Published in final edited form as:

Arterioscler Thromb Vasc Biol. 2021 February ; 41(2): 638–650. doi:10.1161/ATVBAHA.120.315401.

Endothelial Rap1 restricts inflammatory signaling to protect from the progression of atherosclerosis.

Bandana Singh, Ph.D.[a], **Ramoji Kosuru, Ph.D.**[a], **Sribalaji Lakshmikanthan, Ph.D.**[a], **Mary Sorci-Thomas, Ph.D.**[b,c], **David Zhang, Ph.D.**[b], **Rodney Sparapani, Ph.D.**[d,e], **Jeannette Vasquez-Vivar, Ph.D.**[f], **Magdalena Chrzanowska, Ph.D., F.A.H.A.**[a,e,g]

[a]Blood Research Institute, Versiti, Milwaukee, Wisconsin

[b]Department of Medicine, Medical College of Wisconsin, Milwaukee, Wisconsin

[c]Division of Endocrinology, Medical College of Wisconsin, Milwaukee, Wisconsin

[d]Division of Biostatistics, Institute for Health and Equity, Medical College of Wisconsin, Milwaukee, Wisconsin

[e]Cardiovascular Center, Medical College of Wisconsin, Milwaukee, Wisconsin

[f]Department of Biophysics and Redox Biology Program, Medical College of Wisconsin, Milwaukee, Wisconsin

[g]Department of Pharmacology and Toxicology, Medical College of Wisconsin, Milwaukee, Wisconsin

Abstract

OBJECTIVE: Small GTPase Rap1 is a novel, positive regulator of NO release and endothelial function with a potentially key role in mechanosensing of atheroprotective, laminar flow. Our objective was to delineate the role of Rap1 in the progression of atherosclerosis and its specific functions in the presence and absence of laminar flow, to better define its role in endothelial mechanisms contributing to plaque formation and atherogenesis.

APPROACH AND RESULTS: In a mouse atherosclerosis model, endothelial Rap1B deletion exacerbates atherosclerotic plaque formation in the thoracic aorta, where laminar shear stress-induced NO is otherwise atheroprotective, plaque area is increased in Athero-Rap1B$^{i\Delta EC}$ mice. Endothelial Rap1 deficiency also leads to increased plaque size, leukocyte accumulation and increased CAM expression in athero-prone areas, while vascular permeability is unchanged. In endothelial cells, in the absence of protective laminar flow, Rap1 deficiency leads to an increased proinflammatory TNF-α signaling and increased NF-κB activation and elevated inflammatory receptor expression. Interestingly, this increased signaling to NF-κB activation is corrected by AKTVIII, an inhibitor of Akt translocation to the membrane. Together these data implicate Rap1

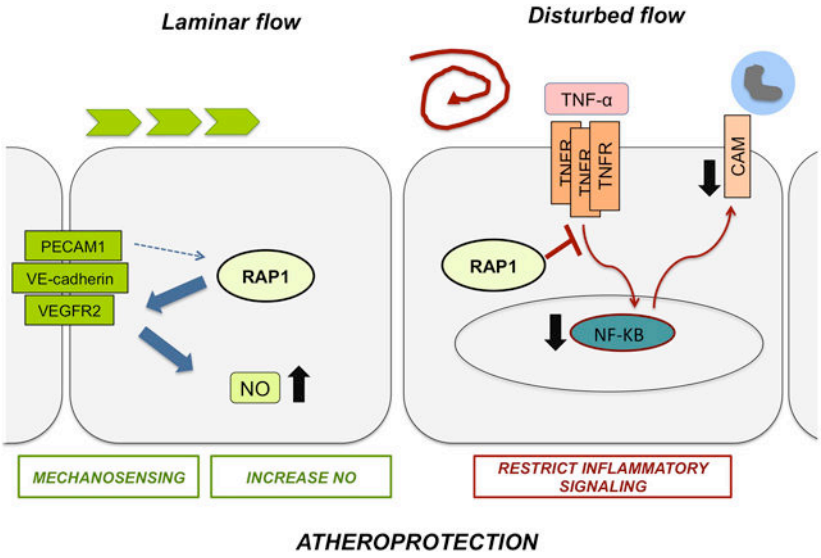
Corresponding Author: Magdalena Chrzanowska, Blood Research Institute, Versiti, Milwaukee, Wisconsin 53201-2178, Department of Pharmacology and Toxicology, Medical College of Wisconsin, Milwaukee, Wisconsin 53226, tel: 414.937.3890, mchrzanowska@versiti.org.

Disclosures: none.

in restricting Akt-dependent signaling, preventing excessive cytokine receptor signaling and proinflammatory NF-κB activation.

CONCLUSIONS: Via two distinct mechanisms, endothelial Rap1 protects from the atherosclerosis progression in the presence and absence of laminar flow; Rap1-stimulated NO release predominates in laminar flow and restriction of proinflammatory signaling predominates in the absence of laminar flow. Our studies provide novel insights into the mechanisms underlying endothelial homeostasis and reveal the importance of Rap1 signaling in cardiovascular disease.

Graphical Abstract



Keywords

Cardiovascular disease; Endothelial function; Hemodynamics; Inflammatory signaling; Small GTPase Rap1

Subject terms:

Endothelium; Vascular type; Nitric Oxide; Hemodynamics; Cell Signaling; Signal Transduction; Atherosclerosis; Animal Models of Human Disease

Introduction

Atherosclerosis, a chronic inflammatory disease of the vessels, and its complications, such as heart disease and stroke, are a leading cause of death in the Western world[1]. The main feature of atherosclerosis, pathological vessel wall remodeling, results from chronic inflammation and is initiated by damage to endothelium occurring before the disease's onset[2–5]. The endothelium responds to physical, chemical, and humoral cues from the environment by releasing bioactive substances that modulate vascular smooth muscle tone, maintain non-adhesive lumen surface, and control inflammatory responses in the vascular wall. Laminar shear flow promotes NO release from endothelial NOS and suppresses proinflammatory signaling. In contrast, perturbed hemodynamic forces resulting from

disturbed flow switch signaling towards proinflammatory effects, impair NO release, promote vascular permeability and immune cell infiltration, and contribute to vascular pathology[6–8]. This differential endothelial response to flow patterns is responsible for a geometrical localization of atherosclerosis with plaques preferentially forming at the sites of vessel bifurcations and vessel curvature, while vessel areas exposed to laminar blood flow and higher shear stress are relatively athero-protected[2, 9–11]. Thus, endothelial response to flow determines vessel lesion size and vulnerability[12].

Several endothelial cell elements are implicated in shear flow sensing[13]; among them, endothelial junctional mechanosensing complex, composed of PECAM-1, VE-cadherin, and Vascular Endothelial Growth Factor Receptor 2 (VEGFR2)[14] plays a key role in hemodynamic regulation of eNOS activity[15] and flow-mediated, ligand-independent VEGFR2 transactivation required for NO release[16]. We recently demonstrated that small GTPase Rap1 is an essential component of endothelial mechanosensing response. Endothelial Rap1 deletion leads to impaired NO release in vitro and endothelial dysfunction in vivo[17]. However, the implications of Rap1 dysfunction in vascular homeostasis and cardiovascular disease (CVD) are unknown.

Conserved and ubiquitously expressed, the best-characterized functions of the two highly homologous Rap1 isoforms: Rap1A and Rap1B pertain to modulation of adhesive and signaling properties of integrins and cadherins[18, 19]. Rap1 is required for vessel stabilization during development and endothelial deletion of both isoforms leads to embryonic lethality due to cardiovascular defects[20]. Postnatally, endothelial Rap1 promotes angiogenesis[21], and dynamic regulation of endothelial barrier[22, 23]. Interestingly, the deletion of both isoforms in existing vessels does not lead to increased basal vascular permeability in most vascular beds[17, 22].

At the molecular level, Rap1 controls endothelial functions by promoting activation and signaling by VEGFR2 with functional consequences for VEGF-induced vessel growth and permeability[23, 24]. Moreover, Rap1 controls laminar flow-induced transactivation of VEGFR2, and transmission of shear-induced signals from the EC junctional mechanosensory complex to eNOS activation in response to laminar flow[17]. These findings implicate Rap1 as a key molecular regulator of EC function.

In this study, we examined the pathological consequences of endothelial deletion of Rap1B, the predominant Rap1 isoform in endothelium, on the development of atherosclerosis in a hypercholesterolemic mouse model (Athero-Rap1B$^{i\Delta EC}$ mice). We hypothesized that Rap1B controls endothelial function and its deletion would accelerate atherosclerosis progression in atheroprotective vessel areas. We examined the effect of EC Rap1B deletion in the athero-prone areas, where decreased vascular barrier and increased inflammatory cell infiltration contribute to accelerated atheroma formation. Interestingly, in the absence of protective laminar flow, EC Rap1 deletion also accelerates the development of the disease, however not by increasing vascular permeability, which is unchanged in Athero-Rap1$^{i\Delta EC}$ mice. Instead, we find that Rap1 deletion lead to enhanced cytokine receptor signaling and proinflammatory NF-κB activation. We describe a novel mechanism through which Rap1 restricts proinflammatory cytokine signaling – a key function in endothelial homeostasis.

Materials and Methods

A detailed description of the experimental procedures and all materials used is included in the Supplementary Materials. The authors will make their raw data, analytic methods, and study materials available to other researchers upon written request.

Animals.

