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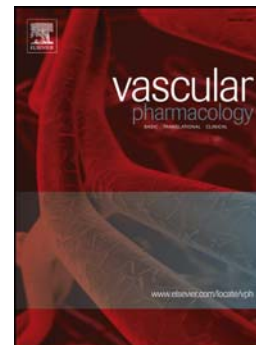
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Cytoprotective pathways in the vascular endothelium. Do they represent a viable therapeutic target?

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Abstract

The vascular endothelium is a critical interface, which separates the organs from the blood and its contents. The endothelium has a wide variety of functions and maintenance of endothelial homeostasis is a multi-dimensional active process, disruption of which has potentially deleterious consequences if not reversed. Vascular injury predisposes to endothelial apoptosis, dysfunction and development of atherosclerosis. Endothelial dysfunction is an end-point, a central feature of which is increased ROS generation, a reduction in endothelial nitric oxide synthase and increased nitric oxide consumption. A dysfunctional endothelium is a common feature of diseases including rheumatoid arthritis, systemic lupus erythematosus, diabetes mellitus and chronic renal impairment. The endothelium is endowed with a variety of constitutive and inducible mechanisms that act to minimise injury and facilitate repair. Endothelial cytoprotection can be enhanced by exogenous factors such as vascular endothelial growth factor, prostacyclin and laminar shear stress. Target genes include endothelial nitric oxide synthase, heme oxygenase-1, A20 and anti-apoptotic members of the B cell lymphoma protein-2 family. In light of the importance of endothelial function, and the link between its disruption and the risk of atherothrombosis, interest has focused on therapeutic conditioning and reversal of endothelial dysfunction. A detailed understanding of cytoprotective signalling pathways, their regulation and target genes is now required to identify novel therapeutic targets. The ultimate aim is to add vasculoprotection to current therapeutic strategies for systemic inflammatory diseases, in an attempt to reduce vascular injury and prevent or retard atherogenesis.

Keywords

Endothelium; vascular injury; shear stress; cytoprotection; endothelial dysfunction.

Abbreviations

AMPK: AMP-activated protein kinase

bFGF: basic fibroblast growth factor

Bcl-2: B cell lymphoma protein

CRE: cAMP response element

CO: carbon monoxide

CV: cardiovascular

DAF: decay-accelerating factor

Dlk1: Notch inhibitor delta-like 1 homolog

EC: endothelial cells

eNOS: endothelial nitric oxide synthase

GR: glutathione reductase

HDAC: histone deacetylase

HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A

HO-1: heme oxygenase-1

ICAM-1: intercellular adhesion molecule-1

IFN γ : Interferon- γ

IL: interleukin

Keap1: kelch-like ECH-associated protein

KLF: Krüppel-like factor

LDL: low-density lipoprotein

LPS: Lipopolysaccharide

LSS: Laminar shear stress

MAPK: mitogen-activated protein kinase

MMPs: matrix metalloproteinases

MCP-1: monocyte chemotactic protein-1

miRs: Micro-RNAs

MnSOD: Manganese superoxide dismutase

mTOR: Mammalian target of rapamycin

NO: nitric oxide

NQO1: NAD(P)H:quinine oxidoreductase 1

NF- κ B: nuclear factor- κ B

Nrf2: NF-E2-related factor-2

OSS: Oscillatory shear stress

PDGF: platelet-derived growth factor

PI-3K; phosphoinositide-3-kinase

PKC: protein kinase C

PLGF: placental growth factor

RA: rheumatoid arthritis

ROS: reactive oxygen species

SLE: systemic lupus erythematosus

Trx-1: thioredoxin reductase-1

TNF- α : tumour necrosis factor- α

VEGFA: vascular endothelial growth factorA

VSMC: vascular smooth muscle cells

VCAM-1: vascular cell adhesion molecule-1

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1. Introduction

The importance of endothelial homeostasis for health is often underestimated. The vascular endothelium provides an effective, regulated barrier between the circulating blood and the tissues, which sustains blood flow and an anti-coagulant, anti-adhesive surface. This constitutive role is combined with a rapid response mode. The latter requires endothelial activation. This process, which has been reviewed elsewhere in detail, comprises Type I and Type II activation (1). Type I is an acute response independent of new gene transcription, with typical mediators including histamine and thrombin. In contrast, Type II activation is more delayed, longer in duration and requires gene transcription. It is well represented by endothelial responses to tumour necrosis factor- α (TNF- α) and interleukin-1 (IL-1) and leads to the upregulation of cellular adhesion molecules E-selectin, vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1. These endothelial responses facilitate the coagulation cascade, angiogenesis, control of vascular tone and permeability and regulation of the leukocyte adhesion cascade (1, 2). Therefore it is unsurprising that factors which compromise endothelial function predispose to vascular diseases.

Endothelial dysfunction is an imprecise term and represents the common end-point of a variety of upstream insults (3). It is a recognised feature of diseases as diverse as diabetes mellitus, familial hyperlipidemia, sepsis, chronic renal impairment, rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). Dysfunction follows excessive endothelial cell injury, which predisposes to apoptosis and impaired homeostatic responses. A common feature is the excess generation of reactive oxygen species (ROS) that consume nitric oxide (NO). This in turn generates peroxynitrite and leads to the oxidation of tetrahydrobiopterin and its subsequent uncoupling from endothelial nitric oxide synthase (eNOS) (4). Additional disease-specific factors that may contribute to endothelial dysfunction include hyperglycemia, advanced glycation end-products, pathogenic antibodies, complement-mediated injury, modified-low-density lipoprotein (LDL), inflammatory cytokines such as TNF- α and activated leukocytes. The associated local inflammatory response drives a vicious cycle of events which, if uncontrolled, leads to barrier breakdown and increased endothelial permeability to monocytes and LDL, which accumulate in the sub-intimal space and initiate fatty streak formation (5, 6). Post-transplantation, immune-mediated endothelial dysfunction results in accelerated arteriosclerosis (7).

Given that endothelial dysfunction is an early adverse biomarker that can be detected by non-invasive techniques (8), therapeutic reversal represents a key clinical goal. However, effective strategies for this have not been easy to identify. This review aims to integrate

basic science advances in the understanding of molecular mechanisms regulating endothelial protection, with potential clinical therapeutic strategies aimed at optimising endothelial function and repair.

2. Inflammation and Cardiovascular disease

The integral role of chronic inflammation in atherosclerotic plaque development is well recognised (9). In addition to acting as a catalyst for intense research efforts, this pathogenic observation has encouraged different clinical disciplines to share their insights with respect to atherosclerosis. Thus cardiologists liaise more closely with rheumatologists, renal physicians, endocrinology/metabolic medicine experts and imaging specialists. The recognition that patients suffering from RA or SLE, chronic renal failure, diabetes mellitus and the metabolic syndrome are at risk from premature cardiovascular events provides an opportunity to advance understanding of the underlying molecular mechanisms.

