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SPOTLIGHT REVIEW

The atherosusceptible endothelium: endothelial phenotypes in complex haemodynamic shear stress regions *in vivo*

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Abstract Atherosclerosis initiates at predictable focal sites and develops to a spatially regional disease with limited distribution. There is compelling evidence that links haemodynamics to the localized origin of atherosclerotic lesions. Arterial flow *in vivo* is unsteady, dynamically complex, and regionally variable. Sites susceptible to atherosclerosis near arterial branches and curves are associated with regions of disturbed blood flow that contain repetitive phases of flow reversal resulting in steep multidirectional temporal and spatial gradients of wall shear stresses. Endothelium in atherosusceptible regions relative to protected sites shows activation of endoplasmic reticulum (ER) stress and the unfolded protein response (UPR), the altered expression of pro-inflammatory Nuclear Factor kappa B (NFκB) and oxidant/antioxidant pathways, and low expression of major protective factors, notably endothelial nitric oxide synthase and Kruppel-like Factors KLF2 and KLF4. At some atherosusceptible locations, reactive oxygen species levels are significantly elevated. Here we describe flow-related phenotypes identified in steady-state *in vivo* and outline some of the molecular mechanisms that may contribute to pre-lesional atherosusceptibility as deduced from complementary cell experiments *in vitro*. We conclude that disturbed flow is a significant local risk factor for atherosclerosis that induces a chronic low-level inflammatory state, an adaptive response to ensure continued function at the expense of increased susceptibility to atherogenesis. Surprisingly, when challenged by short-term hypercholesterolaemia *in vivo*, atherosusceptible endothelial phenotype was resistant to greater pro-inflammatory expression, suggesting that sustained hyperlipidaemia is required to overcome these protective characteristics.

Keywords Endothelial phenotype • Haemodynamics • Atherosclerosis • Inflammation • Genomicsv

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1. Introduction

In mammals, large quantities of blood are transported through the branching geometry of the arterial system under pulsatile pressure. The interactions of blood flow with the vessel geometry create complex haemodynamic characteristics including heterogeneous spatial and temporal mechanical stresses on the vessel wall. As the vascular interface with flow-mediated shear stresses, the arterial endothelium senses changes in local haemodynamic characteristics and responds by initiating acute changes in artery wall vasomotion and chronic structural remodelling.[1] This important repertoire of regulatory *physiological* responses ensures acute adjustments of the vascular system and facilitates development and growth. However, localized regions of highly disturbed arterial flow are associated with metabolic stress in the endothelium that sensitizes the cells to local inflammatory changes that favour a *pathological* outcome, the initiation and development of atherosclerosis.

A biological definition of the characteristics of 'susceptibility' in an *in vivo* context remains challenging. Often treated as the absence or lowering of protective pathways, it also involves the active induction of a pathological stressed state. Stress arises from complex physical deformation forces that modify endothelial mechanotransduction. Furthermore, blood cells and undesirable metabolites [e.g. reactive oxygen species (ROS)] are retained within recirculating eddies with the potential for cell damage. The endothelium adapts by adjusting the equilibria of multiple pathways, but in doing so the cells may be *locally* more susceptible to *systemic* atherogenic risk factors.

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Differential endothelial phenotypes identified at the actual sites where lesions will or will not develop can help identify the mechanisms involved. Here, we outline flow-related endothelial phenotypes *in vivo*, identified initially by transcriptomics, miRNA-omics, and traditional biochemistry, in susceptible and protected arterial sites in domestic swine and mouse models. Complete sequencing of the swine genome has recently provided access to a wealth of data for structural, genomic, epigenomic, translational, post-translational, and functional research in a species that is highly relevant to human physiology and pathology.

2. Disturbed blood flow predicts atherogenesis

In vivo, a tangential flow force component, shear stress, acts at the arterial luminal surface in the direction of flow to induce cyclic deformation of the endothelial surface throughout each cardiac cycle. As has been described for many years, arterial endothelial alignment *in vivo* and *in vitro* generally follows the shear stress direction.[1–3] All flow in arteries is unsteady. In regions spared of atherosclerosis, the blood surges during the cardiac cycle at an increasing then decreasing velocity as the contraction subsides resulting in unsteady but unidirectional laminar flow that is atheroprotective.

In contrast, atherosclerosis develops in spatially predictable regions (*Figure 1*) within large elastic and muscular distributing arteries near branches and bifurcations—where changes of vessel cross-sectional area occur over short distances—or as blood flow attempts to follow the tight inner curvature of the aortic arch. Within these regions, complicated flow patterns, collectively referred to as disturbed flow, originate from the separations of the mainstream to form recirculating eddies that contain non-uniform spatial and temporal gradients of shear stress. Disturbed flow typically includes periods of reciprocating flow reversal that create oscillating wall shear stresses, a characteristic notably absent in atheroprotected flow regions.

The mean velocity of flow—and therefore the shear stress—is much lower in the separated flow sites; however, low shear stress is a gross oversimplification of the spatial and temporal complexity of the motion of fluid, blood cells, and molecules within the regions. Despite the complexity of disturbed flow, laminarity persists—fluid moving in parallel layers with frictional interactions between them—but with very different spatial and temporal relationships than exist outside of the regions. In extreme circumstances associated with congenital strictures, atherosclerotic stenoses, and cardiac valve dysfunction—where the flow path narrows and then expands—a chaotic state (turbulence) may occur during systole. Turbulence favours acute pro-coagulant risk and accelerated vessel-wall dysfunction leading to cardiovascular disease, e.g. downstream of aortic-valve stenosis.

3. Endothelial phenotypes in disturbed flow

To generate an unbiased large-scale site-specific database *in vivo*, we performed 'omic' analyses (particularly transcriptomics and microRNA-omics) of the endothelium.[4–7] The endothelium was isolated from disturbed and undisturbed flow regions (susceptible vs. protected) of pig arteries for nucleic acid extraction. Differential expression (mRNA; miRNA) databases were analysed for gene interactions, hierarchical structures, pathway associations, and other statistical analyses to identify site-specific endothelial interrelationships. The data were then used in the design of *in vitro* reductionist experiments under controlled conditions for insight into the mechanisms responsible. In the best circumstances, new mechanisms arising from the bench experiments were validated *in vivo*, thereby completing a circular strategy guided initially by the unbiased *in vivo* state (*Figure 2*). Such procedures have been used to identify characteristics of endothelial phenotypes in disturbed flow regions,[4,5] to study the effects of risk factors such as hypercholesterolaemia on regional phenotypes,[6,7] and to strengthen the *in vivo* relevance of the large body of literature reporting *in vitro* flow responses. Systems biology analyses have also been successfully applied to *in vitro* flow experiments;[8] a point of discussion is whether the *in vitro* environment is adequately designed to capture sufficient characteristics (chemical as well as biomechanical) of the arterial environment.

4. Endothelial phenotype in atherosusceptible regions *in vivo*

We now outline several regulatory mechanisms in the endothelium for which current *in vivo* evidence is most robust in linking disturbed flow to an atherosusceptible phenotype.

4.1 The 'atherosusceptible endothelium'

Long-known biological differences at disturbed flow arterial sites include increased endothelial permeability to plasma macromolecules,[9] increased (but still very low) endothelial proliferation,[10] and increased immuno-surveillance by monocytes that attach and migrate into the artery wall.[11] These site-specific functional differences do not result in progression to significant inflammation unless additional systemic risk factors (e.g. hypercholesterolemia; hypertension; diabetes; smoking stress) are also present. The atherosusceptible phenotypes, therefore, may be considered to be in a sensitized—or 'primed'[12]—pre-lesional state.