All mouse procedures were performed according to the protocol approved Medical College of Wisconsin Institutional Animal Use and Care Committee. For atherosclerosis studies, the methods adhered to the guidelines for experimental atherosclerosis studies described in the AHA Statement[25]. Generation of endothelial cell (EC)-specific total Rap1 KO mice (Rap1A +Rap1B knockout, Cdh5(PAC)-CreERT2$^{+/0}$; Rap1A$^{f/f}$ Rap1B$^{f/f}$; Rap1$^{i\Delta EC}$) and EC-specific Rap1B-knockout mice (Cdh5(PAC)-CreERT2$^{+/0}$; Rap1A$^{+/+}$ Rap1B$^{f/f}$; Rap1B$^{i\Delta EC}$) has been previously described[17, 23]. Atherogenic: EC-specific Rap1B knockout (Athero-Rap1B$^{i\Delta EC}$) and EC-specific total Rap1 knockout (Athero-Rap1$^{i\Delta EC}$) mice were generated by crossing Rap1B$^{i\Delta EC}$ and Rap1$^{i\Delta EC}$, respectively, with ApoE$^{-/-}$ mice (The Jackson Laboratory #002052) and feeding a Western high-fat diet (21.2% fat, 0.2% cholesterol) for 16 weeks. Following ATVB guidelines, male and female mice were used throughout the study[26]. Consistently with the known sexual dimorphism of the mouse atherosclerosis model, with the initial faster progression of atherosclerosis in females[27, 28], our statistical analysis model confirmed the effect of sex (Supplemental Table S1). Cdh5-Cre-negative mice, or mice injected with carrier oil only, were used as controls. For L-arginine (L-Arg) treatment, drinking water was supplemented with L-Arg (6 g/kg/day) at weaning until the analyses were completed; control mice received plain water. Total serum cholesterol was measured in blood samples collected after six weeks of high-fat diet (HFD) treatment using an enzymatic colorimetric assay (Wako).

Atherosclerotic Plaque Analysis.

At the conclusion of the HFD treatment, atherosclerotic lesions in the aortic arch (disturbed flow, athero-prone vessel area), and thoracic and abdominal sections (laminar flow, atheroprotective vessel area) of dissected whole aortae was visualized by oil red O (ORO) staining and morphometry was performed using a standardized protocol[29] by an operator blinded to the genotype of examined animals, to avoid experimental bias, of particular importance due to expected high variability in plaque formation in ApoE$^{-/-}$ mice. Following ORO staining, the aortae were opened to expose the endothelium and placed on the cover glass with the endothelium facing down. Slides were scanned in digital whole slide scanner. Tiff images were processed and quantified using Image J (NIH) software. The background was subtracted from all images and areas of total aorta, and ORO-stained plaque area in the aortic arch (disturbed flow, athero-prone vessel area), thoracic and abdominal aorta (laminar flow, athero-protective vessel area) and total area of aorta were measured and percentage of lesion coverage per each vessel area were determined.

Cell Culture, Transfection and Treatments, Western blotting.

Primary human aortic ECs (hAECs) cultured with standard methods for no longer than six passages were used for all *in vitro* experiments. 30–40% confluent hAECs monolayers were

transfected with 50nM Rap1 (Rap1A and Rap1B) siGENOME siRNA pool or with non-targeting siRNA pool (Dharmacon) for 6h and cultured for an additional 30h in complete EBM culture medium (Lonza). NO released from siControl and siRap1B hAECs stimulated with VEGF (50 ng/ml), following pretreatment with 300 μM L-Arg or L-N monomethyl arginine (L-NMMA) was estimated from the reduction of nitrite to NO and quantified by NO-Analyzer-chemiluminescence using authentic standards, as previously described[30].

For analysis of NF-κβ signaling pathway activation, cells were serum-starved before TNF-α (50ng/mL) treatment in the presence or absence of 1μM AKT inhibitor VIII (Cayman Chemical). Clarified cell lysates' samples containing equal amount of protein were resolved on 4–12% gradient SDS-PAGE gels, and transferred to PVDF membranes for Western blotting analysis. Following blocking with 5% skim milk/(PBS and 0.05% Tween 20), the following antibodies were used for Western Blot analysis: antibodies against P-IKK (Ser176/180), IKK, P-IκB (Ser32/36), IκB, P-AKT (Ser473), AKT, Rap1 (Cell Signaling Technology), and β-actin (Santa Cruz Biotechnology).

Basal VCAM1 and ICAM1 expression in aortic arches was determined by Western blotting of lysates prepared from aortic arches isolated from $Rap1^{i\Delta EC}$ mice and Cre-negative controls, as previously described[31], using ICAM1- and VCAM1-specific antibodies. Lung lysate obtained from $Rap1b^{+/-}$ mice[32] was used as a positive control.

Horseradish peroxidase-conjugated secondary antibodies were used for chemiluminescence detection using Amersham Imager 600, and analysis software (GE Healthcare) was used for densitometry. Blanked values (following subtraction of the corresponding unloaded lane value), normalized within each experiment, were used to calculate fold change in phosphorylation between Rap1-deficient and control conditions.

In vivo vascular permeability measurements.

Vascular permeability of aortae was determined using Evans blue assay in Athero-$Rap1^{i\Delta EC}$ and Athero-control at the conclusion of HFD treatment and $Rap1^{i\Delta EC}$ in response to acute inflammation, as we previously described[17].

Immunohistochemistry.

At the conclusion of the HFD treatment, aortae, including aortic root, arches, and thoracic aorta, were harvested, fixed in 4% paraformaldehyde/PBS, and processed for immunohistochemistry using standard protocols. For analysis of macrophage infiltration into aortic roots, 5 μm aorta sections were stained with an F4/80-specific antibody and 12 sections from each animal were analyzed as previously described[33]. CD31 and CAM dual immunofluorescence staining of frozen aortic arch sections were obtained at 5 μm intervals was performed as previously described[34]. Macrophage intensity was expressed as number of F4/80-positive cells per aortic root area. For nuclear NF-κB staining of ECs, tissue sections were labeled with NF-κB p65-specific and CD31-specific antibodies, followed by DAPI nuclear staining to identify individual cells, as previously described[35]. Negative controls were obtained by substituting the primary antibody with an isotype control (for monoclonal antibodies) or an irrelevant antibody from the same species as the primary antibody and followed by the same secondary antibody. Images were obtained using Nikon inverted

microscope and quantified using ImageJ software. For endothelial CAM quantification, the number of CAM-positive cells among CD31-positive ECs lining the luminal surface of aortic arch sections was determined. For nuclear NF-κB quantification, the number of NF-κB–positive EC nuclei was determined as a fraction of all CD31-positive nuclei by operators blinded to the genotype of examined animals.

Statistical Analysis.

SAS version 9.4 (SAS Institute Inc., Cary, NC) and GraphPad Prism version 5 (GraphPad Software) software were used. For animal data, the statistical significance of group differences was determined using linear regression models that adjusted for sex. Two-way interactions were considered for inclusion into the models when main effects (treatment, time, or sex) were significant. Data were analyzed by two-way analysis of variance using the Bonferoni-posthoc comparison test. When laminar and disturbed flow data were combined for analysis, a repeated measures model was used. In lieu of determination of normality and equality of variance, challenging with samples sizes available, p-values were calculated applying via the nonparamentric bootstrapping resampling technique. The parametric and nonparametric p-values were close enough such that no changes of statistical significance were discovered. For densitometric analysis of Western blot data, the statistical significance of increased NF-κB signaling in Rap1-deficient ECs (expected outcome) was determined using one tailed unpaired Student's t-test with Welch's correction; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Results

Rap1B protects from the progression of atherosclerosis *in vivo*.

Because our previous findings implicate Rap1 as a key regulator of endothelial function *in vivo*[17], we examined the effect of endothelial deficiency of Rap1B, the major endothelial Rap1 isoform[24], on progression of atherosclerosis in the Apolipoprotein E-deficient (ApoE$^{-/-}$) mice[36]. Rap1$^{i\Delta EC}$ and control, Cdh5-Cre-negative mice or mice injected with carrier oil only, were crossed with ApoE$^{-/-}$ mice. Thus generated Athero-Rap1B$^{i\Delta EC}$ and Athero-control mice were fed a high-fat western diet (HFD, 21.2% fat, 0.2% cholesterol) for 16 weeks to induce hypercholesterolemia. As expected, we found about 3-fold elevated serum cholesterol in Athero-controls, compared to normal laboratory diet-fed control mice (Fig. 1A). Serum cholesterol was also elevated in Athero-Rap1B$^{i\Delta EC}$, with no difference in total cholesterol levels between Athero-Rap1B$^{i\Delta EC}$ and Athero-control mice (Fig. 1A). At the conclusion of the HFD treatment, atherosclerotic lesions were visualized with oil O red staining, and plaque area was quantified in en-face preparations aorta preparations from male and female mice (Fig. 1B). While the overall plaque detected in Athero-control mice was on the low end of the reported range[37], we found overall atherosclerotic lesion area was higher in female mice (Fig. 1C **and** Supplemental Table S1A), consistent with previous reports of sexual dimorphism in this model[26–28]. Notably, the atherosclerotic lesion area significantly increased in Athero-Rap1B$^{i\Delta EC}$ vs. Athero-control (Fig. 1C and Supplemental Table S1A). Basal endothelial NO synthesis is essential for preventing the progression of atherosclerosis. Asymmetric and symmetric dimethyl-L-arginine metabolites can inhibit eNOS by outcompeting endogenous L-Arg and reduce vascular NO. To determine if L-Arg

supplementation can reduce plaque formation in Athero-Rap1B$^{i\Delta EC}$, mice received L-Arg in the diet. After 16 weeks of treatment, we found reduced plaque formation predominantly in Athero-Rap1B$^{i\Delta EC}$ group (Fig. 1C, right panel, Supplemental Table S1A), suggesting that defective NO production is involved in enhanced atherogenesis in the EC-Rap1B deficient model. To directly address L-Arg's effect on NO production in Rap1B-deficient cells, NO release from VEGF-stimulated Rap1B-deficient hAECs was quantified. L-Arg treatment increased NO release from control and siRap1B hAECs, albeit NO release was lower in siRap1B hAECs (Fig. 1D). Furthermore, Rap1B deficiency increased sensitivity to NO inhibitor, L-N monomethyl arginine (L-NMMA), compared to siControl hAECs (Fig. 1E). Together, these data implicate the defective NO production as an underlying factor in enhanced atherogenesis in Athero-Rap1B$^{i\Delta EC}$ mice.

Rap1B and shear stress protects from plaque buildup.

To determine how Rap1 affects flow-pattern shear stress dependent pathology, we quantified plaque in the descending aorta and in the aortic arch, where the flow is laminar and disturbed, respectively. After 16 weeks of HFD, atherosclerotic plaque in the aortic arch accumulates in 5 areas of the in inner curvatures of ascending aorta (AA) and descending aorta (DA) and in the opposite surfaces to branch bifurcations in brachiocephalic artery (BCA), left common carotid artery (LCC) and left subclavian artery (LS)[38] Fig. 1F). We observed significantly increased lesion area in the descending aorta in Athero-Rap1B$^{i\Delta EC}$ mice, compared to Athero-control mice (Fig. 1G, left panel, and Supplemental Table S1B). The plaque in Athero-Rap1B$^{i\Delta EC}$ vessel areas with laminar flow was significantly reduced by L-Arg supplementation in the diet, to levels comparable to Athero-control mice (Fig. 1G, right panel and Supplemental Table S1B).