2.1 Pathology of atherogenesis

Excellent recent reviews have described in detail the complex cellular interactions, immune and inflammatory processes that drive atherogenesis and atherothrombosis (9-11). Pro-inflammatory cytokines including TNF- α and interleukin-1 (IL-1) induce expression of endothelial adhesion molecules including VCAM-1, and act in concert with chemoattractants such as monocyte chemoattractant protein-1 (MCP-1, CCL-2) to facilitate the egress of monocytes and T cells from the bloodstream into the developing atherosclerotic plaque. Subsequent proliferation and maturation of monocytes into macrophages is followed by their uptake of LDL via cell surface scavenger receptors to create foam cells (11). Locally activated vascular smooth muscle cells (VSMC), dendritic cells, mast cells and B cells are present in the developing atherosclerotic plaque. Subsequent apoptosis of VSMC and foam cells leads to the accumulation of cell debris and cholesterol crystals to create a necrotic lipid core which is retained by an overlying fibrous cap.

The importance of cellular adaptive immunity and particularly the pivotal regulatory role of T cells in atherogenesis has emerged over the last decade. Interferon- γ (IFN γ) is a critical pro-atherogenic cytokine. In contrast, the regulatory T cell subset may work in the opposite direction (12). Similarly B cells may be important, with outcomes reflecting the balance between B1 cells, which are reported to be protective, and B2 cells which may accelerate atherogenesis (9, 13, 14).

2.2 Atherothrombosis – endothelial erosion and plaque rupture

Atherosclerotic plaques may impair arterial blood flow and induce tissue ischemia. Life-threatening events are typically the result of atherothrombosis leading to myocardial infarction or stroke, with the latter due to plaque embolisation and occlusion of a distal cerebral artery (5).

Changes in the plaque that predispose to atherothrombosis include endothelial erosion and plaque rupture. Plaques are typically covered by arterial endothelium, which is exposed to high levels of shear stress, and circulating pro-inflammatory mediators including TNF- α , IL-1 β and eicosanoids. The inflammatory milieu predispose to endothelial cell apoptosis and loss of endothelial monolayer integrity. Endothelial erosion is a recognised feature of plaques obtained from up to 40% of patients suffering coronary thrombosis (15). Erosion exposes the pro-thrombotic basement membrane. Plaques with this phenotype are typically less inflamed, incorporate proliferating VSMC and undergo significant neovascularisation of the plaque base (15). Of note, a role for innate immunity in endothelial erosion has been proposed. Plaque endothelial cells express Toll-like receptor 2, ligation of which, by hyaluronan or following bacterial infection, induces endothelial apoptosis (16).

Classical plaque rupture is driven by inflammatory processes within the plaque. TNF- α induces plaque macrophages to generate matrix metalloproteinases (MMPs) which degrade collagen and reduce plaque stability (17). Thinning of the fibrous cap is a critical event and ultimately rupture leads to the release of pro-thrombotic plaque contents and atherothrombosis (10).

2.3 Atherosclerosis and systemic inflammatory diseases

The identification of a role for inflammation and immune mechanisms in atherogenesis and post-transplant arteriosclerosis initiated intense investigation of the factors responsible for the increased risk of CV events in rheumatic diseases. This approach has further defined the roles of inflammatory pathways, MMPs and adaptive immunity in typical atherosclerosis (18). Taking RA and SLE as examples, both diseases represent independent risk factors for the development of atherosclerosis (19, 20). However, while these diseases share certain features, important differences exist between them, including the predominant pathogenic cytokines involved (18).

CV disease in the rheumatologic conditions is not confined to atherosclerosis. Conduction disturbances, myocarditis and other non-ischaemic causes of heart failure are also important (21, 22). However, the question of why these patients are at increased risk of atherothrombosis remains. Contributory factors likely include widespread endothelial injury

secondary to systemic inflammation. This predisposes to inflammation-accelerated plaque propagation and ultimately plaque instability. Furthermore, the enhanced pro-thrombotic environment in RA and SLE represents an additional risk factor (23).

In RA, CV disease is a major contributor to premature mortality. The patients exhibit endothelial dysfunction and reduced circulating progenitor cells (24, 25). In both RA and SLE, microvascular dysfunction is present, with evidence for attenuated myocardial blood flow and reduced coronary flow reserve following adenosine challenge (26). If these changes represent an early event prior to the development of epicardial coronary disease, then effective therapy to reverse endothelial dysfunction might reduce the risk of subsequent CV events. Although this remains to be confirmed, a recent study in a rodent model of inflammatory arthritis demonstrated that microvascular changes do precede macrovascular abnormalities and aortic stiffness (27). Moreover, meta-analysis data are encouraging and suggest that the risk of CV events can be therapeutically reduced in RA (18).

Somewhat counter intuitively, studies of the extent of atheroma in RA and SLE have revealed similar or even less extensive disease than in matched controls (reviewed in (28, 29)). Therefore focus has turned to the plaques themselves and emerging evidence suggests that atherosclerotic plaques in RA are less stable and hence more vulnerable to rupture (30, 31). Mechanistically, intriguing data from murine models of atheroma has reported an 'echo effect' following ischaemic injury. Following injury, atherogenesis in plaques at distant sites in the vasculature is accelerated and the atheroprone animals develop larger lesions (32). Extrapolating these data to RA and SLE leads to the speculation that primary disease flare, or additional insults including intra-arterial interventions or concurrent infection in the pre-existing inflammatory environment may drive atherogenesis and predispose to plaque instability.

Despite the recorded increase in CV risk, the absolute number of events in RA and SLE remains low. This presents challenges to clinicians trying to identify those patients at highest risk, and to academics looking to identify underlying molecular mechanisms. Indeed, completing an interventional controlled clinical trial of the size and duration required represents a major undertaking (18, 33).

3. Protection of the vascular endothelium

Maintenance of the vascular endothelium is an active process involving a variety of mechanisms, mediators and target genes. Endothelial cytoprotection has its origins in accommodation, the process through which ABO incompatible allografts can remain viable

in the face of graft-specific alloantibodies and complement fixation. Various protective genes were identified as contributors to graft protection including heme oxygenase-1 (HO-1), B cell lymphoma protein Bcl-2, Bcl-X_L, A1 and A20 (34-36). The regulation of these within the vascular endothelium has now been subsequently studied in detail (Figure 1).

3.1 *Vascular endothelial growth factor*

The vascular endothelial growth factor (VEGF) family, consisting of VEGFA, B, C, D and placental growth factor (PLGF), is essential for vasculogenesis and angiogenesis, as evidenced by embryonic lethality following deletion of a single VEGFA allele (37). Aside from this, VEGFA plays a central role in the maintenance of endothelial homeostasis in the adult, a fact emphasised by side-effects associated with bevacizumab, an anti-VEGFA mAb, used for the treatment of metastatic colonic carcinoma. These patients are at risk of developing hypertension and thrombosis (38, 39). The importance of VEGFA was also revealed by the development of transgenic mice with an inducible podocyte-specific deletion of *vegfa*. These animals developed endothelial cell swelling, local thrombosis, proteinuria and hypertension (38). Human VEGFA has five isoforms, VEGFA121, VEGFA145, VEGFA165, VEGFA189 and VEGFA206.