4.2 Systems biology approaches to localized endothelial phenotype *in vivo*

Prior to 2003, the association between endothelial phenotype and atherosusceptibility *in vivo* was inferred principally by extrapolation from *in vitro* flow experiments or by *en face* confocal imaging of immunostained candidate proteins within susceptible regions *in situ*. Notably, Cybulsky, Collins, and colleagues[12] first reported increased *in situ* expression of several proteins associated with the pro-inflammatory nuclear factor κB (NFκB) pathway at such sites in mice. Lowered transcription of the vasculo-protective endothelial nitric oxide synthase (eNOS) were also noted.[12,13] However, more comprehensive site-specific gene expression profiling *in vivo* in the mouse is limited by the small sample size available within haemodynamic regions of interest. The adult pig is an attractive model for comparative regional endothelial profiling. Linearly amplified RNA from freshly isolated endothelium of the inner aortic arch and descending thoracic aorta was hybridized to microarrays to profile differential gene expression reflecting the steady-state *in vivo*.[4–8]

4.3 Chronic low-level inflammation in atherosusceptible endothelium in swine

The lower expression of eNOS in disturbed flow regions reported in mice was confirmed in normal swine.[8] Transcript profiles also showed the coexistence of enhanced pro- and anti-inflammatory gene

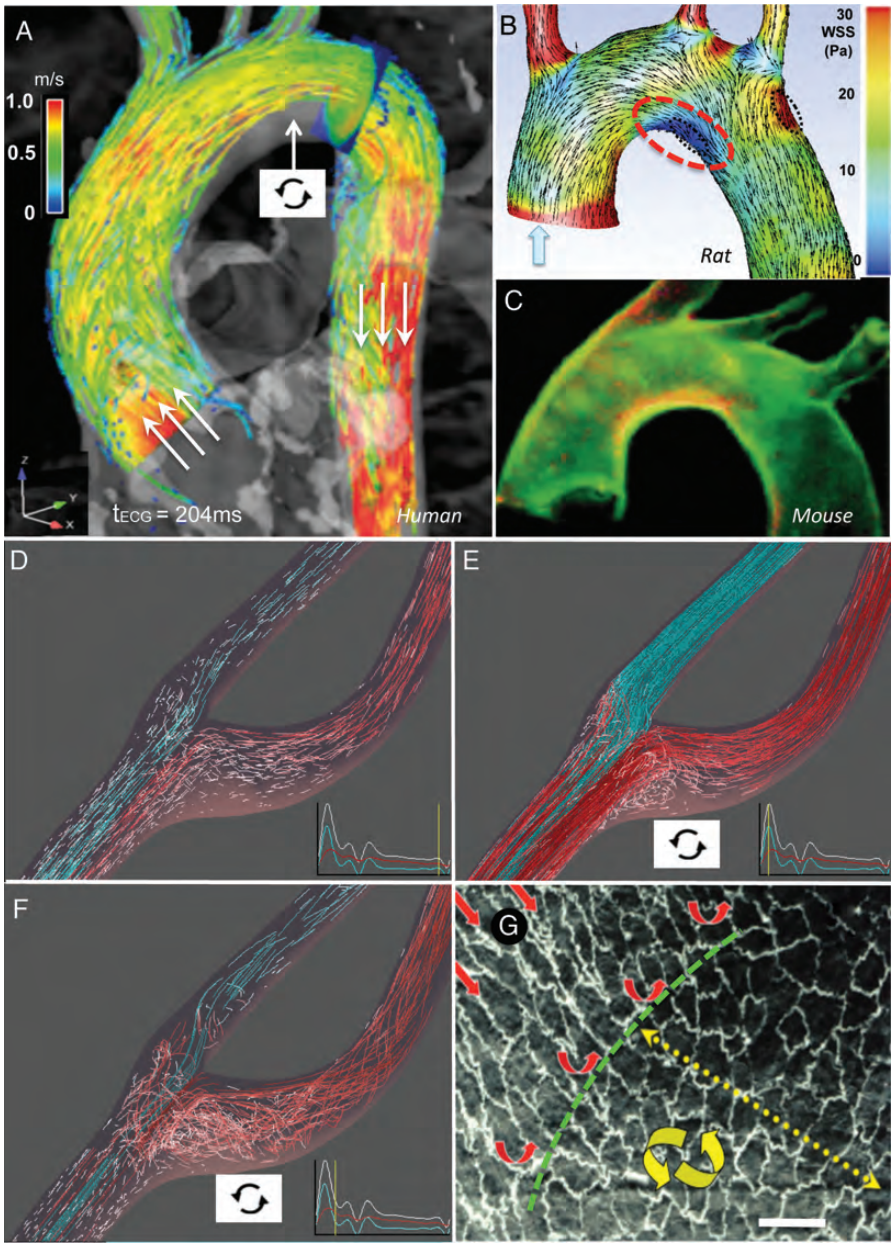

Figure 1 (*A–C*) Flow disturbance at the inner curvature of the aortic arch. (*A*) Flow-velocity profile during systole with flow separation (arrow) in normal human. (*B*) Wall shear stress and velocity distributions (rat). (*C*) VCAM-1 immunostaining (red) in mouse aorta indicative of pro-inflammatory state. (*D–F*) Flow separation and disturbance in the normal human carotid sinus. (*F*) Endothelial cell morphology transitions from aligned (undisturbed flow) to polygonal (disturbed flow) adjacent to a branch artery in primate aorta. Bar 15 μm. From: (*A*) Markl M, Kilner PJ, Ebbers T. *J Cardiovasc Magn Reson* 2011;**13**:7 (Additional File 2).[74] (*A–C*) Adapted from Bjorck *et al.* 2012.[75] (*B*) Bjorck HM, Renner J, Maleki S, Nilsson SF, Kihlberg J, Folkersen L *et al. PLoS One* 2012;**7**:e52227.[75] (*D–F*) Courtesy Professor D.A. Steinman, Biomedical Simulation Lab, University of Toronto. (*G*) Redrawn from Davies.[1] (*A*, *B*, *D–F*) Computational fluid dynamic imaging from MRI.

expression patterns in susceptible endothelium.[8] The differential expression of pro-inflammatory genes was primarily associated with NFκB pathway priming (*Figure 3*). In subsequent experiments, western blots showed a slightly elevated nuclear NFκB and modestly increased phosphorylation of IκBα,[14] both suggestive of low-level inflammation and consistent with candidate gene and protein measurements in the mouse.[12] Other prominent findings in the swine[8] were the suppression of the protective endothelial transcription factors Krüppel-like factor (KLF) 2 and KLF4, and the differential expression of gap junctional gene connexin 43 that disrupted the signalling between cells in flow studies *in vitro*.[15] On the protective side of the balance in the atherosusceptible cells were increased expression of glutathione peroxidase and other antioxidant genes. Increased procoagulant von Willebrand Factor expression was accompanied by protective enhancement of fibrinolysis-related gene expression.[8] However, neither histological evidence of inflammation nor significant differences in the expression of vascular cell adhesion molecule-1 (VCAM-1), E-selectin, and intracellular cell adhesion molecule-1 (ICAM-1)—considered essential for transendothelial