The plaque area, in the aortic arch with characteristic disturbed flow, where protection by NO is limited[8], was not significantly different between Athero-Rap1B$^{i\Delta EC}$ and control mice, (Supplemental Table S1C). The average plaque volume in the Athero-Rap1B$^{i\Delta EC}$, however, was significantly increased in both laminar and disturbed flow areas (Fig. 1H). Macrophage infiltration and degradation of extracellular matrix in fibrous cap contribute to atherosclerotic plaque development (Fig. 1I). The aortic side of the aortic's valvular leaflet is a site of disturbed flow and preferentially susceptible to atherosclerotic plaque development[7]. Since macrophages contribute to local release of proinflammatory cytokines and plaque formation, we quantified macrophage infiltration in the aortic root in both Athero-Rap1B$^{i\Delta EC}$, and Athero-controls. We found a significant increase in macrophage density in Athero-Rap1B$^{i\Delta EC}$ mice (Fig. 1J). These results indicate that endothelial Rap1 confers atheroprotection in laminar and disturbed flow areas by distinct mechanisms.

Endothelial Rap1-deficiency increased proatherogenic response to TNF-α.

A major proinflammatory cytokine in atherosclerosis is TNF. It exerts proatherogenic effects through several mechanisms, including the increased expression of adhesion receptors VCAM1 and ICAM1, which contribute to leukocyte recruitment. We hypothesized that increased leukocyte recruitment might be associated with increased inflammatory CAM expression in Rap1-deficient endothelium. To test this hypothesis, we examined inflammatory CAM expression in situ, in serial sections of aortic arches from Athero-control

and Athero-Rap1$^{i\Delta EC}$ mice fluorescently stained with ICAM1-, VCAM1- and, as an endothelial marker, CD31 (PECAM-1)-specific antibodies (Fig. 2A, B). We found that the expression of both CAMs was significantly increased in aortic arches of Athero-Rap1$^{i\Delta EC}$ compared to Athero-controls (Fig. 2C, D). However, EC-Rap1 deficiency did not increase CAM expression in aortic arches under basal conditions (Supplemental Fig. I), with similar levels of VCAM1 in Rap1$^{i\Delta EC}$ and control mice (Supplemental Fig. IA, C) and undetectable ICAM1 in either mice (Supplemental Fig. IB). To determine if elevated CAM expression is directly linked to the absence of Rap1, we examined CAM expression in cultured control and Rap1-depleted hAECs, following 48 h treatment with TNF-α (Fig. 2 E). TNF-α-induced VCAM1 expression in siRap1 hCAECs (Fig. 2F), demonstrating that Rap1 is critically involved in the expression of endothelial CAMs induced by proinflammatory signaling.

In addition to induction of proinflammatory transcription, TNF-α exerts proatherogenic effects via non-transcriptional mechanisms causing vascular barrier dysfunction[39, 40]. To determine the physiological significance of increased permeability of Rap1-deficient EC barrier *in vivo*, we examined Evans Blue-conjugated BSA extravasation in the aortic roots of the aortae from Athero-control and Athero-Rap1B$^{i\Delta EC}$ mice We found that vascular permeability was not increased under conditions of atherosclerosis, with a similar level of Evans Blue-BSA extravasation in both animals. (Supplemental Fig. IB, Supplemental Table S3). Similar results were obtained by LPS-treatment to induce acute inflammation (Supplemental Fig. IA, Supplemental Table S2). In combination, these results demonstrate increased Rap1 increased CAMs transcription but not vascular permeability.

EC-Rap1 deletion enhances NF-κB signaling.

Elevated CAM expression in vivo depends on the activation of NF-κB transcription factor, in response to elevated cytokines[41, 42]. Therefore, we examined aortic arches of aortae from and for increased NF-κB activation (Fig. 3A). We found elevated nuclear localization of p65 subunit NF-κB in ECs lining aortic arches in Athero-Rap1$^{i\Delta EC}$, compared to Athero-control (Fig. 3B), supportive of increased NF-κB activation in Athero-Rap1$^{i\Delta EC}$ vessels.

In disturbed flow areas, increased NF-κB and proinflammatory protein transcription is mediated by TNF-α receptor, TNFR1[40, 43, 44]. Increased CAM expression (Fig. 2C, D) and NF-κB activation (Fig. 3B) in Athero-Rap1$^{i\Delta EC}$ aortae suggested that Rap1 modulates TNF-α-induced activation. To test this hypothesis, we examined the effects of TNF-α on early signaling events leading to NF-κB activation in ECs in the absence of laminar flow[40, 45]. Under such conditions, TNF-α-induced stimulation of hAECs leads to a rapid phosphorylation of IκB kinase (IKK), and, downstream, IκB phosphorylation on Ser32/36, followed by IκB degradation (Fig. 3C)[40, 45]. Interestingly, when Rap1 was depleted from hAECs, TNF-α-induced IκB phosphorylation was rapidly elevated, as early as 2min following stimulation, the earliest time point tested (Fig. 3D). Consequently, changes in total IκB were transient in siRap1 hAECs, consistently with accelerated phosphorylation-dependent IκB degradation (Fig. 3C). Therefore, we examined TNF-α-induced activation of IκB kinase complex responsible for IκB phosphorylation. We found that in control hAECs TNF-α treatment led to rapid increase in on Ser32/36 IKK phosphorylation, with a

timeframe parallel to IκB phosphorylation (Fig. 3D). Consistently with increased IκB phosphorylation in the absence of Rap1, this TNF-α-induced IKK phosphorylation was significantly elevated in Rap1-depleted hAECs, as compared to WT controls (Fig. 3D). Together, our studies indicate that Rap1 depletion leads to significantly increased IKK activation, accelerating early signaling events leading to NF-κB activation.

Rap1 restricts Akt-mediated IκB phosphorylation.

Signaling by the TNFR complex can be enhanced by recruitment of PI3K and its target, Akt[46]. As part of TNFR complex, Akt phosphorylates IκB, and IKK, inducing NF-κB activity[47, 48]. Previous studies have implicated Rap1 in regulation of PI3K, an upstream Akt activator. Similarly to Ras, Rap1 interacts with PI3K[49] via its effector domain[50], promoting PI3K activation by both a conformational change and PI3K reorientation, increasing its affinity and proximity to its lipid effectors in the plasma membrane[51]. Thus, we hypothesized that in the absence of Rap1, PI3K-Akt interaction may be perturbed, resulting in increased NF-κB activation in siRap1 hAECs. To test this hypothesis, we examined TNF-α-induced IKK and IκB phosphorylation in Rap1-deficient and control ECs in the presence of Akt1/2 selective inhibitor, AKT inhibitor VIII, which inhibits Akt by binding to is plextrin homology domain, preventing Akt association with the membrane[52] (Fig. 4A). TNF-α treatment of hCAECs did not lead to elevated Akt phosphorylation (no global increased Akt activation). As expected, treatment with Akt inhibitor VIII led to a significantly decreased Akt -Ser 473 phosphorylation, consistent with decreased Akt activity in both, WT and siRap1 ECs (Fig. 4B). The presence of AKT inhibitor VIII during TNF-α-treatment did not significantly alter IκB or IKK phosphorylation in WT hAECs (Fig. 4C, D). In contrast, AKT inhibitor VIII decreased excessive TNF-α-induced IκB and IKK phosphorylation siRap1 hAECs, bringing it down to a similar level of that in WT hAECs (Fig. 4C, D). These results demonstrate that restriction of Akt activity in Rap1-deficient cells restores normal ECs response to TNF-α and suggest that Rap1 restricts TNF-α-induced signaling to NF-κB activation by restricting Akt-mediated IKK activation.

Discussion

Progression of atherosclerosis depends on perturbed endothelial homeostasis[2–5]. Altered endothelial function and mechanotransduction, and increased proinflammatory signaling are key culprits of subsequent dysfunctional alteration of endothelial phenotype towards pro-atherogenic endothelium. However, exact understanding of how biochemical and mechanical signals are integrated at the molecular level to maintain endothelial homeostasis remains elusive. We recently discovered that Rap1, a small GTPase best known for modulation of cell adhesion and adhesion signaling[18, 19, 21], plays important role in mechanosensing of laminar flow and is a critical, positive regulator of NO release and endothelial function[17]. These characteristics suggest a role for EC-Rap1 in inflammatory conditions.

We demonstrate that in the atherosclerosis model, EC-Rap1 deficiency leads to increased atherosclerotic burden in the thoracic aorta, where laminar flow shear-induced NO release is atheroprotective in WT animals (Fig. 1C, G). This phenotype is similar to that of eNOS knockout mice on ApoE-null background, with increased extent of plaque deposition[53]. In

the absence of laminar flow, EC Rap1 deletion leads to increased proinflammatory cytokine signaling and NF-κB activation. Through this mechanism Rap1 may be a major modulator of leukocyte infiltration and increased plaque size in hypercholesterolemic conditions (Fig. 4E). In regions of laminar flow, Rap1 enhances eNOS activity. The hitherto unreported anti-atherogenic functions of Rap1 (Fig. 5), provide **novel insights** into the mechanisms of endothelial homeostasis and CVD progression.

Previously, we demonstrated that Rap1 is essential for transmission of protective laminar flow signaling to endothelium, acting as a critical regulator of endothelial junctional mechanosensing complex formation and signaling to eNOS activation. Activated by shear stress, downstream from PECAM- 1, Rap1 transmits signals required for the formation of the junctional mechanosensory complex, VEGFR2 transactivation and signaling to eNOS activation and NO release[17]. Our findings of accelerated atherosclerosis progression *in the laminar flow* area in $Rap1^{i\Delta EC}$ aortae, similar to that in eNOS knockout mice[53, 54], support this mechanism. Significantly, our results show that in the *absence of laminar flow*, Rap1 protects endothelial homeostasis by restricting TNF-α proinflammatory signaling to NF-κB activation. To understand how Rap1 deficiency shifts endothelium towards proinflammatory phenotype, we examined early signaling events leading to proinflammatory activation of NF-κB. We found that Rap1 deficiency led to increased IKK activation and enhanced IκB phosphorylation (Fig. 3D), which is consistent with increased NF-κB activation in vivo (Fig. 3A,B). Interestingly, this elevated IκB activation in Rap1-deficient ECs was normalized to the level of WT cells by pre-treatment with Akt-specific inhibitor, thus implicating elevated Akt in increased IKK activation in the absence of Rap1. Thus, in the *absence of laminar flow,* Rap1 restricts Akt activity, preventing excessive signaling to NF-kB (Fig. 4E).