Vascular endothelial growth factors signal via receptor tyrosine kinases (VEGF receptors 1-3). VEGFA, VEGFB and PLGF bind VEGFR1 and VEGFA also binds VEGFR2, the predominant receptor on EC. VEGFC and D bind VEGFR3, with important modulation by co-receptors including neuropilin and heparan sulfate proteoglycans and a critical role in the regulation of lymphatic endothelial function (40, 41). VEGFA signals to EC via VEGFR2 to activate an array of downstream pathways, which include those regulated by phosphoinositide-3-kinase (PI3K)/Akt (protein kinase B), the mitogen-activated protein kinase (MAPK) enzymes ERK1/2 and protein kinase C (PKC). Intriguingly, autocrine VEGFA signaling triggered within the cell also plays a role in endothelial homeostasis without influencing angiogenesis (42). Exposure of vascular endothelial cells to VEGFA results in the induction of Bcl-2 and A1 (43-45) and HO-1 (46). In addition, increased release of prostacyclin, eNOS expression and NO biosynthesis (47) contributes to the maintenance of an anti-thrombotic endothelial surface. VEGFA also modulates protection of the endothelium against complement-mediated injury via upregulation of the complement inhibitory protein decay-accelerating factor (DAF) (48).

3.2 *Anti-inflammatory and anti-apoptotic genes*

Intrinsic mechanisms regulated by exogenous factors are critical to the maintenance of endothelial homeostasis and vascular health. NO is generated by the actions of eNOS on its

substrate L-arginine and exerts a variety of effects relevant to endothelial homeostasis. These include vasodilation, resistance to inflammation and platelet inhibition. Attenuated synthesis or reduced bioavailability of NO following eNOS uncoupling, results in endothelial dysfunction (4).

The balance between the pro- and anti-apoptotic members of the Bcl-2 family is critical for determining cell fate. Bcl-2, Bcl-X_L and A1 are localised in mitochondria and although predominantly anti-apoptotic, they also exert anti-inflammatory and cytoprotective effects. However, their protective actions may be overcome if pro-apoptotic Bim, Bid and Bad are present in sufficient amounts. They bind to Bcl-2 and Bcl-X_L and result in the release of sequestered Bax and Bak, and the escape of mitochondrial cytochrome c. The resultant apoptosome cleaves and activates apoptosis effector caspases 3, 6 and 7 (49).

A20 is a deubiquitinating enzyme that displays a classic negative feedback function. Induced as a consequence of NF- κ B activation it acts to suppress IKK activity and hence has a potent anti-inflammatory effect (50). Evidence for this is revealed by its targeted deletion, with knockout mice developing severe widespread inflammation and cachexia (51). In the endothelium A20, by limiting NF- κ B activity, reduces the expression of E-selectin, VCAM-1 and ICAM-1, and prevents prolonged leukocyte emigration.

HO-1 activity plays a critical role in endothelial homeostasis, an observation supported by the reports of two *HMOX1*-deficient patients (52, 53). Endothelial injury was an important component of the phenotype. This was associated with elevated levels of plasma von Willebrand factor, coagulation and fibrinolysis defects, generalised inflammation and premature atherosclerosis.

HO-1 is an inducible enzyme which confers anti-inflammatory, anti-oxidant and anti-apoptotic properties to the endothelium, alongside pro-angiogenic and tissue reparative actions (54). HO-1 activity degrades heme to generate carbon monoxide (CO), bilirubin and ferrous iron. The latter is sequestered by intracellular ferritin or actively exported from the cell, while biliverdin reductase generates bilirubin from biliverdin (54, 55). The cytoprotective properties of HO-1 in the vasculature are largely delivered by its enzymatic products CO, biliverdin and bilirubin and are reviewed in detail elsewhere (54, 56). CO is anti-apoptotic and anti-inflammatory (57, 58). Similarly, biliverdin and bilirubin are potently cytoprotective, anti-oxidant and anti-inflammatory (59-61). These actions contribute to the ability of HO-1 activity to modulate atherogenesis and increase atherosclerotic plaque stability (62-65). This in turn has led to significant interest in the therapeutic modulation of HO-1 (55, 66).

3.3 Complement regulation

Although the complement cascade is tightly regulated, tissues are at risk of bystander injury and uncontrolled complement activation plays an important role in a variety of diseases including RA, SLE and glomerulonephritis. The vasculature is exposed to the products of the complement tick-over pathway, and on occasion to excessive complement activation. The latter is associated with endothelial dysfunction, immune-complex-mediated vascular injury, post-transplant vasculopathy and atherosclerosis (7, 67, 68). The importance of molecules able to control complement pathway activation is revealed by mice deficient in decay-accelerating factor (DAF, CD55) and CD59. These mice are more susceptible to ischaemia-reperfusion injury (69) and to atherogenesis when crossed with atherosclerosis-prone animals (70-72). DAF, which accelerates the decay of C3 and C5 convertases, and CD59 which inhibits the formation of the C5b-9 membrane act complex, are both present on the vascular endothelial surface. Importantly their expression, and hence endothelial resistance to complement-mediated injury, can be enhanced in response to specific stimuli including cytokines and growth factors (73-77)

We have reported a link between HO-1 activity and the expression and function of DAF. Endothelial cells derived from *Hmox1*^{-/-} mice had reduced constitutive DAF expression and increased sensitivity to complement activation (78). Inhibition of HO-1 and its products attenuated DAF induction, and we propose that this HO-1-DAF relationship is important for accommodation, resistance to post-transplant arteriosclerosis and atherosclerosis.

3.4 Shear stress

A current paradigm suggests that, at least in part, the geometric nature of atherosclerotic plaque development reflects exposure to different patterns of blood flow. Importantly, following exposure to unidirectional pulsatile laminar high-flow shear stress (LSS, 10-20 dynes/cm²) selective induction of endothelial cell (EC) protective genes including eNOS is observed. This response protects against atherogenesis, while disturbed flow (DF) waveforms, with low shear reversing flow patterns, such as those located at arterial branch points, are pro-inflammatory and pro-atherosclerotic (79, 80). (Figure 1)

The atheroprotected and atheroprone phenotypes found in different regions of the vasculature reflect transcriptional, translational, and post-translational mechanisms (81), alongside local changes in mass transport (82). In response to disturbed flow patterns, the activity of protective transcription factors including NF-E2-related factor-2 (Nrf2) and Krüppel-like factors (KLF)-2 and -4 is diminished (80). Likewise, in the same regions, nuclear factor-

κ B (NF- κ B) is primed for pro-inflammatory responses (83). While eNOS is reduced, the endothelial response to disturbed flow includes increased generation of ROS (84-86) and evidence for activation of endoplasmic reticulum stress and the unfolded protein response (87).

LSS increases the expression of anti-apoptotic genes, anti-oxidant genes including HO-1 and NAD(P)H:quinine oxidoreductase 1 (NQO1) and the anti-thrombotic proteins thrombomodulin and prostacyclin (79, 80). The atheroprotective effects of LSS led us to investigate its ability to enhance resistance to complement-mediated injury. Exposure of EC to LSS for 24 - 48 hours resulted in a significant increase in CD59 expression and enhanced resistance to C9 deposition and complement-mediated lysis (88). In contrast, DF failed to activate the ERK5/KLF2 signalling pathway and was unable to reproduce this response. Further study of the murine aortic endothelium confirmed this differential expression of CD59 (88), while deficiency of CD59 in the *LdlR*^{-/-} mice exacerbated atherosclerosis (72).

