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Taylor, Janice; Allen, Anne Marie; Graham, Ann

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Targeting mitochondrial 18kDa Translocator protein (TSPO) regulates macrophage cholesterol efflux

Janice M W Taylor, Anne-Marie Allen and Annette Graham*

Department of Life Sciences *and* the Diabetes Research Group, Institute for Applied Health Research, Glasgow Caledonian University, Charles Oakley Laboratories, Cowcaddens Road, Glasgow G4 0BA, United Kingdom

Short Title Taylor: TSPO regulates macrophage cholesterol efflux

*Corresponding author: Professor A Graham, Department of Life Sciences, Glasgow Caledonian University, Charles Oakley Laboratories, Cowcaddens Road, Glasgow G4 0BA, UK

T: +44(0) 141 331 3722 F: +44(0) 141 331 3208 E: Ann.Graham@gcu.ac.uk

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Abstract

The aim of this study was to establish mitochondrial cholesterol trafficking 18kDa translocator protein (TSPO) as a potential therapeutic target, capable of increasing macrophage cholesterol efflux to (apo)lipoprotein acceptors.

Expression and activity of TSPO in human (THP-1) macrophages were manipulated genetically, and by the use of selective TSPO ligands. Cellular responses were analysed by quantitative PCR, immunoblotting and radiolabelling, including [^{3}H]cholesterol efflux to apoA-I, HDL and human serum.

Induction of macrophage cholesterol deposition by acetylated LDL (AcLDL) increased expression of TSPO mRNA and protein, reflecting findings in human carotid atherosclerosis. Transient overexpression of TSPO protein enhanced efflux (E%) of [^{3}H]cholesterol to ApoA-I, HDL and human serum, compared with empty vector controls, while gene knockdown of TSPO achieved the converse. Ligation of TSPO (PK11195, FGIN-1-27, flunitrazepam) triggered increases in [^{3}H]cholesterol efflux, an effect which was amplified in TSPO overexpressing macrophages. Overexpression of TSPO induced expression of genes *(PPARA, NR1H3, ABCA1, ABCG4, APOE*) and proteins (ABCA1, PPARα) involved in cholesterol efflux, reduced macrophage neutral lipid mass and lipogenesis, and limited cholesterol esterification following exposure to AcLDL. Thus, targeting TSPO reduces macrophage lipid content and prevents macrophage foam cell formation, via enhanced cholesterol efflux to (apo)lipoprotein acceptors.

Key Words Translocator protein (TSPO), mitochondrial cholesterol trafficking, apoA-I, HDL, macrophage foam cell

Introduction

Mitochondrial dysfunction, associated with increased production of reactive oxygen species, accumulation of mitochondrial DNA damage and progressive loss of respiratory chain function, is found in atherosclerotic lesions in both human studies, and in animal models of atherogenesis[1]. Oxidized low density lipoprotein (OxLDL), ageing, hyperhomocysteinaemia, hyperglycaemia, hypertriglyceridaemia, type 2 diabetes mellitus and cigarette smoking are all associated with mitochondrial damage and increased production of reactive oxygen species[2-4]. Increased arterial oxidative stress modifies LDL to a form recognised by macrophage scavenger receptors, resulting in further mitochondrial damage, and the unregulated accumulation of excess cholesterol and cholesteryl esters within macrophage 'foam cells', hallmark of early and developing atheroma[1-5]. Reversal of this process, regressing and stabilising atherosclerotic lesions, can be effected by efficient removal of cholesterol from 'foam cells', to acceptor particles such as apolipoprotein (apo) A-I, apoE or nascent high density lipoproteins (HDL), which then enter the athero-protective reverse cholesterol transport pathway responsible for delivery of excess cholesterol to the liver for excretion in bile and bile acids[6].

Recently, we[7] and others[8], demonstrated that overexpression of mitochondrial cholesterol trafficking protein, steroidogenic acute regulatory protein (StAR), decreases macrophage lipid content[7,8], represses inflammation[8] and increases macrophage cholesterol efflux[7,8], while a viral vector expressing StAR reduces aortic lipids and atheroma, in apoE$^{-/-}$ mice[9]. Overexpression of StAR enhances the rate-limiting step in mitochondrial generation of oxysterol ligands for LXRα/β: transfer of cholesterol to sterol 27-hydroxylase (CYP27A1) located on the inner mitochondrial membrane[10,11]. Sterol 27-hydroxylase generates oxysterol ligands for Liver X Receptors (LXRα/β), nuclear transcription factors which regulate gene expression of proteins involved in the cholesterol efflux pathway, including ATP binding cassette transporters, ABCA1, ABCG1/ABCG4, which orchestrate transfer of cholesterol and/or phospholipids across the plasma membrane to (apo)lipoproteins, such as apoA-I and apoE[12-14]. Loss of CYP27A1 results in cerebrotendinous xanthomatosis, which can be characterised by increased risk of premature atherosclerosis, despite normal circulating concentrations of plasma LDL[15]. Exploiting the anti-atherogenic functions of StAR may prove problematic, however, due either to induction of lipogenesis[7] or the dearth of small molecules capable of modulating the activity of this protein *in vivo*.

However, StAR interacts with a protein complex, located at contact sites between outer and inner mitochondrial membranes, which includes 18kDa translocator protein (TSPO/peripheral benzodiazepine receptor/PBR), voltage dependent anion channel (VDAC) and adenine nucleotide transporter (ANT), and associated proteins, including cAMP-dependent protein kinase associated protein (PAP7/ Acyl CoA binding domain-3/ACBD3), PBR-associated protein (PRAX1) and its proposed endogenous ligand, diazepam binding inhibitor (DBI/ACBD1)[16,17]. The translocator protein

has five transmembrane domains, and a high affinity cholesterol recognition amino acid (CRAC) binding C-terminal domain[16, 17]; the CRAC peptide was recently used as an interchelating agent to reduce cholesterol and aortic lesions in apoE$^{-/-}$ mice[18]. Knockdown of TSPO induces arrest of mitochondrial cholesterol transport, which can be rescued by TSPO cDNA, while global deletion of *TSPO* is embryonic lethal in mice[16, 17]. Intriguingly, TSPO ligands were recently shown to activate fasting metabolism, reducing lipogenesis and hepatosteatosis in obese mice[19], suggesting that additional activities associate with this protein.

The contribution, and therapeutic potential, of TSPO and associated proteins in regulation of the cholesterol efflux pathway in macrophages and macrophage 'foam cells', remains uninvestigated, despite the potential importance of this pathway in activating the anti-atherogenic cholesterol efflux process. Here, we study gene and protein expression of TSPO during macrophage 'foam cell' formation, and demonstrate the positive impact of TSPO overexpression and/or ligation on macrophage cholesterol efflux; notably, TSPO overexpression causes a decline in macrophage total neutral lipid mass, without induction of lipogenesis, and effectively prevents 'foam cell' formation following exposure to acetylated LDL. We postulate that existing ligands for TSPO may possess anti-atherosclerotic properties, readily amenable to testing in clinical studies.

