Differential effects of chlorinated and oxidized phospholipids in vascular tissue: implications for neointima formation.

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Abstract

The presence of inflammatory cells and MPO (myeloperoxidase) in the arterial wall after vascular injury could increase neointima formation by modification of phospholipids. This study investigates how these phospholipids, in particular oxidized and chlorinated species, are altered within injured vessels and how they affect VSMC (vascular smooth muscle cell) remodeling processes. Vascular injury was induced in C57BL/6 mice and high fat fed ApoE$^{-/-}$ mice by wire denudation and ligation of the left carotid artery. Neointimal and medial composition was assessed using immunohistochemistry and electrospray mass spectrometry. Primary rabbit aortic SMCs were utilized to examine the effects of modified lipids on VSMC proliferation, viability and migration at a cellular level. Neointimal area, measured as intima-to-media ratio was significantly larger in wire-injured ApoE$^{-/-}$ mice (3.62 ± 0.49 *vs.* 0.83 ± 0.25 in C57BL/6 mice, n=3) and there was increased oxidized LDL (oxLDL) infiltration and elevated plasma MPO levels. Relative increases in lysophosphatidylcholines and unsaturated phosphatidylcholines were also observed in wire-injured ApoE$^{-/-}$ carotid arteries. Chlorinated lipids had no effect on VSMC proliferation, viability or migration while chronic incubation with oxidized phospholipids stimulated proliferation in the presence of fetal calf serum (154.8 ± 14.2% of viable cells at 1 μM PGPC *vs.* control, n=6). In conclusion, ApoE$^{-/-}$ mice with an inflammatory phenotype develop more neointima in wire-injured arteries and accumulation of oxidized lipids in the vessel wall may propagate this effect

Abbreviations:

2-ClHDA, 2-chlorohexadecanal; αSMA, alpha smooth muscle actin; BrdU, bromodeoxyuridine; ClOH, chlorohydrin; ESMS, electrospray mass spectrometry; FCS, fetal calf serum; HOCl, hypochlorous acid; LCA, left carotid artery; MPO, myeloperoxidase; PAPC, 1-palmitoyl-2-arachidonoyl-s*n*-glycero-3-phosphocholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PGPC, 1-palmitoyl-2-glutaroyl-s*n*-glycero-3-phosphocholine; POVPC, 1-palmitoyl-2-oxovaleroyl-s*n*-glycero-3-phosphocholine; PS, phosphatidylserine; RCA, right carotid arteries; SOPC, 1-stearoyl-2-oleoyl-s*n*-glycero-3-phosphocholine; VSMC, vascular smooth muscle cell

INTRODUCTION

Revascularization procedures such as balloon angioplasty and stenting often result in neointimal hyperplasia in patients. This can be considered an exaggerated form of wound healing characterized by luminal (re)narrowing due to smooth muscle hyperplasia and rapid vascular remodeling of the instrumented vessel [1]. The formation of neointima is a complex and multifactorial process, but excessive VSMC (vascular smooth muscle cell) proliferation and migration, inflammation and the production of large amounts of ECM (extracellular matrix) are all involved [2].

Inflammation appears to be pivotal in driving neointima formation. In a rabbit model of balloon injury, there is early upregulation of leukocyte adhesion molecules on the injured vessel with increased inflammatory cell adhesion and transmigration [3]. There is also evidence of sustained inflammation, with inflammatory cells remaining in close proximity to the stent struts up to 28 days after placement [4]. In the mouse wire injury model, increased leukocyte adhesion to injured vessels is observed *ex vivo* up to 28 days following surgery [5]. The importance of circulating inflammatory cells was demonstrated in neutropenic rabbits, which had a significantly reduced extent of neointima at 28 days [6]. $ApoE^{-/-}$ mice (deficient in apolipoprotein E) are hyperlipidemic and demonstrate greater neointima formation following vascular injury induced by either wire denudation [7] or *ex vivo* aortic stenting and interpositional grafting [8]. $ApoE^{-/-}$ mice also show increased inflammatory cell adhesion to the injured carotid artery intima and, intriguingly, total plasma cholesterol correlated with neointima formation [7]. However, what remains to be determined is the role that modified lipids present in the vessel wall play in mediating the enhanced neointima formation in hyperlipidemic mice.

Modification of LDL (low density lipoproteins) and formation of oxidized phospholipids contributes to the pathophysiology of atherosclerosis [9]. The phagocytic enzyme MPO

(myeloperoxidase) catalyzes the production of HOCl (hypochlorous acid) from hydrogen peroxide and chloride anions [10] and can cause chlorination of phospholipids [11]. The active form of MPO as well as HOCl-modified LDL are present in human atherosclerotic plaques [12, 13] and correlate with an increase in the intima-to-media ratio in human iliac arteries [14]. Phospholipid chlorohydrins have a multitude of biological effects [15, 16]. Their high polarity can disrupt cell membranes and cause toxicity in myeloid and endothelial cells [17, 18] and can also induce leukocyte adhesion by upregulation of adhesion molecules [19]. Lysophosphatidylcholine-chlorohydrins have been detected in human atherosclerotic vessels with a 60-fold increase in plaques compared to healthy tissue [20], and fatty acid chlorohydrins have been found at high levels in the plasma of patients with acute pancreatitis [21]. Other chlorinated species such as 2-ClHDA (2-chlorohexadecanal) are produced by the action of MPO on plasmalogens [22] and have also been detected in human atherosclerotic plaques [23]. Oxidized phospholipids, such as oxidized PAPC (1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine) and its constituents, POVPC (1-palmitoyl-2-oxovaleroyl-*sn*-glycero-3-phosphocholine) and PGPC (1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphocholine), increase expression of pro-inflammatory genes in endothelial cells [24] and participate in the phenotypic switching of VSMCs that occurs following injury [25]. PAPC also induces monocyte adhesion and inflammation in atherosclerotic mice *in vivo* [26]. Several studies, including some conducted *in vivo*, have demonstrated that oxidized phospholipids can influence many of the processes involved in vascular remodeling such as VSMC proliferation, migration and extracellular matrix production [27-30].

In this study we used a mouse model of vascular injury in normal and hyperlipidemic ApoE$^{-/-}$ mice to generate vessels containing neointima. By comparing the lipid profile in the injured and contralateral uninjured carotid arteries, we were able to study how the presence of modified

phospholipids changes in an injured artery with neointima and how plasma hyperlipidemia influences this. We then studied the effects of some oxidized and chlorinated lipids known to be present in oxLDL (oxidized LDL) on cultured VSMCs, to assess whether the change in lipid profile observed in injured arteries could drive the main processes involved in neointima formation.

MATERIALS AND METHODS

Animals

All experiments were performed in accordance with the United Kingdom Animals (Scientific Procedure) Act of 1986. Weight-matched C57BL/6 and ApoE$^{-/-}$ mice (22-29 g) were 8 to 10 weeks of age at the start of the surgical procedure. Mice were maintained on 12 hour cycles of light and dark and at ambient temperature. C57BL/6 mice were fed a standard chow diet while ApoE$^{-/-}$ mice commenced a high fat diet (21% lard and 0.15% cholesterol, SDS, Essex, U.K.) at 6 weeks of age and continued on this throughout the study period.

Murine carotid artery injury model

Mice were given preoperative analgesia (i.p. (intraperitoneal) buprenorphine; 0.1 mg/kg), antiplatelet therapy (i.p. dipyridamole; 2.5 mg) and saline (0.5 ml subcutaneously). Carotid injury was performed according to an adapted method previously described [5]. General anaesthesia was induced by inhalation of 3% isoflurane supplemented with oxygen and maintained at 1.5% isoflurane throughout the procedure. The LCA (left carotid artery) was exposed and the proximal end ligated. An incision was made and a nylon fishing line (external diameter 0.014 in) with a blunt, spherical tip was inserted and advanced down the vessel with a torquing motion to ensure endothelial damage. The artery was then ligated distal to the

incision site. Mice where the artery was ligated without the insertion of the nylon line were also investigated as ligation alone has been shown to result in formation of neointima [31]. After 14 days, mice were euthanized and carotid arteries harvested and either fixed and embedded in paraffin wax for histological examination or snap frozen for ESMS (electrospray mass spectrometry) analysis.