3.5 Non-coding RNAs

Micro-RNAs (miRs) play an important regulatory role in endothelial homeostasis and may contribute to vascular disease pathogenesis (89, 90). These non-coding RNAs exert their effects predominantly via the inhibition of mRNA translation. The critical nature of miRs is illustrated by their importance in angiogenesis and by the effect of shear stress on the expression of specific miRs.

Individual miRs have been associated with vasculoprotection. In particular miR 126 plays a prominent role. In endothelial cells miR126-5p suppresses Notch inhibitor delta-like 1 homolog (Dlk1) to facilitate proliferation. *In vivo*, this manifests as delayed re-endothelialisation after arterial balloon injury in miR126^{-/-} mice (91). Pulsatile LSS favours induction of miRs including miR23b, miR27a, miR143 and miR145, which are considered atheroprotective (reviewed in (92)), while other miRs are increased by oscillatory shear stress (OSS) and favour atherogenesis. Thus, miR92a is specifically increased in EC located at atherosusceptible sites in the aorta and inhibits the cytoprotective transcription factors KLF2 and KLF4 (93, 94). In contrast, miR10a is reduced at similar sites and loss of its anti-inflammatory actions favours induction of E-selectin and VCAM-1 and release of pro-inflammatory cytokines (95). Recent data shows that the pro-inflammatory miR34a is downregulated by LSS, while its upregulation by OSS favours induction of VCAM-1 and ICAM-1 and subsequent adhesion of monocytes to the endothelium (96).

Microparticles and exosomes have been implicated in direct transfer of miRs to target cells including the endothelium (97, 98). The impact may be injurious or protective, suggesting that under specific circumstances microparticles and exosomes play a role in both homeostasis and disease pathogenesis (99). Intriguingly, over-expression of KLF2 increases EC shedding of extracellular vesicles containing miRs from the miR143/145 cluster. These vesicles transferred miRs to VSMC *in vitro* and were able to exert anti-atherosclerotic actions *in vivo* (100). The influence of miRs on disease phenotypes has been widely explored, with atherosclerosis and diabetes mellitus studied in detail. Loss of endothelial miR126 is a feature of diabetes and delivery of miR126 by endothelial-derived microparticles protects against the deleterious effects of hyperglycaemia in the vasculature (90, 101). In atherogenesis, an increase in miR126 is protective and activates endothelial repair, while in contrast miR145 down-regulation is pro-atherogenic (89).

A detailed understanding of individual miRs and other non-coding RNAs in specific tissues including the vascular endothelium may offer new therapeutic opportunities for a variety of disease states such as endothelial dysfunction and associated vascular pathologies.

4. Cytoprotective signalling in the vasculature

The signalling pathways culminating in the induction of protective genes in the vascular endothelium remain to be fully elucidated. The most widely studied are those involving PI3K/Akt (protein kinase B), the MAPK enzymes ERK1/2, JNK and p38, and NF- κ B (102).

4.1 The PI3K/Akt pathway

In the endothelium the PI3K/Akt pathway has been linked to metabolic activity, cell growth and proliferation, cell survival and resistance to apoptosis. Shear stress, ROS, growth factors including VEGFA, platelet-derived growth factor (PDGF) and basic fibroblast growth factor activate this pathway (103). Following activation of PI3K, Akt is relocated to the cell membrane and is phosphorylated on Thr³⁰⁸ and then Ser⁴⁷³. Downstream effects are mediated via mammalian target of rapamycin (mTOR) and glycogen synthase-3 β pathways.

The ability of Akt to optimise endothelial cell survival is underpinned by its potent anti-apoptotic activity. Akt phosphorylates caspase-9 so inhibiting the cytochrome-c/Apaf-1/caspase-9 apoptosis pathway (104). Akt also phosphorylates BAD so allowing Bcl-2 and Bcl-X_L to exert anti-apoptotic actions (105). This action is enhanced by the interaction between Akt and forkhead (FOXO) transcription factors. Inhibition of FOXO activity attenuates expression of pro-apoptotic Bcl-2 family members (106).

4.2 MAPK pathways

MAPK signalling pathways may exert pro-survival and pro-apoptotic actions which are context and cell type specific (102). ERK1/2 kinase activity enhances EC survival through induction of Bcl-2, Bcl-X_L and MCL-1. ERK1/2 is also the downstream target of exogenous pro-survival mediators including IL-10 and VEGFA (107).

In the vascular endothelium JNK activation is predominantly pro-inflammatory and pro-apoptotic. JNK phosphorylation is induced by TNF- α and differentially by DF (108). Although the mechanisms are not fully elucidated, JNK activity leads to activation of caspase-9 and caspase-3 and induces pro-apoptotic Bcl-2 family members. The activation of p38 MAPK isoforms in the endothelium has been linked to both injurious actions and cytoprotective effects (57, 109-111).

4.3 NF- κ B pathways

NF- κ B is best known as a pro-inflammatory transcription factor. The NF- κ B family comprises RelA (p65), RelB, c-Rel, and precursors p105 and p100, which generate p50 and p52 respectively. The components share a Rel-homology domain and may combine to form homo- and hetero-dimers including p50/RelA (112). NF- κ B exerts context-dependent effects on gene expression including both induction and repression, mediated via specific κ B elements present in promoters and enhancers (113). NF- κ B activation pathways include the canonical pathway, activated by TNF α , IL-1 β , ROS and lipopolysaccharide (LPS). Canonical signalling is dependent upon IKK α / β -NEMO complex activation, leading to I κ B α phosphorylation and degradation, the release of NF- κ B dimers and nuclear translocation. An alternative non-canonical pathway is stimulated by CD40, lymphotoxin β , receptor activator of NF- κ B ligand and LPS. The subsequent activation of IKK α involves NF- κ B-inducing kinase, resulting in the generation of p52-RelB heterodimers (113).

In addition to the regulation of pro-inflammatory genes such as E-selectin, VCAM-1, ICAM-1 and IL-6, NF- κ B activity controls anti-inflammatory, pro-proliferative and cell survival genes. These include anti-apoptotic Bcl-2 family members, cyclin D and A20 (34). A20 is part of a negative feedback loop, important for the prevention of prolonged NF- κ B activity. NF- κ B activation may also inhibit JNK activity and induce anti-oxidant proteins such as manganese superoxide dismutase (MnSOD) and H-ferritin (114).

4.4 Cytoprotective transcription factors

Transcriptional responses are central to the maintenance of endothelial homeostasis and for its response mode. A full account of these diverse and complex mechanisms are beyond the scope of this review, which will therefore focus on four important transcription factors KLF2, KLF4, Nrf2 and CREB.