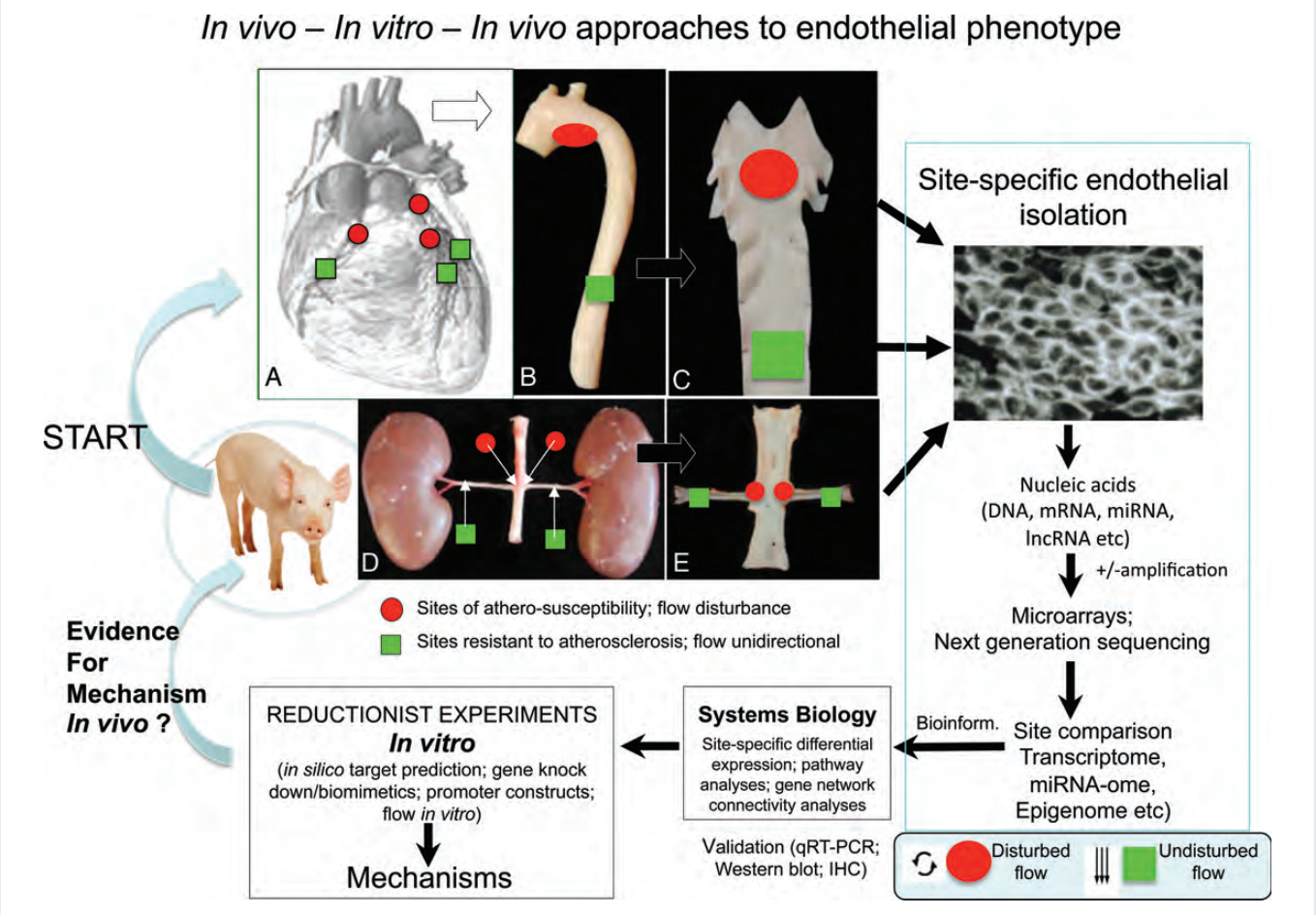

Figure 2 Outline of an *in vivo* 'omics' approach to endothelial phenotyping leading to *in vitro* experiments to investigate mechanisms which can then be probed *in vivo*.

migration of monocytes into the arterial wall—were found in the disturbed flow regions in swine. Nor could measurable nuclear translocation of the NFκB p65 subunit, required to facilitate pro-inflammatory gene expression, be detected by *en face* immunocytochemistry,[4] despite a modest increase measured by western blot. The coexistence of opposing mechanisms for pro/anti-inflammation in the same cells *in vivo* suggested that activation of inflammation is largely kept in check but with measurable biochemical evidence of low-level chronic NFκB activation.

In vitro studies have corroborated and greatly extended the *in vivo* findings, principally through corollary experiments showing protection from inflammation by undisturbed laminar flow.[16–18] *In vivo*, the local geometry of mouse carotid artery has been narrowed by cast or ligation to create flow disturbance resulting in 'cause-effect' inflammatory atherosclerosis[19] and the identification of expected and new differential gene expression.[20]

4.4 Endoplasmic reticulum-stress and the unfolded protein response is a signature endothelial response to flow disturbance at multiple sites *in vivo*

The endothelial phenotype *in vivo* seems to be in dysequilibrium in areas of disturbed flow but without pathological consequences—suggesting successful adaptation to the prevailing haemodynamic conditions. Parallels with cell-adaptive responses to manage multiple kinds of cellular stress suggested to us that biosynthetic load may be increased during disturbed flow leading to endoplasmic reticulum (ER) stress.

4.4.1 ER stress induces unfolded protein response

ER stress occurs in response to excessive protein biosynthesis that interferes with normal peptide folding mechanisms in the ER lumen. If the rate of new synthesis exceeds its protein-folding capacity, unfolded protein response (UPR) activates a co-ordinated transcriptional up-regulation of ER chaperones and folding enzymes to promote the correct assembly of unfolded polypeptides and prevent incompletely folded proteins from aggregating.[21]

In the unstressed state, the ER chaperone-binding protein, BiP (also known as heat shock protein A5, HSPA5 and glucose-related protein 78, GRP78) binds to each of the three ER stress transducers. These are ER transmembrane proteins each having an ER-luminal domain for the sensing of unfolded proteins and a cytosolic domain for signalling. While bound, BiP maintains the inactive state of the transducers; however, when an imbalance occurs in the luminal flux of newly synthesized unfolded or misfolded peptides, UPR is activated (*Figure 4*). To bind unfolded/misfolded polypeptides in the ER lumen, BiP dissociates from the chaperones causing their phosphorylation. Activation of chaperones ATF6α (activating transcription factor 6α), IRE1α (inositol requiring kinase 1α), and PERK (protein kinase-like ER kinase) converge as

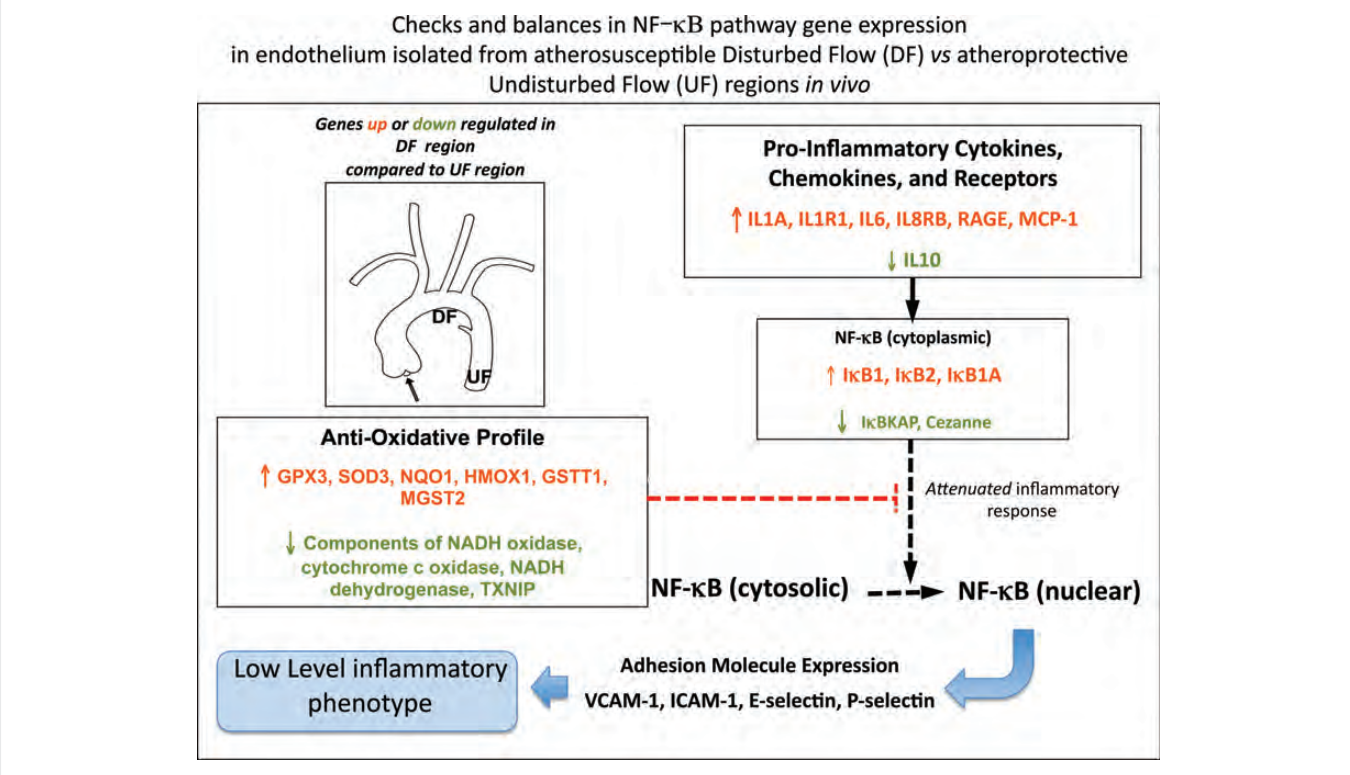

Figure 3 Differential gene expression in the atherosusceptible endothelium of normal swine. Pro-inflammatory NF-kB pathway activation coexisting with an enhanced antioxidative profile attenuates inflammation to a low level. Derived from Passerini *et al.*[4]

transcriptional regulators in the nucleus to up-regulate ER chaperone and UPR transducer synthesis and to ubiquitinate unfolded/misfolded proteins for degradation through the proteosome. Both processes relieve ER stress accumulation of unfolded proteins and restore homeostasis. Failure to restore ER protein equilibrium to a normal range leads to apoptosis through transcriptional induction of CHOP (C/ERB homologous protein), inflammation through activation of NFκB, and the generation of ROS through excessive intracellular protein oxidation in the ER. ROS are also influential in *initiating* ER-stress since extracellular ROS itself promotes ER stress.

