Regulation of Endogenous Apolipoprotein E Secretion by Macrophages

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Abstract—Apolipoprotein E has critical roles in the protection against atherosclerosis and is understood to follow the classical constitutive secretion pathway. Recent studies have indicated that the secretion of apoE from macrophages is a regulated process of unexpected complexity. Cholesterol acceptors such as apolipoprotein A-I, high density lipoprotein, and phospholipid vesicles can stimulate apoE secretion. The ATP binding cassette transporter ABCA1 is involved in basal apoE secretion and in lipidating apoE-containing particles secreted by macrophages. However, the stimulation of apoE secretion by apoA-I is ABCA1-independent, indicating the existence of both ABCA1-dependent and -independent pathways of apoE secretion. The release of apoE under basal conditions is also regulated, requiring intact protein kinase A activity, intracellular calcium, and an intact microtubular network. Mathematical modeling of apoE turnover indicates that whereas some pools of apoE are committed to either secretion or degradation, other pools can be diverted from degradation toward secretion. Targeted inhibition or stimulation of specific apoE trafficking pathways will provide unique opportunities to regulate the biology of this important molecule. (Arterioscler Thromb Vasc Biol. 2008;28:000-000)

Key Words: ●●●

Apolipoprotein E (apoE) is a 34-kDa protein that is produced and secreted by many cell types such as hepatocytes, smooth muscle cells, neuronal cells, and macrophages. As a constituent of plasma lipoproteins, apoE directs movement of lipids from the periphery to the liver, where high affinity binding of apoE to the LDL receptor and other members of the LDL-receptor family facilitates uptake of the lipoprotein particles. A critical role for apoE in the protection against atherosclerosis was clearly established by the development of the apoE knockout mouse and subsequent studies which showed that macrophage-specific expression of apoE was antiatherogenic.1,2 The mechanisms by which macrophage apoE is antiatherogenic may include stimulating the removal of excess cholesterol from macrophage foam cells,3 as well as antiinflammatory,4 antiproliferative,5 and immunomodulatory properties.6,7 The regulation of the secretion of apoE from macrophages is thus of great importance to our understanding of the atherosclerotic process.

The aim of this review is to describe the regulation of the secretion of apoE by macrophages, in light of recent observations indicating that its trafficking and secretion are under the control of signaling pathways. Although recycling of lipoprotein-derived apoE is referenced briefly for comparison, a detailed analysis of apoE recycling is beyond the scope of the present review and the interested reader is directed to recent excellent reviews on this topic.8–10

Basic Biology of ApoE in Macrophages

Transcriptional Regulation of ApoE Synthesis

Transcription of macrophage apoE can be induced by differentiation, exposure to cytokines, hormones, and lipids such as cholesterol or oxysterols. In addition to proximal promoter elements, apoE transcription is modulated by 2 distal flanking sequences termed multienhancer 1 (ME.1) and multienhancer 2 (ME.2), which were shown to direct macrophage and adipose-specific expression in transgenic mice.11 Transcription factors involved in the direct transcriptional regulation of apoE in macrophages included: AP-1,12,13 NF-H9260B,12 LXR,14 and PPARγ.15 The LXR/RXR heterodimer regulates apoE transcription in response to lipids through interaction with conserved LXR response elements in the multienhancer regions. Studies in LXR-deficient mice indicate that LXRs mediate lipid-inducible rather than tissue-specific expression of apoE, and point to an important role of LXR in foam cell-related apoE expression in atherosclerosis.14 Regulation of apoE transcription by PPARγ is mediated via a PPRE element present in the intergenic region between the apoE and apoC1 genes, which is also well outside the proximal promoter.15

Other modulators of macrophage apoE transcription have been described, including transforming growth factor (TGF)-beta which stimulates apoE expression by promoting binding...
of c-Jun to AP-1,13 and lipopolysaccharide (LPS), which represses apoE gene expression via both Tpl-2 and MEKK1 signaling pathways, leading to c-Jun and NF-kB action on distinct regions in the proximal promoter.27

Posttranslational Modifications to ApoE

ApoE is understood to follow the classical secretory pathway, whereby synthesis in the ER is followed by its movement to the Golgi and Trans Golgi network, and incorporation into vesicular structures, before being transported to the plasma membrane and secreted (Figure 1, pathway 1). Secreted apoE is typically released as phospholipid discs16 (Figure 1, pathway 6), however secreted apoE can vary in its density and association with lipid.17–19 As well as being released into the extracellular medium, a proportion of secreted apoE can be bound to the cell surface (Figure 1, pathway 7), particularly in association with heparan sulfate proteoglycans.20,21 Cell surface pools may be re internalized and subsequently degraded (Figure 1, pathways 3 and 4), or transported to the Golgi network for further modification22 (Figure 1, pathways 5), or released into the extracellular medium (Figure 1, pathway 6).

