased (Ishigaki et al., 2008). Thus, saying this, development of atherosclerotic lesions might be initiated by altered hepatic lipid uptake. This further shows the different role of LOX-1 in different tissues. Differences in phenotypes observed in most LOX-1 research could be due to the mouse models being used or the diets that are fed to these mice. Furthermore, endothelial cell-specific knocking out of LOX-1 in mice would elucidate about the signalling pathways involved. In addition, measuring lipid contents in liver could further support the findings found.

A seminal study by Chen and colleagues was one of the first studies that linked diabetes and up-regulation of LOX-1 (Chen et al., 2001c). But then again, the mechanisms causing the up-regulation of LOX-1 in diabetes are not fully understood. Previously, it was also noticed that mice with LOX-1 knockdown had insulin resistance, where glucose metabolism was altered (Mughal, 2015). Skeletal muscle is one of the important organs targeted by insulin. In this study, for the first time, I showed that LOX-1 supresses the activation of Akt and SAPK/JNK, which are responsible for glucose transporter translocation and glycogen synthase activity, respectively (Antonescu et al., 2005; Moxham et al., 1996). This suggests that LOX-1 is making skeletal muscle to be insulin-resistant. Furthermore, altered lipid and glucose metabolism in adipose tissue is linked to obesity and insulin resistance (Fain et al., 2004). Although there was not much change in the signalling activation, LOX-1 is required for the activation of Akt, possibly for the translocation of glucose transporter in the cells. This, again, shows LOX-1 has diverse roles depending where the receptor is being expressed. Although insulin signalling was not specifically studied in my work, insulin resistance could be quantified by performing hyperinsulinaemic clamping studies in mice.

7.3 Concluding remarks

Studies on LOX-1 and LOX-1-based therapy have been on-going for over 20 years. Given existing evidence, LOX-1 is a potential target for oxidised LDL-mediated cardiovascular diseases such as atherosclerosis and thrombosis. LOX-1 is a unique membrane receptor that elicits biological functions via diverse mechanisms, and because of this, it may be hazardous to completely block LOX-1 activity. I have shown here hyperlipidaemia and insulin resistance are potential mechanisms by which atherosclerosis is influenced by LOX-1. Further understanding of the many functions of LOX-1 would help establish a novel therapeutic strategy for the treatment of cardiovascular studies.

REFERENCES

ABDUL ZANI, I., STEPHEN, S. L., MUGHAL, N. A., RUSSELL, D., HOMER-VANNIASINKAM, S., WHEATCROFT, S. B. & PONNAMBALAM, S. (2015). Scavenger receptor structure and function in health and disease. *Cells,* 4**,** 178-201.

ACTON, S. L., SCHERER, P. E., LODISH, H. F. & KRIEGER, M. (1994). Expression cloning of SR-BI, a CD36-related class B scavenger receptor. *J Biol Chem,* 269**,** 21003-9.

ADACHI, H. & TSUJIMOTO, M. (2002). FEEL-1, a novel scavenger receptor with in vitro bacteria-binding and angiogenesis-modulating activities. *J Biol Chem,* 277**,** 34264-70.

ADACHI, H. & TSUJIMOTO, M. (2010). Adaptor protein sorting nexin 17 interacts with the scavenger receptor FEEL-1/stabilin-1 and modulates its expression on the cell surface. *Biochim Biophys Acta,* 1803**,** 553-63.

AKHMEDOV, A., ROZENBERG, I., PANENI, F., CAMICI, G. G., SHI, Y., DOERRIES, C., SLEDZINSKA, A., MOCHARLA, P., BREITENSTEIN, A., LOHMANN, C., STEIN, S., VON LUKOWICZ, T., KURRER, M. O., BOREN, J., BECHER, B., TANNER, F. C., LANDMESSER, U., MATTER, C. M. & LUSCHER, T. F. (2014). Endothelial overexpression of LOX-1 increases plaque formation and promotes atherosclerosis in vivo. *Eur Heart J,* 35**,** 2839-48.

ALON, R., FEIZI, T., YUEN, C. T., FUHLBRIGGE, R. C. & SPRINGER, T. A. (1995). Glycolipid ligands for selectins support leukocyte tethering and rolling under physiologic flow conditions. *J Immunol,* 154**,** 5356-66.

ALONSO, R., BUORS, C., LE DANTEC, C., HILLION, S., PERS, J. O., SARAUX, A., MONTERO, E., MARIANOWSKI, R., LOISEL, S., DEVAUCHELLE, V., YOUINOU, P. & RENAUDINEAU, Y. (2010). Aberrant expression of CD6 on B-cell subsets from patients with Sjogren's syndrome. *Journal of autoimmunity,* 35**,** 336-41.

AMIEL, E., NICHOLSON-DYKSTRA, S., WALTERS, J. J., HIGGS, H. & BERWIN, B. (2007). Scavenger receptor-A functions in phagocytosis of E. coli by bone marrow dendritic cells. *Exp Cell Res,* 313**,** 1438-48.

ANDREWS, B. J., PROTEAU, G. A., BEATTY, L. G. & SADOWSKI, P. D. (1985). The FLP recombinase of the 2 micron circle DNA of yeast: interaction with its target sequences. *Cell,* 40**,** 795-803.

ANDREWS, N. C. & SCHMIDT, P. J. (2007). Iron homeostasis. *Annu Rev Physiol,* 69**,** 69-85.

ANTONESCU, C. N., HUANG, C., NIU, W., LIU, Z., EYERS, P. A., HEIDENREICH, K. A., BILAN, P. J. & KLIP, A. (2005). Reduction of insulin-stimulated glucose uptake in L6 myotubes by the protein kinase inhibitor SB203580 is independent of p38MAPK activity. *Endocrinology,* 146**,** 3773-81.

AOYAMA, T., FUJIWARA, H., MASAKI, T. & SAWAMURA, T. (1999). Induction of lectin-like oxidized LDL receptor by oxidized LDL and lysophosphatidylcholine in cultured endothelial cells. *J Mol Cell Cardiol,* 31**,** 2101-14.

BADIMON, J. J., FUSTER, V., CHESEBRO, J. H. & BADIMON, L. (1993). Coronary atherosclerosis. A multifactorial disease. *Circulation,* 87**,** Ii3-16.

BAIRD, A., FLORKIEWICZ, R. Z., MAHER, P. A., KANER, R. J. & HAJJAR, D. P. (1990). Mediation of virion penetration into vascular cells by association of basic fibroblast growth factor with herpes simplex virus type 1. *Nature,* 348**,** 344-6.

BAKTHAVATSALAM, D., SOUNG, R. H., TWEARDY, D. J., CHIU, W., DIXON, R. A. & WOODSIDE, D. G. (2014). Chaperonin-containing TCP-1 complex directly binds to the cytoplasmic domain of the LOX-1 receptor. *FEBS Lett,* 588**,** 2133-40.

BANCELLS, C., BENITEZ, S., JAUHIAINEN, M., ORDONEZ-LLANOS, J., KOVANEN, P. T., VILLEGAS, S., SANCHEZ-QUESADA, J. L. & OORNI, K. (2009). High binding affinity of electronegative LDL to human aortic proteoglycans depends on its aggregation level. *J Lipid Res,* 50**,** 446-55.

BARRETT, E. J., EGGLESTON, E. M., INYARD, A. C., WANG, H., LI, G., CHAI, W. & LIU, Z. (2009). The vascular actions of insulin control its delivery to muscle and regulate the rate-limiting step in skeletal muscle insulin action. *Diabetologia,* 52**,** 752-64.

BASU, S. K., GOLDSTEIN, J. L., ANDERSON, G. W. & BROWN, M. S. (1976). Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. *Proc Natl Acad Sci U S A,* 73**,** 3178-82.

BECKMAN, J. A., CREAGER, M. A. & LIBBY, P. (2002). Diabetes and atherosclerosis: epidemiology, pathophysiology, and management. *JAMA,* 287**,** 2570-81.

BENITEZ, S., VILLEGAS, V., BANCELLS, C., JORBA, O., GONZALEZ-SASTRE, F., ORDONEZ-LLANOS, J. & SANCHEZ-QUESADA, J. L. (2004). Impaired binding affinity of electronegative low-density lipoprotein (LDL) to the LDL receptor is related to nonesterified fatty

acids and lysophosphatidylcholine content. *Biochemistry,* 43**,** 15863-72.

BERGMAN, R. N. (1989). Lilly lecture 1989. Toward physiological understanding of glucose tolerance. Minimal-model approach. *Diabetes,* 38**,** 1512-27.

BERLINER, J. A., NAVAB, M., FOGELMAN, A. M., FRANK, J. S., DEMER, L. L., EDWARDS, P. A., WATSON, A. D. & LUSIS, A. J. (1995). Atherosclerosis: basic mechanisms. Oxidation, inflammation, and genetics. *Circulation,* 91**,** 2488-96.

BERLINER, J. A., TERRITO, M. C., SEVANIAN, A., RAMIN, S., KIM, J. A., BAMSHAD, B., ESTERSON, M. & FOGELMAN, A. M. (1990). Minimally modified low density lipoprotein stimulates monocyte endothelial interactions. *J Clin Invest,* 85**,** 1260-6.

BICKEL, P. E. & FREEMAN, M. W. (1992). Rabbit aortic smooth muscle cells express inducible macrophage scavenger receptor messenger RNA that is absent from endothelial cells. *J Clin Invest,* 90**,** 1450-7.

BIOCCA, S., FALCONI, M., FILESI, I., BALDINI, F., VECCHIONE, L., MANGO, R., ROMEO, F., FEDERICI, G., DESIDERI, A. & NOVELLI, G. (2009). Functional analysis and molecular dynamics simulation of LOX-1 K167N polymorphism reveal alteration of receptor activity. *PLoS One,* 4**,** e4648.

BLANCO, F. J., VILLEGAS, S., BENITEZ, S., BANCELLS, C., DIERCKS, T., ORDONEZ-LLANOS, J. & SANCHEZ-QUESADA, J. L. (2010). 2D-NMR reveals different populations of exposed lysine residues in the apoB-100 protein of electronegative and electropositive fractions of LDL particles. *J Lipid Res,* 51**,** 1560-5.

BOREN, J., LEE, I., ZHU, W., ARNOLD, K., TAYLOR, S. & INNERARITY, T. L. (1998). Identification of the low density lipoprotein receptor-binding site in apolipoprotein B100 and the modulation of its binding activity by the carboxyl terminus in familial defective apo-B100. *J Clin Invest,* 101**,** 1084-93.

BORING, L., GOSLING, J., CLEARY, M. & CHARO, I. F. (1998). Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature,* 394**,** 894-7.

BRANEN, L., HOVGAARD, L., NITULESCU, M., BENGTSSON, E., NILSSON, J. & JOVINGE, S. (2004). Inhibition of tumor necrosis factor-alpha reduces atherosclerosis in apolipoprotein E knockout mice. *Arterioscler Thromb Vasc Biol,* 24**,** 2137-42.

BRAUNWALD, E. (1997). Shattuck lecture--cardiovascular medicine at the turn of the millennium: triumphs, concerns, and opportunities. *N Engl J Med,* 337**,** 1360-9.

BROWN, M. S. & GOLDSTEIN, J. L. (1986). A receptor-mediated pathway for cholesterol homeostasis. *Science,* 232**,** 34-47.

BROWN, M. S., HO, Y. K. & GOLDSTEIN, J. L. (1976). The low-density lipoprotein pathway in human fibroblasts: relation between cell surface receptor binding and endocytosis of low-density lipoprotein. *Ann N Y Acad Sci,* 275**,** 244-57.

BROWNLEE, M. (2005). The pathobiology of diabetic complications: a unifying mechanism. *Diabetes,* 54**,** 1615-25.

BURDO, T. H., LENTZ, M. R., AUTISSIER, P., KRISHNAN, A., HALPERN, E., LETENDRE, S., ROSENBERG, E. S., ELLIS, R. J. & WILLIAMS, K. C. (2011). Soluble CD163 made by monocyte/macrophages is a novel marker of HIV activity in early and chronic infection prior to and after anti-retroviral therapy. *The Journal of infectious diseases,* 204**,** 154-63.

CALLOW, A. D. (2006). Cardiovascular disease 2005--the global picture. *Vascul Pharmacol,* 45**,** 302-7.

CAMPBELL, L. A., PUOLAKKAINEN, M., LEE, A., ROSENFELD, M. E., GARRIGUES, H. J. & KUO, C. C. (2012). Chlamydia pneumoniae binds to the lectin-like oxidized LDL receptor for infection of endothelial cells. *Microbes and infection / Institut Pasteur,* 14**,** 43-9.

CAMPOS, H., ARNOLD, K. S., BALESTRA, M. E., INNERARITY, T. L. & KRAUSS, R. M. (1996). Differences in receptor binding of LDL subfractions. *Arterioscler Thromb Vasc Biol,* 16**,** 794-801.

CARMENA, R., DURIEZ, P. & FRUCHART, J. C. (2004). Atherogenic lipoprotein particles in atherosclerosis. *Circulation,* 109**,** Iii2-7.

CHABRASHVILI, T., TOJO, A., ONOZATO, M. L., KITIYAKARA, C., QUINN, M. T., FUJITA, T., WELCH, W. J. & WILCOX, C. S. (2002). Expression and cellular localization of classic NADPH oxidase subunits in the spontaneously hypertensive rat kidney. *Hypertension,* 39**,** 269-74.

CHANG, Y. H., ABDALLA, D. S. & SEVANIAN, A. (1997). Characterization of cholesterol oxidation products formed by oxidative modification of low density lipoprotein. *Free Radic Biol Med,* 23**,** 202-14.

CHAPMAN, M. J., GOLDSTEIN, S., LAGRANGE, D. & LAPLAUD, P. M. (1981). A density gradient ultracentrifugal procedure for the isolation of the major lipoprotein classes from human serum. *J Lipid Res,* 22**,** 339-58.

CHEATHAM, B. & KAHN, C. R. (1995). Insulin action and the insulin signaling network. *Endocr Rev,* 16**,** 117-42.

CHEN, C. H., JIANG, T., YANG, J. H., JIANG, W., LU, J., MARATHE, G. K., POWNALL, H. J., BALLANTYNE, C. M., MCINTYRE, T. M., HENRY, P. D. & YANG, C. Y. (2003). Low-density lipoprotein in hypercholesterolemic human plasma induces vascular endothelial cell apoptosis by inhibiting fibroblast growth factor 2 transcription. *Circulation,* 107**,** 2102-8.

CHEN, M., INOUE, K., NARUMIYA, S., MASAKI, T. & SAWAMURA, T. (2001a). Requirements of basic amino acid residues within the lectin-like domain of LOX-1 for the binding of oxidized low-density lipoprotein. *FEBS Lett,* 499**,** 215-9.