Methods

2.1 Materials Human monocytic cell line (THP-1) and murine RAW264.7 macrophages were purchased from the European Cell Culture Collection (Porton Down, UK). Cryopreserved human peripheral blood monocytes (10^7) isolated from fresh blood were purchased from Promocell (UK distributors); the sample of total RNA (50μg) derived from normal human aortas pooled from 4 male/female Caucasians (aged 27-45) who died of sudden death, was purchased from Clontech Laboratories Inc. (CA, USA). Tissue culture reagents were purchased from Lonza (Wokingham, UK); other sources include Amaxa Transfection reagent (Lonza, UK), NuPage gels and buffers (Life Technologies, Paisley, Scotland), antibodies (AbCAM, Cambridge, UK) and primers and probes (Eurogentec, Belgium). Apolipoprotein A-I, high density lipoprotein (HDL) and low density density lipoprotein (LDL) were purchased from Athens Research UK (USA); LDL was acetylated according to Brown *et al*[20]. Radiochemicals ([^{3}H]cholesterol, [^{14}C]acetic acid and [^{3}H]oleic acid) were provided by ICN Biologicals; all other chemicals were provided by Sigma-Aldrich (Poole, Dorset, UK). Mammalian expression vectors (*pCMV6*) encoding *TSPO*, *DBI*, *VDAC* and *ANT* were supplied by Origene, via Cambridge Biosciences (UK); TriFecta Dicer Substrate RNAi duplexes directed against *TSPO* and scrambled control sequences were supplied by Integrated DNA Technologies (IDT, Germany). We are greatly indebted to Professor D.J. Mangelsdorf (South Western Medical Center, USA) for his kind gift of LXRE reporter plasmid (*pCMX.LXRE*).

2.2 Cell culture Human THP-1 monocytes were maintained using a split ratio of 1:10 in RPMI medium supplemented with foetal bovine serum (FBS, 10%, v/v), L-glutamine (200mM) and penicillin/streptomycin (50 μg ml^{-1}; 50 U ml^{-1}, respectively). For experiments, cells were plated onto 12-well tissue culture dishes at a density of $1x10^6$ cells $well^{-1}$, in complete RPMI medium (above) supplemented with 100nM phorbol 12-myristate 13-acetate (PMA) to induce macrophage differentiation. Transfection of monocytes, immediately prior to differentiation, was achieved using the 'Human Monocyte' transfection reagent (VPA-1007) and electroporation protocol (Y001), as per manufacturer's instructions (Amaxa, Lonza), and as previously described[21]; our studies have established a transfection efficiency of 70-80%, without significant (<5%) loss of cell viability, using this system. Mammalian expression vector *pCMV.6_TSPO* (0.5μg) was used to transiently (48h) overexpress the mitochondrial translocator protein, while validated siRNA (10nM) duplexes directed against *TSPO* and *PPARA* were used for gene knockdown (48h). Transient transfections were also performed using the same quantities of vector encoding full length *VDAC*, *ANT* and *DBI*. The empty vector (*pCMV6*), and scrambled siRNA sequences of the same nucleotides, were used as respective controls. Macrophages were treated with TSPO ligands for 48h prior to assessment of cholesterol efflux, using DMSO (<0.01%) as vehicle. Transient expression of *pCMX.LXRE* (0.5μg) was achieved using Fugene6 (6μl: 1μg DNA; 24h) and luciferase activity determined (Britelite Plus, Perkin Elmer) as previously described[7,22, 23]. Cell viabilities were determined by conversion of methyl thiazolyl blue tetrazolium bromide to formazan, as previously[7,22, 23].

2.3 Macrophage lipid analysis and cholesterol efflux Incorporation of [^{14}C]acetic acid (1μCi ml^{-1}; 24h) into fatty acid, phospholipid, cholesterol, cholesteryl ester and triacylglycerol pools was initiated 48h post-transfection, and as previously described[7,22,23]. Esterification of cholesterol, in the presence of acetylated LDL (50 μg ml^{-1}) was monitored by flux of [^{3}H]oleic acid (10μM, 1μCi ml^{-1}) into cholesteryl [^{3}H]oleate, as previously described[7,20,22,23]. Macrophage lipids were extracted using hexane:isopropanol (3:2, v/v) and dried under nitrogen, before separation by t.l.c., using the mobile phases described[7,20,22,23]. Lipids were identified by co-migration with authentic standards, incorporation of radiolabel determined by scintillation counting, with data expressed as nmol incorporation per mg cell protein. Production of $^{14}CO_2$ from [1-^{14}C]oleic acid (3μM; 0.15 μCi ml^{-1}) was assessed as previously described[24]. Neutral lipid mass (total cholesterol, triacylglycerol) in macrophage lipid extracts (above) were determined using colorimetric assays (Infinity™), and expressed as μg lipid per mg cell protein[7, 20,22,23]. Macrophage efflux of [^{3}H]cholesterol to apoA-I (20 μg ml^{-1}), HDL (20 μg ml^{-1}) and human serum (1%, v/v) were determined as previously described[22,23]. Efflux (24h) from radiolabelled macrophages was initiated 48h post-transfection by addition of serum-free RPMI containing each acceptor, and results are expressed as % cholesterol efflux, calculated as $[dpm_{media}/dpm_{(media + cells)}] \times 100\%$.

2.4 Analysis of gene and protein expression Total RNA was isolated (TriFast, Peqlabs) from macrophages and reverse transcribed to cDNA (BioLine, UK) prior to measurement of cellular levels of mRNA encoding the mitochondrial cholesterol trafficking complex (*TSPO*, *VDAC*, *ANT*), lipid responsive transcription factors (*SREBF1*, *SREBF2*, *PPARA*, *PPARG*, *PPARD*, *NR1H3*) and key elements of the cholesterol efflux pathway (*ABCA1*, *ABCG1*, *ABCG4*, *APOE*). Quantitative polymerase chain reaction (Q-PCR), relative to *GAPDH*, was performed using the primers and fluorescent (FAM/TAMRA) probe sequences indicated in **Table 1 (Supplementary Data);** negative reverse transcription controls were performed for all samples. Gene expression of an array of 84 genes implicated in atherosclerosis were analysed in control and TSPO overexpressing macrophages using the RT^2 Profiler Human Atherosclerosis PCR array (Qiagen; PAHS-038A). Assessment of CPT-1A gene expression was performed using forward (ACAGTCGGTGAGGCCTCT TATGAA) and reverse primers (TCTTGCTGCCTGAATGTGAGTTGG), generating a 252 bp product, semi-quantified by densitometry using a GelDoc XR.

Macrophage cell lysates were prepared in RIPA buffer (25mM Tris HCl pH 7.6, 150mM NaCl, 1% (v/v), NP-40, 1% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl suphate) supplemented with Complete™ protease inhibitor cocktail (Roche), and proteins (20-50μg $lane^{-1}$) separated using NuPAGE (10%, w/v) gels. Transfer to a PDVF membrane was followed by immunoblotting using rabbit polyclonal antibodies (1:1000 dilution) against ABCA1 TSPO, PPARα, ACAT1 and GAPDH (1:2000) and detection achieved for the majority of the data presented, using secondary antibodies coupled to the ECL detection system, as previously described[7,22,23]. Densitometry was performed using a GelDoc XR using Image Lab Software for Western Blot Analysis (Biorad, UK). However, the images presented for SiRNA knockdown of TSPO in Figure 2A, and for PPARα following TSPO overexpression in Figure 5B were detected using a donkey anti-rabbit secondary antibody (1:12,500) labelled with IRDye® 800CW (LI-COR Biosciences UK Ltd) and the LI-COR Odyssey Fc Imaging System with LI-COR Image Studio version 3.1.4 software.

All values are mean±SEM, with numbers of independent experiments denoted by *n*. Significant ($p<0.05$) differences were determined using Student's t-test, or by employing analysis of variance (ANOVA) and post *t*-tests, as indicated in the Legends to Figures.