Our results suggest a novel mechanism controlling proinflammatory NF-κB activation in ECs involving Rap1-dependent restriction of Akt activity towards IKK. TNFR1 and other members of TNFR superfamily can directly and indirectly activate PI3K/Akt pathway in different cells[46], although this synergy is best characterized in T-cells. In T-cells, upon ligand binding, TNFR molecules oligomerize to organize signalosomes in membrane lipid domains by recruiting TRAF adaptor proteins (TNFR-association factor) and, via their cytoplasmic domain, PI3K/Akt[46]. The synergy between Akt TNFR is not well understood in ECs and therefore our findings, implicating Rap1 in restricting Akt activity in the context of TNF-α-induced signaling, provide a novel insight into mechanisms modulating proinflammatory signaling in ECs. Furthermore, they speak to a novel role of Rap1-Akt signaling in the context of inflammation.

The **exact mechanism through which Rap1 restricts Akt activity** remains to be fully explained. Nonetheless, there exists evidence for Rap1 interaction with PI3K, an upstream regulator of Akt, as well as for Rap1-Akt. Rap1-PI3K interaction has been characterized *in vitro*[49]. Rap1 directly interacts with PI3K via its effector domain[50]. Rap1-PI3K interaction leads to PI3K activation via an allosteric effect and reorientation, increasing its affinity and proximity to its lipid effectors in the plasma membrane[51]. In ECs, PI3K has been implicated as a direct (RBD-binding) Rap1 effector, however in a functionally different context of Weibel-Pallade Body exocytosis[55]. In the context of angiogenesis, Rap1 promotes afadin-PI3K activation in response to Sphingosine 1-Phosphate[56]. Furthermore, in response to

VEGF, Rap1 has been shown to promote Akt phosphorylation, albeit, most likely, indirectly, via integrin activation[57]. Of relevance to regulation of endothelial function, we demonstrated that Rap1 is essential for normal recruitment of PI3K to endothelial junctional mechanosensory complex, promoting Akt activation and NO release in response to laminar shear flow[17]. Thus, whether Rap1 promotes or restricts Akt activity appears to depend on flow type-specific endothelial mechanotransduction mechanisms. While the exact mechanism through which Rap1 restricts Akt activity in response to inflammatory cytokines in the absence of laminar flow, remains to be fully explained, one intriguing possibility is that Rap1-PI3K interaction may restrict Akt recruitment away from TNFR2 signalosomes, thus preventing potentiation of the proinflammatory signaling.

Alternative and potentially complementary mechanisms can be envisioned. Inhibition of NO bioavailability and increased reactive oxygen species (ROS) generation in Rap1-deficient ECs may additionally elevate TNF-α response, contributing to increased atherosclerotic burden in $Rap1^{i\Delta EC}$ mice. However, *in vitro*, and particularly in the absence of protective laminar flow, human ECs used in these experiments are a poor source of NO and thus, the effect on eNOS is unlikely to be the cause of increased proinflammatory response in the *in vitro* system. Thus, the limitation of the model system allows us to identify a novel component of Rap1 signaling restricting NF-κB activation. TNF-α promotes proinflammatory changes also via ROS generation and specifically, TNF-α-induced NADPH oxidase activation contributes to NF-κB activation[58–60]. We have previously shown that in choroid epithelial cells Rap1 can inhibit NOX-dependent ROS activation[61]. Thus, in future studies, it will be important to determine if it also plays a role in cytokine-induced, NOX-dependent NF-κB activation.

Because increased plaque in the disturbed flow areas is associated with **increased vascular permeability**, we examined if impaired endothelial barrier in Rap1-deficient ECs may be responsible for the observed phenotype. During development[20] and junction reformation after inflammation-induced breakdown[23], Rap1 promotes cell adhesion and cell-cell junction formation. Surprisingly, after development, Rap1 is not essential for EB maintenance in most organs. Here, we found that aortic vascular permeability was not increased under conditions of atherosclerosis (Supplemental Fig. IIB). Thus, altered vascular permeability is an unlikely cause for exacerbated atherosclerosis in the disturbed flow area (Fig. 1F, G).

Our study provides new, important insights into **physiological functions of Rap1** in endothelium and the molecular mechanisms that underlie them. Two highly homologous isoforms of Rap1, Rap1A and Rap1B, have both distinct and isoform-specific functions[21, 23]. Importantly, both Rap1 isoforms have additive effect on endothelial function[17, 62]. Also, both isoforms are involved in cell-cell junction reassembly and formation of the vascular barrier[23]. Thus, we expect pathological defects, described here using Athero-Rap1B mice, would be further increased in double knockouts of both Rap1 isoforms. Interestingly, the atheroprotective function of Rap1 reported here, contrasts with the proinflammatory effect of integrin $\alpha_v\beta_3$[63], one of the better characterized effectors of Rap1[24, 64]. Therefore, the anti-inflammatory Rap1 effects we described here are not likely to be mediated via this integrin. Our mechanistic studies, performed in human aortic

endothelial cells depleted of both Rap1 isoforms, signify the relevance of our novel mechanism for human pathology and identify Akt as a physiologically relevant target of Rap1 under the conditions of inflammation.

Our findings implicate dysregulation of Rap1 in restriction of inflammatory processes, fundamental to the development of CVD. This is novel, *because currently little is known about dysregulation of Rap1 activity in cardiovascular disease*. Unlike its close relative Ras[65], no functional mutants of Rap1 have been identified in human disease. Similar to Ras[66], there is evidence for dysregulated Rap1 activity due to altered expression of Rap1GAP in cancer[67]. Literature search revealed only two studies listing differential expression of Rap1 regulators in CVD: Rap1GAP and RasGRP3 in CVD[68, 69], but their function in CVD are not known. Therefore, our studies provide a rationale for further studies of mechanisms of Rap1 regulation in CVD and atherosclerosis.

In sum, our study identifies a novel mechanism underlying EC homeostasis that involves restriction of proinflammatory signaling by Rap1. Together with promoting NO release the two functions of Rap1 are key novel determinants of endothelial homeostasis. Furthermore, our results implicate endothelial Rap1 signaling as a novel target for treatment of cardiovascular disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements.

We thank Mr. Jon Weiser and Ms. Alexis Visotcky for their excellent technical assistance.

Sources of Funding. This work was funded by NIH grant R01HL111582 (M.C.). M.S.T. is supported by NIH grants R01HL127649 and R0HL138907, D.X. is supported by NIH grant R01HL096647 and J.V.V is supported by NIH grants NS081936 and NS114972.

Non-standard Abbreviations and Acronyms

ApoE$^{-/-}$	Apolipoprotein E deficient
Athero-Rap1B$^{i\Delta EC}$	Atherogenic EC-specific, tamoxifen-inducible Rap1B knockout
Athero-Rap1$^{i\Delta EC}$	Atherogenic EC-specific, tamoxifen-inducible total Rap1 (Rap1A + Rap1B) knockout
CAM	Cell adhesion molecule
CVD	Cardiovascular disease
EC	Endothelial cell
HFD	High fat diet
hAECs	Human aortic endothelial cells

ICAM1	Intercellular adhesion molecule 1
KO	Knockout
L-Arg	L-arginine
NO	Nitric Oxide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ORO	Oil red O
Rap1	Ras-association proximate 1
$Rap1^{i\Delta EC}$	EC-specific, tamoxifen-inducible Rap1A+Rap1B knockout mice
$Rap1B^{i\Delta EC}$	EC-specific, tamoxifen-inducible Rap1B knockout
TNF-α	Tumor necrosis factor alpha
VCAM1	Vascular cell adhesion molecule 1
VEGFR2	Vascular Endothelial Growth Factor Receptor 2

References

1. Benjamin EJ, Virani SS, Callaway CW et al. On behalf of the American Heart Association Council on E, Prevention Statistics C, Stroke Statistics S. Heart disease and stroke statistics - 2018 update: A report from the american heart association. Circulation. 2018;137:E67–E492 [PubMed: 29386200]
2. Widlansky ME, Gokce N, Keaney JF Jr, Vita JA. The clinical implications of endothelial dysfunction. J. Am. Coll. Cardiol 2003;42:1149–1160 [PubMed: 14522472]
3. Pober JS, Sessa WC. Evolving functions of endothelial cells in inflammation. Nat. Rev. Immunol 2007;7:803–815 [PubMed: 17893694]
4. Tabas I, García-Cardeña G, Owens GK. Recent insights into the cellular biology of atherosclerosis. Journal of Cell Biology. 2015;209:13–22
5. Gimbrone MA, García-Cardeña G. Endothelial cell dysfunction and the pathobiology of atherosclerosis. Circulation Research. 2016;118:620–636 [PubMed: 26892962]
6. Berk BC, Min W, Yan C, Surapisitchat J, Liu Y, Hoefen R. Atheroprotective mechanisms activated by fluid shear stress in endothelial cells. Drug News and Perspectives. 2002;15:133–139 [PubMed: 12677255]
7. Chiu JJ, Chien S. Effects of disturbed flow on vascular endothelium: Pathophysiological basis and clinical perspectives. Physiological Reviews. 2011;91:327–387 [PubMed: 21248169]
8. Heo KS, Fujiwara K, Abe JI. Shear stress and atherosclerosis. Molecules and Cells. 2014;37:435–440 [PubMed: 24781409]
9. Giddens DP, Zarins CK, Glagov S. The role of fluid mechanics in the localization and detection of atherosclerosis. Journal of Biomechanical Engineering. 1993;115:588–594 [PubMed: 8302046]
10. Topper JN, Cai J, Falb D, Gimbrone MA Jr. Identification of vascular endothelial genes differentially responsive to fluid mechanical stimuli: Cyclooxygenase-2, manganese superoxide dismutase, and endothelial cell nitric oxide synthase are selectively up-regulated by steady laminar shear stress. Proceedings of the National Academy of Sciences of the United States of America. 1996;93:10417–10422 [PubMed: 8816815]