ApoE is synthesized as a 38,500 Mr protein designated preapoE, containing a NH2-terminal 18-AA extension23 which mediates entry into the ER. After cleavage of the signal peptide in the ER, the protein is trafficked to the Golgi, where apoE is O-glycosylated on threonine 19424 and extensively sialylated.16 Glycosylation and sialylation can therefore act as useful markers of the intracellular trafficking of apoE. Glycosylation and sialylation are uniformly present in apoE freshly secreted from hepatocytes and macrophages.16,25 However, the majority of apoE found in the plasma is at least partially desialylated.16,23 These posttranslational modifications of apoE do not appear to affect receptor binding or lipoprotein clearance but may determine the preferential association with high density lipoprotein (HDL) particles.26 Studies using Chinese Hamster Ovary (CHO) cells defective in O-glycosylation demonstrated that glycosylation/sialylation is not obligatory for secretion of apoE.24,27 This is supported by other studies showing that oleic acid stimulates secretion of macrophage apoE while apparently reducing its sialylation.28 The possibility that glycosylation and sialylation may affect recycling of secreted apoE (see below) remains unexplored.

Cellular Localization in Macrophages

ApoE represents up to 8% to 10% of protein constitutively secreted from macrophages.29 Although a number of studies have investigated the localization of endogenous apoE in hepatocytes,30–32 detailed microscopic studies in macrophages are limited. Werb et al29 showed that apoE was distributed in the Golgi complex, ER, secretory vesicles, and coated vesicles of mouse peritoneal macrophages. Kruth et al33 demonstrated that cellular apoE can localize to invaginations of the plasma membrane containing cholesterol crystals or modified LDL, in so-called “surface-connected” compartments where it may facilitate cholesterol solubilization. In the studies of Werb, apoE was released from secretory storage granules in response to frustrated phagocytosis that occurred after contact with immobilized immune complexes.34 The same investigators also described reuptake and secretion of secreted apoE.29 This observation of apparent “recycling” of macrophage-derived apoE has been confirmed and extended by several groups (see below). ApoE does not accumulate in lysosomes under normal circumstances, consistent with usually efficient lysosomal degradation, but may be observed when lysosomal activity is inhibited.35

The quantitative importance of cell surface–bound apoE, relative to internal vesicular apoE, as a source for secreted apoE varies between cell types. In hepatocytes (studies principally performed in HepG2 cells) up to 54% of total cellular apoE at steady-state is at the cell surface,36 existing in distinct heparinase- and lipid-releasable pools.20 In contrast, in primary macrophages,17,22,37 less than 10% of cell apoE is on the cell surface. This suggests that the binding of endogenous apoE to cell-surface proteoglycans varies between cell types and appears to be quantitatively more important in HepG2 cells than in macrophages. There is also variation between primary macrophages of different species, as primary murine macrophages have a larger cell surface pool of apoE (sensitive to pronase) than do primary human macrophages.38 In primary human macrophages heparinase has no effect on apoE secretion,37,38 suggesting that the cell-surface binding of apoE to proteoglycans may be a quantitatively minor regulator of the rate of apoE secretion under steady state conditions.

Recycling of Macrophage-Derived ApoE in Macrophages

Reuptake of endogenously synthesized and secreted apoE by macrophages was first observed by Werb et al.29 Recent
Intracellular Degradation

