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Synergistic Therapeutic Vascular Cytoprotection against Complement-Mediated Injury Induced via a PKCα-, AMPK-, and CREB-Dependent Pathway

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Endothelial injury and dysfunction precede accelerated arterial disease in allograft vasculopathy and systemic autoimmune diseases and involve pathogenic Abs and complement. Recent reports suggest that switching to rapamycin from calcineurin antagonists reduces posttransplant vasculopathy and prolongs survival following cardiac transplantation. The majority of these patients also receive statin therapy. We examined potential mechanisms underlying this protective response in human endothelial cells and identified synergy between rapamycin and atorvastatin. Mechanistically, atorvastatin and rapamycin activated a protein kinase Ca, AMP-activated kinase, and CREB-dependent vasculoprotective pathway, which induced decay-accelerating factor (DAF) promoter activity via binding to the cAMP response element, mutation of which attenuated promoter activity. This response significantly increased endothelial cell surface DAF and enhanced protection against complement-mediated injury. Synergy with rapamycin was reproduced by simvastatin, whereas combining atorvastatin with cyclosporine or mycophenolate in place of rapamycin was ineffective. Importantly, synergy was reproduced in vivo, in which only atorvastatin and rapamycin therapy in combination was sufficient to induce DAF on murine aortic endothelium. We believe this pathway represents an important therapeutically inducible vasculoprotective mechanism for diseases mediated by pathogenic Abs and complement, including posttransplant vasculopathy and systemic lupus erythematosus. Although our study focuses on the vascular endothelium, the findings are likely to be broadly applicable, given the diverse cellular expression of DAF. *The Journal of Immunology, 2014, 192: 4316–4327.

Located at a critical tissue interface, the vascular endothelium is constantly exposed to injurious stimuli and has evolved a variety of protective mechanisms for maintenance of vascular homeostasis. Endothelial injury and dysfunction is a recognized initiating event in vascular pathology associated with atherosclerosis, and also systemic inflammatory diseases, posttransplant vasculopathy, diabetes mellitus, and renal failure. Endothelial injury and dysfunction are characterized by an inflammatory response, reduced NO bioavailability, often associated with excess reactive oxygen species (ROS), endothelial apoptosis, increased permeability to lipids, monocyte recruitment to the subintimal space, and, subsequently, the development of atherosclerotic plaques. Given their importance, we propose that a detailed understanding of molecular mechanisms controlling endothelial homeostasis and cytoprotection may offer novel therapeutic opportunities for the reversal of endothelial dysfunction.

The complement cascade, activated by the classical, alternative, or mannose-binding lectin pathways, provides innate defense against bacterial infection and bridges between innate and adaptive immunity. However, uncontrolled complement activation can provoke bystander injury in the vasculature and has been implicated in endothelial dysfunction, immune complex-mediated vasculitis, posttransplant arteriosclerosis, and atherogenesis (1, 2). The importance of regulating distal complement pathway activity is well illustrated by the study of mice deficient in the membrane-bound complement-regulatory proteins decay-accelerating factor (DAF), CD55 or CD59. These rodents may demonstrate increased susceptibility to microvascular injury after ischemia/reperfusion and accelerated atherosclerosis in DAF−/−/Adlr−/−, CD55−/−/Adlr−/−, and CD59−/−/ApoE−/− animals (3–6).

The role of DAF as a complement regulator is long established, with anti-inflammatory and cytoprotective effects mediated by...
restricting generation of C3 and C5 convertases and minimizing deposition of C3 and generation of C3a, C5a, and C5b-9 (7). Structurally, DAF comprises a proximal serine/threonine-enriched spacer domain and four complement control protein (CCP) repeats, which are principally tethered to the cell membrane by a GPI anchor, although transmembrane and secreted forms have been described (7). Following binding of DAF to C3, the CCP2 and CCP3 domains act to prevent the formation and accelerate the decay of the C3 convertases C3bBb (alternative pathway) and C4b2a (classical and mannose-binding lectin pathways) (8). Thus, during inflammation DAF induction on the endothelial surface represents an important response mechanism for the maintenance of vascular integrity (9–11).

AMPK represents a heterotrimeric complex including liver kinase B1 and Ca2+/calmodulin-dependent protein kinase kinase (12, 13). AMPK represents an important response mechanism for the maintenance of vascular integrity (9–11).

AMPK (Thr172) is activated in response to cellular stress such as that associated with hypoxia, exercise, and starvation. Rising concentrations of intracellular AMP and the AMP:ATP ratio result in the increased phosphorylation and activation of AMPK (Thr172) via upstream kinases, including liver kinase B1 and Ca2+/calmodulin-dependent protein kinase kinase (12, 13). AMPK represents a heterotrimeric complex of two catalytic α subunits and the regulatory β and γ subunits (12). Reports demonstrating that AMPK is activated in vascular endothelial cells (EC) by laminar shear stress (14, 15), vascular endothelial growth factor (VEGF) (16), and peroxynitrite (17) have led to interest in whether AMPK plays a role in endothelial cytoprotection (15). Furthermore, the observation that AMPK may be activated indirectly by drugs, including metformin, statins, and the thiazolidinediones, has led to its role as a novel therapeutic target for endothelial dysfunction and inflammation associated with metabolic syndrome and atherosclerosis (12, 13).

The importance of DAF for protection against posttransplant vasculopathy, allograft rejection, ischemia-reperfusion injury, and atherosclerosis (3, 18) also suggests that therapeutic induction might be of clinical benefit. We have demonstrated in vitro that 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase antagonists (statins) increase human EC DAF expression, a response enhanced by pharmacological inhibition of PI3K (19). The mammalian target of rapamycin (mTOR) is a downstream target of PI3K–activated proliferation pathways and is inhibited by the immunosuppressant drug rapamycin (sirolimus), through prevention of FKBP12 binding to the FKBP-rapamycin–binding domain of mTOR (20). Statin treatment also activates EC AMPK (21, 22), and increased AMPK activity independently inhibits both mTOR (23) and HMG-CoA reductase (24). These observations led to the hypothesis that combination therapy with atorvastatin and rapamycin maximally induces DAF-mediated vascular cytoprotection and that AMPK activation plays a key role in this therapeutic response.

