Apolipoprotein E (apoE) isoforms differentially induce nitric oxide production in endothelial cells

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Abstract Although apolipoprotein E3 (apoE3) is atheroprotective, two common isoforms, apoE2 and apoE4, produce recessive and dominant hyperlipidaemias, respectively. Using a fluorescent assay, we report herein that apoE3 particles secreted from recombinant cells stimulate more nitric oxide release in cultured human EA.hy926 endothelial cells than apoE2 or apoE4 (141% more than controls vs. 61 or 11%). Phosphatidylinositol (PI) 3-kinase inhibitors suppressed the apoE effect, while apoE receptor 2 (apoER2) was tyrosine phosphorylated. We conclude that apoE stimulates endothelial nitric oxide release in an isoform-dependent manner, and propose that tyrosine phosphorylation of apoER2 initiates PI3-kinase signalling and activation of nitric oxide synthase.

Key words: Apolipoprotein E receptor 2; EA.hy926 cells; Nitric oxide synthase; PI3-kinase; Tyrosine phosphorylation

1. Introduction

Apolipoprotein E (apoE) is a 34-kDa polypeptide synthesised predominantly by the liver and helps protect against atherosclerosis, in part by mediating hepatic clearance of remnant plasma lipoproteins [1,2]. When apoE is absent or dysfunctional, severe hyperlipidaemia and atherosclerosis ensue, while infusion of apoE or hepatic gene overexpression are protective [3–5]. ApoE has three common isoforms that arise from single nucleotide polymorphisms [1,2]. The rarest variant, apoE2, differs from wild-type apoE3 by an Arg158Cys substitution and causes Type III hyperlipidaemia [6], while apoE4 (Cys112Arg) produces a dominant hyperlipidaemia and is a risk factor for restenosis [7] and smoking-related heart disease [8].

ApoE is also abundant in atherosclerotic lesions, secreted by resident cholesterol-loaded macrophages [9]. This locally produced apoE is atheroprotective by contributing to reverse cholesterol transport [10,11], inhibiting smooth muscle cell proliferation [12], preventing oxidation [13], and restricting platelet aggregation [14]. In platelets, the binding of apoE by apoE receptor 2 (apoER2) appears to initiate a signal transduction cascade to upregulate nitric oxide synthase (NOS) [14–16]. Moreover, we have recently reported that in human umbilical vein endothelial cells (HUVECs), cell-derived apoE inhibits expression of vascular cell adhesion molecule-1 (VCAM-1) [17], a key pro-atherogenic molecule involved in the recruitment of monocytes to lesions [18,19]. Suppression of endothelial activation by apoE occurred via stimulation of NOS, and we proposed that apoE secreted locally at lesion sites by macrophages is anti-inflammatory by stimulating the endothelium to release NO and suppress VCAM-1 expression [17].

Here, we have studied the apoE-NO link in EA.hy926 cells, a hybrid cell line derived by fusion of HUVECs with A549 epithelial cells [20], which is known to retain many of the differentiated functions characteristic of endothelial cells [20–23]. We confirm that apoE stimulates endothelial NO release by incubating EA.hy926 cells with conditioned medium from recombinant Chinese hamster ovary (CHO) cells that secrete apoE particles, similar to those secreted by macrophages [17]. We then show that the three apoE isoforms have a differential ability to stimulate NO release by using media from $CHO^{apoE2/3/4}$ cells secreting apoE2, apoE3 and apoE4 particles, respectively, and report a heterogeneity in apoE particle size that may influence this activity. Finally, we implicate phosphatidylinositol (PI) 3-kinase signalling in apoE-induced NOS activation and suggest that this pathway is activated by tyrosine phosphorylation of cytoplasmic apoER2 upon endothelial exposure to apoE.

2. Materials and methods

2.1. Cell culture

EA.hy926 human endothelial cells were generously provided by Dr Edgell, University of North Carolina and were maintained in DMEM media supplemented with 10% (v/v) fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.1 mM sodium hypoxanthine, 0.4 μM aminopterin and 16 μM thymidine. Recombinant CHO cells stably secreting human apoE2, apoE3 or apoE4 isoforms (CHO^{apoE2}, CHO^{apoE3} and CHO^{apoE4} cells) were produced as described elsewhere [24]. They were maintained in Iscove's selection media plus 2 mM

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Abbreviations: apo, apolipoprotein; apoER2, apolipoprotein E receptor 2; CHO, Chinese hamster ovary; DAF-2 DA, 4,5-diaminofluorescein diacetate; ELISA, enzyme-linked immunosorbent assay; Ethyl-ITU, 2-ethyl-2-isothiopseudourea; FBS, fetal bovine serum; NDGE, non-denaturing gel electrophoresis; NO, nitric oxide; NOS, nitric oxide synthase; PI3-kinase, phosphatidylinositol 3-kinase; MAP kinase, mitogen-activated protein kinase; VCAM-1, vascular cell adhesion molecule-1