KLF2 and KLF4 are members of a seventeen zinc-finger transcription factor family. They are both induced in by the exposure of human cultured EC to unidirectional LSS, a response that is suppressed by DF (115, 116). This pattern is reproduced *in vivo* (93). KLF2 and KLF4 activity is a critical regulator of cytoprotective genes including eNOS, thrombomodulin and HO-1 (116-120). They also exert an anti-inflammatory action suppressing the upregulation of E-selectin, VCAM-1 and ICAM-1 and attenuating leukocyte adhesion (116, 121). LSS activates MAPK kinase/MEK5, leading to phosphorylation of ERK5 and activation of myocyte enhancer factor-2 (MEF2) which binds the KLF2 promoter (118), while shear induction of KLF4 shares the MEK5/MEF2 pathway with KLF2 (120). Activation of AMP-activated kinase (AMPK) by LSS is an important additional component of this response and lies upstream of ERK5 (122). There is a close relationship between the expression of KLF2 and KLF4 and susceptibility to atherosclerosis (80). Hemizygous deficiency of KLF2 resulted in enhanced atherogenesis in a murine model (123). Similarly, endothelial-specific deletion of KLF4 exacerbated atherosclerosis, while targeted over-expression had the opposite effect (121).

The cap'n'collar basic region leucine zipper cytoprotective transcription factor Nrf2 is retained in the cytoplasm by kelch-like ECH-associated protein (Keap1). Various stimuli including oxidative stress, toxins, growth factors and LSS lead to the dissociation of the Nrf2-Keap1 complex. Free Nrf2 translocates to the nucleus, where it binds to the anti-oxidant response element (ARE) and controls expression of phase II detoxification enzymes and anti-oxidant proteins including HO-1, (NQO1), ferritin heavy chain (FHC), glutathione reductase (GR) and thioredoxin reductase-1 (Trx-1) (84, 124, 125). Alternative Nrf2 regulatory mechanisms and cross-talk with other signalling pathways including AMPK and NF- κ B have also been reported recently (126-129).

In contrast to LSS, DF does not lead to translocation and binding of Nrf2 to the ARE. Of note DF increases nuclear localisation of Class I and II histone deacetylases (HDACs), while LSS leads to Class II HDAC export. Thus, DF associates HDAC-1, 2 and 3 with Nrf2 and HDAC-3, 5 and 7 with MEF2 resulting in the reduced expression of KLF2 and NQO1 (130, 131). In the murine aorta, the different pattern of Nrf2 localisation in EC exposed to LSS and DF is preserved. At LSS-exposed sites *in vivo*, where Nrf2 is localised to the nucleus, p38 MAPK signaling is suppressed. This protective response is lost in DF sites leading to pro-

inflammatory changes (132, 133). The relationship between Nrf2 and atherosclerosis is complex and yet to be fully resolved. Studies performed in murine models have demonstrated both pro- and anti-atherogenic actions of Nrf2 and further studies are awaited with interest, as this pathway has important therapeutic potential (134).

Members of the CREB family of transcription factors include CREB1 and activating transcription factor-1 (135). CREB is activated by phosphorylation of serine 133 in response to stimuli including protein kinase A, PKC and calmodulin kinases (136). Binding to the cAMP response element CRE and interactions with CREB binding protein or p300 drives the transcription of CREB-responsive genes (135, 136). CREB activity regulates a variety of functions including cell proliferation and survival, inflammatory and immune responses. CREB has been identified as an AMPK target (137), a response important for the vasculoprotective actions of methotrexate and atorvastatin + rapamycin combination therapy (138, 139). Likewise CREB is an important target of the PKC ϵ kinase cascade (45, 140, 141).

CREB has an important protective role in the cardiovascular system. Increased activity of AMPK and CREB inhibits proliferation and migration of vascular smooth muscle cells (142). In the endothelium CREB activation is central to VEGFA-driven cytoprotection, angiogenesis and endothelial barrier function (143, 144). CREB target genes Bcl-2 and HO-1 are likely to be important in these responses. The exact nature of CREB's role in the vasculature may be cell type and context-dependent. However, its protective function is illustrated by the fact that depletion in the rodent aorta predisposes to hypertension, atherosclerosis and insulin resistance (142). Likewise, cardiac-specific delivery of DN-CREB increases oxidative stress, mitochondrial dysfunction, apoptosis, and mortality (145).

4.4 Protein kinase C

PKC is a family of serine/threonine kinases, comprising classical (α , β , γ), novel (δ , ϵ , η , θ) and atypical (ζ , ι , ν) isoforms. Unique functions of individual PKC isoforms reflect differences within isoform structure, subcellular compartmentalisation and PKC-target protein interactions (146). For example, activation of PKC ϵ leads to translocation to specific subcellular compartments (Golgi, plasma membrane, mitochondria), and close association with its enzymatic substrates via anchoring to receptors for activated kinases. In the cardiovascular system, interest in PKC ϵ began with the recognition that its activation in cardiomyocytes during ischaemic pre-conditioning (IPC) exerts a potent protective effect (147). Genetic disruption of PKC ϵ inhibits cardioprotective actions of IPC, while over-expression and PKC ϵ agonists enhance the response (148, 149).

Intriguingly, PKC ϵ activity exerts opposite effects in VSMC and EC. In VSMC, PKC ϵ increases proliferation, while its inhibition attenuates neointimal hyperplasia following balloon injury. Importantly, no adverse effect on re-endothelialisation was seen (150). Relevant to this we have found that PKC ϵ activity increases EC migration without significantly increasing proliferation (44). PKC ϵ plays an important role in endothelial homeostasis through induction of cell survival genes and suppression of pro-inflammatory transcripts. Initial findings demonstrated that when exposed to chronic hypoxia PKC ϵ ^{-/-} mice exhibit endothelial dysfunction, with reduced eNOS, increased vascular tone, accentuated right ventricular size and pulmonary hypertension, which can be reduced by NO inhalation (151).

We have reported that PKC ϵ forms a signalling complex and acts co-operatively with Akt to protect human vascular EC against apoptosis, through induction of Bcl-2 and inhibition of caspase-3 cleavage (44). PKC ϵ also plays a role in VEGFA-induced Akt and eNOS activation (152), protecting EC against serum starvation via modulation of eNOS activity (153). Our subsequent work has demonstrated that PKC ϵ activates ERK1/2 and inhibits JNK MAPK in EC leading to diversion of the NF- κ B pathway away from pro-inflammatory signalling to cytoprotection (45) (Figure 2). At least in part this reflects activation of and co-operation between CREB and Nrf2, leading to induction of protective genes including HO-1, A1, A20, Bcl-2 and MnSOD (45, 140). Further understanding of PKC ϵ signalling in the vasculature seems likely to reveal specific therapeutic targets that may allow reversal of endothelial dysfunction and hence slow the progress of atherosclerosis.

In the arterial endothelium PKC ϵ and PKC ζ exert opposite effects. Exposure to LSS activates PKC ϵ (154). In contrast, disturbed flow activates PKC ζ which phosphorylates ERK5, decreases eNOS stability and increases apoptosis to enhance atherogenesis (155, 156). PKC ζ activity is also pro-inflammatory and has been implicated in JNK activation and TNF- α -mediated induction of ICAM-1 (157). Thus targeted activation of PKC ϵ and/or inhibition PKC ζ has the potential to significantly improve arterial endothelial function (Figure 3). However, their actions may vary in different vascular beds. While PKC ϵ activity increased HO-1 expression in arterial and venous EC and PKC ζ is pro-inflammatory in arterial endothelium, PKC ζ also plays an important role in angiogenesis. This latter function also involves HO-1. Thus, stromal-derived growth factor-1 induced angiogenesis is dependent upon a PKC ζ -HO-1 signalling pathway (158).