Histological analysis

Paraffin embedded LCA and RCA (right carotid arteries) were serially sectioned at 4 μm and stained with haematoxylin and eosin to visualize and quantify medial and neointimal areas. To investigate the presence of proliferating and apoptotic cells as well as identify VSMCs in the vessel wall, immunohistochemistry was utilized. Briefly, sections were rehydrated and antigens retrieved by microwave pressure cooking in citric acid. Endogenous peroxidase and non-specific binding was blocked prior to primary antibody incubation overnight at 4 ^{o}C. The primary antibodies were diluted in 1% (w/v) BSA in PBS and used at the following dilutions: αSMA (alpha smooth muscle actin), 1:200, active caspase 3, 1:50, Ki67, 1:100 and E06, 1:100. Secondary antibody (anti-rabbit IgG antibody; Vector Laboratories, U.K.) or, in the case of E06, anti-mouse IgG (Abcam, U.K.) was added for 1 hour at room temperature before DAB chromagen solution (3,3-diaminobenzidine and hydrogen peroxidase solution; Vector Laboratories, U.K.) was added for between 2-5 minutes to develop. Slides were then counterstained with haematoxylin, dehydrated and coverslipped. In all cases, a negative control where the primary antibody was omitted was run concurrently. Staining was visualized using a light microscope and analyzed using QCapture Pro 6.0 software.

Measurement of plasma MPO

The MPO content of plasma from C57BL/6 and ApoE$^{-/-}$ mice was analyzed using a mouse MPO ELISA kit (Hycult Biotech Inc., Netherlands). Plasma samples were diluted 1 in 16 in dilution buffer and the assay was performed as per the manufacturer's instructions. A standard curve ranging from 1.6 ng/ml to 100 ng/ml was included for each experiment. Absorbance values were measured spectrophotometrically at 450 nm using a SpectraMax® M2 microplate reader.

ESMS of phospholipids extracted from vessels

The lipid content of uninjured, ligated and wire-injured arteries was investigated using a modified version of the Bligh-Dyer procedure [32] for lipid extraction from vessels. Briefly, LCA and RCA were placed in methanol containing 100 µg/ml of butylated hydroxytoluene, vortexed and sonicated for 15 minutes. An equal volume of chloroform was then added, vortexed and left overnight at 4 ºC. An aqueous layer of 0.88% (w/v) KCl was added to the mixture, vortexed and incubated at 4 ºC for 20 minutes. The layers were separated by centrifugation (13800 x *g* for 1 minute) and the chloroform phase removed and dried under a steady flow of oxygen-free nitrogen gas. Dried lipid extracts were reconstituted in 20% (v/v) chloroform in methanol and then diluted in methanol. Samples were run in either positive- or negative-ion mode by direct infusion on a QTRAP® 5500 mass spectrometer (AB SCIEX, Warrington, U.K.). For analysis using positive-ion mode, samples were diluted in 1% (v/v) aqueous formic acid in methanol, giving a final dilution of the lipid extracts of 1 in 500 for LCA and 1 in 250 for RCA. Spectra, in the range of m/z 400-1000, were acquired for 2 minutes. Precursor ion scanning was performed for m/z 184.1 which is selective for PCs (phosphatidylcholines) and a neutral ion loss scan of 141.1 Da for PEs (phosphatidylethanolamines). For analysis using negative-ion mode, samples were diluted in 10% (v/v) 5 mM ammonium acetate in methanol, giving a final dilution of the lipid extracts of

1 in 50 for LCA and 1 in 25 for RCA. Spectra, in the range of m/z 400-1000, were acquired for 4 minutes. Neutral loss scan of 87.0 Da was performed for PSs (phosphatidylserines). Samples were analyzed by looking for consistent differences observed between spectra from different sample groups.

Preparation of chlorinated lipids

Native phospholipid (SOPC, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) and the oxidized lipids, PGPC and POVPC, were purchased from Avanti Polar Lipids (Alabama, U.S.A.). Formation of chlorohydrins was assessed by the loss of the native phospholipid and the addition of m/z 52 or 54 (differences being due to the pattern of the chloride isotope, either ^{35}Cl or ^{37}Cl) to the native phospholipid which was seen in its sodiated form (an additional 22 m/z to the value compared to the protonated form).

The alpha-chloro fatty aldehyde, 2-ClHDA, was synthesized and provided by Professor Andrew R. Pitt (Aston University, U.K.) following an adapted method previously described [33]. Briefly, hexadecanol was oxidized to hexadecanal using pyridinium chlorochromate in oxygen-free CH_2Cl_2, followed by reaction with methanol and catalytic *p*-toluenesulfonic acid to form the dimethyl acetal. The dimethyl acetal was then α-chlorinated utilizing MnO_2-trimethylchlorosilane. 2-ClHDA was finally produced by refluxing the chloroacetal in 1:1 trifluoroacetic acid:dichloromethane and purified by flash chromatography using silica eluting with 7:3 hexane:dichloromethane to give solid 2-ClHDA (17% overall yield).

VSMC proliferation, viability and migration

Ideally, the *in vitro* effects of chlorinated and oxidized lipids would have been tested in mouse VSMCs. However, in order to provide a sufficient quantity of VSMCs for all assays, rabbit aortic SMCs were utilized and grown from explants. Although derived from a different species,

this allowed us to test several oxidized and chlorinated lipids, several incubation times and a range of concentrations, which would not have been possible using mouse cells. Use of primary cells from explants also avoided the use of immortalised cell lines or use of cells at a high passage where features associated with VSMCs may have been lost. Briefly, aortae from male New Zealand white rabbits (2.5 to 3.5 kg body weight) were excised and cleaned of any connective tissue and fat. The outer and inner surface of the vessel was scraped, removing the adventitia and endothelium respectively. VSMCs were cultured in in 1:1 Waymouth's MB 752/1 and Ham's F12 with GlutaMAX supplemented with 1% (v/v) penicillin-streptomycin solution and 10% (v/v) FCS (fetal calf serum) and used between passages 3 to 8. Cells were identified as VSMCs by positive immunofluorescent staining for αSMA (data not shown). All oxidized or chlorinated lipids were resuspended in serum-free VSMC medium at a concentration of $1x10^{-2}$ M by vortexing and sonicating before diluting to the required concentration for cell treatment. Under these conditions, the lipids form multilamellar vesicles rather than micelles or liposomes.

The effect of modified lipids on cell proliferation was measured using a BrdU (bromodeoxyuridine) assay kit (Calbiochem, U.K.). Cells were seeded in 96 well plates at a density of 10,000 cells per well and quiesced in 0.1% (v/v) FCS-containing medium for 24 hours. Cells were incubated with the chlorinated or oxidized lipids (1-100 μM) for either 2 hours in 0.1% FCS-containing medium for acute experiments or, in the case of chronic exposure, for 24 hours in the presence of 10% FCS. VSMCs were then stimulated with 10% FCS-containing medium and addition of BrdU 24 hours prior to terminating the experiment. The assay was performed as per the manufacturer's instructions and proliferation was detected using a spectrophotometric measurement of absorbance at dual wavelengths of 450 nm and 540 nm using a SpectraMax M2 microplate reader.

Bioluminescent detection of cellular ATP using a ViaLight™ Plus kit (Lonza, Switzerland) was employed to determine cell viability after either 2 or 6 hours in 0.1% FCS-containing medium or 24 hours exposure to the modified lipids in the presence of FCS. This assay enables a comparison to be made between cells incubated under control conditions with those incubated with lipids. A reduction in total cellular ATP is indicative of a degree of cell apoptosis or necrosis though it cannot distinguish between the two. Cells were prepared as for the proliferation assay then lysis buffer was added to release cellular ATP. Following this, ATP Monitoring Reagent Plus containing luciferase was added to the lysed cells to catalyze the conversion of ATP and luciferin into emitted light. Luminescence was measured using a POLARstar OPTIMA microplate reader (BMG Labtech, Germany).

Cell migration was measured using a CHEMICON® QCM chemotaxis cell migration assay containing an 8 μm pore membrane (Millipore, Watford, U.K.). Cells were quiesced in 0.1% FCS-containing medium for 24 hours prior to stimulation. For pretreatment experiments, cells were incubated for 2 hours with either the chlorinated or oxidized lipids then harvested and added at 30 000 cells/well to the top chamber in serum-free medium. 10% FCS-containing medium was utilized as the chemotactic agent in the lower chamber. After 24 hours, the remaining cells and media present in the upper chamber were aspirated and non-migrated cells were removed from the interior side of the insert. The membrane was then stained, placed in the extraction buffer and the absorbance measured at 560 nm using a SpectraMax M2 microplate reader. For chronic experiments, the lipid was present in the top chamber for the full 24 hour stimulation period.

