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REVIEW

Mitochondrial function and regulation of macrophage sterol metabolism and inflammatory responses

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Abstract

The aim of this review is to explore the role of mitochondria in regulating macrophage sterol homeostasis and inflammatory responses within the aetiology of atherosclerosis. Macrophage generation of oxysterol activators of liver X receptors (LXRs), *via* sterol 27-hydroxylase, is regulated by the rate of flux of cholesterol to the inner mitochondrial membrane, *via* a complex of cholesterol trafficking proteins. Oxysterols are key signalling molecules, regulating the transcriptional activity of LXRs which coordinate macrophage sterol metabolism and cytokine production, key features influencing the impact of these cells within atherosclerotic lesions. The precise identity of the complex of proteins mediating mitochondrial cholesterol trafficking in macrophages remains a matter of debate, but may include steroidogenic acute regulatory protein and translocator protein. There is clear evidence that targeting either of these proteins enhances removal of cholesterol *via* LXRα-dependent induction of ATP binding cassette transporters (ABCA1, ABCG1) and limits the production of inflammatory cytokines; interventions which influence mitochondrial structure and bioenergetics also impact on removal of cholesterol from macrophages. Thus, molecules which can sustain or improve mitochondrial structure, the function of the electron transport chain, or increase the activity of components of the protein complex involved in cholesterol transfer, may therefore have utility in limiting or regressing atheroma development, reducing the incidence of coronary heart disease and myocardial infarction.

Key words: Atherosclerosis; Macrophage; Cholesterol; High density lipoproteins; Apolipoproteins; ATP binding cassette transporters; Scavenger receptor B1; Mitochondria (dys)function; Sterol 27-hydroxylase; Liver X receptors

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Core tip: Mitochondrial cholesterol trafficking to CYP27A1 located on the inner mitochondrial membrane regulates the formation of oxysterol ligands for liver X receptors (LXRs) in sterol-laden macrophage "foam" cells. In turn, ligation of LXRα has profound implications for sterol removal and inflammatory responses in macrophage "foam" cells, both factors which may contribute to the effective resolution of atherosclerotic lesions and reductions in the incidence of coronary heart disease and its sequelae.

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INTRODUCTION

Coronary heart disease (CHD) is the major cause of morbidity and mortality worldwide, and the single largest cause of disease burden, determined according to disability-adjusted life years, the sum of life lost and years lived with disability[1,2]. Genetic factors contribute to coronary heart disease, fuelled by behavioural (smoking, physical inactivity, unhealthy diet, excess alcohol intake), metabolic (hypertension, diabetes, elevated serum cholesterol, overweight and obesity) and environmental (poverty, stress, educational status) factors[1-3].

Atherosclerosis is the primary cause of coronary heart disease characterised by chronic and unresolved inflammatory responses at sites of perturbed laminar blood flow in large and medium-sized arteries[4-6]. Activation of the arterial endothelial layer allows the accumulation of low density lipoprotein (LDL) within the intima of the vessel, where it can become modified *via* oxidation or crosslinking, triggering the recruitment of monocytes, neutrophils, lymphocytes and circulating stem cells to sites of inflammation[4-6]. Within this complex microenvironment, monocytes differentiate into macrophages which lie within a broad phenotypic spectrum, ranging from pro- (M1) to anti-inflammatory (M2)[6].

Arterial macrophages become laden with excess cholesterol and cholesteryl esters, part *via* the unregulated uptake of modified LDL by scavenger receptors (*e.g.*, CD36, CD68, LOX-1 and SR-AI/AII), and by phagocytosis of apoptotic cells, resulting in formation of "foam cells", a hallmark of early "fatty streak", developing, and unstable atherosclerotic lesions[7-10]. During the early phase of lesion development, this process may represent a protective mechanism; however, in more advanced lesions, cholesterol-laden macrophages, by releasing inflammatory cytokines and matrix metalloproteinases, contribute to chronic unresolved inflammation[10], accelerating the disease process and acute thrombotic events such as cerebrovascular stroke or myocardial infarction.