In this study, we have demonstrated that, in combination, atorvastatin and rapamycin activate a protein kinase C (PKCα), AMPKα, CREB-dependent signaling pathway, which transcriptionally induces daf promoter activity via binding to the cAMP response element (CRE). Synergy between atorvastatin and rapamycin resulted in optimal DAF induction on the vascular endothelium in vitro and in vivo. This response effectively protected the endothelium against complement-mediated injury. Thus, in combination, these drugs provide direct vasculoprotective effects of particular relevance for the treatment of diseases mediated by pathogenic Abs and complement, including allograft vasculopathy and systemic lupus erythematosus.

Materials and Methods

Reagents

Abs were used to anti-human DAF HH anti-human membrane cofactor protein (CD46); TRA-2-10 (gifts of D. Lublin and J. Atkinson, respectively; Washington University School of Medicine, St. Louis, MO); anti-human CD59 Bric 229 (Blood Group Reference Laboratory, Bristol, U.K.); anti-human endothelin RMAC8 (gift of A. dePace, St. Vincent’s Hospital, Fitzroy, Australia); anti-murine DAF MD-1 (gift of C. Harris University of Wales College of Medicine, Cardiff, U.K.); anti-murine CD31 EA2 (gift of B. Imhof, University of Geneva, Geneva, Switzerland); anti-PKCα, anti-PKC β, and anti-PKCζ (Santa Cruz Biotechnology, Santa Cruz, CA); anti-phospho-PKCα (Thr636) and anti-phospho-PKCζ (Thr655) (Upstate, Dundee, U.K.); anti-phospho-PKCα (Thr657) and anti-PKCζ; anti-phospho-AMPKα (Thr172), anti-CREB, anti–phospho-CREB (Thr181, Gln183) (Cell Signaling, Danvers, MA); actinomycin D, cycloheximide, phorbol 12,13-dibutyrate, PGE2, and 5-aminoimidazole-4-carboxamide-1-β-riboside (AICAR), GFI0203X, and Go6976 (Merck, Nottingham, U.K.); atorvastatin, simvastatin, mycophenolic acid, compound C, 5-aminooimidazole-4-carboxamide-1-β-riboside, Z-riboside, 5'-phosphate (AICAR), and Go6976 were prepared and stored, as previously described (9). Cyclosporin A (CsA) and rapamycin were from LC Laboratories (Woburn, MA). In all experiments, EC were treated with the appropriate drug vehicle controls.

EC isolation and culture

HUVEC were isolated and cultured in M199/20% FBS, 2 mM t-glutamine, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin (Invitrogen, Paisley, U.K.), and 30 μg/ml endothelial growth factor (SigaUtdish, CA and Germany) and human microvascular EC (gift of E. Ades, Center for Disease Control, Atlanta, GA) were cultured, as described (9). The use of human EC was approved by Hamersham Hospitals Research Ethics Committee (ref. 06/Q0086/21).

Recombinant adenoviruses

The use of recombinant adenoviruses expressing dominant-negative (DN) PKCα (gift of M. Ohba, Showa University), constitutively active AMPK (CA-AMPK) (gift of D. Carling, Imperial College, London, U.K.), β-galactosidase, and the empty vector Ad0 has been described elsewhere (11, 25, 26).

RNA interference

Test and control short-interfering RNAs (siRNA) (50 nM) were complexed with geneFECToR (Venn-Nova) and added to HUVEC for 6 h in OptiMEM. HUVEC were washed and cultured overnight in EC basal medium (Cambrex BioScience, Wokingham, U.K.), prior to culture in M199/10% FCS for up to 48 h. The validated siRNA sequences targeting AMPKα1 and AMPKα2 catalytic subunits (Hs_PKRAA1_5_5HP; Hs_PKRAA2_6_5HP) and nontargeting scrambled control sequences were purchased from Qiagen (Valencia, CA).

Immunoblotting

As described previously (11), immunoblots were probed overnight with primary mAbs, detected with HRP-conjugated secondary Abs, and visualized with a chemiluminescence substrate (Pierce, Rockford, IL). To determine the equivalent sample loading, membranes were reprobed with control Abs. Relative levels of protein expression were quantified using ImageJ 1.29 software (National Institutes of Health, Bethesda, MD).

Real-time PCR

Quantitative real-time PCR was performed using an iCycler (Bio-Rad, Hercules, CA). β-actin was the housekeeping gene, with data calculated in relation to β-actin and verified using GAPDH. DNase–digested total RNA (1 μg) was reverse transcribed using 1 μM oligo(dT) and Superscript reverse transcriptase (Invitrogen). cDNA was amplified in a 25 μl reaction containing 5 μl cDNA template, 12.5 μl iSYBR supermix, 0.5 pM sense and antisense gene-specific primers, and double dh2O. Cycling parameters were 3 min at 95°C, 40 cycles of 95°C for 10 s, and 56°C for 45 s. Primer sequences are as follows: DAF forward, 5′-CCCTCAAACAGCCTTA- GGTTCC-3′ and reverse, 5′-AAATGGCACCCTGCTTATCATACT-3′;

Measurement of cellular ROS

Chloromethylketone-di-chloro-dihydro-fluorescein-diacetate (DCFH-DA) was used to detect intracellular peroxides. DCFH-DA is converted to
DCFH, generating a green fluorescent product 2'-7'-dichlorofluorescein (514 nm excitation/585 nm emission). HUVEC were treated with drugs for up to 24 h and loaded with DCFH-DA (5 μM) for 30 min, followed by exposure to H$_2$O$_2$ and analysis by flow cytometry.

**Flow cytometry**

Flow cytometry was performed, as previously described, using Epics XL-MCL and Cyan ADP flow cytometers (Beckman Coulter) (9). Results are expressed as the relative fluorescent intensity (RFI), representing mean fluorescent intensity (MFI) with test mAb divided by the MFI using an isotype-matched irrelevant mAb.