Statistical analysis

All results are expressed as mean ± SEM. Data were analyzed with GraphPad Prism 5.0 software (California, U.S.A.) using either a one-way ANOVA followed by a Dunnett's or two-

way ANOVA followed by Bonferroni's post hoc test as appropriate. In all cases, $p<0.05$ was considered statistically significant.

RESULTS

Extent of neointima formation in C57BL/6 and ApoE$^{-/-}$ mice

Ligation of the LCA without wire injury resulted in formation of measurable neointima compared to the contralateral, uninjured RCA in both C57BL/6 and ApoE$^{-/-}$ mice (Figure 1A). There was significantly more neointima, measured as the intima-to-media ratio, present in injured ApoE$^{-/-}$ LCA compared to both injured C57BL/6 vessels and ligated ApoE$^{-/-}$ vessels (Figure 1B). Measurement of the external elastic lamina length revealed no difference in vessel circumference between groups and microscopic examination of ApoE$^{-/-}$ aortae revealed no atherosclerotic plaques (Figure 1A). However, ApoE$^{-/-}$ mice did display a generalized inflammation characterized by significantly increased plasma MPO compared to C57BL/6 mice in both ligated and injured groups (Figure 1C) and a significantly increased spleen weight relative to total body weight (data not shown). Immunohistochemical analysis of neointima at 14 days revealed no difference in the presence of VSMCs (αSMA; Figure 2A) or proliferating cells (Ki67; Figure 2B). Minimal staining for active caspase 3, a marker of apoptosis was found in all groups (Figure 2C).

Presence of modified lipids in the arterial wall after acute vascular injury

In the uninjured RCA, neither strain showed any positive staining for oxLDL using the E06 antibody (Figure 3). In injured vessels, ApoE$^{-/-}$ mice had a greater abundance of oxLDL present compared to C57BL/6 mice where expression was lower and more variable (Figure 3). Using ESMS, the ratios of different phospholipids in uninjured, ligated and injured arteries from

C57BL/6 and ApoE$^{-/-}$ mice were studied. Some differences in lipid profile between C57BL/6 and ApoE$^{-/-}$ carotid arteries were noted, but more importantly, the response to ligation and injury differed. Significantly higher relative levels of lysophosphatidylcholines were found in both ligated and injured ApoE$^{-/-}$ carotid arteries compared with the uninjured RCA while no change in lysophosphatidylcholines were seen in C57BL/6 mice after vascular injury (Figure 4). Precursor ion scanning for m/z 184.1 displayed small elevations in the relative levels of PCs, m/z 734.9 (C32:0) in ligated and injured carotid arteries of C57BL/6 mice and m/z 810.9 (C38:4) in ligated carotid arteries (Figure 4A and C). In ApoE$^{-/-}$ mice, m/z 734.9 (C32:0) was also relatively increased in ligated arteries, as was m/z 758.9 (C34:2) and 786.9 (C36:2) in both ligated and injured vessels compared to the uninjured RCA (Figure 4B and D). In comparison, there was a reduction in the relative levels of m/z 782.9 (C36:4) and 810.9 (C38:4) in injured ApoE$^{-/-}$ carotid arteries. No detectable changes were observed in chain-shortened PCs. PEs and PSs were present at considerably lower concentrations than PCs and there were no significant changes across the groups in either strain of mice (data not shown).

Effect of modified lipids on VSMC remodeling processes

As we found changes in lysophosphatidylcholines and other PCs in the injured vessel wall, and since raised plasma MPO may form modified lipids which could contribute to the greater neointima formation in ApoE$^{-/-}$ mice, we assessed the effect of selected chlorinated and oxidized lipids on VSMC remodeling processes. Following acute exposure (2 hours in 0.1% FCS-containing medium) neither the phospholipid chlorohydrin, SOPC ClOH nor the alpha-chloro fatty aldehyde, 2-ClHDA had any effect on VSMC proliferation, viability or migration, even at concentrations as high as 100 μM (Figure 5). In contrast the oxidized lipids POVPC or PGPC caused a significant reduction in VSMC proliferation and viability at concentrations of 25 μM (PGPC) or 50 μM (POVPC) and above. At lower, non-toxic concentrations neither

oxidized lipid affected VSMC migration (Figure 6). No morphological changes were observed following either chlorinated lipid treatment (Figure 5E); however, substantial cell death was evident after incubation with 50 μM of either oxidized phospholipid (Figure 6E). A longer incubation (6 hours in 0.1% FCS-containing medium) with SOPC ClOH caused a concentration-dependent reduction in VSMC viability at 100 μM, whereas 2-ClHDA had no effect (data not shown). As expected, 6 hours incubation with POVPC caused a dramatic reduction in VSMC viability (data not shown).

Chronic incubation (24 hours in 10% FCS-containing medium) with SOPC ClOH or 2-ClHDA had no effect on VSMC proliferation, viability or migration (data not shown). Paradoxically, the decline in proliferation and viability seen after 2 hours treatment with POVPC was reversed with chronic incubation (Figure 7A-C) while PGPC significantly increased VSMC proliferation and viability (Figure 7B-D). However, both POVPC and PGPC caused a marked reduction in migration at the highest concentration studied (Figure 7F-G). No significant changes in morphology were observed with either treatment (Figure 7E).

DISCUSSION

Here we characterize for the first time the phospholipid content of the arterial wall of C57BL/6 and ApoE$^{-/-}$ mice after wire injury. Increased neointima formation was observed in injured arteries in ApoE$^{-/-}$ mice, as well as elevated plasma MPO levels, increased oxLDL and an altered lipid profile in the arterial wall in comparison with C57BL/6 mice and also in comparison with the uninjured contralateral carotid artery. In addition, this study is the first to investigate the effects of chlorinated lipids on VSMC remodeling processes and highlight the divergent effects of chlorinated and oxidized lipid species. Chlorinated lipids had no effect on VSMC proliferation, viability or migration, whereas oxidized phospholipids caused a

concentration-dependent reduction in all of these VSMC remodeling processes, which was reversed in the presence of FCS.

In this study, neointima formation was dramatically increased in injured ApoE$^{-/-}$ arteries compared to carotid ligation without injury, while no significant differences were observed in C57BL/6 mice. Increased neointima formation has previously been observed following both wire and balloon injuries in ApoE$^{-/-}$ mice [7, 34, 35] suggesting there is an increased susceptibility for neointima formation in atherosclerotic or hyperlipidemic mice. Elevated plasma MPO content could contribute to the enhanced inflammatory state of these mice and result in an increase in the production of reactive oxidants. Indeed, increased oxLDL was detected in the neointima of ApoE$^{-/-}$ mice, which may well have occurred through the heightened inflammation and/or MPO in the vessel wall after injury. Incubation of MPO and its product, HOCl, in a temporarily isolated carotid artery induced intimal and medial apoptosis which was followed by a proliferative response and neointima hyperplasia in a rat model [36]. In this study, only the plasma MPO was measured and therefore the level of MPO in the neointima is unknown. However, greater infiltration of inflammatory cells at the site of injury in ApoE$^{-/-}$ mice is likely to enhance the secretion of MPO in the developing neointima.

MPO is a known route for producing both chlorinated and oxidized lipid species *in vivo*. Intact phospholipid chlorohydrins have yet to be observed in diseased vessels *in vivo* while lysophosphatidylcholine-chlorohydrins have been detected in human atherosclerotic vessels with a 60-fold increase in plaques compared to healthy tissue [20]. We observed a marked elevation in the relative levels of lysolipids at m/z 496.7 (lyso-palmitoyl PC) and 524.7 (lyso-stearoyl PC) in ligated and injured ApoE$^{-/-}$ carotid arteries in comparison to the uninjured vessels and C57BL/6 mice. Lysophosphatidylcholine has previously been found to induce apoptosis in VSMCs and promote recruitment of monocytes [37, 38], which could lead to an exacerbated inflammatory response at the site of injury. The precursor ion scan for m/z 184.1

displayed an increase in relative abundance of unsaturated PCs such as m/z 758.9 (C34:2) and 786.9 (C36:2) in ligated and injured LCA of ApoE$^{-/-}$ mice and a reduction in m/z 782.9 (C36:4) and 810.9 (C38:4). This suggests there is an increase in di-unsaturated species in ApoE$^{-/-}$ mice following vascular injury but a reduction in arachidonate-containing PCs in the injured arteries. These unsaturated PCs could then undergo modification by MPO leading to a further increase in modified lipids present at the site of injury. However, chain-shortened PCs were not detected, which could be due to low concentrations compared to their native phospholipids within these samples, or they may be metabolized or detoxified *in vivo* [39]. The high levels of plasma MPO in ApoE$^{-/-}$ mice suggests the concept of increased levels of HOCl and therefore of chlorinated lipids in the vessels, although without a more extensive investigation by LC-MSMS and GCMS, and quantification of MPO within the vessel itself it is not possible to confirm this.