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Glutamax and 10% (v/v) dialysed FBS, while media for control CHO^{dhfr-} cells was supplemented with 0.1 mM hypoxanthine and 16 μM thymidine [24]. Cell-conditioned media was collected by incubating CHO^{apoE2}, CHO^{apoE3}, CHO^{apoE4} or CHO^{dhfr-} cells (each ~80% confluent) for 24 h in M199 media containing 5% (v/v) FBS when the yields were typically 8, 4 and 2 μg apoE/ml, respectively. The apoE media were adjusted to contain the same amount of apoE by addition of CHO^{dhfr-}-conditioned medium and, after concentrating three-fold in Vivaspin concentrators (30 000 molecular weight cut off; Vivaspin Ltd, Lincoln, UK), sterile filtered (0.2 μM).

2.2. Intracellular NO measurement

Intracellular endothelial NO production was measured directly in triplicate wells using the cell-permeable fluorescent indicator 4,5-diaminofluorescein diacetate (DAF-2 DA; Calbiochem, Nottingham, UK) [25]. Confluent EA.hy926 cells in 48-well plates were incubated with 10 μM DAF-2 DA for 1 h at 37°C. To inhibit NOS activity, wells were pre-incubated for 2 h with 20 μM 2-ethyl-2-isothiopseudourea (ethyl-ITU; Alexis Corporation Ltd, Nottingham, UK) [26]. Other wells were pre-incubated for 1 h with either wortmannin (50–200 nM range; Calbiochem, Nottingham, UK) or LY294002 (5–20 μM range; Sigma) prior to addition of DAF-2 DA. Monolayers were washed with warm phosphate-buffered saline (PBS) and then incubated with CHO cell-conditioned media (from either CHO^{apoE2}, CHO^{apoE3}, CHO^{apoE4} or CHO^{dhfr-} cells) with some wells supplemented with 20 μM ethyl-ITU, 50–200 nM wortmannin or 5–20 μM LY294002. Fluorescent emissions at 530 nm (bandwidth of 25 nm) were read using a Cytofluor Series 400 plate reader (PerSeptive Biosystems, Framingham, MA, USA) upon excitation at 485 nm (bandwidth of 20 nm). Results are expressed as change in relative DAF-2 DA fluorescence (arbitrary units) over a 4 h time course as a percentage of the fluorescence signal from control wells treated with CHO^{dhfr-} cell-conditioned media.

2.3. Electrophoretic characterisation of apoE particles

ApoE particles in CHO cell-conditioned media (from CHO^{apoE2}, CHO^{apoE3} and CHO^{apoE4} cells) were analysed for electrophoretic mobility in agarose hydragels (Sebia, Issy-les-Moulineanx, France) using immunoblotting for detection [17]. In addition, particle diameter was assessed by non-denaturing gel electrophoresis (NDGE) on a 20% Tris–borate (pH 8.3) mini-gel (Invitrogen, The Netherlands) that had been pre-run at 100 V for 30 min at 4°C. Native high molecular weight protein standards (Amersham Biosciences UK Ltd, Little Chalfont, UK) were used as size standards [27]. Non-boiled and non-reduced samples were loaded in a buffer containing 10% sucrose and 0.016% bromophenol blue. Electrophoresis was performed with the following step-wise voltage scheme: 25 V for 15 min, 50 V for 15 min, 75 V for 15 min and then 100 V for 18 h whilst being maintained at 4°C. Proteins were blotted to a nitrocellulose membrane and probed with polyclonal goat anti-human apoE antibody (DiaSorin Inc., Stillwater, MN, USA). Immunoreactive bands were visualised by enhanced chemiluminescence (Amersham Biosciences UK Ltd), while native molecular size standards were detected by Coomassie staining the gel.

2.4. Quantification of apoE by enzyme-linked immunosorbent assay (ELISA)

ApoE concentration in CHO cell-conditioned media was measured by a two-antibody sandwich ELISA [5]. Briefly, polyclonal goat anti-human apoE antibodies were used both for capture (DiaSorin Inc.) and, after biotinylation using a commercial kit (Amersham Biosciences UK Ltd), for detection (Biogenesis Ltd, Poole, Dorset, UK). Human serum containing a known amount of apoE was used as a standard (Technoclone Ltd, Dorking, UK). Standard and media samples were diluted in assay buffer; 150 mM NaCl, pH 7.4 containing 0.5% (w/v) bovine serum albumin, 0.05% (w/v) gamma-globulin, 0.01% (v/v) Tween 40 and 50 mM Tris.