Results

An indication of the levels of gene expression of *TSPO*, *VDAC* and *ANT*, relative to the housekeeping gene *GAPDH*, in peripheral blood monocytes and in normal heart aorta are shown in **Figure 1A**, to provide a physiological context and comparison for the studies carried out using macrophages derived from the human THP-1 monocytic cell line; mRNA levels of these genes in differentiated THP-1 macrophages and THP-1 macrophage 'foam cells' are shown in **Figure 1B.** Exposure of THP-1

macrophages to two doses of 50 μg ml^{-1} AcLDL (24h), led to a modest but significant increase (27%; $p<0.05$) in total cholesterol mass (control 58.0±1.7 μg mg^{-1} cell protein versus AcLDL 73.7±7.2 μg mg^{-1} cell protein; $p<0.05$; *n=4*), a mild cholesterol loading condition that avoids the overt toxicity associated with exposure to higher concentrations of modified LDL[25]. Levels of mRNA encoding TSPO were increased by 1.8±0.3-fold following exposure to AcLDL, reflected in increased levels of TSPO protein (37%; $p<0.05$; *n=3*), relative to VDAC or GAPDH (**Figure 1B).** Analysis (MatInspector) of the -3kb upstream promoter region of *TSPO* revealed a number of putative transcription factor binding sites, including sterol regulatory elements (-2113-2097; -1957-1922; -1783-1769) and peroxisome proliferator activated receptor (PPAR) responsive elements (-2214-2191; -366-344).

Overexpression and gene knockdown of TSPO in THP-1 macrophages: impact on cholesterol efflux

Overexpression and knockdown of dimeric TSPO protein (44kDa) relative to GAPDH, and compared with the empty vector controls (EV; pCMV.6) or SiRNA negative control, are shown in **Figure 2A** and **2B**, measured as detailed in the Methods. No changes in cell viability were associated with TSPO overexpression (EV 37.8±0.9 μM formazan *versus* TSPO 36.2 ±0.9 μM formazan, *n*=3; *NS*). Overexpression of TSPO significantly increased efflux (24h), of [^{3}H]cholesterol, to 20 μg ml^{-1} ApoA-I (60±27.4%; $p<0.01$; *n=3*), 20 μg ml^{-1} HDL (74± 13.2%; $p<0.05$) and 1% (v/v) human serum (51.7±21.2%; $p<0.05$; *n=3*) (**Figure 2C**), when compared with cells transfected with the empty vector. Conversely, gene knockdown of *TSPO* subsequently reduced [^{3}H]cholesterol efflux (24h) to ApoA-I (20 μg ml^{-1}) and HDL (20 μg ml^{-1}) by 15.7±5.7% ($p<0.05$) and 14.3±9.2% ($p<0.05$), respectively, when compared with the scrambled SiRNA negative control (**Figure 2D**).

Transfection with the same quantity of vector encoding the proposed endogenous ligand for TSPO, DBI induced a small increase in efflux to apoA-I, which did not quite reach significance (EV 5.1±0.78% *versus* DBI 6.4±0.3%; $p=0.06$; *n=3*), but did significantly ($p<0.01$) increase efflux of [^{3}H]cholesterol to HDL (EV 19.8±1.8% *versus* DBI 21.3±1.9%; *n=3*). By contrast, equivalent transfections with vector encoding either VDAC or ANT, did not significantly alter efflux of [^{3}H]cholesterol to either apoA-I (EV 4.0±0.2% *versus* VDAC 4.2±0.3% *versus* ANT 4.4±0.4%; NS; *n=3*) or HDL (EV 8.8±0.7% *versus* VDAC 8.8±0.8% *versus* ANT 9.7±0.8%; NS; *n=3*), when tested under the same conditions.

Ligation of TSPO increases macrophage cholesterol efflux to apoA-I and HDL

Wild type THP-1 macrophages were treated with established TSPO ligands, PK11195, FGIN-1-27 and flunitrazepem, using concentrations commonly utilised to increase mitochondrial cholesterol trafficking and steroidogenesis [16, 17]. Significant increases in [^{3}H]cholesterol efflux to apoA-I (20μg

ml^{-1}; **Figure 3**) were noted following treatment with PK11195 (30μM; $p<0.05$; *n=3*), flunitrazepam (30μM; $p<0.01$; *n=3*) and FGIN-1-27 (10μM; $p<0.05$*; n=3*) (**Figures 3A, 3B** and **3C**). By contrast, only PK11195 ($p<0.01$; *n=3*) and FGIN-1-27 ($p<0.01$; *n=3*) increased efflux of cholesterol to HDL (20μg ml^{-1}) (**Figures 3A, 3B** and **3C)**. Notably, in macrophages overexpressing TSPO, treatment with FGIN-1-27 increased efflux to apoA-I by a further 2.2±0.2-fold ($p<0.001$; *n=3*) resulting in a 5.2±0.6 fold ($p<0.001$; *n=3*) increase in efflux above basal levels (**Figure 3D**). Equally, knockdown of TSPO abrogated the stimulatory (31.7±8.6%) effect of FGIN-1-27 (10μM) on cholesterol efflux to apoA-I (scrambled control 8.3±0.7% versus siRNA to TSPO 6.8±0.6%; $p<0.05$; *n=3*).

Overexpression of TSPO: impact on lipid phenotype

Transient TSPO overexpression (48h) was associated with a marked loss of macrophage neutral lipid mass, due to reductions in total cholesterol (52.6±8.9%; $p<0.05$, *n=4*) and triacylglycerol (34.2±10.9%; $p<0.05$, *n=4*) content (**Figure 4A**). However, we previously reported that overexpression of mitochondrial cholesterol trafficking protein, steroidogenic acute regulatory protein (StAR; STARD1) is associated with induction of lipogenesis in macrophages[7]. Accordingly, we investigated the impact of transient TSPO overexpression (48h) on the subsequent incorporation of [^{14}C]acetate into macrophage lipid pools (24h) (**Figure 4B**). A small reduction in total lipogenesis (10±3.1%; $p<0.05$; *n=6*) was noted, predominantly due to a 15±6.2% ($p<0.05$; *n=6*) reduction in incorporation of [^{14}C]acetate into the fatty acid pool. Increased oxidation of [1-^{14}C]oleic acid, expressed as a percentage of cellular uptake, (EV 0.30±0.03% h^{-1} *versus* TSPO 0.52±0.05% h^{-1}; $p<0.05$; *n=3*), was associated with a 2-fold induction of gene expression of CPT-1A (arbitrary fluorescence units: EV 61.2±6.7 *versus* TSPO 119.8±2.5; *n=3*; $p< 0.01$).

The ability of TSPO overexpression (48h) to increase cholesterol efflux, and protect macrophages against accumulation of cholesteryl ester was investigated following exposure to AcLDL (50μg ml^{-1}) for 24h (**Figures 4C** and **4D**). Following a cholesterol load, apoA-I stimulated cholesterol efflux by 1.9±0.2-fold ($p<0.05$) above basal (empty vector) control. By contrast, in TSPO overexpressing cells, apoA-I increased cholesterol efflux by 3.8±0.4-fold ($p<0.001$), an increase of 1.6±0.1-fold ($p<0.01$) compared with the EV control (**Figure 4C**). Equally, while addition of AcLDL (50μg ml^{-1}) increased incorporation of [^{3}H]oleate into the cholesteryl ester pool by 81±12.0% ($p<0.01$, *n=3*) in macrophages transfected with the empty vector, overexpression of TSPO prevented significant increases in formation of cholesteryl [^{3}H]oleate following treatment with this modified lipoprotein (**Figure 4D**, top panel). Equally, TSPO overexpression effectively prevented the increase in cholesterol mass following AcLDL (50μg ml^{-1}; 24h) treatment (**Figure 4D**, bottom panel), compared with the empty vector control. A small decrease in expression of ACAT1 protein was noted in TSPO-overexpressing cells (8.9± 1.8%; NS, *n=3*) (**Figure 5B**).

Overexpression of TSPO: changes in expression of genes and proteins involved in the cholesterol efflux pathway

Gene expression of the sterol responsive transcription factors, *SREBF2*, *PPARD* and *PPARG* were repressed in cells overexpressing TSPO, while expression of *NR1H3* was significantly increased, this last reflected in increased LXRE reporter activity (TSPO 184.8±23.2% of control; $p<0.05$; *n=4*). Gene expression of *PPARA* **(Figure 5A)** was increased, accompanied by an increase in PPARα protein (32± 5.7%; $p<0.05$, *n=3*) in macrophages transiently expressing TSPO compared with GAPDH (**Figure 5B**). Treatment with FGIN-1-27 (10μM; 24h) also increased mRNA levels of *PPARA* (2.0±0.2-fold; *n=3*) and *NR1H3* (1.5±0.5-fold; *n=7*), replicating observations in TSPO overexpressing macrophages.