CHEN, M., KAKUTANI, M., NARUKO, T., UEDA, M., NARUMIYA, S., MASAKI, T. & SAWAMURA, T. (2001b). Activation-dependent surface expression of LOX-1 in human platelets. *Biochem Biophys Res Commun,* 282**,** 153-8.

CHEN, M., NAGASE, M., FUJITA, T., NARUMIYA, S., MASAKI, T. & SAWAMURA, T. (2001c). Diabetes enhances lectin-like oxidized LDL receptor-1 (LOX-1) expression in the vascular endothelium: possible role of LOX-1 ligand and AGE. *Biochem Biophys Res Commun,* 287**,** 962-8.

CHEN, S. H., YANG, C. Y., CHEN, P. F., SETZER, D., TANIMURA, M., LI, W. H., GOTTO, A. M., JR. & CHAN, L. (1986). The complete cDNA and amino acid sequence of human apolipoprotein B-100. *J Biol Chem,* 261**,** 12918-21.

CHEN, Y., WANG, X., BEN, J., YUE, S., BAI, H., GUAN, X., BAI, X., JIANG, L., JI, Y., FAN, L. & CHEN, Q. (2006). The di-leucine motif contributes to class a scavenger receptor-mediated internalization of acetylated lipoproteins. *Arterioscler Thromb Vasc Biol,* 26**,** 1317-22.

CHIEN, M. W., CHIEN, C. S., HSIAO, L. D., LIN, C. H. & YANG, C. M. (2003). OxLDL induces mitogen-activated protein kinase activation mediated via PI3-kinase/Akt in vascular smooth muscle cells. *J Lipid Res,* 44**,** 1667-75.

CHUI, P. C., GUAN, H. P., LEHRKE, M. & LAZAR, M. A. (2005). PPARgamma regulates adipocyte cholesterol metabolism via oxidized LDL receptor 1. *J Clin Invest,* 115**,** 2244-56.

CLARKE, M. C., FIGG, N., MAGUIRE, J. J., DAVENPORT, A. P., GODDARD, M., LITTLEWOOD, T. D. & BENNETT, M. R. (2006). Apoptosis of vascular smooth muscle cells induces features of plaque vulnerability in atherosclerosis. *Nat Med,* 12**,** 1075-80.

COMINACINI, L., PASINI, A. F., GARBIN, U., DAVOLI, A., TOSETTI, M. L., CAMPAGNOLA, M., RIGONI, A., PASTORINO, A. M., LO CASCIO, V. & SAWAMURA, T. (2000). Oxidized low density

lipoprotein (ox-LDL) binding to ox-LDL receptor-1 in endothelial cells induces the activation of NF-kappaB through an increased production of intracellular reactive oxygen species. *J Biol Chem,* 275**,** 12633-8.

COMINACINI, L., RIGONI, A., PASINI, A. F., GARBIN, U., DAVOLI, A., CAMPAGNOLA, M., PASTORINO, A. M., LO CASCIO, V. & SAWAMURA, T. (2001). The binding of oxidized low density lipoprotein (ox-LDL) to ox-LDL receptor-1 reduces the intracellular concentration of nitric oxide in endothelial cells through an increased production of superoxide. *J Biol Chem,* 276**,** 13750-5.

COX, R. A. & GARCIA-PALMIERI, M. R. 1990. Cholesterol, Triglycerides, and Associated Lipoproteins. *In:* WALKER, H. K., HALL, W. D. & HURST, J. W. (eds.) *Clinical Methods: The History, Physical, and Laboratory Examinations.* Boston: Butterworth Publishers, a division of Reed Publishing.

CYRUS, T., WITZTUM, J. L., RADER, D. J., TANGIRALA, R., FAZIO, S., LINTON, M. F. & FUNK, C. D. (1999). Disruption of the 12/15-lipoxygenase gene diminishes atherosclerosis in apo E-deficient mice. *J Clin Invest,* 103**,** 1597-604.

DA SILVA, R. P. & GORDON, S. (1999). Phagocytosis stimulates alternative glycosylation of macrosialin (mouse CD68), a macrophage-specific endosomal protein. *Biochem J,* 338 (Pt 3)**,** 687-94.

DE BEER, M. C., ZHAO, Z., WEBB, N. R., VAN DER WESTHUYZEN, D. R. & DE VILLIERS, W. J. (2003). Lack of a direct role for macrosialin in oxidized LDL metabolism. *J Lipid Res,* 44**,** 674-85.

DE CATERINA, R., LIBBY, P., PENG, H. B., THANNICKAL, V. J., RAJAVASHISTH, T. B., GIMBRONE, M. A., JR., SHIN, W. S. & LIAO, J. K. (1995). Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *J Clin Invest,* 96**,** 60-8.

DERDAK, Z., LANG, C. H., VILLEGAS, K. A., TONG, M., MARK, N. M., DE LA MONTE, S. M. & WANDS, J. R. (2011). Activation of p53 enhances apoptosis and insulin resistance in a rat model of alcoholic liver disease. *J Hepatol,* 54**,** 164-72.

DESPRES, J. P., LEMIEUX, I. & PRUD'HOMME, D. (2001). Treatment of obesity: need to focus on high risk abdominally obese patients. *BMJ,* 322**,** 716-20.

DHALIWAL, B. S. & STEINBRECHER, U. P. (1999). Scavenger receptors and oxidized low density lipoproteins. *Clin Chim Acta,* 286**,** 191-205.

DIMMELER, S., FLEMING, I., FISSLTHALER, B., HERMANN, C., BUSSE, R. & ZEIHER, A. M. (1999). Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature,* 399**,** 601-5.

DOMINICZAK, M. H. & CASLAKE, M. J. (2011). Apolipoproteins: metabolic role and clinical biochemistry applications. *Ann Clin Biochem,* 48**,** 498-515.

DONG, Z. M., CHAPMAN, S. M., BROWN, A. A., FRENETTE, P. S., HYNES, R. O. & WAGNER, D. D. (1998). The combined role of P- and E-selectins in atherosclerosis. *J Clin Invest,* 102**,** 145-52.

DONGIOVANNI, P., FRACANZANI, A. L., FARGION, S. & VALENTI, L. (2011). Iron in fatty liver and in the metabolic syndrome: a promising therapeutic target. *J Hepatol,* 55**,** 920-32.

DRAUDE, G., HRBOTICKY, N. & LORENZ, R. L. (1999). The expression of the lectin-like oxidized low-density lipoprotein receptor (LOX-1) on human vascular smooth muscle cells and monocytes and its down-regulation by lovastatin. *Biochem Pharmacol,* 57**,** 383-6.

DRAUDE, G. & LORENZ, R. L. (2000). TGF-beta1 downregulates CD36 and scavenger receptor A but upregulates LOX-1 in human macrophages. *Am J Physiol Heart Circ Physiol,* 278**,** H1042-8.

DUDEK, H., DATTA, S. R., FRANKE, T. F., BIRNBAUM, M. J., YAO, R., COOPER, G. M., SEGAL, R. A., KAPLAN, D. R. & GREENBERG, M. E. (1997). Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science,* 275**,** 661-5.

DUFRESNE, S. D., BJORBAEK, C., EL-HASCHIMI, K., ZHAO, Y., ASCHENBACH, W. G., MOLLER, D. E. & GOODYEAR, L. J. (2001). Altered extracellular signal-regulated kinase signaling and glycogen metabolism in skeletal muscle from p90 ribosomal S6 kinase 2 knockout mice. *Mol Cell Biol,* 21**,** 81-7.

DUNN, S., VOHRA, R. S., MURPHY, J. E., HOMER-VANNIASINKAM, S., WALKER, J. H. & PONNAMBALAM, S. (2008). The lectin-like oxidized low-density-lipoprotein receptor: a pro-inflammatory factor in vascular disease. *Biochem J,* 409**,** 349-55.

ECKHARDT, E. R., CAI, L., SHETTY, S., ZHAO, Z., SZANTO, A., WEBB, N. R. & VAN DER WESTHUYZEN, D. R. (2006). High density lipoprotein endocytosis by scavenger receptor SR-BII is clathrin-dependent and requires a carboxyl-terminal dileucine motif. *J Biol Chem,* 281**,** 4348-53.

ECKHARDT, E. R., CAI, L., SUN, B., WEBB, N. R. & VAN DER WESTHUYZEN, D. R. (2004). High density lipoprotein uptake by scavenger receptor SR-BII. *J Biol Chem,* 279**,** 14372-81.

EHARA, S., UEDA, M., NARUKO, T., HAZE, K., ITOH, A., OTSUKA, M., KOMATSU, R., MATSUO, T., ITABE, H., TAKANO, T., TSUKAMOTO, Y., YOSHIYAMA, M., TAKEUCHI, K., YOSHIKAWA, J. & BECKER, A. E. (2001). Elevated levels of oxidized low density lipoprotein show a positive relationship with the severity of acute coronary syndromes. *Circulation,* 103**,** 1955-60.

ESSLER, M., RETZER, M., BAUER, M., HEEMSKERK, J. W., AEPFELBACHER, M. & SIESS, W. (1999). Mildly oxidized low density lipoprotein induces contraction of human endothelial cells through activation of Rho/Rho kinase and inhibition of myosin light chain phosphatase. *J Biol Chem,* 274**,** 30361-4.

ESTERBAUER, H., GEBICKI, J., PUHL, H. & JURGENS, G. (1992). The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Radic Biol Med,* 13**,** 341-90.

ETO, H., MIYATA, M., KUME, N., MINAMI, M., ITABE, H., ORIHARA, K., HAMASAKI, S., BIRO, S., OTSUJI, Y., KITA, T. & TEI, C. (2006). Expression of lectin-like oxidized LDL receptor-1 in smooth muscle cells after vascular injury. *Biochem Biophys Res Commun,* 341**,** 591-8.

ETZERODT, A. & MOESTRUP, S. K. (2013). CD163 and inflammation: biological, diagnostic, and therapeutic aspects. *Antioxidants & redox signaling,* 18**,** 2352-63.

EZEKOWITZ, B., ALAN, R. & HOFFMAN, A. J. (2003). *Innate Immunity,* New Jersey, Humana.

FAIN, J. N., MADAN, A. K., HILER, M. L., CHEEMA, P. & BAHOUTH, S. W. (2004). Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. *Endocrinology,* 145**,** 2273-82.

FARGION, S., MATTIOLI, M., FRACANZANI, A. L., SAMPIETRO, M., TAVAZZI, D., FOCIANI, P., TAIOLI, E., VALENTI, L. & FIORELLI, G. (2001). Hyperferritinemia, iron overload, and multiple metabolic alterations identify patients at risk for nonalcoholic steatohepatitis. *Am J Gastroenterol,* 96**,** 2448-55.

FAXON, D. P., FUSTER, V., LIBBY, P., BECKMAN, J. A., HIATT, W. R., THOMPSON, R. W., TOPPER, J. N., ANNEX, B. H., RUNDBACK, J. H., FABUNMI, R. P., ROBERTSON, R. M. & LOSCALZO, J. (2004). Atherosclerotic Vascular Disease Conference: Writing Group III: pathophysiology. *Circulation,* 109**,** 2617-25.

FEBBRAIO, M., PODREZ, E. A., SMITH, J. D., HAJJAR, D. P., HAZEN, S. L., HOFF, H. F., SHARMA, K. & SILVERSTEIN, R. L. (2000).

Targeted disruption of the class B scavenger receptor CD36 protects against atherosclerotic lesion development in mice. *J Clin Invest,* 105**,** 1049-56.

FERRANNINI, E. (2000). Insulin resistance, iron, and the liver. *Lancet,* 355**,** 2181-2.

FONG, L. G., PARTHASARATHY, S., WITZTUM, J. L. & STEINBERG, D. (1987). Nonenzymatic oxidative cleavage of peptide bonds in apoprotein B-100. *J Lipid Res,* 28**,** 1466-77.

FRANGOS, S. G., GAHTAN, V. & SUMPIO, B. (1999). Localization of atherosclerosis: role of hemodynamics. *Arch Surg,* 134**,** 1142-9.

GABRIELSEN, J. S., GAO, Y., SIMCOX, J. A., HUANG, J., THORUP, D., JONES, D., COOKSEY, R. C., GABRIELSEN, D., ADAMS, T. D., HUNT, S. C., HOPKINS, P. N., CEFALU, W. T. & MCCLAIN, D. A. (2012). Adipocyte iron regulates adiponectin and insulin sensitivity. *J Clin Invest,* 122**,** 3529-40.

GABRIELY, I. & BARZILAI, N. (2003). Surgical removal of visceral adipose tissue: effects on insulin action. *Curr Diab Rep,* 3**,** 201-6.

GABRIELY, I., MA, X. H., YANG, X. M., ATZMON, G., RAJALA, M. W., BERG, A. H., SCHERER, P., ROSSETTI, L. & BARZILAI, N. (2002). Removal of visceral fat prevents insulin resistance and glucose intolerance of aging: an adipokine-mediated process? *Diabetes,* 51**,** 2951-8.

GEHART, H., KUMPF, S., ITTNER, A. & RICCI, R. (2010). MAPK signalling in cellular metabolism: stress or wellness? *EMBO Rep,* 11**,** 834-40.

GERHARD, G. T. & DUELL, P. B. (1999). Homocysteine and atherosclerosis. *Curr Opin Lipidol,* 10**,** 417-28.

GIMBRONE, M. A., JR. (1999). Vascular endothelium, hemodynamic forces, and atherogenesis. *Am J Pathol,* 155**,** 1-5.

GLASS, C. K. & WITZTUM, J. L. (2001). Atherosclerosis. the road ahead. *Cell,* 104**,** 503-16.

GOLDSTEIN, J. L., HO, Y. K., BASU, S. K. & BROWN, M. S. (1979). Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc Natl Acad Sci U S A,* 76**,** 333-7.

GOWEN, B. B., BORG, T. K., GHAFFAR, A. & MAYER, E. P. (2001). The collagenous domain of class A scavenger receptors is involved in macrophage adhesion to collagens. *J Leukoc Biol,* 69**,** 575-82.

GOWN, A. M., TSUKADA, T. & ROSS, R. (1986). Human atherosclerosis. II. Immunocytochemical analysis of the cellular composition of human atherosclerotic lesions. *Am J Pathol,* 125**,** 191-207.

GRAHAM, J. M., HIGGINS, J. A., GILLOTT, T., TAYLOR, T., WILKINSON, J., FORD, T. & BILLINGTON, D. (1996). A novel method for the rapid separation of plasma lipoproteins using self-generating gradients of iodixanol. *Atherosclerosis,* 124**,** 125-35.