2.5. Identification of apoER2 in EA.hy926 cells

EA.hy926 cells were incubated for 4 h with either CHO^{dhfr-}or CHO^{apoE2} cell-conditioned media, washed with PBS (4°C) and then lysed with lysis buffer [1% (v/v) Triton X-100, 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA and protease inhibitor cocktail tablets (Complete™; Roche Diagnostics, Welwyn Garden City, UK)], supplemented with 1 mM dithiothreitol and 1 mM sodium orthovanadate. Lysates were clarified and the supernatant precleared for 90 min with anti-rabbit IgG-coated Dynabeads (Dynal, Bromborough, UK). The cleared sample was then incubated overnight at 4°C with rabbit anti-apoER2 antisera directed against a cytoplasmic tail sequence common to all splice variants [16] (1:100 dilution) and then with anti-rabbit IgG-coated Dynabeads for 90 min. Immunocomplexes were collected, washed with lysis buffer and boiled in sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer. Proteins were separated by 8% or 4–20% gradient SDS–PAGE, transferred to nitrocellulose and immunoblotted for either phosphotyrosine using polyclonal anti-phosphotyrosine (PY20; Zymed Laboratories, San Francisco, CA, USA) or for apoER2 using goat polyclonal anti-apoER2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

To confirm that apoER2 was present in plasma membranes, confluent EA.hy926 cell monolayers were washed with warm PBS and cell-surface proteins biotinylated using sulpho-NHS-LC-biotin at 0.5 mg/ml (Pierce, Tattenhall, UK) for 30 min at room temperature. Cells were then washed, incubated with 10 mM glycine for 20 min to quench any unbound biotin and lysed. Samples were clarified by centrifugation and the supernatants were incubated with streptavidin beads (Sigma) for 2 h at 4°C on a rotating wheel, after which time the beads were washed with lysis buffer and then boiled in SDS–PAGE buffer. Proteins were separated by 8% SDS–PAGE, transferred to nitrocellulose and probed for apoER2 using our own rabbit polyclonal anti-apoER2 antibody (anti-apoER2Ins) [15].

2.6. Statistical analysis

Statistical analysis for $n = 3$ independent experiments, which were done in triplicate for each point in the data, was performed using Student's t-test. The results are shown as mean ± S.E. of the mean values from the three independent experiments and $P < 0.05$ was considered significant.

3. Results

3.1. ApoER2 and eNOS are expressed in the EA.hy926 human endothelial cell line

ApoER2 is localised to caveolae signalling microdomains within plasma membranes [16]; it is thought to be an 'outside-in' signal transducer in brain (28–30) and to mediate apoE-NO signaling in platelets and endothelial cells [15–17,31]. ApoER2 differs from other members of the low-density lipoprotein receptor (LDL-R) gene family by having several splice variants in mRNA from human tissues [32–34]. Here, we have immunoprecipitated whole cell lysates to detect apoER2 protein in the hybrid HUVEC–epithelial cell line,

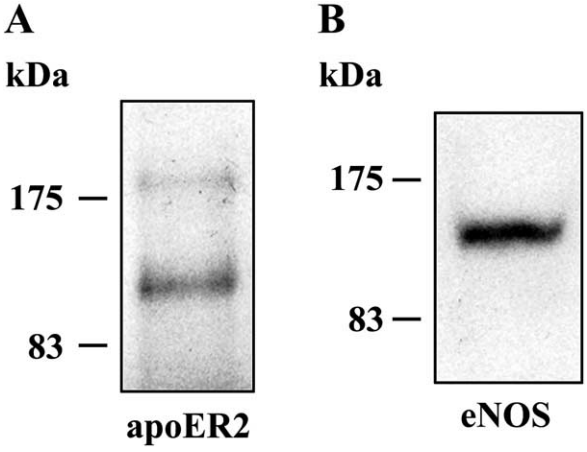

Fig. 1. ApoER2 and eNOS are expressed in EA.hy926 cells. A: Immunoprecipitation of EA.hy926 cell lysate with antisera against the cytoplasmic tail of apoER2 verified that apoER2Δ4–6 (132 kDa) was expressed and also a smaller amount of full-length apoER2 (180 kDa). B: Western blotting of cell lysates confirmed expression of eNOS (140 kDa).