Increases in the expression of genes encoding proteins involved in the cholesterol efflux pathway were noted in macrophages overexpressing TSPO (**Figure 5A**). Levels of *ABCA1* mRNA increased by more than 6.5±2.3-fold (*n=6*), and this was reflected in increased levels of ABCA1 protein (39± 9.3 %; $p<0.05$; *n=3*; **Figure 5B**) compared with GAPDH. Gene expression of *ABCG4* and the endogenously produced cholesterol acceptor, *APOE*, were also increased by 2 to 3-fold (*n=6*) in macrophages overexpressing TSPO (48h). Analysis of an array of genes implicated in atherosclerosis, relative to five housekeeping genes, revealed only two more genes upregulated by >3-fold following TSPO overexpression: *FN1* and *PDGFB*.

Overexpression and ligation of TSPO: blockade by PPARα antagonism

Since induction of PPARα is implicated in the pathway by which TSPO overexpression or ligation stimulates the cholesterol efflux, a short series of experiments were performed using PPARα antagonists. Induction of *ABCA1* gene expression by TSPO (ratio to GAPDH: control 0.98 *versus* TSPO 2.45) was blocked by addition of 10μM GW6471[26] (ratio to GAPDH: control 0.85 *versus* TSPO 0.98) and partially reduced by treatment with GGPP[7] (10μM) (ratio to GAPDH: control 1.05 *versus* TSPO 1.99). Induction of *NR1H3* by TSPO (ratio to GAPDH: control 1.14 *versus* TSPO 3.89) was effectively inhibited by both 10μM GW6471 (ratio to GAPDH: control 0.58 *versus* TSPO 0.39) and 10μM GGPP[7] (ratio to GAPDH: control 0.10 *versus* TSPO 0.31); the values are averages from a single representative experiment, which was performed three times.

Further, the stimulatory effect of TSPO overexpression on [^{3}H]cholesterol efflux to apoA-I (20μg ml^{-1}; 24h) was effectively blocked by the addition of PPARα antagonist, GW6471[26] (10μM) (**Figure 6A**) and by treatment with siRNA (10nM) directed against PPARα (**Figure 6B**), allow us to posit the pathway described in **Figure 6C**.

Discussion

This study investigated the relationship between components of the mitochondrial cholesterol trafficking and macrophage cholesterol homeostasis, and demonstrated that overexpression, gene silencing and ligation of mitochondrial TSPO modulate cholesterol efflux, with TSPO overexpression channelling cholesterol away from the cholesteryl ester pool and reducing macrophage total neutral lipid mass, without triggering compensatory increases in lipogenesis. These effects appear to be related to the induction and/or activation of LXRα and PPARα caused by TSPO overexpression or ligation: blockade of either pathway using antagonists and/or siRNA directed against these transcription factors prevented the TSPO-dependent increases in cholesterol efflux.

Overexpression of the 18kDa translocator protein (TSPO) is associated with increased efflux of cholesterol to apoA-I and HDL, and induction of expression of genes involved in the cholesterol efflux pathway[7,8], including *NR1H3*, *ABCA1*, *ABCG4*, but not *ABCG1* (**Figure 5**). The reason for this distinctive regulatory pattern is not clear at present, but dissociation of *ABCA1* and *ABCG1* gene expression has been noted in bovine mammary tissue during the switch from lactating to dry to periods[27]. Induction of LXRα is consistent with sequestration of SREBP2 protein at the endoplasmic reticulum, and loss of *SREBF2* expression (**Figure 5A**): both may contribute to increased induction of ABCA1 mRNA and protein[29-31]. Expression of *ABCG4* in human monocyte-macrophages lies under the control of oxysterols and retinoids[31] suggesting this gene and *ABCA1* may both be regulated by LXRα activation or induction (**Figure 5A**), and one mechanism by which [^{3}H]cholesterol efflux to HDL is increased (**Figure 2B**).

Moreover, overexpression of TSPO and/or ligation of this protein by FGIN-1-27, induced PPAR alpha gene and/or protein levels, previously associated with increased ABCA1 expression and cholesterol efflux[32-35]. Expression of PPARα can be regulated by stress, hormones and starvation in rodents, while human PPARα can mediate its own expression, and is induced during macrophage differentiation in response to high glucose levels[33]. Notably, the PPARα promoter can be activated by PPAR agonists in an LXRα dependent manner, and LXRα is a PPARα target[35], so that these transcription factors can work together in a cooperative manner to control ABCA1 expression and cholesterol efflux[32]. Overexpression of TSPO induces expression of LXRα and PPARα, while use of either SiRNA or an antagonist directed against PPARα (GW6471) (**Figure 6**) indicates an obligate role for PPARα in mediating TSPO-dependent increases in [^{3}H]cholesterol efflux to apoA-I and possibly other cholesterol acceptors (**Figure 2B**).

Indeed, the coordinated induction of PPARα and LXRα by TSPO may be a key factor in abrogating the adverse effects previously associated with activation of LXRα by agonists such as T091317[36]. In particular, our previous study[7], reported that overexpression and activation of mitochondrial

cholesterol trafficking protein, StAR, in murine macrophages, was associated not only with enhanced cholesterol efflux and increased expression of ABCA1, but also with substantive induction of lipogenesis, thereby limiting the potential therapeutic utility of this approach[7, 36]. By contrast, overexpression of TSPO was associated not only with increased cholesterol efflux to acceptors (**Figure 2B**) but with a marked loss of macrophage neutral lipid content (**Fig 4A**), and a small reduction in lipogenesis, primarily within the fatty acid pool (**Fig 4B**). Further, and consistent with PPARα induction, levels of CPT-1A gene expression were increased, reflected in enhanced fatty acid oxidation, in TSPO overexpressing cells. Activation of PPARα is known to induce modest increases in lipogenesis, possibly via participation in the generation of an endogenous LXRα ligand[35], and enhances fatty acid oxidation, by inducing the expression of long chain fatty acyl CoA synthetases and carnitine palmitoyl transferase-1, essential for generating fatty acyl CoA and facilitating the entry of fatty acyl carnitine into mitochondria for β-oxidation[32-34]. These data provide a plausible explanation for the observed reductions in macrophage triacylglycerol content (**Figure 4A**) and [^{14}C]fatty acid levels (**Fig 4B**). Overexpression of TSPO was also associated with reduced incorporation of [^{3}H]oleate into the cholesteryl ester pool, and with decreased total cholesterol mass, following exposure to modified LDL (**Fig 4D**), which may reflect the reduced availability of *de novo* synthesized fatty acids for esterification (**Fig 4B**)[32-34]; indeed, activation of PPARα and LXRα have previously been associated reductions in ACAT activity and cholesterol esterification in human macrophages[32,33].

Notably, treatment with acetylated LDL appears to reflect the induction of TSPO observed in human carotid atherosclerotic plaques, wherein binding of TSPO ligands such as [^{3}H]PK11195 and [^{3}H]DAA1106 have been shown to significantly correlate with macrophage burden[37]. Lipid regulation of TSPO gene expression has not previously been reported during macrophage cholesterol loading, but *Tspo* can be regulated by protein kinase Cε, by downstream signalling pathways involving mitogen-activated protein kinase MAPK (Raf-ERK1/2), and by acting on targets such as c-Jun, and the signal transducer and activator of transcription 3 (STAT3)[16,17]. It is known that STAT3 gene delivery can reduce atherosclerotic lesions in LDL receptor knockout mice fed a high cholesterol diet[37], but whether induction of TSPO forms part of that response is not established. However, it is known that expression of TSPO is modulated by PPARs in a tissue specific manner, with transrepression of *Tspo* by PPARα cited in Leydig cells, but the reverse noted in non-steroidogenic tissues[17]. Given the presence of a number of putative PPAR response elements within the promoter region of TSPO, it is possible to speculate that induction of PPARα in human macrophages may form part of a feed-forward regulatory loop, sustaining or amplifying *TSPO* expression (**Figure 6**).