GRAHAM, S. A., ANTONOPOULOS, A., HITCHEN, P. G., HASLAM, S. M., DELL, A., DRICKAMER, K. & TAYLOR, M. E. (2011). Identification of neutrophil granule glycoproteins as Lewis(x)-containing ligands cleared by the scavenger receptor C-type lectin. *J Biol Chem,* 286**,** 24336-49.

GRATCHEV, A., SOBENIN, I., OREKHOV, A. & KZHYSHKOWSKA, J. (2012). Monocytes as a diagnostic marker of cardiovascular diseases. *Immunobiology,* 217**,** 476-82.

GU, L., OKADA, Y., CLINTON, S. K., GERARD, C., SUKHOVA, G. K., LIBBY, P. & ROLLINS, B. J. (1998). Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. *Mol Cell,* 2**,** 275-81.

GUO, L., CHEN, M., SONG, Z., DAUGHERTY, A. & LI, X. A. (2011). C323 of SR-BI is required for SR-BI-mediated HDL binding and cholesteryl ester uptake. *J Lipid Res,* 52**,** 2272-8.

GURSEL, M., GURSEL, I., MOSTOWSKI, H. S. & KLINMAN, D. M. (2006). CXCL16 influences the nature and specificity of CpG-induced immune activation. *J Immunol,* 177**,** 1575-80.

GUTWEIN, P., ABDEL-BAKKY, M. S., SCHRAMME, A., DOBERSTEIN, K., KAMPFER-KOLB, N., AMANN, K., HAUSER, I. A., OBERMULLER, N., BARTEL, C., ABDEL-AZIZ, A. A., EL SAYED EL, S. M. & PFEILSCHIFTER, J. (2009). CXCL16 is expressed in podocytes and acts as a scavenger receptor for oxidized low-density lipoprotein. *Am J Pathol,* 174**,** 2061-72.

HALLIWELL, B. (2006). Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. *Plant Physiol,* 141**,** 312-22.

HARJA, E., BU, D. X., HUDSON, B. I., CHANG, J. S., SHEN, X., HALLAM, K., KALEA, A. Z., LU, Y., ROSARIO, R. H., ORUGANTI, S., NIKOLLA, Z., BELOV, D., LALLA, E., RAMASAMY, R., YAN, S. F. & SCHMIDT, A. M. (2008). Vascular and inflammatory stresses mediate atherosclerosis via RAGE and its ligands in apoE-/- mice. *The Journal of clinical investigation,* 118**,** 183-94.

HAYASHIDA, K., KUME, N., MINAMI, M. & KITA, T. (2002). Lectin-like oxidized LDL receptor-1 (LOX-1) supports adhesion of

mononuclear leukocytes and a monocyte-like cell line THP-1 cells under static and flow conditions. *FEBS Lett,* 511**,** 133-8.

HE, M., KUBO, H., MORIMOTO, K., FUJINO, N., SUZUKI, T., TAKAHASI, T., YAMADA, M., YAMAYA, M., MAEKAWA, T., YAMAMOTO, Y. & YAMAMOTO, H. (2011). Receptor for advanced glycation end products binds to phosphatidylserine and assists in the clearance of apoptotic cells. *EMBO reports,* 12**,** 358-64.

HELDIN, C. H. & WESTERMARK, B. (1999). Mechanism of action and in vivo role of platelet-derived growth factor. *Physiol Rev,* 79**,** 1283-316.

HILLEN, W. & BERENS, C. (1994). Mechanisms underlying expression of Tn10 encoded tetracycline resistance. *Annu Rev Microbiol,* 48**,** 345-69.

HIRSCH, H. A., ILIOPOULOS, D., JOSHI, A., ZHANG, Y., JAEGER, S. A., BULYK, M., TSICHLIS, P. N., SHIRLEY LIU, X. & STRUHL, K. (2010). A transcriptional signature and common gene networks link cancer with lipid metabolism and diverse human diseases. *Cancer cell,* 17**,** 348-61.

HOFF, H. F., HEIDEMAN, C. L., GOTTO, A. M., JR. & GAUBATZ, J. W. (1977). Apolipoprotein B retention in the grossly normal and atherosclerotic human aorta. *Circ Res,* 41**,** 684-90.

HOFMANN, M. A., DRURY, S., FU, C., QU, W., TAGUCHI, A., LU, Y., AVILA, C., KAMBHAM, N., BIERHAUS, A., NAWROTH, P., NEURATH, M. F., SLATTERY, T., BEACH, D., MCCLARY, J., NAGASHIMA, M., MORSER, J., STERN, D. & SCHMIDT, A. M. (1999). RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. *Cell,* 97**,** 889-901.

HOLMAN, R. L., MC, G. H., JR., STRONG, J. P. & GEER, J. C. (1958). The natural history of atherosclerosis: the early aortic lesions as seen in New Orleans in the middle of the of the 20th century. *Am J Pathol,* 34**,** 209-35.

HOLNESS, C. L. & SIMMONS, D. L. (1993). Molecular cloning of CD68, a human macrophage marker related to lysosomal glycoproteins. *Blood,* 81**,** 1607-13.

HOLVOET, P., DE KEYZER, D. & JACOBS, D. R., JR. (2008). Oxidized LDL and the metabolic syndrome. *Future Lipidol,* 3**,** 637-649.

HONJO, M., NAKAMURA, K., YAMASHIRO, K., KIRYU, J., TANIHARA, H., MCEVOY, L. M., HONDA, Y., BUTCHER, E. C., MASAKI, T. & SAWAMURA, T. (2003). Lectin-like oxidized LDL receptor-1 is a

cell-adhesion molecule involved in endotoxin-induced inflammation. *Proc Natl Acad Sci U S A,* 100**,** 1274-9.

HORI, O., BRETT, J., SLATTERY, T., CAO, R., ZHANG, J., CHEN, J. X., NAGASHIMA, M., LUNDH, E. R., VIJAY, S., NITECKI, D. & ET AL. (1995). The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphoterin. Mediation of neurite outgrowth and co-expression of rage and amphoterin in the developing nervous system. *The Journal of biological chemistry,* 270**,** 25752-61.

HSIEH, C. C., YEN, M. H., YEN, C. H. & LAU, Y. T. (2001). Oxidized low density lipoprotein induces apoptosis via generation of reactive oxygen species in vascular smooth muscle cells. *Cardiovasc Res,* 49**,** 135-45.

HU, C., CHEN, J., DANDAPAT, A., FUJITA, Y., INOUE, N., KAWASE, Y., JISHAGE, K., SUZUKI, H., LI, D., HERMONAT, P. L., SAWAMURA, T. & MEHTA, J. L. (2008a). LOX-1 abrogation reduces myocardial ischemia-reperfusion injury in mice. *J Mol Cell Cardiol,* 44**,** 76-83.

HU, C., DANDAPAT, A., SUN, L., CHEN, J., MARWALI, M. R., ROMEO, F., SAWAMURA, T. & MEHTA, J. L. (2008b). LOX-1 deletion decreases collagen accumulation in atherosclerotic plaque in low-density lipoprotein receptor knockout mice fed a high-cholesterol diet. *Cardiovasc Res,* 79**,** 287-93.

HUANG, Y. H., RONNELID, J. & FROSTEGARD, J. (1995). Oxidized LDL induces enhanced antibody formation and MHC class II-dependent IFN-gamma production in lymphocytes from healthy individuals. *Arterioscler Thromb Vasc Biol,* 15**,** 1577-83.

INOUE, K., ARAI, Y., KURIHARA, H., KITA, T. & SAWAMURA, T. (2005). Overexpression of lectin-like oxidized low-density lipoprotein receptor-1 induces intramyocardial vasculopathy in apolipoprotein E-null mice. *Circ Res,* 97**,** 176-84.

ISHIGAKI, Y., KATAGIRI, H., GAO, J., YAMADA, T., IMAI, J., UNO, K., HASEGAWA, Y., KANEKO, K., OGIHARA, T., ISHIHARA, H., SATO, Y., TAKIKAWA, K., NISHIMICHI, N., MATSUDA, H., SAWAMURA, T. & OKA, Y. (2008). Impact of plasma oxidized low-density lipoprotein removal on atherosclerosis. *Circulation,* 118**,** 75-83.

ISHII, J., ADACHI, H., AOKI, J., KOIZUMI, H., TOMITA, S., SUZUKI, T., TSUJIMOTO, M., INOUE, K. & ARAI, H. (2002). SREC-II, a new member of the scavenger receptor type F family, trans-interacts with SREC-I through its extracellular domain. *J Biol Chem,* 277**,** 39696-702.

ISHIKAWA, M., ITO, H., AKIYOSHI, M., KUME, N., YOSHITOMI, H., MITSUOKA, H., TANIDA, S., MURATA, K., SHIBUYA, H., KASAHARA, T., KAKINO, A., FUJITA, Y., SAWAMURA, T., YASUDA, T. & NAKAMURA, T. (2012). Lectin-like oxidized low-density lipoprotein receptor 1 signal is a potent biomarker and therapeutic target for human rheumatoid arthritis. *Arthritis and rheumatism,* 64**,** 1024-34.

ISHIKAWA, Y., KIMURA-MATSUMOTO, M., MURAKAMI, M., YAMAMOTO, K., AKASAKA, Y., UZUKI, M., YURI, Y., INOMATA, N., YOKOO, T. & ISHII, T. (2009). Distribution of smooth muscle cells and macrophages expressing scavenger receptor BI/II in atherosclerosis. *J Atheroscler Thromb,* 16**,** 829-39.

ITABE, H., OBAMA, T. & KATO, R. (2011). The Dynamics of Oxidized LDL during Atherogenesis. *J Lipids,* 2011**,** 418313.

ITABE, H., TAKESHIMA, E., IWASAKI, H., KIMURA, J., YOSHIDA, Y., IMANAKA, T. & TAKANO, T. (1994). A monoclonal antibody against oxidized lipoprotein recognizes foam cells in atherosclerotic lesions. Complex formation of oxidized phosphatidylcholines and polypeptides. *J Biol Chem,* 269**,** 15274-9.

IVANDIC, B., CASTELLANI, L. W., WANG, X. P., QIAO, J. H., MEHRABIAN, M., NAVAB, M., FOGELMAN, A. M., GRASS, D. S., SWANSON, M. E., DE BEER, M. C., DE BEER, F. & LUSIS, A. J. (1999). Role of group II secretory phospholipase A2 in atherosclerosis: 1. Increased atherogenesis and altered lipoproteins in transgenic mice expressing group IIa phospholipase A2. *Arterioscler Thromb Vasc Biol,* 19**,** 1284-90.

IWAI-KANAI, E., HASEGAWA, K., SAWAMURA, T., FUJITA, M., YANAZUME, T., TOYOKUNI, S., ADACHI, S., KIHARA, Y. & SASAYAMA, S. (2001). Activation of lectin-like oxidized low-density lipoprotein receptor-1 induces apoptosis in cultured neonatal rat cardiac myocytes. *Circulation,* 104**,** 2948-54.

JANG, S., OHTANI, K., FUKUOH, A., YOSHIZAKI, T., FUKUDA, M., MOTOMURA, W., MORI, K., FUKUZAWA, J., KITAMOTO, N., YOSHIDA, I., SUZUKI, Y. & WAKAMIYA, N. (2009). Scavenger receptor collectin placenta 1 (CL-P1) predominantly mediates zymosan phagocytosis by human vascular endothelial cells. *J Biol Chem,* 284**,** 3956-65.

JENSEN, J. & LAI, Y. C. (2009). Regulation of muscle glycogen synthase phosphorylation and kinetic properties by insulin, exercise, adrenaline and role in insulin resistance. *Arch Physiol Biochem,* 115**,** 13-21.

JING, Q., XIN, S. M., CHENG, Z. J., ZHANG, W. B., ZHANG, R., QIN, Y. W. & PEI, G. (1999). Activation of p38 mitogen-activated protein

kinase by oxidized LDL in vascular smooth muscle cells: mediation via pertussis toxin-sensitive G proteins and association with oxidized LDL-induced cytotoxicity. *Circ Res,* 84**,** 831-9.

JONASSON, L., HOLM, J., SKALLI, O., BONDJERS, G. & HANSSON, G. K. (1986). Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque. *Arteriosclerosis,* 6**,** 131-8.

JOZEFOWSKI, S., ARREDOUANI, M., SULAHIAN, T. & KOBZIK, L. (2005). Disparate regulation and function of the class A scavenger receptors SR-AI/II and MARCO. *J Immunol,* 175**,** 8032-41.

KAKINUMA, T., YASUDA, T., NAKAGAWA, T., HIRAMITSU, T., AKIYOSHI, M., AKAGI, M., SAWAMURA, T. & NAKAMURA, T. (2004). Lectin-like oxidized low-density lipoprotein receptor 1 mediates matrix metalloproteinase 3 synthesis enhanced by oxidized low-density lipoprotein in rheumatoid arthritis cartilage. *Arthritis and rheumatism,* 50**,** 3495-503.

KAKUTANI, M., MASAKI, T. & SAWAMURA, T. (2000). A platelet-endothelium interaction mediated by lectin-like oxidized low-density lipoprotein receptor-1. *Proc Natl Acad Sci U S A,* 97**,** 360-4.

KAKUTANI, M., UEDA, M., NARUKO, T., MASAKI, T. & SAWAMURA, T. (2001). Accumulation of LOX-1 ligand in plasma and atherosclerotic lesions of Watanabe heritable hyperlipidemic rabbits: identification by a novel enzyme immunoassay. *Biochem Biophys Res Commun,* 282**,** 180-5.

KAMADA, N., KODAMA, T. & SUZUKI, H. (2001). Macrophage scavenger receptor (SR-A I/II) deficiency reduced diet-induced atherosclerosis in C57BL/6J mice. *J Atheroscler Thromb,* 8**,** 1-6.

KANGAS, M., BRANNSTROM, A., ELOMAA, O., MATSUDA, Y., EDDY, R., SHOWS, T. B. & TRYGGVASON, K. (1999). Structure and chromosomal localization of the human and murine genes for the macrophage MARCO receptor. *Genomics,* 58**,** 82-9.

KATAOKA, H., KUME, N., MIYAMOTO, S., MINAMI, M., MORIMOTO, M., HAYASHIDA, K., HASHIMOTO, N. & KITA, T. (2001). Oxidized LDL modulates Bax/Bcl-2 through the lectinlike Ox-LDL receptor-1 in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol,* 21**,** 955-60.

KATAOKA, H., KUME, N., MIYAMOTO, S., MINAMI, M., MORIWAKI, H., MURASE, T., SAWAMURA, T., MASAKI, T., HASHIMOTO, N. & KITA, T. (1999). Expression of lectinlike oxidized low-density lipoprotein receptor-1 in human atherosclerotic lesions. *Circulation,* 99**,** 3110-7.