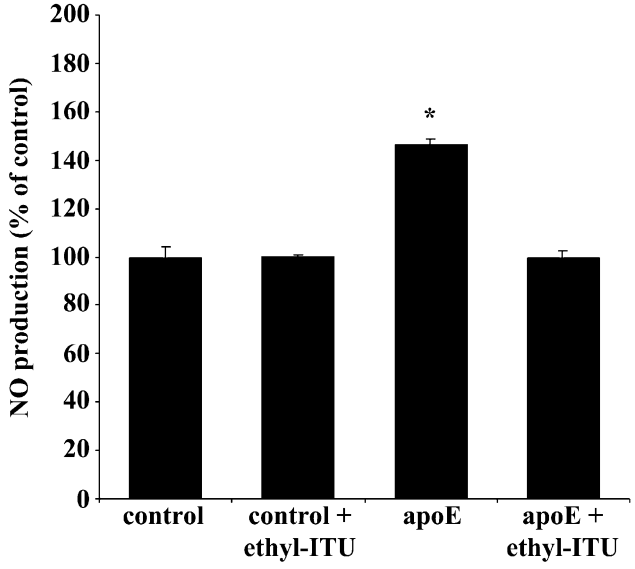

Fig. 2. ApoE stimulates NO production in EA.hy926 cells. Intracellular NO production was measured directly using a fluorescent DAF-2 DA assay. There was a 46.7 ± 2.0% increase in NO from basal levels of 100.0 ± 4.2% (in EA.hy926 cells exposed to controlled conditioned media) to 146.7 ± 2.0% (*$P < 0.001$, $n = 3$) during a 4 h incubation with CHO^{apoE2} cell-conditioned media containing 17.3 ± 2.6 μg apoE/ml. This increase was blocked by pre-incubation with 20 μM of the NOS inhibitor, ethyl-ITU (*$P < 0.001$, $n = 3$). Results are expressed as a percentage of the relative fluorescence (arbitrary units) in wells treated with control CHO^{dhfr-} cell-conditioned media. Data are shown from three independent experiments performed in triplicate using three different preparations of cell-conditioned media.

EA.hy926 (Fig. 1A). Two bands were detected, a major one at 132 kDa corresponding to apoER2Δ4–6 (receptor lacking ligand-binding repeats 4–6) and a minor one at 180 kDa representing the full-length receptor. When cell-surface expression of the receptor was monitored, using the biotinylation of intact cells [15], the 132-kDa band was also the predominant form (data not shown). To confirm that EA.hy926 cells express eNOS, cell lysates were immunoblotted with anti-eNOS; the predicted immunoreactive band at 140 kDa was detected (Fig. 1B).

3.2. ApoE stimulates NO production in EA.hy926 endothelial cells

Intracellular NO production in EA.hy926 cells was measured directly using the cell-permeable fluorescent indicator, DAF-2 DA. This reagent is hydrolysed intracellularly to the active form, DAF-2, which then reacts with NO to produce highly fluorescent DAF-2T (triazolofluorescein). Measurement of DAF-2T includes the simultaneous detection of DAF-2 and, although its contribution is negligible when NO production is high, in systems such as endothelial cells with low NO output, DAF-2 auto-fluorescence may contribute significantly to the signal; the cells themselves and media may also contribute. Indeed, the basal production of NO by EA.hy926 cells appeared negligible; when we added the NOS inhibitor, ethyl-ITU, the signal was not reduced, implying that it was derived from the combined auto-fluorescent background of DAF-2, cells and media rather than NO (Fig. 2).

To investigate effects of apoE on release of NO by EA.hy926 cells, we initially used conditioned media from CHO^{apoE2} cells; this clone was our highest producing cell line (~8 μg apoE/ml/24 h), while any role for the LDL-R-related protein can be discounted as both these receptors bind apoE2 poorly [17]. During a 4 h incubation with CHO^{apoE2} cell-conditioned media containing 17.3 ± 2.6 μg apoE/ml, the fluorescent signal increased by almost 50% above basal levels, from 100.0 ± 4.2% in EA.hy926 cells incubated with control-conditioned media to 146.7 ± 2.0% ($P < 0.001$, $n = 3$) (Fig. 2). Moreover, this effect was completely blocked by pretreating the cells with the NOS inhibitor, ethyl-ITU ($P < 0.001$, $n = 3$), confirming that the increased signal was due to NO release.