Finally, the ability of TSPO to moderate gene transcription, has been observed in lower species, and thus may be evolutionarily conserved in mammalian cells. For example, the bacterial ortholog, TspO

outer membrane protein, found in *Rhodobacter sphaeroides*, which is involved in effluxing intermediates of the porphyrin biosynthetic pathway, acts as a co-activator of transcription of a number of genes, encoding enzymes involved in photopigment biosynthesis[39,40]. Arabidopsis TSPO is also linked to porphyrin metabolism, and appears to act as a signal linking water-related stress to transient increases in gene expression of the plant stress hormone, absicisc acid[41].

Summary

We propose a model wherein a modest cholesterol load induces macrophage overexpression of TSPO mRNA and protein, facilitating cholesterol efflux to apoA-I, HDL and human serum, inducing the expression of *NR1H3, PPARA, ABCA1, ABCG4* and *APOE*, and reducing macrophage cholesterol and triacylglycerol mass. In turn, we speculate that loss of this protective TSPO-coordinated response, exemplified in **Figure 6C**, may be part of the pathophysiology of 'foam cell' formation; certainly, chronic high fat, high cholesterol supplementation, and associated oxidative stress, decreases TSPO binding capacity in rodent hepatic and aortic tissues[42]. Targeting mitochondrial TSPO activity or maintaining its function could prove therapeutically useful in regressing atherosclerotic lesions, particularly since bioavailable TSPO ligands, without obvious side effects, are currently in development for other disease conditions[43].

Author contribution

The study was originated and designed by AG, in consultation with JMT; JMT performed the majority of the laboratory work, with additional input from AMA. All three authors have been involved in preparation of the manuscript.

Funding

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Acknowledgments

None

Conflict of Interest

All of the authors named confirm that no potential conflict of interest exists which might constitute an embarrassment to any of the authors, if it were not to be declared and were to emerge after publication.

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Figure Legends

Figure Legends

Figure 1 Gene expressions of components of the basal mitochondrial complex, *TSPO*, *VDAC* and *ANT*, relative to the housekeeping gene *GAPDH*, in human peripheral blood monocytes (PBMC; 10^7) and pooled human heart aortas (50μg) (Materials and Methods) are shown in Figure 1A. In each case, a single sample was analysed. The levels of mRNA for *TSPO* (*n=6*), *VDAC* (*n=5*) and *ANT* (*n=5*).relative to the housekeeping gene *GAPDH*, in THP-1 macrophages (THP-1 Mϕ) and in THP-1 macrophage 'foam cells' (THP-1 Foam Mϕ) generated by two consecutive treatments (24h) with 50μg ml^{-1} AcLDL, is shown in Figure 1B. Values are mean±SEM, and significance between expression in macrophages and macrophage 'foam' cells was determined by Student's t-test. Figure 1C shows AcLDL-induced expression of TSPO protein, relative to VDAC and GAPDH (*n=3;* *$p<0.05$) as assessed by ECL detection.

Figure 2 Figure 2A shows the extent of overexpression (24h) of TSPO (0.5μg pCMV_TSPO) compared with empty vector (EV) control; values are mean±SEM for *n=3* experiments. Figure 2B shows the knockdown (24h) of TSPO protein achieved using SiRNA (10nM) directed against TSPO, compared with scrambled SiRNA control (10nM); values are mean±SEM for *n=3* experiments. Overexpression of TSPO was detected using ECL reagents, while the image for SiRNA knockdown of TSPO was generated using fluorescently labelled secondary antibodies and the LI-COR detection

system (**Methods**). *p<0.05 indicates a significant difference from the EV and SiRNA controls, determined using Students' t-test. The effect of overexpression of TSPO protein, on the efflux (24h) of [^{3}H]cholesterol to apoA-I (20μg ml^{-1}; *n=3*), HDL (20μg ml^{-1}; *n=3*) or human serum (1%, v/v; *n=3*) is shown in Figure 2C. Figure 2D shows the effect of siRNA (10nM) directed against TSPO on efflux of [^{3}H]cholesterol to apoA-I (20μg ml^{-1}; *n=4*) and HDL (20μg ml^{-1}; *n=4*). Values are mean±SEM; significant differences due to genetic manipulation of TSPO were determined using Students' t-test and are as indicated on Figure 2C and 2D.

Figure 3 The effect of TSPO ligands, PK11195 (30μM; *n=3*) and flunitrazepam (30μM; *n=3*), on the efflux of [^{3}H]cholesterol to apoA-I (20μg ml^{-1}) and HDL (20μg ml^{-1}) are shown in Figure 3A and 3B, respectively. Figure 3C shows the effect of treatment with FGIN-1-27 (10μM; *n=3*) on cholesterol efflux to ApoA-I and HDL at the same concentrations. Significance levels in Fig3A to 3C were determined using one-way ANOVA and Bonferroni post-test: *p<0.05; ***p<0.001 compared with the control incubation; † p<0.05 and †† p<0.01 compared with apoA-I or HDL alone. Figure 3D shows efflux to apoA-I (20μg ml^{-1}), measured in the absence (*n=6*) or presence (*n=3*) of FGIN-1-27 (10μM), in empty vector and TSPO overexpressing cells. Significant differences between empty vector and TSPO overexpressing cells were determined using Student's t-test.

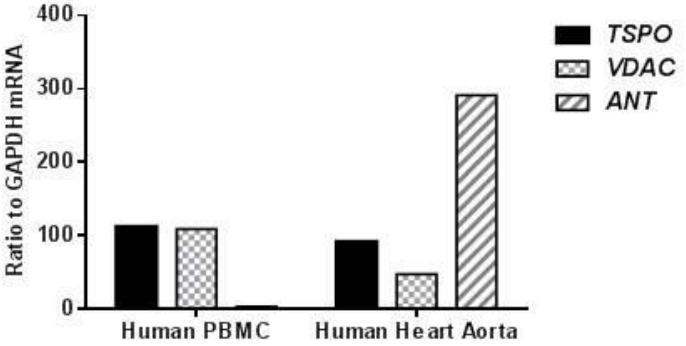
Figure 4 Figure 4A shows the effect of TSPO overexpression on macrophage triacylglycerol and total cholesterol mass; four independent experiments were performed, and statistical significance determined using Student's t-test. The impact of TSPO overexpression on nmol incorporation mg^{-1} protein of [^{14}C]acetate (1μCi ml^{-1}) into phospholipid, triacylglycerol, cholesteryl ester, cholesterol and fatty acid lipid pools, compared with the empty vector (EV) control is shown in Figure 4B; *n=6*, with significance determined by Student's t-test. The efflux of cholesterol to apoA-I (20 μg ml^{-1}; 24h; *n=4*) in empty vector and TSPO-transfected macrophages previously exposed to a cholesterol load (AcLDL, 24h, 50μg ml^{-1}) is shown in Figure 4C; significant differences were determined by one way ANOVA and Bonferroni post-test. The incorporation of [^{3}H]oleate (1μCi ml^{-1}; 10μM; *n=3*) into the cholesteryl ester pool in empty vector and TSPO transfected macrophages, and in the absence or presence of AcLDL (50μg ml^{-1}; 24h) is shown in the upper panel of Figure 4D, while the total cholesterol mass, investigated in the presence of AcLDL (50μg ml^{-1}; 24h; *n=3*), is shown in the lower panel of Figure 4D; significant differences were determined using one-way ANOVA and Bonferroni post t-test. In all cases, *p<0.05, **p<0.01 or ***p|<0.001 compared with the relevant control incubation; other significant differences are indicated on each Figure.