KAUFFMANN-ZEH, A., RODRIGUEZ-VICIANA, P., ULRICH, E., GILBERT, C., COFFER, P., DOWNWARD, J. & EVAN, G. (1997). Suppression of c-Myc-induced apoptosis by Ras signalling through PI(3)K and PKB. *Nature,* 385**,** 544-8.

KELLEY, J. L. & KRUSKI, A. W. (1986). Density gradient ultracentrifugation of serum lipoproteins in a swinging bucket rotor. *Methods in enzymology,* 128**,** 170-81.

KHAIDAKOV, M., MITRA, S., WANG, X., DING, Z., BORA, N., LYZOGUBOV, V., ROMEO, F., SCHICHMAN, S. A. & MEHTA, J. L. (2012). Large impact of low concentration oxidized LDL on angiogenic potential of human endothelial cells: a microarray study. *PLoS One,* 7**,** e47421.

KNOTT, T. J., PEASE, R. J., POWELL, L. M., WALLIS, S. C., RALL, S. C., JR., INNERARITY, T. L., BLACKHART, B., TAYLOR, W. H., MARCEL, Y., MILNE, R. & ET AL. (1986). Complete protein sequence and identification of structural domains of human apolipoprotein B. *Nature,* 323**,** 734-8.

KNOWLES, J. W., REDDICK, R. L., JENNETTE, J. C., SHESELY, E. G., SMITHIES, O. & MAEDA, N. (2000). Enhanced atherosclerosis and kidney dysfunction in eNOS(-/-)Apoe(-/-) mice are ameliorated by enalapril treatment. *J Clin Invest,* 105**,** 451-8.

KODAMA, T., FREEMAN, M., ROHRER, L., ZABRECKY, J., MATSUDAIRA, P. & KRIEGER, M. (1990). Type I macrophage scavenger receptor contains alpha-helical and collagen-like coiled coils. *Nature,* 343**,** 531-5.

KRAAL, G., VAN DER LAAN, L. J., ELOMAA, O. & TRYGGVASON, K. (2000). The macrophage receptor MARCO. *Microbes Infect,* 2**,** 313-6.

KRISTIANSEN, M., GRAVERSEN, J. H., JACOBSEN, C., SONNE, O., HOFFMAN, H. J., LAW, S. K. & MOESTRUP, S. K. (2001). Identification of the haemoglobin scavenger receptor. *Nature,* 409**,** 198-201.

KUME, N. & KITA, T. (2001). Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) in atherogenesis. *Trends Cardiovasc Med,* 11**,** 22-5.

KUME, N., MURASE, T., MORIWAKI, H., AOYAMA, T., SAWAMURA, T., MASAKI, T. & KITA, T. (1998). Inducible expression of lectin-like oxidized LDL receptor-1 in vascular endothelial cells. *Circ Res,* 83**,** 322-7.

KUSUHARA, M., CHAIT, A., CADER, A. & BERK, B. C. (1997). Oxidized LDL stimulates mitogen-activated protein kinases in smooth muscle cells and macrophages. *Arterioscler Thromb Vasc Biol,* 17**,** 141-8.

LAWRENCE, T. (2009). The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harb Perspect Biol,* 1**,** a001651.

LEVITAN, I., VOLKOV, S. & SUBBAIAH, P. V. (2010). Oxidized LDL: diversity, patterns of recognition, and pathophysiology. *Antioxidants & redox signaling,* 13**,** 39-75.

LI, A. C., GUIDEZ, F. R., COLLIER, J. G. & GLASS, C. K. (1998). The macrosialin promoter directs high levels of transcriptional activity in macrophages dependent on combinatorial interactions between PU.1 and c-Jun. *J Biol Chem,* 273**,** 5389-99.

LI, D., CHEN, H., ROMEO, F., SAWAMURA, T., SALDEEN, T. & MEHTA, J. L. (2002a). Statins modulate oxidized low-density lipoprotein-mediated adhesion molecule expression in human coronary artery endothelial cells: role of LOX-1. *J Pharmacol Exp Ther,* 302**,** 601-5.

LI, D., LIU, L., CHEN, H., SAWAMURA, T. & MEHTA, J. L. (2003a). LOX-1, an oxidized LDL endothelial receptor, induces CD40/CD40L signaling in human coronary artery endothelial cells. *Arterioscler Thromb Vasc Biol,* 23**,** 816-21.

LI, D. & MEHTA, J. L. (2000a). Antisense to LOX-1 inhibits oxidized LDL-mediated upregulation of monocyte chemoattractant protein-1 and monocyte adhesion to human coronary artery endothelial cells. *Circulation,* 101**,** 2889-95.

LI, D. & MEHTA, J. L. (2000b). Upregulation of endothelial receptor for oxidized LDL (LOX-1) by oxidized LDL and implications in apoptosis of human coronary artery endothelial cells: evidence from use of antisense LOX-1 mRNA and chemical inhibitors. *Arterioscler Thromb Vasc Biol,* 20**,** 1116-22.

LI, D. & MEHTA, J. L. (2009). Intracellular signaling of LOX-1 in endothelial cell apoptosis. *Circ Res,* 104**,** 566-8.

LI, D., SINGH, R. M., LIU, L., CHEN, H., SINGH, B. M., KAZZAZ, N. & MEHTA, J. L. (2003b). Oxidized-LDL through LOX-1 increases the expression of angiotensin converting enzyme in human coronary artery endothelial cells. *Cardiovasc Res,* 57**,** 238-43.

LI, D., WILLIAMS, V., LIU, L., CHEN, H., SAWAMURA, T., ANTAKLI, T. & MEHTA, J. L. (2002b). LOX-1 inhibition in myocardial ischemia-reperfusion injury: modulation of MMP-1 and inflammation. *Am J Physiol Heart Circ Physiol,* 283**,** H1795-801.

LI, D., WILLIAMS, V., LIU, L., CHEN, H., SAWAMURA, T., ROMEO, F. & MEHTA, J. L. (2003c). Expression of lectin-like oxidized low-density

lipoprotein receptors during ischemia-reperfusion and its role in determination of apoptosis and left ventricular dysfunction. *Journal of the American College of Cardiology,* 41**,** 1048-55.

LI, D. Y., CHEN, H. J. & MEHTA, J. L. (2001). Statins inhibit oxidized-LDL-mediated LOX-1 expression, uptake of oxidized-LDL and reduction in PKB phosphorylation. *Cardiovasc Res,* 52**,** 130-5.

LI, D. Y., ZHANG, Y. C., PHILIPS, M. I., SAWAMURA, T. & MEHTA, J. L. (1999a). Upregulation of endothelial receptor for oxidized low-density lipoprotein (LOX-1) in cultured human coronary artery endothelial cells by angiotensin II type 1 receptor activation. *Circ Res,* 84**,** 1043-9.

LI, D. Y., ZHANG, Y. C., PHILIPS, M. I., SAWAMURA, T. & MEHTA, J. L. (1999b). Upregulation of endothelial receptor for oxidized low-density lipoprotein (LOX-1) in cultured human coronary artery endothelial cells by angiotensin II type 1 receptor activation. *Circulation research,* 84**,** 1043-9.

LI, J. & SCHMIDT, A. M. (1997). Characterization and functional analysis of the promoter of RAGE, the receptor for advanced glycation end products. *The Journal of biological chemistry,* 272**,** 16498-506.

LI, L., SAWAMURA, T. & RENIER, G. (2003d). Glucose enhances endothelial LOX-1 expression: role for LOX-1 in glucose-induced human monocyte adhesion to endothelium. *Diabetes,* 52**,** 1843-50.

LI, L., SAWAMURA, T. & RENIER, G. (2004). Glucose enhances human macrophage LOX-1 expression: role for LOX-1 in glucose-induced macrophage foam cell formation. *Circulation research,* 94**,** 892-901.

LIANI, R., HALVORSEN, B., SESTILI, S., HANDBERG, A., SANTILLI, F., VAZZANA, N., FORMOSO, G., AUKRUST, P. & DAVI, G. (2012). Plasma levels of soluble CD36, platelet activation, inflammation, and oxidative stress are increased in type 2 diabetic patients. *Free Radic Biol Med,* 52**,** 1318-24.

LIAO, L., AW, T. Y., KVIETYS, P. R. & GRANGER, D. N. (1995). Oxidized LDL-induced microvascular dysfunction. Dependence on oxidation procedure. *Arterioscler Thromb Vasc Biol,* 15**,** 2305-11.

LIBBY, P. (2003). Vascular biology of atherosclerosis: overview and state of the art. *Am J Cardiol,* 91**,** 3a-6a.

LIBBY, P., RIDKER, P. M. & HANSSON, G. K. (2011). Progress and challenges in translating the biology of atherosclerosis. *Nature,* 473**,** 317-25.

LIBBY, P., RIDKER, P. M. & MASERI, A. (2002). Inflammation and atherosclerosis. *Circulation,* 105**,** 1135-43.

LING, W., LOUGHEED, M., SUZUKI, H., BUCHAN, A., KODAMA, T. & STEINBRECHER, U. P. (1997). Oxidized or acetylated low density lipoproteins are rapidly cleared by the liver in mice with disruption of the scavenger receptor class A type I/II gene. *J Clin Invest,* 100**,** 244-52.

LONG, Y. C. & ZIERATH, J. R. (2008). Influence of AMP-activated protein kinase and calcineurin on metabolic networks in skeletal muscle. *Am J Physiol Endocrinol Metab,* 295**,** E545-52.

LOUGHEED, M., MOORE, E. D., SCRIVEN, D. R. & STEINBRECHER, U. P. (1999). Uptake of oxidized LDL by macrophages differs from that of acetyl LDL and leads to expansion of an acidic endolysosomal compartment. *Arterioscler Thromb Vasc Biol,* 19**,** 1881-90.

LU, J., MITRA, S., WANG, X., KHAIDAKOV, M. & MEHTA, J. L. (2011). Oxidative stress and lectin-like ox-LDL-receptor LOX-1 in atherogenesis and tumorigenesis. *Antioxidants & redox signaling,* 15**,** 2301-33.

LU, J., YANG, J. H., BURNS, A. R., CHEN, H. H., TANG, D., WALTERSCHEID, J. P., SUZUKI, S., YANG, C. Y., SAWAMURA, T. & CHEN, C. H. (2009). Mediation of electronegative low-density lipoprotein signaling by LOX-1: a possible mechanism of endothelial apoptosis. *Circ Res,* 104**,** 619-27.

LUO, P., YAN, M., FROHLICH, E. D., MEHTA, J. L. & HU, C. (2011). Novel concepts in the genesis of hypertension: role of LOX-1. *Cardiovascular drugs and therapy / sponsored by the International Society of Cardiovascular Pharmacotherapy,* 25**,** 441-9.

LUSIS, A. J. (2000). Atherosclerosis. *Nature,* 407**,** 233-41.

MACKENZIE, R. W. & ELLIOTT, B. T. (2014). Akt/PKB activation and insulin signaling: a novel insulin signaling pathway in the treatment of type 2 diabetes. *Diabetes Metab Syndr Obes,* 7**,** 55-64.

MAKINEN, P. I., LAPPALAINEN, J. P., HEINONEN, S. E., LEPPANEN, P., LAHTEENVUO, M. T., AARNIO, J. V., HEIKKILA, J., TURUNEN, M. P. & YLA-HERTTUALA, S. (2010). Silencing of either SR-A or CD36 reduces atherosclerosis in hyperlipidaemic mice and reveals reciprocal upregulation of these receptors. *Cardiovasc Res,* 88**,** 530-8.

MANGO, R., BIOCCA, S., DEL VECCHIO, F., CLEMENTI, F., SANGIUOLO, F., AMATI, F., FILARETO, A., GRELLI, S., SPITALIERI, P., FILESI, I., FAVALLI, C., LAURO, R., MEHTA, J. L., ROMEO, F. & NOVELLI, G. (2005). In vivo and in vitro studies support that a new splicing isoform of OLR1 gene is protective against acute myocardial infarction. *Circulation research,* 97**,** 152-8.

MANNING-TOBIN, J. J., MOORE, K. J., SEIMON, T. A., BELL, S. A., SHARUK, M., ALVAREZ-LEITE, J. I., DE WINTHER, M. P., TABAS, I. & FREEMAN, M. W. (2009). Loss of SR-A and CD36 activity reduces atherosclerotic lesion complexity without abrogating foam cell formation in hyperlipidemic mice. *Arterioscler Thromb Vasc Biol,* 29**,** 19-26.

MARATHE, S., KURIAKOSE, G., WILLIAMS, K. J. & TABAS, I. (1999). Sphingomyelinase, an enzyme implicated in atherogenesis, is present in atherosclerotic lesions and binds to specific components of the subendothelial extracellular matrix. *Arterioscler Thromb Vasc Biol,* 19**,** 2648-58.

MASUDA, J. & ROSS, R. (1990a). Atherogenesis during low level hypercholesterolemia in the nonhuman primate. I. Fatty streak formation. *Arteriosclerosis,* 10**,** 164-77.

MASUDA, J. & ROSS, R. (1990b). Atherogenesis during low level hypercholesterolemia in the nonhuman primate. II. Fatty streak conversion to fibrous plaque. *Arteriosclerosis,* 10**,** 178-87.

MAZIERE, C., MORLIERE, P., SANTUS, R., MARCHEUX, V., LOUANDRE, C., CONTE, M. A. & MAZIERE, J. C. (2004). Inhibition of insulin signaling by oxidized low density lipoprotein. Protective effect of the antioxidant Vitamin E. *Atherosclerosis,* 175**,** 23-30.

MAZZONE, T., CHAIT, A. & PLUTZKY, J. (2008). Cardiovascular disease risk in type 2 diabetes mellitus: insights from mechanistic studies. *Lancet,* 371**,** 1800-9.

MCGILL, H. C., JR. (1984). George Lyman Duff memorial lecture. Persistent problems in the pathogenesis of atherosclerosis. *Arteriosclerosis,* 4**,** 443-51.

MCGILL, H. C., JR., MCMAHAN, C. A., HERDERICK, E. E., MALCOM, G. T., TRACY, R. E. & STRONG, J. P. (2000). Origin of atherosclerosis in childhood and adolescence. *Am J Clin Nutr,* 72**,** 1307s-1315s.

MCNAMARA, J. R., SMALL, D. M., LI, Z. & SCHAEFER, E. J. (1996). Differences in LDL subspecies involve alterations in lipid composition and conformational changes in apolipoprotein B. *J Lipid Res,* 37**,** 1924-35.

MEHTA, J. L. (2006). Oxidized or native low-density lipoprotein cholesterol: which is more important in atherogenesis? *J Am Coll Cardiol,* 48**,** 980-2.

