Secretion of Apolipoprotein E From Macrophages Occurs via a Protein Kinase A– and Calcium-Dependent Pathway Along the Microtubule Network


Abstract—Macrophage-specific expression of apolipoprotein (apo)E protects against atherosclerosis; however, the signaling and trafficking pathways regulating secretion of apoE are unknown. We investigated the roles of the actin skeleton, microtubules, protein kinase A (PKA) and calcium (Ca²⁺) in regulating apoE secretion from macrophages. Disrupting microtubules with vinblastine or colchicine inhibited basal secretion of apoE substantially, whereas disruption of the actin skeleton had no effect. Structurally distinct inhibitors of PKA (H89, KT5720, inhibitory peptide PKI14–22) all decreased basal secretion of apoE by between 50% to 80% (P<0.01). Pulse-chase experiments demonstrated that inhibition of PKA reduced the rate of apoE secretion without affecting its degradation. Confocal microscopy and live cell imaging of apoE–green fluorescent protein–transfected RAW macrophages identified apoE–green fluorescent protein in vesicles colocalized with the microtubular network, and inhibition of PKA markedly inhibited vesicular movement. Chelation of intracellular calcium ([Ca²⁺]) with 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetate-acetoxymethyl ester (BAPTA-AM) inhibited apoE secretion by 77.2% (P<0.01). Injection of c57Bl6 apoE⁺/− bone marrow–derived macrophages into the peritoneum of apoE⁻/− C57Bl6 mice resulted in time-dependent secretion of apoE into plasma, which was significantly inhibited by transient exposure of macrophages to BAPTA-AM and colchicine and less effectively inhibited by H89. We conclude that macrophage secretion of apoE occurs via a PKA- and calcium-dependent pathway along the microtubule network. (Circ Res. 2007;101:607-616.)

Key Words: apolipoprotein E ▪ atherosclerosis ▪ macrophages ▪ signaling pathways

A polipoprotein (apo)E is a 34-kDa protein that has important roles in remnant lipoprotein clearance,¹ Alzheimcr’s disease, and lymphocyte activation.² ApoE represents a large proportion of total protein constitutively secreted from macrophages.³ Vessel-specific and macrophage-specific secretion of apoE protects against atherosclerosis.⁴⁻⁵ ApoE expression is increased during macrophage differentiation and by cholesterol loading⁶⁻⁷ and is decreased by interleukin-1, granulocyte/macrophage-colony stimulating factor, lipopolysaccharide, and interferon-γ.⁸⁻¹⁰ After translation, the protein is transported from the endoplasmic reticulum to the Golgi, where it undergoes O-linked glycosylation,¹¹ after which apoE is either secreted or degraded.¹² A proportion of secreted apoE binds to cell surface proteoglycans and can be released from the cell surface or internalized and then degraded or recycled to the cell surface.¹³ Intracellular apoE has a half-life of 22 minutes,¹⁴ and most of the apoE synthesized is rapidly degraded.¹² Secretion redirects apoE away from intracellular degradation, thereby increasing the total pool in the arterial wall.¹⁴⁻¹⁵ Recycling of exogenous apoE derived from very-low-density lipoprotein has also been described,¹⁶ but there are important differences between the trafficking of exogenous and endogenous apoE.¹⁷ HDL,¹⁴ reconstituted A-I phospholipid particles, and apoA-I⁵⁺¹⁸,¹⁹,²⁰ stimulate secretion of apoE. The ATP-binding cassette transporter ABCA1 is implicated in basal apoE secretion, but not that stimulated by apoA-I.¹⁵,²⁰ and a recent report suggests involvement of ABCG1 in basal secretion of apoE.²¹ The signaling pathways regulating constitutive and stimulated traffic/secretion of apoE are unknown.

In general terms, constitutive and stimulus-regulated secretion of proteins involves transport of proteins within secretory vesicles from the endoplasmic reticulum via the Golgi to the plasma membrane.²² Vesicular traffic occurs along actin microfilaments and/or microtubules.²³,²⁴ Regulation involves interactions between signaling molecules and motor proteins.
associated with either actin or tubulin. 23,25 Protein kinase (PK)A regulates traffic of many proteins at different steps along the constitutive secretory pathway, including transport from endoplasmic reticulum to Golgi, exit out of the Golgi, and transport from the trans Golgi network to the plasma membrane. 26,27 Calcium is another important regulator and is required for the regulated secretion of neurotransmitters and hormones from endocrine and neuronal cells. 28,29

We investigated the role of these pathways in the secretion of apoE from macrophages. apoE secretion is demonstrated to be PKA- and calcium-dependent and to involve the microtubular network.