Thus, removal of cholesterol from macrophage "foam cells" may achieve successful regression and stabilisation of atheroma, and the importance of this pathway in protecting against CHD is supported by epidemiological studies in humans, and in genetically modified mice in which components of this pathway have been overexpressed or deleted. For example, HDL-cholesterol (HDL-C) emerged as an independent risk factor for cardiovascular disease in the Framingham Heart Study, offering a risk reduction of 2%-3% for each 1 mg/dL increase in HDL-C concentration[11,12]. HDL particles also possess antioxidant, anti-thrombotic and pro-fibrinolytic properties, and can counteract the chronic inflammation[13-16], proliferation of haematopoietic stem cells[17] and leucocytosis[10,18] which promote atherosclerosis. However, increasing the level of HDL-C, with niacin[19,20], fibrates[20] or dolcetrapib (dal-OUTCOMES Ⅲ trial)[20,21], does not necessarily confer protection against CHD[19-21] and in patients with systemic inflammation, coronary heart disease, chronic renal disease or diabetes, the protective properties of HDL are lost, and the particles transformed into those with pro-atherogenic potential[22-24]. Thus, it is not just the level, but the quality, composition (including levels of cargo molecules such as sphingosine-1-phosphate)[25] and function of HDL particles that are important.

Some, but not all, of the beneficial effects associated with HDL are mediated *via* the interaction of ATP binding cassette (ABC) transporters, such as ABCA1, ABCG1 and ABCG4, with apolipoproteins and HDL (Figure 1). While ABCA1 promotes efflux of cholesterol and phospholipids to lipid-poor apolipoproteins, such as apoA-I and apoE[13], ABCG1 and ABCG4 promote efflux of cholesterol, oxysterols and desmosterol to HDL[26]. Thus, these transporters together in a sequential manner to generate nascent HDL, which can then mature to HDL_3 and HDL_2 within the reverse cholesterol transport pathway in the bloodstream[25].

Both rare and common genetic variations in ABCA1 influence the levels of HDL-C[26] and risk of ischaemic heart disease (IHD). However, the association between ABCA1 variants and coronary disease seem to be independent of the plasma level of HDL-C[27]. Instead, cholesterol efflux from macrophages is strongly linked to atherosclerosis and provides a novel way of assessing cardiovascular risk that provides a greater level of prediction than HDL-C[28]. Thus the expression and activity of the ABCA1 protein, and the quality and functionality of the nascent HDL generated, may prove valuable discriminants of the risk of cardiovascular disease[29].

Importantly, macrophage ABCA1 expression and cholesterol accumulation are intrinsically linked to the inflammatory status of these cells. Excess cholesterol proves cytotoxic and pro-inflammatory if recycling *via* ABCA1 is disrupted in macrophages[30-33]. Enhanced Toll-like receptor signalling is noted in ABCA1/ABCG1 null macrophages, resulting in increased expression of pro-inflammatory genes, and free cholesterol accumulation[34], while activation of Toll-like receptors 3 and 4 represses induction of ABCA1 and reduces macrophage cholesterol efflux[35]. Conversely, interleukin-6 (IL-6) attenuates pro-inflammatory responses and stimulates efflux of cholesterol *via* ABCA1 in human macrophages[36]. In good agreement with this integrated paradigm, macrophage ABCA1 limits inflammatory responses *via* ApoA-I dependent activation of the Jak2/Stat3 pathway[37,38], while macrophage sterol