MEHTA, J. L., CHEN, J., HERMONAT, P. L., ROMEO, F. & NOVELLI, G. (2006). Lectin-like, oxidized low-density lipoprotein receptor-1

(LOX-1): a critical player in the development of atherosclerosis and related disorders. *Cardiovasc Res,* 69**,** 36-45.

MEHTA, J. L., CHEN, J., YU, F. & LI, D. Y. (2004). Aspirin inhibits ox-LDL-mediated LOX-1 expression and metalloproteinase-1 in human coronary endothelial cells. *Cardiovasc Res,* 64**,** 243-9.

MEHTA, J. L. & LI, D. (2002). Identification, regulation and function of a novel lectin-like oxidized low-density lipoprotein receptor. *Journal of the American College of Cardiology,* 39**,** 1429-35.

MEHTA, J. L., SANADA, N., HU, C. P., CHEN, J., DANDAPAT, A., SUGAWARA, F., SATOH, H., INOUE, K., KAWASE, Y., JISHAGE, K., SUZUKI, H., TAKEYA, M., SCHNACKENBERG, L., BEGER, R., HERMONAT, P. L., THOMAS, M. & SAWAMURA, T. (2007). Deletion of LOX-1 reduces atherogenesis in LDLR knockout mice fed high cholesterol diet. *Circ Res,* 100**,** 1634-42.

MEMON, R. A., STAPRANS, I., NOOR, M., HOLLERAN, W. M., UCHIDA, Y., MOSER, A. H., FEINGOLD, K. R. & GRUNFELD, C. (2000). Infection and inflammation induce LDL oxidation in vivo. *Arterioscler Thromb Vasc Biol,* 20**,** 1536-42.

MERHI-SOUSSI, F., BERTI, M., WEHRLE-HALLER, B. & GABAY, C. (2005). Intracellular interleukin-1 receptor antagonist type 1 antagonizes the stimulatory effect of interleukin-1 alpha precursor on cell motility. *Cytokine,* 32**,** 163-70.

MIETUS-SNYDER, M., FRIERA, A., GLASS, C. K. & PITAS, R. E. (1997). Regulation of scavenger receptor expression in smooth muscle cells by protein kinase C: a role for oxidative stress. *Arterioscler Thromb Vasc Biol,* 17**,** 969-78.

MINAMI, M., KUME, N., KATAOKA, H., MORIMOTO, M., HAYASHIDA, K., SAWAMURA, T., MASAKI, T. & KITA, T. (2000). Transforming growth factor-beta(1) increases the expression of lectin-like oxidized low-density lipoprotein receptor-1. *Biochem Biophys Res Commun,* 272**,** 357-61.

MITRA, S., GOYAL, T. & MEHTA, J. L. (2011). Oxidized LDL, LOX-1 and atherosclerosis. *Cardiovasc Drugs Ther,* 25**,** 419-29.

MOELLER, J. B., NIELSEN, M. J., REICHHARDT, M. P., SCHLOSSER, A., SORENSEN, G. L., NIELSEN, O., TORNOE, I., GRONLUND, J., NIELSEN, M. E., JORGENSEN, J. S., JENSEN, O. N., MOLLENHAUER, J., MOESTRUP, S. K. & HOLMSKOV, U. (2012). CD163-L1 is an endocytic macrophage protein strongly regulated by mediators in the inflammatory response. *Journal of immunology (Baltimore, Md. : 1950),* 188**,** 2399-409.

MOIRAND, R., MORTAJI, A. M., LOREAL, O., PAILLARD, F., BRISSOT, P. & DEUGNIER, Y. (1997). A new syndrome of liver iron overload with normal transferrin saturation. *Lancet,* 349**,** 95-7.

MORAWIETZ, H. (2007). LOX-1 and atherosclerosis: proof of concept in LOX-1-knockout mice. *Circ Res,* 100**,** 1534-6.

MORAWIETZ, H., RUECKSCHLOSS, U., NIEMANN, B., DUERRSCHMIDT, N., GALLE, J., HAKIM, K., ZERKOWSKI, H. R., SAWAMURA, T. & HOLTZ, J. (1999). Angiotensin II induces LOX-1, the human endothelial receptor for oxidized low-density lipoprotein. *Circulation,* 100**,** 899-902.

MORIWAKI, H., KUME, N., SAWAMURA, T., AOYAMA, T., HOSHIKAWA, H., OCHI, H., NISHI, E., MASAKI, T. & KITA, T. (1998). Ligand specificity of LOX-1, a novel endothelial receptor for oxidized low density lipoprotein. *Arterioscler Thromb Vasc Biol,* 18**,** 1541-7.

MOVVA, R. & RADER, D. J. (2008). Laboratory assessment of HDL heterogeneity and function. *Clin Chem,* 54**,** 788-800.

MOXHAM, C. M., TABRIZCHI, A., DAVIS, R. J. & MALBON, C. C. (1996). Jun N-terminal kinase mediates activation of skeletal muscle glycogen synthase by insulin in vivo. *J Biol Chem,* 271**,** 30765-73.

MUGHAL, N. (2015). MD, University of Leeds.

MUKHOPADHYAY, S., VARIN, A., CHEN, Y., LIU, B., TRYGGVASON, K. & GORDON, S. (2011). SR-A/MARCO-mediated ligand delivery enhances intracellular TLR and NLR function, but ligand scavenging from cell surface limits TLR4 response to pathogens. *Blood,* 117**,** 1319-28.

MURASE, T., KUME, N., KORENAGA, R., ANDO, J., SAWAMURA, T., MASAKI, T. & KITA, T. (1998). Fluid shear stress transcriptionally induces lectin-like oxidized LDL receptor-1 in vascular endothelial cells. *Circ Res,* 83**,** 328-33.

MURPHY, J. E., TACON, D., TEDBURY, P. R., HADDEN, J. M., KNOWLING, S., SAWAMURA, T., PECKHAM, M., PHILLIPS, S. E., WALKER, J. H. & PONNAMBALAM, S. (2006). LOX-1 scavenger receptor mediates calcium-dependent recognition of phosphatidylserine and apoptotic cells. *Biochem J,* 393**,** 107-15.

MURPHY, J. E., TEDBURY, P. R., HOMER-VANNIASINKAM, S., WALKER, J. H. & PONNAMBALAM, S. (2005). Biochemistry and cell biology of mammalian scavenger receptors. *Atherosclerosis,* 182**,** 1-15.

MURPHY, J. E., VOHRA, R. S., DUNN, S., HOLLOWAY, Z. G., MONACO, A. P., HOMER-VANNIASINKAM, S., WALKER, J. H. & PONNAMBALAM, S. (2008). Oxidised LDL internalisation by the

LOX-1 scavenger receptor is dependent on a novel cytoplasmic motif and is regulated by dynamin-2. *J Cell Sci,* 121**,** 2136-47.

MURSHID, A., GONG, J. & CALDERWOOD, S. K. (2010). Heat shock protein 90 mediates efficient antigen cross presentation through the scavenger receptor expressed by endothelial cells-I. *J Immunol,* 185**,** 2903-17.

NAGASE, M., ABE, J., TAKAHASHI, K., ANDO, J., HIROSE, S. & FUJITA, T. (1998). Genomic organization and regulation of expression of the lectin-like oxidized low-density lipoprotein receptor (LOX-1) gene. *J Biol Chem,* 273**,** 33702-7.

NAGASE, M., ANDO, K., NAGASE, T., KANAME, S., SAWAMURA, T. & FUJITA, T. (2001). Redox-sensitive regulation of lox-1 gene expression in vascular endothelium. *Biochem Biophys Res Commun,* 281**,** 720-5.

NAGASE, M., HIROSE, S., SAWAMURA, T., MASAKI, T. & FUJITA, T. (1997a). Enhanced expression of endothelial oxidized low-density lipoprotein receptor (LOX-1) in hypertensive rats. *Biochemical and biophysical research communications,* 237**,** 496-8.

NAGASE, M., HIROSE, S., SAWAMURA, T., MASAKI, T. & FUJITA, T. (1997b). Enhanced expression of endothelial oxidized low-density lipoprotein receptor (LOX-1) in hypertensive rats. *Biochem Biophys Res Commun,* 237**,** 496-8.

NAGASE, M., KANAME, S., NAGASE, T., WANG, G., ANDO, K., SAWAMURA, T. & FUJITA, T. (2000). Expression of LOX-1, an oxidized low-density lipoprotein receptor, in experimental hypertensive glomerulosclerosis. *J Am Soc Nephrol,* 11**,** 1826-36.

NAGEL, T., RESNICK, N., ATKINSON, W. J., DEWEY, C. F., JR. & GIMBRONE, M. A., JR. (1994). Shear stress selectively upregulates intercellular adhesion molecule-1 expression in cultured human vascular endothelial cells. *J Clin Invest,* 94**,** 885-91.

NATHAN, D. M., CLEARY, P. A., BACKLUND, J. Y., GENUTH, S. M., LACHIN, J. M., ORCHARD, T. J., RASKIN, P. & ZINMAN, B. (2005). Intensive diabetes treatment and cardiovascular disease in patients with type 1 diabetes. *N Engl J Med,* 353**,** 2643-53.

NEGORO, N., KANAYAMA, Y., HARAGUCHI, M., UMETANI, N., NISHIMURA, M., KONISHI, Y., IWAI, J., OKAMURA, M., INOUE, T. & TAKEDA, T. (1995). Blood pressure regulates platelet-derived growth factor A-chain gene expression in vascular smooth muscle cells in vivo. An autocrine mechanism promoting hypertensive vascular hypertrophy. *J Clin Invest,* 95**,** 1140-50.

NIELSEN, M. J., MADSEN, M., MOLLER, H. J. & MOESTRUP, S. K. (2006). The macrophage scavenger receptor CD163: endocytic properties of cytoplasmic tail variants. *Journal of leukocyte biology,* 79**,** 837-45.

NIEVELSTEIN, P. F., FOGELMAN, A. M., MOTTINO, G. & FRANK, J. S. (1991). Lipid accumulation in rabbit aortic intima 2 hours after bolus infusion of low density lipoprotein. A deep-etch and immunolocalization study of ultrarapidly frozen tissue. *Arterioscler Thromb,* 11**,** 1795-805.

NIKI, E. (2011). Do free radicals play causal role in atherosclerosis? Low density lipoprotein oxidation and vitamin E revisited. *J Clin Biochem Nutr,* 48**,** 3-7.

NISHI, K., ITABE, H., UNO, M., KITAZATO, K. T., HORIGUCHI, H., SHINNO, K. & NAGAHIRO, S. (2002). Oxidized LDL in carotid plaques and plasma associates with plaque instability. *Arterioscler Thromb Vasc Biol,* 22**,** 1649-54.

NISHIKAWA, S., SUGIMOTO, J., OKADA, M., SAKAIRI, T. & TAKAGI, S. (2012). Gene expression in livers of BALB/C and C57BL/6J mice fed a high-fat diet. *Toxicol Pathol,* 40**,** 71-82.

NOWICKI, M., KOSACKA, J., SERKE, H., BLUHER, M. & SPANEL-BOROWSKI, K. (2012). Altered sciatic nerve fiber morphology and endoneural microvessels in mouse models relevant for obesity, peripheral diabetic polyneuropathy, and the metabolic syndrome. *Journal of neuroscience research,* 90**,** 122-31.

O'GORMAN, S., FOX, D. T. & WAHL, G. M. (1991). Recombinase-mediated gene activation and site-specific integration in mammalian cells. *Science,* 251**,** 1351-5.

OGURA, S., KAKINO, A., SATO, Y., FUJITA, Y., IWAMOTO, S., OTSUI, K., YOSHIMOTO, R. & SAWAMURA, T. (2009). Lox-1: the multifunctional receptor underlying cardiovascular dysfunction. *Circ J,* 73**,** 1993-9.

OHNISHI, M., KATO, S. & RAZZAQUE, M. S. (2011). Genetic induction of phosphate toxicity significantly reduces the survival of hypercholesterolemic obese mice. *Biochem Biophys Res Commun,* 415**,** 434-8.

OHTANI, K., SUZUKI, Y., EDA, S., KAWAI, T., KASE, T., KESHI, H., SAKAI, Y., FUKUOH, A., SAKAMOTO, T., ITABE, H., SUZUTANI, T., OGASAWARA, M., YOSHIDA, I. & WAKAMIYA, N. (2001). The membrane-type collectin CL-P1 is a scavenger receptor on vascular endothelial cells. *J Biol Chem,* 276**,** 44222-8.

OJALA, J. R., PIKKARAINEN, T., TUUTTILA, A., SANDALOVA, T. & TRYGGVASON, K. (2007). Crystal structure of the cysteine-rich domain of scavenger receptor MARCO reveals the presence of a basic and an acidic cluster that both contribute to ligand recognition. *J Biol Chem,* 282**,** 16654-66.

OKA, K., SAWAMURA, T., KIKUTA, K., ITOKAWA, S., KUME, N., KITA, T. & MASAKI, T. (1998). Lectin-like oxidized low-density lipoprotein receptor 1 mediates phagocytosis of aged/apoptotic cells in endothelial cells. *Proc Natl Acad Sci U S A,* 95**,** 9535-40.

OLIVEIRA, C. L., SANTOS, P. R., MONTEIRO, A. M. & FIGUEIREDO NETO, A. M. (2014). Effect of oxidation on the structure of human low- and high-density lipoproteins. *Biophys J,* 106**,** 2595-605.

ORR, A. W., STOCKTON, R., SIMMERS, M. B., SANDERS, J. M., SAREMBOCK, I. J., BLACKMAN, B. R. & SCHWARTZ, M. A. (2007). Matrix-specific p21-activated kinase activation regulates vascular permeability in atherogenesis. *J Cell Biol,* 176**,** 719-27.

PALINSKI, W., HORKKO, S., MILLER, E., STEINBRECHER, U. P., POWELL, H. C., CURTISS, L. K. & WITZTUM, J. L. (1996). Cloning of monoclonal autoantibodies to epitopes of oxidized lipoproteins from apolipoprotein E-deficient mice. Demonstration of epitopes of oxidized low density lipoprotein in human plasma. *J Clin Invest,* 98**,** 800-14.

PARASASSI, T., BITTOLO-BON, G., BRUNELLI, R., CAZZOLATO, G., KRASNOWSKA, E. K., MEI, G., SEVANIAN, A. & URSINI, F. (2001). Loss of apoB-100 secondary structure and conformation in hydroperoxide rich, electronegative LDL(-). *Free Radic Biol Med,* 31**,** 82-9.

PARK, Y. M., DRAZBA, J. A., VASANJI, A., EGELHOFF, T., FEBBRAIO, M. & SILVERSTEIN, R. L. (2012). Oxidized LDL/CD36 interaction induces loss of cell polarity and inhibits macrophage locomotion. *Mol Biol Cell*.