### Materials and Methods

#### Reagents

Vinblastine, colchicine, cytochalasin D, H89, KT 5720, PKA inhibitory fragment 14-22, and 8-bromoadenosine-3',5'-cyclic-monophosphate (8-bromo-cAMP), 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetae-actoxymethyl ester (BAPTA-AM), and EGTA were obtained from Sigma. Latrunculin B, forskolin, 3'-isobutylmethylxanthine (IBMX), U73122, U73343, 2-aminoethoxydiphenylborate (2-APB), and thapsigargin were purchased from Calbiochem, and the St-Ht31 inhibitory and control peptides were from Promega. Molecular Probes (IBMX), U73122, U73343, 2-aminoethoxydiphenylborate (2-APB), and thapsigargin were purchased from Calbiochem, and the St-Ht31 inhibitory and control peptides were from Promega. Molecular Probes supplied Fura-2-AM. Human apoA-I, LDL, acetylated LDL, and inhibitory and control peptides were from Promega. Molecular Probes

#### Secretion of apoE In Vivo

All animal procedures were performed at the Central Animal Facility of the Medical Faculty of La Pitié Hospital, with approval from the Direction Départementale des Services Vétérinaires, Paris, France, under strict compliance with European Community Regulations. Bone marrow–derived macrophages (BMDMs) were obtained from wild-type female C57BL/6 mice and seeded in corning dishes in L929 conditioned medium. 34 Matured macrophages (4 days in culture) were cholesterol loaded by incubation in with 50 \( \mu \)g/mL acetylated LDL for 48 hours. Cells were detached from the plates using Accutase, washed, resuspended in RPMI medium 1640 containing 0.1% (wt/vol) BSA at 8 \( \times 10^6 \) cells/mL and exposed to the indicated concentrations of H89, colchicine, and BAPTA-AM for 30 minutes at 37°C. After treatment, cells were washed, an aliquot was stored at -20°C, and 4 \( \times 10^6 \) cells in 500 \( \mu \)L of PBS were injected intraperitoneally into fasted female apoE-deficient mice (n=6 for each condition; Charles River Laboratories, Wilmington, Mass). Blood was collected in heparin and EDTA before and 1, 2, and 3 hours after intraperitoneal injection by retroorbital puncture. At 3 hours, mice were euthanized, peritoneal lavage (PBS) was collected, and cells and supernatant were separated by ultracentrifugation and stored in RIPA buffer.

### Isolation and Culture of Human Monocyte-Derived Macrophages

Human monocytes were isolated from white cell concentrates of healthy donors of the New South Wales Red Cross blood transfusion service, Sydney, Australia, using density gradient centrifugation after layering on Ficoll–Paque Plus (Amersham). 35 After differentiation for 6 days, the cells were enriched with cholesterol by incubation in RPMI medium 1640 containing 10% lipoprotein-deficient serum (vol/vol) and acetylated LDL (50 \( \mu \)g/mL) for 2 days. 35

#### Inhibitor Treatments

Cells were washed twice and incubated with or without various treatments in RPMI medium 1640 containing 0.1% BSA. After the indicated times, media were transferred to Eppendorf tubes, mixed with Complete protease inhibitor (Roche Applied Science), and 0.02 trypsin inhibitory units of aprotinin (Sigma), and spun 5 minutes at 1300 g to remove any detached cells. The cultures were washed twice with PBS and then scraped and lysed in 0.1% Triton containing Complete protease inhibitor and 0.02 thrombin inhibitory units of aprotinin.

### Measurement of apoE Secretion

apoE secreted into the medium was measured by ELISA and confirmed by Western blot analysis. 15 Cellular apoE levels were determined by Western blot analysis. 15 For murine in vivo studies, apoE in cells, peritoneal lavage supernatant and infranatant, and plasma were determined by Western blot analysis using a rabbit anti-mouse antibody (Biodesign) and using mouse plasma as a standard.

### Analysis of apoE mRNA by Real-Time PCR

Total RNA was isolated, and apoE mRNA levels were analyzed by quantitative RT-PCR. 15

### Pulse-Chase Experiments and Metabolic Labeling of Cell Proteins With [35S]-Methionine/Cysteine

Pulse-chase studies were performed as described previously. 15 Secretion of total [35S]-labeled protein was measured by trichloroacetic acid precipitation, whereas [35S]-labeled apoE in cell lysates and medium was immunoprecipitated using a goat antibody to human apoE and protein A–Sepharose (Amersham Biosciences). Secreted lysozyme was determined by Western blotting using a rabbit anti-human lysozyme antibody (Dako). Immunoprecipitated [35S]-labeled apoE from cell lysates was separated by SDS-PAGE. The 34-kDa band was quantified by phosphorimaging (Photostimulated Luminescence, Fujix BAS-1000) and expressed as arbitrary units of [35S]-apoE per milligram of cell protein. 15

### Immunofluorescence Microscopy

Immunofluorescence was performed as described. 32 Transiently transfected Raw 264.7 cells expressing apoE-GFP were fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in blocking buffer (PBS containing 0.5% BSA). Primary antibodies to mouse anti-α-tubulin (Molecular Probes), Alexa 488 phalloidin (Molecular Probes), mouse anti-GM130 (BD Biosciences), and cy3-conjugated secondary antibodies (The Jackson Laboratory) were used in blocking buffer. Epifluorescence microscopy was performed using an Olympus Provis BX51 microscope equipped with a \( \times 100 \) oil objective and a DP70 camera. Confocal microscopy was performed using an LSM 510 META (Carl Zeiss Microscope Systems).