ApoE undergoes intracellular degradation in a post Golgi compartment in macrophages (Figure 1, pathway 4). HepG2 and CHO cells,\textsuperscript{45,46} Newly synthesized apoE has a half-life of 22 minutes in macrophages\textsuperscript{47} and of 2.6 hours in HepG2 cells.\textsuperscript{48} Some studies have concluded degradation only occurs within lysosomes, whereas others have shown that degradation is sensitive to both lysosomal and proteasomal chemical inhibitors.\textsuperscript{35,46,48} Brefeldin A, which perturbs ER to Golgi transport, inhibits apoE degradation and causes intracellular accumulation of unglycosylated apoE with lower molecular weight.\textsuperscript{48} This suggests that entry of apoE into the Golgi network, and glycosylation of apoE, precede entry into the degradatory compartment.\textsuperscript{35} Degradation of apoE is inhibited by ALLN, an inhibitor of Ca\textsuperscript{2+}-dependent proteases, in HepG2 cells, macrophages, and CHO cells expressing apoE.\textsuperscript{46,48} Furthermore, the degradation of apoE by HepG2 cells is inhibited by incubation in Ca\textsuperscript{2+}-free medium. This has been interpreted as indicating that Ca\textsuperscript{2+}-dependent proteases may contribute to intracellular degradation.\textsuperscript{46} However, interpretation is complicated by the effects of Ca\textsuperscript{2+} on intracellular signaling, and more recently, its direct role in apoE secretion.\textsuperscript{49}

ApoE degradation has been inhibited by up to 60% to 70%\textsuperscript{16} by the use of lysosomal chemical inhibitors. Concentrations of the lysosomal inhibitor chloroquine, which effectively inhibited apoE degradation (25 to 100 \(\mu\)mol/L), did not stimulate apoE secretion.\textsuperscript{35} In other studies, a cysteine protease inhibitor (ALLN) and lysosomal inhibitors did not stimulate apoE secretion despite effectively inhibiting degradation.\textsuperscript{21} The lack of stimulation of apoE secretion when degradation is inhibited suggests that some cellular pools of apoE are committed either to degradation or secretion.\textsuperscript{21} In this model, committed entry into the lysosomal or other degradatory compartment precludes reentry into the secretory compartment. However, as a number of studies have shown that stimulation of apoE secretion by HDL and apoA-I decreases net degradation of apoE,\textsuperscript{47,50} it is likely that there are other “uncommitted” pools of apoE otherwise destined for degradation which can be redirected to secretion.

We have recently modeled the secretion and degradation of apoE in human macrophages measured by \([^{35}\text{S}]\)-methionine labeling pulse-chase turnover studies and fitting the data to a first order equation (Figure 2A).\textsuperscript{17} The data best fit a model in which \([^{35}\text{S}]\)-apoE could exist within mobile (Em) or stable (Es) pools, where Em, but not Es, was available for secretion or degradation. In response to stimulation of apoE secretion with apoA-I (see below), the rate of degradation decreased, whereas Es was unchanged. These results support that some
E₄ pools are “uncommitted” and accessible to either secretory or degradatory pathways.

**Stimulated Secretion of ApoE**

As distinct from factors that increase apoE secretion by stimulating its synthesis (eg, LXR/RXR ligands, cholesterol enrichment, cytokines), a number of agents have been shown to directly increase apoE secretion (independent of transcription). These include, LDL, HDL, apoA-I, A-II, A-IV, apoE, phospholipid vesicles (PLV), heparinase, oleic acid, and lactoferrin. Phospholipid discs containing apoA-I are more potent stimuli of apoE secretion than phospholipid vesicles or lipid-free apoA-I. Such stimulation presumably occurs at the level of the Golgi or post-Golgi compartments, as the secreted apoE is almost always to some extent glycosylated and sialylated. Most studies to date have not been designed to discriminate between stimulated de novo secretion of endogenous apoE and stimulated rescission of reinternalized endogenous apoE.

The observation that apoE can stimulate its own secretion from macrophages raises the potential of a positive feedback loop, which may be of particular importance in the microenvironment of the arterial wall. Physiological concentrations of apoE in the arterial wall have been demonstrated to be atheroprotective. The natural isoforms of apoE vary in their ability to exogenously stimulate secretion. Exogenous apoE is less effective at stimulating apoE secretion from macrophages than are apoE₃ and apoE₂, which may be relevant. Exogenous apoE is less effective at stimulating apoE secretion from macrophages than are apoE₃ and apoE₂, which may be relevant. Exogenous apoE is less effective at stimulating apoE secretion from macrophages than are apoE₃ and apoE₂. Such stimulation presumably occurs at the level of the Golgi or post-Golgi compartments, as the secreted apoE is almost always to some extent glycosylated and sialylated. Most studies to date have not been designed to discriminate between stimulated de novo secretion of endogenous apoE and stimulated rescission of reinternalized endogenous apoE.