11. Gimbrone MA Jr, Topper JN, Nagel T, Anderson KR, Garcia-Cardeña G. Endothelial dysfunction, hemodynamic forces, and atherogenesis. Annals of the New York Academy of Sciences. 2000;902:230–240 [PubMed: 10865843]
12. Cheng C, Tempel D, Van Haperen R, Van Der Baan A, Grosveld F, Daemen MJAP, Krams R, De Crom R. Atherosclerotic lesion size and vulnerability are determined by patterns of fluid shear stress. Circulation. 2006;113:2744–2753 [PubMed: 16754802]
13. Hahn C, Schwartz MA. Mechanotransduction in vascular physiology and atherogenesis. Nature Reviews Molecular Cell Biology. 2009;10:53–62 [PubMed: 19197332]
14. Tzima E, Irani-Tehrani M, Kiosses WB, Dejana E, Schultz DA, Engelhardt B, Cao G, DeLisser H, Schwartz MA. A mechanosensory complex that mediates the endothelial cell response to fluid shear stress. Nature. 2005;437:426–431 [PubMed: 16163360]
15. Conway D, Schwartz MA. Lessons from the endothelial junctional mechanosensory complex. F1000 Biology Reports. 2012;4
16. Jin ZG. Ligand independent activation of vegf receptor 2 by fluid shear stress regulates activation of endothelial nitric oxide synthesis. Circ. Res 2003;93:354–363 [PubMed: 12893742]
17. Lakshmikanthan S, Zheng X, Nishijima Y, Sobczak M, Szabo A, Vasquez-Vivar J, Zhang DX, Chrzanowska-Wodnicka M. Rap1 promotes endothelial mechanosensing complex formation, NO release and normal endothelial function. EMBO Reports. 2015;16:628–637 [PubMed: 25807985]
18. Boettner B, Van Aelst L. Control of cell adhesion dynamics by Rap1 signaling. Current Opinion in Cell Biology. 2009;21:684–693 [PubMed: 19615876]
19. Pannekoek WJ, Post A, Bos JL. Rap1 signaling in endothelial barrier control. Cell Adhesion and Migration. 2014;8:100–107 [PubMed: 24714377]
20. Chrzanowska-Wodnicka M, White GC, Quilliam LA, Whitehead KJ. Small GTPase Rap1 is essential for mouse development and formation of functional vasculature. PLoS ONE. 2015;10
21. Chrzanowska-Wodnicka M Rap1 in endothelial biology. Current Opinion in Hematology. 2017;24:248–255 [PubMed: 28178039]
22. Birukova AA, Meng F, Tian Y, Meliton A, Sarich N, Quilliam LA, Birukov KG. Prostacyclin post-treatment improves LPS-induced acute lung injury and endothelial barrier recovery via Rap1. Biochimica et Biophysica Acta - Molecular Basis of Disease. 2015;1852:778–791
23. Lakshmikanthan S, Sobczak M, Li Calzi S, Shaw L, Grant MB, Chrzanowska-Wodnicka M. Rap1b promotes vegf-induced endothelial permeability and is required for dynamic regulation of the endothelial barrier. Journal of Cell Science. 2018;131
24. Lakshmikanthan S, Sobczak M, Chun C, Henschel A, Dargatz J, Ramchandran R, Chrzanowska-Wodnicka M. Rap1 promotes VEGFR2 activation and angiogenesis by a mechanism involving integrin alphavbeta(3). Blood. 2011;118:2015–2026 [PubMed: 21636859]
25. Daugherty A, Tall Alan R, Daemen Mat JAP, Falk E, Fisher Edward A, García-Cardeña G, Lusis Aldons J, Owens AP, Rosenfeld Michael E, Virmani R. Recommendation on design, execution, and reporting of animal atherosclerosis studies: A scientific statement from the american heart association. Arteriosclerosis, Thrombosis, and Vascular Biology. 2017;37:e131–e157
26. Robinet P, Milewicz DM, Cassis LA, Leeper NJ, Lu HS, Smith JD. Consideration of sex differences in design and reporting of experimental arterial pathology studies-statement from ATVB council. Arteriosclerosis, Thrombosis, and Vascular Biology. 2018;38:292–303
27. Caligiuri G, Nicoletti A, Zhou X, Törnberg I, Hansson GK. Effects of sex and age on atherosclerosis and autoimmunity in apoe-deficient mice. Atherosclerosis. 1999;145:301–308 [PubMed: 10488957]
28. Maeda N, Johnson L, Kim S, Hagaman J, Friedman M, Reddick R. Anatomical differences and atherosclerosis in apolipoprotein E-deficient mice with 129/SVEV and C57Bl/6 genetic backgrounds. Atherosclerosis. 2007;195:75–82 [PubMed: 17275002]
29. Mohanta S, Yin C, Weber C, Hu D, Habenicht AJR. Aorta atherosclerosis lesion analysis in hyperlipidemic mice. Bio-protocol. 2016;6:e1833 [PubMed: 27366759]
30. Cornelius J, Tran T, Turner N, Piazza A, Mills L, Slack R, Hauser S, Alexander JS, Grisham MB, Feelisch M, Rodriguez J. Isotope tracing enhancement of chemiluminescence assays for nitric oxide research. Biological Chemistry. 2009;390:181–189 [PubMed: 19040352]

31. Liu CW, Sung HC, Lin SR, Wu CW, Lee CW, Lee IT, Yang YF, Yu IS, Lin SW, Chiang MH, Liang CJ, Chen YL. Resveratrol attenuates ICAM-1 expression and monocyte adhesiveness to TNF-α-treated endothelial cells: Evidence for an anti-inflammatory cascade mediated by the mir-221/222/AMPK/p38/NF-κB pathway. Scientific Reports. 2017;7
32. Chrzanowska-Wodnicka M, Smyth SS, Schoenwaelder SM, Fischer TH, White GC. Rap1b is required for normal platelet function and hemostasis in mice. Journal of Clinical Investigation. 2005;115:2296–2296
33. Menon P, Fisher EA. Immunostaining of macrophages, endothelial cells, and smooth muscle cells in the atherosclerotic mouse aorta. Methods in Molecular Biology. 2015;1339:131–148 [PubMed: 26445786]
34. Michalska M, MacHtoub L, Manthey HD, Bauer E, Herold V, Krohne G, Lykowsky G, Hildenbrand M, Kampf T, Jakob P, Zernecke A, Bauer WR. Visualization of vascular inflammation in the atherosclerotic mouse by ultrasmall superparamagnetic iron oxide vascular cell adhesion molecule-1-specific nanoparticles. Arteriosclerosis, Thrombosis, and Vascular Biology. 2012;32:2350–2357
35. Ko KI, Syverson AL, Kralik RM, Choi J, DerGarabedian BP, Chen C, Graves DT. Diabetes-induced NF-κB dysregulation in skeletal stem cells prevents resolution of inflammation. Diabetes. 2019;68:2095 [PubMed: 31439641]
36. Getz GS, Reardon CA. Thematic review series: Living history of lipids: ApoE knockout and knockin mice: The history of their contribution to the understanding of atherogenesis. Journal of Lipid Research. 2016;57:758–766 [PubMed: 27015743]
37. Meir KS, Leitersdorf E. Atherosclerosis in the apolipoprotein e-deficient mouse: A decade of progress. Arteriosclerosis, Thrombosis, and Vascular Biology. 2004;24:1006–1014
38. Assemat P, Siu KK, Armitage JA, Hokke SN, Dart A, Chin-Dusting J, Hourigan K. Haemodynamical stress in mouse aortic arch with atherosclerotic plaques: Preliminary study of plaque progression. Computational and Structural Biotechnology Journal. 2014;10:98–106 [PubMed: 25349678]
39. Brett J, Gerlach H, Nawroth P, Steinberg S, Godman G, Stern D. Tumor necrosis factor/cachectin increases permeability of endothelial cell monolayers by a mechanism involving regulatory G proteins. The Journal of Experimental Medicine. 1989;169:1977 [PubMed: 2499653]
40. Urschel K, Cicha I. TNF-α in the cardiovascular system: From physiology to therapy. International Journal of Interferon, Cytokine and Mediator Research. 2015;7:9–25
41. Urschel K, Wörner A, Daniel WG, Garlichs CD, Cicha I. Role of shear stress patterns in the TNF-α-induced atherogenic protein expression and monocytic cell adhesion to endothelium. Clinical Hemorheology and Microcirculation. 2010;46:203–210 [PubMed: 21135495]
42. Partridge J, Carlsen H, Enesa K, Chaudhury H, Zakkar M, Luong L, Kinderlerer A, Johns M, Blomhoff R, Mason JC, Haskard DO, Evans PC. Laminar shear stress acts as a switch to regulate divergent functions of NF-κB in endothelial cells. The FASEB Journal. 2007;21:3553–3561 [PubMed: 17557931]
43. Zhou Z, Connell MC, MacEwan DJ. TNFR1-induced NF-κB, but not ERK, p38 MAPK or JNK activation, mediates TNF-induced ICAM-1 and VCAM-1 expression on endothelial cells. Cellular Signalling. 2007;19:1238–1248 [PubMed: 17292586]
44. Rajan S, Ye J, Bai S, Huang F, Guo YL. NF-κB, but not p38 MAP kinase, is required for TNF-α-induced expression of cell adhesion molecules in endothelial cells. Journal of Cellular Biochemistry. 2008;105:477–486 [PubMed: 18613029]
45. Brigelius-Flohé R, Flohé L. Basic principles and emerging concepts in the redox control of transcription factors. Antioxidants and Redox Signaling. 2011;15:2335–2381 [PubMed: 21194351]
46. So T, Croft M. Regulation of PI-3-kinase and akt signaling in T lymphocytes and other cells by TNFR family molecules. Frontiers in Immunology. 2013;4
47. Bai D, Ueno L, Vogt PK. Akt-mediated regulation of NF-κB and the essentialness of NF-κB for the oncogenicity of PI3K and Akt. International Journal of Cancer. 2009;125:2863–2870 [PubMed: 19609947]