4.4.2 Site-specific endothelial ER-stress/UPR *in vivo* is linked to atherosusceptibility

The association of ER stress with inflammatory genes in cultured endothelial cells following exposure to oxidized lipids was reported in 2006.[22] Civelek *et al.*[23] rediscovered the prominence of ER stress and the UPR *in vivo* during analyses of transcription profiles in a multi-site study in normal adult swine. Endothelium in the susceptible regions of the aortic arch, proximal brachiocephalic artery, aorto-renal branch region, and abdominal aorta were analysed relative to protected sites of the common carotid artery, descending thoracic aorta, and the distal renal artery. Each of the atherosusceptible regions is associated with complex disturbed blood flow. The most abundant common feature of the endothelium in atherosusceptibility regions was the up-regulation of genes associated with ER processing of proteins, ER stress, and the UPR. Differential gene-expression analysis identified 133 genes, 73% of which were involved in ER protein processing and folding and which form a highly connected and co-ordinated network up-regulated in the susceptible regions. Three independent and unbiased pathway mining approaches—Gene Ontology using the program DAVID, GSEA, and Ingenuity Pathway Analysis—identified ER stress and the UPR to be over-represented functional categories in atherosusceptible endothelium together with genes that function in protein folding, synthesis, and post-translational protein modification.[23]

To validate the genomics analyses, endothelial cell proteins were isolated from the aortic arch and descending thoracic aorta as well as from the atherosusceptible aorto-renal branch and the protected distal renal artery. At each atherosusceptible disturbed flow site, BiP transcript and/or protein expression was significantly up-regulated. Western blot demonstrated significantly elevated phospho-ATF6α (*Figure 4A*), phospho-IRE1α, and its target, spliced XBP-1 (*Figure 4B*). However, the third transducer pathway PERK was not activated (*Figure 4C*). A second study examined the disturbed and undisturbed flow regions of the coronary arteries, the endothelial origin of which is developmentally distinct from non-coronary arteries. Here, the role(s) of ER stress in the endothelium of coronary arteries may be particularly important. Civelek *et al.*[5] used gene connectivity analyses to discriminate between coronary and non-coronary endothelial transcript profiles. Differential expression of 1300 endothelial genes were identified in the coronary artery endothelium with highest significance expressed in the gene modules enriched for biological functions related to ER stress and UPR, regulation of transcription and translation, and redox homeostasis. ROS load appeared to be heavier and antioxidant protection lower in coronary arteries. Overall, these studies, approached without pre-conceived expectations of differential expression of genes and proteins associated with ER stress/UPR, suggest that stresses associated with flow disturbance *in vivo* elicit partial activation of the UPR and that chronic ER stress is a signature for atherosusceptible endothelial phenotype *in vivo*.

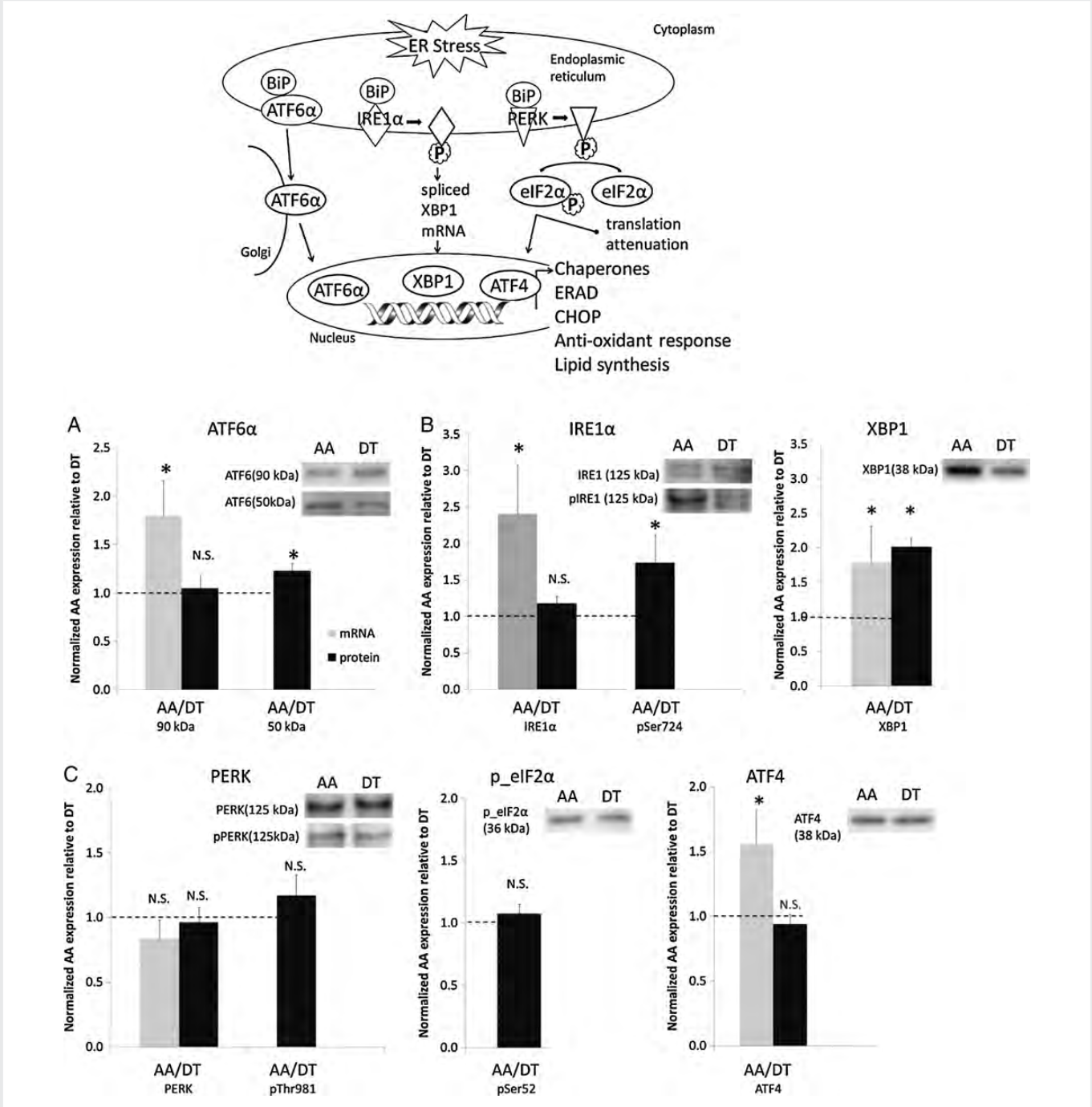

Figure 4 Top panel: Schematic of ER-stress/UPR. (*A*–*C*) Activation of UPR chaperones ATF6α (*A*), IRE1α (*B*) but not of PERK (*C*) in atherosusceptible endothelium *in vivo*. From Civelek *et al.*[23]

Around the same time, connections between ER stress and the biomechanics of disturbed flow were reported from *in vitro* experiments.[24,25]

4.4.3 Flow characteristics *in vitro* induce BiP (GRP78; HSPA5) activation

Feaver *et al.* (2008)[24] used *in vitro* flow to simulate human arterial shear stress waveforms. Atherosusceptible disturbed flow or atheroprotective laminar flow was applied to human endothelial cells. BiP (GRP78) was found to be significantly up-regulated in a sustained manner under atherosusceptible, but not atheroprotective flow up to 24 h. This response was dependent on both sustained activation of p38, as well as integrin α2β1. Increased BiP expression correlated with the activation of the ER stress sensing element promoter by atherosusceptible flow as a marker of the UPR. Shear stress regulation of BiP was through increased protein stability when compared with other flow-regulated proteins, such as connexin-43 and VCAM-1. Increased endothelial expression of BiP was also observed in atherosusceptible vs. atheroprotective regions of C57BL6 mice. These findings support a role for the haemodynamic environment in the preferential induction of ER stress and UPR in atherosusceptible regions.