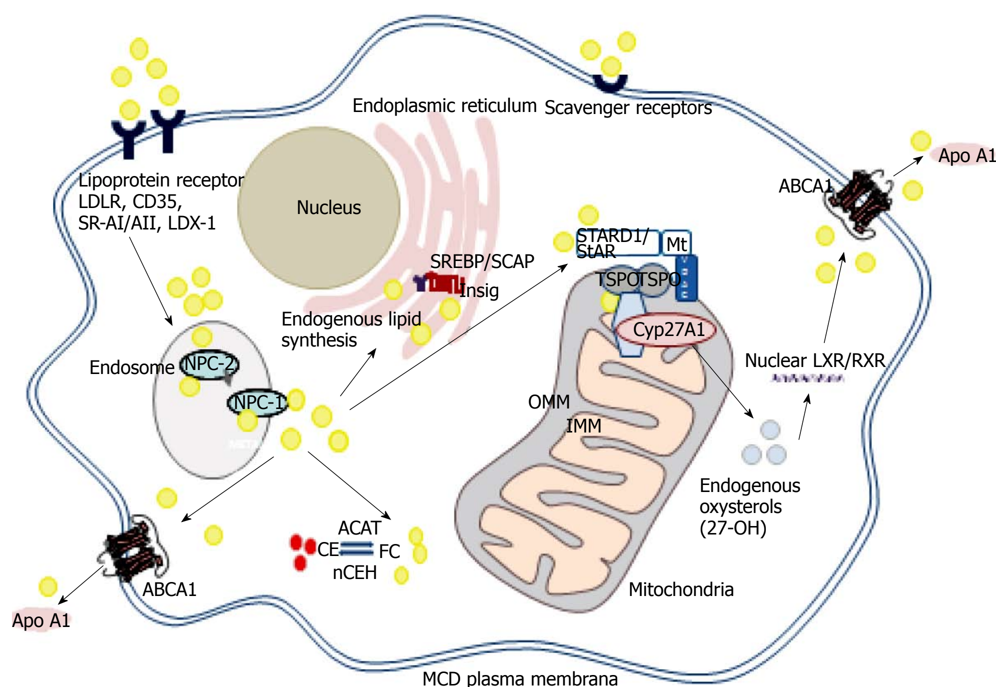

Figure 1 The role of mitochondrial cholesterol trafficking in regulation of macrophage sterol metabolism. Increased expression of steroidogenic acute regulatory protein (StAR, STARD1) or 18 kDa translocator protein (TSPO) drive cholesterol trafficking to mitochondrial sterol 27-hydroxylase (CYP27A1), enhancing endogenous production of oxysterols (24-, 25- and 27-hydroxycholesterol), in turn activating liver X receptors (LXR) and enhancing cholesterol efflux to apolipoprotein A-I (Apo AI) *via* ATP binding cassette transporter A1 (ABCA1). One current model for cholesterol transfer from the outer (OMM) to inner (IMM) mitochondrial membrane, derived from studies in steroidogenic cells, involves a complex of proteins, including StAR, TSPO, voltage-dependent anion channel (VDAC), regulatory subunits of protein kinase A (PKA-R1α), acyl CoA binding domains-1 and -3, ATPase family AAA domain-containing protein 3A (ASTAD3A) and optic atrophy type 1 proteins. Exogenous cholesterol delivered to the endocytic pathway *via* lipoprotein or scavenger receptors is transported either to the plasma membrane, enhancing cholesterol efflux *via* ABCA1, to lipid poor acceptors such as Apo AI or Apo E, or delivered to the endoplasmic reticulum (ER), retaining the Sterol Regulatory Element Binding Protein (SREBP)/SREBP-cleavage activating protein (SCAP) complex, in turn reducing cholesterol biosynthesis. Oxysterols enhance this process by binding to Insig-1/2 (insulin-induced gene-1 or -2). Excess cholesterol is esterified *via* Acyl CoA: Cholesterol Acyltransferase-1 (ACAT-1), and stored in lipid droplets within the cytoplasm as "foamy" droplets. nCEH: Neutral cholesteryl ester hydrolase; FC: Free cholesterol; CE: Cholesteryl ester: NPC-1/NPC-2: Niemann-Pick C1/C2 protein; StAR: Steroidogenic acute regulatory protein: RXR: Retinoic acid receptor.

accumulation activates Liver X Receptor nuclear (LXR) transcription factors, achieving induction of ABCA1 and ABCG1 and repression of inflammation (below)[39,40].