48. Balwani S, Chaudhuri R, Nandi D, Jaisankar P, Agrawal A, Ghosh B. Regulation of NF-κB activation through a novel PI-3K-independent and PKA/Akt-dependent pathway in human umbilical vein endothelial cells. PLOS ONE. 2012;7:e46528 [PubMed: 23071583]
49. Herrmann C Ras-effector interactions: After one decade. Current Opinion in Structural Biology. 2003;13:122–129 [PubMed: 12581669]
50. Pacold ME, Suire S, Perisic O, Lara-Gonzalez S, Davis CT, Walker EH, Hawkins PT, Stephens L, Eccleston JF, Williams RL. Crystal structure and functional analysis of ras binding to its effector phosphoinositide 3-kinase γ. Cell. 2000;103:931–944 [PubMed: 11136978]
51. Suire S, Hawkins P, Stephens L. Activation of phosphoinositide 3-kinase γ by Ras. Current Biology. 2002;12:1068–1075 [PubMed: 12121613]
52. Lindsley CW, Zhao Z, Leister WH, Robinson RG, Barnett SF, Defeo-Jones D, Jones RE, Hartman GD, Huff JR, Huber HE, Duggan ME. Allosteric Akt (PKB) inhibitors: Discovery and sar of isozyme selective inhibitors. Bioorganic and Medicinal Chemistry Letters. 2005;15:761–764 [PubMed: 15664853]
53. Kuhlencordt PJ, Gyurko R, Han F, Scherrer-Crosbie M, Aretz TH, Hajjar R, Picard MH, Huang PL. Accelerated atherosclerosis, aortic aneurysm formation, and ischemic heart disease in apolipoprotein E/endothelial nitric oxide synthase double-knockout mice. Circulation. 2001;104:448–454 [PubMed: 11468208]
54. Knowles JW, Reddick RL, Jennette JC, Shesely EG, Smithies O, Maeda N. Enhanced atherosclerosis and kidney dysfunction in $eNos^{-/-}ApoE^{-/-}$ mice are ameliorated by enalapril treatment. The Journal of Clinical Investigation. 2000;105:451–458 [PubMed: 10683374]
55. van Hooren KWEM, van Breevoort D, Fernandez-Borja M, Meijer AB, Eikenboom J, Bierings R, Voorberg J. Phosphatidylinositol-3,4,5-triphosphate-dependent Rac Exchange Factor 1 regulates epinephrine-induced exocytosis of Weibel-Palade bodies. Journal of Thrombosis and Haemostasis. 2014;12:273–281 [PubMed: 24283667]
56. Tawa H, Rikitake Y, Takahashi M, Amano H, Miyata M, Satomi-Kobayashi S, Kinugasa M, Nagamatsu Y, Majima T, Ogita H, Miyoshi J, Hirata K-i, Takai Y. Role of afadin in Vascular Endothelial Growth Factor- and sphingosine 1-phosphate-induced angiogenesis. Circ Res. 2010;106:1731–1742 [PubMed: 20413783]
57. Carmona G, Gottig S, Orlandi A, Scheele J, Bauerle T, Jugold M, Kiessling F, Henschler R, Zeiher AM, Dimmeler S, Chavakis E. Role of the small GTPase Rap1 for integrin activity regulation in endothelial cells and angiogenesis. Blood. 2009;113:488–497 [PubMed: 18805968]
58. Pober JS. Activation and injury of endothelial cells by cytokines. Pathologie Biologie. 1998;46:159–163 [PubMed: 9769910]
59. Griendling KK, Sorescu D, Ushio-Fukai M. NAD(P)H oxidase - role in cardiovascular biology and disease. Circulation Research. 2000;86:494–501 [PubMed: 10720409]
60. Gertzberg N, Neumann P, Rizzo V, Johnson A. NAD(P)H oxidase mediates the endothelial barrier dysfunction induced by tnf-α. American Journal of Physiology - Lung Cellular and Molecular Physiology. 2004;286:L37–L48 [PubMed: 12807699]
61. Wang H, Jiang Y, Shi D, Quilliam LA, Chrzanowska-Wodnicka M, Wittchen ES, Li DY, Hartnett ME. Activation of Rap1 inhibits NAD(P)H oxidase-dependent ROS generation in retinal pigment epithelium and reduces choroidal neovascularization. FASEB Journal. 2014;28:265–274 [PubMed: 24043260]
62. Lakshmikanthan S, Zieba BJ, Ge ZD, Momotani K, Zheng X, Lund H, Artamonov MV, Maas JE, Szabo A, Zhang DX, Auchampach JA, Mattson DL, Somlyo AV, Chrzanowska-Wodnicka M. Rap1b in smooth muscle and endothelium is required for maintenance of vascular tone and normal blood pressure. Arteriosclerosis, Thrombosis, and Vascular Biology. 2014;34:1486–1494
63. Chen J, Green J, Yurdagul A Jr., Albert P, McInnis MC, Orr AW. αvβ3 integrins mediate flow-induced NF-κB activation, proinflammatory gene expression, and early atherogenic inflammation. American Journal of Pathology. 2015;185:2575–2589
64. Lagarrigue F, Kim C, Ginsberg MH. The Rap1-Riam-talin axis of integrin activation and blood cell function. Blood. 2016;128:479–487 [PubMed: 27207789]
65. Cox AD, Der CJ. Ras history. Small GTPases. 2010;1:2–27 [PubMed: 21686117]

66. Maertens O, Cichowski K. An expanding role for Ras GTPase activating proteins (Ras GAPs) in cancer. Advances in Biological Regulation. 2014;55:1–14 [PubMed: 24814062]

67. Zhang L, Cui M, Song L, Zhang M, Zhangd J. Function, significance, and regulation of rap1b in malignancy. Critical Reviews in Eukaryotic Gene Expression. 2019;29:151–160 [PubMed: 31679270]

68. Yang H-C, Liang Y-J, Wu Y-L et al. Genome-wide association study of young-onset hypertension in the Han chinese population of Taiwan. PloS one. 2009;4:e5459–e5459 [PubMed: 19421330]

69. Rajkumar R, Konishi K, Richards TJ, Ishizawar DC, Wiechert AC, Kaminski N, Ahmad F. Genomewide RNA expression profiling in lung identifies distinct signatures in idiopathic pulmonary arterial hypertension and secondary pulmonary hypertension. Am J Physiol Heart Circ Physiol. 2010;298:H1235–1248 [PubMed: 20081107]

Highlights

- Deletion of Rap1 in endothelium exacerbates the progression of atherosclerosis.
- In laminar flow areas, increased plaque deposition in Athero-Rap1B$^{i\Delta EC}$ mice is attenuated by enhanced NO bioavailability.
- In the absence of laminar flow, in athero-prone, disturbed aortic flow areas, endothelial deletion of Rap1 increases macrophage infiltration.
- In the absence of laminar flow, Rap1 restricts Akt-mediated phosphorylation and the activation of NF-κB proinflammatory signaling, damping proinflammatory receptor expression and leukocyte infiltration.

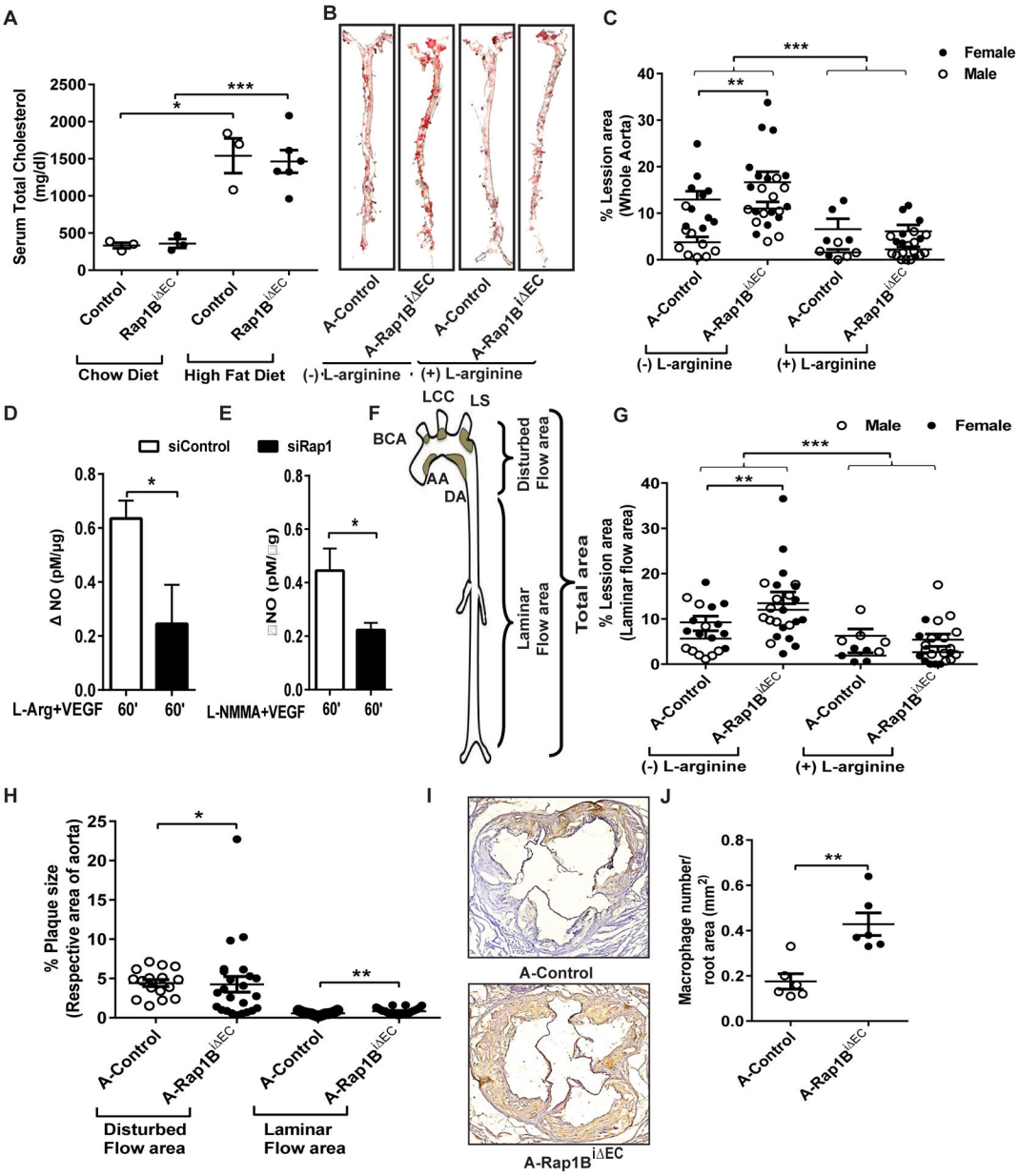


Figure 1. Endothelial Rap1B-deficiency exaggerates atherosclerotic plaque deposition in the presence and absence of laminar flow.