### Cell Viability

Cell viability (always between 85% and 100%) was routinely assessed by light microscopic morphology, by estimation of cell protein using the BCA method 30 and by measuring leakage of lactate dehydrogenase (LDH assay) into the medium. 15

### Data Analysis

The degradation and secretion of cellular apoE from pulse-chase experiments were simultaneously fitted to a first-order rate equation, with \( k_i \) and \( k_j \) describing the rate constants for secretion and degradation, respectively.

\[
\frac{\partial E_n}{\partial t} = -(k_i + k_j) \times E_n
\]

The first-order rate equation (Equation), was fitted to the experimental secretion and degradation data using a nonlinear least-squares fitting program (Solver, Microsoft Excel). The quality of the fit was evaluated by an error function as described. 15,33 Cellular apoE was previously shown to exist in stable (Es) and mobile pools (Em). 15 Data presented are means±SD of triplicate cultures from single experiments representative of at least 2 to 3 independent experiments. A significant difference between control and multiple treat-
ment groups was assessed by ANOVA using Dunnett post hoc test for multiple comparisons. Comparisons of two groups were performed by unpaired Student’s *t*-test or Mann Whitney-U test as appropriate. IC50 values were determined by nonlinear regression using Graphpad Prism. Differences were considered significant at *P* < 0.05. Mouse studies are presented as the means±SEM of *n* = 6 mice for each treatment group.

**Results**

**Basal Secretion of ApoE Is Dependent on an Intact Microtubule Network**

We investigated whether secretion of apoE from human monocyte-derived macrophages (HMDMs) was dependent on an intact microtubule network and/or an intact actin skeleton by using 2 mechanistically distinct disruptors of the microtubule network (colchicine and vinblastine) and of the actin skeleton (cytochalasin D and Latrunculin B). Secretion of apoE was markedly inhibited by both colchicine and vinblastine but was unaffected by disruption of the actin skeleton (cytochalasin D and Latrunculin B). Secretion of apoE was markedly inhibited by both colchicine and vinblastine but was unaffected by disruption of the actin skeleton (cytochalasin D and Latrunculin B). Secretion of apoE was markedly inhibited by both colchicine and vinblastine but was unaffected by disruption of the actin skeleton (cytochalasin D and Latrunculin B). Secretion of apoE was markedly inhibited by both colchicine and vinblastine but was unaffected by disruption of the actin skeleton (cytochalasin D and Latrunculin B). Secretion of apoE was markedly inhibited by both colchicine and vinblastine but was unaffected by disruption of the actin skeleton (cytochalasin D and Latrunculin B).

**Inhibition of PKA Decreases Basal ApoE Secretion**

H89, an inhibitor of PKA, markedly and dose-dependently (0 to 40 μmol/L) inhibited secretion of apoE, reaching maximal inhibition of 74.9±4.6% (Figure 1A) without cytotoxicity (data not shown). Inhibition of apoE secretion by H89 was also time dependent (data not shown).

Although it is possible PKA inhibition may decrease apoE transcription via activator protein-2,34 under the present experimental conditions, H89 had no effect on total macrophage apoE mRNA or protein levels (data not shown). The effect of H89 was attributable to its inhibition of PKA, and not off-target effects, as two other PKA inhibitors (KT5720 and PKI14–22) also decreased apoE secretion (Figure 1B). Spatial and temporal regulation of PKA is mediated by its binding to the A-kinase anchoring proteins (AKAPs).35 Figure 1C shows that disruption of PKA-AKAP anchoring using St-Ht31 inhibitory peptide (Ht31i) inhibited apoE secretion by 66.6±4.6% (100 μmol/L), whereas the inactive St-Ht31 control peptide had no effect, providing independent confir-

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**TABLE 1. Effect of Microtubule and Actin Skeleton Disruption on ApoE Secretion**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Secreted ApoE (% of Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Microtubules</strong></td>
<td></td>
</tr>
<tr>
<td>Colchicine</td>
<td></td>
</tr>
<tr>
<td>0 μmol/L</td>
<td>100±11.1</td>
</tr>
<tr>
<td>20 μmol/L</td>
<td>51.5±10.6*</td>
</tr>
<tr>
<td>40 μmol/L</td>
<td>26.0±16.5*</td>
</tr>
<tr>
<td>80 μmol/L</td>
<td>16.8±8.2*</td>
</tr>
<tr>
<td>Vinblastine</td>
<td></td>
</tr>
<tr>
<td>0 μmol/L</td>
<td>100±1.0</td>
</tr>
<tr>
<td>5 μmol/L</td>
<td>38.4±1.8*</td>
</tr>
<tr>
<td>10 μmol/L</td>
<td>31.8±2.2*</td>
</tr>
<tr>
<td>20 μmol/L</td>
<td>27.7±1.1*</td>
</tr>
<tr>
<td><strong>Actin skeleton</strong></td>
<td></td>
</tr>
<tr>
<td>Cytochalasin D</td>
<td>100±10.7</td>
</tr>
<tr>
<td>5 μmol/L</td>
<td>96.9±10.9</td>
</tr>
<tr>
<td>10 μmol/L</td>
<td>94.2±11.6</td>
</tr>
<tr>
<td>Latrunculin B</td>
<td>100±4.4</td>
</tr>
<tr>
<td>5 μmol/L</td>
<td>99.7±0.7</td>
</tr>
<tr>
<td>10 μmol/L</td>
<td>91.5±3.0</td>
</tr>
<tr>
<td>20 μmol/L</td>
<td>94.9±5.5</td>
</tr>
</tbody>
</table>

HMDMs were treated with the indicated concentrations of inhibitors for 1 hour. *P* < 0.05 relative to control (0 μmol/L).