3.3. NO production is dependent on apoE isoform

To compare the NO production stimulated by different isoforms of apoE in parallel wells, media were collected from

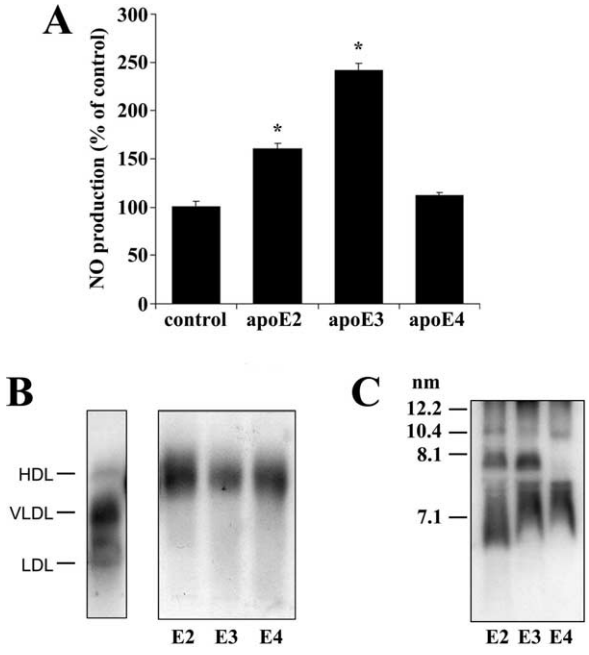

Fig. 3. Production of NO is apoE isoform dependent. A: EA.hy926 cells were incubated with cell-conditioned media containing approximately equal concentrations of apoE2, apoE3 or apoE4 (2.7 ± 0.4, 2.8 ± 0.5, 2.8 ± 0.3 μg apoE/ml, respectively) for 4 h, and intracellular NO production was measured using a fluorescent DAF-2 DA assay. ApoE3 caused the greatest increase in NO release (141.0 ± 6.9%, *$P < 0.001$, $n = 3$) over the control baseline of 100.0 ± 5.2%, while the increase caused by apoE2 was less pronounced (61 ± 5.3% over control, *$P < 0.01$, $n = 3$). In marked contrast, apoE4 did not significantly increase NO production (11.0 ± 2.8%, $P > 0.05$, $n = 3$). Results are expressed as a percentage of the relative DAF-2 DA fluorescence (arbitrary units) in wells treated with CHO^{dhfr-} cell-conditioned media. Data are from three independent experiments performed in triplicate, with parallel wells for each isoform, using three different preparations of cell-conditioned media. B: ApoE particles secreted from CHO$^{apoE2/3/4}$ cells were separated by native agarose gel electrophoresis and immunoblotted for apoE. Equal amounts of apoE were used for each isoform. The mobility of particles was compared to a sample of human plasma. Most cell-derived apoE particles had pre-α mobility. C: ApoE particles from CHO^{apoE2}, CHO^{apoE3} and CHO^{apoE4} cell-conditioned media (all at approximately 2.9 μg apoE/ml) were characterised by NDGE using immunoblotting for detection. Hydrated diameters were compared to native high molecular weight standards. Three main particle populations with diameters of 7.6, 8.0 and 10.4 nm could be identified, but were distributed heterogeneously between the different apoE isoforms.