A. Induction of atherosclerosis in Rap1B$^{i\Delta EC}$ mice: Quantification of serum cholesterol levels in Rap1B$^{i\Delta EC}$ and control (Cdh5-Cre-negative, or injected with carrier oil only) mice on ApoE$^{-/-}$ background measured after 16 weeks of HFD (Athero-mice), compared to serum cholesterol levels in mice fed normal laboratory diet. Data are means ± s.e.m. (n=5).

B, C. Increased atherosclerotic lesion area in Athero-Rap1B$^{i\Delta EC}$ mice. B. Representative images of Oil O red (ORO)-stained *en face* preparations of aortae from Athero-control and Athero-Rap1B$^{i\Delta EC}$ mice without or with L-Arg supplementation in the diet. **C.** Quantification of atherosclerotic plaque area expressed as percentage of ORO-positive areas vs. total aorta area in Athero Rap1B$^{i\Delta EC}$ compared to Athero-control male and female mice. Individual values and mean ±s.e.m. for each sex are shown. **D. E.** VEGF-induced NO

release from Rap1B-deficient or control hAECs in the presence L-Arg (D) or L-NMMA. Mean values of NO concentration after 60 min of VEGF stimulation calculated using authentic standards were normalized to total protein in each sample. n=4. **F. Quantification scheme of atherosclerotic lesions in predominantly laminar-and disturbed-flow areas of mouse aortae:** ascending aorta (AA), descending aorta (DA), branchiocephalic artery (BCA), left common carotid artery (LCC) and left subclavian artery (LS). **G. Quantification of atherosclerotic plaque area within laminar flow area in male and female Athero-Rap1B$^{i\Delta EC}$ mice, compared to Athero-control mice, without or with L-Arg supplementation.** Individual values, and mean and s.e.m. for each sex are shown. **H. Average plaque size** in each flow region vs. total area of vessel region. Values are mean ± s.e.m. (n=9–14; p values were calculated by using Welch-corrected one-sided t-test). **I, J. Increased macrophage infiltration into aortic root in Athero-Rap1B$^{i\Delta EC}$ mice. I.** Representative expression of F4/80 marker detected by immunohistochemistry in the aortic root of Athero-control and Athero-Rap1B$^{i\Delta EC}$ mice; scale bar=100 um. **J.** Quantification of F4/80-positive macrophages in aortic roots of Athero-control and Athero Rap1B$^{i\Delta EC}$ mice (n≥5), expressed relative to aortic root area. Values are means ± s.e.m. p values - as in Supplemental Table S1; * indicates $p<0.05$; ** $p<0.01$; *** $p<0.001$.

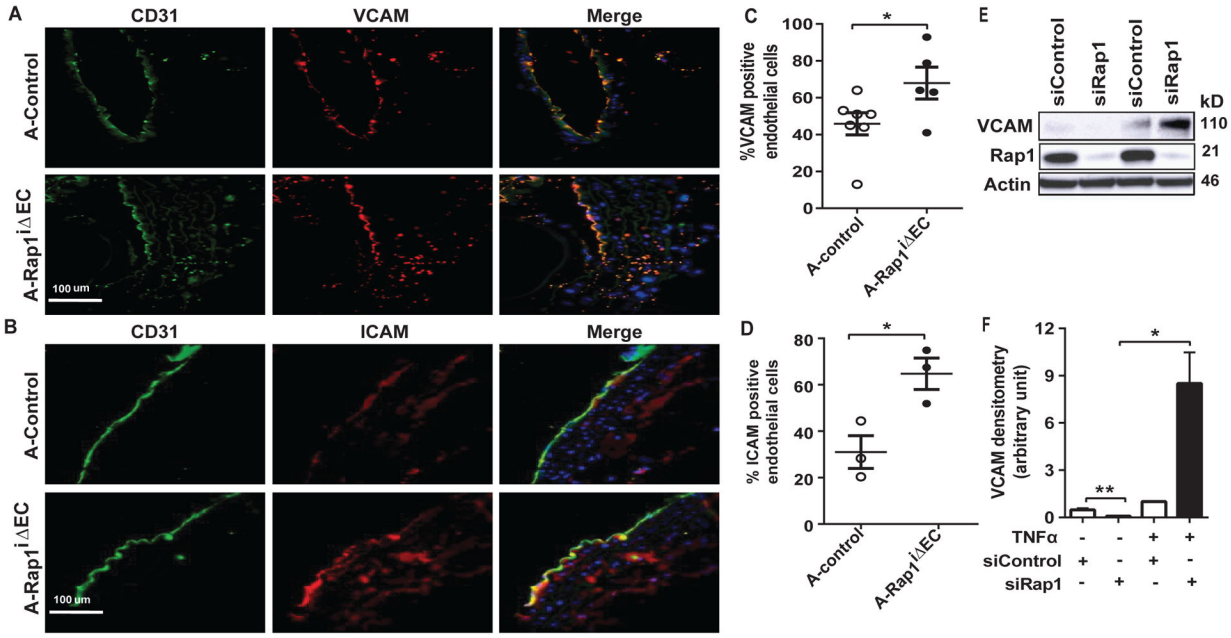


Figure 2. In the absence of laminar flow, EC Rap1-deletion elevates inflammatory receptors (CAM) expression.

A-D. Upregulation of inflammatory leukocyte cell adhesion receptors in Athero Rap1$^{i\Delta EC}$ mice *in vivo*. Representative immunohistological analysis of VCAM1 (**A**) and ICAM1 (**B**) and CD31 expression in serial sections of aortic arches from Athero-Rap1$^{i\Delta EC}$ or Athero-control mice. **C, D.** Quantification of CAM-positive ECs, obtained from ≥100 ECs per mouse. Data are represented as mean ± s.e.m. (n=3–6). **E, F. Upregulation of VCAM1 expression in Rap1-deficient hAECs in response to TNF-α *in vitro*. H.** Representative Western blot of VCAM1 expression in siRap1 or siControl RNA-transfected hAECs following TNF-α treatment for 48 h. **I.** Quantification of fold change in TNF-α-induced VCAM1 expression, normalized to actin, in siRap1 and siControl hAECs. Data are mean ± s.e.m. (n=3) (* indicates p≤ 0.05).

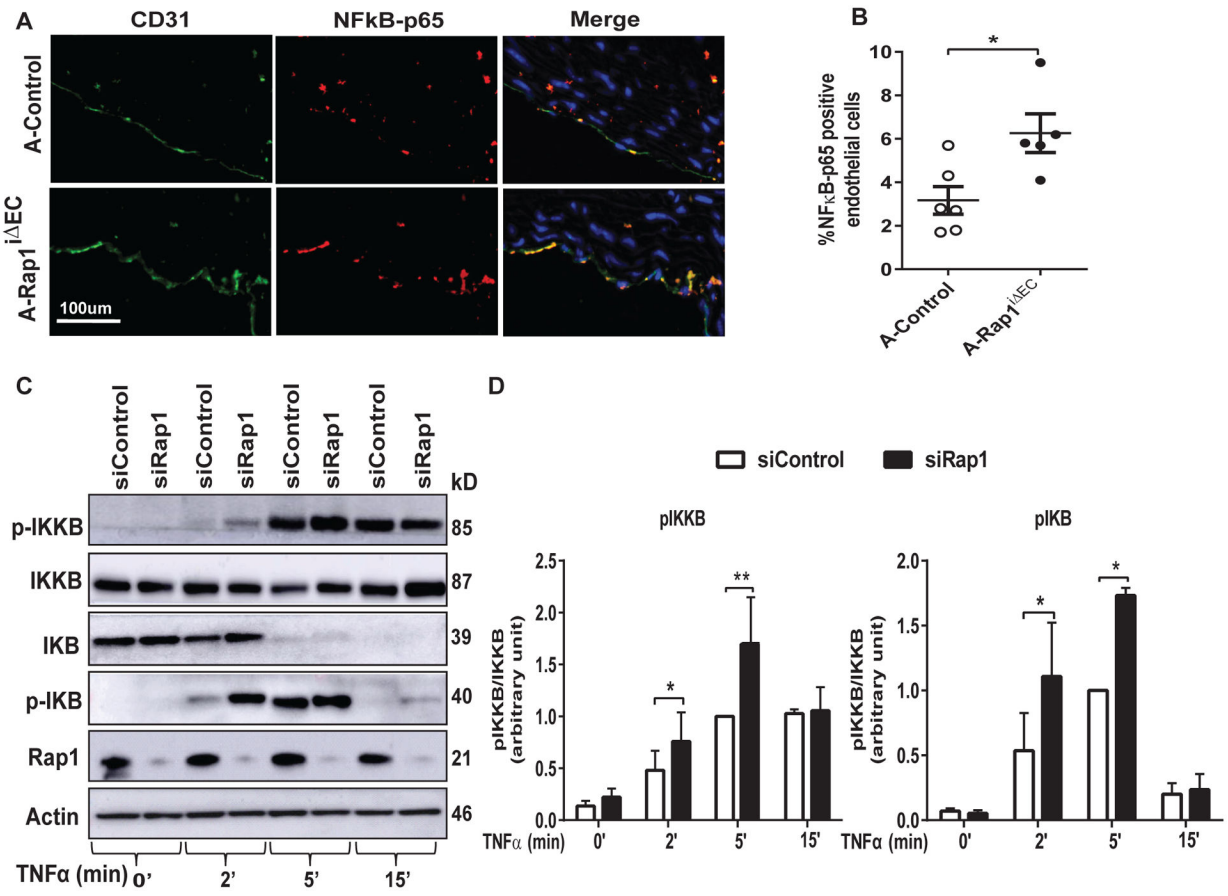


Figure 3. In the absence of laminar flow, Rap1 deficiency leads to Increased NF-κB activation.