4.4.4 Spliced XBP-1 chaperone pathway of UPR

Further *in vitro* evidence centres on spliced XBP-1 (sXBP-1), one of the three transduction arms of the UPR response. sXBP-1 encodes the XBP-1 transcription factor that translocates to the nucleus to activate selective pro-apoptotic target genes. Following the observation of endothelial expression of the XBP-1 pathway of UPR in the branching regions of ApoE$^{-/-}$ mice arteries and in atherosclerotic lesions that developed there, Zheng *et al.*[25] reported that atherosusceptible flow waveforms induced XBP-1 splicing in cultured endothelial cells and that overexpression of (activated) sXBP-1 induced apoptosis. To extend the findings to an *in vivo* assay for atherogenesis, adenoviral-mediated overexpression of sXBP-1 was induced in an ApoE$^{-/-}$ murine aortic isograft model. In these animals, enhanced intimal hyperplasia and atherosclerosis developed in normally protected regions of the aorta, suggesting that when the XBP-1 UPR pathway is greatly over-stimulated, the adaptive protective function of UPR reverts to a pathological imbalance. While over-expression was not limited to the endothelium in the isograft model, the data are supportive of a prominent role for endothelial sXBP-1.

The role of ROS in arterial ER stress is further supported by the effects of oxidized and glycated lipoproteins which were recently shown to induce endothelial ER stress *in vitro* through a mechanism of sarcoplasmic/ER Ca^{2+} ATPase (SERCA) oxidation.[26] This was inhibited by the activation of AMPK and evidence for such a mechanism *in vivo* was obtained by antioxidant administration resulting in the attenuation of impaired endothelial-mediated vasorelaxation. In ApoE$^{-/-}$ and ApoE/AMPK double knock-out mice fed a high-fat diet, antioxidants attenuated SERCA oxidation, ER stress, and atherosclerosis. A similar AMPK mechanism may influence ER stress in vascular smooth muscle,[27] and it is clear that oxidation-related ER stress pathways are very important in macrophages for the ongoing development of atherosclerosis.[28] These complementary approaches to endothelial ER stress provide compelling evidence for the existence of site-specific chronic adaptive UPR in atherosusceptible endothelium *in vivo* regulated through haemodynamics characteristics.

4.5 Regulation of eNOS function

Consideration of flow mechanisms responsible for the regulation of eNOS remains instructive despite most studies reporting responses to the transition from no-flow to flow *in vitro*.

4.5.1 Shear stress and eNOS phosphorylation

In contrast to Ca^{2+}-dependent agonist stimulation of endothelial eNOS, the response to fluid shear stress does not require constant elevation in intracellular Ca^{2+}. Rather the sensitivity of eNOS to intracellular Ca^{2+} is amplified by the phosphorylation of the enzyme. Of numerous eNOS phosphorylation sites, most is known about the functional consequences of phosphorylation of a serine residue (human eNOS sequence: Ser1177) in the reductase domain and a threonine residue (human eNOS sequence Thr495: bovine Thr497) within the calmodulin (CaM)-binding domain.

In cultured endothelial cells, Ser11 77 is rapidly phosphorylated after the application of fluid shear stress,[29,30] VEGF,[31,32] or bradykinin.[33] The kinases involved vary with the stimuli applied and while shear stress elicits the phosphorylation of Ser1177 via protein kinase A,[34,35] insulin, oestrogen, and VEGF mainly phosphorylate eNOS in the endothelial cells via Akt.[31] The serine phosphorylation of eNOS alone is not sufficient to increase enzyme activity or NO production. This is because under basal conditions, the enzyme is constitutively phosphorylated on a threonine residue (human eNOS sequence: Thr495) within the CaM-binding domain and only when Thr495 is dephosphorylated can calmodulin bind to eNOS and NO production increase. Indeed most eNOS activating stimuli elicit the reciprocal regulation of Ser1177 and Thr495,[32,33,36] although to different extents as receptor-dependent agonists elicit the almost complete dephosphorylation of this residue, while it remains largely phosphorylated in endothelial cells exposed to shear stress (in undisturbed flow). However, the status of this regulatory balance in endothelium subjected to disturbed flow has not been reported.

More recent studies suggest that the phosphorylation of an additional residue, i.e. Tyr 657 (human sequence) also acts like a brake to decrease NO generation.[37] Indeed, this residue is phosphorylated in the endothelial cells exposed to shear stress in undisturbed flow but not to receptor-dependent agonists, and unlike the phosphorylation of Ser1177, the consequence of eNOS Tyr657 phosphorylation, is a complete loss of enzyme activity. A clue to why this particular tyrosine residue could have such dramatic effects can be found by considering the mechanisms known to regulate the activity of the neuronal NOS (nNOS), which was reported to be determined by preventing a large-scale swinging motion of the FMN domain to deliver electrons to the catalytic module in the holoenzyme essentially locking the FMN domain into its electron-accepting position, thus inhibiting enzyme activity.[38] Since Tyr657 is the equivalent tyrosine residue in the human eNOS sequence, it is highly likely that its phosphorylation would be associated with a loss of NO production.

4.5.2 PYK2 and eNOS regulation

The kinase reported to phosphorylate eNOS Tyr657 is the focal adhesion protein tyrosine kinase, proline-rich tyrosine kinase 2 (PYK2), which is a rather unusual tyrosine kinase in that it contains no Src homology 2 (SH2) or SH3 domains. PYK2 is reported to mediate endothelial-cadherin-based cell–cell adhesion by tyrosine phosphorylation of β-catenin,[39] has been implicated in regulating the organization of the actin cytoskeleton and can be activated by integrin stimulation (reviewed in Orr and Murphy-Ullrich[40]). Each of these mechanisms occurs in subcellular locations highly responsive in mechanotransduction.[41] Whether or not Ca^{2+} influx alone, without concomitant integrin activation, increases PYK2 activity in endothelial cells remains controversial.[42] PYK2 is also generally considered to be a redox-sensitive kinase that is activated following stimulation in situations associated with elevated oxidative stress,[42,43] itself a hallmark of atherosusceptible sites and of most cardiovascular diseases and associated with impaired endothelial function. It is tempting to speculate that direct inactivation of eNOS via its tyrosine phosphorylation by PYK2 contributes to the phenomenon of endothelial dysfunction including sites of atherosusceptibility.