Figure 5 Fold changes in gene expression of lipid-responsive transcription factors in macrophages transiently expressing TSPO, relative to the empty vector (EV) control, are shown in Figure 5A. Increased expressions of ABCA1 and PPARα, relative to ACAT1 and GAPDH, in TSPO-

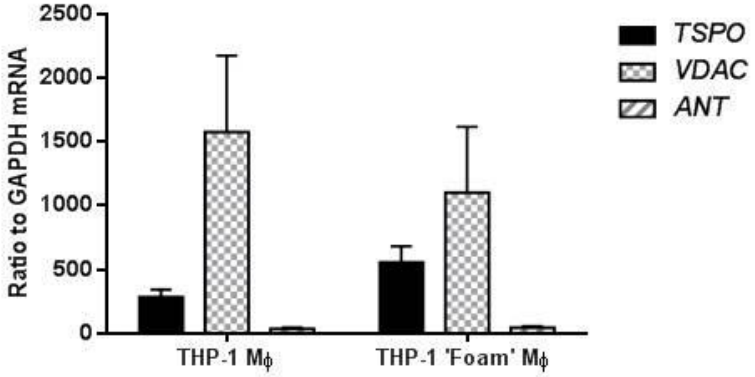
overexpressing cells, compared with EV controls, are shown in Figure 5B (*n=3*; *p<0.05). The immunoblots for ABCA1 and ACAT1 were generated using ECL detection, while that for PPARα utilised fluorescently labelled secondary antibody and LI-COR software (**Methods**).

Figure 6 The impact of PPARα antagonist GW6471 (10μM) on [^{3}H]cholesterol efflux to apoA-I (20μg ml^{-1}; 24h; *n=4*), in empty vector (EV) and TSPO transfected macrophages is shown in Figure 6A. Significant differences were determined by one-way ANOVA and Bonferroni post-test. Efflux to apoA-I (20μg ml^{-1}; 24h; *n=3*) in TSPO transfected macrophages simultaneously treated with either negative SiRNA (10nM) or SiRNA (10nM) directed against PPARα is shown in Figure 6B. Significance between negative SiRNA and PPARα SiRNA in TSPO transfected cells was determined by Student's t-test. In all cases, *p<0.05 compared with the empty vector control; other significant differences are indicated on the Figures. Figure 6C shows a schematic model illustrating the putative pathways by which TSPO overexpression may interface with LXRα and PPARα pathways to mediate the observed effects on macrophage lipid phenotype.

Figure 1A



1B



1C

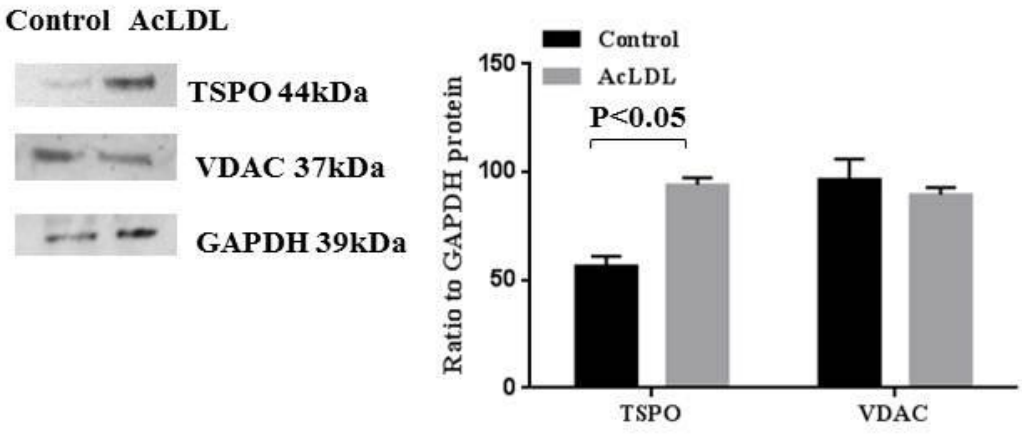
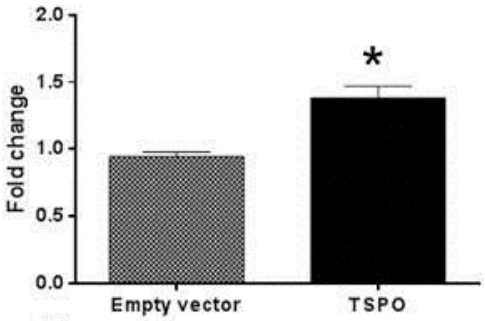
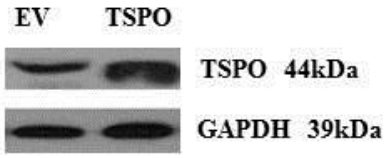
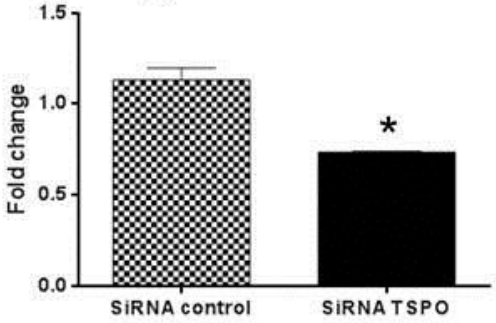