5. Therapy and vascular cytoprotection

Recognition that endothelial injury is a critical initial step in atherogenesis, and that endothelial dysfunction is an early and persistent feature in many diseases associated with premature atherosclerosis, suggests that therapeutic conditioning of the vascular endothelium might slow or prevent atherogenesis (Table 1).

5.1 Clinical evidence

There is emerging evidence from clinical scenarios that modulation of endothelial function can alter cardiovascular risk. This is exemplified by the endothelial actions of the statins, which include anti-inflammatory, anti-adhesive, immunomodulatory, anti-oxidant and anti-thrombotic actions, above and beyond their ability to lower LDL-cholesterol (159, 160)

In the treatment of systemic inflammatory diseases, recent evidence suggests that a combination of immunosuppression and vasculoprotection is an important goal. Rapamycin, which inhibits mTOR, may achieve this under certain circumstances. In a cardiac transplant cohort, 45 patients were switched to rapamycin and 58 remained on calcineurin inhibitors. Subsequent intravascular ultrasound imaging revealed reduced post-transplant vasculopathy, increased vascular remodelling and fewer cardiovascular events, leading to improved 5 year survival in the rapamycin group (161). Mechanisms underlying this protection are multifactorial and include inhibition of complement activation and myofibroblast proliferation. Of note, rapamycin is reported to induce DAF expression in this setting and the drug may also increase HO-1 activity (162, 163) (Table 1).

In RA, biological therapy targeting TNF- α reduces aortic inflammation and stiffness (164) and may reduce future CV events when compared to non-biologic therapies (165). A recent meta-analysis has illustrated the importance of drug therapy selection, particularly when considering the effect on future CV outcomes (166). Thus, treatment of RA with TNF- α antagonists or methotrexate resulted in a reduction in the risk of CV events of 30% and 28% respectively. In contrast, the use of corticosteroids led to a 47% increase (166).

5.2 Endothelial conditioning

We have studied established drugs used in the treatment of patients with systemic inflammatory diseases, to identify the mechanisms through which they might condition and protect the endothelium.

The statins inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, so reducing cholesterol synthesis and serum LDL-cholesterol. Statin-mediated inhibition of isoprenoid lipid production, protein prenylation and the activity of signaling proteins such as

the small GTPases, underlie many of their LDL-cholesterol independent actions (159, 167). Indeed, a reduction in Rho-associated protein kinase activity and improved endothelial function was demonstrated in patients receiving high-dose statins when compared to those prescribed low-dose statins or ezetimibe (168). These cholesterol-independent actions of statins result in significant improvement in endothelial function in both hyper- and normocholesterolaemic patients with atherosclerosis (167). In vascular endothelium, statins increase eNOS mRNA stability and NO biosynthesis, leading to inhibition of leukocyte trafficking, a response that is absent in eNOS-deficient mice (169). Additionally, statin therapy may induce endothelial DAF and CD59 expression and enhance resistance to complement-mediated injury (77, 170).

There is a considerable body of evidence to suggest that statins can enhance HO-1 expression in the vascular endothelium. However, some variability between study outcomes does exist, likely reflecting differences in the vascular bed studied, the source and concentration of statin and the methodology adopted. Simvastatin, atorvastatin and rosuvastatin have been reported to increase *HMOX1* promoter activity and mRNA levels, to induce HO-1 enzyme activity and to increase anti-oxidant capacity (119, 171-174). *In vivo* atorvastatin therapy increased HO-1 expression in the murine aortic endothelium (124). These data raise the possibility that HO-1 induction contributes to the clinical benefits associated with statin therapy.

VEGFA also induces DAF expression on the vascular endothelium and increases resistance to complement. This response was inhibited by the immunosuppressive drug cyclosporine A (Mason 2004), but not by rapamycin. Following cardiac transplantation, rapamycin and statins are often co-prescribed. The efficacy of rapamycin for protection against transplant vasculopathy is illustrated by studies in which it replaced calcineurin antagonists (161, 175). Statin therapy also activates AMPK in EC and this in turn inhibits mTOR and HMG-CoA reductase (176-178). These findings, alongside the importance of DAF for protection against post-transplant arteriosclerosis, allograft rejection and atherosclerosis (7, 70), led us to the hypothesis that in combination atorvastatin and rapamycin therapy maximally induces DAF via increased AMPK activity.

We subsequently demonstrated synergistic induction of endothelial DAF by atorvastatin and rapamycin. Although this response was reproduced by simvastatin in place of atorvastatin, replacement of rapamycin with CsA was ineffective (138). Mechanistically, a PKC α , AMPK and CREB-dependent pathway was activated by the combination therapy to induce DAF expression. The synergistic induction translated to the *in vivo* setting in which only

atorvastatin and rapamycin in combination significantly upregulated DAF on the murine vascular endothelium (138).

Methotrexate therapy improves clinical markers of endothelial dysfunction and reduces cardiovascular events and mortality in RA (166, 179-181). While these in part reflect an anti-atherosclerosis effect of adenosine signalling via the A_{2A} receptor, we have recently identified an additional mechanism. Methotrexate is able to activate EC AMPK at concentrations achieved in plasma following therapeutic dosing. Activation of CREB resulted in the transcriptional regulation of protective genes including HO-1 and MnSOD. In an *in vivo* model of inflammatory vasculopathy, methotrexate upregulated protective genes, attenuated intramyocardial arterial injury and end-organ damage (139). This suggests that the activity of the AMPK-CREB signalling pathway is vasculoprotective and merits further investigation as a specific therapeutic target.

5.3 Effect of shear stress on therapeutic responses

Using HO-1 as a transcriptional target we found differential induction of HO-1 by atorvastatin at atherosusceptible and atheroprotective sites of the murine aorta. *In vitro*, LSS significantly reduced the concentration of atorvastatin required to upregulate HO-1 and to protect EC against ROS-mediated injury. The synergy between LSS and atorvastatin involved Akt phosphorylation, activation of KLF2 and Nrf2 and increased NO synthase activity. Importantly, in EC at DF sites, HO-1 induction by atorvastatin was markedly reduced (124). Similar findings in EC exposed to LSS and DF have been reported for thrombomodulin and KLF2 induction by simvastatin (182). The data suggest that beneficial statin pleiotropy may be suboptimal at atherosusceptible sites in the vasculature.

6. Future perspectives

Inflammatory reactions within the arterial wall are tightly regulated. When excessive they predispose to endothelial apoptosis and dysfunction, VSMC proliferation, atherogenesis, atherothrombosis, myofibroblast-mediated arterial occlusion and aneurysm formation. Vascular endothelial injury is multifactorial and enhanced by diseases with a systemic or localised inflammatory component including SLE, RA, diabetes mellitus, transplant vasculopathy and atherosclerosis. In light of this and the international morbidity and mortality burden associated with CV disease, considerable interest has been directed at therapeutic prevention and reversal of vascular injury and endothelial dysfunction, with specific focus on anti-inflammatory strategies (183, 184).