MACROPHAGE LIPID METABOLISM AND INFLAMMATION ARE REGULATED BY LIVER X RECEPTORS

Activation of nuclear LXRs (LXRα/β) is marshals cellular responses to increasing levels of sterol, promoting cholesterol efflux (above)[39-43]. Liver X receptors form heterodimeric complexes with retinoic acid receptors (RXRs), and bind to imperfect direct repeats of the nuclear receptor half-site TGACCT[39-43]. Ligand binding dissociates co-repressor proteins, destined for ubiquitination and proteasomal degradation, and engages co-activator proteins such as histone demethylases and G-protein pathway suppressor-2 (GPS2), stimulating target gene transcription[44].

Activation of LXRα also represses cholesterol biosynthesis *via* novel negative LXR DNA-response elements within the promoter region of squalene synthase and lanosterol 14α-demethylase and suppresses uptake of LDL[45,46]. Oxysterols also bind to Insig-1/2, facilitating sequestration of sterol-regulatory element binding proteins (SREBPs) at the endoplasmic reticulum, ensuring repression of cholesterol biosynthesis and uptake[45]. Deletions of LXRα and LXRβ in murine models of atheroma cause lipid accumulation within the aortic root, even in the absence of an atherogenic diet[47,48].

It is also evident that LXRs modulate innate and adaptive immune responses mediated by macrophages, neutrophils, lymphocytes, neutrophils and dendritic cells[45], decreasing cytokine-mediated expression of a range of pro-inflammatory genes. This is achieved *via* a mechanism involving nuclear receptor co-repressor (NCoR), silencing mediator of retinoid and thyroid receptors (SMRT) and inhibition of nuclear factor kappa B (NFκB) signalling[45,48,49]. Activation of LXRs is also achieved by phagocytosis of apoptotic cells by macrophages increasing expression of receptor tyrosine kinase (*Mertk*), amplifying phagocytosis and cell clearance, and reducing production of inflammatory mediators[50]. Absence of LXR signalling enhances the apoptosis of macrophages challenged with *Listeria monocytogenes*, *Escherichia coli* or *Salmonella typhinurium*, *via* loss of the anti-apoptotic factor AIM/

Spα[51,52].

MACROPHAGE GENERATION OF OXYSTEROL LIGANDS FOR LIVER X RECEPTORS

High levels of mitochondrial sterol 27-hydroxylase (CYP27A1) are found in human macrophages, and this enzyme can produce modified sterols, proven to act as LXR ligands *in vitro* and *in vivo*[53-56]. Loss of CYP27A1 leads to the lipid storage disease, cerebrotendinous xanthomatosis (CTX), which triggers accumulation of cholesterol and cholestanol in brain and tendons, progressive neurological deterioration, xanthomas and, as a secondary complication, accelerated atherosclerosis[57,58].

The rate-limiting step controlling CYP27A1 activity is the flux of cholesterol from the outer to the inner mitochondrial membrane, *via* a mitochondrial cholesterol trafficking complex (discussed below)[59]. Mitochondrial oxysterols therefore act as key cell signalling molecules, the levels of which can be moderated by sulfation (SULT2B1b), esterification (ACAT-1) or metabolism to soluble bile acid derivatives[53]. Conceivably, this process could be "uncoupled" by accumulation of free cholesterol at the interface between endoplasmic reticulum (ER) and mitochondrial membranes, triggering ER stress and proteasomal degradation of ABCA1, and opening of the permeability transition pore in mitochondria[53]. Esterification of excess oxysterols may then result: over 85% of the 27-hydroxycholesterol in human atherosclerotic lesions is esterified and incapable of activating LXRα and its downstream pathways[60,61]. Loss of this protective pathway predicates mitochondrial damage, apoptosis and cytotoxicity, features associated with addition of exogenous atheroma-relevant oxysterols ($\geqslant$ 20 μmol/L) to cultured cells[62].