PENG, Y., AKMENTIN, W., CONNELLY, M. A., LUND-KATZ, S., PHILLIPS, M. C. & WILLIAMS, D. L. (2004). Scavenger receptor BI (SR-BI) clustered on microvillar extensions suggests that this plasma membrane domain is a way station for cholesterol trafficking between cells and high-density lipoprotein. *Mol Biol Cell,* 15**,** 384-96.

PESSIN, J. E. & SALTIEL, A. R. (2000). Signaling pathways in insulin action: molecular targets of insulin resistance. *J Clin Invest,* 106**,** 165-9.

PETERSEN, K. F., DUFOUR, S., BEFROY, D., GARCIA, R. & SHULMAN, G. I. (2004). Impaired mitochondrial activity in the insulin-resistant

offspring of patients with type 2 diabetes. *N Engl J Med,* 350**,** 664-71.

PIETERS, M. N., ESBACH, S., SCHOUTEN, D., BROUWER, A., KNOOK, D. L. & VAN BERKEL, T. J. (1994). Cholesteryl esters from oxidized low-density lipoproteins are in vivo rapidly hydrolyzed in rat Kupffer cells and transported to liver parenchymal cells and bile. *Hepatology,* 19**,** 1459-67.

PIGA, R., NAITO, Y., KOKURA, S., HANDA, O. & YOSHIKAWA, T. (2007). Short-term high glucose exposure induces monocyte-endothelial cells adhesion and transmigration by increasing VCAM-1 and MCP-1 expression in human aortic endothelial cells. *Atherosclerosis,* 193**,** 328-34.

PIKKARAINEN, T., BRANNSTROM, A. & TRYGGVASON, K. (1999). Expression of macrophage MARCO receptor induces formation of dendritic plasma membrane processes. *J Biol Chem,* 274**,** 10975-82.

PODREZ, E. A., FEBBRAIO, M., SHEIBANI, N., SCHMITT, D., SILVERSTEIN, R. L., HAJJAR, D. P., COHEN, P. A., FRAZIER, W. A., HOFF, H. F. & HAZEN, S. L. (2000). Macrophage scavenger receptor CD36 is the major receptor for LDL modified by monocyte-generated reactive nitrogen species. *J Clin Invest,* 105**,** 1095-108.

POLI, G. (2000). Pathogenesis of liver fibrosis: role of oxidative stress. *Mol Aspects Med,* 21**,** 49-98.

PUCCETTI, L., PASQUI, A. L., PASTORELLI, M., CIANI, F., PALAZZUOLI, A., GIOFFRE, W., AUTERI, A. & BRUNI, F. (2005). 3'UTR/T polymorphism of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) is associated with modified anti-platelet activity of atorvastatin in hypercholesterolemic subjects. *Atherosclerosis,* 183**,** 322-8.

QUAN, Z., YANG, H., YANG, Y., YAN, B., CAO, R., WEN, G., LIU, C. & XU, Y. (2007). Construction and functional analysis of a lentiviral expression vector containing a scavenger receptor (SR-PSOX) that binds uniquely phosphatidylserine and oxidized lipoprotein. *Acta Biochim Biophys Sin (Shanghai),* 39**,** 208-16.

QUINN, M. T., PARTHASARATHY, S., FONG, L. G. & STEINBERG, D. (1987). Oxidatively modified low density lipoproteins: a potential role in recruitment and retention of monocyte/macrophages during atherogenesis. *Proc Natl Acad Sci U S A,* 84**,** 2995-8.

RAMASAMY, R., YAN, S. F. & SCHMIDT, A. M. (2009). RAGE: therapeutic target and biomarker of the inflammatory response--the evidence mounts. *Journal of leukocyte biology,* 86**,** 505-12.

RAMPRASAD, M. P., TERPSTRA, V., KONDRATENKO, N., QUEHENBERGER, O. & STEINBERG, D. (1996). Cell surface expression of mouse macrosialin and human CD68 and their role as macrophage receptors for oxidized low density lipoprotein. *Proc Natl Acad Sci U S A,* 93**,** 14833-8.

RANGASWAMY, S., PENN, M. S., SAIDEL, G. M. & CHISOLM, G. M. (1997). Exogenous oxidized low-density lipoprotein injures and alters the barrier function of endothelium in rats in vivo. *Circ Res,* 80**,** 37-44.

RIBE, D., YANG, J., PATEL, S., KOUMANOV, F., CUSHMAN, S. W. & HOLMAN, G. D. (2005). Endofacial competitive inhibition of glucose transporter-4 intrinsic activity by the mitogen-activated protein kinase inhibitor SB203580. *Endocrinology,* 146**,** 1713-7.

RICCI, R., SUMARA, G., SUMARA, I., ROZENBERG, I., KURRER, M., AKHMEDOV, A., HERSBERGER, M., ERIKSSON, U., EBERLI, F. R., BECHER, B., BOREN, J., CHEN, M., CYBULSKY, M. I., MOORE, K. J., FREEMAN, M. W., WAGNER, E. F., MATTER, C. M. & LUSCHER, T. F. (2004). Requirement of JNK2 for scavenger receptor A-mediated foam cell formation in atherogenesis. *Science,* 306**,** 1558-61.

ROBBESYN, F., SALVAYRE, R. & NEGRE-SALVAYRE, A. (2004). Dual role of oxidized LDL on the NF-kappaB signaling pathway. *Free Radic Res,* 38**,** 541-51.

ROSS, R. (1993). Atherosclerosis: current understanding of mechanisms and future strategies in therapy. *Transplant Proc,* 25**,** 2041-3.

ROSS, R. (1999). Atherosclerosis--an inflammatory disease. *N Engl J Med,* 340**,** 115-26.

SALTIEL, A. R. & KAHN, C. R. (2001). Insulin signalling and the regulation of glucose and lipid metabolism. *Nature,* 414**,** 799-806.

SANCHEZ-QUESADA, J. L., CAMACHO, M., ANTON, R., BENITEZ, S., VILA, L. & ORDONEZ-LLANOS, J. (2003). Electronegative LDL of FH subjects: chemical characterization and induction of chemokine release from human endothelial cells. *Atherosclerosis,* 166**,** 261-70.

SANO, M., KOREKANE, H., OHTSUBO, K., YAMAGUCHI, Y., KATO, M., SHIBUKAWA, Y., TAJIRI, M., ADACHI, H., WADA, Y., ASAHI, M. & TANIGUCHI, N. (2012). N-glycans of SREC-I (scavenger receptor expressed by endothelial cells): essential role for ligand binding, trafficking and stability. *Glycobiology,* 22**,** 714-24.

SAWAMURA, T., KUME, N., AOYAMA, T., MORIWAKI, H., HOSHIKAWA, H., AIBA, Y., TANAKA, T., MIWA, S., KATSURA, Y., KITA, T. &

MASAKI, T. (1997). An endothelial receptor for oxidized low-density lipoprotein. *Nature,* 386**,** 73-7.

SCARSELLI, E., ANSUINI, H., CERINO, R., ROCCASECCA, R. M., ACALI, S., FILOCAMO, G., TRABONI, C., NICOSIA, A., CORTESE, R. & VITELLI, A. (2002). The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *EMBO J,* 21**,** 5017-25.

SCAZZOCCHIO, B., VARI, R., D'ARCHIVIO, M., SANTANGELO, C., FILESI, C., GIOVANNINI, C. & MASELLA, R. (2009). Oxidized LDL impair adipocyte response to insulin by activating serine/threonine kinases. *J Lipid Res,* 50**,** 832-45.

SCHNEIDERHAN, W., SCHMID-KOTSAS, A., ZHAO, J., GRUNERT, A., NUSSLER, A., WEIDENBACH, H., MENKE, A., SCHMID, R. M., ADLER, G. & BACHEM, M. G. (2001). Oxidized low-density lipoproteins bind to the scavenger receptor, CD36, of hepatic stellate cells and stimulate extracellular matrix synthesis. *Hepatology,* 34**,** 729-37.

SCHNEIDERMAN, J., BORDIN, G. M., ADAR, R., SMOLINSKY, A., SEIFFERT, D., ENGELBERG, I., DILLEY, R. B., THINNES, T. & LOSKUTOFF, D. J. (1998). Patterns of expression of fibrinolytic genes and matrix metalloproteinase-9 in dissecting aortic aneurysms. *Am J Pathol,* 152**,** 703-10.

SCHWENKE, D. C. & CAREW, T. E. (1989). Initiation of atherosclerotic lesions in cholesterol-fed rabbits. I. Focal increases in arterial LDL concentration precede development of fatty streak lesions. *Arteriosclerosis,* 9**,** 895-907.

SEGREST, J. P., JONES, M. K., DE LOOF, H., BROUILLETTE, C. G., VENKATACHALAPATHI, Y. V. & ANANTHARAMAIAH, G. M. (1992). The amphipathic helix in the exchangeable apolipoproteins: a review of secondary structure and function. *J Lipid Res,* 33**,** 141-66.

SEGREST, J. P., JONES, M. K., DE LOOF, H. & DASHTI, N. (2001). Structure of apolipoprotein B-100 in low density lipoproteins. *J Lipid Res,* 42**,** 1346-67.

SHEIKINE, Y. & SIRSJO, A. (2008). CXCL16/SR-PSOX--a friend or a foe in atherosclerosis? *Atherosclerosis,* 197**,** 487-95.

SHETTY, S., WESTON, C. J., OO, Y. H., WESTERLUND, N., STAMATAKI, Z., YOUSTER, J., HUBSCHER, S. G., SALMI, M., JALKANEN, S., LALOR, P. F. & ADAMS, D. H. (2011). Common lymphatic endothelial and vascular endothelial receptor-1 mediates the transmigration of regulatory T cells across human hepatic sinusoidal endothelium. *J Immunol,* 186**,** 4147-55.

SHIH, P. T., ELICES, M. J., FANG, Z. T., UGAROVA, T. P., STRAHL, D., TERRITO, M. C., FRANK, J. S., KOVACH, N. L., CABANAS, C., BERLINER, J. A. & VORA, D. K. (1999). Minimally modified low-density lipoprotein induces monocyte adhesion to endothelial connecting segment-1 by activating beta1 integrin. *J Clin Invest,* 103**,** 613-25.

SHIMAOKA, T., KUME, N., MINAMI, M., HAYASHIDA, K., SAWAMURA, T., KITA, T. & YONEHARA, S. (2001). LOX-1 supports adhesion of Gram-positive and Gram-negative bacteria. *J Immunol,* 166**,** 5108-14.

SHIMAOKA, T., NAKAYAMA, T., FUKUMOTO, N., KUME, N., TAKAHASHI, S., YAMAGUCHI, J., MINAMI, M., HAYASHIDA, K., KITA, T., OHSUMI, J., YOSHIE, O. & YONEHARA, S. (2004). Cell surface-anchored SR-PSOX/CXC chemokine ligand 16 mediates firm adhesion of CXC chemokine receptor 6-expressing cells. *J Leukoc Biol,* 75**,** 267-74.

SIGURDARDOTTIR, V., FAGERBERG, B. & HULTHE, J. (2002). Circulating oxidized low-density lipoprotein (LDL) is associated with risk factors of the metabolic syndrome and LDL size in clinically healthy 58-year-old men (AIR study). *J Intern Med,* 252**,** 440-7.

SILVERSTEIN, R. L. & FEBBRAIO, M. (2009). CD36, a scavenger receptor involved in immunity, metabolism, angiogenesis, and behavior. *Sci Signal,* 2**,** re3.

SILVERSTEIN, R. L., LI, W., PARK, Y. M. & RAHAMAN, S. O. (2010). Mechanisms of cell signaling by the scavenger receptor CD36: implications in atherosclerosis and thrombosis. *Trans Am Clin Climatol Assoc,* 121**,** 206-20.

SIMPSON, C. F. & HARMS, R. H. (1983). Aortic atherosclerosis in nonlaying hens with fatty liver syndrome. *Avian Dis,* 27**,** 652-9.

SJOSTRAND, M., GUDBJORNSDOTTIR, S., HOLMANG, A., LONN, L., STRINDBERG, L. & LONNROTH, P. (2002). Delayed transcapillary transport of insulin to muscle interstitial fluid in obese subjects. *Diabetes,* 51**,** 2742-8.

SMITH, J. D., TROGAN, E., GINSBERG, M., GRIGAUX, C., TIAN, J. & MIYATA, M. (1995). Decreased atherosclerosis in mice deficient in both macrophage colony-stimulating factor (op) and apolipoprotein E. *Proc Natl Acad Sci U S A,* 92**,** 8264-8.

SONG, L., LEE, C. & SCHINDLER, C. (2011). Deletion of the murine scavenger receptor CD68. *J Lipid Res,* 52**,** 1542-50.

STARY, H. C. (1989). Evolution and progression of atherosclerotic lesions in coronary arteries of children and young adults. *Arteriosclerosis,* 9**,** I19-32.

STEINBERG, D., PARTHASARATHY, S., CAREW, T. E., KHOO, J. C. & WITZTUM, J. L. (1989). Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med,* 320**,** 915-24.

STEINBRECHER, U. P., PARTHASARATHY, S., LEAKE, D. S., WITZTUM, J. L. & STEINBERG, D. (1984). Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc Natl Acad Sci U S A,* 81**,** 3883-7.

STERN, M. P., WILLIAMS, K. & HUNT, K. J. (2005). Impact of diabetes/metabolic syndrome in patients with established cardiovascular disease. *Atheroscler Suppl,* 6**,** 3-6.

STEWART, C. R., STUART, L. M., WILKINSON, K., VAN GILS, J. M., DENG, J., HALLE, A., RAYNER, K. J., BOYER, L., ZHONG, R., FRAZIER, W. A., LACY-HULBERT, A., EL KHOURY, J., GOLENBOCK, D. T. & MOORE, K. J. (2010). CD36 ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimer. *Nature immunology,* 11**,** 155-61.

STOCKER, R. & KEANEY, J. F., JR. (2005). New insights on oxidative stress in the artery wall. *J Thromb Haemost,* 3**,** 1825-34.

SUARNA, C., DEAN, R. T., MAY, J. & STOCKER, R. (1995). Human atherosclerotic plaque contains both oxidized lipids and relatively large amounts of alpha-tocopherol and ascorbate. *Arterioscler Thromb Vasc Biol,* 15**,** 1616-24.

SUN, B., BOYANOVSKY, B. B., CONNELLY, M. A., SHRIDAS, P., VAN DER WESTHUYZEN, D. R. & WEBB, N. R. (2007). Distinct mechanisms for OxLDL uptake and cellular trafficking by class B scavenger receptors CD36 and SR-BI. *J Lipid Res,* 48**,** 2560-70.