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**Figure 1.** Inhibition of PKA decreases apoE secretion. HMDMs were incubated with increasing concentrations of H89 (A) or 20 μmol/L H89, KT5720, or PKI14–22 (PKI) (B) or peptide inhibitors of AKAP-PKA-binding Ht31i and Ht31c (C). After 40 minutes, secreted apoE was measured. *P* < 0.05 relative to control.
mation of PKA involvement. Cellular apoE levels were not affected by St-Ht31i (data not shown).

To determine whether PKA directly controls apoE secretion, or acts indirectly through control of its proteolytic turnover, macrophage proteins were pulse-labeled with [35S]-methionine/cysteine, and the kinetics of [35S]-apoE secretion and degradation were determined. H89 markedly decreased the secretion of [35S]-apoE in 1 hour from 19.1+/−3.3% to 5.7+/−2.5% of total (Figure 2). Under the same conditions, cellular [35S]-apoE decreased by 74.5+/−5.0% in control cells and by 66.8+/−5.0% in H89-treated cells. Net degradation of [35S]-apoE, calculated by subtracting residual [35S]-apoE in cell and media from [35S]-apoE in cells at time 0 (T0), was unchanged by H89.

In previous studies, modeling apoE turnover and secretion, we identified that macrophage apoE exists in relatively “mobile” (Em) and “stable” (Es) pools (Figure 2D), the secretion (k1) and degradation (k2) of which can be described with a first-order rate equation (Equation).15 Fitting the experimental data in Figure 2 to the same first-order rate equation showed that k1 was 3.6-fold higher under control conditions than when PKA was inhibited but that inhibition of PKA did not affect k2 (Table 2). Concomitantly, H89 treatment increased the stable pool of cellular apoE (Es).

Modeling data without an increase in Es, ie, assuming Es remains unaltered by H89, was incompatible with prespecified error functions of the model,15 confirming increased Es was an important consequence of PKA inhibition. Thus, PKA directs apoE secretion but not apoE degradation.

### Visualization of ApoE Trafficking

To visualize the effect of PKA inhibition, RAW macrophages were transiently transfected with apoE-GFP. Preliminary studies established that apoE and apoE-GFP stably transfected into CHO-K1 cells were secreted with similar efficiency and were equally inhibited by H89 and stimulated by apoA-I, thereby indicating that apoE-GFP was a valid surrogate for apoE (Figure I in the online data supplement). ApoE-GFP demonstrated a typical perinuclear Golgi distri-

**TABLE 2. Summary of Modeling Parameters for ApoE Secretion**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>H89</th>
</tr>
</thead>
<tbody>
<tr>
<td>k1 (min⁻¹)</td>
<td>0.018±0.003</td>
<td>0.005±0.003</td>
</tr>
<tr>
<td>k2 (min⁻¹)</td>
<td>0.037±0.001</td>
<td>0.038±0.001</td>
</tr>
<tr>
<td>Es (%)</td>
<td>26.3±1.7</td>
<td>39.8±5.5</td>
</tr>
</tbody>
</table>
bution, colocalizing with the Golgi marker GM130 (Figure 3), as well as being present in large vesicular structures distributed throughout the cell. In cells stained to depict F-actin, there was no apparent association of apoE-GFP vesicles with microfilaments. In cells with labeled microtubules, apoE-GFP vesicles were seen associated with microtubules in the cell periphery. H89 did not appreciably alter the overall distribution of apoE in live (see supplemental Movies 1 and 2) or in fixed cells (data not shown). Live cell imaging demonstrated apoE-GFP–containing vesicles moving out of the Golgi toward the cell surface and other vesicles with multidirectional trajectories (supplemental Movie 1). Total movement of apoE-containing vesicles was dramatically inhibited by H89 (P<0.0001; supplemental Movie 2), indicating PKA regulates the traffic of post-Golgi apoE-containing vesicles to and from the plasma membrane.

Figure 3. PKA regulates post-Golgi traffic of apoE-containing vesicles. Raw264.7 cells expressing apoE-GFP (green) and immuno-stained for GM130 (blue) (A), alexa-488 phalloidin (red) (A), or anti-α-tubulin (red) (B) to stain cis/medial Golgi structures, actin skeleton, or microtubules, respectively. A, Some apoE-GFP is present in the Golgi, whereas bright green vesicles represent apoE-GFP scattered through the cytoplasm. B, ApoE-GFP vesicular structures scattered throughout the cell, and in multiple instances, these are closely aligned with microtubules (arrows). C, Graph describing movement of 50 apoE-GFP–containing vesicles during live cell imaging; horizontal lines represent median movement for each treatment group. *P<0.001 relative to control (Mann–Whitney U test). Vesicle movements are demonstrated in an example shown in a sequence of still images from supplemental Movie 1 (D). ApoE-GFP is seen as a single large vesicle that moves out of the Golgi toward the periphery. Scale bar=5 μm (A and B).
Intracellular Calcium Signaling Is Required for Macrophage ApoE Secretion