**Complement deposition and lysis assay**

For analysis of C3 binding, HUVEC were opsonized with anti-endoglin mAb RAMAC8 prior to incubation for up to 180 min at 37°C with 10% NHS or heat-inactivated human serum (HHS) in veronal-buffered saline containing 0.1% gelatin. C3 deposition was detected with FITC-conjugated anti-C3 (DAKO) and quantified by flow cytometry. To estimate complement-mediated cell lysis, human EC were opsonized with RAMAC8 or plain medium alone, washed, and incubated with 10% normal rabbit serum (Serotec, Oxford, U.K.), prior to washing and resuspension in veronal-buffered saline containing 0.1% gelatin. Propidium iodide (PI) was added to a final concentration of 80 μg/ml EC were analyzed in triplicate samples by flow cytometry, with lysis calculated as the number of PI-positive cells expressed as a percentage of the total number of cells. For DAF inhibition studies, anti-DAF inhibitory mAb I4H4 was added to achieve a final concentration of 15 μg/ml (26).

**Chromatin immunoprecipitation and luciferase reporter assays**

Chromatin immunoprecipitation (ChIP) was performed using ChIP-IT (Active Motif, Rixensart, Belgium). HUVEC were fixed in 1% formaldehyde, lysed, and homogenized. Chromatin was sheared by enzyme digestion, precipitated, and added to 2 μg CREB Ab (Santa Cruz) or negative control rabbit IgG and rotated overnight at 4°C. The chromatin-immune complex was bound to protein G beads and washed, and the immunoprecipitated DNA was eluted and purified. PCR was performed using oligonucleotide sequences flanking the CRE site in the human daf promoter (forward, 5'-GGACCCTCTGACCACAACAAA-3' and reverse, 5'-CGAGGCTTCTGCTTACTGC-3') and a control site 3000 bp downstream from the daf promoter (forward, 5'-ACAGGTAAATTGCAAGCA-3' and reverse, 5'-CCCTCTCTCAAAAGACCACTT-3'). The daf promoter luciferase reporter plasmid pGL3-DAF A and 5' daf promoter deletion constructs have been previously described (27). EC were transfected in triplicate with pGL3-basic or pGL3-DAF A constructs using Genetuec (Merck Biosciences) and analyzed for luciferase activity 16 h posttreatment using a dual luciferase reporter assay (Promega).

**Animals**

C57BL/6 mice (Harlan Olac, Bicester, Oxford, U.K.) were housed under controlled climactic conditions in microisolator cages with autoclaved bedding. Irradiated food and drinking water were readily available. All animals were studied according to U.K. Home Office guidelines and with ethical approval from Imperial College London (License PPL 70/6722).

**Immunohistochemistry**

Six-week-old C57BL/6 mice were treated twice daily with i.p. atorvastatin (5 mg/kg), and/or once daily with rapamycin (5 mg/kg) over 48 h. Mice were killed by cervical dislocation, the aorta, aortic arch, and descending aorta were dissected, equilibrated in OCT, and frozen in isopentane. Serial cryostat sections were incubated with 20% normal rabbit serum, and incubated with rat anti-mouse DAF (MD1), rat anti-mouse CD31 (EA3), or isotype-matched control Ig for 45 min. Following washing, sections were incubated with biotinylated rabbit anti-rat Ab with 2% normal mouse serum (NMS) for 40 min, followed by streptavidin-AlexaFluor 568 (Invitrogen) with 2% NMS for 1 h. Following washing and incubation for 1 min with Drag-5 (1:1000), sections were mounted with Hydromount (National Diagnostics, Atlanta, GA) and examined using a Zeiss LSM 510 Meta inverted confocal microscope (Thornwood, NY). Scan and photomultiplier settings were set to optimize signal/noise ratio for each emission wavelength. Using these photomultiplier settings, there was no detectable crossover between channels. However, to eliminate any possibility of data skew by signal contamination quantification was performed on DAF-only stained sections. Processing was with Zeiss LSM Image Browser, and quantification by export of the images into Image J. The regions of interest were selected, and the histogram function was used to calculate the distribution of pixel intensities in the red channel (corresponding to AlexaFluor 568).

**Statistical analysis**

All data are expressed as the mean of the individual experiments ± SEM. Data were grouped according to treatment and analyzed using ANOVA with Bonferroni’s correction, or an unpaired Student’s t test or the Mann-Whitney U test for nonparametric data sets. Single-sample analysis for nonparametric data was performed using the Wilcoxon’s signed-rank test (GraphPad Prism, San Diego, CA).

**Results**

**Atorvastatin and rapamycin synergistically induce DAF**

The initial observation that atorvastatin-induced DAF expression on the surface of cultured vascular EC is enhanced by PI3K blockade (19) led us to explore the effect of combining atorvastatin with the immunosuppressant drug rapamycin, an antagonist of mTOR that lies downstream of PI3K. First, concentration curves were performed, quantifying EC DAF by flow cytometry after 24 h. A significant increase in expression was detectable following treatment with low concentrations of atorvastatin (0.5 μM) or rapamycin (1 μM) alone, with maximal induction seen following exposure to supratherapeutic concentrations, 10 and 7.5 μM, respectively (Supplemental Fig. 1A, 1B). Next, the regulation of DAF was explored following exposure of EC to both atorvastatin and rapamycin. Importantly, in combination with 1 μM rapamycin, 0.5 μM atorvastatin optimally enhanced EC DAF expression, with concentrations of rapamycin above this having no further effect (Fig. 1A). The induction of DAF observed at low concentrations was synergistic and sustained up to 72 h (Fig. 1B). Similar changes in DAF mRNA were seen, first detectable at 6 h and maximal at 18 h after addition of atorvastatin and rapamycin (Supplemental Fig. 1C), with a 5- to 8-fold synergistic induction observed at 18 h (Supplemental Fig. 1D). Mevalonate was used to confirm the role of HMG-CoA reductase inhibition, and its inclusion inhibited both the atorvastatin-induced and synergistic responses (Supplemental Fig. 1E). Although atorvastatin led to EC shape change, no toxicity was observed with atorvastatin and rapamycin alone or in combination (Supplemental Fig. 2A). An isobologram (Fig. 1C) was used to confirm the synergistic effect of 0.5 μM atorvastatin and 1 μM rapamycin. The isobologram indicates the concentration of each individual drug required to achieve 80% of the maximum dose response, namely 6 μM atorvastatin and 3 μM rapamycin. Synergy is indicated by drug dose pairs, which also achieve 80% of the maximum response and which lie below the additive line (Fig. 1C).