Thus, it is clear that the biological impact of oxysterols are not solely restricted to LXR activation[63-67]. For example, oxysterols also serve as endogenous ligands for G-protein coupled receptor 183 (Epstein-Barr virus-induced gene 2, *EBI2*)[63], function as selective estrogen receptor modulators[64], bind to the Smoothened molecule to modulate Hedgehog signalling[65], while CYP27A1-derived 7α and 7β, 27-hydroxycholesterol modify innate and adaptive immune responses by acting as agonists of retinoic acid-related (RAR) orphan receptor gamma t (ROPRγT)[66].

Acute exposure of macrophages to exogenous oxysterols induce rapid (< second) oscillations in cytoplasmic [Ca^{2+}] triggered by influx from the extracellular medium, followed by sustained increases in [Ca^{2+}] mediated by translocation of TRPC1 (transient receptor potential, canonical) channels into lipid rafts in the plasma membrane[68]. Calcium transfer between ER and mitochondria is facilitated by mitochondria-associated membranes, which act as a hub for lipid transfer, regulation of mitochondrial morphology (fission, fusion and trafficking), apoptosis, autophagy and ER stress[69], although the role of endogenously generated oxysterols in these processes remains unknown at present. Certainly, chronic exposure to exogenous oxysterol congeners can activate calcium release from the ER, increasing dephosphorylation of Bcl-2 antagonist of cell death by the calcium-dependent phosphatase calcineurin, and promoting apoptosis[68].

TARGETING PROTEIN CONSTITUENTS OF THE MITOCHONDRIAL CHOLESTEROL TRAFFICKING COMPLEX: IMPACT ON MACROPHAGE STEROL METABOLISM AND INFLAMMATION

Despite intensive investigations in steroidogenic cells and tissues, the nature of the mitochondrial cholesterol trafficking complex remains a matter of debate. One recent model suggests a basal complex, forming contact sites between the outer and inner mitochondrial membranes, composed of the 18 kDa translocator protein (TSPO), adenine nucleotide transporter (ANT) and voltage-dependent anion channel (VDAC)[70-72]. In hormone-stimulated steroidogenic tissues, a "transduceosome" complex is formed, involving recruitment of the regulatory subunits of protein kinase A (PKA-R1α) and acyl CoA binding domain proteins-1 and -3. Elevated levels of cyclic adenosine monophosphate (cAMP) release PKA catalytic subunits to phosphorylate 37 kDa steroidogenic acute regulatory protein at the outer mitochondrial membrane; import of both StAR and cholesterol into the inner mitochondrial membrane and matrix facilitate both proteolytic processing of StAR to its 30 kDa form, and conversion of cholesterol into pregnenolone by CYP11A1[70-72]. However, a dynamic 800 kDa bioactive protein complex in steroidogenic cells has also been described, which does not involve ANT, but is composed of TSPO, VDAC, CYP11A1, ATPase family AAA domain-containing protein 3A (ASTAD3A) and optic atrophy type 1 proteins[73]; in this model, StAR facilitated binding of cholesterol to the 800 kDa complex, enhancing steroidogenesis.

Importantly, there is a growing realisation that key mitochondrial cholesterol trafficking proteins, such as StAR, play an important role in non-steroidogenic tissues[74]. This, combined with conflicting results regarding the impact of genetic deletion of TSPO on steroidogenesis and viability in mice[75-78], may lead to increased consideration of alternate functions for these proteins[74]. For example, StAR is expressed in endothelial cells, monocytes and macrophages[79-82], albeit at levels far lower than those found in adrenal or gonadal tissues[74]. By contrast, other components of the mitochondrial trafficking complex, such as TSPO, are widely expressed in a variety of tissues, including macrophages[78,81].