Calcium signaling can be stimulated by PKA and in turn can regulate PKA activity. We therefore investigated whether calcium is involved in apoE secretion from HMDMs. Chelation of $[\text{Ca}^{2+}]$, with BAPTA-AM decreased secretion of apoE in a concentration-dependent manner (Figure 5A), whereas chelation of extracellular Ca$^{2+}$ using EGTA had no effect. Similar results were obtained when cells were treated with BAPTA-AM or EGTA in calcium-free medium, supporting the finding that apoE secretion is not dependent on an extracellular Ca$^{2+}$ pool.

To determine whether $[\text{Ca}^{2+}]$, was mobilized through activation of Phospholipase C (PLC) and subsequent inositol 3-phosphate (IP$_3$) generation, cells were treated with the PLC inhibitor U73122, its inactive structural analog U73343, and an IP$_3$ receptor (IP$_3$R) antagonist, 2-APB. U73122 (10 $\mu$mol/L) inhibited basal apoE secretion by 53.0$\pm$11.4% and inhibited apoA-I–stimulated apoE secretion to a lesser extent (by 24$\pm$3%; data not shown), whereas the inactive analogue had no effect (Figure 5). 2-APB also dose-dependently inhibited apoE secretion. These data support a role for PLC and IP$_3$ in the mobilization of $[\text{Ca}^{2+}]$, during apoE secretion.

BAPTA-AM inhibited basal and apoA-I–stimulated apoE secretion similarly (77.2$\pm$2.8% and 78.3$\pm$3.6% inhibition, respectively). The possibility that apoA-I stimulates apoE secretion by triggering release of $[\text{Ca}^{2+}]$, was investigated with fluorescence microscopy using Fura-2-AM. ApoA-I did not trigger release of $[\text{Ca}^{2+}]$, (supplemental Figure III). Although positive controls ATP, thapsigargin, and the calcium ionophore A23187 all achieved marked increases in $[\text{Ca}^{2+}]$, none of these compounds stimulated apoE secretion.

Generalized Inhibition of the Constitutive Secretory Pathway in HMDMs

The effect of microtubule disruption, PKA inhibition, and chelation of $[\text{Ca}^{2+}]$, on other proteins constitutively secreted by HMDMs was investigated. Colchicine, H89, and BAPTA-AM inhibited total protein secretion by 22.7%, 57.1%, and 64.8%, respectively. Secretion of total protein, $[^{35}\text{S}]$-apoE, and lysozyme were concurrently inhibited (supplemental Table I). None of the inhibitors affected total cell-associated trichloroacetic acid–precipitable material (not shown). Thus the pathway inhibitors can be concluded to affect HMDM constitutive protein secretion in general.

PKA, Microtubules, and Intracellular Ca$^{2+}$ Play a Role in ApoE Secretion From Macrophages

In Vivo

To study macrophage-mediated apoE secretion in vivo, BM-DMs obtained from wild-type C57Bl/6 mice were treated with H89, colchicine, and BAPTA-AM in vitro for 30 minutes, washed, and then injected into the peritoneal cavity of apoE$^{-/-}$ mice. Blood was collected from the apoE$^{-/-}$ mice over 3 hours, and plasma was analyzed for the appearance of macrophage-derived apoE. ApoE was secreted in vivo, appearing in plasma time dependently (Figure 6), the peak rate of rise occurring between 1 and 2 hours, reaching a plateau...
between 2 and 3 hours. In vitro and in vivo rates of secretion of apoE (combining peritoneal lavage supernatant and total plasma apoE at 3 hours) from murine macrophages were comparable (0.32 ± 0.01 versus 0.78 ± 0.01 arbitrary units of apoE per milligram of cell protein per hour, respectively). Pretreatment of BMDMs with H89, colchicine, and BAPTA-AM decreased apoE secretion in vivo, with BAPTA-AM being almost completely inhibitory. Colchicine significantly inhibited apoE secretion at 1 hour by 29.3 ± 14.5% but was not significantly inhibitory by 2 hours. H89 decreased apoE levels by 22.3 ± 3.2% at 1 hour; however, this did not reach statistical significance (P = 0.17).

As in vivo inhibition diminished over time, we investigated whether differences between in vivo and in vitro efficacy may be explained by reversible inhibition following cell washing (supplemental Table II). In vitro inhibition was indeed reversible after removal of H89 and colchicine but not after removal of BAPTA-AM.

**Discussion**

The present study is the first to characterize the major signaling pathways required for apoE secretion from macrophages. We have identified that apoE is secreted along the microtubular network and involves PKA and intracellular calcium signaling.