Circumstantial evidence to suggest that such a mechanism may exist includes thrombin-induced activation of PYK2 that leads to the activation of NFκB and its target genes, including VCAM-1 and MCP-1.[44] Interestingly, PYK2 knock-down impaired the thrombin-induced activation of the IκB kinase and attenuated the release and the transcriptional capacity of RelA/p65.[44] To what extent these effects can be explained by changes in NO production, which also inhibits NFκB activity,[45] is unclear. Angiotensin-II-induced endothelial dysfunction is PYK2- and NADPH oxidase-dependent[46] and the attenuated response to acetylcholine in arteries treated with a low concentration of H_2O_2 can be restored to control levels by the overexpression of the dominant negative PYK2 mutant.[46] Moreover, aortic PYK2 was found to be activated as early as

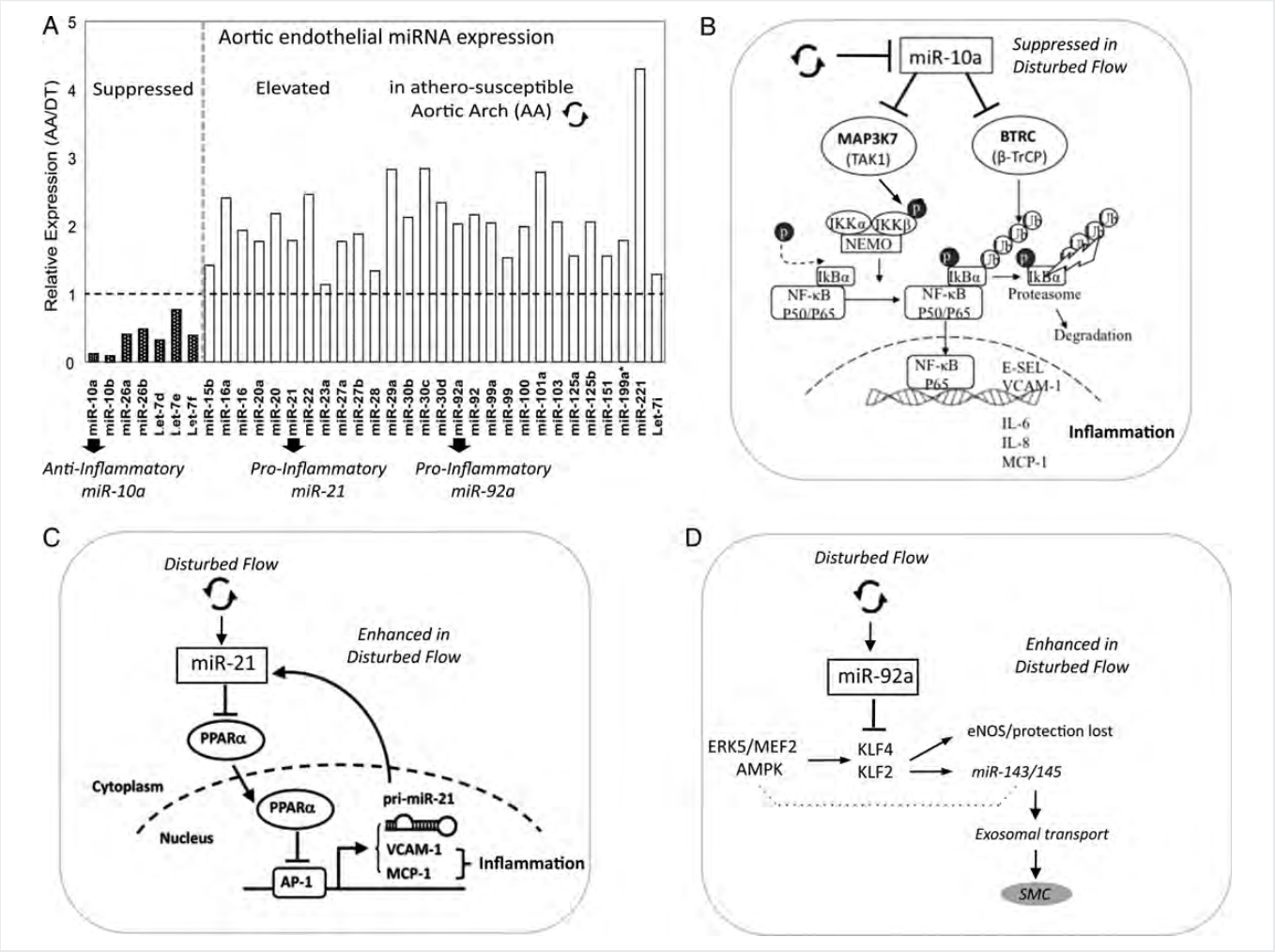

Figure 5 (*A*) Differentially expressed miRNAs in atherosusceptible endothelium *in vivo*. (*B*–*D*) Three miRNAs promote inflammation, one by suppressed expression and two by enhanced expression. (*B*) miR-10a, (*C*) miR-21, (*D*) miR-92a. Modified from references (*A* and *B*)[52] and Zhou *et al.* (*C*).[56]

7 days after starting a Western diet (i.e. before inflammatory cell activation) and PYK2 knockout mice bred onto an apolipoprotein E (ApoE)-deficient background demonstrated markedly decreased aortic lesions after 8 weeks of a Western diet.

Thus, it seems that shear stress elicits the phosphorylation of a tyrosine residue that negatively regulates eNOS activity and it is tempting to speculate that this event plays a key role in keeping NO output low and reducing the risk of co-factor, i.e. tetrahydrobiopterin depletion and eNOS uncoupling. Phosphorylation of the same residue in pathophysiological conditions may be responsible for decreasing NO generation. However, to what extent the latter mechanism competes with or complements the changes associated with eNOS uncoupling remain to be determined. Nevertheless, PYK2 may be an attractive therapeutic target as its inhibition would be expected to increase NO production.

4.6 Differential microRNA expression in atherosusceptible endothelium *in vivo*

MicroRNAs (miRNAs) are highly-conserved, small non-coding RNAs of 19–26 nucleotides that post-transcriptionally regulate gene expression. Mammalian miRNAs usually bind to the 3′ untranslated region (3′UTR) of target mRNAs, promoting mRNA degradation and/or inhibiting translation of the protein-coding genes.[47] Evolutionarily conserved Watson–Crick pairing between cognate mRNA 3′ UTR and miRNA 5′ regions centred on seed nucleotides (nt2–7) primarily determines miRNA target selection.[48] Emerging evidence suggests that individual miRNAs fine-tune the synthesis of many genes and that miRNA-mediated proteomes are typically mirrored by transcriptomes.[49,50] Given the widespread scope but modest repression of transcriptomes/proteomes by individual miRNAs, phenotypical consequences may arise by co-ordinated actions on multiple targets by single miRNAs or by an integrated regulatory effect on key pathways by multiple miRNAs.[51] A subset of differentially expressed endothelial miRNAs was identified in atherosusceptible inner aortic arch compared with protected regions of thoracic aorta in normal swine,[14,52] several of which show regulatory properties for NFκB pathway and the expression of transcription factors KLF2 and KLF4 (*Figure 5A*).

4.6.1 miRNA-10a regulation of NFκB pathway

Endothelial miR-10a in swine aortic arch is suppressed by 70–80% compared with undisturbed flow regions of the thoracic aorta.[52] A combination of genomic profiling, *in silico* analyses, miR manipulations, and molecular analyses in freshly isolated arterial endothelium and in

cultured human endothelial cells (HAEC) demonstrated miR-10a as a novel negative regulator of NFκB signalling.[52] Whole-genome transcriptome was profiled in cultured HAEC after the knock-down of endogenous miR-10a. Gene Set Enrichment Analysis (GSEA) identified IκB/NFκB-mediated inflammation as the top category of up-regulated biological processes. Phosphorylation of IκBα, a prerequisite for IκBα proteolysis and NFκB activation, was significantly up-regulated in miR-10a knock-down HAEC and was accompanied by increased nuclear translocation of NFκB p65. The inflammatory biomarkers monocyte chemoattractant protein-1 (MCP-1), interleukins IL-6, IL-8, VCAM-1, and E-selectin were elevated following miR-10a knock-down. Conversely, knock-in of miR-10a (a conservative 25-fold increase) inhibited the basal expression of VCAM-1 and E-selectin in HAEC. To investigate the molecular mechanisms underlying miR-10a suppression of pro-inflammatory molecules, genes in the canonical NFκB pathway were interrogated with miR-10a putative downstream targets predicted by TargetScan 5.1 that considers evolutionary conservation of miR seed sites.[53] The *in silico* analyses identified two molecules: mitogen-activated protein kinase kinase kinase 7 (MAP3K7; also known as TAK1) gene, and beta-transducin repeat containing gene (BTRC; also known as β-TrCP). Both promote proteasomal degradation of IκBα and p65 nuclear translocation and contain evolutionarily conserved miR-10a binding sites in the 3′UTRs. MAP3K7 protein directly phosphorylates and activates IκB kinase β (IKKβ) stimulating phosphorylation of IκBs. BTRC recognizes phosphorylated IκBa and mediates phosphorylation-dependent ubiquitination leading to IκB proteolysis. Knock-down of endothelial miR-10a in HAEC significantly up-regulated MAP3K7 and BTRC expressions and the evidence of direct miR-10a binding to the 3′-UTR was demonstrated (*Figure 5B*).