The amount of apoE bound to, and released from, the cell surface can be expected to affect the stimulation achieved by various agents. Increased secretion when proteoglycan synthesis is inhibited suggests that cell surface binding can sequester apoE and inhibit its secretion. Cell surface apoE can also be bound to lipoprotein receptors, such that antisera to the LDL receptor (LDLr) and suramin (which blocks ligand binding to the LDLr-related protein LRP) can reduce sequestration on the macrophage surface and increase apoE secretion. Simple displacement of apoE from the macrophage surface is unlikely to fully explain apoA-I–stimulated apoE secretion.

As cell surface pools are relatively small, rapidly internalized, and degraded, vesicular traffic and exocytosis of apoE probably continuously replenish cell surface pools of apoE, in which case cell surface and intracellular apoE transport mechanisms may need to be considered as closely connected. Recent studies from our own group indicate that inhibition of intracellular vesicular trafficking results in very rapid inhibition of apoE secretion, implying that residence time on the cell surface is either relatively brief in primary human macrophages or contributes relatively little to apoE secretion.

**ATP-Binding Cassette Transporters and ApoE Secretion**

**ABCA1, Constitutive Macrophage ApoE Secretion and Cholesterol Efflux**

The ATP binding cassette transporter ABCA1 has been identified as the key regulator of cholesterol efflux to apoA-I and the protein which is defective in Tangier disease. ABCA1 activity contributes to basal constitutive secretion of apoE, as macrophages from patients with Tangier disease and normal macrophages in which ABCA1 activity or expression is inhibited show reduced apoE secretion. Similar observations were made in the brain, where murine ABCA1 deficiency was associated with lower tissue apoE levels and a markedly reduced secretion of apoE from astrocytes and microglial cells. The mechanism by which ABCA1 regulates apoE secretion is unclear. Cells from patients with Tangier disease and mice with ABCA1 deficiency have a morphologically expanded Golgi complex, in parallel with impaired intracellular transport, likely attributable to perturbed vesicular budding between the Golgi and the plasma membrane, and these characteristics would be expected to disturb apoE trafficking. The ABCA1 transporter has also been reported to modulate late endocytic trafficking of cholesterol and the Niemann Pick Type 1 (NPC1) protein, raising the additional possibility that late endosomal dysfunction could perturb apoE secretion in Tangier disease.

Although the pathways stimulating apoE secretion and cholesterol efflux are distinct, apoE, whether added to cells or during its secretion from cells, can itself mediate cholesterol efflux. ABCA1 primarily, but not exclusively, mediates cholesterol efflux to delipidated or lipid-poor apolipoproteins. There is evidence to suggest that exogenous apoE removes cholesterol via an ABCA1-mediated pathway, whereas secreted apoE may also remove cholesterol in an ABCA1-independent manner. Increasing lipidation of cholesterol acceptors decreases the contribution of ABCA1 to cholesterol efflux relative to that of ATP-binding cassette transporter G1 (ABCG1). This raises the possibility that ABCG1 may contribute to cholesterol efflux mediated by phospholipid-containing secreted apoE particles.

**ABCG1 and ApoE Secretion**

ABCG1 is a major mediator of cholesterol efflux to phospholipid-containing acceptor particles such as HDL, contributes to reverse cholesterol transport of macropophage cholesterol (reviewed in), and may have a role in apoE expression or secretion. Deletion of macrophage ABCG1 has recently been shown to be associated with increased expression of ABCA1, increased cellular apoE levels, and increased secretion of apoE. Transplantation of these ABCG1-deficient macrophages into LDLr−/− mice led to an increase in plasma apoE concentrations. However, in another study, total body ABCG1-deletion had no effect on plasma levels of apoE. The discrepancy between the 2 studies may arise from differences between macrophage-specific and total body ABCG1-deletion, differences between LDLr-deficient and LDLr-replete animal models, or from differences in the severity of hypercholesterolemia achieved in the 2 studies. Assuming that there are circumstances in which ABCG1-
ApoE secretion, expanded by the involvement of secretory granules containing accumulated secretory products that are released on cellular recognition of appropriate stimuli. A third, lysosomal secretory pathway, is used by cathepsins and involves a subset of specialized lysosomes with unique morphology and content that release their constituents in response to external stimuli. Finally, a nonclassical or ER/Golgi-independent secretory pathway is described. This is relevant to proteins which do not possess a signal sequence necessary for translocation to the ER, and this pathway is not inhibited by brefeldin A or monensin. IL-1 and high mobility group box-1 (HMGB1) protein are secreted from macrophages via this route. Although constitutive secretion and regulated exocytosis of proteins have traditionally been considered quite distinct, apoE appears to combine constitutive secretion with acute regulation.