The levels of modified lipids found in pathophysiological conditions have previously proved hard to quantify, owing to the limited availability of suitable controls and the large number of different structures present. A limitation of the present study is the lack of internal or external standards in the ESMS analysis. In this study, the sizes and weights of the arteries varied thus the lipid concentrations could not be accurately calculated. However, the amount of lipid present is likely to vary within the artery therefore, while standards may indicate the absolute concentration, all lipids should fluctuate depending on the total lipid content extracted. It should also be noted that ESMS is not a quantitative technique in itself, due to the different ionization efficiencies of lipid classes, therefore PC levels cannot be directly compared with PEs or PSs. However, all these differences should fluctuate concurrently between samples, thus it is valid to look for changes in relative intensities and patterns between samples. In areas of inflammation such as rheumatoid arthritis, the presence of OCl^-, the anion present in HOCl, has been found to reach concentrations of up to 300 μM, due to the presence of neutrophils

releasing MPO [40]. The levels of oxidized phospholipids have also been found within the micromolar range in atherosclerotic plaques in both human and animal tissue [41-43].

Within this concentration range, chlorinated lipids were found to have little to no effect on VSMCs. The majority of work published previously has observed toxicity with incubation of chlorohydrins thought to be caused by disruption of the membrane due to their high polarity [17]. However, necrotic cell death has also been reported in endothelial cells and an increase in caspase 3 levels suggesting apoptosis in myeloid cells [18, 44]. The lack of cytotoxicity described in this study may be due to the different cell type used, as VSMCs could be more resistant to the effects of chlorohydrins than other cell types, or potential species differences. In contrast to chlorohydrins, very little is known about the actions of alpha-chloro fatty aldehydes as the few studies conducted focussed primarily on endothelial cells and nitric oxide biosynthesis [45, 46]. 2-ClHDA was thought to be an exciting prospect with its identification in atherosclerotic lesions *in vivo*; however, no effects were observed on the vascular remodeling processes examined in this study.

In stark contrast, oxidized phospholipids caused a dramatic concentration-dependent reduction in VSMC proliferation and viability after 2 hours incubation. These lipids have been found to induce apoptotic signaling pathways by the activation of sphingomyelinase and, in particular, the acid form of the enzyme which is known to be involved in the earlier stages of apoptosis resulting in phosphorylation of MAPK and caspase 3 signaling [47, 48]. A biphasic response of oxidized phospholipids has been reported previously with proliferation occurring at low concentration and apoptosis predominating at high concentrations [27, 29]. In the present study, chronic incubation of oxidized phospholipids in the presence of FCS abolished cell death and induced an increase in proliferation and viability. This would suggest that the presence of serum phospholipases causes a breakdown of oxidized phospholipids into a product with anti-apoptotic effects. This is in partial agreement with a previous study; however, the anti-

proliferative nature of the lipids was still seen with the oxidized phospholipid treatment in the presence of FCS [28]. PGPC rather than POVPC had the greater effect in all our experiments, despite the fact that the latter has been suggested to be the more potent of the two truncated oxidation products of PAPC [28, 47]. However, recently PGPC has been reported to have a higher toxicity in cultured macrophages due to more efficient membrane blebbing in apoptotic cells [49].

Previous studies have found apoptosis to peak at about 24 hours after vascular injury while proliferation occurs later in the process, at around 4 days after injury [7, 36]. This correlates with the oxidized phospholipid data described in this study, where VSMC death was induced after a short incubation time of only 2 hours. Modified lipids could also be involved in the latter proliferative stages of vascular injury leading to the formation of neointima; however, this would be difficult to measure *in vitro*. Longer incubations would require medium containing growth supplements such as FCS which would lead to the breakdown of these lipids as shown in this study and by Fruhwirth *et al,* (2006).

In conclusion, we have detected alterations in several modified lipids in ligated and wire-injured carotid arteries from ApoE$^{-/-}$ mice compared to uninjured vessels and C57BL/6 controls. These differences are likely to be a consequence of heightened inflammatory state in the plasma of hyperlipidemic ApoE$^{-/-}$ mice coupled with inflammatory cell infiltration following wire injury. Our *in vitro* data suggest that chlorinated lipids are unlikely to be involved in vascular smooth muscle remodeling processes which lead to neointima formation. However, incubation of oxidized lipids in the presence of FCS led to increased proliferation and accumulation of these species in the artery wall following injury and so they may be involved in propagating neointima formation. This hypothesis requires to be tested *in vivo*.

CLINICAL PERSPECTIVES

- Abnormal VSMC proliferation and migration contribute to restenosis in patients following revascularization techniques such as balloon angioplasty or stent implantation. MPO (myeloperoxidase) and modified lipids have been found to be vital mediators in the propagation of the inflammatory response and plaque formation, suggesting they could be viable targets in preventing restenosis.
- The present study shows the altered phospholipid expression within the injured arterial wall of hyperlipidemic mice and elevated MPO levels. In addition, there were divergent effects of modified lipids on VSMC remodeling processes.
- These findings suggest that changes in unsaturated phospholipids could be involved in initiating and maintaining VSMC remodeling processes which are critical in restenosis in patients and therefore could be targeted for future therapies.

AUTHOR CONTRIBUTION

Fiona Greig, Corinne Spickett and Simon Kennedy were responsible for study conception and design. All authors were responsible for data collection, interpretation and analysis. Fiona Greig, Corinne Spickett and Simon Kennedy were responsible for manuscript drafting.

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FIGURE LEGENDS

Figure 1 Effect of vascular injury on the intima-to-media ratio and plasma MPO levels in C57BL/6 and ApoE$^{-/-}$ mice.

(**A**) Representative histological sections of RCA and LCA from ligated and injured C57BL/6 and ApoE$^{-/-}$ mice using haematoxylin and eosin staining. Scale bar for LCA and RCA = 100 μm, magnification x 10. Scale for aorta = 250 μm, magnification x 4. (**B**) Neointimal growth in LCA after vascular injury was assessed as the intima-to-media ratio. $^{‡‡}p<0.01$ *vs.* C57BL/6 injured, $^{††}p<0.01$ *vs.* ApoE$^{-/-}$ ligated. Each bar represents the mean ± S.E.M. of 3 or 4 animals. (**C**) The MPO content of mouse plasma was measured using a mouse MPO ELISA kit. *******$p<0.001$ *vs.* C57BL/6 ligated, $^{‡‡‡}p<0.001$ *vs.* C57BL/6 injured. Each bar represents the mean ± S.E.M. of six animals.

Figure 2 Effect of vascular injury on smooth muscle, proliferative and apoptotic markers in C57BL/6 and ApoE$^{-/-}$ mice.

Representative histological sections of injured LCA of C57BL/6 and ApoE$^{-/-}$ mice which were stained with (**A**) anti-αSMA, (**B**) anti-Ki67 and (**C**) anti-active caspase 3 then counterstained with haematoxylin. Positive immunoreactivity for αSMA, Ki67 or active caspase 3 was measured as a percentage of positive DAB staining divided by the neointimal area. Scale bar = 100 μm, magnification x 10. Each bar represents mean ± S.E.M. of three animals.

Figure 3 Detection of oxLDL in the carotid arteries of C57BL/6 and ApoE$^{-/-}$ mice.

Representative histological sections of RCA and LCA of C57BL/6 (top) and ApoE$^{-/-}$ mice (bottom) which were stained with E06 to detect oxLDL. Scale bar = 100 μm, magnification x 20 (left) or x 10 (right).

Figure 4 Effect of vascular injury on modified lipids present in the neointima and arterial wall of C57BL/6 and ApoE$^{-/-}$ mice.