Importantly, both StAR and TSPO appear to impact on macrophage lipid and inflammatory phenotype, in

part *via* the pathway involving sterol 27-hydroxylase, activation of LXRα and upregulation of ABCA1/ABCG1 mRNA and protein[81-83], arguing a functional role for these proteins in mediating cholesterol supply to CYP27A1. Overexpression of StAR decreased macrophage lipid content[82,83], repressed inflammation[82] and apoptosis[84] and increased macrophage cholesterol efflux[82,83], while a viral vector expressing StAR reduced aortic lipids and atheroma in apoE$^{-/-}$ mice[85]. However, exploiting any putative anti-atherogenic properties of StAR could prove problematic, due to the associated induction of lipogenesis in macrophages[83,86], presumably *via* LXRα dependent induction of *Srebp1c*[87].

This led to focus on other components of the mitochondrial cholesterol trafficking complex and, in particular, TSPO[81]. Transient overexpression of TSPO in human (THP-1) macrophages increased the levels of ABCA1 mRNA and protein, and enhanced efflux of cholesterol to apoA-I, HDL and human serum, a finding reversed by gene knockdown of TSPO. Small molecule TSPO ligands also increased cholesterol efflux, an effect that was amplified in macrophages genetically engineered to overexpress TSPO[81]. Notably, TSPO overexpression caused a decline in macrophage total neutral lipid mass, without induction of lipogenesis, and effectively prevented "foam cell" formation following exposure to modified LDL[81]. These effects were associated with induction of both LXRα and PPARα the latter providing a plausible mechanism for the observed reductions in macrophage lipid mass[81]. Notably, overexpression of some of the other proposed components of the mitochondrial cholesterol trafficking complex, such as VDAC, ANT and ACBD1, discussed above, exerted minimal effects on the macrophage cholesterol efflux pathway[81].

Expression of TSPO is upregulated by exposure to modified LDL in human macrophages[81], and TSPO ligands have been used to image vascular inflammation in CD68 positive macrophages at sites of disturbed flow in murine carotid arteries[88], and macrophage burden[89] and intraplaque inflammation[90] within human carotid atherosclerotic lesions. Despite this evident association with inflammation, it appears that upregulation of TSPO, or signalling *via* this protein, may represent an adaptive mechanism designed to limit tissue damage. Overexpression of TSPO in microglia decreased production of pro-inflammatory cytokines, reflected in increased expression of alternately activated M2 stage-related genes and mediated *via* repression of NF-κB activation[91]. Similarly, TSPO ligands inhibited the proliferation of retinal microglial cells, and repressed the output of reactive oxygen species and TNFα[92]. In good agreement, levels of TSPO are higher in dystrophic murine retina, and in microglia treated with LPS, while TSPO ligand XBD173 repressed the expression of chemokine (C-C motif) ligand 2 (CCL2), IL-6 and iNOS[93]. The TSPO ligand, PK11195 has proved effective in ameliorating the severity of disease in an experimental murine model of multiple sclerosis, by reducing inflammatory responses and promoting oligodendroglial regeneration[94]. TSPO has also been posited as a novel target for Alzheimer's disease[95], anxiety, psychiatric and neurologic disorders[96-99], pain[100], cancer[101] and vascular dysfunction[88-90,102]. At present, it is not known how many of these effects are related to the cholesterol trafficking function of TSPO, although LXRs influence expression of an array of genes involved in cholesterol homeostasis, glucose metabolism, inflammation and Alzheimer's disease[103]. It is also clear that some of the reported effects of TSPO and its ligands may require re-evaluation, given the lack of phenotype recently reported in healthy TSPO$^{-/-}$ mice[75,76].

MITOCHONDRIAL STRUCTURE AND BIOENERGETICS: IMPACT ON CHOLESTEROL HOMEOSTASIS

Mitochondria exhibit constant movement, fusion and fission[104]. The mitochondrial membrane protein mitofusin (Mfn2) is involved in maintaining mitochondrial morphology, energy provision, and cellular growth and apoptosis[105-107]. Recently, Mfn2 has emerged as a regulator of macrophage cholesterol efflux, *via* upregulation of peroxisome proliferator activated receptor-γ (PPARγ) ABCA1, ABCG1 and scavenger receptor-B1 (SR-B1), reflected in marked reductions in cholesterol mass[107]. Overexpression of Mfn2 attenuates the formation of atherosclerotic lesions in rabbit carotid arteries, and levels of Mfn2 are progressively reduced during lesion formation in apoE$^{-/-}$ mice during atherogenesis; levels of Mfn2 are also reduced in atherosclerotic, compared with non-atherosclerotic, human arteries[107].