A, B. Increased nuclear NF-κB p65 localization in aortic arch ECs in Athero-Rap1$^{i\Delta EC}$ vs. Athero-control mice. **A**. Representative immunohistological analysis of NF-κB p65, DAPI and CD31 expression. **B**. Quantification of NF-κB p65-positive EC nuclei (stained blue in panel A), represented as a fraction of all scored ECs. Mean ± s.e.m. from 148 ±20 cells per mouse are shown. **C. Increased TNF-α-induced IKK activation, IκB phosphorylation and IκB degradation in Rap1-depleted hAECs. C.** Representative Western blots of IKK Ser 176/180 phosphorylation, IκB Ser 32/36 phosphorylation from cells lysates prepared from siControl or siRap1 (siRap1A + siRap1B)-transfected hAECs, treated with TNF-α (50ng/ml), as indicated. **D.** Quantification of fold change in TNF-α-induced phosphorylation of IKK and IκB, and of total IκB and phospho- IκB vs. actin, as a measure of IκB degradation, in siRap1 vs siControl-transfected hAECs. The values are ratios of phosphorylated- to total IKK and IκB, or total or phospho- IκB to actin densitometry values of specific bands, normalized to ““0 min”“ siControl values. Rap1 expression is shown to demonstrate siRap1 knockdown efficiency. (n=4).

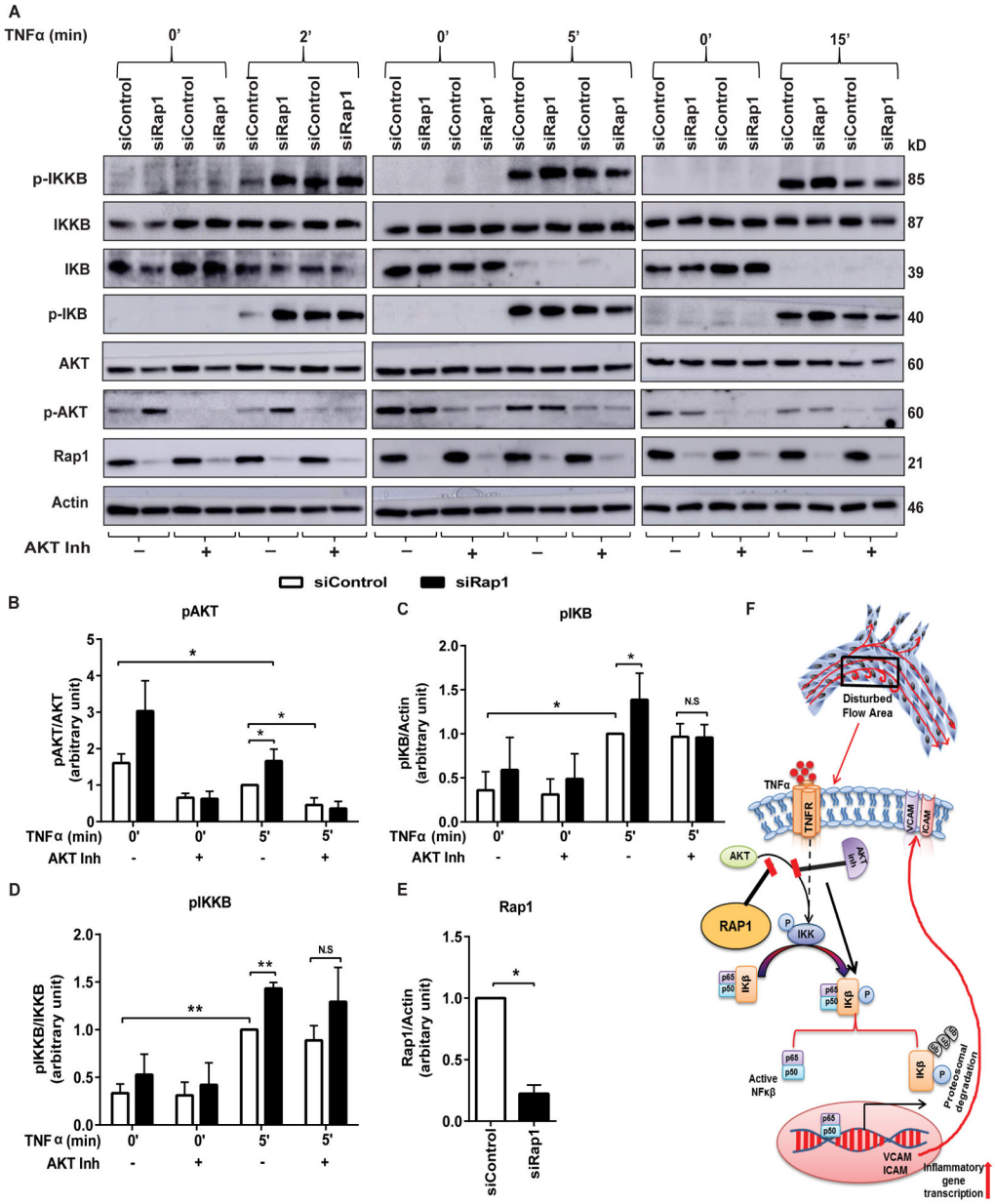


Figure 4. In the absence of laminar flow, Rap1 restricts inflammatory signaling to NF-κB via inhibition of Akt.

A-D. Akt inhibitor decreases IκB phosphorylation and degradation in Rap1-depleted hAECs. **A.** Representative Western blots of IKK Ser 176/180 phosphorylation and IκB Ser 32/36 phosphorylation from siControl or siRap1 transfected hAECs pretreated with DMSO or an AKT inhibitor AKTVIII (1uM; ""AKT Inh"") for 30 min prior to TNF-α (50ng/ml) treatment for indicated times. **B-D.** Quantification of fold TNF-α-induced phosphorylation, normalized to total protein, vs. non-treated control at the 5 min timepoint. **E.** Rap1 expression is shown to demonstrate knockdown efficiency. (n=4). **F. Proposed model of the protective effect of Rap1 in the athero-prone vessel area in the absence of laminar flow.** In the absence of laminar flow, Rap1 restricts Akt-mediated phosphorylation and activation in response to inflammatory cytokines such as TNF-α, resulting in-induced NF-κB

activation through inhibition of IKK and IκB phosphorylation. In the absence of Rap1, enhanced IκB phosphorylation and degradation leads to increased NF-κB activation and increased expression of leukocyte inflammatory adhesion receptors on activated ECs, promoting leukocyte infiltration and atherosclerosis progression.

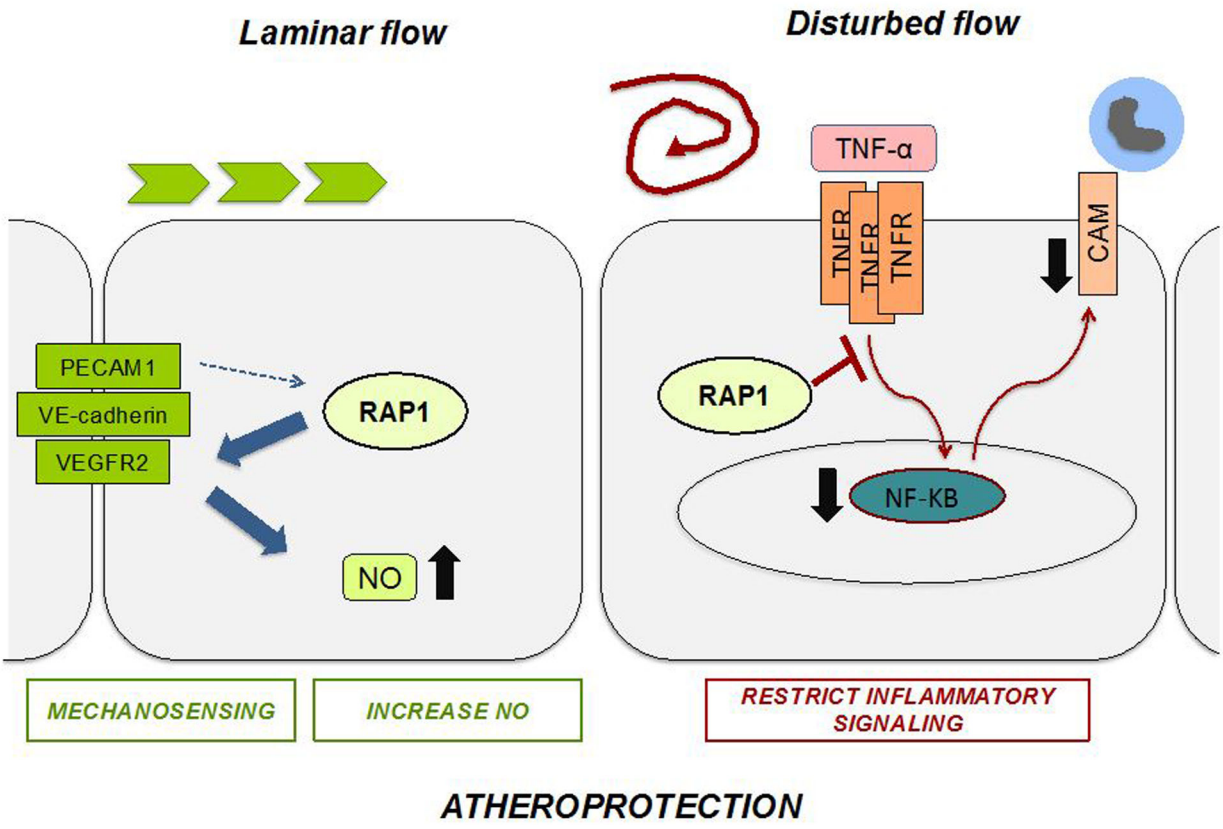


Figure 5. Via discrete mechanisms, endothelial Rap1 protects from atherosclerosis in the presence and absence of laminar flow.

Left: In response to laminar flow, Rap1 promotes NO release via its mechanosensing function. *Right*: In the absence of laminar flow, or in the presence of disturbed flow, Rap1 restricts proinflammatory cytokine signaling, NF-κB activation and proinflammatory gene expression in endothelium.