The role of these newly identified molecules *in vivo* was investigated in the endothelium from atherosusceptible aortic arch and atheroprotected descending thoracic aorta. MAP3K7, BTRC, phospho-IκBα, and nuclear p65 expression were all significantly up-regulated in atherosusceptible endothelium, where miR-10a expression is low. Collectively, these data demonstrate that differential expression of miR-10a contributes to the regulation of pro-inflammatory atherosusceptible endothelial phenotypes *in vivo*.

Regulation by miRs is multilayered; other mechanisms of control are emerging. Endothelial miR-126 has been shown to suppress VCAM-1 expression *in vitro*[54] further implicating miRNAs in the control of endothelial responses to vascular perturbation; however, this has not been tested *in vivo*. Furthermore, flow-sensitive miR-663 and miR-21 have been demonstrated to provoke endothelial inflammation,[55,56] the latter promoting activator protein 1 (AP-1) expression through the inhibition of PPARα (*Figure 5C*). eNOS has recently been reported as a direct target of miR-155.[57] TNFα-induction of inflammation up-regulated miR-155 resulting in the reduced expression of eNOS, an effect that was reversed by simvastatin. However, differential expression of miR-155 in atherosusceptible endothelium has not been reported and this mechanism may require the development of atherosclerosis.

4.6.2 miRNA-92a regulation of transcription factors KLF2 and KLF4

We postulated that other differentially expressed miRs may target molecules known to be important in endothelial regions predisposed to atherosusceptibility or protection. The Kruppel-like family members KLF2 and KLF4 are endothelial transcription factors induced by (undisturbed, atheroprotective) laminar shear stress *in vitro*.[58,59] KLF2 and KLF4 expression *in vitro* is suppressed by disturbed flow but this biomechanical regulation can be reversed by overexpression of KLF2 or KLF4, resulting in the induction of atheroprotective genes despite disturbed flow.[60,61] KLF2 and KLF4 inhibit proinflammatory induction of VCAM-1 and E-selectin and attenuate leucocyte adhesion, induce thrombomodulin, and inhibit PAI-1 expression.[20,60] Conversely, KLF4 deficiency enhanced the expression of cytokine-induced inflammatory VCAM-1, ICAM-1, and MCP-1 and suppressed eNOS,[61] effects that were not mitigated by the simultaneous up-regulation of KLF2, suggesting some diversity in their regulatory mechanisms. Consistent with atherosusceptibility *in vivo*, KLF2 and KLF4 expression is low in endothelium within the regions of disturbed flow in swine and mouse.[14,17]

Both KLF2 and KLF4 induce eNOS, the up-regulation of which is itself anti-inflammatory and anti-coagulant.[62] Transgenic experiments in mice demonstrated enhanced atherosclerosis in hemizygous KLF2- deficient mice.[63] Recent endothelial-specific gain- and loss-of-function studies of human KLF4 in mice fed a high-fat diet demonstrated significant reduction in atherosclerosis lesion area in mice in which there was sustained expression of KLF4, while endothelial deletion of KLF4 notably augmented the atherosclerotic burden.[61] KLF4 was also shown to interact with p300 transcriptional co-activator and does so in competition with other transcription factors (including NFκB) allowing differential transcriptional regulation through co-activator competition. This provides another layer of regulation that could result in the induction of some downstream KLF targets while inhibiting others.[61]

In contrast to miR-10a, miR-92a is expressed at a higher level in atherosusceptible disturbed flow regions in swine than in protected regions.[14] *In silico* analyses predicted highly conserved binding sites in the 3′-untranslated region (3′UTR) of KLF4 for five miRs of the subset of site-specific differentially expressed miRs (miR-26a, -26b, -29a, -92a, and -103) and a single binding site for an miR-92a complex in the 3′UTR of KLF2. However, only miR-92a knock-down and knock-in resulted in responses of KLF4 and KLF2 expression in human arterial endothelial cells. Dual luciferase reporter assays demonstrated functional interactions of miR-92a with full-length 3′UTR sequences of both KLF4 and KLF2s and with the predicted binding elements. Two evolutionarily conserved miR-92a sites in KLF4 3′UTR and one site in KLF2 3′UTR were functionally validated.[14] Knock-down of miR-92a in endothelial cells resulted in partial rescue from cytokine-induced inflammatory marker expression (MCP-1, VCAM-1, E-selectin, and eNOS), and the effects were attributable to enhanced KLF4 expression. Leucocyte-human arterial endothelial cell-adhesion experiments supported this conclusion.[14] In atherosusceptible swine aortic arch endothelium, a site of elevated miR-92a expression, both KLFs were expressed at low levels consistent with miR-92a targeting (*Figure 5D*).

Wu *et al.*[64] recently reported that an atheroprone flow waveform *in vitro* increased not only the level of endothelial miR-92a but also the association of miR-92a and KLF2 mRNA with both Ago1 and Ago2 proteins that are associated with the RNA-induced silencing complex. These findings are consistent with the above *in vivo* findings suggesting that KLF2 and KLF4 expression is regulated by the local haemodynamics through miR-92a (*Figure 5D*).

4.6.3 Flow-induced KLF2 regulates the expression of protective miR-143/145

In addition to direct miRNA regulation of KLF2 and KLF4 expression discussed earlier, miRNAs miR-143/145—downstream of KLF2—are themselves regulated by this transcription factor. miR-143/145 control the vascular smooth muscle phenotype (contractile/synthetic) during development;[65] however, their expression is increased in