Remodelling of the inner mitochondrial membrane by optic atrophy 1 (OPA1) also alters the efficiency of mitochondrial cholesterol trafficking, at least in steroidogenic cells[108,109]. Increased steroidogenesis is reported in trophoblasts undergoing syncytilisation, which express increased levels of the pro-fission mitochondrial shaping protein Drp1 increased, and decreased levels of Opa1 and mitofusin. An inverse relationship between levels of Opa1 and steroidogenesis were also evidenced in cells genetically manipulated to express higher levels of Opa1, while accumulation of cholesterol at the inner mitochondrial membrane was observed in mitochondria lacking Opa1[108,109].

Finally, it is self-evident that ATP is needed to mount an effective non-adaptive immune response, and to fuel cholesterol biosynthesis and the activity of ABC transporters that determine the rate of macrophage cholesterol efflux. However, more subtle changes in mitochondrial function or loss of bioenergetic capacity, the emerging concept of the Bioenergetic Health Index (BHI)[110], have been shown to reduce the efficiency of mitochondrial cholesterol trafficking and hormone biosynthesis in steroidogenic tissues[111,112]. Dissipation of the mitochondrial membrane potential ($\Delta\psi_m$ using carbonyl cyanide *m*-chlorophenylhydrazone), inhibition of electron transport at complex Ⅲ (using antimycin), reduction of pH (nigericin) and inhibition

of ATP synthase (oligomycin) blocked the formation of progesterone and synthesis or import of StAR protein in Leydig cells[111,112].

A parallel study in macrophages supports the notion that acute loss of mitochondrial function is also associated with dysregulated cholesterol homeostasis[113]. Cholesterol efflux was inhibited by nigericin and oligomycin in RAW 264.7 macrophages; levels of ABCA1 protein decreased in response to oligomycin treatment, despite paradoxical increases in *Abca1* mRNA[113,114], reflecting findings in carotid atherosclerotic lesions[114] Further, while oligomycin treatment did not alter cholesterol biosynthesis, cholesterol esterification was significantly inhibited, promoting apoptosis. Oligomycin induced expression of genes involved in cholesterol efflux (*Abca1, Abcg4, Stard1*) and cholesterol biosynthesis *(Hmgr, Mvk, Scap, Srepb2*) arguing that loss of coordinated regulation of sterol homeostasis is caused by loss of mitochondrial ATP generation[113]. In turn, accumulation of free cholesterol or fatty acids can trigger mitochondrial dysfunction, which could promote inflammation *via* loss of LXRα-dependent repression of NF-κB (above) and upregulation of cytokine expression, but also by NLRP3 inflammasome-dependent and –independent pathways[115].

QUESTIONS FOR THE FUTURE

This review summarizes the current evidence that, in part, macrophage sterol homeostasis, and inflammatory responses, can be linked to mitochondrial cholesterol trafficking, and mitochondrial structure and bioenergetics. Whether proteins involved in mitochondrial structure, fission, fusion or organelle dynamics can also impact on these processes is currently uninvestigated and an area of keen interest. More particularly, mitochondria-mediated hormetic effects in aging[116,117] suggest a retrograde signalling pathway by which mitochondrial dysfunction in a single distinct tissue elicits the mitochondrial stress response in some (but not all) distal tissues. In turn, this suggests that loss of effective mitochondrial function, such as that caused by hepatic insulin resistance for example, may be transmitted *via* "mitokines" to peripheral tissues, promoting vascular dysfunction and cardiovascular disease. These exciting findings offer some intriguing possibilities for therapeutic strategies aimed at sustaining or improving mitochondrial function.

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