(A) Precursor ion scanning for m/z 184.1 by positive-ionization ESMS identified the distribution of PCs in uninjured RCA, ligated and injured LCA of C57BL/6 mice. Spectra shown are representative. The signal at m/z 564.0 was not identified as a phospholipid species and is thought to be a contaminant from the HPLC system. (**B**) Precursor ion scanning for m/z 184.1 by positive-ionization ESMS identified the distribution of PCs in uninjured RCA, ligated and injured LCA of ApoE$^{-/-}$ mice. Spectra shown are representative. (**C**) Relative intensities of detected PCs as a percentage of the largest peak in C57BL/6 mice. *****$p<0.05$, ******$p<0.01$ and *******$p<0.001$ *vs.* uninjured. Each bar represents the mean ± S.E.M. of three animals. (**D**) Relative intensities of detected PCs as a percentage of the largest peak in ApoE$^{-/-}$ mice. *****$p<0.05$, ******$p<0.01$ and *******$p<0.001$ *vs.* uninjured. Each bar represents the mean ± S.E.M. of three animals.

Figure 5 Effect of acute treatment of chlorinated lipids on VSMC remodeling processes.

VSMCs were incubated with 1-100 μM of SOPC ClOH or 2-ClHDA for 2 hours prior to stimulation with 10% FCS-containing medium. (**A-B**) VSMC proliferation was assessed by incorporation of BrdU. (**C-D**) Cell viability was measured by detection of cellular ATP. (**E**) Representative images of VSMCs treated with chlorinated lipids to visualize morphological changes. (**F-G**) Cell migration was measured using a chemotaxis assay. Each bar represents the mean ± S.E.M. of six animals and performed in triplicate.

Figure 6 Effect of acute treatment of oxidized phospholipids on VSMC remodeling processes.

VSMCs were incubated with 1-100 μM of POVPC or PGPC for 2 hours prior to stimulation with 10% FCS-containing medium. (**A-B**) VSMC proliferation was assessed by incorporation of BrdU. (**C-D**) Cell viability was measured by detection of cellular ATP. (**E**) Representative images of VSMCs treated with oxidized phospholipids to visualize morphological changes. (**F-G**) Cell migration was measured using a chemotaxis assay. *****p<0.05, ******p<0.01 and *******p<0.001 *vs.* 10% FCS control. Each bar represents the mean ± S.E.M. of six animals and performed in triplicate.

Figure 7 Effect of chronic incubation of oxidized phospholipids on VSMC remodeling processes.

(**A-B**) VSMC proliferation was assessed by incorporation of BrdU. (**C-D**) Cell viability was measured by detection of cellular ATP. (**E**) Representative images of VSMCs treated with oxidized phospholipids to visualize morphological changes. (**F-G**) Cell migration was measured using a chemotaxis assay. *****p<0.05 and *******p<0.001 *vs.* 10% FCS control. Each bar represents the mean ± S.E.M. of six animals and performed in triplicate.

SUMMARY STATEMENT

Many patients have to undergo revascularization techniques following stent implantation due to neointima hyperplasia. The present study highlights a link between modified lipids and vascular smooth muscle remodeling processes, which are critical in this neointima formation.

Figure 1

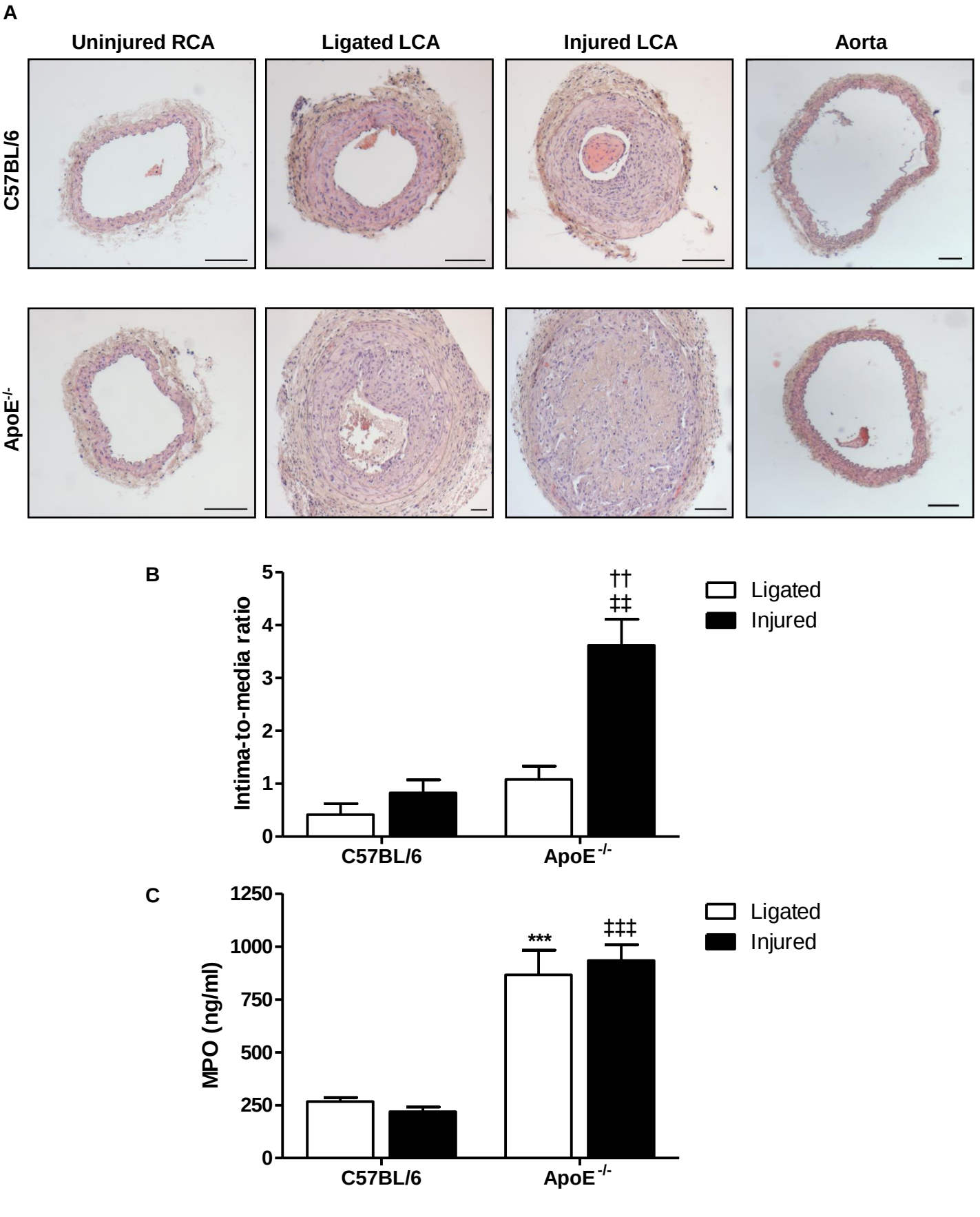

A
Uninjured RCA
Ligated LCA
Injured LCA
Aorta
C57BL/6
ApoE-/-
B
Intima-to-media ratio
0
1
2
3
4
5
††
‡‡
Ligated
Injured
C57BL/6
ApoE-/-
C
MPO (ng/ml)
0
250
500
750
1000
1250
***
‡‡‡
Ligated
Injured
C57BL/6
ApoE-/-