Secretion of apoE is important in the pathogenesis of atherosclerosis and other disease states. Macrophage-specific secretion of apoE increases cholesterol clearance and protects against the development of atherosclerosis.37,38 Removal of excess macrophage cholesterol and the antiinflammatory, antiproliferative, and immune-modulating properties of apoE are understood to contribute.2

ApoE is constitutively secreted from macrophages.3 Constitutive secretion has been understood as a continuous movement of vesicles to the plasma membrane followed by unregulated release of vesicular contents. Regulated macrophage protein secretion, for example after stimulation by cytokines, is distinct from constitutive secretion. For example, whereas tumor necrosis factor-α release is stimulated by lipopolysaccharide exposure,39 apoE synthesis and secretion are inhibited by lipopolysaccharide, interferon-γ, and tumor necrosis factor-α, and apoE travels via different intracellular vesicular routes.39 Our data using trichloroacetic acid precipitation and quantification of lysozyme secretion indicate that other constitutively released proteins are PKA-, microtubule-, and calcium-dependent. Thus apoE secretion can be considered a prototype for the understanding of the secretion of other macrophage proteins. Recent studies have demonstrated that constitutive secretion is regulated,40,41 involving kinases, phosphatases, receptors, and, typically, the microtubule network.42 Microtubules mediate long-range transport in vesicle traffic via kinesin and dynein motor proteins.45 Most likely, cytoplasmic vesicle-bound PKA complexes with dynein and kinesin, as has been reported for the intracellular transport of pigment granules.43 In our study, interfering with the actin skeleton had no effect on apoE secretion, suggesting a distinction between actin-mediated phagocytosis or migration44,45 and macrophage-mediated protein secretion.
Although cAMP/PKA can modulate apoE transcription, our data demonstrate PKA modulates apoE secretion under conditions in which mRNA or protein are unaltered. We therefore conclude that secretion is more sensitively and rapidly inhibited by inhibition of PKA than are apoE transcription and synthesis. This distinction may be important in vivo, as PKA-dependent signaling pathways can be rapidly regulated.

Pulse-chase studies confirmed that PKA affects secretion of preformed \[^{35}S\]-apoE. As H89 decreased the rate constant for apoE secretion without diminishing the rate constant for degradation, this indicates PKA selectively modulates secretory trafficking of apoE without affecting degradation. The markedly reduced movement of vesicles containing apoE-GFP after H89 suggests that relatively immobile vesicles contribute to the increased stable pool of apoE during PKA inhibition.

ApoE secretion was inhibited by chelation of \([Ca^{2+}]\). As extracellular \(Ca^{2+}\) was not required for apoE secretion, triggered internalization of extracellular \(Ca^{2+}\) is unlikely to be involved in this process. Roles for both PKA and \(Ca^{2+}\) have been observed for many other secretory processes, and many levels of crosstalk between the 2 pathways exist. PKA potentiates the \(Ca^{2+}\) response by controlling PLC activity through its phosphorylation. PKA can also alter the properties of IP$_3$Rs by phosphorylation and phosphorylates \(Ca^{2+}\) channels, regulating \(Ca^{2+}\) entry directly. \(Ca^{2+}\) can also affect the cAMP/PKA response, and several adenyl cyclases are \(Ca^{2+}\)-dependent.

Although macrophage apoE secretion appears to inhibit atheroma formation, a recent study suggests plasma apoE concentrations, which are principally liver-derived, correlate with cardiovascular risk. Thus comparative in vitro and in vivo studies must account for cellular specificity. We adapted a recently validated model of in vivo macrophage cholesterol efflux to establish an in vivo model of macrophage apoE secretion. In this model, the peritoneal space represents the interstitial space of the arterial wall, and all apoE appearing in plasma of apoE-deficient mice derives from intraperitoneally injected apoE/macrophages. Time-dependent secretion of apoE into plasma was most sensitive to BAPTA-AM and less sensitive to colchicine and H89. Although the effects of H89 and colchicine were limited by transient exposure and reversible inhibition, the data do provide support for microtubule-, calcium-, and probably PKA-dependent macrophage apoE secretion in vivo. Definitive confirmation will require stable, sustained, and nontoxic in vivo inhibition of each of these pathways.
The difference in IC50 for H89-mediated inhibition of basal and apoA-I-induced apoE secretion may be explained by the presence of 2 pathways: one that is in common to basal secretion and apoA-I and another that is exclusive to apoA-I-induced apoE secretion. Stimulation of PKA activity, addition of 8-Br-cAMP or dibutyryl-cAMP (M.K., L.K. unpublished observations, 2007) and increased [Ca2+]i, all failed to stimulate apoE secretion. Thus [Ca2+]i, and PKA can be considered permissive but are, in themselves, insufficient to stimulate apoE secretion and do not explain apoA-I–specific apoE secretion.

Conclusions
Macrophage secretion of apoE occurs via a PKA and calcium-dependent pathway along the microtubule network.

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Disclosures
None.

References


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Supplementary Material and Methods

Cloning of ApoE-GFP

Full-length human apoE3 cDNA (pcDNA3.1/Zeo⁺-apoE) was kindly provided by Mary LaDu (Evanston Northwestern Healthcare Research Institute, USA). An apoE-GFP construct was generated in house (T. Sabaretnam) by removing the stop codon on the apoE cDNA and tagging the C-terminus of the complete human apoE gene to the N-terminus of pEGFP-N1 (BD Clontech), in frame. The apoE coding region was amplified by PCR to incorporate a 5′-EcoR1 site and 3′-BamH1 site using specific primers 5′-ACGAATTCCATGAAGTTCTGTGGGCTGC-3′ and 5′-GTGGATCCGCGTGATTGTCGCTGGGCAC-3′, respectively (Proligo, NSW, Australia), and a purified apoE product was ligated into EcoR1 and BamH1 sites of the poly linker region of the pEGFP-N1 vector using T4-ligase (Invitrogen), obtaining a apoE-GFP with a C-terminal GFP tag.