Figure 2A

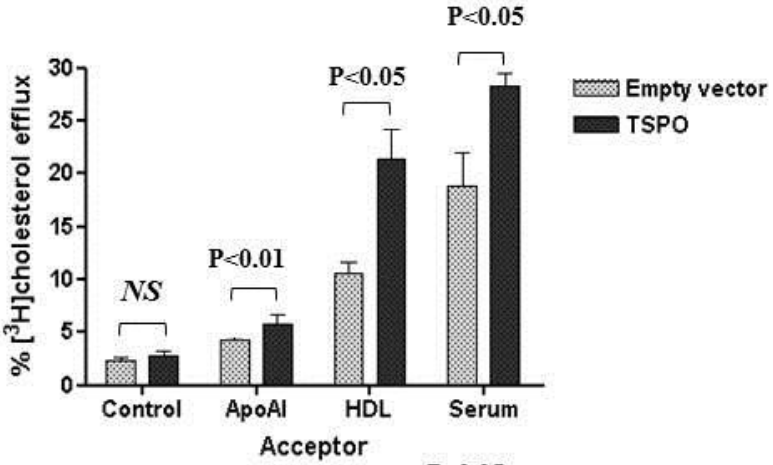




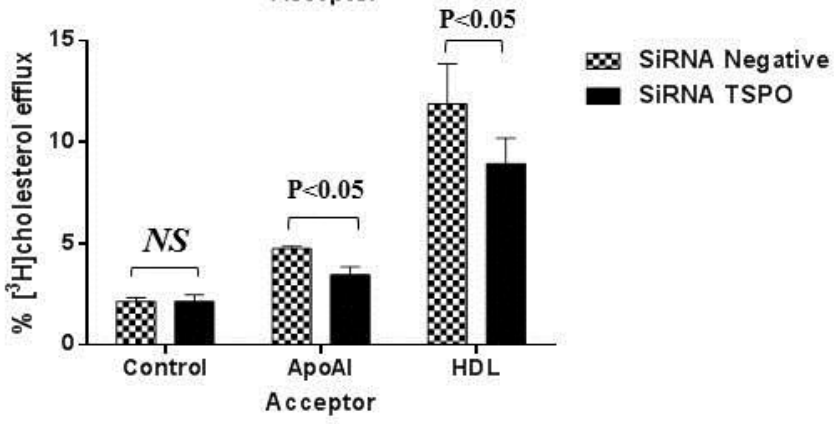
2B

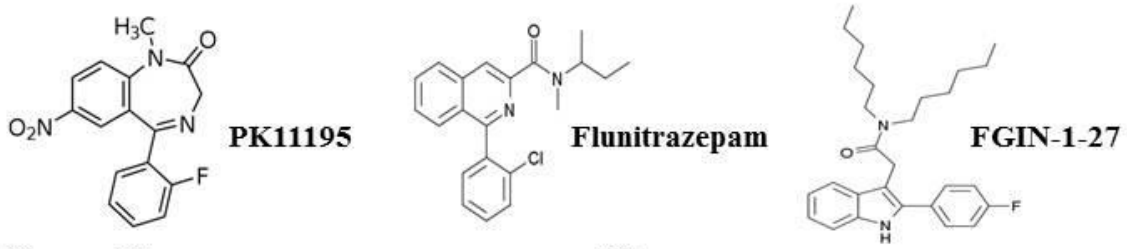


2C



2D




PK11195
Flunitrazepam
FGIN-1-27

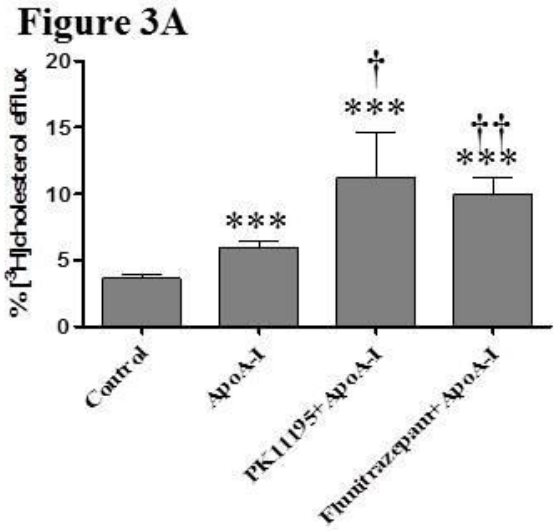
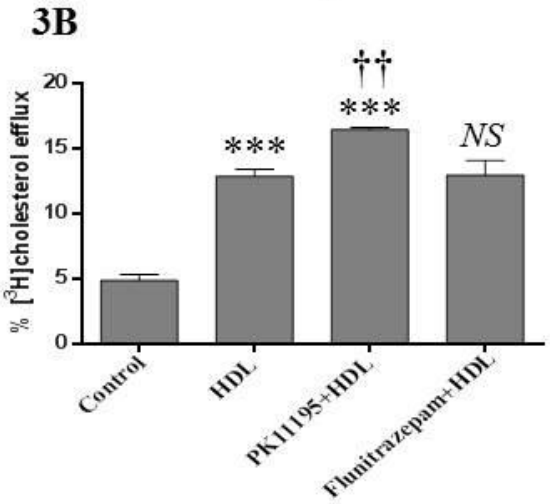
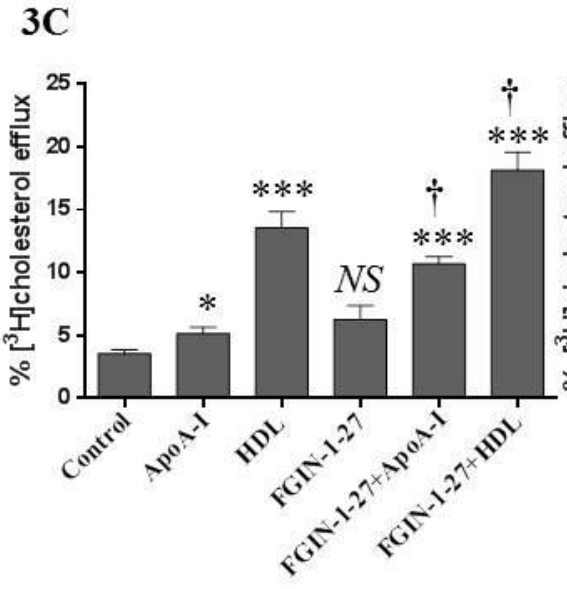


Figure 3A
% [3H]cholesterol efflux
Control
ApoA-I
PK11195+ApoA-I
Flunitrazepam+ApoA-I


3B
% [3H]cholesterol efflux
Control
HDL
PK11195+HDL
Flunitrazepam+HDL
NS


3C
% [3H]cholesterol efflux
Control
ApoA-I
HDL
FGIN-1-27
FGIN-1-27+ApoA-I
FGIN-1-27+HDL
NS

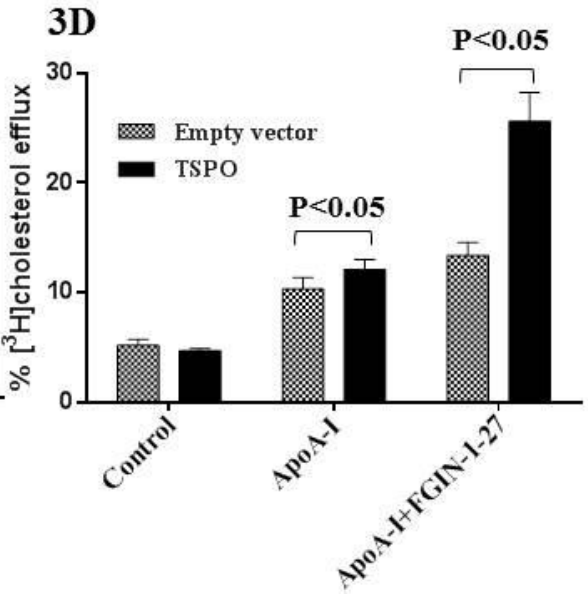
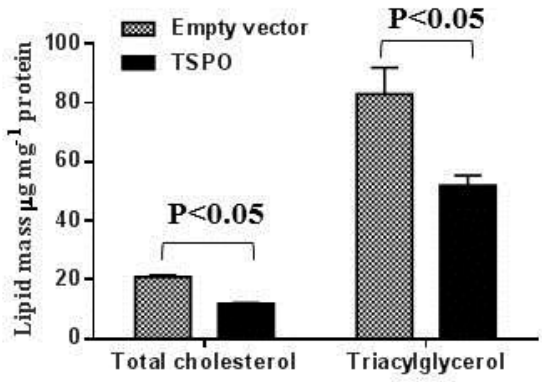
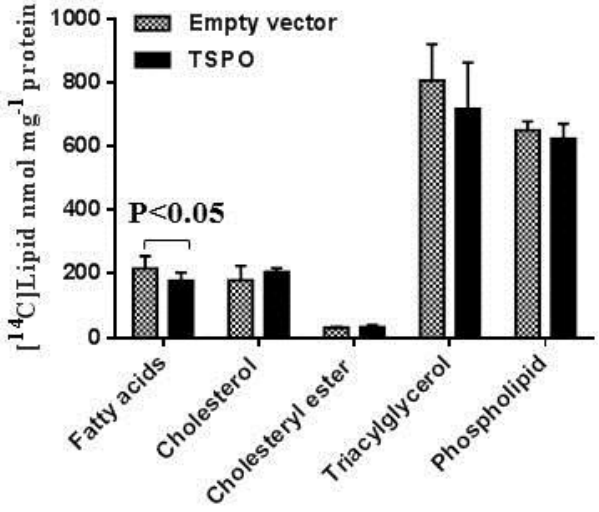

3D
% [3H]cholesterol efflux
Empty vector
TSPO
P<0.05
P<0.05
Control
ApoA-I
ApoA-I+FGIN-1-27