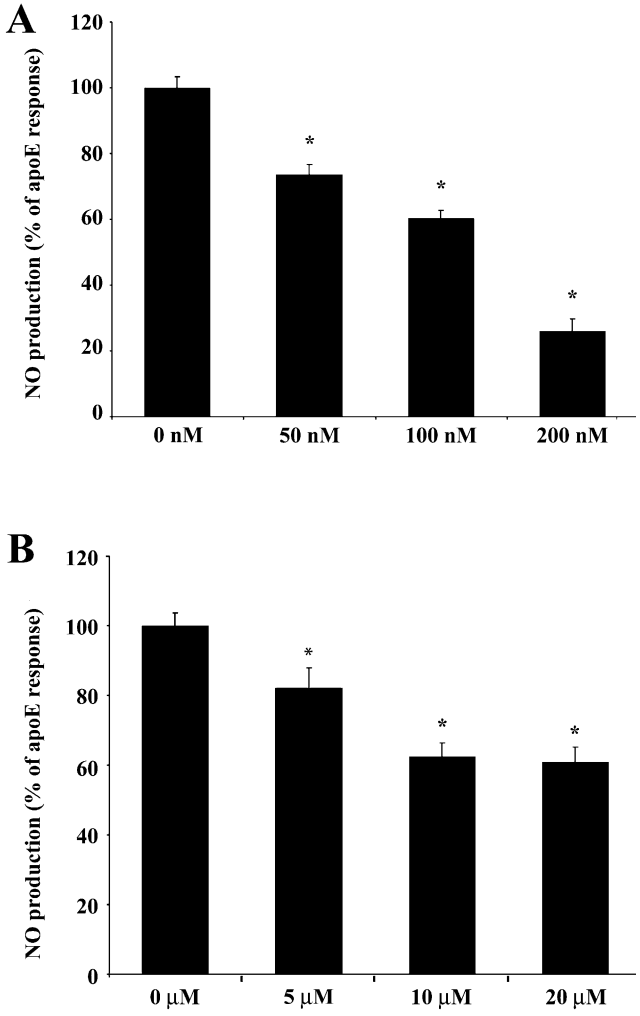

Fig. 4. P13-kinase inhibitors suppress apoE-induced NO production. A: EA.hy926 cells were pre-incubated with the general kinase inhibitor, wortmannin (50–200 nM), and then incubated with conditioned media containing 16.9 ± 2.1 μg apoE2/ml for 4 h. NO release was monitored using the fluorescent DAF-2 DA assay and data are shown as 'NO production as a percentage of maximal apoE response' (values from control cell-conditioned media were subtracted from all data and the maximal apoE response was normalised to 100%; 100.0 ± 3.2%, $n = 3$). ApoE-induced NO production was inhibited by 26.3 ± 2.9%, 39.6 ± 2.4% and 73.8 ± 3.6% when cells were incubated with 50, 100 and 200 nM wortmannin, respectively (all $^*P < 0.001$, $n = 3$). B: EA.hy926 cells were pretreated with the specific PI3-kinase inhibitor, LY294002 (5–20 μM), and then incubated with conditioned media containing 15.8 ± 2.3 μg apoE2/ml for 4 h. ApoE-induced NO response was 100.0 ± 3.7% ($n = 3$) and was inhibited by 17.9 ± 5.6%, 37.6 ± 4.1% and 39.1 ± 4.2% when cells were incubated with 5, 10 and 20 μM LY294002, respectively (all $^*P < 0.001$, $n = 3$). Data sets shown in both A and B were from three independent experiments performed in triplicate using three different preparations of cell-conditioned media.

CHO^{apoE2}, CHO^{apoE3} and CHO^{apoE4} cells and added to the EA.hy926 cells at equivalent concentrations (2.7 ± 0.4, 2.8 ± 0.5 and 2.8 ± 0.3 μg apoE/ml, respectively) as measured by apoE ELISA. Over the 4 h incubation, apoE3 caused the greatest increase (141 ± 6.9%) in NO release over control media ($P < 0.001$, $n = 3$, Fig. 3A), with apoE2 stimulating less than half the increase of apoE3 (61.0 ± 5.3%, $P < 0.01$, $n = 3$). In contrast, apoE4 did not stimulate a significant increase in NO production (11.0 ± 2.8%, $P > 0.05$, $n = 3$).

3.4. Characterisation of apoE particles

Previously, we have shown that the apoE particles secreted by recombinant CHO cells have several similarities to those secreted by human blood monocyte-derived macrophages [17]. They were spheres of a similar diameter, as judged by negative scanning electron microscopy, and had comparable profiles by agarose gel electrophoresis and gel filtration [17]. Here, we have directly compared the apoE particles secreted from $CHO^{apoE2/3/4}$ cells using agarose gel electrophoresis and apoE immunoblotting. Most of the cell-derived apoE particles had pre-α mobility, and all three isoforms gave similar patterns (Fig. 3B). However, some differences between the isoforms were noted when equal amounts of apoE were analysed using NDGE. Three main apoE particle populations were identified with hydrated diameters of 7.6, 8.0 and 10.4 nm (Fig. 3C). The 7.6 nm particle was common to all three isoforms but appeared to be less abundant in the apoE2 sample. The 8 nm particle was common to apoE2 and apoE3, though more prominent in the apoE3 sample, but appeared to be completely absent from apoE4 media. The 10.2 nm particle was present in each sample, although much lower in apoE3 media.

3.5. Inhibitors of PI3-kinase partially suppress apoE-induced release of NO

Wortmannin is an irreversible inhibitor of PI3-kinase at low concentrations [35]. Pretreatment of EA.hy926 cells with wortmannin (50–200 nM) caused a dose-dependent inhibition of NO release when cells were incubated with 16.9 ± 2.1 μg/ml apoE2 for 4 h (Fig. 4A). NO production is shown as a percentage of the NO response produced by apoE alone (above the level of the control-conditioned media) normalised to 100% (100.0 ± 3.2%, $n = 3$). Cells incubated with 50 and 100 nM wortmannin had production of NO inhibited by 26.3 ± 2.9% and 39.6 ± 2.4%, respectively (both $P < 0.001$, $n = 3$). Further inhibition of apoE-stimulated NO release (73.8 ± 3.6%, $P < 0.001$, $n = 3$) was detected using 200 nM wortmannin. However, at this higher dose, wortmannin is not a specific inhibitor of P13-kinase, and inhibition of NO could be due to effects on other signalling pathways, such as the mitogen-activated protein (MAP) kinase pathway [36].