Generation of stable CHO-apoE and CHO-apoE-EGFP transfectants

CHO-K1 (ATCC, Manassas) were transfected with pcDNA3.1/Zeo⁺-apoE or pEGFP-apoE using lipofectamine (Invitrogen) according to the manufactures instructions. CHO-K1 cells stably expressing apoE or apoE-EGFP were selected using zeocin (Invitrogen) or geneticin (Invitrogen), respectively.

Live Cell Imaging.

Transiently transfected cells were cultured on 25-mm coverslips incubated in CO2-independent media on a stage-mounted heating block warmed to 37°C on an Olympus IX71 inverted microscope equipped with an Olympus 100 X oil objective lens. Images were captured with an IMAGO Super VGA 12 bit 1280 X 1024 pixel CCD camera.
(T.I.L.L. Photonics, Germany). Movie frame capture rate was 0.5 seconds, with total capture periods ranging from 5 to 30 minutes after addition of H89. Movies were cropped, constructed and analysed using Image J v1.35 and Volocity v3.6. Movies were exported as Quicktime movies with a playback speed of 20 frames per second.

**PKA Activity Assay**

PKA activity was measured using SignaTECT® cAMP-dependent protein kinase assay system (Promega) according to manufacturer’s instructions with minor modifications. PKA specific phosphorylation was assessed performing a duplicate reaction in the presence of a PKA inhibitory peptide (Promega). PKA-specific activity was calculated by subtracting kinase activity in the presence of the inhibitor from total kinase activity.

**Calcium Measurement by Fluorescence Microscopy**

Calcium measurements were performed with the Olympus Cell® system using an Olympus IX81 microscope equipped for fluorescence and MT20 light source. Cholesterol-loaded macrophages grown on slide dishes (MatTek) were placed in Hank’s balanced salts solution (HBSS) without phenol red (Invitrogen) and loaded with 5µM fura-2-AM for 30 minutes at 37°C. Cells were equilibrated in HBSS and fluorescence at 510 nm was recorded after excitation at 340 nm and 380 nm. The change in [Ca^{2+}] was expressed as the fluorescence ratio at 340/380nm and normalized to the baseline ratio recorded during the first minute.
**Supplementary Legends**

**Supplementary Figure 1: Secretion of ApoE-GFP is comparable to that of wild type apoE.**
CHO-K1 cells stably expressing apoE or apoE-GFP secrete similar amounts of apoE in 4 hours (A). Stable transfectants were incubated with 20\(\mu\)mol/L H89 for 2 hours (B) or 25\(\mu\)g/ml apoA-I for 6 hours (C). Secreted apoE-GFP and apoE are shown by Western blot and quantified by ELISA for apoE and by Western blot analysis for apoE-GFP as described under “Materials and Methods”. st; human apoE standard *\(p < 0.05\) relative to control.

**Supplementary Movie 1 and 2: H89 abolishes trafficking of apoE containing vesicles**
Live cell imaging of RAW264.7 cells expressing apoE-GFP in the absence (Movie 1) and presence (Movie 2) of 40\(\mu\)mol/L H89. Frames were captured at 0.5 second intervals with total capture periods ranging from 5 to 30 minutes after addition of H89.

**Supplementary Figure 2: PKA activation does not increase apoE secretion**
Human macrophages were incubated with 50\(\mu\)mol/L Forskolin in the presence of 75\(\mu\)mol/L IBMX or with 25\(\mu\)g/mL apoA-I. After 10 minutes secreted apoE (A) and PKA activity (B) were determined. *, \(p < 0.05\) relative to control.

**Supplementary Figure 3: ApoA-I does not trigger release of intracellular Ca\(^{2+}\).** (A)
Cells were loaded with fura-2-AM and [Ca\(^{2+}\)]\(_i\) levels were recorded as described under “Materials and Methods”. ApoA-I was added at 1 minute to a final concentration of
25µg/ml, control cells received a similar volume of saline (Ctrl). At 6 minutes ATP was added to a final concentration of 100µmol/L. Values are mean ± SEM of 9 and 10 cells for Ctrl and apoA-I, respectively. Data shown is a representative of n=3 independent experiments performed in duplicate. (B) Effect of agents stimulating [Ca^{2+}]_{i} on apoE secretion. Human macrophages were incubated with 100µmol/L ATP, 1µmol/L thapsigargin or 1µmol/L A23187 for 30 minutes. Secreted apoE was determined by ELISA as described under “Materials and Methods”. Secreted apoE from control cells was set at 100%. *, p < 0.05 relative to control.
### Supplementary Table 1: Effect of H89, Colchicine and BAPTA-AM on total and specific protein secretion