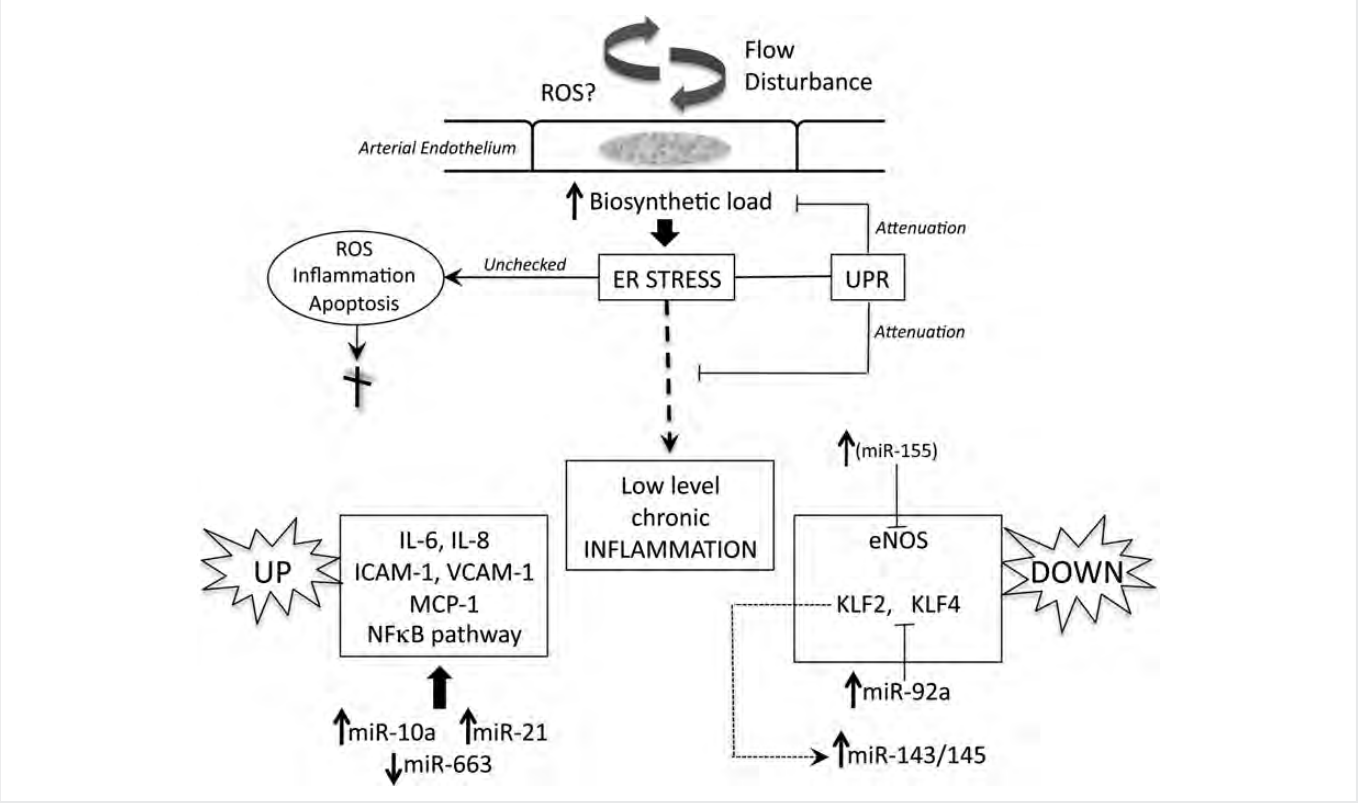

Figure 6 A schema of regulatory mechanisms of endothelial atherosusceptibility discussed in the text. Flow disturbance in regions susceptible to atherogenesis places a stress load on the biosynthetic capacity of the endothelial endoplasmic reticulum (ER) that leads to ER stress. Activation of the unfolded protein response (UPR) compensates to maintain normal cell function but a residual low-level chronic inflammatory state persists. Pro-inflammatory molecules are up-regulated (left) and protective molecules down-regulated (right). MicroRNAs (miRs) regulated up or down by disturbed flow regulate (at least in part) the pro-inflammatory state and are shown in a reciprocal transcriptional relationship to their targets. Notes: eNOS is a direct target of miR-155, inhibiting eNOS in cytokine-induced inflammation, however, differential miR-155 expression has not yet been demonstrated in disturbed flow. miR-143/145 is increased by flow-induced suppression of KLF2 and is shed in endothelial exosomes (*Figure 5D*). *Not shown* in this schema or discussed in the text (because of space limitations) are other regulatory mechanisms believed to contribute to endothelial pro-inflammatory phenotype through the MAPK pathway that mimics cytokine-induced mechanisms and through a low level of protective Nrf2 in atherosusceptible endothelium in mice.[17]

endothelial cells exposed to shear stress *in vitro*.[66] Furthermore, extracellular endothelium-derived microparticles (exosomes) secreted by miR-143/145-expressing endothelial cells were able to control target gene expression in co-cultured smooth-muscle cells (*Figure 5D*). Although the initial observation linked the endothelial expression of miR-143/145 with KLF2,[66] a more recent study demonstrated that the shear stress-induced up-regulation of miR-143/145 can be attributed to the activation of the AMP-activated protein kinase (AMPK), the subsequent phosphorylation and activation of p53 and the post-transcriptional up-regulation of miR-143/145.[18] These findings are significant inasmuch as metabolic diseases in general are associated with dysregulation of AMPK. Given that the AMPK is also activated by shear stress as well as an imbalance in the cellular AMP:ATP ratio, robust links are developing between haemodynamic stimuli, endothelial metabolism, post-transcriptional miRNA maturation, and the aspects of atherogenesis as a metabolic disease.

4.6.4 Interactions

How the mechanisms discussed earlier may interact to influence susceptibility *in vivo* remains speculative. *Figure 6* broadly outlines some connections. Despite the profiles being an average of a regional steady state, endothelial phenotype heterogeneity within a region of flow disturbance will likely result in a spectrum of individual cell susceptibilities.

4.7 Transition of endothelial atherosusceptibility to inflammatory atherogenesis *in vivo*

The aforementioned outlines describe the regulation of some of the *spatial* differences of endothelial phenotype associated with atherosusceptibility. However, critical *in vivo temporal* information about endothelial phenotype change is lacking in longitudinal studies of the initiation of atherosclerosis. Studies of endothelial phenotype transition during progressive induction of atherosclerosis by hypercholesterolaemia are in an early stage and those conducted so far—involving short-duration high-fat diet in swine—revealed surprising resistance of the endothelium to the development of pathological phenotypes.[6,7]

4.7.1 Endothelial phenotype transition is resistant to short-duration hypercholesterolaemia

The effect of short duration (2 weeks) hypercholesterolaemia was tested in swine.[6] Despite substantial elevation of circulating lipoprotein

levels, endothelial ER stress/UPR status (vs. normocholesterolaemic controls) at comparative sites *in vivo* remained unchanged despite histological evidence of enhanced lipid permeability at the atherosusceptible sites. However, intimal macrophages were rarely observed despite subendothelial extracellular matrix-bound lipid. At face value, this result suggests that accumulation of a critical concentration of subendothelial lipoproteins may be necessary to trigger mechanisms that attract and facilitate the entry of monocyte-derived macrophages. Since monocyte transmigration requires the expression of adhesion molecules on the surface of endothelium, the rate-limiting step of site-specific transition to an overt inflammatory endothelial phenotype may be differential increase of endothelial permeability to circulating lipoproteins and monocyte-derived macrophages (which enter the arterial wall during normal immuno-surveillance at a higher rate in atherosusceptible sites[11,67]). Locally secreted macrophage cytokines may then act on the endothelium to develop the inflammatory phenotype signature, recruit more macrophages, and promote lesion development.

Is disturbed flow regulating endothelial permeability? Circumstantial evidence includes the enhanced turnover of the endothelial monolayer in turbulent flow that decreases junctional stability,[68] the naturally increased immunosurveillance dynamics of monocytes in the regions of flow disturbance *in vivo,*[11,67] the inhibitory effect of disturbed flow upon gap-junctional communication *in vitro*[15] and *in vivo*,[69] the KLF2-induced expression of connexin (Cx)37 in protective laminar flow *in vitro* and Cx37 suppression in disturbed flow *in vivo*,[70] and perturbation of endothelial junctional molecule claudin-5 in disturbed flow regions *in vivo*[71] and its regulation by SOX-18 *in vitro*.[72]

Limited investigation of the phenotypes of endothelial cells overlying clinical human atherosclerotic lesions obtained either as surgical by-products or as autopsy material[73] suggests significant enhancement of inflammatory genes and pathways consistent with the general inflammatory profiles of the underlying lesions. Missing is knowledge of the phenotype-transition mechanisms (susceptible-to-inflammatory) and an understanding of the timing and dynamics involved. Nor should Nature's 'checks and balances' be ignored; in aortic-valve endothelium, significantly larger numbers of genes and pathways were changed following short-duration hypercholesterolaemia on the side of the valve leaflet susceptible to calcific valvular sclerosis.[7] Most interestingly, however, the changes demonstrated the induction of *protective* rather than inflammatory pathways by hypercholesterolaemia despite enhanced lipid insudation. Unlike valve endothelium, significant site-specific protective networks were not induced in the arterial endothelium by hypercholesterolaemia. However, protective genes were enhanced in response to hypercholesterolaemia in all arterial endothelial sites studied irrespective of whether the sites were susceptible or protective;[6] these included increased expression of reverse cholesterol transport regulators and antioxidant genes. Clarification of the timing and dynamics of inflammatory and protective transitions during hypercholesterolaemia are likely to emerge from longer periods of exposure to hypercholesterolaemia.

4.8 Future directions

Techniques to more thoroughly investigate cell phenotype than are reported in this review are already available through systems-targeted microarrays and whole genome sequencing in tandem with increasingly sophisticated statistical and bioinformatics analyses. Spatial and temporal accessibility to phenotype profiling is available over a range of scales down to the single cell embedded in vascular tissue. Measurement of the local mechanobiology of cells and extracellular matrices has rapidly advanced. Next-generation sequencing for analysis of entire systems (epigenetic, environmental, transcription factors, higher level chromatin organization, etc.), including the identification of genomic and epigenomic elements that regulate specific phenotypes, is now routinely accessible and can as readily be applied to vascular pathophysiology as to cell transformation, stem-cell differentiation, diabetes, and other metabolic diseases. Precise measurements of the complex arterial haemodynamics present *in vivo,* particularly, in the important coronary circulation, remain challenging. In addition to impacting mechanotransduction dynamics, these are also required to address the potential contributions of flow-dependent effects on the transport and local concentrations of potent signalling molecules, particularly, labile peptides and free radicals that undoubtedly play a significant role in arterial pathophysiology.

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