The synergy observed between atorvastatin and rapamycin was not confined to venous EC, with the response of HAEC directly below the additive line (Fig. 2A). Comparison with simvastatin, another clinically important statin, confirmed that the synergistic response with rapamycin was not unique to atorvastatin. Simvastatin concentration-response studies revealed a significant increase in DAF expression with a maximal plateau of 80% at concentrations of 0.75–1 μM. The addition of 1 μM rapamycin led to synergistic DAF induction to levels in excess of 200% above untreated cells (Fig. 2B).

Mycohenolate mofetil (MMF), a purine synthesis inhibitor, and calcineurin antagonist CsA are clinical alternatives to rapamycin for the prevention of rejection following organ transplantation. Although treatment with MMF (given as the active form myco-phenolic acid) resulted in a small and nonsignificant induction of DAF, addition of atorvastatin and 10 μM MMF led to an additive
increase, with synergy seen with 50 μM MMF. However, this response was modest when compared with that seen with atorvastatin and rapamycin (Fig. 2C). Treatment with CsA alone failed to induce DAF expression and did not affect the response to atorvastatin (Fig. 2D).

We next considered whether a change in the cellular redox environment or NO bioavailability might influence DAF regulation in response to atorvastatin and rapamycin. First, inclusion of NO synthase antagonist Nω-monomethyl-L-arginine failed to inhibit DAF induction (Supplemental Fig. 2B). Second, to analyze ROS generation, EC were loaded with 2′,7′-dichlorofluorescein. However, subsequent exposure to rapamycin and atorvastatin alone or in combination did not affect ROS generation (Supplemental Fig. 2C). Likewise, inclusion of ROS scavenger N-acetylcysteine failed to significantly inhibit DAF upregulation (Supplemental Fig. 2D), suggesting induction is independent of ROS.

**Atorvastatin- and rapamycin-induced DAF expression is cytoprotective**

In contrast to DAF, neither atorvastatin nor rapamycin, alone or in combination, altered expression of the membrane-bound complement-regulatory proteins CD46 and CD59 (Fig. 3A). This suggests a change in DAF alone was likely to account for any increased resistance to complement. To investigate this further, initial experiments used a C3 deposition assay (26). HUVEC were treated for 24 h with atorvastatin and rapamycin, opsonized with anti-endoglin mAb RMAC8, and exposed to NHS or HIHS. Analysis of C3 deposition by flow cytometry confirmed that exposure to atorvastatin and rapamycin resulted in a 40–50% decrease in cell surface C3, when compared with vehicle-treated cells (Fig. 3B, 3C). Moreover, the inclusion of the inhibitory anti-DAF mAb 1H4 reversed the protective effect, suggesting atorvastatin- and rapamycin-mediated induction of DAF was responsible for reduced C3 deposition. To exclude the possibility that the augmented C3 deposition in the presence of 1H4 reflected immune complex formation and enhanced complement activation, rather than a DAF inhibitory action, we compared the ability of RMAC8 (IgG2a) and 1H4 (IgG1) to fix complement. As seen in Supplemental Fig. 3A, when bound to the surface of EC, only the IgG2a mAb RMAC8 was able to significantly fix complement and deposit C3 on the cell surface. This response was inhibited by inclusion of EDTA, consistent with a role for complement activation. Opsonization with 1H4 resulted in C3 deposition equivalent to that on nonopsonized EC exposed to NHS. These data suggest that the increased cell surface C3 in the presence of the mAb1H4 reflects the specific ability of the Ab to inhibit the function of DAF.

To assess the physiological relevance of the reduced C3 binding, a complement-mediated EC cytotoxicity assay was used. EC opsonized with RMAC8 were exposed to rabbit serum, and complement-mediated EC lysis was quantified. Rapamycin treatment alone decreased EC lysis by 20%, whereas atorvastatin monotherapy reduced cell death by up to 40%. However, atorvastatin and rapamycin combination therapy reduced cell lysis by up to 65% (Fig. 3D, gray bars). The role of DAF was confirmed by inclusion of 1H4, which abrogated drug-induced cytoprotection (Fig. 3D, black bars).

**Atorvastatin and rapamycin synergistically induce DAF in vivo**

To investigate the efficacy of atorvastatin and rapamycin in vivo, C57BL/6 mice were dosed i.p. with vehicle alone, atorvastatin (5 mg/kg, twice daily), and/or rapamycin (5 mg/kg, once daily) for 48 h, drug doses comparable to those previously reported (28, 29). DAF and CD31 protein expression in the aortae of treated animals was assessed by quantitative confocal analysis. Whereas CD31 staining revealed an intact luminal aortic endothelium in vehicle-treated animals, there was minimal endothelial DAF detectable, with DAF only expressed robustly in the adventitia. Likewise, following dosing with either atorvastatin or rapamycin alone, DAF was only abundant in the adventitia (Fig. 4). However, combined treatment with atorvastatin and rapamycin led to a significant 3.5-fold induction of DAF on the aortic endothelium, confirming the synergistic relationship of the drugs in vivo (Fig. 4).
Induction of DAF is AMPK dependent