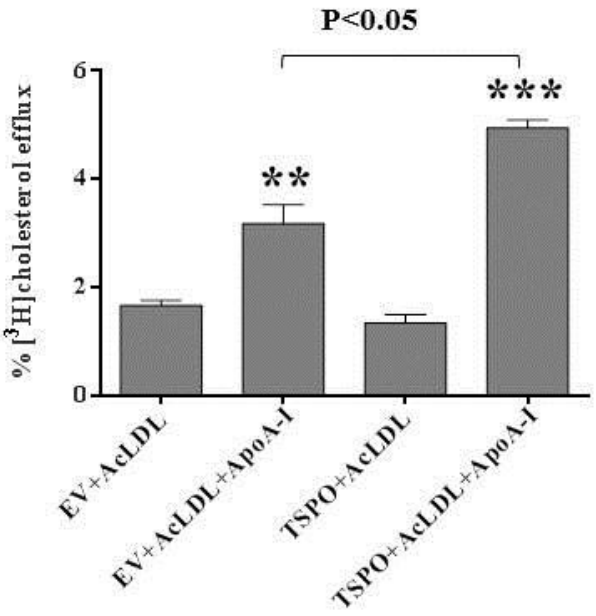
Figure 4A



4B



4C



4D

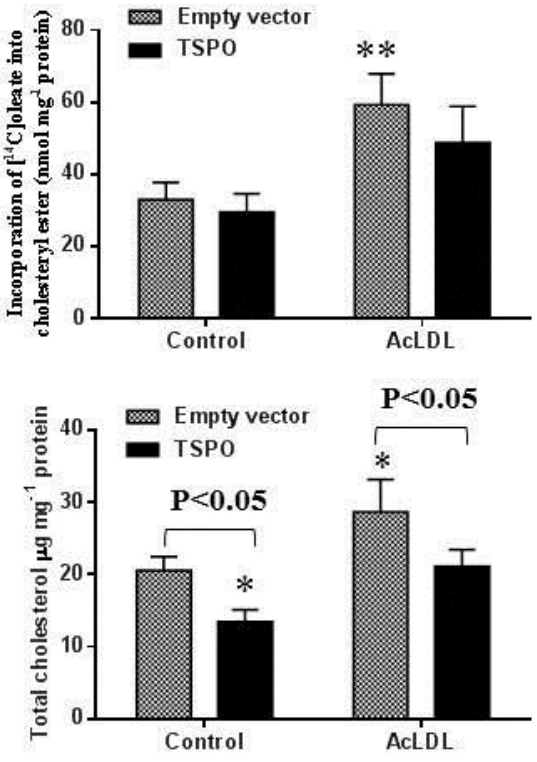
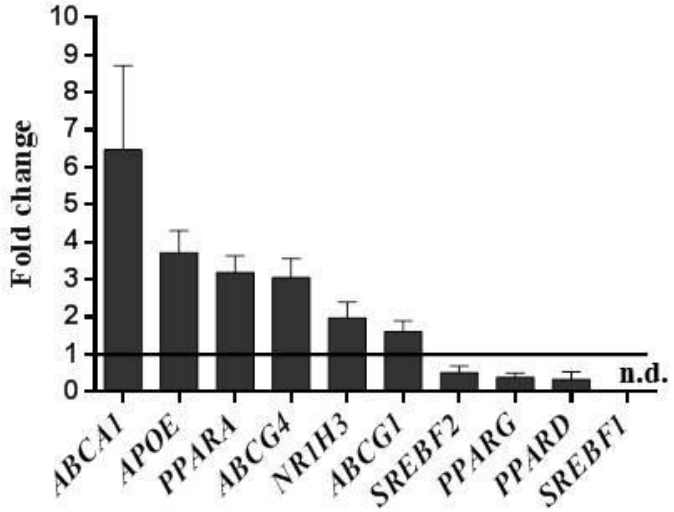
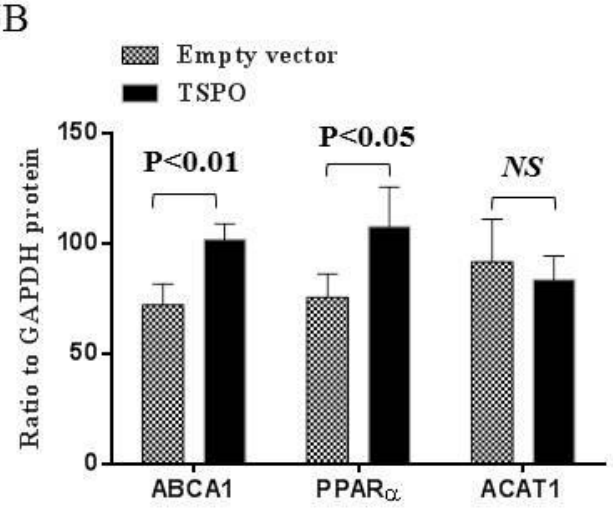


Figure 5A


Fold change
10
9
8
7
6
5
4
3
2
1
0
n.d.
ABCA1
APOE
PPARA
ABCG4
NR1H3
ABCG1
SREBF2
PPARG
PPARD
SREBF1

5B


Empty vector
TSPO
Ratio to GAPDH protein
150
100
50
0
P<0.01
P<0.05
NS
ABCA1
PPARα
ACAT1

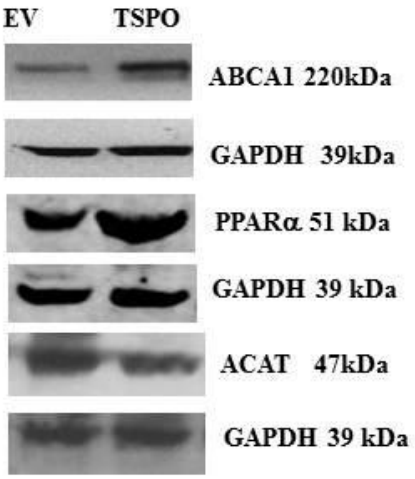
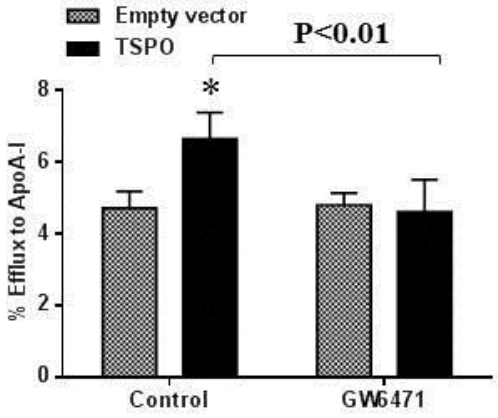
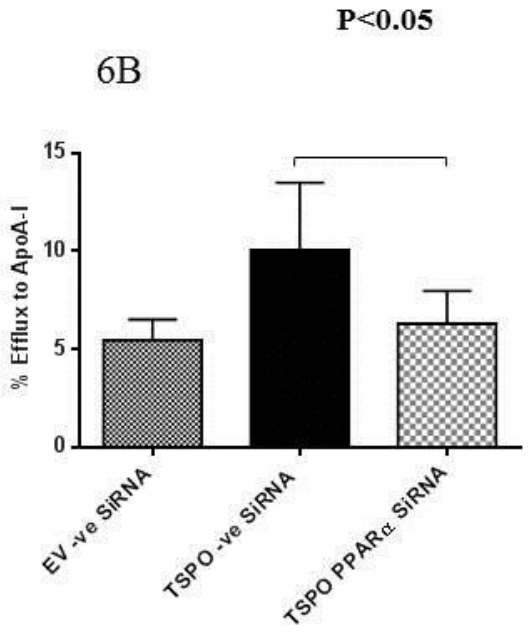

EV
TSPO
ABCA1 220kDa
GAPDH 39kDa
PPARα 51 kDa
GAPDH 39 kDa
ACAT 47kDa
GAPDH 39 kDa

Figure 6A



6B



6C