SUPERKO, H. R. (2009). Advanced lipoprotein testing and subfractionation are clinically useful. *Circulation,* 119**,** 2383-95.

SUZUKI, H., KURIHARA, Y., TAKEYA, M., KAMADA, N., KATAOKA, M., JISHAGE, K., UEDA, O., SAKAGUCHI, H., HIGASHI, T., SUZUKI, T., TAKASHIMA, Y., KAWABE, Y., CYNSHI, O., WADA, Y., HONDA, M., KURIHARA, H., ABURATANI, H., DOI, T., MATSUMOTO, A., AZUMA, S., NODA, T., TOYODA, Y., ITAKURA, H., YAZAKI, Y., KODAMA, T. & ET AL. (1997). A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. *Nature,* 386**,** 292-6.

TABAS, I., WILLIAMS, K. J. & BOREN, J. (2007). Subendothelial lipoprotein retention as the initiating process in atherosclerosis: update and therapeutic implications. *Circulation,* 116**,** 1832-44.

TAKANABE-MORI, R., ONO, K., SOWA, N., WADA, H., TAKAYA, T., HORIE, T., SATOH-ASAHARA, N., SHIMATSU, A., FUJITA, M., SAWAMURA, T. & HASEGAWA, K. (2010). Lectin-like oxidized low-density lipoprotein receptor-1 is required for the adipose tissue expression of proinflammatory cytokines in high-fat diet-induced obese mice. *Biochem Biophys Res Commun,* 398**,** 576-80.

TAKAYA, T., WADA, H., MORIMOTO, T., SUNAGAWA, Y., KAWAMURA, T., TAKANABE-MORI, R., SHIMATSU, A., FUJITA, Y., SATO, Y., FUJITA, M., KIMURA, T., SAWAMURA, T. & HASEGAWA, K. (2010). Left ventricular expression of lectin-like oxidized low-density lipoprotein receptor-1 in failing rat hearts. *Circulation journal : official journal of the Japanese Circulation Society,* 74**,** 723-9.

TALL, A. R. & YVAN-CHARVET, L. (2015). Cholesterol, inflammation and innate immunity. *Nat Rev Immunol,* 15**,** 104-16.

TEDDER, T. F., STEEBER, D. A., CHEN, A. & ENGEL, P. (1995). The selectins: vascular adhesion molecules. *Faseb j,* 9**,** 866-73.

THAKKAR, S., WANG, X., KHAIDAKOV, M., DAI, Y., GOKULAN, K., MEHTA, J. L. & VARUGHESE, K. I. (2015). Structure-based Design Targeted at LOX-1, a Receptor for Oxidized Low-Density Lipoprotein. *Sci Rep,* 5**,** 16740.

THELEN, T., HAO, Y., MEDEIROS, A. I., CURTIS, J. L., SEREZANI, C. H., KOBZIK, L., HARRIS, L. H. & ARONOFF, D. M. (2010). The class A scavenger receptor, macrophage receptor with collagenous structure, is the major phagocytic receptor for Clostridium sordellii expressed by human decidual macrophages. *J Immunol,* 185**,** 4328-35.

THOMAS, H., SENKEL, S., ERDMANN, S., ARNDT, T., TURAN, G., KLEIN-HITPASS, L. & RYFFEL, G. U. (2004). Pattern of genes influenced by conditional expression of the transcription factors HNF6, HNF4alpha and HNF1beta in a pancreatic beta-cell line. *Nucleic Acids Res,* 32**,** e150.

THOMSEN, J. H., ETZERODT, A., SVENDSEN, P. & MOESTRUP, S. K. (2013). The haptoglobin-CD163-heme oxygenase-1 pathway for hemoglobin scavenging. *Oxidative medicine and cellular longevity,* 2013**,** 523652.

THORNE, A., LONNQVIST, F., APELMAN, J., HELLERS, G. & ARNER, P. (2002). A pilot study of long-term effects of a novel obesity treatment: omentectomy in connection with adjustable gastric banding. *Int J Obes Relat Metab Disord,* 26**,** 193-9.

TSIMIKAS, S., AIKAWA, M., MILLER, F. J., JR., MILLER, E. R., TORZEWSKI, M., LENTZ, S. R., BERGMARK, C., HEISTAD, D. D., LIBBY, P. & WITZTUM, J. L. (2007). Increased plasma oxidized phospholipid:apolipoprotein B-100 ratio with concomitant depletion of oxidized phospholipids from atherosclerotic lesions after dietary lipid-lowering: a potential biomarker of early atherosclerosis regression. *Arterioscler Thromb Vasc Biol,* 27**,** 175-81.

TWIGG, M. W., FREESTONE, K., HOMER-VANNIASINKAM, S. & PONNAMBALAM, S. (2012). The LOX-1 Scavenger Receptor and Its Implications in the Treatment of Vascular Disease. *Cardiol Res Pract,* 2012**,** 632408.

UZA, N., NAKASE, H., YAMAMOTO, S., YOSHINO, T., TAKEDA, Y., UENO, S., INOUE, S., MIKAMI, S., MATSUURA, M., SHIMAOKA, T., KUME, N., MINAMI, M., YONEHARA, S., IKEUCHI, H. & CHIBA, T. (2011). SR-PSOX/CXCL16 plays a critical role in the progression of colonic inflammation. *Gut,* 60**,** 1494-505.

WANG, X., PHILLIPS, M. I. & MEHTA, J. L. (2011). LOX-1 and angiotensin receptors, and their interplay. *Cardiovasc Drugs Ther,* 25**,** 401-17.

WANG, X., SUN, Y., YANG, H., LU, Y. & LI, L. (2016). Oxidized Low-Density Lipoprotein Induces Apoptosis in Cultured Neonatal Rat Cardiomyocytes by Modulating the TLR4/NF-kappaB Pathway. *Sci Rep,* 6**,** 27866.

WEN, Y. & LEAKE, D. S. (2007). Low density lipoprotein undergoes oxidation within lysosomes in cells. *Circ Res,* 100**,** 1337-43.

WHITE, S. J., SALA-NEWBY, G. B. & NEWBY, A. C. (2011). Overexpression of scavenger receptor LOX-1 in endothelial cells promotes atherogenesis in the ApoE(-/-) mouse model. *Cardiovasc Pathol,* 20**,** 369-73.

WHO (2014). The top 10 causes of death in the world, 2000 and 2012.

WIDMANN, C., GIBSON, S., JARPE, M. B. & JOHNSON, G. L. (1999). Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol Rev,* 79**,** 143-80.

WILLIAMS, K. J. & TABAS, I. (1995). The response-to-retention hypothesis of early atherogenesis. *Arterioscler Thromb Vasc Biol,* 15**,** 551-61.

WILLIAMS, M. J., POULTON, R. & WILLIAMS, S. (2002). Relationship of serum ferritin with cardiovascular risk factors and inflammation in young men and women. *Atherosclerosis,* 165**,** 179-84.

WINYARD, P. G., TATZBER, F., ESTERBAUER, H., KUS, M. L., BLAKE, D. R. & MORRIS, C. J. (1993). Presence of foam cells containing

oxidised low density lipoprotein in the synovial membrane from patients with rheumatoid arthritis. *Annals of the rheumatic diseases,* 52**,** 677-80.

WLAZLO, N., VAN GREEVENBROEK, M. M., FERREIRA, I., JANSEN, E. H., FESKENS, E. J., VAN DER KALLEN, C. J., SCHALKWIJK, C. G., BRAVENBOER, B. & STEHOUWER, C. D. (2013). Iron metabolism is associated with adipocyte insulin resistance and plasma adiponectin: the Cohort on Diabetes and Atherosclerosis Maastricht (CODAM) study. *Diabetes Care,* 36**,** 309-15.

WU, T., XIE, C., WANG, H. W., ZHOU, X. J., SCHWARTZ, N., CALIXTO, S., MACKAY, M., ARANOW, C., PUTTERMAN, C. & MOHAN, C. (2007). Elevated urinary VCAM-1, P-selectin, soluble TNF receptor-1, and CXC chemokine ligand 16 in multiple murine lupus strains and human lupus nephritis. *J Immunol,* 179**,** 7166-75.

XIE, J., MENDEZ, J. D., MENDEZ-VALENZUELA, V. & AGUILAR-HERNANDEZ, M. M. (2013). Cellular signalling of the receptor for advanced glycation end products (RAGE). *Cellular signalling,* 25**,** 2185-97.

XIE, Q., MATSUNAGA, S., NIIMI, S., OGAWA, S., TOKUYASU, K., SAKAKIBARA, Y. & MACHIDA, S. (2004). Human lectin-like oxidized low-density lipoprotein receptor-1 functions as a dimer in living cells. *DNA Cell Biol,* 23**,** 111-7.

XU, S., LIU, Z., HUANG, Y., LE, K., TANG, F., HUANG, H., OGURA, S., LITTLE, P. J., SHEN, X. & LIU, P. (2012). Tanshinone II-A inhibits oxidized LDL-induced LOX-1 expression in macrophages by reducing intracellular superoxide radical generation and NF-kappaB activation. *Transl Res,* 160**,** 114-24.

XU, S., OGURA, S., CHEN, J., LITTLE, P. J., MOSS, J. & LIU, P. (2013). LOX-1 in atherosclerosis: biological functions and pharmacological modifiers. *Cell Mol Life Sci,* 70**,** 2859-72.

YAMADA, Y., DOI, T., HAMAKUBO, T. & KODAMA, T. (1998). Scavenger receptor family proteins: roles for atherosclerosis, host defence and disorders of the central nervous system. *Cell Mol Life Sci,* 54**,** 628-40.

YAMAMOTO, K., KAKINO, A., TAKESHITA, H., HAYASHI, N., LI, L., NAKANO, A., HANASAKI-YAMAMOTO, H., FUJITA, Y., IMAIZUMI, Y., TOYAMA-YOKOYAMA, S., NAKAMA, C., KAWAI, T., TAKEDA, M., HONGYO, K., OGURO, R., MAEKAWA, Y., ITOH, N., TAKAMI, Y., ONISHI, M., TAKEYA, Y., SUGIMOTO, K., KAMIDE, K., NAKAGAMI, H., OHISHI, M., KURTZ, T. W., SAWAMURA, T. & RAKUGI, H. (2015). Oxidized LDL (oxLDL) activates the angiotensin II type 1 receptor by binding to the lectin-like oxLDL receptor. *FASEB J,* 29**,** 3342-56.

YAMAMOTO, N., TOYODA, M., ABE, M., KOBAYASHI, T., KOBAYASHI, K., KATO, M., MIYAUCHI, M., KIMURA, M., UMEZONO, T. & SUZUKI, D. (2009). Lectin-like oxidized LDL receptor-1 (LOX-1) expression in the tubulointerstitial area likely plays an important role in human diabetic nephropathy. *Internal medicine (Tokyo, Japan),* 48**,** 189-94.

YAMAUCHI, R., TANAKA, M., KUME, N., MINAMI, M., KAWAMOTO, T., TOGI, K., SHIMAOKA, T., TAKAHASHI, S., YAMAGUCHI, J., NISHINA, T., KITAICHI, M., KOMEDA, M., MANABE, T., YONEHARA, S. & KITA, T. (2004). Upregulation of SR-PSOX/CXCL16 and recruitment of CD8+ T cells in cardiac valves during inflammatory valvular heart disease. *Arterioscler Thromb Vasc Biol,* 24**,** 282-7.

YAMAYOSHI, S., FUJII, K. & KOIKE, S. (2012a). Scavenger receptor b2 as a receptor for hand, foot, and mouth disease and severe neurological diseases. *Front Microbiol,* 3**,** 32.

YAMAYOSHI, S., IIZUKA, S., YAMASHITA, T., MINAGAWA, H., MIZUTA, K., OKAMOTO, M., NISHIMURA, H., SANJOH, K., KATSUSHIMA, N., ITAGAKI, T., NAGAI, Y., FUJII, K. & KOIKE, S. (2012b). Human SCARB2-dependent infection by coxsackievirus A7, A14, and A16 and enterovirus 71. *J Virol,* 86**,** 5686-96.

YAN, S. D., CHEN, X., FU, J., CHEN, M., ZHU, H., ROHER, A., SLATTERY, T., ZHAO, L., NAGASHIMA, M., MORSER, J., MIGHELI, A., NAWROTH, P., STERN, D. & SCHMIDT, A. M. (1996). RAGE and amyloid-beta peptide neurotoxicity in Alzheimer's disease. *Nature,* 382**,** 685-91.

YAN, S. D., SCHMIDT, A. M., ANDERSON, G. M., ZHANG, J., BRETT, J., ZOU, Y. S., PINSKY, D. & STERN, D. (1994). Enhanced cellular oxidant stress by the interaction of advanced glycation end products with their receptors/binding proteins. *J Biol Chem,* 269**,** 9889-97.

YAO, D. & BROWNLEE, M. (2010). Hyperglycemia-induced reactive oxygen species increase expression of the receptor for advanced glycation end products (RAGE) and RAGE ligands. *Diabetes,* 59**,** 249-55.

YAO, F., SVENSJO, T., WINKLER, T., LU, M., ERIKSSON, C. & ERIKSSON, E. (1998). Tetracycline repressor, tetR, rather than the tetR-mammalian cell transcription factor fusion derivatives, regulates inducible gene expression in mammalian cells. *Hum Gene Ther,* 9**,** 1939-50.

YATES, M., KOLMAKOVA, A., ZHAO, Y. & RODRIGUEZ, A. (2011). Clinical impact of scavenger receptor class B type I gene polymorphisms on human female fertility. *Hum Reprod,* 26**,** 1910-6.

YEH, C. H., STURGIS, L., HAIDACHER, J., ZHANG, X. N., SHERWOOD, S. J., BJERCKE, R. J., JUHASZ, O., CROW, M. T., TILTON, R. G. & DENNER, L. (2001). Requirement for p38 and p44/p42 mitogen-activated protein kinases in RAGE-mediated nuclear factor-kappaB transcriptional activation and cytokine secretion. *Diabetes,* 50**,** 1495-504.

YOSHIDA, H., QUEHENBERGER, O., KONDRATENKO, N., GREEN, S. & STEINBERG, D. (1998). Minimally oxidized low-density lipoprotein increases expression of scavenger receptor A, CD36, and macrosialin in resident mouse peritoneal macrophages. *Arterioscler Thromb Vasc Biol,* 18**,** 794-802.

YOSHIDA, T., TSURUTA, Y., IWASAKI, M., YAMANE, S., OCHI, T. & SUZUKI, R. (2003). SRCL/CL-P1 recognizes GalNAc and a carcinoma-associated antigen, Tn antigen. *J Biochem,* 133**,** 271-7.