Figure 2

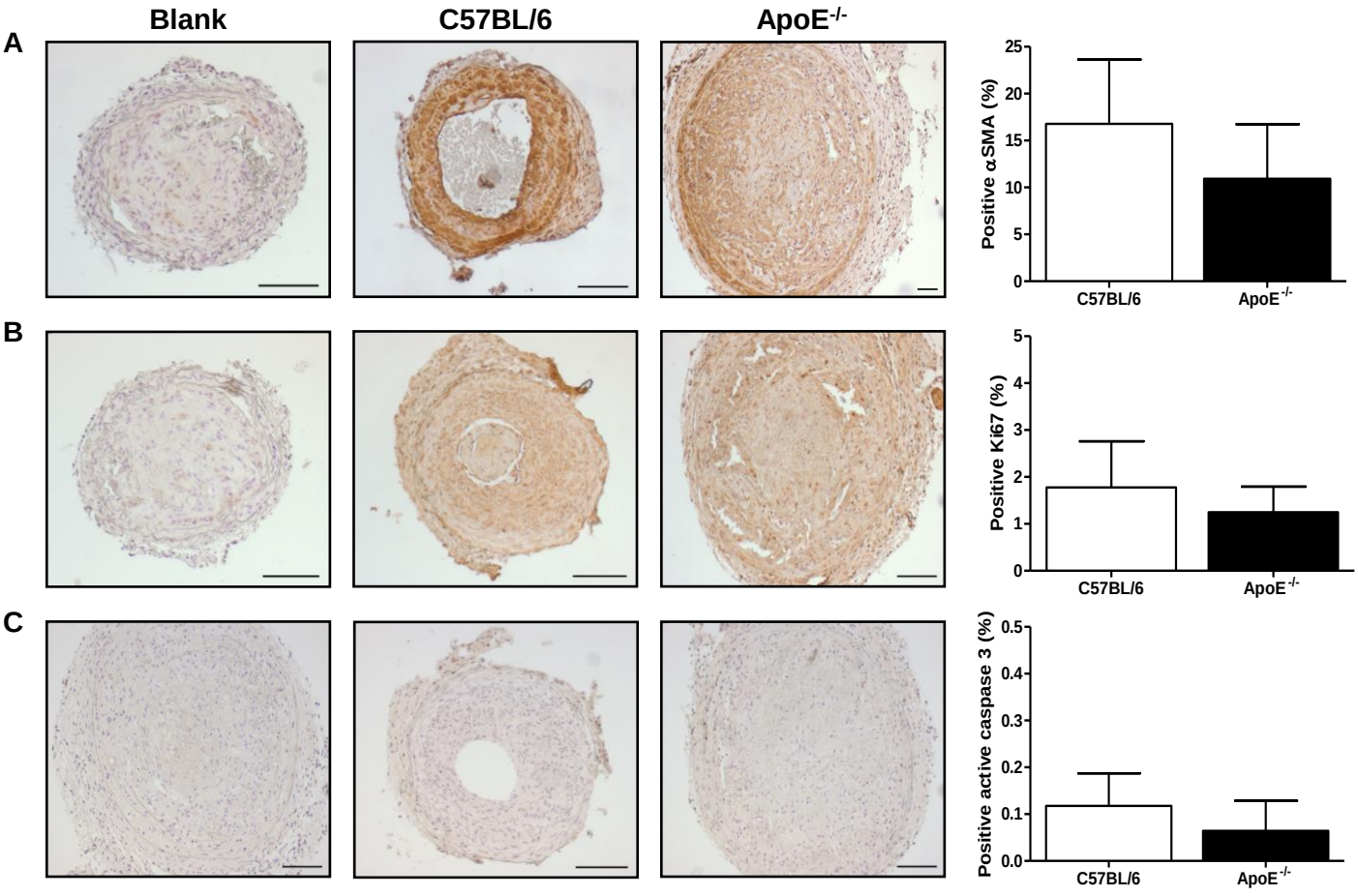

Blank
C57BL/6
ApoE-/-
A
B
C
Positive αSMA (%)
25
20
15
10
5
0
C57BL/6
ApoE-/-
Positive Ki67 (%)
5
4
3
2
1
0
C57BL/6
ApoE-/-
Positive active caspase 3 (%)
0.5
0.4
0.3
0.2
0.1
0.0
C57BL/6
ApoE-/-