In human EC, DAF induction is typically transcriptional (9, 10, 19), and treatment with actinomycin D attenuated atorvastatin- and rapamycin-induced DAF upregulation (Supplemental Fig. 3A). Cycloheximide exerted a comparable effect (Supplemental Fig. 3B), suggesting that the synergistic induction of DAF is dependent upon gene transcription and de novo protein synthesis. Furthermore, subsequent actinomycin D chase experiments showed no significant difference in DAF mRNA stability (Supplemental Fig. 3C). To explore the role of mTOR inhibition further, an siRNA approach was used. However, mTOR depletion (Supplemental Fig. 3D) did not enhance the effect of atorvastatin and reduced DAF induction somewhat (Supplemental Fig. 3E). Alternative mechanistic pathways were investigated, starting with AMPK and PKC signaling. Statin treatment phosphorylates endothelial AMPKα in vitro and in vivo (21, 22), whereas we have identified a role for the activation of PKCα and PKCδ in DAF regulation (11). Initial experiments with AMPK agonist AICAR revealed a 3- to 4-fold increase in DAF expression at 24 h (Fig. 5A). Although more modest, overexpression of a constitutively active mutant of AMPK (CA-AMPKα) using an adenoviral vector (Supplemental Fig. 4A) also increased DAF, a response not seen with the control virus (Fig. 5B). Optimal AMPKα(Thr172) phosphorylation is reported following treatment with 1–10 μM atorvastatin or simvastatin (21, 22). We found that atorvastatin (0.5 μM) or rapamycin (1 μM) alone had minimal effect on AMPKα phosphorylation (data not shown). However, in combination at these concentrations, the drugs increased AMPKα(Thr172) phosphorylation by 2-fold after 30–60 min (Fig. 5C). Moreover, inhibition of AMPK signaling using two separate approaches, the antagonist compound C and AMPKα1 siRNA, attenuated synergistic DAF induction, confirming the role of AMPK (Supplemental Fig. 4B, Fig. 5D, 5E).

Relationship between AMPK and CREB

The transcription factor CREB is a recognized AMPK target (30), and expression of CA-AMPKα resulted in a 4-fold increase in CREB(Ser133) phosphorylation in human EC (Fig. 5F). Similarly, treatment of EC with atorvastatin and rapamycin resulted in maximal CREB phosphorylation at 60–90 min, a response not seen with either drug alone (Fig. 5G, 5H). The role of AMPK was

![Figure 2](http://www.jimmunol.org/)
demonstrated by inclusion of compound C, which attenuated CREB phosphorylation in cells treated with atorvastatin and rapamycin (Fig. 5I).

Induction of DAF by atorvastatin and rapamycin requires PKCa activation. GF109203X, an antagonist of classical and novel PKC isoforms, inhibited CREB phosphorylation by atorvastatin and rapamycin (Fig. 6A) and the synergistic induction of DAF (data not shown). Treatment of EC with either atorvastatin (0.5 μM) or rapamycin (1 μM) had no significant effect on PKCa(Ser657) phosphorylation (data not shown), whereas in combination the drugs increased phosphorylation 2-fold, maximal after 30–90 min (Fig. 6B). In contrast, neither PKCd(Thr505) nor PKCe(Ser729) was phosphorylated (Supplemental Fig. 4C, 4D). The functional role of PKCa was confirmed using a cell-permeable peptide antagonist of PKCa/b (myr-PKC) and a DN-PKCa-Adv, both of which inhibited DAF induction by atorvastatin and rapamycin and prevented synergy (Fig. 6C, 6D). Furthermore, inhibition of PKCa also significantly attenuated atorvastatin- and rapamycin-induced CREB phosphorylation (Fig. 6E), consistent with PKCa acting upstream of CREB.

Promoter analysis

To study the effects of atorvastatin and rapamycin on daf promoter activity, human 5′ daf promoter deletion constructs were used (27). Transfection of the complete daf promoter construct (~724/+80) into HUVEC significantly enhanced luciferase activity, consistent with basal DAF protein expression, and activity was further enhanced by atorvastatin and rapamycin (Fig. 7A). Known DAF inducers PGE2 and phorbol dibutyrate increased luciferase activity by 3- and 5-fold, respectively, whereas atorvastatin and rapamycin treatment resulted in a 2- to 3-fold increase (Fig. 7B). HUVEC transfected with daf promoter deletion constructs displayed a progressive decline in basal luciferase activity (Fig. 7B). However, there was no significant change in the increased activity seen following exposure to atorvastatin and rapamycin. As construct −126/+80 into HUVEC was lost, confirming the role of CRE in the synergistic response (Fig. 7B). Finally, using a ChIP

FIGURE 3. Atorvastatin- and rapamycin-induced DAF protects against complement-mediated injury. (A) HUVEC were left untreated (UT) or treated with atorvastatin (AT) (0.5 μM) or rapamycin (R) (1 μM), alone and in combination for 24 h. DAF, CD59, and membrane cofactor protein expression was measured by flow cytometry, and data are presented as the median RFI ± SEM, expressed relative to untreated EC (n = 3 experiments). (B and C) HUVEC were treated with AT (0.5 μM) and/or rapamycin (R) (1 μM) for 24 h and then harvested and opsonized with anti-endoglin mAb RMAC8, in the presence or absence of inhibitory DAF mAb 1H4 or plain medium alone. Following incubation with NHS or HIHS for up to 180 min, deposition of C3 was quantified by flow cytometry. Data are presented as median fluorescent intensity ± SEM (B) and as a percentage of the maximal C3 deposition seen on opsonized unprotected control cells (C). (D) HUVEC were treated with AT (0.5 μM) and/or rapamycin (Rap) (1 μM) for 24 h, harvested, and opsonized with anti-endoglin mAb RMAC8, in the presence or absence of inhibitory DAF mAb 1H4 (black bars) or plain medium alone (gray bars) for 30 min at 4˚C. Cells were then exposed to 10% rabbit serum (RS) for 30 min, prior to addition of PI and quantification of EC lysis using flow cytometry. Percentage lysis was calculated as the number of PI-positive cells expressed as a percentage of the total number of cells and relative to lysis seen in untreated opsonized EC exposed to 10% RS (mean ± SEM, n = 5 experiments). C1, IgG2a control; C2, heat-inactivated rabbit serum. *p < 0.05.
assay, we found that CREB binding to the CRE in the daf promoter (Supplemental Fig. 4E) was enhanced 4-fold following treatment with atorvastatin and rapamycin (Fig. 7C). PCR amplification was also performed on the same samples using primers specific to the distal 3’ region of the daf open reading frame as a negative control (Fig. 7C, Supplemental Fig. 4E).