Protein kinase A (PKA) regulates traffic of several proteins at different steps in the constitutive secretory pathway, and a range of PKA inhibitors decreased secretion of apoE. Live cell imaging of apoE-GFP–transfected RAW macrophages indicated that PKA is required for the movement of apoE-containing vesicles. Pulse-chase apoE turnover studies showed that inhibition of PKA specifically reduces the rate of apoE secretion, expanding the size of the intracellular apoE pool, but does not affect its degradation. This suggests that apoE-containing secretory vesicles accumulate within the cell in response to PKA inhibition, at a site inaccessible to lysosomal degradation. Importantly, an extracellular PKA agonist did not stimulate apoE secretion, indicating constitutive PKA activity is sufficient.

Another important regulator of secretion is the second messenger calcium, which is also required for the regulated secretion of neurotransmitters and hormones from endocrine cells.
and neuronal cells.\textsuperscript{46–48} Chelation of intracellular calcium ([\(\text{Ca}^{2+}\)]) with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N"-tetraacetate-acetoxymethyl ester (BAPTA-AM) inhibited apoE secretion, but chelation of extracellular calcium with EGTA had no effect. As inhibitors of phospholipase C (PLC) and the inositol triphosphate (IP3) receptor (IP3R) also decreased secretion of apoE, a role for PLC and IP3R in the mobilization of [\(\text{Ca}^{2+}\)], during secretion of apoE is most likely.\textsuperscript{49}

Although PKA and [\(\text{Ca}^{2+}\)], are required for apoE secretion, neither stimulation of PKA activity nor increased [\(\text{Ca}^{2+}\)], stimulated basal apoE secretion from macrophages; conversely apoA1-stimulated apoE secretion was not accompanied by an increase in cellular PKA activity or [\(\text{Ca}^{2+}\)]. Thus [\(\text{Ca}^{2+}\)], and PKA can be considered permissive but do not explain how apoE secretion is stimulated. The IC\textsubscript{50} for inhibiting basal apoE secretion with the PKA inhibitor H89 is half that for its inhibition of apoA1-stimulated apoE secretion.\textsuperscript{49} Therefore it is likely that ABCA1-mediated basal apoE secretion occurs predominantly via a PKA-sensitive pathway, and that apoA1-stimulated secretion additionally involves a contribution from an as yet undefined PKA-insensitive pathway (Figure 2B). Whether PKA and calcium interact during apoE secretion as described elsewhere,\textsuperscript{46,48,50} or act at different steps in the secretory pathway, requires further investigation.

As constitutive release of lysozyme and other proteins from macrophages were also found to be PKA-, microtubule-, and calcium-dependent,\textsuperscript{49} the regulation of apoE secretion can be considered a prototype for understanding the processes regulating the secretion of other macrophage proteins.

**Future Directions**

The regulation of apoE secretion from macrophages provides an important avenue for rapidly regulating the concentration of apoE in local cellular environments such as the arterial wall, and potentially in the plasma. However there are quite major limitations to our current understanding of how secretion of apoE is regulated. The intracellular routes for recycling of macrophage-secreted apoE require further careful mapping and comparison made with those required for recycling of lipoprotein-derived apoE. Once these routes are defined, the relative quantitative contribution of resecretion of macrophage-derived apoE and secretion of Golgi-network-derived de novo apoE under basal and apoA1–induced conditions can be understood. The signaling and vesicular pathways regulating apoE secretion in macrophages clearly need deeper exploration and will require identifying the different pathways involved and how they interact, including the signaling partners and subcellular sites of action for PKA. The relevance of these pathways to cells other than macrophages cannot be automatically assumed and will require in vitro evaluation before targeted manipulation of apoE secretion in defined tissues can be undertaken. Targeted inhibition or stimulation of specific apoE trafficking pathways in vivo will provide unique opportunities to test directly the role of secreted macrophage-derived apoE in regulating atherosclerosis.

**Disclosures**

None.

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