Figure 3

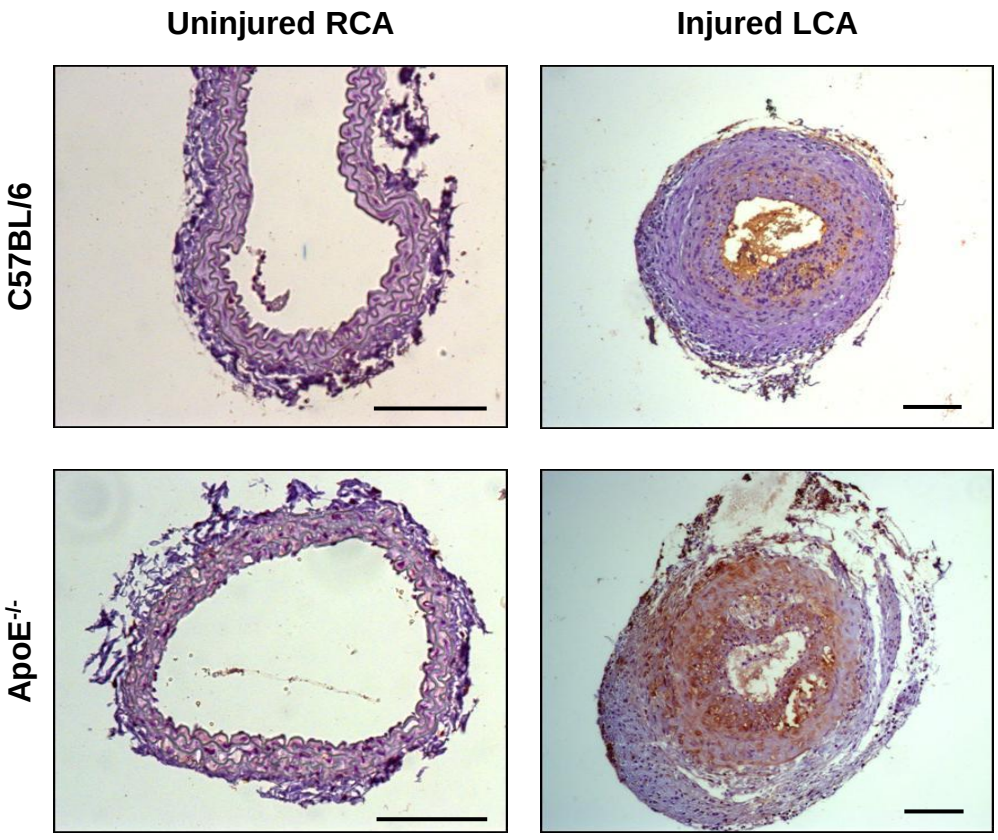


Figure 4

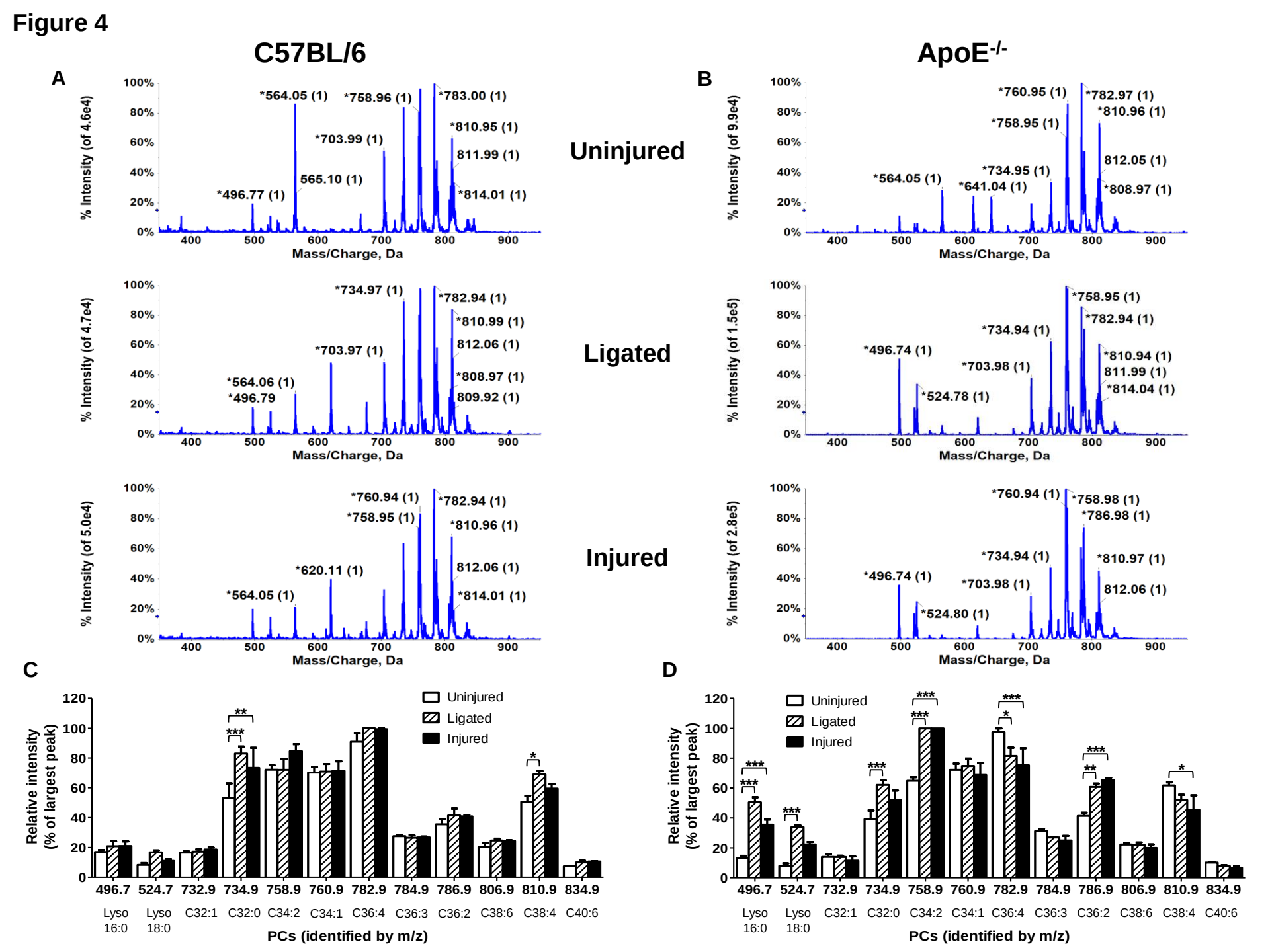

C57BL/6
ApoE-/-
A
B
Uninjured
Ligated
Injured
% Intensity (of 4.6e4)
*564.05 (1)
*758.96 (1)
*783.00 (1)
*703.99 (1)
*810.95 (1)
811.99 (1)
565.10 (1)
*496.77 (1)
*814.01 (1)
% Intensity (of 9.9e4)
*760.95 (1)
*782.97 (1)
*810.96 (1)
*758.95 (1)
812.05 (1)
*734.95 (1)
*564.05 (1)
*641.04 (1)
*808.97 (1)
% Intensity (of 4.7e4)
*734.97 (1)
*782.94 (1)
*810.99 (1)
*703.97 (1)
812.06 (1)
*808.97 (1)
*564.06 (1)
*496.79
809.92 (1)
% Intensity (of 1.5e5)
*758.95 (1)
*782.94 (1)
*734.94 (1)
*496.74 (1)
*810.94 (1)
*703.98 (1)
811.99 (1)
*524.78 (1)
*814.04 (1)
% Intensity (of 5.0e4)
*760.94 (1)
*758.95 (1)
*782.94 (1)
*810.96 (1)
*620.11 (1)
812.06 (1)
*564.05 (1)
*814.01 (1)
% Intensity (of 2.8e5)
*760.94 (1)
*758.98 (1)
*786.98 (1)
*734.94 (1)
*810.97 (1)
*496.74 (1)
*703.98 (1)
812.06 (1)
*524.80 (1)
Mass/Charge, Da
C
D
Uninjured
Ligated
Injured
Relative intensity (% of largest peak)
496.7 Lyso 16:0
524.7 Lyso 18:0
732.9 C32:1
734.9 C32:0
758.9 C34:2
760.9 C34:1
782.9 C36:4
784.9 C36:3
786.9 C36:2
806.9 C38:6
810.9 C38:4
834.9 C40:6
PCs (identified by m/z)

Figure 5

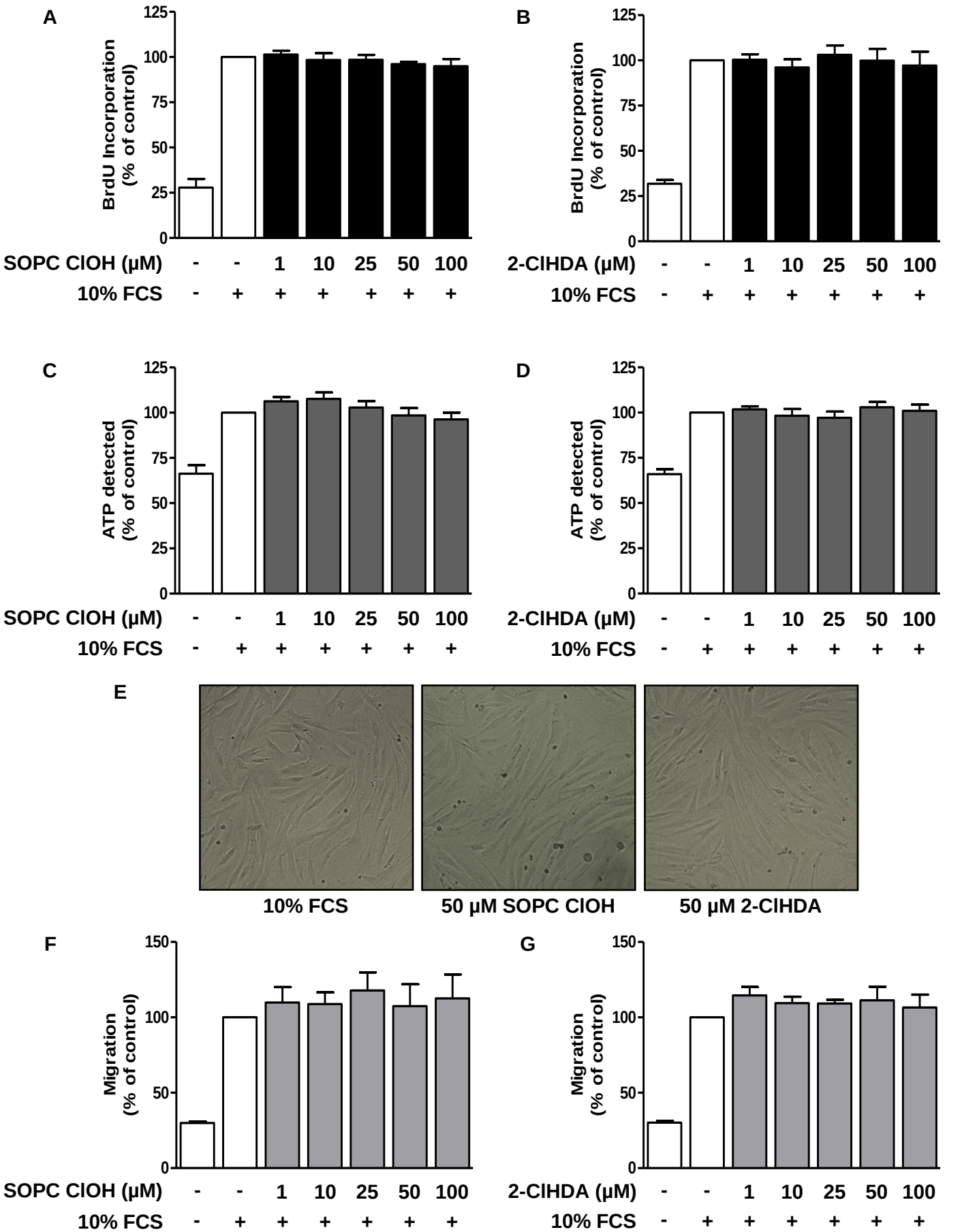
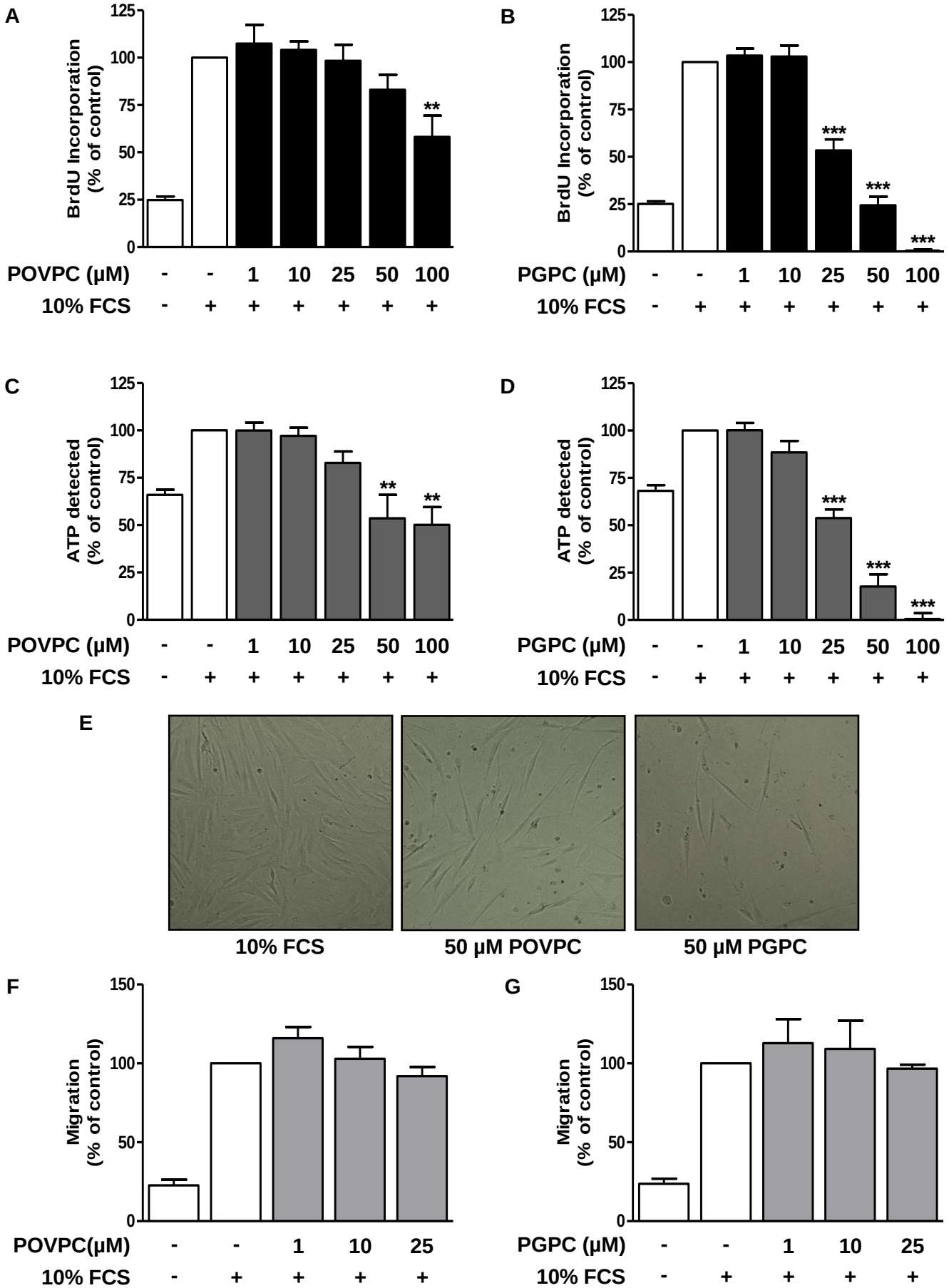