Discussion
Vascular injury leading to endothelial dysfunction is an important early event in atherogenesis, posttransplant vasculopathy, and graft rejection. The importance of the complement-regulatory protein DAF in vasculoprotection has been highlighted (3, 9, 18), and we initially reported that statins upregulate endothelial DAF (19). However, optimal in vitro induction required supratherapeutic drug concentrations, leading us to consider the therapeutic potential of drug synergy. This was prompted by the finding that PI3K inhibition enhanced statin-mediated DAF induction (19) and the fact that rapamycin, which inhibits mTOR, a downstream target of PI3K, is established in clinical practice. These preliminary observations, alongside emerging evidence that an early switch to rapamycin from calcineurin inhibitors confers significant protection against allograft vasculopathy following cardiac transplantation (31–33), led us to explore the efficacy of atorvastatin and rapamycin in combination. Although rapamycin or atorvastatin monotherapy led to some protection, combination resulted in a maximal 65% fall in cell lysis, emphasizing the significance of DAF induction. Of note, atorvastatin and rapamycin failed to induce either CD46 or CD59, suggesting that despite their complementary functions, CD46 and CD59 regulatory pathways are distinct from DAF. Importantly, the effects of atorvastatin and rapamycin were also observed in vivo. Whereas monotherapy failed to upregulate DAF on murine aortic endothelium, their combination led to a marked synergistic increase. DAF induction by VEGF, thrombin, and proinflammatory cytokines is important for the maintenance of endothelial integrity during inflammation (9–11, 26), and the current study suggests this response might be enhanced therapeutically to increase protection against proinflammatory, prothrombotic, and bystander injury effects of complement activation.

To imitate the effect of rapamycin, we depleted EC of mTOR and exposed them to atorvastatin. However, this failed to reproduce synergistic induction of DAF. Rapamycin, a macrolide lactone, forms a complex with FKBP12, binds the FKBP-rapamycin-binding domain of mTOR, and disrupts the formation and function of mTORC1 by preventing Raptor binding. In contrast, mTORC2 is only inhibited after >24-h treatment with rapamycin (34). This acute effect on mTORC1 alone may be relevant to the synergy

FIGURE 4. Atorvastatin and rapamycin synergistically enhance DAF expression on murine aortic endothelium. (A) C57BL/6 mice (n = 5 per treatment) were treated twice daily with vehicle alone, 5 mg/kg atorvastatin (AT), and/or once daily 5 mg/kg rapamycin (R) for 48 h. Aortic endothelial DAF or CD31 expression was assessed using immunofluorescence confocal microscopy on a Zeiss LSM 510 Meta inverted confocal microscope. Images shown are at original magnification ×20. DAF or CD31 (green), nuclei (red), elastin (purple) arrows indicate luminal expression. (B) Enlarged image of the area indicated by box in (A) (original magnification ×40), demonstrating aortic endothelial DAF expression following treatment with AT + R. (C) Regions of interest were selected, and the histogram function in Image J was used to calculate the distribution of pixel intensities in the red channel (corresponding to AlexaFluor 568). The histogram shows pooled quantification data from image analysis, representing DAF expression as relative fluorescence versus matched isotype control. Data are presented as fold change ± SEM of mice treated with vehicle control. **p < 0.01. ns, not significant.
observed, given that mTORC2 is important for the stability and function of PKCα (35). Furthermore, statins also inhibit mTORC1 (36). The PKC family is divided on the basis of structure and response to phosphatidylserine, calcium, and diacylglycerol, into classical (α, βI, βII, γ), novel (δ, ε, η, θ), and atypical (ζ, τ, γ) isoforms. Unique cell-specific functions for individual isoforms have emerged, reflecting differences within isoform structure, subcellular compartmentalization, and PKC-target protein interactions (37). In combination, atorvastatin and rapamycin-phosphorylated PKCα (Thr172) (pPKCα) assessed by immunoblotting. (D) HUVEC were left untreated (UT) or treated with AT (0.5 μM) alone and in combination for 24 h, in the presence or absence of compound C (CC) (5 μM) with DAF expression quantified by flow cytometry. (E) HUVEC were left untreated (UT) or transfected with control (Ctrl) or AMPKα siRNA (50 nM). After 48 h, EC were treated with AT (0.5 μM) or R (1 μM), alone and in combination for 24 h, and analyzed by flow cytometry. (F) HUVEC were left untreated (UT) or infected with an adenovirus expressing CA-AMPK or a control adenovirus (Ad0) (multiplicity of infection 100), and were analyzed after 16 h for phospho-CREB(Ser133) by immunoblotting. (G) HUVEC were left untreated or treated with AT (0.5 μM) alone or in combination with R (1 μM) for 45 min, lysed, and immunoblotted with anti–phospho-CREB(Ser133) and anti-CREB Abs. (H) HUVEC were left untreated or treated with AT and R for 15–90 min, lysed, and immunoblotted for phospho-CREB and total CREB. (I) HUVEC were treated with vehicle alone (UT) or compound C (5 μM) for 30 min prior to addition of 0.5 μM AT and 1 μM R for 60 min. EC were then lysed, and phospho-CREB was analyzed by immunoblotting. Representative immunoblots are shown with the histograms representing pooled quantification data corrected for the loading controls, with data presented as fold change relative to untreated EC n = 3–4 experiments. *p < 0.05, **p < 0.01.
FIGURE 6. Atorvastatin and rapamycin specifically activate PKCα. (A) HUVEC were treated with vehicle alone (UT) or with the PKC inhibitor GF-109203X (GF) (3 μM) for 30 min prior to the addition of atorvastatin (AT) (0.5 μM) and rapamycin (R) (1 μM) for 45 min. EC were lysed and immunoblotted with anti–phospho-CREB(Ser 133) or anti-CREB Abs. (B) HUVEC were left untreated (UT) or treated with AT (0.5 μM) and rapamycin (R) (1 μM) for up to 120 min, lysed, and immunoblotted with anti–phospho-PKCα(Ser657) or anti-PKCα. (C and D) HUVEC were left untreated (UT) or in (C) treated with myr-ψPKC, a classical PKC isoyme-specific peptide inhibitor, or in (D) transfected with a dominant-negative PKCα adenovirus (DN-PKCα) or control adenovirus (Ad0). After 24 h, EC were treated with vehicle alone, atorvastatin (AT) (0.5 μM), and/or rapamycin (R) (1 μM) for 24 h, and DAF expression was analyzed by flow cytometry. (E) HUVEC were left untreated (UT) or treated with myr-ψPKC prior to addition of AT + R for 60 min, prior to lysis and immunoblotting with anti–phospho-CREB(Ser 133) or anti-CREB Abs. Representative immunoblots are shown, with the histograms representing pooled quantification data corrected for the loading controls. Data are derived from three experiments and expressed as fold change relative to untreated cells and presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.
its role in the synergistic induction of DAF. AMPK is a metabolic regulator that senses and responds to cellular stress. Recent evidence suggests that, in addition to playing an important regulatory role in endothelial metabolism, AMPK may also contribute to endothelial homeostasis and cytoprotection (13). EC AMPK may be activated via liver kinase B1 and alternatively by Ca²⁺-dependent and AMP-independent processes involving phosphorylation of calmodulin-dependent protein kinase kinase B (38). Classically, AMPK acts to inhibit gene transcription and activate catabolic pathways to generate ATP. However, it may increase gene transcription (13), as demonstrated by the increase in DAF. The importance of AMPK to EC homeostasis is supported by the report that AMPKα1 depletion increases oxidative stress and upregulates antioxidant genes in EC (39), by its activation by VEGF (16), its role in laminar shear stress-induced upregulation of KLF2 (40), and eNOSSer1177 phosphorylation (41).