In the last decade remarkable progress has been made towards identifying molecular mechanisms that regulate the activity of protective genes within the endothelium and broader vasculature. Over the next decade it seems likely that these insights will reveal novel therapeutic targets and lead to the development of new drugs. Meanwhile, physicians need to develop strategies capable of identifying those patients most at risk of CV events in order to mitigate risk. This may require further advances in non-invasive imaging and the development of specific CV disease biomarkers in patients with systemic inflammatory diseases (18). However, considered use of currently available therapies and development of personalised medicine to optimise immunosuppression and vasculoprotection may prove to be a significant move in the right direction.

To this end two exciting clinical trials are in progress which will test for the first time the inflammatory hypothesis of atherothrombosis. The CANTOS (Canakinumab Anti-inflammatory Thrombosis Outcomes Trial, NCT01327846) targets IL-1 β , a cytokine known to drive IL-6 pathways, to activate endothelium and to be produced locally within the atherosclerotic plaque. Canakinumab is an inhibitory monoclonal antibody and the trial will test its efficacy for reducing CV events in patients with myocardial infarction and subsequent revascularisation associated with a CRP >2mg/L (185). Similarly, the CIRT trial (Cardiovascular Inflammation Reduction Trial, NCT01594333) will take forward the positive vascular protective effects of methotrexate reported in patients with RA and test its efficacy in patients without autoimmune disease. Low-dose methotrexate (15-20mg per week) will be administered to patients with stable coronary artery disease and diabetes or the metabolic syndrome. The trial will seek a positive effect of methotrexate on recurrent myocardial infarction, stroke and CV death (186). The outcomes of these exciting studies are awaited with great interest.

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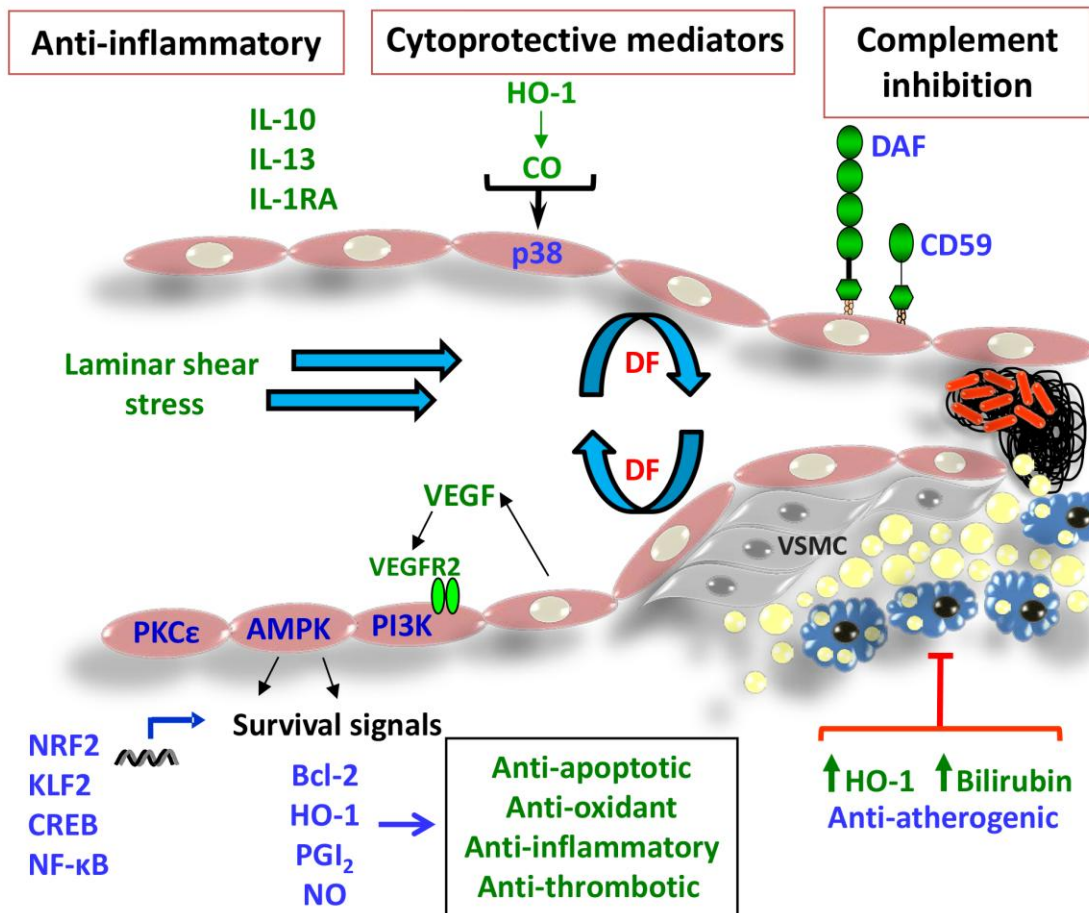


Figure 1. Cytoprotection in the vasculature. The vascular endothelium is protected by exogenous anti-inflammatory and pro-survival factors including IL-10, IL-13, IL-1RA and VEGF. Induction of intrinsic cytoprotective genes via PKC ϵ , AMPK and PI-3K/Akt-dependent pathways results in enhanced protection against apoptosis, oxidative stress, thrombosis and complement-mediated injury expression. AMPK = AMP kinase; IL = interleukin; IL-1RA = IL-1 receptor antagonist; NO = nitric oxide; PKC = protein kinase C; VEGF = vascular endothelial growth factor; HO-1 = heme oxygenase-1; PGI₂ = prostacyclin; DAF = decay-accelerating factor.

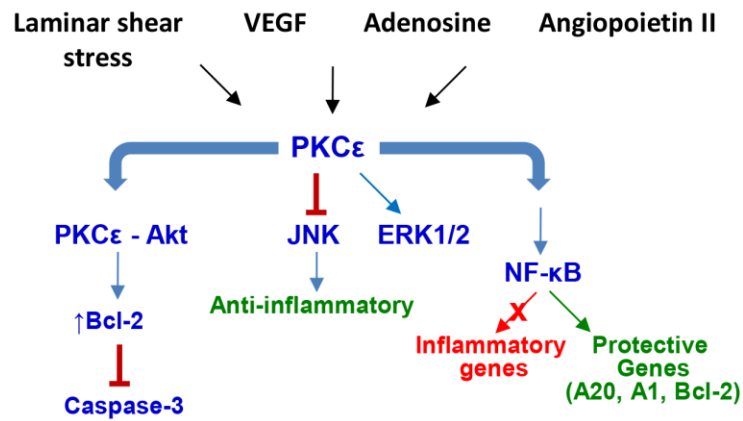


Figure 2: Protective PKC ϵ signalling. The physiological ligands of PKC ϵ in the endothelium are poorly understood. They include laminar shear stress (LSS), vascular endothelial growth factor (VEGF), angiopoietin II and adenosine. Activation of PKC ϵ leads to the formation of a signalling module with Akt, which induces Bcl-2 to inhibit apoptosis by attenuating caspase-3 activity. PKC ϵ activity inhibits JNK to exert anti-inflammatory effects. In contrast, activation of ERK1/2 and NF- κ B diverts NF- κ B signalling towards the selective transcription of protective genes.