LY294002 is a specific PI3-kinase inhibitor which acts at the ATP-binding site of the enzyme [37]. Pretreatment of EA.hy926 cells with LY294002 (5–20 μM) caused an inhibition of NO production when cells were incubated with 15.8 ± 2.3 μg apoE/ml for 4 h (Fig. 4B). Cells incubated with 5 and 10 μM LY294002 showed apoE-induced NO production inhibited by 17.9 ± 5.6% and 37.6 ± 4.1%, respectively (both $P < 0.001$, $n = 3$). A higher dose of inhibitor did not cause any additional inhibition of NO production indicating that a maximal effect had been reached; 20 μM LY294002 led to a decrease in NO production by apoE of 39.1 ± 4.2% ($P < 0.001$, $n = 3$). This inhibition was comparable to that achieved by using 100 nM wortmannin (39.6 ± 2.4%).

3.6. ApoER2 is tyrosine phosphorylated upon exposure to apoE

ApoER2 has two tyrosine residues within its cytoplasmic tail. To investigate tyrosine phosphorylation of cytoplasmic apoER2 as a potential signalling step, EA.hy926 cells were exposed to apoE for 0, 2.5, 5 and 10 min. Cells were then lysed, immunoprecipitated for apoER2 and immunoblotted for phosphotyrosine. Equal amounts of protein were loaded

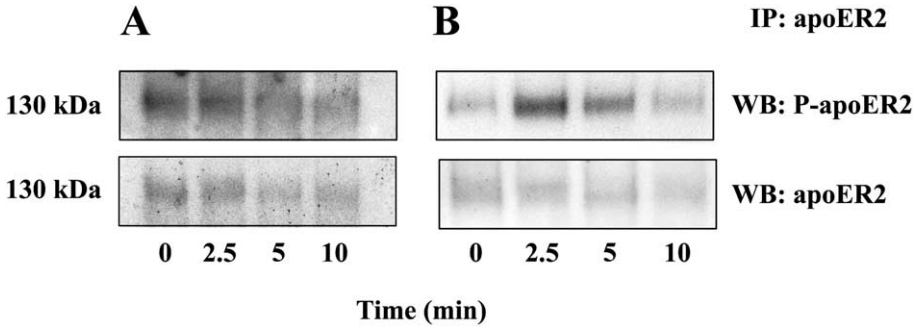

Fig. 5. ApoER2 is tyrosine phosphorylated upon exposure to apoE. EA.hy926 cells were incubated with conditioned media from either CHO^{dhfr-} (A) or CHO^{apoE2} (B) cells for up to 10 min. At each time point, cells were lysed and apoER2 isolated by immunoprecipitation. The immunoprecipitates were separated by 8% SDS–PAGE gel and immunoblotted for apoER2 and phosphotyrosine (PY20). Equal amounts of protein were loaded in each lane and this was confirmed by immunoblotting for apoER2. A: EA.hy926 cells incubated with control cell-conditioned media from CHO^{dhfr-} cells showed no change in tyrosine phosphorylation. B: Incubation with apoE, however, stimulated tyrosine phosphorylation. The peak level was seen at 2.5 min, which then began to decrease at 5 min until phosphorylation levels returned to those in resting cells by 10 min.

in each lane and this was confirmed by immunoblotting for apoER2. EA.hy926 cells were also treated in parallel with control-conditioned media from CHO^{dhfr-} cells; no change in the level of tyrosine phosphorylation was detected (Fig. 5A). In contrast, exposure to apoE2 (16.3 ± 2.2 μg apoE/ml) caused an increase in tyrosine phosphorylation above the level in resting cells. Maximal tyrosine phosphorylation was detected by 2.5 min which then began to decrease at 5 min, until phosphorylation levels returned to those in resting cells by 10 min (Fig. 5B).

4. Discussion

This study in EA.hy926 cells confirms our earlier report, carried out in a different model of human endothelial cells, HUVECs, that apoE activates NOS to release NO [17]. In extending this work, we now report a marked differential isoform effect on stimulating NO production with apoE3 > apoE2 ≫ apoE4, a finding consistent with the potential of apoE2 and apoE4 to confer increased risk for developing coronary heart disease [2,7,38]. In addition, we have implicated tyrosine phosphorylation of apoER2 and PI3-kinase signalling as being involved in apoE-mediated activation of eNOS.