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>H89</th>
<th>Colchicine</th>
<th>BAPTA-AM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total protein secreted</strong></td>
<td>8799.2 ± 1586.2</td>
<td>3776.3 ± 225.7</td>
<td>6891.5 ± 1456.6</td>
<td>3096.7 ± 250.9</td>
</tr>
<tr>
<td>cpm/µg cell protein</td>
<td>100.0 ± 18.0</td>
<td>42.9 ± 2.6</td>
<td>78.3 ± 16.5</td>
<td>35.2 ± 2.9</td>
</tr>
<tr>
<td><strong>% of control</strong></td>
<td>100.0 ± 18.0</td>
<td>42.9 ± 2.6</td>
<td>78.3 ± 16.5</td>
<td>35.2 ± 2.9</td>
</tr>
<tr>
<td><strong>ApoE</strong></td>
<td>387.2 ± 24.3</td>
<td>160.0 ± 4.6</td>
<td>289.9 ± 12.6</td>
<td>131.3 ± 4.8</td>
</tr>
<tr>
<td>cpm/µg cell protein</td>
<td>100.0 ± 6.3</td>
<td>41.3 ± 1.2</td>
<td>74.9 ± 3.2</td>
<td>33.9 ± 3.8</td>
</tr>
<tr>
<td><strong>% of control</strong></td>
<td>100.0 ± 6.3</td>
<td>41.3 ± 1.2</td>
<td>74.9 ± 3.2</td>
<td>33.9 ± 3.8</td>
</tr>
<tr>
<td><strong>Lysozyme</strong></td>
<td>82.9 ± 9.2</td>
<td>31.3 ± 1.5</td>
<td>52.7 ± 5.1</td>
<td>38.3 ± 6.0</td>
</tr>
<tr>
<td>AU/µg cell protein</td>
<td>100.0 ± 11.1</td>
<td>37.7 ± 1.8</td>
<td>63.6 ± 6.1</td>
<td>46.1 ± 7.3</td>
</tr>
</tbody>
</table>

Human macrophages were incubated in methionine/cysteine free DMEM with 250µCi/mL [³⁵S]methionine/cysteine for 3 h. Cells were then washed and chased in medium without or with 40µmol/L colchicine, 40µmol/L H89 or 100µmol/L BAPTA-AM for 1 h. Total [³⁵S]-labeled protein was determined by TCA precipitation, with T=0 being 391528.4 ± 42510.9 cpm/µg cell protein.

²[³⁵S]-ApoE was determined by immunoprecipitation using apoE antibodies

³Lysozyme was measured using Western Blotting analysis

*<i>p < 0.05 relative to control</i>
**Supplementary Table 2: Inhibition of apoE secretion by H89 and colchicine is reversible**

<table>
<thead>
<tr>
<th>Inhibitor (min pre-incubation / min chase)</th>
<th>Secreted apoE (% of control)</th>
<th>Secreted apoE (% of control)</th>
<th>Secreted apoE (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30/0</td>
<td>30/30</td>
<td>30/120</td>
</tr>
</tbody>
</table>

*H89*

+ / -  
57.1 ± 6.9  
49.7 ± 6.2  
70.8 ± 8.9

+ /+  
57.1 ± 6.9  
19.3 ± 1.5  
9.9 ± 0.7

*Colchicine*

+ / -  
57.6 ± 4.7  
59.4 ± 5.4  
73.8 ± 2.9

+ /+  
57.6 ± 4.7  
61.7 ± 2.6  
48.3 ± 1.4

*BAPTA-AM*

+ / -  
46.6 ± 4.1  
45.1 ± 4.1  
49.3 ± 6.6

+ /+  
46.6 ± 4.1  
12.3 ± 0.4  
4.5 ± 1.4

Human macrophages were incubated with control medium, 40µmol/L H89, 40µmol/L colchicine or 100µmol/L Bapta-AM for 30 minutes, washed and chased in control medium or the same concentration of inhibitor for the time indicated.

*p < 0.05 relative to control*
Supplementary Movies

Movie 1
See additional movie file 1

Movie 2
see additional movie file 2
Supplementary Figure 1

A

B

C

Secreted apoE (%)

Ctrl   H89   Ctrl   H89
apoE-GFP   apoE

Secreted apoE (%)

Ctrl   apoA-I   Ctrl   apoA-I
apoE-GFP   apoE

* *
Supplementary Figure 2

A

PKA activity (pmol/min/µg cell protein)

B

Secreted apoE (ng/mg cell protein)

Ctrl  Fors  apoA-I

Ctrl  Fors  apoA-I

0.0  0.3  0.6  0.9  1.2

0  500  1000  1500  2000
Supplementary Figure 3

A

[Ca^{2+}]_i
Ratio 340/380

Time (min)

apoA-I/
saline
ATP

B

Secreted apoE (%)

0 20 40 60 80 100 120

Ctrl ATP Thap A2317
Supplementary References


2. Bishara NB, Murphy TV, Hill MA. Capacitative Ca(2+) entry in vascular endothelial cells is mediated via pathways sensitive to 2 aminoethoxydiphenylborate and xestospongin C. *Br J Pharmacol.* 2002;135:119-128.