To investigate the role of AMPK, a gain- and loss-of-function approach was adopted. First, AMPK activation by AICAR or CA-AMPK upregulated DAF. Second, in combination, atorvastatin and rapamycin phosphorylated AMPKα1, and, finally, both AMPK antagonist compound C and AMPKα siRNA attenuated DAF induction by the drug combination. AMPK activity is also anti-inflammatory, inhibiting JNK phosphorylation, NF-κB activation, cellular adhesion molecule upregulation, and leukocyte adhesion to EC (13, 42). These findings further emphasize the therapeutic potential of AMPK activation and suggest that, alongside DAF induction and enhanced complement resistance, treatment with

**FIGURE 7.** Synergistic induction of DAF requires activation of PKCα and CREB. HUVEC were treated with atorvastatin (AT) and/or rapamycin (R) following transfection with (A) the pGL4.73[hRluc/SV40] plasmid and the complete daf promoter luciferase reporter construct (−724→+80), or (B) with the pGL4.73[hRluc/SV40] plasmid and either complete daf promoter luciferase reporter construct (−724→+80) or specific deletion constructs. The pGL4 basic vector was used as a negative control. EC were lysed, and transcriptional activity was measured using a dual luciferase reporter assay. Phorbol dibutyrate (PDBu) or PGE₂ were used as positive controls. Results are expressed as a ratio of luminescence generated by firefly luciferase produced by DAF promoter constructs relative to Renilla luciferase produced by pGL4.73 (hRluc/SV40). All results are normalized to the untreated complete daf promoter construct. (C) HUVEC were left untreated or exposed to AT (0.5 μM) and R (1 μM) for 60 min. Following immunoprecipitation with mouse anti-CREB mAb or negative control IgG, genomic DNA obtained was used in a PCR with primers spanning a region flanking the CREB binding site within the daf promoter. To ensure specificity, PCR amplification was also performed using primers specific to the 3’ region of the daf locus. Densitometry was performed relative to input DNA and expressed as fold change (n = 3). Data in the figure are presented as mean ± SEM derived from three or more experiments. *p < 0.05, **p < 0.01, ***p < 0.001. ns, not significant compared with the untreated control.
atorvastatin and rapamycin may activate additional endothelial cytoprotective genes.

Members of the CREB family of transcription factors, including CREB1, have been identified as AMPK targets (30), a response we demonstrated in EC using a CA-AMPK vector. Interdependence between AMPK, PKCo, and CREB in the induction of DAF by atorvastatin and rapamycin was revealed. First, exposure of EC to the drugs in combination resulted in sustained phosphorylation of Ser133 in the CREB1 kinase-inducible domain, a response shown to be AMPK and PKCo dependent. CREB1 binds to a 5’-TGACGTCACA-3’ DNA sequence, and the importance of this CRE site for the synergistic induction of DAF, and the binding of CREB to it, was confirmed using specific daf promoter mutants and ChIP analysis.

In addition to its role in DAF regulation, activation of an AMPK-CREB pathway by atorvastatin and rapamycin is likely to have broader significance in terms of vasculoprotection. Increased activity of AMPK and CREB has been shown to exert antiproliferative, anti-inflammatory effects on vascular smooth muscle cells (43). Additional AMPK targets include those with endothelial protective actions such as eNOS, hypoxia-inducible factor-1α, and VEGF (13). Likewise, increasing evidence points toward an important role for CREB in the maintenance of a healthy arterial wall, with downstream targets, including the antiapoptotic protein Bcl-2 and the antioxidant, anti-inflammatory enzyme heme oxygenase-1. Although the precise role of CREB in the vasculature remains to be determined, and may to some extent be cell type and context dependent, its protective credentials are strengthened by the report that CREB downregulation in the aorta is associated with vascular disease in rodent models of hypertension, atherosclerosis, and insulin resistance (43). Furthermore, cardiac-specific expression of DN-CREB increases oxidative stress, mitochondrial dysfunction, apoptosis, and mortality (44). CREB also plays an important role in VEGF-driven cytoprotection, angiogenesis, and endothelial barrier function (45, 46).