A
BrdU Incorporation (% of control)
125
100
75
50
25
0
SOPC ClOH (μM) - - 1 10 25 50 100
10% FCS - + + + + + +
B
BrdU Incorporation (% of control)
2-ClHDA (μM) - - 1 10 25 50 100
10% FCS - + + + + + +
C
ATP detected (% of control)
SOPC ClOH (μM) - - 1 10 25 50 100
10% FCS - + + + + + +
D
ATP detected (% of control)
2-ClHDA (μM) - - 1 10 25 50 100
10% FCS - + + + + + +
E
10% FCS
50 μM SOPC ClOH
50 μM 2-ClHDA
F
Migration (% of control)
150
100
50
0
SOPC ClOH (μM) - - 1 10 25 50 100
10% FCS - + + + + + +
G
Migration (% of control)
2-ClHDA (μM) - - 1 10 25 50 100
10% FCS - + + + + + +

Figure 6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BrdU Incorporation (% of control)
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POVPC (μM) - - 1 10 25 50 100
10% FCS - + + + + + +
B
BrdU Incorporation (% of control)
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PGPC (μM) - - 1 10 25 50 100
10% FCS - + + + + + +
C
ATP detected (% of control)
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POVPC (μM) - - 1 10 25 50 100
10% FCS - + + + + + +
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PGPC (μM) - - 1 10 25 50 100
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10% FCS
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50 μM PGPC
F
Migration (% of control)
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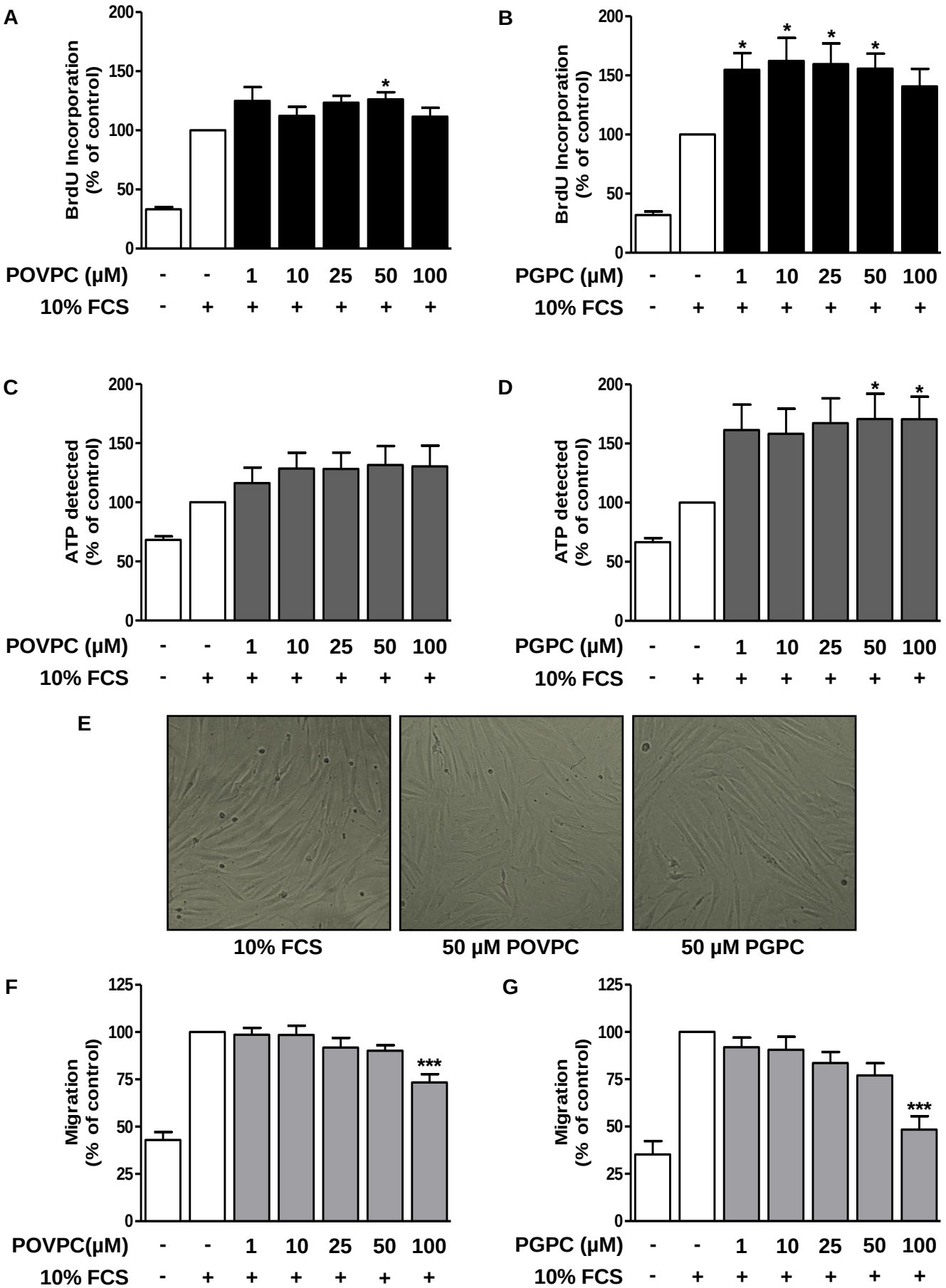
POVPC(μM) - - 1 10 25
10% FCS - + + + +
G
Migration (% of control)
PGPC (μM) - - 1 10 25
10% FCS - + + + +

Figure 7


A
BrdU Incorporation (% of control)
200
150
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*
POVPC (μM) - - 1 10 25 50 100
10% FCS - + + + + + +
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BrdU Incorporation (% of control)
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PGPC (μM) - - 1 10 25 50 100
10% FCS - + + + + + +
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POVPC (μM) - - 1 10 25 50 100
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10% FCS
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Migration (% of control)
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POVPC(μM) - - 1 10 25 50 100
10% FCS - + + + + + +
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Migration (% of control)
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PGPC (μM) - - 1 10 25 50 100
10% FCS - + + + + + +