The EA.hy926 cell line is a hybrid of primary HUVECs fused with A549 epithelial cells [20], which perform many of the differentiated functions characteristic of endothelial cells, such as angiogenesis, haemostasis, and inflammation [20–23]. They have been used before in NO signalling studies [21] and, importantly, in future studies they will be easier to manipulate genetically than primary cells. We confirmed that EA.hy926 cells expressed eNOS protein and, as we had previously implicated apoER2 as the receptor mediating apoE-induced release of NO in HUVECs [17], screened for this protein using two different immunological techniques. We found that EA.hy926 cells expressed two forms of the receptor; as in HUVECs the splice variant lacking ligand-binding repeats 4–6, apoER2Δ4–6, predominated. Moreover, when we incubated EA.hy926 cells with apoE2-containing conditioned media from CHO^{apoE2} cells, we found a clear increase in NO production, as previously reported in HUVECs [17]. This response was due to activation of eNOS, since the inhibitor ethyl-ITU blocked the effect.

Recombinant cell-secreted apoE has biological activities similar to native apoE [17,39] and, therefore, any difference in the ability of apoE isoforms to activate eNOS might be relevant to susceptibility to certain diseases. ApoE2 is the genetic cause of Type III hyperlipidaemia, but protects against Alzheimer's disease [40]; apoE4 is a risk factor for restenosis [7] and smoking-related heart disease [8], and is strongly linked to late-onset Alzheimer's disease and other neurodegenerative diseases [40–43]. Intriguingly, these two isoforms stimulated less production of NO than wild-type apoE3. Indeed, apoE2 stimulated less than half the NO released by apoE3, while apoE4 failed to significantly increase NO levels above the control. NO has multiple atheroprotective effects [44–46], some of which are common to apoE including inhibition of platelet adhesion and aggregation [28,47], smooth muscle proliferation and migration [12,48,49], and inhibition of endothelial cell adhesion molecules [17,50]. NO is also an important molecule in the brain, acting as an intracellular messenger, regulating blood flow and contributing to morphogenesis and neuronal plasticity [51]. It is also crucial for neurite outgrowth [52] and, as this process is impaired in Alzheimer's disease [53] it will be of interest to test whether apoE isoforms differ in their ability to stimulate NOS in neuronal cells.

We propose that some of the differential effects of apoE isoforms on eNOS activation may reflect heterogeneity in secreted particles, rather than protein sequence per se. ApoE conformation and receptor binding activity are markedly influenced by lipid environment and particle characteristics [39,54]. Characterisation of apoE in $CHO^{apoE2/3/4}$ cell media revealed that although the majority of particles had pre-α mobility, a particle population of 8 nm in diameter was prominent in apoE3 media, less abundant in apoE2 media and absent in apoE4 media. Further experiments are needed to establish whether the 8 nm apoE-containing particles are the active cell-derived factors that stimulate the release of NO.

A variety of signal transduction cascades activate eNOS, including the P13-kinase pathway [55]. We found that wortmannin, and the specific inhibitor of PI3-kinase, LY294002, consistently suppressed release of NO from apoE-stimulated endothelial cells. However, this inhibition was not complete; a maximal inhibition of ~40% was achieved by LY294002, with higher doses unable to block further NO release. By

contrast, higher doses of wortmannin, which are known to block other signalling enzymes in addition to PI3-kinase, including MAP kinase, myosin light chain kinase, PI4-kinase and phospholipase D [56], did cause further inhibition. This suggests that more than one pathway might relay the apoE-triggered signal for activating eNOS.

The initial step most likely involves binding of apoE by apoER2 [15,57], although we cannot entirely exclude involvement of other LDL-R family members or heparan sulphated proteoglycans [2,58], and we report herein that endothelial apoER2 was rapidly tyrosine phosphorylated when cells were exposed to apoE. Although apoER2 has homology to other members of the LDL-R family, its cellular role is unlikely to involve lipoprotein uptake and degradation [30,59]. Indeed, the cytoplasmic sequence NPxY, common to all LDL-R family members and originally identified as the recognition motif for endocytosis [60], also serves as a ligand for the phosphotyrosine binding and PDZ domains of signalling molecules [60]. Current evidence implicates apoER2 as a signal transducer molecule, regulating neuronal cell migration during brain development [28–30,60,61], and suppressing platelet aggregation in the vasculature [15,16,31]. Signalling appears to require interaction between cytoplasmic apoER2 and adapter molecules [28–30,60,61] and crucially apoER2 and intermediates of potential signalling cascades, including eNOS, are present in specialised plasma membrane microdomains, caveolae [16,62]. Cytoplasmic apoER2 contains two tyrosine residues, though as yet we do not know which residue(s) are phosphorylated. It is tempting, however, to speculate that this apoE-induced phosphorylation event may constitute an early step in an 'outside-in' signal transduction cascade that involves P13-kinase and ends in eNOS activation. Clearly, further work is needed to verify this possibility and to delineate the rest of the signalling pathway.

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