The efficacy of rapamycin in protection against allograft vasculopathy following cardiac transplantation has been demonstrated by studies in which 90–100% of the patients were coprescribed a statin. Mancini et al. (32) randomized patients with angiographically proven transplant vasculopathy to either receive rapamycin or continue azathioprine or MMF and reported a significant benefit against accelerated atherogenesis associated with systemic inflammatory diseases.

In conclusion, in combination atorvastatin and rapamycin act synergistically to activate a PKCo-AMPK-CREB–dependent pathway that induces DAF expression and resistance to complement-mediated injury in the vascular endothelium. In addition to immunomodulatory, lipid-lowering, and complement-inhibitory actions, activation of AMPK and CREB may also exert antioxidant, anti-inflammatory, antiproliferative, and antiapoptotic effects. Thus, we propose that combination therapy with statins and rapamycin therapeutically conditions the vascular endothelium, resulting in enhanced protection against ischemia–reperfusion injury, posttransplant vasculopathy, and graft rejection, and has the potential to protect against accelerated atherogenesis associated with systemic inflammatory diseases.

Disclosures
The authors have no financial conflicts of interest.

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Supplementary Figure 1. Atorvastatin and rapamycin induce DAF mRNA and protein. HUVEC were left untreated (UT) or treated for 24h with vehicle alone or increasing concentrations of (A) atorvastatin, or (B) rapamycin. DAF expression was measured by flow-cytometry and is presented as fold increase in relative fluorescent intensity above control cells exposed to vehicle alone. (C) HUVEC were treated with atorvastatin (AT) (0.5µM) and rapamycin (R) (1µM) for up to 24h and (D) with AT and/or R at the same concentrations for 18h. Changes in DAF expression were quantified by qPCR. (E) HUVEC were left untreated or treated with mevalonate (100µM) for 30 min prior to the addition of AT or R, alone or in combination for 24h, followed by analysis of DAF expression by flow-cytometry. The figure shows data from at least 3 independent experiments. *p<0.05, **p<0.01 compared to untreated control.
**Supplementary Figure 2. Role of nitric oxide and redox signaling in atorvastatin and rapamycin-induced DAF expression.** (A) HUVEC monolayers were treated with 0.5µM atorvastatin (AT) and/or 1µM rapamycin (R) for 24h or vehicle alone. EC were imaged under a phase-contrast microscope and photographed at 20X magnification. No significant toxicity is apparent. HUVEC were left untreated (UT) or treated for 24h with atorvastatin (AT) (0.5µM) and/or rapamycin (R) (1µM) in the presence or absence of (B) nitric oxide synthase antagonist L-NMMA (1mM). DAF expression was analyzed by flow-cytometry and presented as percent change in RFI relative to UT cells. (C) HUVEC were left UT or treated for 24h with AT and/or R and then loaded with chloromethylketone-di-chloro-dihydro-fluorescein-diacetate (DCF) (5µM) for 30 min followed by treatment with vehicle alone (black bars) or H₂O₂ (5µM) (grey bars) for 45 min and analysis of DCF fluorescence by flow-cytometry. (D) HUVEC were treated for 24h with AT and/or R in the presence or absence of I-acetylcysteine (NAc) (10mM) and changes in DAF expression analyzed by flow-cytometry. Data are presented as mean ± SEM of 3 independent experiments. *p<0.05, **p<0.01.
Supplementary Figure 3. Role of transcription, translation and mTOR in DAF induction.

(A) HUVEC were left untreated (UT), exposed to normal human serum (NHS) alone, or opsonized with anti-DAF mAb 1H4 or anti-endoglin mAb RMAC8 prior to exposure to NHS or heat-inactivated serum (HIHS). Following incubation for 180 min, C3 deposition was quantified by flow-cytometry. HUVEC were left UT or treated with (B) actinomycin D (0.2μM) (Act D), or (C) cycloheximide (3μM) (CHX), for 30 min prior to the addition of atorvastatin (AT) (0.5μM) and/or rapamycin (R) (1μM) for 24h. DAF expression was quantified using flow-cytometry. (D) HUVEC were left UT or treated with AT + R for 18h prior to the addition of actinomycin D (0.2μM) or vehicle. EC were harvested after 0-120 mins and DAF mRNA quantified using PCR. The data is plotted as a percentage of mRNA expression prior to the addition of Act D. (E and F) HUVEC were left UT or transfected with control (Ctl) or mTOR siRNA (30nM) (TOR) for 48h prior to the addition of vehicle or AT (0.5μM) for 18h. EC were analyzed for (E) mTOR RNA or (F) DAF mRNA levels using qPCR. Data are expressed as fold change relative to UT cells exposed to vehicle alone and normalized to actin mRNA levels. Data are presented as mean ± SEM of 3 experiments. *p<0.05, **p<0.01, ***p<0.001, ns = not significant.
Supplementary Figure 4. Analysis of PKC and AMPK signaling. HUVEC were left untreated (UT) or infected with an adenovirus expressing CA-AMPK or a control adenovirus (Ad0) (MOI 25-100). After 24h the EC were lysed and immunblotted for phospho-AMPKα (Thr 172) and GAPDH. (B) HUVEC were UT or transfected with control (Ctrl) or AMPKα siRNA (50nM) and immunblotted after 48h for total AMPKα and GAPDH. (C and D) HUVEC were left UT or infected with AT (0.5µM) and R (1µM) for up to 120 min, lysed and immunoblotted for (C) anti-phospho-PKCε (Ser729) and total PKCε, and (D) anti-phospho-PKCδ (Thr505) and total PKCδ. Data are derived from 3 separate experiments and expressed as fold change relative to untreated cells exposed to vehicle alone and presented as mean ± SEM. **p<0.01, ***p<0.001. (E) The genomic DNA sequence of the proximal 5’ region of the human DAF gene is shown. The approximate transcription start site is designated +1 and the ATG translation initiation site is indicated in bold. The CRE binding site is over scored. Oligonucleotide sequences flanking the CRE site and 3000 base pairs downstream from the DAF promoter used in ChIP assays are indicated by arrows.