ZEIBIG, S., LI, Z., WAGNER, S., HOLTHOFF, H. P., UNGERER, M., BULTMANN, A., UHLAND, K., VOGELMANN, J., SIMMET, T., GAWAZ, M. & MUNCH, G. (2011). Effect of the oxLDL binding protein Fc-CD68 on plaque extension and vulnerability in atherosclerosis. *Circ Res,* 108**,** 695-703.

ZHANG, L., LIU, H. J., LI, T. J., YANG, Y., GUO, X. L., WU, M. C., RUI, Y. C. & WEI, L. X. (2008). Lentiviral vector-mediated siRNA knockdown of SR-PSOX inhibits foam cell formation in vitro. *Acta Pharmacol Sin,* 29**,** 847-52.

ZHANG, Q., LIU, J., LIU, J., HUANG, W., TIAN, L., QUAN, J., WANG, Y. & NIU, R. (2014). oxLDL induces injury and defenestration of human liver sinusoidal endothelial cells via LOX1. *J Mol Endocrinol,* 53**,** 281-93.

ZHANG, W. & LIU, H. T. (2002). MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Res,* 12**,** 9-18.

ZHANG, Y., ZANOTTI, I., REILLY, M. P., GLICK, J. M., ROTHBLAT, G. H. & RADER, D. J. (2003). Overexpression of apolipoprotein A-I promotes reverse transport of cholesterol from macrophages to feces in vivo. *Circulation,* 108**,** 661-3.

Appendix A

Publications and Conference proceedings

Publications (5):

Abdul Zani, I, Stephen, SL, Mughal, NA, Russel, DA, Homer-Vanniasinkam, S, Wheatcroft, SB and Ponnambalam, S. (2015) Scavenger receptor structure and function in health and disease. *Cells* 4(2):178-201.

De Siqueira, J, **Abdul Zani, I,** Russel, DA, Wheatcroft, SB, Ponnambalam, S and Homer-Vanniasinkam, S. (2015) Clinical and pre-clinical use of LOX-1-specific antibodies in diagnostics and therapeutics. *J Cardiovasc Transl Res* 8(8):458-465.

Smith, GA, Fearnley, GW, **Abdul Zani, I,** Wheatcroft, SB, Tomlinson, DC, Harrison, MA, and Ponnambalam, S. (2016) VEGFR2 trafficking, signalling and proteolysis is regulated by the ubiquitin isopeptidase USP8. *Traffic* 17(1):53-65.

Fearnley, GW, Smith, GA, **Abdul Zani, I,** Yuldasheva, N, Mughal, NA, Homer-Vanniasinkam, S, Kearney, MT, Zachary, IC, Tomlinson, DC, Harrison, MA, Wheatcroft, SB, and Ponnambalam, S. (2016) VEGF-A isoforms program differential VEGFR2 signal transduction, trafficking and proteolysis. *Biol Open* 5(5):571-583.

Smith, GA, Fearnley, GW, **Abdul Zani, I**, Wheatcroft, SB, Tomlinson, DC, Harrison, MA, and Ponnambalam, S. (2017) Ubiquitination of basal VEGFR2 regulates signal transduction and endothelial function. *Biol Open.*

Manuscripts in preparation (2):

Abdul Zani, I, Mughal, N, Stephen, SL, Yuldasheva, N, Fearnely, GW, Gage, MC, Skromna, A, Sawamura, T, Kearney, MT, Wheatcroft, SB, and Ponnambalam, S. (2017) LOX-1 scavenger receptor signal transduction and trafficking regulates atherosclerosis.

Lacey, K, Mandal, P, Sakonsiri, C, Vohra, RS, **Abdul Zani, I**, Stephen, SL, Smith, D, Wheatcroft, SB, Homer-Vanniasinkam, S, Turnbull, WB, and Ponnambalam, S. (2017) LOX-1 scavenger receptor binding to oxidised LDL particles is mediated by recognition of galactose-containing carbohydrates.

Meeting presentations & participation:

Oral presentations:

Abdul Zani, I, Wheatcroft, SB, Ponnambalam, S. LOX-1 scavenger receptor signal transduction and trafficking in atherosclerosis. *North of England Cell Biology (NECB) meeting, Norcroft Centre, University of Bradford, UK*: **13th Sep. 2016 (20 min talk)**

Abdul Zani, I, Wheatcroft, SB, Ponnambalam, S. Oxidized LDL and LOX-1 scavenger receptor regulation of pro-atherogenic signal transduction. *Faculty of Biological Sciences Postgraduate Symposium, University of Leeds, UK*: **6th-7th July 2016 (10 min talk)**

Chair sessions:

North of England Cell biology (NECB) meeting, University of York, UK. **7th Sept 2015.**

Poster presentations:

Abdul Zani, I, Mughal, NA, Stephen, SL, Homer-Vanniasinkam, S, Wheatcroft, SB, Ponnambalam, S. Oxidized LDL and LOX-1 scavenger receptor in signal transduction and atherosclerosis. *British Atherosclerosis*

Society (BAS) conference, Murray Edwards College, University of Cambridge, UK: **15th-16th Sep. 2016.**

Abdul Zani, I, Mughal, NA, Stephen, SL, Homer-Vanniasinkam, S, Wheatcroft, SB, Ponnambalam, S. Oxidized LDL and LOX-1 scavenger receptor in signal transduction and atherosclerosis. *North of England Cell Biology (NECB) meeting, Norcroft Centre, University of Bradford, UK*: **13th Sep. 2016.**

Abdul Zani, I, Mughal, NA, Stephen, SL, Homer-Vanniasinkam, S, Wheatcroft, SB, Ponnambalam, S. Oxidized LDL and LOX-1 scavenger receptor in signal transduction and atherosclerosis. *British Society for Cardiovascular Research (BSCR), University of Leeds, UK*: **5th-6th Sep. 2016.**

Abdul Zani, I, Wheatcroft, SB, Ponnambalam, S. The LOX-1 scavenger receptor regulates pro-inflammatory signal transduction pathways. *Northern Cardiovascular Research Group (NCRG) meeting, University of Leeds, UK*: **14th Apr. 2016.**

Abdul Zani, I, Wheatcroft, SB, Ponnambalam, S. The LOX-1 scavenger receptor regulates pro-inflammatory signal transduction pathways. *British Atherosclerosis Society (BAS) conference, Murray Edwards College, University of Cambridge, UK*: **10th-11th Sep. 2015.**

Abdul Zani, I, Wheatcroft, SB, Ponnambalam, S. LOX-1 scavenger receptor regulating pro-inflammatory signal transduction. *North of England Cell Biology (NECB) meeting, University of York, UK*: **7th Sep. 2015.**

Abdul Zani, I, Wheatcroft, SB, Ponnambalam, S. LOX-1 scavenger receptor regulating pro-inflammatory signal transduction. *Faculty of Biological Sciences Postgraduate Symposium, University of Leeds, UK*: **7th-8th July 2015.**

Abdul Zani, I, Wheatcroft, SB, Ponnambalam, S. LOX-1-mediated intracellular signalling pathway. *Faculty of Biological Sciences Postgraduate Symposium, University of Leeds, UK*: **9th-10th Apr 2014.**

Appendix B

Supplementary Figures

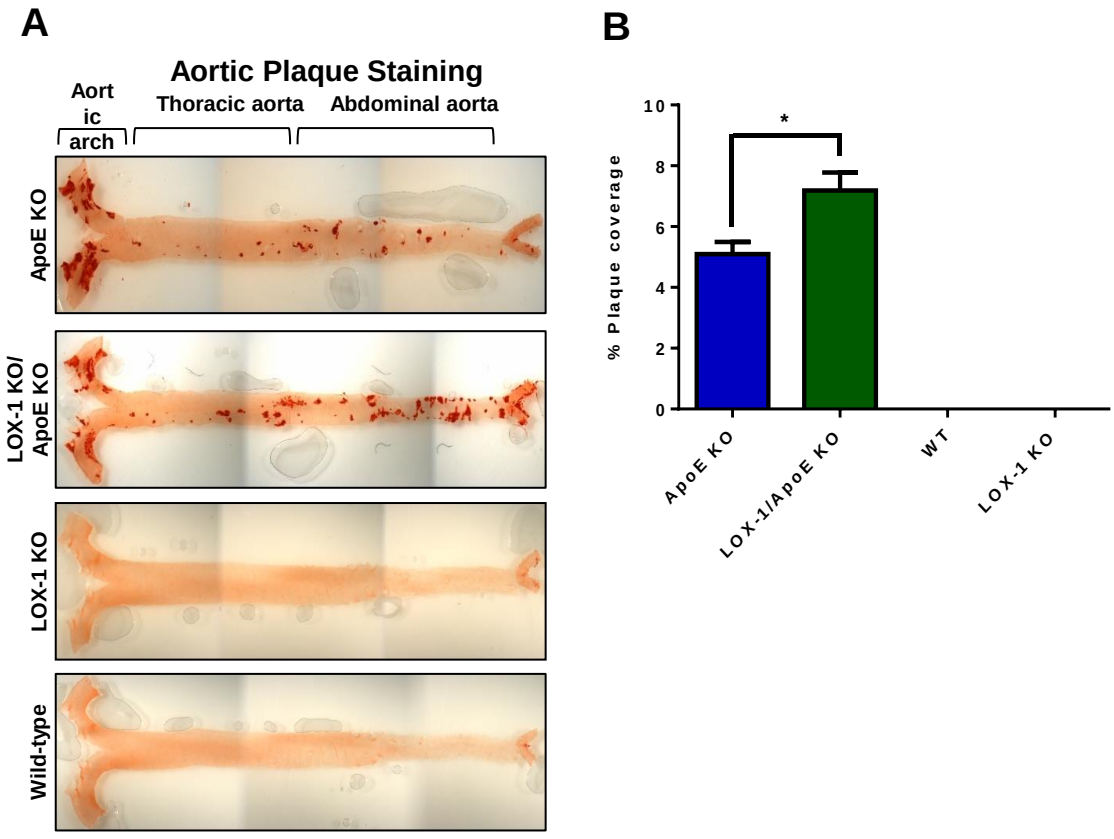

Figure B1. Loss of functional LOX-1 modulates plaque formation. (A) Wild-type and transgenic mice fed on a lipid- and fat-rich diet had aortas harvested and stained with Oil Red O to visualize plaques. (B) The plaque surface area for the different groups measured as % of total aortic surface area. Error bars denote ±SEM (n=4-12), with significance indicated as: *, $p<0.05$; **, $p<0.01$. **Data provided by Nadeem Mughal (University of Leeds, UK).**

Appendix B

Supplementary Figures

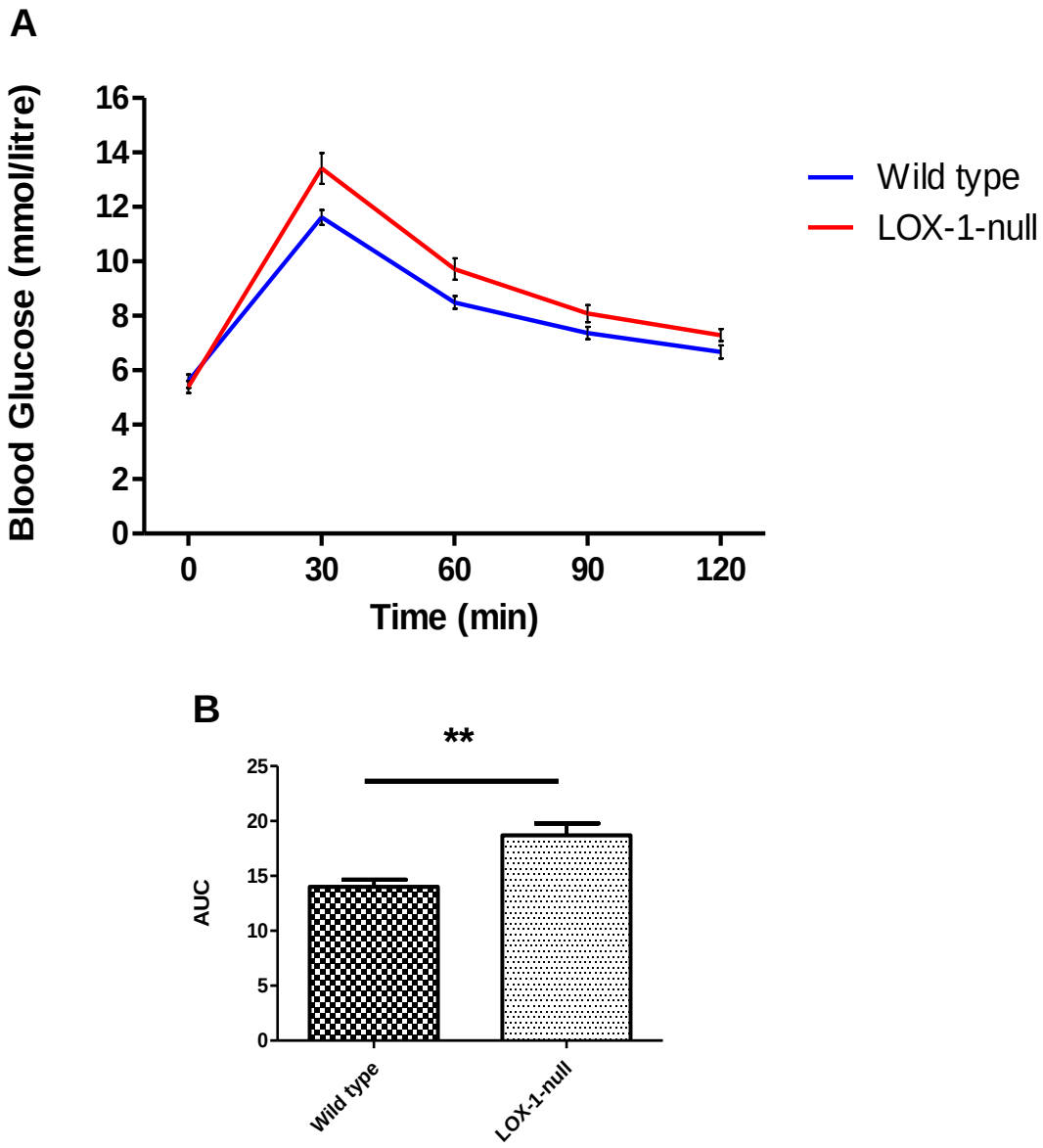

Figure B2. *LOX-1* influence on glucose tolerance. (A) Wild type and *LOX-1*-null mice were injected with a weight-specific bolus of glucose and blood glucose measured at 0 (baseline), 30, 60, 90 and 120 min post-treatment. (B) Area under the curve (AUC) analysis was used to calculate the difference between groups. Error bars denote ± SEM (n=12; ** $p<0.01$). **Data provided by Nadeem Mughal (University of Leeds, UK).**