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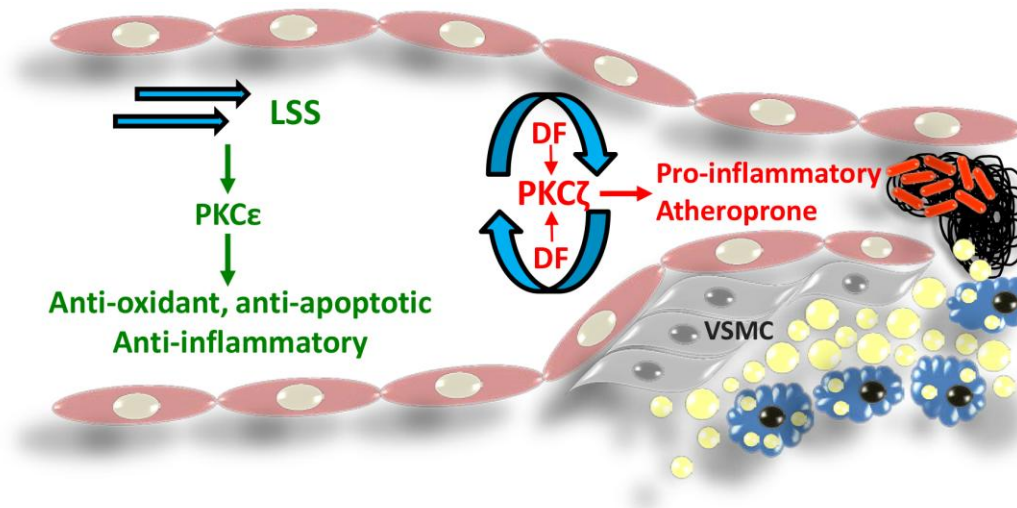
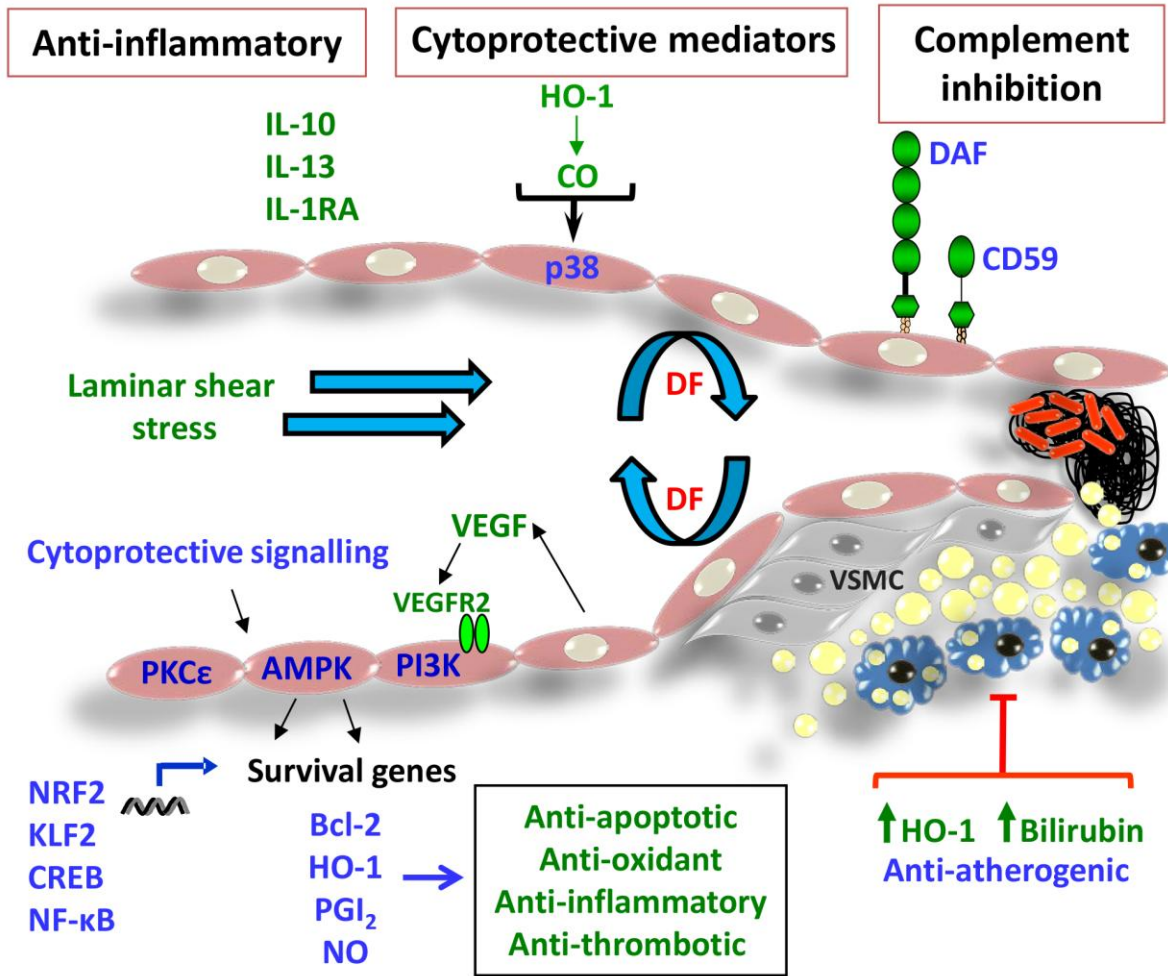


Figure 3. Opposing actions of PKC ϵ and PKC ζ in the vasculature. Laminar shear stress (LSS) activates PKC ϵ in the vascular endothelium. PKC ϵ activity is linked to the induction of protective genes that exert anti-inflammatory, anti-oxidant and anti-apoptotic actions. In contrast at disturbed flow sites, PKC ζ activation is linked with pro-inflammatory changes include leukocyte adhesion molecule up regulation, with PKC ζ activity linked to atherosclerosis.

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Disease	Current therapies that may confer vasculoprotection	Active areas of research
Rheumatoid arthritis	Methotrexate ^{18,21} TNF- α blockade ²⁵	Role of MnSOD and HO-1 induction? ¹³⁹ Is IL-6 blockade vasculoprotective? ¹⁸
Systemic lupus erythematosus	Glucocorticoids ^{18,22} Hydroxychloroquine Mycophenolate mofetil	How should steroids be optimally used? Is B cell targeting vasculoprotective?
Atherosclerosis	Aspirin ⁵ Statins	Cantos Trial of anti-IL-1 ¹⁸⁵ CIRT trial of methotrexate ¹⁸⁶
Vasculitides	Rituximab ²² Methotrexate	Is there a role for anti-proliferative agents eg. Rapamycin?
Cardiac transplants	Rapamycin ¹⁷⁵ Statins	How important is the induction of DAF and HO-1 in graft protection? ^{138, 163}

Table 1: Current therapies and their potential roles in vasculoprotection are shown. Some outstanding questions, current and future avenues for research are listed.



Graphical abstract